

The effects of undercarboxylated osteocalcin on oxidative *versus* glycolytic skeletal muscle

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Abstract

The regulation of glucose uptake and mass in skeletal muscle are essential for the maintenance of functional capacity, general health, and quality of life. Skeletal muscle can be categorised into two major types, glycolytic and oxidative muscles. The regulation of muscle glucose uptake varies between the two muscle types, dependent on specific conditions, such as whether or not insulin is present, and whether the muscle is at rest or following exercise. Furthermore, muscle atrophy in response to disuse, disease or aging also occurs in a muscle type-specific manner. However, the exact mechanisms underlying these muscle type specificities still remain unclear.

Emerging evidence indicates that undercarboxylated osteocalcin (ucOC), a hormone secreted from bone, may play a role in the regulation of muscle glucose uptake and muscle mass. The signalling pathways underlying the ucOC effect on muscle are not clear, but may include G protein-coupled receptor, class C, group 6, member A (GPRC6A) as the receptor, along with the activation of protein kinase B (Akt), extracellular signal-regulated kinases (ERK), 5' adenosine monophosphate-activated protein kinase (AMPK), protein kinase C (PKC), Akt substrate of 160kD (AS160), mammalian target of rapamycin complex 1 (mTORC1), and/or the class O of forkhead box transcription factors (FOXOs). It is also not clear whether the effect of ucOC on muscle glucose uptake and the loss of ucOC signalling during muscle atrophy are muscle type-specific, contributing to the muscle type specificities in glucose uptake regulation and muscle wasting, respectively. Therefore, this PhD thesis aimed to explore the link between ucOC and muscle glucose uptake (under various conditions) and muscle mass in glycolytic and oxidative muscles, as well as investigate the underlying mechanisms.

The thesis contains four separate but related studies, all of which have been published in top journals in the area of bone and mineral research.

Study 1

It was reported that exercise increased circulating ucOC levels in humans, which was related to improved insulin sensitivity. However, whether ucOC exerts direct effects on muscle insulin sensitivity following exercise is unknown. Thus, we first tested the hypothesis that ucOC increases insulin-stimulated glucose uptake post-*ex vivo* contraction in a muscle type-specific manner in mice. Muscle glucose uptake and signalling proteins in a glycolytic muscle - extensor digitorum longus muscle (EDL), and an oxidative muscle - soleus muscle (Soleus), were examined in different conditions: with or without ucOC and/or insulin stimulation at rest or following contraction. In EDL but not in Soleus, ucOC (10 ng/mL) moderately increased ($P < 0.05$) insulin-stimulated muscle glucose uptake and phosphorylated (p-)AS160 (Threonine (Thr)642) 2.5 hr following muscle contraction. These data indicate that ucOC directly enhances insulin sensitivity following exercise in glycolytic muscles, but not in oxidative muscles, possibly due to the enhancement of insulin-stimulated AS160 phosphorylation. This study has been published in Osteoporos Int.

Study 2

In Study 1 we reported that ucOC did not alter glucose uptake in EDL at rest, with or without insulin. However, we hypothesised that the lack of ucOC effect is likely due to the use of intact muscles, which limits the contact of ucOC with myotubes. As such, in this study we examined the effect of ucOC alone (0, 0.3, 1, 3, 30 ng/mL) on glucose uptake (without insulin) in mouse EDL and Soleus that were longitudinally divided into halves. ucOC increased muscle glucose uptake in both EDL (48.4 %, $P < 0.05$) and Soleus (33.3 %, $P < 0.01$). It also triggered the phosphorylation of ERK2 (Thr202/ Tyrosine (Tyr)204) and AS160 (Thr642) in both muscle types. ucOC had no significant effect on basal phosphorylation levels of Akt (Serine (Ser)473), AMPK α (Thr172) or PKC δ/θ (Ser643/676). The inhibition of ucOC-induced ERK phosphorylation, with the pretreatment of ERK inhibitor U0126, had limited effects on ucOC-stimulated glucose uptake and AS160 phosphorylation in both muscle types. Taken together, ucOC *per se* triggers non-insulin-stimulated glucose uptake in both EDL and Soleus, likely due to the enhancement of AS160 phosphorylation. This study

has been published in Front Endocrinol.

Study 3

Following Study 2, we then tested whether ucOC also directly increases glucose uptake in insulin-stimulated muscles at rest. Muscle glucose uptake and signalling proteins were analysed in mouse EDL and Soleus (divided into halves, no contraction) treated with ucOC (0, 0.3, 1, 3, 30 ng/mL), followed by insulin addition. ucOC treatment at 30 ng/ml enhanced muscle glucose uptake in insulin-stimulated Soleus (17.5 %, $P < 0.05$), but not in EDL. In insulin-stimulated Soleus only, ucOC treatment (3 and 30 ng/ml) increased the phosphorylation of AS160 (Thr642) and ERK2 (Thr202/Tyr204), but not Akt (Ser473) or AMPK α (Thr172). Overall, ucOC enhances glucose uptake and in insulin-stimulated Soleus but not EDL (at rest), attributed to the increase of AS160 phosphorylation in insulin-stimulated muscles. This study has been published in Calcif Tissue Int.

Study 4

ucOC has been reported to benefit muscle growth, probably via increasing protein synthesis, inhibiting protein degradation, and promoting myotube formation. As both hindlimb immobilisation and testosterone depletion have adverse effects on bone formation, we tested the hypothesis that the loss of ucOC actions is associated with the loss of muscle mass and strength during hindlimb immobilisation and castration, as well as associated with changes in anabolic and/or catabolic pathways. Rats underwent castration surgery and 10 d later, they were subjected to 7 d of hindlimb immobilisation. Hindlimb immobilisation, but not castration, resulted in reductions in serum ucOC concentration (~30 %, $P < 0.05$), as well as in muscle mass and strength of EDL (~10%, $P < 0.05$) and Soleus (~50%, $P < 0.001$). Lower ucOC correlated with the degree of muscle loss and muscle weakness in both EDL and Soleus. Furthermore, hindlimb immobilisation results in a reduction in GPRC6A expression (~50 %, $P < 0.001$) associated with muscle wasting, only in Soleus. In addition, ucOC levels, GPRC6A expression, as well as the activity of ERK and AMPK were associated with

the protein expression and activity of a number of proteins in the mTORC1 and FOXO signalling pathways in a muscle type-specific manner. Collectively, a greater loss of the ucOC/GPRC6A cascade in Soleus is associated with a higher degree of muscle atrophy during hindlimb immobilisation. This study has been published in J Bone Miner Res.

General conclusion

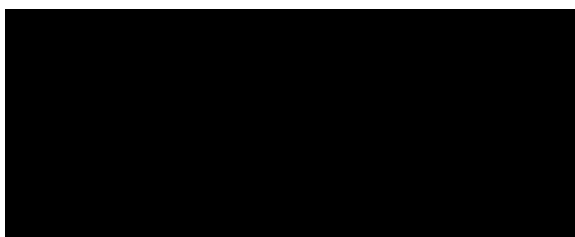
ucOC triggers non-insulin-stimulated glucose uptake in both muscle types, and enhances insulin-stimulated glucose uptake in a muscle type-specific manner (at rest in Soleus and following contraction in EDL), likely due to the increase of AS160 phosphorylation. In addition, a greater reduction in the ucOC/GPRC6A cascade in Soleus is associated with a higher degree of muscle atrophy during hindlimb immobilisation, implying that ucOC regulation may be important for muscle mass maintenance in oxidative muscles. The findings of this thesis provide novel evidence on muscle type-specific effect of ucOC on muscle glucose uptake, and on muscle type-specific loss of ucOC signalling associated with muscle wasting. These results suggest there is a therapeutic potential of ucOC administration for insulin resistance and muscle atrophy, particularly in oxidative muscles.

Student declaration

“I, Xuzhu Lin, declare that the PhD thesis by publication entitled “The effects of undercarboxylated osteocalcin on oxidative *versus* glycolytic skeletal muscle” is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.”

Signature:

Date: 6/3/2018



Thesis by publication

All doctoral students at Victoria University are permitted to submit a thesis by publication. The thesis by publication is a thesis format that includes manuscripts that have been prepared or accepted for publication. These manuscripts may have more than one author in which signatures from co-authors are required. The thesis must reflect a sustained and cohesive theme and framing or substantial linking text normally required in introducing the research and linking the chapter and manuscripts.

The current thesis contains four studies, all of which have been published. My specific contributions to these publications are detailed at the beginning of each chapter.

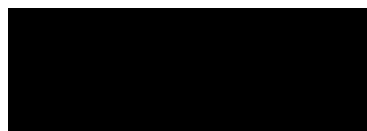
Details of included papers: thesis by publication

Chapter No.	Paper title	Publication status	Publication title and details
3	The effects of muscle contraction and recombinant osteocalcin on insulin sensitivity ex vivo	Published	Osteoporos Int, 27, 653-663, 2016 Impact factor: 3.591 Quartile ranking: Q1
4	Recombinant uncarboxylated osteocalcin <i>per se</i> enhances mouse skeletal muscle glucose uptake in both extensor digitorum longus and soleus muscles	Published	Front Endocrinol, 8, 2017 Impact factor: 3.675 Quartile ranking: Q1
5	Uncarboxylated osteocalcin enhances glucose uptake ex vivo in insulin-stimulated mouse oxidative but not glycolytic muscle	Published	Calcif Tissue Int, 9; 1-8, 2018 Impact factor: 3.275 Quartile ranking: Q1
6	Hindlimb immobilization, but not castration, induces reduction of undercarboxylated osteocalcin associated with muscle atrophy in rats	Published	J Bone Miner Res, 31; 1967-1978, 2016 Impact factor: 6.284 Quartile ranking: Q1

Declaration by:

Xuzhu Lin

Signature:



Date:

6/3/2018

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List of abbreviations

2-DG: 2-Deoxy-D-glucose

ACC: acetyl-coenzyme A carboxylase

ADT: androgen deprivation treatment

AICAR: 5-Aminoimidazole-4-carboxamide ribonucleotide

Akt: protein kinase B

AMPK: 5' adenosine monophosphate-activated protein kinase

ApoE-KO: Apolipoprotein E-deficient

AS160: Akt substrate of 160kD

ATF4: activating transcription factor 4

ATP: Adenosine Triphosphate

BSA: bovine serum albumin

CKD: chronic kidney disease

cOC: carboxylated osteocalcin

CoL: contralateral leg

CREB: cAMP response element-binding protein

d: day(s)

DAG: diacylglycerol

DAPI: 2-(4-amidinophenyl)-1H-indole-6-carboxamide

DMEM: Dulbecco's modified eagle medium

DMSO: dimethyl sulfoxide

DTT: dithiothreitol

ECL: chemiluminescent

EDL: extensor digitorum longus muscle(s)

EGF: epidermal growth factor

ERK: extracellular signal-regulated kinases

ER: endoplasmic reticulum

FA: fatty acid

FDL: flexor digitorum longus

FFA: free fatty acid

FOXO: the class O of forkhead box transcription factor

GLP-1: Glucagon-like peptide-1

Glut4: glucose transporter 4

GM: gene modification

GPRC6A: G protein-coupled receptor, class C, group 6, member A

HDAC4: histone deacetylase 4

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFD: high fat diet,

HGF: hepatocyte growth factor

hr: hour(s)

HSL: hormone sensitive lipase

IGF-1: insulin-like growth factor 1

IL-6: interleukin 6

ImmL: immobilised leg

InsR: insulin receptor

IR: insulin resistance

IRS-1: insulin receptor substrate 1

ISGU: insulin-stimulated glucose uptake

KHB buffer: Krebs-Henseleit buffer

KRP: Krebs Ringer Phosphate

LDL-C: low-density lipoprotein cholesterol

LHRH: luteinising hormone releasing hormone

MAFbx/Fbx32: muscle atrophy F-box

MAPK: mitogen-activated protein kinase

Mcad: Medium-chain acyl-coenzyme A dehydrogenase

MEK: MAPK kinase

min: minute(s)

mTOR: mammalian target of rapamycin

mTORC1/2: mammalian target of rapamycin complex 1/2

MuRF1: muscle RING-finger protein-1

MW/BW: muscle weight/body weight

MyHC: myosin heavy chain

NF- κ B: nuclear factor κ B

NIS: non-insulin-stimulated

NR: non-immobilised-rats

Nrf1: Nuclear respiratory factor 1

OC: osteocalcin

OPG: osteoprotegrin

OST-PTP: osteotesticular protein tyrosine phosphatase

p-: phosphorylated

P/C: post-contraction

P70S6K: ribosomal protein S6 kinase

PGC-1 α : peroxisome proliferator-activated receptor- γ coactivator-1 α

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

c/nPKC: conventional/novel PKC

aPKC: atypical PKC

PLC: Phospholipase C

PMA: phorbol 12-myristate 13-acetate

PTH: parathyroid hormone

PVDF: polyvinylidene fluoride

RG: red gastrocnemius

RIPA: radioimmunoprecipitation assay

RQ: red quadriceps

ROS: reactive species oxygen

RUNX2: runt-related transcription factor 2

SDS: sodium dodecyl sulfate

SEM: standard error of mean

Ser: Serine

SOL: Soleus

Soleus: soleus muscle(s)

T2DM: type 2 diabetes

TBST: Tris-buffered saline with 0.1 % Tween-20

TCA: tricarboxylic acid

TG: triglycerides

Thr: Threonine

TWIST2: twist family BHLH transcription factor 2

Tyr: Tyrosine

ucOC: undercarboxylated osteocalcin

ucOC %: undercarboxylation percentage of osteocalcin

Ucp1: Uncoupling protein 1

ULK1: Unc-51 like autophagy activating kinase 1

VL: vastus lateralis

w: week(s)

WG: white gastrocnemius

WQ: white quadriceps

WT: wide type

List of publications and conference presentations

Peer reviewed journal publications related to the thesis:

1. Levinger I*, **Lin X***, Zhang X, Brennan-Speranza T.C, Volpato B, Hayes A, Jerums G, Seeman E. McConell G. 2016. The effects of muscle contraction and recombinant osteocalcin on insulin sensitivity *ex vivo*. Osteoporos Int, 27; 653-663. doi: 10.1007/s00198-015-3273-0. (*: Levinger I and **Lin X** are co-first authors)
2. **Lin X**, Parker L, McLennan E, Zhang X, Hayes A, McConell G, Brennan-Speranza T.C, Levinger I. 2017. Recombinant uncarboxylated osteocalcin *per se* enhances mouse skeletal muscle glucose uptake in both extensor digitorum longus and soleus muscles. Front Endocrinol, 8. doi: 10.3389/fendo.2017.00330.
3. **Lin X**, Parker L, McLennan E, Zhang X, Hayes A, McConell G, Brennan-Speranza T.C, Levinger I. 2018. Uncarboxylated osteocalcin enhances glucose uptake *ex vivo* in insulin-stimulated mouse oxidative but not glycolytic muscle. Calcif Tissue Int, 9; 1-8. doi: 10.1007/s00223-018-0400-x.
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1. Levinger I, **Lin X**, Zhang X, Hayes A, Jerums G, Ferron M, Karsenty G, Seeman E,

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 3. **Lin X**, Hanson E, Betik A.C, Brennan-Speranza T.C, Hayes A, Levinger I. Hindlimb immobilization, but not castration, induces reduction of undercarboxylated osteocalcin associated with muscle atrophy in rats. American Society for Bone and Mineral Research Annual Meeting, Atlanta, Georgia, USA (2016). (Selected as a plenary poster)
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Chapter 1. Introduction

Skeletal muscle constitutes approximately 40 % of total body mass. It plays a major role in the body locomotion and also contributes to the maintenance of whole-body energy homeostasis [1-3]. As such, metabolic and/or functional disorders of skeletal muscle are often linked with poor general health, low quality of life, and premature mortality [4-6]. Healthy muscle function and metabolism considerably depend on muscle glucose uptake and muscle mass maintenance [7-9]. Therefore, a better understanding of the regulation of these two biological processes contributes not only to the knowledge of muscle function modulation, but also to the therapeutic design for pathological conditions such as type 2 diabetes (T2DM) and muscle wasting disorders.

Skeletal muscle can be categorised into two major types, glycolytic and oxidative muscles, based on the types of muscle fibres of which the muscle majorly consists [2, 10, 11]. Muscle glucose uptake of these two muscle types is differently regulated [12-14]. Under various physiological conditions, such as whether or not insulin is present, and whether muscle is at rest or following exercise, the regulation of muscle glucose uptake in a specific muscle type can also be different [12-17]. Furthermore, muscle mass is affected by muscle atrophy in a muscle type-selective manner [18, 19]. However, the exact mechanisms behind the muscle type specificities are still unclear.

Bone and muscle largely constitute the musculoskeletal system [20]. Recent research suggests that the interaction between them goes beyond simple physical attachment and force production [21, 22]. ucOC is a hormone secreted from bone, and may be involved in the regulation of muscle function and metabolism [23-28]. Very recently, it was reported that ucOC enhanced glucose uptake in muscles at rest [23, 29]. Furthermore, following acute exercise, the increase of circulating ucOC was associated with the enhancement of muscle insulin sensitivity, implying a possible role of ucOC in the insulin-sensitising effect after exercise [30, 31].

ucOC may also benefit muscle growth via modulating muscle protein synthesis,

protein degradation, and myotube formation [26, 27, 32]. Therefore, the elucidation of these ucOC effects on muscle can considerably enrich our understanding of the interaction between bone and muscle, and the modulation of muscle glucose uptake and muscle mass. However, the evidence for a direct effect of ucOC on muscle cells is very limited. Moreover, whether the ucOC regulation is muscle type-specific is not clear.

Understanding the molecular mechanisms involved in ucOC effects on muscle cells is important for the identification of protein targets for future drug design. To date limited data suggests that the signalling pathway may include GPRC6A as the postulated receptor of ucOC, as well as Akt, ERK, AMPK, PKC, AS160, mTORC1, and/or FOXOs as downstream proteins [23, 26, 27, 29, 33, 34]. However, current evidence is scant and findings are contradictory, and it is also not known whether the signalling pathway(s) triggered by ucOC is (are) also muscle type-specific.

Accordingly, the primary aim of current thesis was to explore the link between ucOC and muscle glucose uptake (with or without insulin stimulation, at rest or following muscle contraction) and muscle mass in both glycolytic and oxidative muscles, as well as to investigate the related mechanisms. The current thesis includes four studies, all of which have been published. Studies 1, 2, and 3 (**Chapter 3, 4, and 5**), which focus on the ucOC effect on muscle glucose uptake, are reported in the order in which they were conducted. Study 4 (**Chapter 6**) explores the loss of ucOC regulation in muscle mass during atrophic conditions.

Chapter 2. Literature review

This chapter comprises four main sections. **Section 2.1** covers general knowledge regarding skeletal muscle, in terms of muscle morphology, muscle type, and muscle function. **Section 2.2** describes the production, modification, and function of osteocalcin with a focus on its effect on muscle energy metabolism and muscle mass, including the potential signalling pathways. **Section 2.3** describes muscle type differences in muscle glucose uptake under various conditions, and in muscle wasting during muscle atrophy, as well as the possible involvement of ucOC in the underlying mechanisms. Finally, **Section 2.4** outlines the aims and hypotheses of the thesis.

2.1 Skeletal muscle overview

Skeletal, cardiac and smooth muscles are the three major muscle types in the body. Unlike the other two muscle types, skeletal muscle, which is voluntarily controlled by the somatic nervous system, is responsible for all conscious movements of the body [35]. Skeletal muscle tissue is also one of the major constituents of the human body, comprising approximately 40 % of total body mass [3]. As such, metabolic and functional disorders of skeletal muscle are often associated with poor general health, low quality of life, and premature mortality [4-6].

2.1.1 Skeletal muscle morphology

Skeletal muscle is an organ with a highly-organised structure, formed by its predominant component skeletal muscle tissue and a small amount of multiple other tissues (**Figure 2.1**).

The formation of skeletal muscle tissue occurs via myogenesis during embryonic development [36]. In this process, embryonic muscle cells called myoblasts, fuse into striated multinucleated cells termed muscle fibres, which are basic structural units in mature muscle tissue [37]. In a skeletal muscle, muscle fibres are clustered together into numerous bundles, which are defined as fascicles [38].

Connective tissue is another important constituent of skeletal muscle includes the endomysium, perimysium, and epimysium layers. These connective tissue layers closely wrap each muscle fibre and fascicle, as well as the whole muscle [36]. In addition, nerves, neuronal branches, arteries, veins, and capillaries, which penetrate through connective tissue layers, are also critical components of this organ that supply muscle fibres with innervation, oxygen, nutrients, and regulatory factors [36, 39, 40].

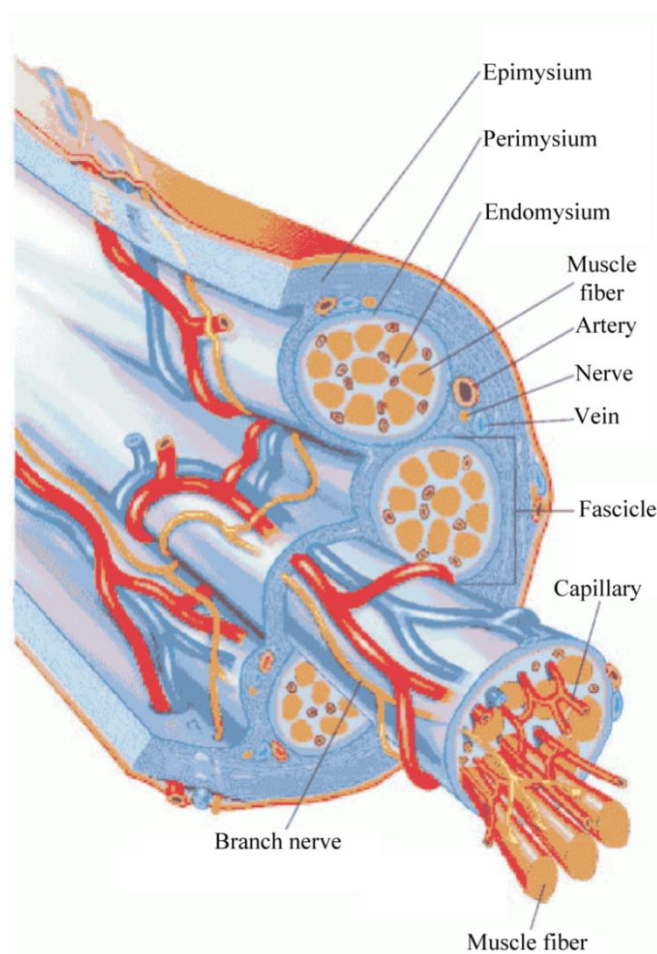


Figure 2.1. The morphological structure of skeletal muscle [41]. Skeletal muscle fibres are cylindrical multinucleated cells and are clustered into fascicles. An individual skeletal muscle consists of numerous fascicles and wrapped by multiple connective tissue layers. First, each muscle is surrounded by a connective tissue sheath called the epimysium. Furthermore, each fascicle is surrounded by a layer of connective tissue called the perimysium. Within the fascicle, each individual muscle fibre is surrounded by connective tissue called the endomysium.

Skeletal muscles have an abundant supply of blood vessels and nerves. Generally, an artery and at least one vein accompany each nerve that penetrates the epimysium of a skeletal muscle. Muscle fibres are directly innervated and nourished via branches of the nerve and capillaries.

2.1.2 Skeletal muscle fibre types

Vertebrate skeletal muscle has evolved into a heterogeneous tissue containing different fibre types with various molecular, mechanical, and metabolic traits [42, 43]. In general, two muscle fibre types exist in mammalian skeletal muscle, fast-twitch (white) fibres and slow-twitch (red) and fibres [44].

Fast-twitch fibres are also named type II fibres (subcategorised into type IIx/d, type IIa, and type IIb), as they express type II myosin heavy chain (MyHC) molecules. They predominantly utilise glycolytic metabolism, in which glucose is catabolised via glycolysis, and are characterised by low mitochondrial content, low capillary density, and vast amounts of glycolytic enzymes [2, 45].

Fast-twitch fibres express type I MyHC molecules, so they are also termed type I fibres. Compared to type II fibres, type I fibres mainly rely on oxidative metabolism. They possess a large number of mitochondria, high capillary density, and abundant expression of glucose and fatty acid (FA) oxidative enzymes [2, 45].

The heterogeneity of muscle fibres results in varying skeletal muscle types with differing abilities to adapt and generate various mechanical functions [2]. For example, glycolytic muscles, such as EDL located within the anterior group of lower leg muscles, predominantly contain type II fibres (98 % type II fibres in mouse EDL [46]), and are mainly recruited during high-intensity exercise since glycolytic metabolism quickly generates adenosine triphosphate (ATP) for a short duration. On the other hand, oxidative muscles such as Soleus located within the posterior group of lower leg muscles, are enriched with type I fibres (55 % type I fibres in mouse Soleus [46]), and are mainly recruited for low-intensity movements and body posture maintenance as oxidative metabolism slowly but efficiently provides long-lasting ATP production [10, 11]. Therefore, it is likely that the metabolic regulation in skeletal muscle has evolved into a muscle type-specific manner to fulfil particular tasks with optimum efficiency [14, 43]. This muscle type-specific modulation is further described in **Section 2.3.1**. Furthermore, the muscle type specificity appears to be associated with pathological

conditions such as muscle atrophy [18], which is described in **Section 2.3.2**.

2.1.3 Skeletal muscle functions

Skeletal muscle contributes to multiple functions in the body. From a mechanical perspective, it enables locomotion by generating forces [2]. From a metabolic point of view, it contributes to energy homeostasis via both storage and metabolism of nutrients [1].

Two factors are critical for skeletal muscle to achieve optimum performance for its functions. Firstly, muscle contraction requires glucose - one of the key sources of energy, and skeletal muscle is the major site for the disposal of circulating glucose in response to insulin stimulation [9]. Therefore, the modulation of glucose uptake in skeletal muscle cells plays an essential role in energy metabolism during exercise/muscle contraction, as well as whole-body glycaemic control in the postprandial state [47, 48]. Secondly, the maintenance of a normal volume of muscle mass is the other important factor in both mechanical and metabolic functions of skeletal muscle [7, 8]. In the body, both muscle glucose uptake and muscle mass are tightly controlled via various modulations, of which hormones play an important role [7]. Recent evidence has shown that bone may participate in the endocrine regulation of skeletal muscle via the actions of a small, bone-derived polypeptide called osteocalcin [23, 27].

2.2 Osteocalcin

Bone and skeletal muscle largely constitute the musculoskeletal system [20]. The crosstalk between these two organs is widely known, and traditionally researched and interpreted as a biomechanical one [49]. From this point of view, bone offers muscle attachment sites, while muscle influences bone with mechanical loading [49]. However, the evidence that has emerged over the last decade has highlighted both bone and muscle as possible endocrine organs, and thus the biochemical interaction

between these two tissues has been increasingly investigated [21, 22]. Recent reports indicated that ucOC, which is secreted from bone and traditionally described as a serum marker for bone remodelling [50], may participate in the biochemical bone-muscle crosstalk by upregulating muscle glucose uptake and muscle mass (see below for details) [23-28]. Therefore, the delineation of ucOC effects on muscle not only enriches the knowledge of bone-muscle interaction and muscle biology, but is also important for the design of new therapeutics for pathological conditions such as T2DM and muscle atrophy.

2.2.1 Osteocalcin overview

Osteocalcin (OC) is a 49 amino acid protein (in human) with a low molecular weight at 5.7 KDa. It is also named gamma-carboxyglutamic acid-containing protein or bone gla protein [51]. OC is the most abundant non-collagenous protein within the bone matrix, primarily produced by osteoblasts during the late stage of their differentiation [52]. After protein translation at the osteoblast endoplasmic reticulum, OC undergoes carboxylation at the 17, 21 and 24 Glu residues by γ -glutamyl carboxylase in the presence of vitamin K. This post-translational modification changes the conformation of OC protein, resulting in increased affinity for calcium ions exposed at the surface of the hydroxyapatite crystal in the bone matrix [52, 53]. However, not all OC in bone is carboxylated, as a small percentage of OC still remains in its undercarboxylated form.

Some OC, including around 40 % ucOC, is released from bone into the general circulation [54]. Importantly, it has been suggested that the circulating ucOC is a multifunctional hormone that regulates various physiological processes and pathological responses [55]. In addition to its modulation in muscle energy metabolism and muscle mass, ucOC has also been reported to regulate whole-body metabolism [56, 57], male fertility [58], brain development [59], cognition [59], cardiovascular health [60], and anti-tumour immunity [61]. In **Section 2.2.3.2** and **2.2.4**, the effects of ucOC on muscle energy metabolism and muscle mass are

described in detail.

2.2.2 Regulation of ucOC production in bone

The regulation of ucOC secretion from bone is important for its endocrine functions. A reciprocal regulation between bone and pancreas, which has been reported by several groups, leads to the increase of ucOC production [34, 62-66]. In this putative regulatory mechanism, insulin, of which secretion is enhanced by the effect of ucOC on pancreatic β -cells (**Section 2.2.3.1**), stimulates the secretion of ucOC from bone via two signalling pathways (**Figure 2.2**).

Firstly, the insulin action blocks the twist family BHLH transcription factor 2 (TWIST2), which attenuates runt-related transcription factor 2 (RUNX2) [64]. As RUNX2 is the key regulator of *Osteocalcin* gene (*Oc*) transcription in bone, the increase of RUNX2 activity by insulin leads to elevated *Oc* encoding in osteoblasts and increased OC content in the extracellular matrix [52, 64]. However, most of the OC secreted into the bone matrix is bio-inactive, as it has already undergone carboxylation within osteoblasts [52, 67].

In the other multi-cellular signalling cascade, the activity of FOXO1 is reduced in response to insulin stimulation [63, 66]. In the nucleus of osteoblasts, FOXO1 functions synergistically with another nuclear factor activating transcription factor 4 (ATF4) via direct binding [66]. The compromise of FOXO1/ATF4 transcriptional activity by insulin results in reduced osteoprotegerin (OPG) expression in the bone matrix [66]. As OPG is a negative regulator of osteoclast function, its reduction leads to elevated bone resorption which was reported to be majorly responsible for the decarboxylation of OC within the extracellular matrix [66]. Consequently, the insulin action in osteoblasts leads to increased ucOC production and release. Simultaneously, the inhibition of FOXO1 also leads to a positive-feedback on the insulin action by decreasing the transcription of gene *ESP* and the expression of osteotesticular protein tyrosine phosphatase (OST-PTP), which down-regulates the insulin signalling in osteoblasts [65].

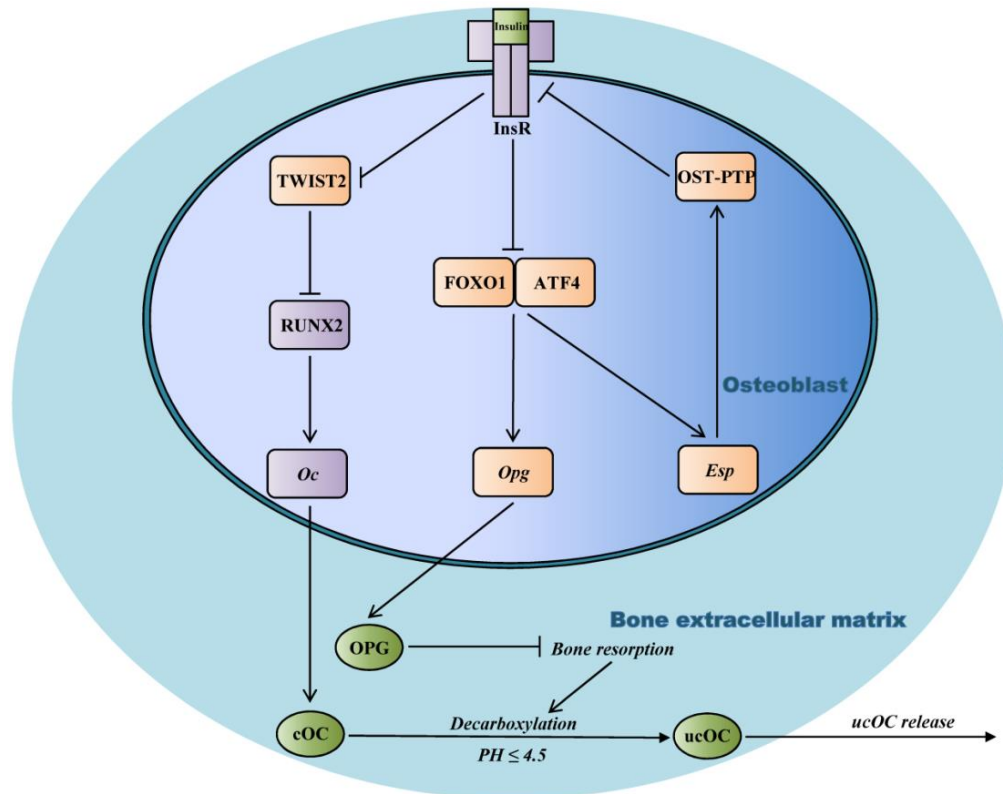


Figure 2.2. Suggested mechanisms underlying the regulation of ucOC secretion from bone. The ucOC secretion in bone is modulated via two signalling pathways. In the first pathway, the activation of insulin signalling negatively regulates TWIST2 and then attenuates its inhibitory action on RUNX2, leading to elevated *Oc* encoding in osteoblasts and a subsequent increase of OC content in the extracellular matrix. In the other multi-cellular signalling cascade, the activity of FOXO1, which synergistically functions with another nuclear factor ATF4 via direct binding, is reduced in response to insulin stimulation. The attenuation of FOXO1/ATF4 transcriptional activity results in reduced OPG expression in the bone matrix, leading to elevated bone resorption. As bone resorption favours the decarboxylation of cOC within extracellular matrix, the insulin action in osteoblasts eventually results in increased ucOC production. Simultaneously, the inhibition of FOXO1 also leads to a positive-feedback on insulin action by decreasing the transcription of *Esp* and the expression of OST-PTP, which downregulates the insulin signalling in osteoblasts.

InsR: insulin receptor; TWIST2: twist family BHLH transcription factor 2; RUNX2: runt-related transcription factor 2; FOXO1: the class O of forkhead box transcription factor 1; ATF4: activating transcription factor 4; OPG: osteoprotegerin; OST-PTP: osteotesticular protein tyrosine phosphatase; cOC: carboxylated osteocalcin; ucOC: undercarboxylated osteocalcin; *Oc*: osteocalcin gene; *Opg*: osteoprotegerin gene; *Esp*: OST-PTP gene.

Protein/gene in blue: protein/gene that promotes ucOC production;

Protein/gene in orange: protein/gene that inhibits ucOC production;

Protein in green: hormone/secreted factor;

→ : activation or promotion;

—| : inhibition;

→ : decarboxylation/translocation;

Italicised text: process or event.

The discovery of the mutual dependence of bone and pancreas suggests the possibility of crosstalk between bone and other ucOC target tissues, such as the testis. A recent report suggested that ucOC upregulates the secretion of testosterone via its actions in testis Leydig cells [58]. Similar to the insulin receptor (InsR), androgen receptors are expressed in osteoblasts [68, 69]. Furthermore, testosterone has long been found to benefit bone quality [70, 71]. Therefore, it is possible that testosterone can reciprocally influence ucOC production in bone, similar to the action of insulin. In this thesis, this specific hypothesis is tested via measuring serum ucOC levels in castrated animals (**Chapter 6**).

2.2.3 Functions of ucOC in energy metabolism

2.2.3.1 Metabolic effects of ucOC on the whole body and peripheral tissues

It has been reported that, in mice, the increase of serum levels of ucOC via genetic modification or ucOC administration exerts beneficial effects on whole-body glucose metabolism, fat metabolism, and energy expenditure in both healthy and obese animals [24, 25, 56, 72-75]. Firstly, the rise of ucOC levels led to reduced blood glucose levels, decreased glucose tolerance, and enhanced whole-body insulin sensitivity [24, 56, 72, 74, 75]. Furthermore, mice with higher ucOC levels exhibited lower fat mass, serum total cholesterol levels, serum low-density lipoprotein cholesterol (LDL-C) levels, serum triglycerides (TG) levels, and serum free fatty acid (FFA) concentrations [25, 56, 72, 74]. In addition, although food intake and activity levels were not affected, energy expenditure in mice was significantly enhanced by elevated ucOC levels [24, 25, 72, 73].

The whole-body effect of ucOC on energy metabolism is possibly attributed to the integration of ucOC regulations in multiple organs and tissues. Recent studies have suggested that these ucOC-targeted organs and tissues include the pancreas, intestine, liver, adipose tissue, and skeletal muscle, as shown in **Figure 2.3**.

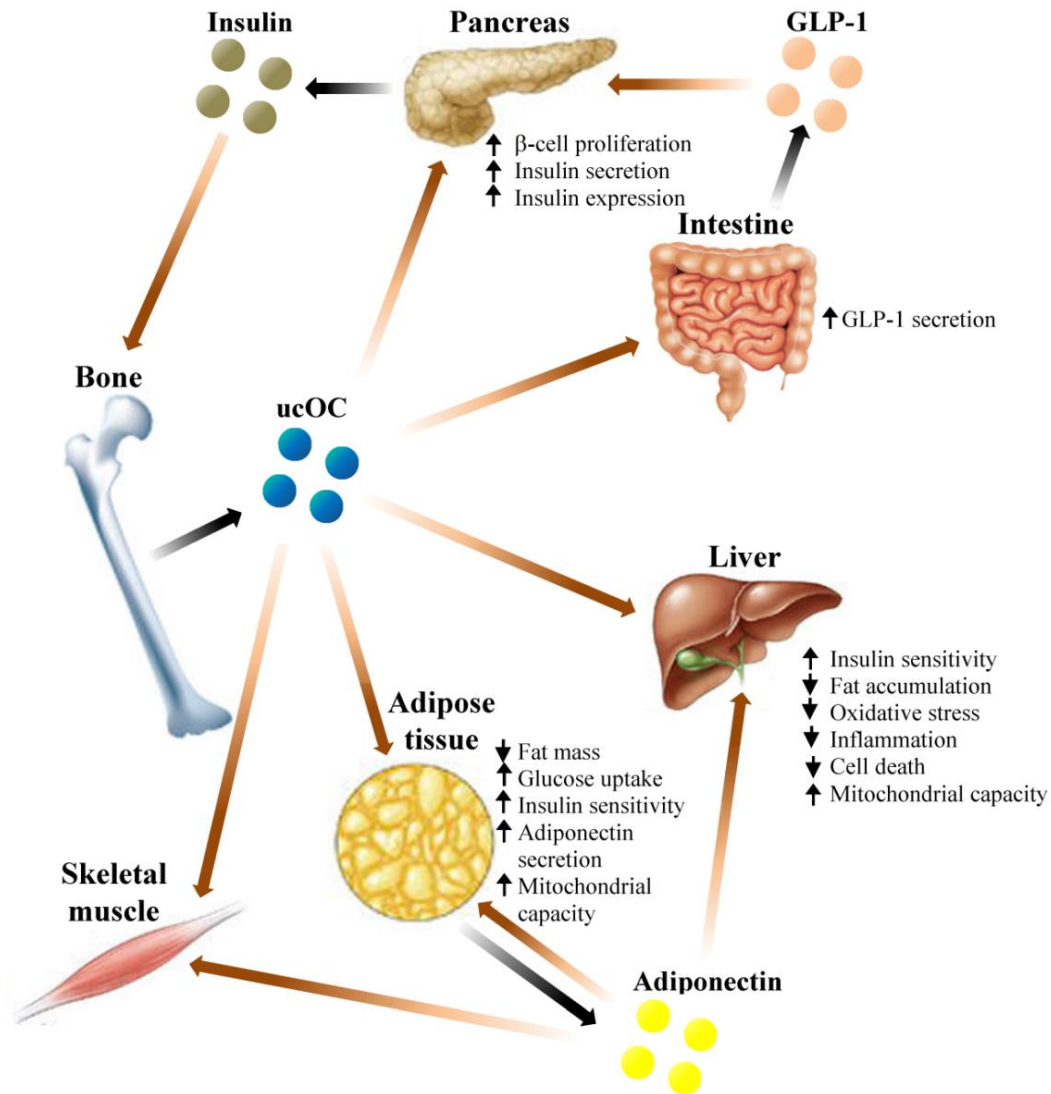


Figure 2.3. Energy metabolic upregulations of ucOC on distal organs and tissues [76]. ucOC, a hormone produced by bone, regulates whole-body energy metabolism via its actions on peripheral tissues and organs.

In the pancreas, ucOC favours the production of insulin, which in turn upregulates ucOC production in bone, by increasing cell proliferation, *Insulin* expression, and insulin secretion in β -cells. Furthermore, ucOC also indirectly enhances insulin production by increasing GLP-1 production in intestines.

In liver cells, ucOC increases insulin sensitivity and mitochondrial capacity. Furthermore, it attenuates fat accumulation, ROS production, inflammatory reactions, and cell death that are induced by HFD.

In adipocytes, ucOC enhances cellular glucose uptake, insulin sensitivity, and mitochondrial capacity. Moreover, in response to HFD challenge, ucOC reduces fat gaining. In addition, ucOC also increases the production of adiponectin, which promotes the insulin sensitivity in peripheral tissues.

The role of ucOC in skeletal muscle is described in **Figure 2.4**.

ucOC: undercarboxylated osteocalcin; GLP-1: glucagon-like peptide-1. Black arrows represent secretion. Brown arrows represent regulation.

In the pancreas, ucOC was reported to directly increase the proliferation of β -cells [25]. Furthermore, it also enhanced *Insulin* gene expression and insulin secretion in these cells [25, 77]. These direct effects of ucOC may lead to increased levels of serum insulin. In addition, ucOC was also suggested to favour serum insulin levels by increasing the production of glucagon-like peptide-1 (GLP-1) from intestines, which resulted in enhanced secretion of insulin in the pancreas [78, 79].

In liver tissue, ucOC *per se* enhanced insulin sensitivity [72]. Furthermore, HFD-induced oxidative stress, inflammation, cell death, fat deposition, and the loss of mitochondrial capacity, were alleviated by ucOC treatment [72, 80, 81].

In adipocytes, the treatment of ucOC was found to directly enhance glucose uptake and insulin sensitivity [72, 82]. Moreover, ucOC administration attenuated HFD-induced fat mass increase and mitochondria loss in mice fat tissue [24, 72]. In addition, ucOC was also reported to directly trigger the production of adiponectin, an adipokine that is known to enhance the insulin sensitivity of peripheral tissues [25, 57].

2.2.3.2 Metabolic effects of ucOC on skeletal muscle

Skeletal muscle is responsible for around 80 % of whole-body glucose uptake in the postprandial state [9]. Furthermore, skeletal muscle is also a major site for nutrient storage and energy expenditure [36, 43]. Therefore, the possible regulation by ucOC in muscle energy metabolism has great importance for maintaining whole-body energy homeostasis. These suggested ucOC effects include the regulation of insulin sensitivity at rest (**Section 2.2.3.2.1.2**) or after acute exercise (**Section 2.2.3.2.1.3**), the instigation of non-insulin-stimulated glucose uptake (**Section 2.2.3.2.2.2**), as well as other effects. The effects are illustrated in **Figure 2.4** and listed in **Table 2.1**.

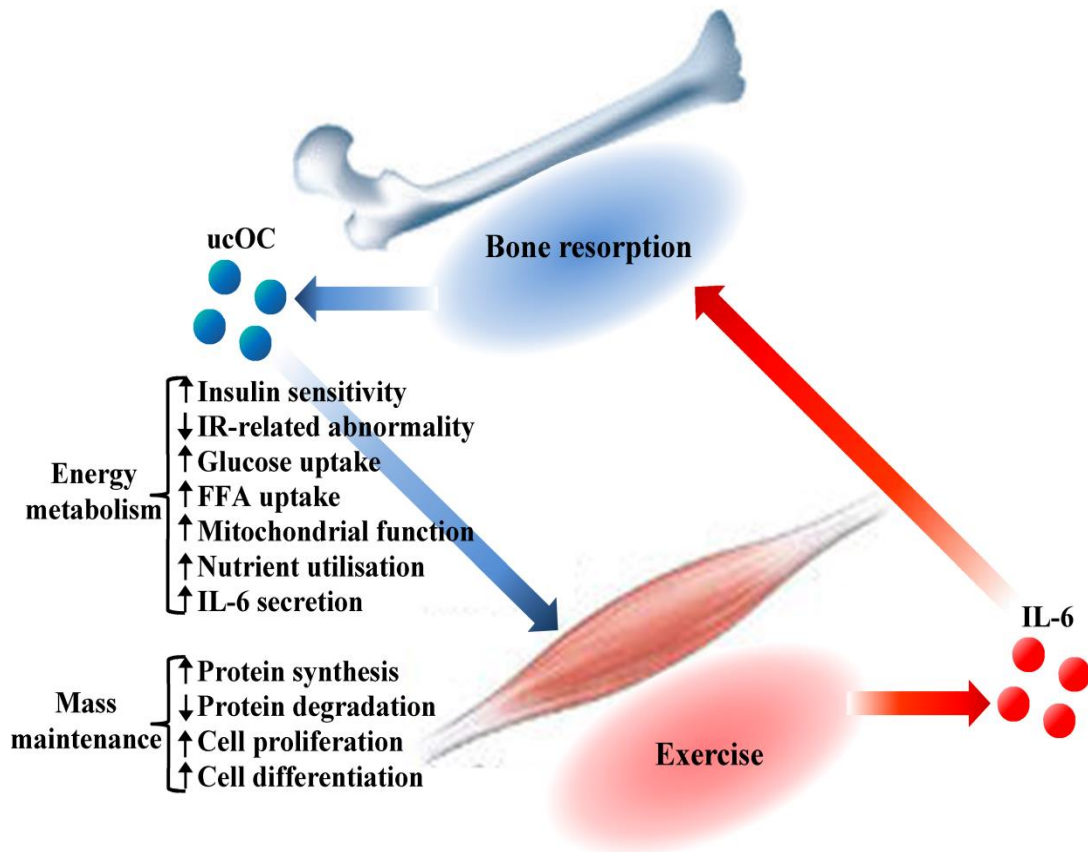


Figure 2.4. Suggested effects of ucOC on skeletal muscle. The actions of ucOC in skeletal muscle favour its energy metabolism and mass growth.

ucOC increases muscle insulin sensitivity both at rest and following exercise. Furthermore, it increases the uptake of glucose and FFA, promotes mitochondrial function, and enhances nutrient utilisation. During exercise, ucOC increases the secretion of IL-6, which in turn favours the production of ucOC in bone. In addition, ucOC attenuates HFD-induced insulin resistance and related abnormalities, including ER stress, fat accumulation, and autophagy.

As to the upregulation of muscle mass, ucOC increases protein synthesis, inhibits protein degradation, and promotes myotube formation.

ucOC: undercarboxylated osteocalcin; IR: insulin resistance; FFA: free fatty acid; IL-6: interleukin 6.

Table 2.1. Reported metabolic effects of ucOC on skeletal muscle.

		ucOC level/concentration change	ucOC change-induced alterations in muscle energy metabolism							
			Insulin sensitivity		IR-related abnormality	NIS glucose uptake	FFA transportation	Mitochondrial function	Nutrient utilisation	IL-6 secretion
			Insulin target gene expression	Insulin-stimulated glucose uptake						
<i>In vivo</i> models	WT mice	↑	↑ [25, 72, 83]				↑ [23]		↑ Glycogenolysis; ↑ TCA cycle; ↑ Lipolysis; ↑ FFA catabolism [23]	↑ [23]
	HFD-fed mice	↑	↑ [72, 83]		↓ ER stress; ↓ Lipid accumulation; ↓ Autophagy [72, 83]			↑ Mitochondria l capacity [24, 72, 83]		
	Oc ^{-/-} mice	↓	↓ [57]				↓ [23]		↓ Glycogenolysis [23]	
	<i>Esp</i> ^{-/-} mice; <i>Esp_{osb}</i> ^{-/-} mice; <i>Atf4_{osb}</i> ^{-/-} mice; <i>Foxo1_{osb}</i> ^{-/-} mice	↑	↑ [34, 57, 65]	↑ [57]				↑ Mitochondria l capacity; ↑ Oxidative phosphorylation [57, 65]		

<i>In vitro</i> models	<i>Ex vivo</i> muscles (oxidative muscle; glycolytic muscles)	↑				↑(Oxidative muscles) ↔ (Glycolytic muscles) [23]				
	Normal myotubes (C2C12; Primary)	↑		↑ [29]		↑ [23]	↑ [23]		↑ Glycolysis; ↑ FFA oxidation [23]	
	IR (tunicamycin; Palmitate;FFA) myotubes (C2C12 L6)	↑			↓ ER stress; ↓ Autophagy [72, 83]					

Metabolic effects of ucOC on skeletal muscle, that have been recently reported in both *in vivo* and *in vitro* studies, are listed in this table.

ucOC: undercarboxylated osteocalcin; IR: insulin resistance; NIS: non-insulin-stimulated; FFA: free fatty acid; IL-6: interleukin 6; WT; wide type; HFD: high fat diet; ER: endoplasmic reticulum; TCA: tricarboxylic acid.

2.2.3.2.1 Regulation of muscle insulin sensitivity by ucOC

2.2.3.2.1.1 Muscle insulin sensitivity - overview

In the postprandial state, in response to increased blood glucose levels, insulin is abundantly secreted from pancreatic β -cells into the general circulation, primarily to restore normal circulatory glucose levels [84, 85]. In skeletal muscle, insulin has several roles in addition to glucose uptake, including glycogen and protein synthesis, cell survival, cell growth, and cell differentiation [86-90].

Muscle insulin sensitivity is defined as the responsiveness of skeletal muscle cells to the stimulation of insulin. Impaired muscle insulin sensitivity (muscle insulin resistance), a hallmark of T2DM, contributing to chronic hyperglycaemia [91]. Therefore, understanding the regulation of muscle insulin sensitivity is important, not only for the contribution to the knowledge, but also for the design of new therapeutic strategies for muscle insulin resistance.

Physical activity, including exercise, is an evolutionarily conserved function that is essential for the survival of most vertebrates [92]. Exercise is known to benefit muscle health, including muscle glucose metabolism/insulin sensitivity [93, 94]. Even a single bout of exercise (acute exercise), which increases muscle glucose uptake during exercise, also improves insulin sensitivity for up to 48 hr following exercise [95-97].

Acute exercise enhances glucose uptake (during exercise) and insulin sensitivity (post-exercise) in muscles of not only healthy people, but also those with insulin resistance, as the mechanisms by which exercise favours glucose uptake/insulin sensitivity are largely independent of the insulin signalling pathway and are usually intact in patients with T2DM [97-99]. Therefore, a full understanding of these mechanisms may allow the design of new therapeutic strategies that “bypass” the impaired insulin pathway in insulin resistance/T2DM patients.

2.2.3.2.1.2 Effects of ucOC on insulin sensitivity in resting muscles

The effects of ucOC on muscle insulin sensitivity were first suggested by several *in*

in vivo studies in mice. For instance, evidence showed that the increase of serum ucOC levels due to gene modification (the deficiency of *Esp*, *Atf4* or *Foxo1* in osteoblasts), led to enhanced expression of insulin target genes in skeletal muscle [34, 65]. A study using an insulin clamp showed higher insulin-stimulated muscle glucose uptake in *Esp*^{-/-} mice compared to WT littermates. ucOC administration via osmotic pump infusion or daily injections elevated insulin target gene expression in mouse skeletal muscle [25, 72, 83]. In muscle from mice challenged with high fat diet (HFD), ucOC administration alleviated compromised gene expression and insulin resistance-induced abnormalities such as endoplasmic reticulum (ER) stress, fat accumulation, and autophagy [73, 83]. However, it should be noted that ucOC was also able to exert effects on cells of other distal organs and tissues such as the pancreas [25, 57], adipose tissue [25, 100], and intestines [75, 78]. These effects enhanced the secretion of insulin, adiponectin, and GLP-1, the latter two of which are also known to benefit insulin action in skeletal muscle [101, 102]. Thus, the insulin-sensitising effect of ucOC on muscle indicated by *in vivo* studies could be an indirect effect rather than a direct one.

In vitro studies are important for revealing direct beneficial effects of ucOC on insulin sensitivity, without confounding factors such as circulating hormones, cytokines, and microvascular blood flow. The evidence for direct effects of ucOC on muscle cells is very limited. In C2C12 myotubes, a long-term (72 hr) ucOC exposure at physiological concentration (10 ng/mL) led to a 2 to 3-fold increase in insulin-stimulated glucose uptake [29]. Whether ucOC regulates glucose uptake in insulin-stimulated muscles in a muscle type-specific manner is also unknown. Thus, the direct effect of ucOC on glucose uptake in insulin-stimulated oxidative and glycolytic muscles is investigated in **Chapter 3, 4, and 5** of this thesis.

2.2.3.2.1.3 *Effects of ucOC on muscle insulin sensitivity following acute exercise*

Current studies suggested that the insulin-sensitising effect post-exercise may be

attributed to the alteration of humoral factors, as the effect of *ex vivo* muscle contraction (without humoral factor alterations) on subsequent *ex vivo* insulin-stimulated glucose uptake was lower than the effect of *in vivo* exercise [97, 103]. Recent evidence implied that ucOC may be one of these factors [30, 31].

Bone is a dynamic tissue that responds to mechanical loading, and unloading, by modifying its mass and strength [104]. As such, it was hypothesised that exercise, which stresses the bone via increased mechanical loading, would have an effect on markers of bone formation, such as OC and ucOC [49, 105]. The evidence for the effect of exercise on OC (total) is currently contradictory as some researchers reported that acute exercise increases OC levels [106, 107], while other reported lower [108], or unchanged [109, 110] OC levels shortly after exercise. This existence of these contradictory data may be due to different exercise modes or intensities, or different time points of blood sampling during/following exercise [105, 111]. However, acute exercise seems to favour circulating ucOC levels. In human, ucOC levels were reported to be increased by 6 – 14 % promptly after a single session of high intensity aerobic exercise [30, 31, 112, 113]. In mice, a much higher increase, up to 2.5-fold, was observed during and shortly after a single bout of aerobic treadmill running. [23].

The enhancement of ucOC levels after exercise may be clinically important, as exercise improves insulin sensitivity and glycaemic control, indicating that the ucOC increase may contribute to the insulin-sensitising effect following exercise. Indeed it was reported that in obese men a single session of high-intensity exercise (95.1 ± 1.9 % of HR_{peak}) increased ucOC, which was correlated with enhanced insulin sensitivity following exercise [30, 31]. Furthermore, ucOC levels were also associated with higher insulin signalling activity in the vastus lateralis muscle following exercise [30, 31].

However, the above studies were performed *in vivo*, with confounding factors, such as blood flow and other hormones, which may affect the results. In addition, the correlation of ucOC with higher insulin sensitivity does not provide evidence for a causative relationship. As such, in this thesis (**Chapter 3**), the direct

insulin-sensitising effect of ucOC on particular muscles post-*ex vivo* muscle contraction is examined.

2.2.3.2.2 Effects of ucOC on muscle glucose uptake independent of insulin

2.2.3.2.2.1 Non-insulin-stimulated muscle glucose uptake - overview

Skeletal muscle is known to be able to take up glucose from the general circulation without the requirement for insulin [8]. There are several stimuli that can trigger insulin-independent muscle glucose uptake, including acute exercise/muscle contraction, passive muscle stretching, reactive oxygen species (ROS) generation, and hypoxic stress [17, 47, 114, 115]. Furthermore, it is reported that administration of some recombinant proteins such as insulin-like growth factor 1 (IGF-1) and adiponectin, as well as chemical compounds such as insulin mimetics, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), and phorbol 12-myristate 13-acetate (PMA), are able to stimulate muscle glucose uptake in the absence of insulin [23, 116-118].

Identifying new agents that trigger muscle glucose uptake independent of insulin and understanding the mechanisms underlying non-insulin-dependent muscle glucose uptake, are important for the development of potential new therapies aimed at countering insulin-resistant conditions.

2.2.3.2.2.2 ucOC-triggered non-insulin-stimulated muscle glucose uptake

To date there is limited evidence for a direct effect of ucOC on muscle glucose uptake independent of insulin. To date, only one study has reported that ucOC is capable of triggering non-insulin-stimulated muscle glucose uptake. Mera et al. [23] reported that ucOC treatment enhanced glucose uptake in cultured muscle myotubes and glucose uptake in isolated oxidative muscles such as soleus and red quadriceps by 20 – 50 % in the absence of insulin. Furthermore, it was also suggested that acute

exercise-stimulated muscle glucose uptake independent of insulin was due to the exercise-induced increase of ucOC [23]. However, more investigations are warranted to verify this ucOC effect, and to understand the underlying mechanism. Therefore, the direct effect of ucOC on non-insulin-stimulated glucose uptake in EDL and Soleus is examined in **Chapter 4**.

As shown in **Table 2.1** and **Figure 2.4**, current literature suggests that ucOC favours muscle energy metabolism also via other means in addition to glucose uptake, such as increasing mitochondrial capacity and activity [23, 24, 65, 72, 83], upregulating nutrient utilisation and energy production during exercise [23], as well as increasing the production and secretion of interleukin 6 (IL-6) which is a myokine responsible for exercise-induced ucOC enhancement in bone [23].

2.2.4 Functions of ucOC in muscle mass regulation

2.2.4.1 Muscle mass regulation and muscle atrophy - overview

Adequate muscle mass and strength are important for healthy muscle function, general health, and quality of life [119]. In healthy individuals, skeletal muscle mass is maintained via a coordinated equilibrium between muscle protein synthesis (muscle anabolism) and muscle protein degradation (muscle catabolism) [7, 120, 121]. With some stimuli, such as resistance training [122], there is a shift of this delicate balance towards net protein synthesis, resulting in muscle hypertrophy. Conversely, in conditions of muscle disuse, androgen deprivation treatment (ADT), aging, and some diseases [120, 123, 124], there is a shift favouring net protein loss leading to muscle wasting, which is also known as muscle atrophy [120].

Muscle disuse-induced atrophy is common with bed rest and immobilisation during the recovery period after injuries, primarily due to mechanical unloading of muscles [125]. As a common model of disuse atrophy, hindlimb immobilisation is

widely used in animals to investigate the impact of disuse on skeletal muscle and bone [126, 127]. It has been suggested that the loss of muscle mass and strength caused by hindlimb immobilisation is an integrated consequence of various factors caused by low mechanical loading [125]. However, the identity and action of the factors responsible for the muscle loss are still not fully understood.

Treatment with ADT via orchidectomy or luteinising hormone releasing hormone (LHRH) agonist administration targeting prostate cancer is a standard clinical practice worldwide [128]. Since androgens, such as testosterone, favour muscle mass by shifting the protein balance towards net protein accretion [129], patients receiving ADT often suffer considerable loss of muscle mass [128]. Although it has been suggested that the ADT-induced muscle wasting is due to the loss of direct effects of androgens on muscle cells [129], the perturbation of circulatory factors may also play a role [130-132]. Nevertheless, most of these factors are currently unknown.

Therefore, a thorough understanding of the mechanisms underlying muscle atrophy, caused by muscle disuse and androgen deprivation, is necessary for designing better treatment for patients following injuries and receiving ADT. Of note, it has been demonstrated that both hindlimb immobilisation and androgen depletion exert detrimental effects on bone formation [126, 128]. Thus, it is possible that they cause the loss of muscle mass and strength by affecting the circulatory levels of bone-secreted factors, which may include ucOC. Indeed, currently several mechanistic and correlation studies (shown in **Table 2.2**) have implied that ucOC possibly contributes to muscle mass and strength in both humans and mice.

Table 2.2. Studies investigating effects of ucOC on muscle mass and strength.

Study	Study type	Animals/Cell line/Subjects	ucOC change/administration	Observations
[32]	Mechanistic	C57BL/6 mice	HFD-induced ucOC decrease	Increased ubiquitination-mediated protein degradation in mice fed with HFD
[28]	Mechanistic	<i>Cx43^{osb/osc}</i> ^{-/-} mice; C2C12 myotubes	GM-induced ucOC decrease ; ucOC administration with injection; <i>In vitro</i> ucOC treatment	Decreased muscle volume , mass , and strength in <i>Cx43^{osb/osc}</i> ^{-/-} mice; Normalised muscle volume , mass , and strength in <i>Cx43^{osb/osc}</i> ^{-/-} mice by ucOC injection; Increased myotube formation in C2C12 myotubes treated with ucOC
[27]	Mechanistic	<i>Oc</i> ^{-/-} mice; Primary mice myotubes	GM-induced ucOC decrease ; ucOC administration with osmotic pumps; <i>In vitro</i> ucOC treatment ;	Decreased muscle volume and mass in older <i>Oc</i> ^{-/-} mice; Improved muscle volume and mass in older <i>Oc</i> ^{-/-} mice; Increased protein synthesis rate by ucOC treatment in myotubes
[26]	Mechanistic	C2C12 myoblasts	<i>In vitro</i> ucOC treatment	Increased proliferation and differentiation by ucOC treatment in C2C12 myoblasts
[133]	Correlation	2317 elder women (age ≥ 70)	/	Positive correlation between ucOC % and quadriceps muscle strength
[134]	Correlation	194 postmenopausal women	/	Positive correlation between ucOC and muscle mass
[135]	Correlation	62 patients with hypoparathyroidism	PTH treatment-induced ucOC increase	Positive correlation between change in ucOC % and change in elbow extension force
[136]	Correlation	39 obese patients with CKD	/	Negative correlation between ucOC and muscle mass

Effects of ucOC on muscle mass and strength, that have been implied by both mechanistic and correlation studies, are listed in this table.

Cx43: connexin 43; OSB: osteoblast; OSC: osteocyte; OC: osteocalcin; ucOC: undercarboxylated osteocalcin; HFD: high fat diet; PTH: parathyroid hormone; GM: gene modification; CKD: chronic kidney disease.

2.2.4.2 ucOC function in muscle mass regulation - evidence from correlation studies

There have been a number of studies that have linked ucOC with the regulation of muscle mass and strength. In a cross-sectional study involving 2317 elder women aged ≥ 70 , a positive correlation was found between undercarboxylation percentage of osteocalcin (ucOC %) and quadricep muscle strength [133]. Similarly, in another cross-sectional study with 194 postmenopausal women, ucOC level was positively correlated with muscle mass [134]. In an interventional study in 62 patients with hypoparathyroidism, parathyroid hormone (PTH) treatment-induced ucOC % increase was positively associated with the change of maximum force generated in elbow extension [135]. However, a recent study also showed a negative correlation between ucOC and muscle mass in 39 obese patients with chronic kidney disease (CKD) [136]. Since CKD patients are known to have impaired levels of serum Vitamin K and thus abnormally high levels of serum ucOC [137], it is possible that the negative correlation just only exists in this specific pathological condition and further investigations are required.

2.2.4.3 ucOC function in muscle mass regulation - evidence from mechanistic studies

Consistently, results from mechanistic studies indicate that ucOC may favour increased muscle mass and force in mice. In mice with osteoblast/osteocyte-specific deletion of a gap junction protein connexin 43 (Cx43), reduced ucOC levels were accompanied with decreased muscle mass, volume, and strength, and this loss of muscle function was reversed by ucOC treatment [28]. Mera et al. [27] showed that osteocalcin deficiency resulted in lower muscle mass and volume in old mice compared to their WT counterparts in a similar age. Moreover, muscle volume in old mice with long-term ucOC treatment was greater than that in old mice treated with vehicle.

Studies also suggested that ucOC promotes muscle growth by increasing protein synthesis, cell proliferation, and myogenic differentiation, as well as inhibiting protein degradation in muscle cells. Mera et al. [27] found that in primary myotubes, a 2 hr treatment with ucOC at physiological levels increased the protein synthesis rate by up

to 50 %. In another recent study using C2C12 myoblasts, researchers reported positive effects of ucOC on cell proliferation and differentiation, which are critical for myotube formation during muscle growth [26]. In mice challenged with HFD, ucOC decreases were aligned with protein degradation regulated by ubiquitination in muscle, indicating a possible role of ucOC in countering muscle protein degradation [32].

Collectively for **Section 2.2.4**, although limited evidence exists, current studies tend to characterise ucOC as an important regulator for muscle growth via modulating protein synthesis, protein degradation, and myotube formation. Therefore, the reduction in ucOC regulation is likely one of the factors responsible for muscle atrophy caused by hindlimb immobilisation and testosterone depletion. This hypothesis is addressed in **Chapter 6** of this PhD thesis.

2.2.5 Signalling pathways underlying ucOC effects on glucose uptake and muscle mass in skeletal muscle

Understanding the mechanisms by which ucOC regulates glucose uptake and muscle mass is important, not only for the enrichment of understanding of muscle physiology and pathology, but also for the identification of new drug targets for conditions such as insulin resistance and T2DM and muscle atrophy. Current evidence suggested that the molecular mechanism behind the actions of ucOC involve GPRC6A as the receptor [23], and several major downstream signalling proteins such as Akt [29, 34], ERK [26, 29], AMPK [23], and PKCs [33]. However, the exact mechanisms underlying ucOC effects on muscle are still poorly known, therefore it is a major focus of the current thesis.

2.2.5.1 G protein-coupled receptor, class C, group 6, member A (GPRC6A)

GPRC6A is currently the only putative receptor of ucOC [138-140]. It belongs to the C family of G protein-coupled receptors and is widely expressed in cells of a number of human tissues such as brain, lung, liver, heart, kidney, pancreas, skeletal muscle, placenta, spleen, ovary, testis, prostate, leukocyte and monocyte [139]. It was speculated that GPRC6A is the ucOC receptor in skeletal muscle, as its deficiency blunted ucOC effects and the activation of downstream signalling proteins [23, 27,

83].

The role of GPRC6A in modulating ucOC effects on muscle energy metabolism has been reported in a couple of very recent investigations. Mera et al. [23] showed that either global or muscle-specific deletion of GPRC6A blocked the effects of ucOC on the transportation and utilisation of nutrients, such as glycogen, glucose, triglyceride, and FA, in mouse muscle during exercise. In another study using insulin-resistant C2C12 myotubes, the knockdown of GPRC6A blunted the effect of ucOC in antagonising insulin resistance-related ER stress and autophagy [83].

GPRC6A was also suggested to mediate the effects of ucOC on muscle mass. In another study carried out by Mera et al. [27], both global and muscle-specific deletion of GPRC6A led to a lower muscle weight in old mice, similar to what was reported in osteocalcin knockout mice. Furthermore, the enhancing effect of long-term ucOC administration on muscle volume in WT old mice was absent in old mice with GPRC6A ablation. In addition, ucOC-induced protein synthesis in cultured myotubes was inhibited by GPRC6A deficiency.

The activation of postulated ucOC downstream targets seemed dependent on the presence of GPRC6A [23]. In muscle during exercise, the deficiency of GPRC6A blocked ucOC-induced phosphorylation of Akt, AMPK, cAMP response element-binding protein (CREB), acetyl-coenzyme A carboxylase (ACC), and hormone sensitive lipase (HSL) [23]. In C2C12 myoblasts, ucOC-stimulated activation of Akt, p38, and ERK was downregulated by *Gprc6a* gene silencing [26].

Taken together, although there are no studies showing direct binding between ucOC and GPRC6A in muscle cells, current evidence indicates that GPRC6A is at least a crucial upstream regulator, if not the direct receptor, in the ucOC-triggered signalling cascade in muscle cells. In this thesis, we illustrate the presence of GPRC6A on the plasma membrane of muscle cells (**Chapter 3**), as well as explore the association of GPRC6A with muscle mass, muscle strength, and the activity of related signalling proteins in both glycolytic and oxidative muscles (**Chapter 6**).

2.2.5.2 Protein kinase B (Akt)

2.2.5.2.1 Akt overview

Protein kinase B, also known as Akt, is a serine/threonine-specific protein kinase that plays an important role in multiple cellular processes such as glucose metabolism, cell proliferation, gene transcription, and cell migration [141]. It has been demonstrated that Akt is a crucial regulator of muscle glucose uptake [142, 143]. In response to insulin stimulation, Akt is phosphorylated at Thr308 via the activation of phosphoinositide 3-kinase (PI3K) cascade, followed by the Ser473 phosphorylation (full activation of Akt) with the facilitation of mammalian target of rapamycin complex 2 (mTORC2) [144-146]. The activation of Akt results in the Thr642 and Ser588 phosphorylation of AS160, leading to the translocation of glucose transporter 4 (Glut4) from intracellular vesicles to the plasma membrane and finally resulting in enhanced glucose uptake [147-149]. Several stimuli may trigger muscle glucose uptake via activating Akt in the absence of insulin, including IGF-1, epidermal growth factor (EGF), and hepatocyte growth factor (HGF) [116, 150, 151].

Akt is also an important modulator for skeletal muscle growth. The phosphorylation of Akt induced by insulin/IGF-1 leads to the activation of mTORC1, leading to the increase in protein synthesis via the phosphorylation of ribosomal protein S6 kinase (P70S6K) [129, 152], as well as the reduction in cellular autophagy via the phosphorylation of Unc-51 like autophagy activating kinase 1 (ULK1) [153]. Moreover, the activation of Akt leads to the deactivation of FOXOs, including FOXO1, FOXO3a, and FOXO4, via phosphorylation at multiple residues [129, 154]. Subsequently, the activity loss of FOXOs reduces the expression of muscle RING-finger protein-1 (MuRF1) and muscle atrophy F-box (MAFbx/Fbx32), which are muscle-specific E3 ligases, resulting in attenuated protein degradation [129, 154].

2.2.5.2.2 The role of Akt in ucOC actions in skeletal muscle

Akt may also be activated by ucOC in skeletal muscle, as ucOC-induced increases in Akt phosphorylation was discovered in muscle cells, with or without the presence of insulin [23, 26, 29, 34]. Furthermore, some downstream cascades/proteins of Akt, such as mTORC1/P70S6K, nuclear factor κ B (NF- κ B), and p38, were recently reported to be activated by ucOC (shown in **Figure 2.5**). The Akt-mediated signalling

pathway induced by ucOC was suggested to contribute to ucOC effects on muscle [29, 34, 72, 81, 83].

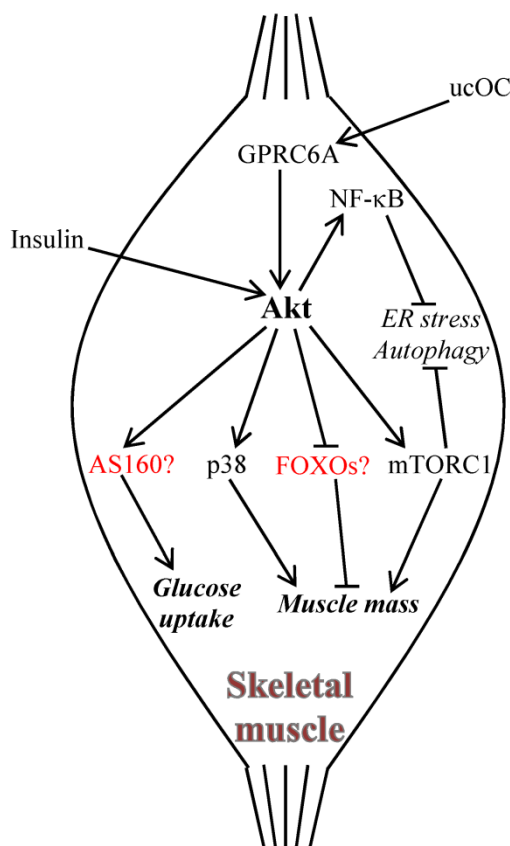


Figure 2.5. Possible Akt-mediated signalling pathways triggered by the ucOC/GPRC6A cascade in skeletal muscle. The increase of Akt phosphorylation via the ucOC/GPRC6A cascade (with or without insulin stimulation), enhances muscle glucose uptake possibly via the enhancement of AS160 phosphorylation. Furthermore, the increase of Akt activity favours muscle mass growth by increasing the activity of p38 and mTORC1, and possibly by inhibiting the activity of FOXOs. In addition, the increase in the activity of NF-κB and mTORC1, via the ucOC/GPRC6A/Akt cascade, antagonises ER stress and autophagy induced by HFD.

ucOC: undercarboxylated osteocalcin; GPRC6A: G protein-coupled receptor, class C, group 6, member A; Akt: protein kinase B; NF-κB: nuclear factor κB; ER: endoplasmic reticulum; AS160: Akt substrate of 160 kD; FOXO: the class O of forkhead box transcription factors; mTORC1: mammalian target of rapamycin complex 1. Red colour with question mark represents unexplored proteins in current studies.

—————> : activation or promotion;

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Italicised text: process or event.

The ucOC-induced increase in insulin-stimulated Akt activation was suggested to be linked to enhanced muscle insulin sensitivity. It was reported that insulin-stimulated phosphorylation of Akt (Ser473) was enhanced via *Atf4* ablation-induced increases in serum ucOC in mouse muscle, or via long-term (72 hr)

ucOC treatment in C2C12 myotubes [29, 34]. In both mouse muscle and cultured myotubes with insulin resistance, ucOC administration restored impaired insulin action on insulin receptor substrate 1 the (IRS-1)/PI3K/Akt cascade [72, 81, 83]. The effect of ucOC on insulin-stimulated Akt phosphorylation is likely to be due to its effect on basal Akt phosphorylation independent of insulin. *In vivo*, Akt phosphorylation at Ser473 was elevated by *Atf4* deficiency-induced ucOC increases in mouse muscle [34]. In C2C12 cells and primary mouse myotubes, ucOC treatment alone seemed to enhance Akt phosphorylation [23, 26, 29]. In contrast, it was reported that in mice or cultured myotubes with normal insulin sensitivity, insulin-stimulated Akt phosphorylation was unchanged in response to ucOC administration [72, 81, 83]. However, in these studies high doses of insulin were used, thus it is possible that, even without ucOC treatment, the Akt phosphorylation was already saturated by insulin stimulation.

There is evidence showing that the activation of the mTORC1/p70S6K cascade accounted for increases in protein synthesis induced by ucOC treatment in primary mouse myotubes, likely via the PI3K/Akt pathway [27]. The activation of mTORC1 by ucOC may result in other responses. In the muscles of insulin-resistant mouse, the activation of mTORC1 by Akt, along with the activation of another Akt downstream protein NF- κ B, were suggested to be responsible for ucOC-mediated antagonism of ER stress and autophagy [72, 83]. The p38 mitogen-activated protein kinase (MAPK) signalling pathway participates in a variety of physiological processes in several types of tissues including skeletal muscle [155, 156]. It has been suggested that in C2C12 myoblasts, ucOC induces the activation of p38 via the phosphorylation of Akt [26]. Thus p38 activation may participate in muscle cell proliferation [26].

Overall, Akt-mediated pathways may be involved in muscle glucose uptake, protein synthesis, cell proliferation, and alleviation of HFD-induced ER stress and autophagy in skeletal muscle cells. However, whether ucOC induces the increase in Akt phosphorylation still remains controversial. Furthermore, while the activation of Akt has been linked with ucOC-induced enhancement of muscle glucose uptake, the activation of AS160, which plays a central role in Akt-mediated Glut4 translocation, has not been assessed in ucOC-treated muscles. Similarly, the effect of ucOC on another important Akt downstream target, FOXOs, which regulate muscle protein degradation, has not been assessed but may be important for the regulation of muscle

mass. As such, in this thesis, the direct effect of ucOC on the phosphorylation of Akt and AS160 in *ex vivo* EDL and Soleus are assessed (**Chapter 3, 4, and 5**). Furthermore, the association of ucOC levels and GPRC6A expression with the phosphorylation of Akt, mTORC1/P70S6K, and FOXOs, under atrophic conditions, is analysed in **Chapter 6** in both muscle types.

2.2.5.3 Extracellular signal-regulated kinases (ERK)

ERK is known as one of the three main MAPKs, which regulate a number of cellular functions in skeletal muscle [157]. The activation of ERK has been linked with non-insulin-stimulated muscle glucose uptake triggered by AICAR, palmitic acid, and HGF [151, 158-160]. Furthermore, it has also been suggested that ERK contributes to muscle growth by activating mTORC1 in response to IGF-1 and androgens [129, 152].

The activation of ERK by ucOC treatment in skeletal muscle was recently reported in C2C12 myotubes [26, 29]. Thus, it is possible that this ERK activation

contributes to the ucOC effect on muscle cells. This hypothesis is illustrated in **Figure 2.6**.

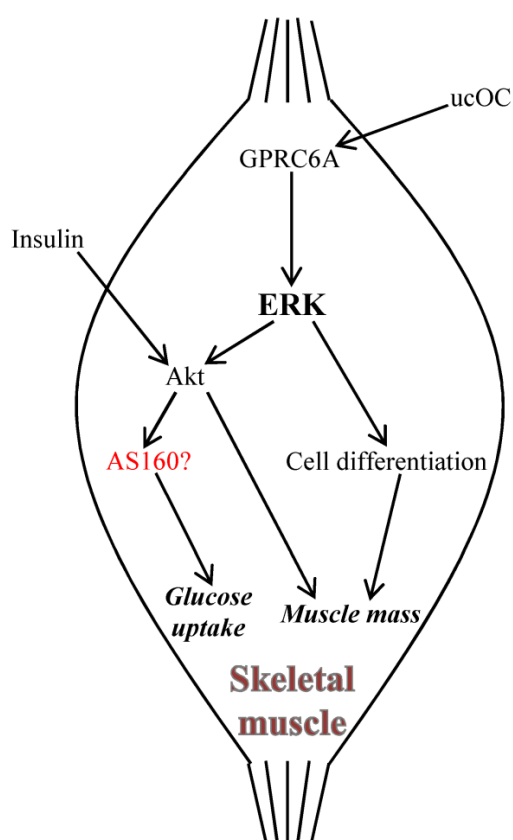


Figure 2.6. Possible ERK-mediated signalling pathways triggered by the ucOC/GPRC6A cascade in skeletal muscle. The activation of ERK via the ucOC/GPRC6A cascade, enhances muscle glucose uptake and muscle mass by increasing the activity of Akt signalling pathways. Furthermore, the ERK activation also favours muscle mass growth by promoting muscle cell differentiation.

ucOC: undercarboxylated osteocalcin; GPRC6A: G protein-coupled receptor, class C, group 6, member A; ERK: extracellular regulated kinase; Akt: protein kinase B; AS160: Akt substrate of 160 kD. Red colour with question mark represents unexplored proteins in current.

→: activation or promotion;
Italicised text: process or event.

In insulin-stimulated C2C12 myotubes, it has been shown that the inhibition of ERK via ERK inhibitor U0126 attenuates the effect of ucOC on Akt phosphorylation,

indicating a role of ERK in ucOC-induced enhancement of Akt activation and glucose uptake in insulin-stimulated muscle cells [29]. Furthermore, the activation of ERK in C2C12 myoblasts is suggested to play a major role in ucOC-induced myogenic differentiation of muscle precursor cells [26]. In contrast, it was also shown that the addition of U0126 did not influence the rescue effect of ucOC on insulin-stimulated activation of the IRS-1/Akt/mTORC1 axis in insulin-resistant C2C12 and L6 myotubes, suggesting ERK may not be involved in ucOC action in insulin-resistant muscle cells [72, 83].

Taken together, the limited evidence may suggest that ERK is activated by ucOC in skeletal muscle. Such ERK activation may enhance glucose uptake and muscle growth, at least in part, by activating Akt. In this thesis, the link between ERK and muscle glucose uptake in the absence and presence of insulin in both muscle types is explored (**Chapter 4** and **5**). Furthermore, the association between ERK and muscle mass and strength is also investigated (**Chapter 6**).

2.2.5.4 5' adenosine monophosphate-activated protein kinase (AMPK)

2.2.5.4.1 AMPK overview

AMPK has been characterised as a master regulator of energy metabolism in many types of tissues [161]. It is a heterotrimeric protein complex which includes α , β , and γ subunits [161]. Two isoforms have been discovered for each of the α and β subunits ($\alpha 1$ and $\alpha 2$, and $\beta 1$ and $\beta 2$), whereas three isoforms for the γ subunit are currently known ($\gamma 1$, $\gamma 2$ and $\gamma 3$) [162]. The α subunit possesses catalytic activity of AMPK, which is indicated by the phosphorylation at Thr172 [163].

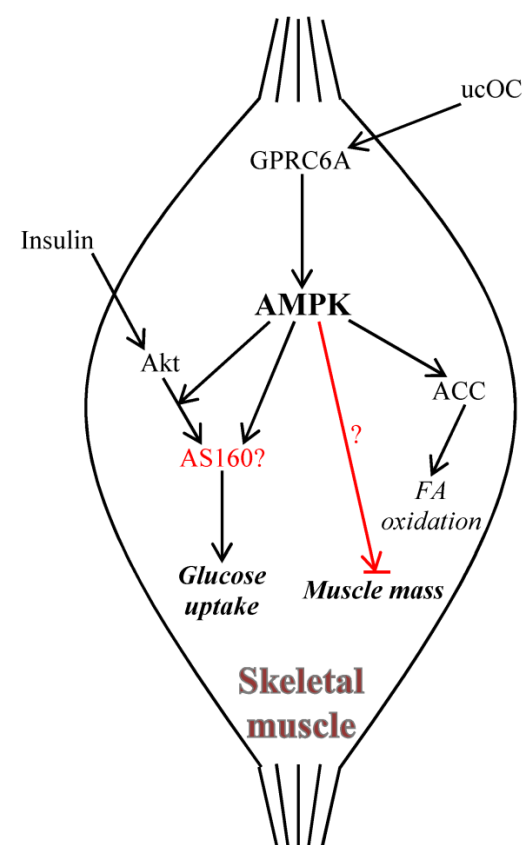
In skeletal muscle, the activation of AMPK is closely linked with muscle glucose uptake. It was recently reported that the activation of AMPK $\alpha 2\beta 2\gamma 3$ was responsible for the enhancement of insulin-stimulated AS160 phosphorylation and glucose uptake several hr following *in situ* contraction or AICAR stimulation [12, 164, 165]. Furthermore, the activation of AMPK was also linked with non-insulin-stimulated glucose uptake in skeletal muscle induced by exercise/contraction, adiponectin treatment, and hypoxia, in an AS160-mediated and Akt-independent manner [17, 118, 166-168].

The relationship between AMPK and muscle mass still remains controversial. The activation of AMPK has been traditionally reported as a negative regulator for muscle growth by inhibiting mTORC1, as well as activating FOXOs and ULK1 [120, 125, 169, 170]. However, recent reports showed that reduced AMPK phosphorylation was accompanied by atrophic conditions caused by hindlimb immobilisation [171, 172] and tail suspension [173-177]. This decrease of AMPK activity may be responsible for the loss of muscle mass via the histone deacetylase 4 (HDAC4)/FOXO cascade [171, 172, 174, 178, 179]. Moreover, in some atrophic conditions low AMPK activity has been linked with insulin resistance and impaired energy metabolism [173, 176, 177], which could lead to muscle wasting due to the loss of IGF-1 action and insufficient energy production for protein synthesis [161, 173, 180].

2.2.5.4.2 The role of AMPK in ucOC effects on skeletal muscle

The link between ucOC and AMPK is not well understood. Some reports suggested that AMPK can be activated by ucOC in skeletal muscle, which may contribute to

ucOC effects on glucose uptake and muscle mass, the proposed signalling is illustrated in **Figure 2.7**.



—> : activation or promotion;
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Figure 2.7. Possible AMPK-mediated signalling pathways triggered by the ucOC/GPRC6A cascade in skeletal muscle. The activation of AMPK via the ucOC/GPRC6A cascade increases insulin-stimulated muscle glucose uptake, as well as directly triggers muscle glucose uptake, probably via the enhancement of AS160 phosphorylation. Furthermore, the activation of AMPK induces FA oxidation via the activation of ACC. However, how the regulation of AMPK by ucOC influences muscle mass currently remains unclear.

ucOC: undercarboxylated osteocalcin; GPRC6A: G protein-coupled receptor, class C, group 6, member A; AMPK: 5' adenosine monophosphate-activated protein kinase; Akt: protein kinase B; AS160: Akt substrate of 160 kD; FA: fatty acid. Red colour with question mark represents unexplored proteins/links in current studies.

Mera et al. showed that the injection of ucOC prior to exercise enhanced exercise-induced AMPK phosphorylation (Thr172), as well as the phosphorylation of its downstream target ACC [23]. This enhancement of activation of the AMPK/ACC cascade may lead to increased FA utilisation to fulfil muscle energy requirements during exercise, since the deficiency of catalytic unit AMPK α 2 inhibited ucOC-induced enhancement of FA oxidation in cultured myotubes [23]. In contrast, it was reported that the loss of AMPK α 2 did not affect ucOC-induced glucose uptake in cultured myotubes, indicating the AMPK activation was not responsible for muscle glucose uptake triggered by ucOC [23]. In addition, another study reported that ucOC treatment failed to increase the phosphorylation of AMPK in C2C12 myotubes, indicating that AMPK may not be involved in ucOC effects in muscle cells [29].

Taken together, current findings regarding ucOC-mediated AMPK activation in skeletal muscle appear contradictory. In addition, limited evidence also suggests that this AMPK activation may only contribute to FA oxidation but not muscle glucose uptake. In the current thesis, further evidence of the ucOC effect on AMPK phosphorylation, in both EDL and Soleus, is provided, and the association between AMPK and muscle glucose uptake is investigated (**Chapter 4** and **5**). Furthermore, since AMPK was reported to either favour or suppress muscle mass growth, the role of ucOC-regulated AMPK activity in muscle mass and strength in different muscle types is explored (**Chapter 6**).

2.2.5.5 Protein kinase C (PKC)

PKCs are a big kinase family involving multiple isoforms. They are majorly classified as conventional/novel PKC (c/nPKC) and atypical PKC (aPKC), based on the dependency of diacylglycerol (DAG) for enzyme activation [181]. In skeletal muscle, the activation of aPKC, via PI3K, contributes to the insulin-stimulated Glut4 translocation without the involvement of AS160 [182-184]. Furthermore, the activation of either c/nPKC or aPKC isoforms has been reported to be responsible for non-insulin stimulated glucose uptake induced by exercise/contraction (c/nPKC), PMA (c/nPKC), or metformin (aPKC) [185-187].

Therefore, it is possible that ucOC influences muscle glucose uptake by activating

certain isoforms of PKC (shown in **Figure 2.8**). However, currently the only evidence linking ucOC with PKC was reported in rat pancreatic β -cells, where ucOC favours insulin secretion in the Phospholipase C (PLC)/PKC/Ras/MAPK kinase (MEK)/Kv channel signalling pathway [33]. In this thesis, the effect of ucOC on PKC δ / θ activation, for the first time, is examined in EDL and Soleus (**Chapter 4**)

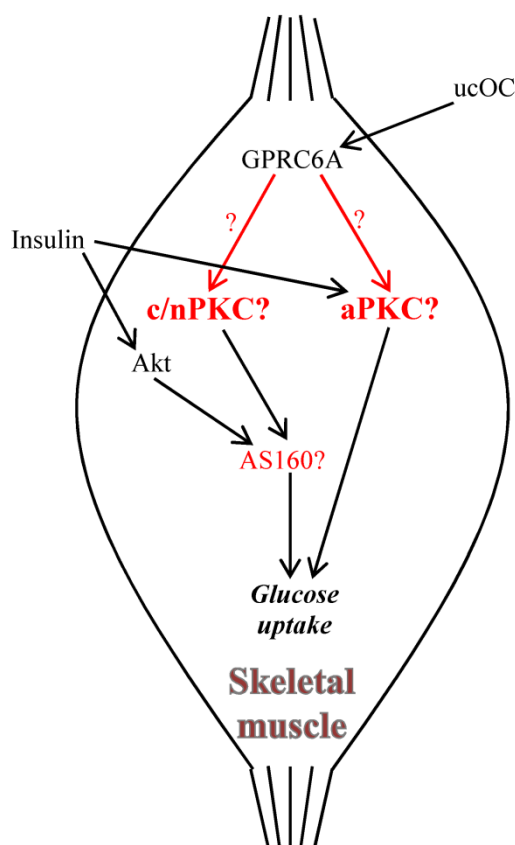


Figure 2.8. Possible PKC-mediated signalling pathways triggered by the ucOC/GPRC6A cascade in skeletal muscle. The ucOC/GPRC6A cascade probably increases muscle glucose uptake by activating the c/nPKC/AS160 cascade, or by activating aPKC, independent of insulin actions.

ucOC: undercarboxylated osteocalcin; GPRC6A: G protein-coupled receptor, class C, group 6, member A; c/nPKC: classical/novel protein kinase C; aPKC: atypical protein kinase C; Akt: protein kinase B; AS160: Akt substrate of 160 kD. Red colour with question mark represents unexplored proteins/links in current studies.

—————> : activation or promotion;

Italicised text: process or event.

2.3 Muscle type differences in muscle glucose uptake and muscle atrophy - the role of ucOC modulation

2.3.1 Muscle type differences in muscle glucose uptake

As described in **Section 2.1.2**, to accomplish different tasks with high energy efficiencies, skeletal muscle is metabolically regulated in a muscle type-specific manner. Indeed, evidence for this exists, at least in insulin-stimulated glucose uptake at rest and following exercise [12-14]. Moreover, the muscle type specificity in glucose uptake may be linked with muscle type-selective regulation of ucOC.

2.3.1.1 Muscle type difference in insulin-stimulated glucose uptake in resting muscles

At rest, insulin-stimulated muscle glucose uptake in oxidative muscles, such as Soleus and red gastrocnemius, is higher than in glycolytic muscles, such as EDL and epitrochlearis [14]. This difference between muscle types can be explained, at least in part, by the observation that insulin-resistant individuals tend to have a type I-to-type II shift in their muscle type distribution [14, 188]. Therefore, the delineation of this muscle type discrepancy in insulin-stimulated glucose uptake has considerable significance for pathological and therapeutic research.

Recent studies have proposed several explanations for the muscle type-selective effect of insulin action. For example, it was suggested that the difference of insulin-stimulated glucose uptake may be ascribed to the variance of Glut4 expression among different fibre types [189, 190]. Furthermore, it was shown that type I fibres have quicker and greater activation of InsR/IRS/PI3K cascade in response to insulin stimulation than the type II counterpart [191]. However, the exact mechanisms are still not well understood.

The comparison of muscle glucose uptake results, between *in vivo* studies using euglycaemic clamps and *ex vivo* studies using *in vitro* insulin stimulation, may help clarify the mechanisms involved. In general, *in vivo* studies report higher levels of insulin-stimulated glucose uptake than *in vitro* ones (**Table 2.3**). This inconsistency between *in vivo* and *ex vivo* studies was previously interpreted as the variance of microvascular blood flow between muscle types, as oxidative muscles have higher

capillary density than glycolytic counterparts [192]. Of note, in studies using *in situ* perfusion with Krebs-Henseleit buffer (KHB), the muscle type differences of insulin-stimulated glucose uptake were similar to those reported in *ex vivo* studies (Table 2.3). This similarity between two study types led to the hypothesis that muscle type-specific humoral factor regulations, rather than blood flow variance, may play an important role in this muscle type difference in insulin-stimulated muscle glucose uptake. In Chapter 4 and 5, whether ucOC in fact belongs to one of these humoral factors is explored.

Table 2.3. Differences in insulin-stimulated glucose uptake between oxidative and glycolytic muscles in *in vivo*, *in situ*, and *ex vivo* models.

Studies	Species	Insulin stimulation models	Oxidative muscle ISGU	Glycolytic muscle ISGU	% Difference
[193]	Rat	<i>In vivo</i> clamp (4.1 or 33 mg/kg/min)	SOL: 18 or 28 $\mu\text{mol}/100\text{g}/\text{min}$; RG: 12 or 19 $\mu\text{mol}/100\text{g}/\text{min}$	EDL: 8 or 11 $\mu\text{mol}/100\text{g}/\text{min}$; WG: 3 or 8 $\mu\text{mol}/100\text{g}/\text{min}$	SOL 125 % (4.1 mg/kg/min) or 155 % (33 mg/kg/min) greater than EDL
[194]	Rat	<i>In vivo</i> clamp (5 mg/kg/min)	SOL: 84 dpm/mg; RG: 78 dpm/mg	EDL: 35 dpm/mg; WG: 15 dpm/mg	SOL 140 % greater than EDL
[192]	Rat	<i>In vivo</i> clamp (4 mg/kg/min)	SOL: 1400 nmol/g/min	EDL: 700 nmol/g/min	SOL 100 % greater than EDL
[195]	Rat	<i>In vivo</i> clamp (4 mg/kg/min)	SOL: 1300 nmol/g/min	EDL: 600 nmol/g/min	SOL 117 % greater than EDL
[196]	Mouse	<i>In vivo</i> clamp (2.5 mg/kg/min)	SOL: 80 $\mu\text{mol}/100\text{g}/\text{min}$	VL: 14 $\mu\text{mol}/100\text{g}/\text{min}$	SOL 471 % greater than VL
[197]	Rat	<i>In situ</i> perfusion (100 nM)	SOL: 15 $\mu\text{mol}/100\text{g}/\text{min}$	EDL: 9.8 $\mu\text{mol}/100\text{g}/\text{min}$	SOL 53 % greater than EDL
[198]	Rat	<i>In situ</i> perfusion (1.5 U/rat)	RQ & RG & SOL: 9 $\mu\text{mol}/\text{g}/\text{hr}$	WQ & WG: 6.5 $\mu\text{mol}/\text{g}/\text{hr}$	RQ & RG & SOL 38 % greater than WQ & WG
[199]	Rat	<i>In situ</i> perfusion (10 mU/mL)	SOL: 24 $\mu\text{mol}/\text{h}/\text{g}$; RG: 26 $\mu\text{mol}/\text{h}/\text{g}$	EDL: 20 $\mu\text{mol}/\text{h}/\text{g}$; WG: 10 $\mu\text{mol}/\text{h}/\text{g}$	SOL 20 % greater than EDL
[189]	Rat	<i>Ex vivo</i> stimulation (2 mU/mL)	SOL: 2.5 $\mu\text{mol}/\text{mL}/20\text{min}$	EDL: 1.7 $\mu\text{mol}/\text{mL}/20\text{min}$	SOL 47.1 % greater than EDL
[197]	Rat	<i>Ex vivo</i> stimulation (100 nM)	SOL: 2.5 $\mu\text{mol}/100\text{g}/\text{min}$	EDL: 2.3 $\mu\text{mol}/100\text{g}/\text{min}$	SOL 8.7 % greater than EDL
[200]	Mouse	<i>Ex vivo</i> stimulation (0.33 or 13.3 nM)	SOL: 1.4 or 1.4 $\mu\text{mol}/\text{mL}/10\text{min}$	EDL: 1.1 or 1.2 $\mu\text{mol}/\text{mL}/10\text{min}$	SOL 27.3 % (0.33 nM) or 16.7 % (13.3 nM) greater than EDL
[201]	Mouse	<i>Ex vivo</i> stimulation (60, 120, or 20000 $\mu\text{U}/\text{mL}$)	SOL: 0.7, 1.0, or 1.1 $\mu\text{mol}/\text{g}/15\text{min}$	EDL: 0.6, 0.7, or 0.8 $\mu\text{mol}/\text{g}/15\text{min}$	SOL 16.7 % (60 $\mu\text{U}/\text{mL}$), 42.9 % (120 $\mu\text{U}/\text{mL}$), or 37.5 % (20000 $\mu\text{U}/\text{mL}$) greater than EDL
[202]	Mouse	<i>Ex vivo</i> stimulation (60 $\mu\text{U}/\text{mL}$)	SOL: 0.8 $\mu\text{mol}/\text{g}/20\text{min}$	EDL: 0.7 $\mu\text{mol}/\text{g}/20\text{min}$	SOL 14.3 % greater than EDL

ISGU: insulin-stimulated glucose uptake; SOL: Soleus; RG: red gastrocnemius; WG: white gastrocnemius; VL: vastus lateralis; RQ: red quadriceps; WQ: white quadriceps.

2.3.1.2 Muscle type difference in insulin-sensitising effect observed following acute exercise

Acute exercise-induced subsequent insulin-sensitising effects in skeletal muscle may occur in a muscle type-selective manner, however results from available studies remain controversial [12, 162, 201, 203]. Some researchers reported that in mice, the enhancement of insulin-stimulated glucose uptake following *in situ* contraction or acute exercise was only detected in EDL but not in Soleus [12, 13]. Kjøbsted et al. suggested that this muscle type specificity might be due to the fact that EDL has much higher expression of AMPK α 2 β 2 γ 3 (EDL 20 % *versus* Soleus 2 %, of all AMPK complexes) [12, 162]. As described in **Section 2.2.5.4.1**, AMPK α 2 β 2 γ 3 is currently the only reported AMPK complex likely responsible for the insulin-sensitising effect of acute exercise. Consistent with this interpretation, the activation of AMPK induced by the treatment of AICAR or IL-6, which mimics the effect of exercise, also led to increased insulin-stimulated muscle uptake in epitrochlearis muscle and EDL but not in Soleus several hr following stimulation [164, 204, 205].

In contrast, a different study in mice reported that following exercise, insulin-stimulated glucose uptake was enhanced in both epitrochlearis muscle and Soleus, suggesting that the insulin sensitisation occurred in both muscle types [201]. Furthermore, findings from research in rats also for the most part did not support a muscle type difference of insulin-sensitising effect, by showing increased insulin-stimulated glucose uptake post exercise in both epitrochlearis muscle and Soleus [203], or in both type I and type II fibres [206].

Taken together, it is possible that acute exercise exerts muscle type-selective insulin-sensitising effect in skeletal muscle, at least in mice, but to date there is limited and contradictory data. As such, in this PhD thesis, the role of ucOC in the possible muscle type difference in this insulin-sensitising effect is examined by testing whether ucOC has a muscle type-specific effect on insulin-stimulated glucose uptake in muscles post-*ex vivo* contraction (**Chapter 3**).

2.3.1.3 Muscle type difference in non-insulin-stimulated muscle glucose uptake

Understanding the muscle type difference in muscle glucose uptake independent of insulin may be important for future development of therapeutics to prevent or manage insulin resistance in specific muscle types. However, current evidence, although quite limited, indicates that non-insulin-stimulated muscle glucose uptake, induced by various stimuli including exercise/contraction [15-17], stretching [207, 208], and hypoxia [17, 209], is very similar in both oxidative and glycolytic muscles.

As described in **Section 2.2.3.2.2.2**, ucOC may trigger muscle glucose uptake independent of insulin in mouse oxidative muscles, which played an important role in exercise-induced muscle glucose uptake. Of note, this ucOC effect was not found in glycolytic muscles, indicating a muscle type variance in regards to ucOC-triggered muscle glucose uptake [23]. This phenomenon was ascribed to the biological variance of GPRC6A expression between muscle types [23]. However, since exercise triggers muscle glucose uptake equally in both muscle types, the reported muscle type-specific ucOC effect is actually contradictory to its role in exercise-induced muscle glucose uptake suggested by the same study. Thus, in **Chapter 4** of this PhD thesis, the existence of possible muscle type-specific ucOC regulation in muscle glucose uptake independent of insulin is verified.

2.3.2 Muscle type differences in muscle atrophy

Loss of muscle mass and strength during atrophic conditions occurs in a muscle type-specific manner [18, 19]. In general, glycolytic muscles are more susceptible to the atrophy caused by cancer/aging cachexia, sepsis, and diabetes, whereas oxidative muscle atrophy is primarily affected by muscle disuse and long-term microgravity [18, 19]. The identification of mechanisms responsible for the fibre type-specific muscle loss can contribute to the knowledge about pathogenesis of disuse atrophy, as well as to the design of therapeutic interventions appropriate for the specific disorders.

Several proteins have been suggested to be involved in the mechanisms of greater

mass loss in oxidative muscles during muscle disuse-caused muscle atrophy. Peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), which is a well described factor that controls mitochondrial biogenesis and is required for the mass maintenance of slow-twitch fibres probably via the inhibition of FOXO3a [210, 211], is a likely candidate. It was reported that denervation caused a rapid loss of PGC-1 α , leading to the greater mass loss of oxidative muscles [212]. Furthermore, hindlimb immobilisation induced inflammatory reactions and cell apoptosis primarily in soleus, resulting in greater wasting of the muscle [213]. Of note, it was reported that during hindlimb unloading, the reduction of AMPK activity in Soleus was much more severe than that in EDL, associated with greater loss of muscle mass and MyHC I expression [173, 175]. This greater loss of AMPK activity may be associated with more pronounced atrophic conditions, due to the possible role of AMPK in muscle growth by inhibiting the HDAC4/FOXO cascade and maintaining energy metabolism (**Section 2.2.5.4.1**).

Overall, muscle atrophy occurs in a muscle type-specific manner and muscle disuse/hindlimb immobilisation-caused muscle atrophy is predominantly observed in oxidative muscles. This muscle type specificity of disuse atrophy may be due to greater inflammation and apoptosis, as well as lower activity of PGC-1 α and AMPK. However, currently the exact mechanisms underlying this muscle type-selective muscle loss during disuse atrophy are largely unknown. Since AMPK activity is possibly regulated by ucOC, it is plausible that the muscle type-selective loss of ucOC regulation in skeletal muscle plays a role. This hypothesis is tested in **Chapter 6**.

2.4 Aims and hypotheses

Collectively, in skeletal muscle, the regulation of glucose uptake and disuse-induced muscle loss are likely to be muscle type-selective (oxidative or glycolytic). The muscle type specificity in muscle glucose uptake is also condition-dependent (with or without insulin, at rest or following exercise). These effects may be, at least in part, due to the muscle type-specific regulation of ucOC. The mechanisms underlying ucOC modulation

in muscle glucose uptake and muscle mass are not clear, but GPRC6A, Akt, ERK, AMPK, PKC, mTORC1, and/or FOXOs are implicated. Therefore, it is important to study the role of ucOC in muscle glucose uptake and muscle mass regulation in different muscle types under various conditions, as well as the underlying signalling pathways. These investigations not only improve the understanding of muscle glucose uptake and muscle mass modulations, but also provide molecular targets for designing new therapies for pathological conditions such as insulin resistance and muscle atrophy.

The effects of ucOC and its related signalling pathways are investigated through four independent, but related, studies. The specific aims of these four studies are:

Study 1 (Chapter 3)

1. To test the hypothesis that ucOC directly enhances insulin-stimulated muscle glucose uptake following *ex vivo* muscle contraction in EDL but not Soleus.
2. To test the hypothesis that ucOC enhances insulin-stimulated phosphorylation of AS160 following *ex vivo* muscle contraction in EDL, with or without the enhancement of Akt phosphorylation.

Study 2 (Chapter 4)

1. To test the hypothesis that ucOC triggers non-insulin-stimulated muscle glucose uptake in resting muscles, in both EDL and Soleus.
2. To test the hypothesis that ucOC triggers the phosphorylation of AS160, probably along with the activation of ERK/Akt, AMPK, and/or PKC δ / θ .

Study 3 (Chapter 5)

1. To test the hypothesis that ucOC increases glucose uptake in insulin-stimulated resting muscles in Soleus but not EDL.
2. To test the hypothesis that ucOC enhances the phosphorylation of AS160 in insulin-stimulated muscles, along with the enhancement of phosphorylation of ERK/Akt and/or AMPK.

Study 4 (Chapter 6)

1. To test the hypothesis that both hindlimb immobilisation and androgen deprivation reduce the production of ucOC, thus indicating a feed-forward loop between ucOC and testosterone.
2. To test the hypothesis that both hindlimb immobilisation and androgen deprivation lead to impaired ucOC signalling, and that the loss of ucOC signalling activity is greater in Soleus than EDL.
3. To test the hypothesis that reduced ucOC levels and/or impaired activity of proteins in ucOC signalling pathway are associated with decreased muscle mass and strength in atrophic muscles.
4. To test the hypothesis that reduced ucOC levels and/or impaired activity of proteins in the proposed ucOC signalling pathway are associated with lower activity of proteins in the muscle anabolic pathway, as well as with higher activity of proteins in the muscle catabolic pathway.

Chapter 3. Study 1: The effects of muscle contraction and recombinant OC on insulin sensitivity *ex vivo*

This study, with the exclusion of **Figure 3.7** which is added to this chapter, has been published as follows:

Levinger I*, **Lin X***, Zhang X, Brennan-Speranza T.C, Volpato B, Hayes A, Jerums G, Seeman E, McConell G. 2016. The effects of muscle contraction and recombinant osteocalcin on insulin sensitivity *ex vivo*. *Osteoporos Int*, 27; 653-663. doi: 10.1007/s00198-015-3273-0. (*: Itamar Levinger and **Lin X** are co-first authors in this publication. **Lin X** majorly contributed to the data collection, data analysis, result interpretation, and manuscript writing for this study)

This study has been presented at an international conference:

.Levinger I, **Lin X**, Zhang X, Hayes A, Jerums G, Ferron M, Karsenty G, Seeman E, McConell G. Recombinant osteocalcin improves insulin-stimulated glucose uptake following muscle contraction. American Society for Bone and Mineral Research Annual Meeting, Baltimore, Maryland, USA (2013).

Declaration of co-authorship and co-contribution

1. PUBLICATION DETAILS

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

	6/3/2018
Signature	Date

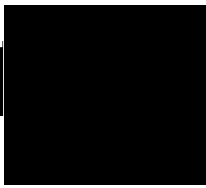
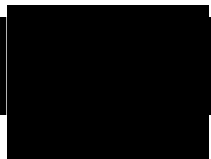
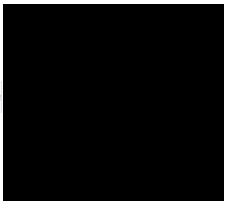
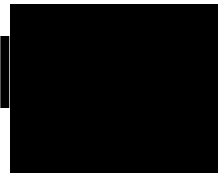
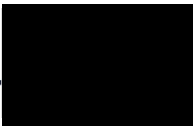
3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

Itamar Levinger, Xuzhu Lin, Xinmei Zhang, Tara Brennan-Speranza, Bianca Valpato, George Jerums, Ego Seeman, Glenn McConell

Name(s) of Co-Author(s)	Nature of Contribution	Signature	Date
Xuzhu Lin	Designed the study; Collected samples; Analysed and interpreted the data; Wrote the manuscript; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Itamar Levinger	Designed the study; Analysed and interpreted the data; Wrote the manuscript; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Xinmei Zhang	Designed the study; Collected samples; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Tara Brennan-Speranza	Collected samples; Analysed and interpreted the data; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Bianca Valpato	Collected samples; Undertook critical revisions for important intellectual content; Approved the final version of		6/3/2018

	the paper.		
George Jerums	Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Ego Seeman	Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Glenn McConell	Designed the study; Analysed and interpreted the data; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018

3.0 General introduction

Acute exercise enhances muscle insulin sensitivity up to 48 hr following exercise [95-97]. However, the mechanisms underlying this effect of exercise still remain unclear.

Previous *in vivo* studies performed in our lab have shown that acute exercise increases the levels of circulating ucOC in humans [30, 31, 112, 113], which are associated with increased global insulin sensitivity measured via insulin clamps several hr after the exercise [30, 31]. Furthermore, we have also shown that exercise-enhanced ucOC levels are correlated with increased post-exercise insulin-stimulated Akt phosphorylation (Ser473) in muscle tissue [30, 31]. These results may suggest that, the increase in ucOC levels induced by exercise, may contribute to the insulin-sensitising effect. Thus, we designed an *ex vivo* study, to exclude confounding factors such as changes in other hormones and/or blood flow, to examine the possible direct effect of ucOC on muscle glucose uptake post-contraction.

Recent studies have suggested that exercise may enhance subsequent muscle insulin sensitivity in glycolytic muscles but not in oxidative muscles, at least in mice [12, 13]. However, the evidence regarding the muscle type specificity in the insulin-sensitising effect of exercise is limited and controversial, and the underlying mechanisms are also poorly understood.

Therefore, in Study 1 we aimed to provide novel evidence for the role of ucOC in the muscle type-specific insulin-sensitising effect of exercise, by testing the hypothesis that ucOC *per se* increases post-contraction insulin-stimulated glucose uptake in EDL but not in Soleus, and investigating the involved signalling pathway(s).

3.1 Abstract

Acute exercise increases skeletal muscle insulin sensitivity. In humans, exercise increases circulating ucOC, a hormone that increases insulin sensitivity in rodents. We tested whether GPRC6A, the putative receptor of ucOC, is present in mouse muscle and whether recombinant ucOC increases insulin sensitivity in both C2C12 myotubes and whole mouse muscle following *ex vivo* muscle contraction. Glucose uptake was examined in C2C12 myotubes that express GPRC6A following treatment with insulin alone or with insulin and increasing ucOC concentrations (0.3, 3, 10 and 30 ng/mL). In addition, glucose uptake, phosphorylated (p-)Akt and/or p-AS160 were examined *ex vivo* in EDL and Soleus dissected from C57BL/6J wild-type mice, at rest, following insulin alone, after muscle contraction followed by insulin, and after muscle contraction followed by recombinant ucOC then insulin exposure. We observed protein expression of the likely receptor for ucOC, GPRC6A, in whole muscle sections and differentiated mouse myotubes. We observed reduced GPRC6A expression following siRNA transfection. ucOC significantly increased insulin-stimulated glucose uptake dose-dependently up to 10 ng/mL, in differentiated mouse C2C12 myotubes. Insulin increased EDL glucose uptake (~30 %, $P < 0.05$) and p-Akt and p-Akt/Akt compared with rest (all $P < 0.05$). Contraction prior to insulin increased EDL muscle glucose uptake (~25 %, $P < 0.05$), p-Akt, p-Akt/Akt, p-AS160 and p-AS160/AS160 compared with contraction alone (all $P < 0.05$). ucOC after contraction increased insulin-stimulated muscle glucose uptake (~12 % $P < 0.05$) in EDL but not in Soleus. ucOC after contraction also increased insulin-stimulated p-AS160 ($P < 0.05$) more than contraction plus insulin alone but without effect on p-Akt in EDL. In the absence of insulin and/or of contraction, ucOC had no significant effect on muscle glucose uptake. In conclusion, GPRC6A, the likely receptor of ucOC, is expressed in mouse muscle. ucOC treatment augments insulin-stimulated skeletal muscle glucose uptake in C2C12 myotubes and following *ex vivo* muscle contraction. ucOC may partly account for the insulin sensitising effect of exercise.

3.2 Introduction

Exercise plays a major role in the prevention and management of insulin resistance and type 2 diabetes [214]. Understanding the mechanisms responsible for the increase in insulin sensitivity post-exercise is important for the development of new interventions for the prevention and management of insulin resistance and diabetes.

An acute bout of exercise increases insulin sensitivity in skeletal muscle, a major site for glucose disposal, for several hr after the cessation of exercise [215, 216]. The mechanisms responsible for this increase in insulin sensitivity in muscle, however, are not well defined. Studies in murine models and in human subjects suggest that the skeleton participates in energy metabolism and glucose homeostasis through a mechanism involving osteocalcin [57].

Osteocalcin, a protein secreted from osteoblasts, undergoes post-translational modification whereby glutamic acids at positions 17, 21 and 24 are carboxylated to form γ -carboxylated glutamic acid or Gla residues [217]. A small amount of ucOC circulates and contributes to energy metabolism and glucose homeostasis in mice by acting on islet cells to stimulate β -cell proliferation and increase insulin secretion and insulin sensitivity [57, 218, 219].

This effect of ucOC on targeted organs is most likely through a member of GPCR family, namely: GPCR6A [57, 58, 65, 66]. Whether the GPCR6A is present in mouse skeletal muscle at the protein level and whether the increase in insulin sensitivity is partly mediated via the effects of ucOC on skeletal muscle are not clear.

A muscle specific action of ucOC on insulin sensitivity after exercise is plausible given that in obese men, serum ucOC increases after the completion of an acute bout of moderate (45 min cycling at 75 % of VO_{2peak}) and high (4 sets $\times$ 4 min cycling at 95 % of HR_{peak}) intensity aerobic exercise [30, 31]. This increase in insulin sensitivity correlates with a reduction in serum glucose and increase in insulin sensitivity in obese men [30, 31].

We investigated whether GPRC6A is present in mouse skeletal muscle sections and the C2C12 mouse muscle cell line, as well as the effects of ucOC on insulin-stimulated glucose uptake by determining the levels and activity (phosphorylation) of AS160 following *ex vivo* muscle contraction. This method allows examination of muscle contraction in whole muscle tissue in a physiological compartment free from confounding circulating hormonal factors. Akt and AS160 are downstream proteins in the PI3K pathway which is responsible for most of the metabolic actions of insulin signalling in muscle fibres [220]. AS160 may represent a common convergence point between the pathways regulating insulin- and contraction-stimulated Glut4 translocation [221, 222]. We hypothesised that as the GPRC6A is expressed in mouse skeletal muscle, and that administration of recombinant ucOC improves muscle insulin sensitivity following muscle contraction induced *ex vivo* compared to that produced by insulin and contraction alone.

3.3 Material and methods

3.3.1 Study design

For *ex vivo* contraction studies, EDL and Soleus were isolated from C57BL/6J mice and stimulated *ex vivo* to contract for 10 min. We compared glucose uptake, p-Akt and p-AS160 at rest (no contraction, insulin or recombinant ucOC) and 120 min after (a) insulin alone (no contraction or ucOC), (b) 120 min post-contraction with insulin added 90 min after exercise and (c) 120 min post-contraction with recombinant ucOC added 45 min after contraction and insulin added 90 min after contraction (**Figure 3.1**). This study design was based on our hypothesis that exercise (muscle contraction), increases ucOC 30 – 60 min after exercise and this, in turn, increases insulin sensitivity [31].

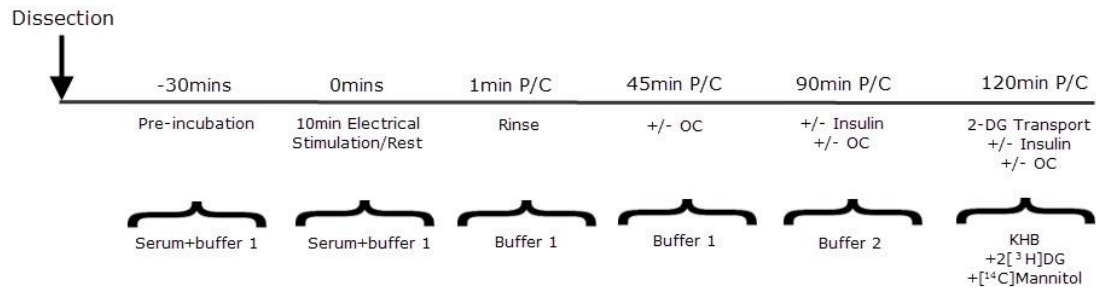


Figure 3.1. A flow chart of the experiment design. P/C: post-contraction; OC: osteocalcin; KHB: Krebs-Henseleit buffer. 2[³H]DG: 2-Deoxy-D-[1,2-³H]-glucose.

3.3.2 Materials

All chemicals were purchased from Sigma unless otherwise stated. Recombinant ucOC for the *ex vivo* study was donated by Professor Gerard Karsenty (Columbia University, NY, USA). Purification of bacterially produced mouse recombinant ucOC was performed as described previously [25]. Recombinant ucOC for the *in vitro* study was purchased from Bachem (Bubendorf, Switzerland). 2-Deoxy-D-[1,2-³H]-glucose and D-[1-¹⁴C] Mannitol were from Perkin Elmer (Australia). RED 660 Protein Assay Reagent Kit and Neutralizer were purchased from GBiosciences. SuperSignal West Femto Chemiluminescent Substrate was provided by Thermo Scientific. Primary antibodies including p-Akt (Thr308, 13038), Akt (4685), p-AS160 (Thr642, 8881), AS160 (2670) and β -Actin (4970) used in Western Blotting were purchased from Cell Signaling. GPRC6A primary antibody, control rabbit IgG and mountant with 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) were from Santa Cruz Biotechnologies. HRP conjugated Goat anti-Rabbit IgG (H + L) secondary Antibody was from Thermo Scientific. Fluorescent secondary anti-rabbit IgG alexa fluoro-488 was purchased from Life Technologies and protease inhibitor mini tablets were from Pierce.

3.3.3 Animals

Eight wk old male C57BL/6J mice ($N = 86$) were purchased from Animal Resources Centre (ARC, Perth, WA, Australia). All mice were housed with a 12-hr light/12-hr dark cycle and fed standard laboratory chow (Barastoc mouse food cubes, Ridley AgriProducts, Melbourne, Australia, that contained 20 % protein, 4.8 % fat and the rest carbohydrate and fibres) and water *ad libitum* until 10 – 12 wk old. The study was approved by both the Animal Experimentation Ethics Committee and the Human Research Ethics Committee of Victoria University and conformed to the Australian National Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.3.4 Muscle dissection

Mice were fasted for 4 hr before deep anaesthetisation with 60 mg/kg intraperitoneal pentobarbital. Left and right EDL, Soleus, and flexor digitorum longus (FDL) muscles were excised within 30 min of anaesthesia. Left and right EDL and Soleus were tied tendon to tendon with 5/0 silk suture, and placed into KHB (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, PH 7.4) to keep the muscle moist. FDL muscles were fixed in 4 % paraformaldehyde overnight, soaked in 10 % sucrose in phosphate buffered saline overnight then embedded in paraffin.

3.3.5 Immunodetection of GPRC6A

Paraffin-embedded mouse FDL muscles were sectioned at 5 μ m thickness and adhered to normal glass microscope slides. Sections were permeabilised, blocked then incubated with primary GPRC6A rabbit polyclonal anti-mouse IgG antibody and negative control rabbit IgG fraction (normal serum) isotype followed by goat

anti-rabbit IgG alexa fluoro-488 and Ultracruz mounting medium with DAPI then visualised under a Zeiss Deconvolution fluorescence microscope.

3.3.6 C2C12 myotube differentiation

C2C12 myoblast cells were cultured in 6-well plates in 10 % Fetal Calf Serum (FCS) in Dulbecco's modified eagle medium (DMEM) (+ NaHCO₃) until ~70 % confluent. Media was then refreshed with low glucose (5.5 mM) DMEM with 2 % horse serum for differentiation. Media was changed every alternate day for 7 d by which time, all cells had differentiated into myotubes.

3.3.7 C2C12 myotube 2-DG uptake assays

Myotubes were serum starved in Krebs Ringer Phosphate (KRP) buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM Mg SO₄, 1 mM CaCl₂, 0.4 mM NaH₂PO₄, 0.6 mM Na₂HPO₄) containing 0.2 % bovine serum albumin for 2 hr at 37 °C. Cells were then incubated in 950 µL of KRP buffer with the relevant concentration of osteocalcin (0, 0.3, 3, 10 or 30 ng/mL, Bachem, Germany) for 60 min. At the 45 min mark, insulin was spiked into the well from a 1000X concentration (1 µL of 60 mU/mL stock into the KRP buffer in the well) to achieve a final activity of 60uU/mL and wells were left at 37 °C for the remaining 15 min. 2-Deoxy-D-glucose (2-DG) uptake was measured as follows: the assay was initiated by the addition of 50 ul of 1 mM 2-DG (20 uCi/mmol) and relevant osteocalcin concentrations. After 4 min, the assay was terminated by washing the cells rapidly three times with ice-cold PBS. Cells were subsequently solubilised in 1 % Triton X-100, and ³H was quantified by scintillation counting (Packard 1900CA liquid scintillation analyser, Packard Instrument Co.). 50 µL of the lysates were also used to run a BCA (Pierce) with a bovine serum albumin (BSA)

standard curve from 2 mg/mL to 0 to determine the total cell protein concentrations in mg/mL for corrections.

3.3.8 C2C12 myotube GPRC6A siRNA knock-down assays

Differentiated myotubes were transfected with complexes of 60 nM siRNA directed at GPRC6A, scrambled sequence and 1 % (v/v) Life Technologies Lipofectamine RNAiMAX transfection reagent contained in OptiMEM medium (Life Technologies). Transfection occurred over 24 hr, after which time transfection medium was aspirated and replaced with 10 % OptiMEM for a further 24 hr. Cells were then prepared for western blotting.

3.3.9 *Ex vivo* muscle contraction and 2-DG uptake measurement

The method used was similar to that described by Funai et al [103] in rat muscle with some modifications. Specifically, EDL and Soleus were transferred to chambers of a specific muscle contraction apparatus (Zultek Engineering, VIC). They were pre-incubated in 50 % human serum diluted with KHB + 0.1 % BSA + 2mM D-glucose + 8mM Mannitol (buffer 1) for 30 min. In a pilot study (unpublished) we observed that in mouse muscle, as in rat muscle, serum was required to be present to observe an insulin-sensitising effect on *ex vivo* skeletal muscle contractions, despite the finding that serum alone without contraction does not stimulate glucose uptake into these muscles as described below. Four healthy recreationally active males that were involved in the study (age: 29, 35, 39 and 50 yr) signed an informed consent and donated blood for the experiment. Blood sampling was performed after an overnight fast. The serum from all four males was mixed to avoid individual variation. Serum OC levels in the human serum was not measured. However, the serum used across all experiments was from the same individuals. Importantly, to ensure that the serum

itself was not increasing muscle glucose uptake we performed an experiment with resting EDL and Soleus incubated in buffer (with no serum) or in human serum for 10 min (identical to the protocol we used for the effects of osteocalcin on muscle glucose uptake, see below) and examined muscle glucose uptake immediately after incubation and 2 hr post incubation. Muscle glucose uptake in EDL between buffer incubation and serum incubation was not different immediately post-incubation (buffer: 2.02 ± 0.23 versus serum: 2.07 ± 0.08 $\mu\text{mol/g/hr}$, $P = 0.81$) and 2 hr after incubation (buffer: 2.76 ± 0.15 versus serum: 2.62 ± 0.14 , $\mu\text{mol/g/hr}$, $P = 0.41$).

For all incubation steps, solutions were maintained at 30 °C and gassed with 95 % O₂-5 % CO₂. After pre-incubation, muscles were mounted with 0.5 g tension before contractions were induced by square wave pulse electrical stimulation (Frequency: 100 Hz, Pulse Duration: 0.2 ms, Train Duration: 350 ms, Train interval: 5 sec, Voltage: 12 V), or muscles remained at rest, for 10 min [223]. Immediately after electrical stimulation or rest, all muscles were rinsed with fresh, fully gassed buffer 1 for 1 min at 30 °C. Muscles were then transferred to new chambers containing ‘buffer 1’ for 90 min. During this step, for muscles treated with recombinant ucOC; 10 ng/mL ucOC was added into the buffer from 45 min to 90 min. This concentration was chosen as it is within the physiological range of ucOC levels, has been used successfully by others and concentrations beyond 10 ng/mL have been shown to reverse the effect of osteocalcin on some of the osteocalcin-induced gene expression outputs [24, 25, 57]. We also observed this concentration to significantly increase C2C12 myotube uptake of 2-DG in vitro (**Figure 3.3**). After 90 min, muscles were transferred to chambers containing buffer 2 (KHB + 0.1 % BSA + 2 mM pyruvate + 8 mM mannitol) for 30 min with or without 10 ng/mL ucOC, in the presence or absence of 60 $\mu\text{U/mL}$ of insulin. After incubation in ‘buffer 2’, muscles were transferred to chambers containing KHB + 0.1 % BSA + 2 mM 2-Deoxy-D-[1,2-³H]-glucose and 16 mM D-[1-¹⁴C] Mannitol with or without 60 $\mu\text{U/mL}$ insulin (see **Figure 3.1**). After 10 min, muscles were rapidly rinsed with freezing KHB buffer, then trimmed of tendons and cut in half with a blade. The half used for immunoblotting was immediately frozen in

liquid nitrogen, and the other half was homogenised in 1M NaOH solution at 95 °C for 10 min. After neutralisation by addition of the same amount of 1 M HCl, homogenates were centrifuged (16,000 g) for 5 min. The supernatant was pipetted into vials with scintillation cocktail for scintillation counting (β -counter) with Tri-Carb 2910TR Liquid Scintillation Analyzer (Perkin Elmer, Australia) the following day.

3.3.10 Sample preparation for Western blotting

Expression of GPRC6A in C2C12 cell was determined as follows: cells were grown to 80 % confluence in a 75 cm² culture flask and lysed in laemmli loading buffer (2 % sodium dodecyl sulfate (SDS), 5 % β -2-mercaptoethanol, 10 % glycerol, 0.002 % bromophenol blue, 0.125 M Tris HCl with the addition of a protease inhibitor mini tablet (Pierce) and briefly sonicated to reduce sample viscosity. Sample preparation method for whole muscle was initially described by Mollica et al. [224]. Briefly, the frozen EDL halves were sectioned at 20 μ m in a cryostat and dissolved into sample Buffer (0.125 M Tris-Cl [pH 6.8], 4 % [w/v] SDS, 10 % glycerol, 10 mM EGTA, 0.1 M dithiothreitol (DTT)). After performing a protein assay using Red 660 Assay Kit to assess protein concentration, 0.01 % bromophenol blue was added to the homogenates. C2C12 myoblast cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 % TritonX 100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate (SDS), and a Pierce protease inhibitor mini-tablet). After calculating total cellular protein with a BCA, equal amounts of protein were then combined with 1x laemmli loading buffer (2 % SDS), 5% β -2-mercaptoethanol, 10 % glycerol, 0.002 % bromophenol blue, 0.125 M Tris HCl), heated at 95 °C for 3 min and centrifuged for 5 min at 2000 rpm. All samples were then immediately used for immunoblotting assay.

3.3.11 Western blotting

Briefly, proteins (5 µg for each well) were separated on 10 % SDS-PAGE gels by gel electrophoresis. After that, proteins in the gels were wet-transferred to Polyvinylidene fluoride (PVDF) (0.45 µm pore) for 120 min at 100 V in a circulating ice-cooled bath. Following transfer, the membrane was blocked with 5 % (w/v) skim milk powder, or 1 % (w/v) heat denatured casein for GPRC6A detection, dissolved in Tris-buffered saline with 0.1 % Tween-20 (TBST) at room temperature for 1 hr. Primary antibodies, diluted in 5% (w/v) BSA in TBST or blocking buffer, were applied and incubated overnight at 4 °C . The dilution of the specific primary antibody was as follows, GPRC6A: 1:250 (or 2 µg/mL), Akt: 1:5,000, p-Akt (Thr308): 1:1,000, AS160: 1:500, p-AS160 (Thr642): 1:1,000, Actin, 1:20,000. After 1 hr incubation with secondary antibody (dilution: Akt: 1:50,000, p-Akt: 1:50,000, AS160: 1:10,000, p-AS160: 1:20,000, Actin, 1:100,000) at room temperature, images were collected following exposure to enhanced chemiluminescent (ECL) detection reagents for GPRC6A and SuperSignal West Femto Chemiluminescent Substrate for all other immunoblots using VersaDoc™ Imaging System (Bio-Rad laboratories, Hercules, CA, USA). Densitometry was performed using the Quantity One software (Bio-Rad laboratories, Hercules, CA, USA).

3.3.12 Statistical analyses

A repeated measures ANOVA, with a post hoc analysis (LSD) was used to determine changes in C2C12 myotube glucose uptake across different ucOC concentrations. Mice *ex vivo* study: An ANOVA was performed to determine overall differences across treatments: rest, insulin, contraction plus insulin and contraction with ucOC plus insulin. Changes between each paired EDL set: left and right dissected from the same animal (left: for instance, contraction versus right: contraction plus insulin), were also analysed with a paired t-test following a significant ANOVA results. The latter analysis

provides a specific control by using the controlateral EDL. Data were analysed using IBM SPSS version 20. All data are reported as mean $\pm$ standard error of mean (SEM) and all statistical analyses were conducted at the 95 % level of significance.

3.4 Results

3.4.1 Protein expression of GPRC6A in mouse muscle sections and differentiated myotubes

Immunofluorescent microscopy showed GPRC6A expression in mouse muscle sections in the periphery of myofibres observed from cross sectional slices of FDL muscles (**Figure 3.2A**). No staining was seen in the isotype control (**Figure 3.2B**), and in differentiated C2C12 myotubes (Green Alexa 488 with DAPI-stained nuclei; **Figure 3.2C**) and negative isotype controls (**Figure 3.2D**). We also detected GPRC6A by western blot in protein extracts from differentiated mouse C2C12 myotubes (**Figure 3.2E**; β -actin used as a loading control). This expression was reduced in cultures that were transfected with siRNA directed at GPRC6A (lanes 5 and 6; siGPRC6A) but not in the non-transfected cultures (lanes 1 and 2) or in the control siRNA transfected cultures (lanes 3 and 4; siScram).

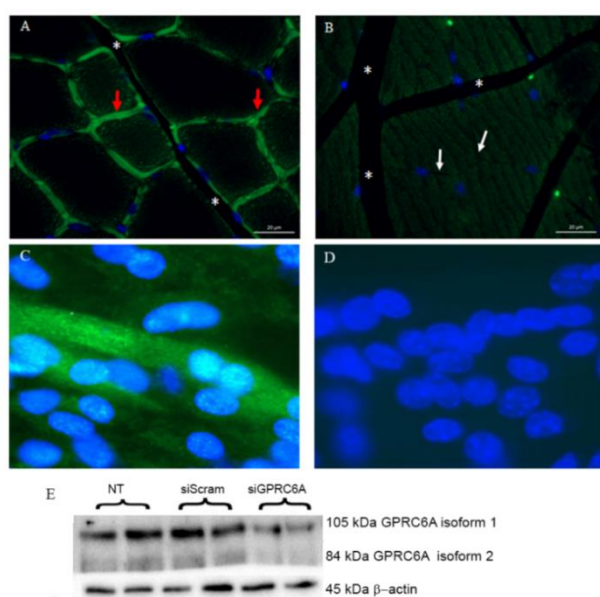


Figure 3.2. GPRC6A is expressed in mice myotubes. Deconvolution micrographs of GPRC6A in differentiated C2C12 myotubes (green Alexafluor 488 fluorescence) with DAPI stained nuclei 63X (A) and isotype control 63X (B), and mouse muscle tissues section, both at 20X (C and D). Red arrows show intense staining around myofibril

periphery, indicating the presence of the GPRC6A receptor. (B) Negative isotype control, contains no green fluorescence, indicating a lack of receptor staining. Note that the outline of the myofibres is still present in the isotype control as observed by the thin black spacing between the fibres (white arrows). Large spacing between whole myobundles can be observed in both micrographs (white stars), nuclei are stained blue with DAPI and scale bars are 20 μm . (E) Protein expression of two isoforms of GPRC6A (105 kDa and 84 kDa) analysed by western blot from cultures of differentiated C2C12 myotubes that were transfected with siRNA directed at GPRC6A (lanes 5 and 6; siGPRC6A), non-transfected cultures (lanes 1 and 2) and control siRNA transfected cultures (lanes 3 and 4; siScram). β -actin was used as a loading control.

3.4.2 Effects of ucOC on insulin-stimulated glucose uptake in mouse myotubes C2C12

ucOC significantly improved insulin-stimulated glucose uptake in the mouse myotube C2C12 line in a dose-dependent manner for concentrations between 0.3 and 10 ng/mL ($P < 0.05$, **Figure 3.3**). Compared to 10 ng/mL, 30 ng/mL ucOC did not significantly increased myotube glucose uptake (**Figure 3.3**).

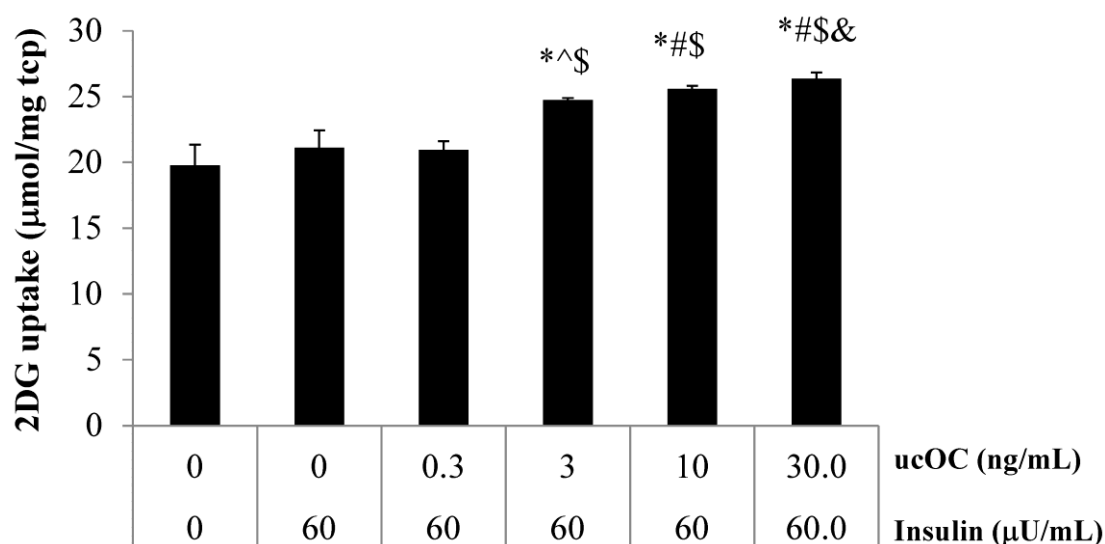


Figure 3.3: Dose-response effects of ucOC on insulin-stimulated glucose uptake in mouse myotubes C2C12. * indicates $P < 0.05$ compared to control (no insulin and no ucOC), # indicates $P < 0.05$ compared to insulin alone, ^ indicates 0.051 compared to insulin alone. \$ indicates $P < 0.01$ compared to 0.3 ng/mL ucOC. & indicates $P < 0.05$ compared to 3 ng/mL ucOC.

3.4.3 Effects of contraction alone

In EDL, two hr post-contraction alone, muscle glucose uptake was not statistically increased compared to rest (2.4 ± 0.5 versus 2.0 ± 0.5 $\mu\text{mol/g/hr}$, respectively, $P = 0.18$). p-AS160 (Thr642) was not significantly lower 2 hr post-contraction, (-35 ± 9 %, $P = 0.10$) and p-Akt (Thr308) was similar compared to rest (9 ± 20 %, $P = 0.72$).

3.4.4 Effects of insulin

Comparisons of paired resting EDL from the same animal revealed that insulin increased EDL glucose uptake (1.9 ± 0.1 versus 1.5 ± 0.1 $\mu\text{mol/g/hr}$, respectively $P < 0.05$, **Figure 3.4A**, expressed as fold-changes). In addition, p-Akt (Thr308, **Figure 3.4B**), p-Akt/Akt (~ 700 %, $P < 0.01$, **Figure 3.4D**) and p-AS160/AS160 (~ 240 %, $P < 0.04$, **Figure 3.4G**) were higher after insulin treatment. There were no significant differences in total Akt, AS160 and p-AS160 (Thr642), although mean change p-AS160 (Thr642) was numerically but not statistically higher (**Figure 3.4E**).

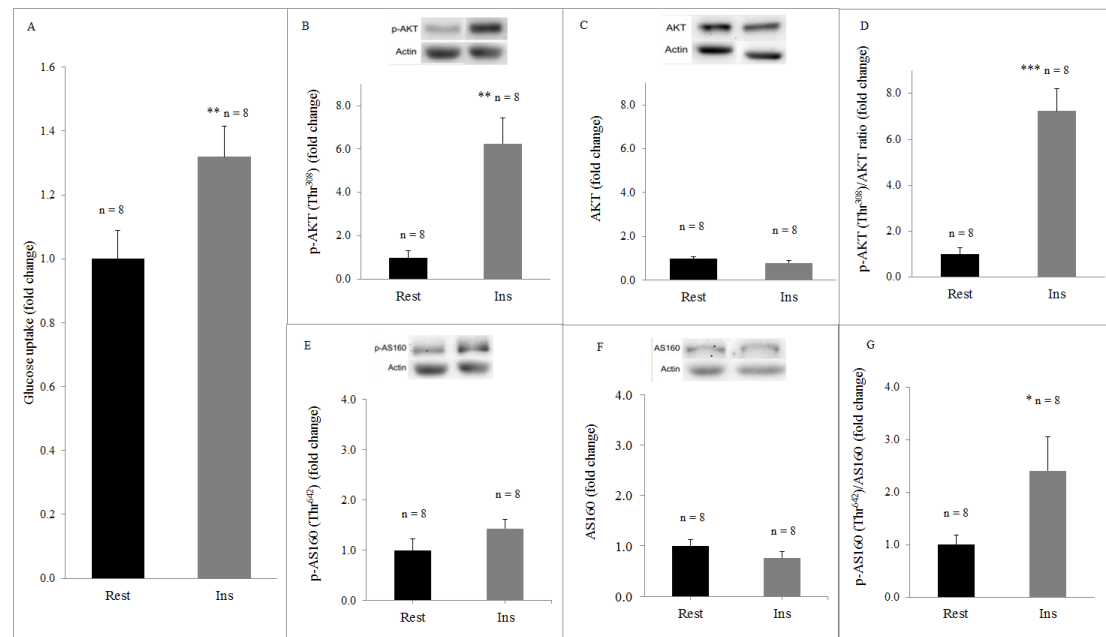


Figure 3.4. Comparison of paired EDL from the same animal at rest compared to post-insulin treatment. Glucose uptake (A), p-Akt (B), Akt (C), p-Akt/Akt (D), p-AS160 (E), AS160 (F) and p-AS160/AS160 (G). * indicates $P < 0.05$. ** indicates $P < 0.01$.

3.4.5 Effects of insulin - post-contraction

In EDL, contraction 90 min prior to insulin treatment increased muscle glucose uptake compared to contraction alone 2.3 ± 0.2 versus 1.8 ± 0.2 $\mu\text{mol/g/hr}$, respectively ($P < 0.05$, **Figure 3.5A**). In addition, p-Akt, the ratio p-Akt/Akt, p-AS160 and p-AS160/AS160 were all higher with insulin post-contraction compared to contraction alone (all $P < 0.05$, **Figure 3.5**).

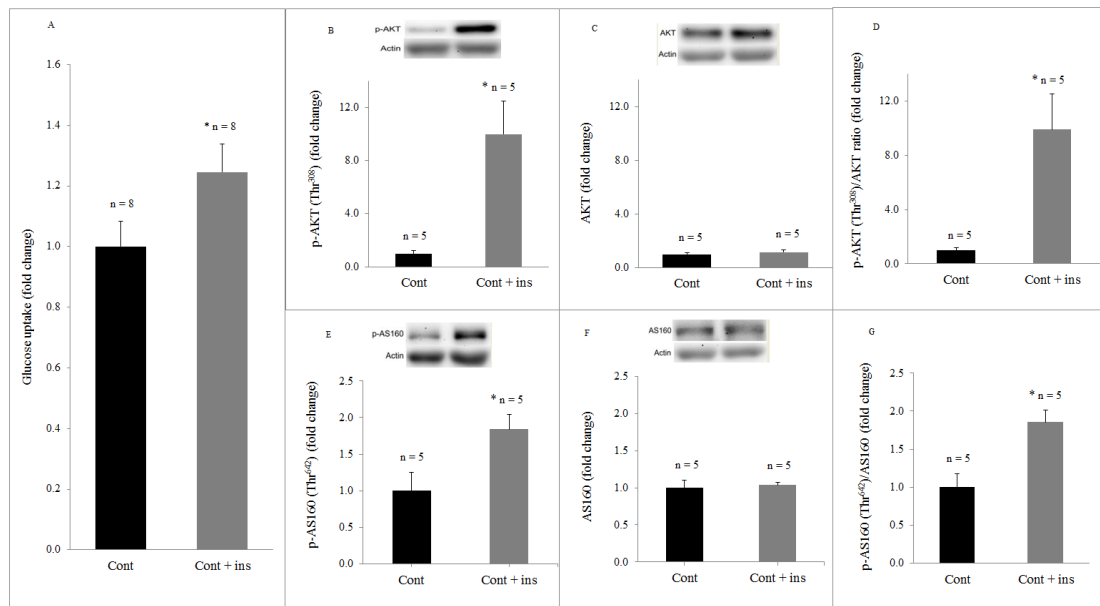


Figure 3.5. Comparison of paired EDL from the same animal post-contraction compared to post-contraction plus insulin. Glucose uptake (A), p-Akt (B), Akt (C), p-Akt/Akt (D), p-AS160 (E), AS160 (F) and p-AS160/AS160 (G). * indicates $P < 0.05$.

3.4.6 Effects of contraction and recombinant ucOC on insulin sensitivity

In EDL, contraction plus ucOC plus insulin increased muscle glucose uptake compared to contraction plus insulin (2.6 ± 0.1 versus 2.3 ± 0.1 $\mu\text{mol/g/hr}$, respectively, $P < 0.05$, **Figure 3.6A**). Likewise, p-AS160 (Thr642) was higher ($\sim 30\%$, $P < 0.05$, **Figure 3.6E**) in contraction plus ucOC plus insulin compared with contraction plus insulin. Akt was also higher ($P < 0.05$) in contraction plus ucOC plus insulin (**Figure 3.6C**) but there was no difference in p-Akt and p-Akt/total Akt.

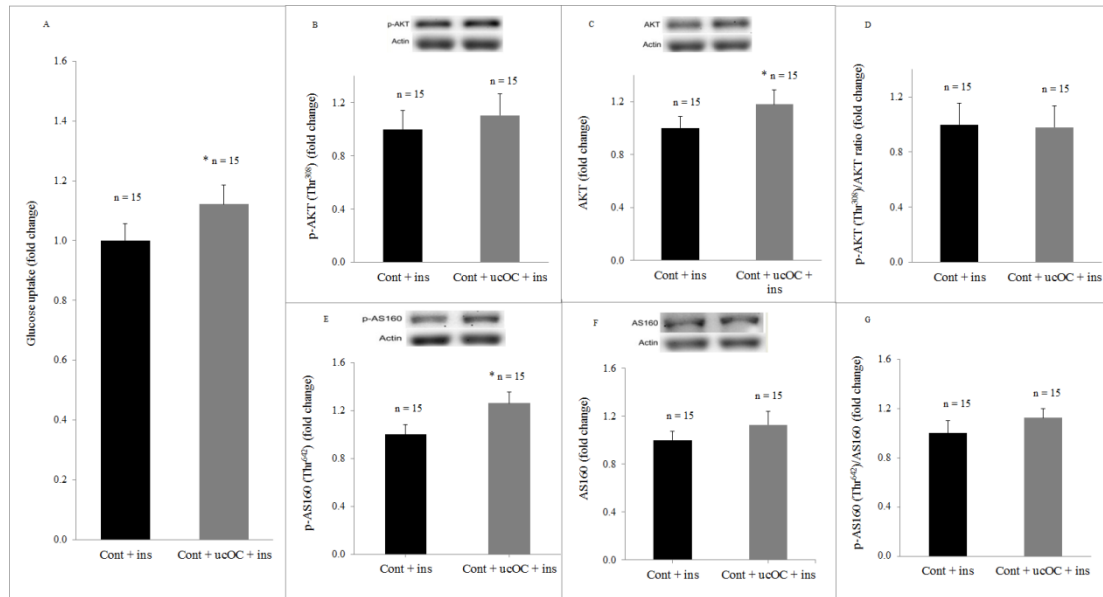


Figure 3.6. Comparison of paired EDL from the same animal post-contraction plus insulin compared to post-contraction plus ucOC plus insulin. Glucose uptake (A), p-AKT (B), Akt (C), p-Akt/Akt (D), p-AS160 (E), AS160 (F) and p-AS160/AS160 (G). * indicates $P < 0.05$.

In Soleus, contraction plus ucOC plus insulin did not alter muscle glucose uptake compared to contraction plus insulin (1.6 ± 0.2 versus 1.6 ± 0.1 $\mu\text{mol/g/hr}$, respectively. $P > 0.1$, **Figure 3.7**)

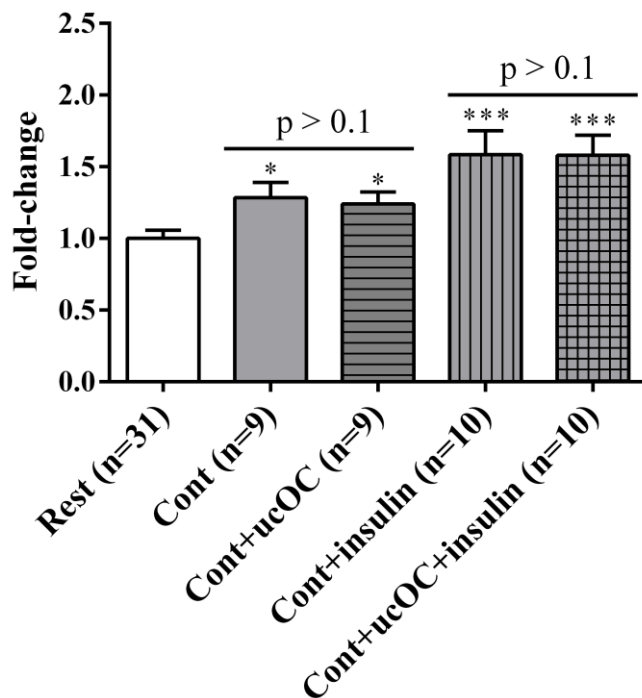


Figure 3.7. Comparison of paired Soleus from the same animal post-contraction plus insulin compared to post-contraction plus ucOC plus insulin. The glucose uptake was detected in Soleus which were at rest, post-contraction, treated with ucOC post-contraction, treated with insulin post-contraction, or treated with ucOC plus insulin post-contraction. The muscles in Cont group were paired with those muscles in Cont + ucOC group. The muscles in Cont + insulin group were paired with those muscles in Cont + ucOC + insulin group. * and *** indicate $P < 0.05$ and $P < 0.001$ compared with Rest in T-test.

3.4.7 Effects of recombinant ucOC

In EDL, ucOC alone did not significantly increase whole muscle glucose uptake from baseline (1.8 ± 0.2 versus 1.6 ± 0.2 $\mu\text{mol/g/hr}$, respectively, $P = 0.32$). ucOC post *ex vivo* muscle contraction (without insulin) had no effect on muscle glucose uptake compared to contraction alone (2.2 ± 0.2 versus 2.2 ± 0.2 $\mu\text{mol/g/hr}$, respectively, $P = 0.96$). p-AKT (12 ± 16 %, $P = 0.20$) and p-AS160 (33 ± 29 %, $P = 0.24$) were also not changed post-contraction with ucOC compared to contraction alone. Finally, ucOC had no effect on resting (no contraction) insulin sensitivity ($P = 0.05$, **Figure 3.8 A – G**).

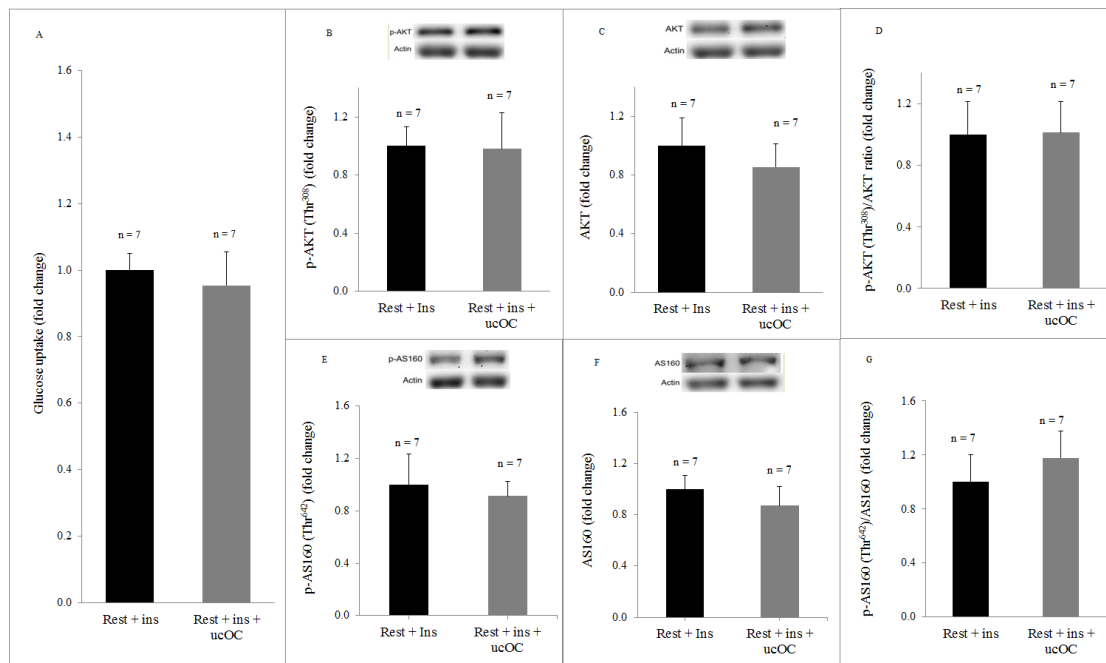


Figure 3.8. Comparison of paired EDL from the same animal post-insulin treatment compared to ucOC plus insulin (no contraction). Glucose uptake (A), p-Akt (B), Akt (C), p-Akt/Akt (D), p-AS160 (E), AS160 (F) and p-AS160/AS160 (G).

3.5 Discussion

Here we show, for the first time at the protein level, that the most likely candidate receptor for osteocalcin, GPPRC6A, is expressed in both sections of whole mouse muscle and in differentiated C2C12 myotubes. Silencing GPRC6A with siRNA directed against GPRC6A reduces the protein expression as analysed by western blot.

We also demonstrate that ucOC improves insulin stimulated glucose uptake in C2C12 myotubes at concentrations of 10 ng/mL and above. Furthermore, we report that recombinant ucOC potentiated the effect of *ex vivo* contraction on insulin sensitivity in mouse muscle. In the absence of insulin and/or in the absence of contraction, ucOC produced only a limited effect on muscle glucose uptake and the activity of signalling molecules known to be activated during insulin-stimulated glucose uptake: p-Akt or AS160 post-*ex vivo* contraction.

Previous studies have identified that ucOC is involved in the metabolic effects on target organs [24, 25, 58]. In addition, we previously observed that ucOC was significantly increased in obese men following exercise whereas total osteocalcin levels remained unchanged [31]. We thus chose to test the effects of ucOC on muscle glucose uptake *in vitro* and post contraction *ex vivo* in the current study.

Exercise increases insulin sensitivity for several hr after exercise [215, 216, 225], however, the mechanisms underlying this effect are not completely understood. In mice, ucOC improves insulin sensitivity in the periphery [57] and improves insulin sensitivity and glucose tolerance in glucocorticoid treated mice [56]. In a cohort of obese men, acute exercise increased ucOC which correlates with the reduction in serum glucose and the increase in insulin sensitivity [30, 31]. Here, we report that ucOC improves insulin sensitivity after contraction via a direct effect on EDL. We observed an increase in insulin-stimulated muscle glucose uptake, p-AS160 (Thr642) and to a lesser degree increased p-Akt (Thr308). This indicates ucOC participates in the increased insulin sensitivity following exercise/contraction through a mechanism involving the key insulin signalling proteins p-AS160 (Thr642) in mouse muscle.

Previous research indicated that p-AS160 (Thr642), is a convergence point between the pathways that regulate insulin- and contraction-stimulated Glut4 translocation to the membrane, thus playing an important role in skeletal muscle glucose uptake both post-contraction as well as post-insulin stimulation [221, 222, 226-228]. The results of the current study add important information regarding the

potential effect of ucOC on skeletal muscle, as our results are from an *ex vivo* model which is independent of neural inputs, blood flow, circulating hormones and other important yet possibly confounding factors.

The mechanism by which ucOC enhances insulin sensitivity in skeletal muscle post-contraction is not clear although previous reports showed ucOC affects insulin target genes in skeletal muscle including *Pgc1-α*, *Nuclear respiratory factor 1 (Nrf1)* and *Medium-chain acyl-coenzyme A dehydrogenase (Mcad)* [57]. GPCR6A is the main candidate receptor for osteocalcin in several tissues including the Leydig cells of the testes as well as β-cells and possibly adipocytes and in the liver [229]. It is therefore likely that the GPCR6A is a receptor for ucOC in skeletal muscle. While mRNA levels of GPCR6A have previously been shown in skeletal muscle [140, 230], here we demonstrated for the first time GPCR6A protein expression in both sections of mouse FDL muscle and differentiated C2C12 myotubes by both immunofluorescence and western blot. Furthermore, GPCR6A^{-/-} mice have glucose intolerance [231].

We have shown that ucOC treatment increases insulin-stimulated glucose uptake in C2C12 myotubes at concentrations of 10 ng/mL and higher. In contrast, in the *ex vivo* model ucOC treatment in the absence of insulin or in the absence of contraction had no or a minimal effect on muscle glucose uptake. It is not clear why glucose uptake *ex vivo* was not enhanced by ucOC in the absence of both contraction and insulin, given that previous investigators have reported that ucOC can reduce circulating glucose levels in mice [24, 25, 57, 66] and stimulate glucose transport at rest (basal) both with and without insulin in L6 myocytes [82], however, it may be due to the *ex vivo* model, which has not been tested before under these conditions. Importantly, muscle insulin sensitivity 4 hr post single leg exercise is much higher in the exercised than the rested leg during a euglycaemic-hyperinsulinemic clamp in humans [232]. Given that both legs would be exposed to the same concentration of ucOC and insulin, it is possible that ucOC has effects only in previously contracted

muscle exposed to insulin. It is also possible that the protocol used to identify changes in insulin sensitivity following ucOC treatment, at the resting state, was not optimal to provoke changes in muscle glucose uptake.

As we observed large differences in muscle glucose uptake across animals, even when mice were treated with the same treatment, we determined the effect of each intervention compared to the contralateral control (no intervention) EDL from the same mouse. We performed an ANOVA across all groups and obtained a variable difference of $P = 0.001$. However, due to inter-animal variability, and the problem of introducing a larger control group when compared to the intervention groups (as every mouse contributed their contralateral leg to the control group), the post-hoc tests were limited and delivered a P value of 0.08 for the comparison between contraction plus insulin and contraction with OC plus insulin. A more specific test, therefore, was to perform individual t-tests between each treatment group and the contralateral control leg (non-intervention) which produced a P value of 0.04 and a difference of ~12 % for these same two groups.

In the current study, we chose to use 10 ng/mL in the *ex vivo* experiments, as this was the optimal dose we observed in our C2C12 myotube glucose uptake experiments. We have also used doses of 0.3 ng/mL and 3 ng/mL with no effect on muscle glucose uptake *ex vivo* (data not shown). While higher doses may be expected to elicit greater responses, previous studies have shown a biphasic effect of increasing osteocalcin concentrations, with concentrations beyond 10 ng/mL having a reversal effect on some of the gene expression outputs known to be stimulated by osteocalcin including *Adiponectin*, *Pgc1- α* and *Uncoupling protein 1 (Ucp1)* [25]. In addition, contraction alone (in the absence of insulin) increases muscle glucose uptake during and immediately after contraction for 1 – 2 hr [103]. In contrast, insulin sensitivity may be enhanced after contraction for 24 – 48 hr [215, 216]. In the current study, muscle glucose uptake and phosphorylation of both Akt and AS160 tended to remain elevated 2 hr post-contraction alone (10 – 30 %; non-significant), when compared with rest

(basal). This may have interfered with the effect of ucOC treatment. Future studies with increased duration to 3 – 4 hr post-contraction to ensure that glucose uptake has returned to baseline are warranted [103]. Finally, only Akt and AS160 were studied due to the amount of muscle sample available. Future studies should determine: (a) the exact mechanism by which ucOC improves insulin stimulated glucose uptake in skeletal muscle and (b) whether ucOC acts directly via GPCR6A as well as examine other factors including insulin receptor expression, IRS1 and 2, TBC1D1, p-AMPK, and Glut4 that may also interact with ucOC.

In conclusion, the most likely candidate receptor for osteocalcin, GPPRC6A, is expressed in both sections of whole mouse muscle and in the mouse myoblast cell line, C2C12, and fully differentiated C2C12 myotubes. ucOC treatment augments insulin-stimulated skeletal muscle glucose uptake in C2C12 myotubes and it potentiates the insulin-sensitising effect of prior *ex vivo* muscle contraction in mouse muscle. ucOC elevation may partly account for the insulin-sensitising effect of exercise.

Chapter 4. Study 2: Recombinant uncarboxylated osteocalcin *per se* enhances mouse skeletal muscle glucose uptake in both extensor digitorum longus and soleus muscles

This study has been published as follows:

Lin X, Parker L, McLennan E, Zhang X, Hayes A, McConell G, Brennan-Speranza T.C, Levinger I. 2017. Recombinant uncarboxylated osteocalcin *per se* enhances mouse skeletal muscle glucose uptake in both extensor digitorum longus and soleus muscles. *Front Endocrinol*, 8. doi: 10.3389/fendo.2017.00330.

This study has been presented at these conferences:

Lin X, McLennan E, Parker L, McConell G, Zhang X, Hayes A, Brennan-Speranza T.C, Levinger I. Undercarboxylated osteocalcin enhances basal and insulin-stimulated glucose uptake in isolated mouse EDL and soleus muscles. *Metabolic Diseases: Breakthrough Discoveries in Diabetes and Obesity*, St Kilda, Victoria, Australia (2016).

Lin X, McLennan E, Parker L, McConell G, Zhang X, Hayes A, Brennan-Speranza T.C, Levinger I. Undercarboxylated osteocalcin enhances basal and insulin-stimulated glucose uptake in isolated mouse muscles *ex vivo*. *Australian and New Zealand Bone and Mineral Society Annual Meeting*, Brisbane, Queensland, Australia (2017).

Lin X, McLennan E, Parker L, McConell G, Zhang X, Hayes A, Brennan-Speranza T.C, Levinger I. Undercarboxylated osteocalcin enhances basal and insulin-stimulated glucose uptake in isolated mouse muscles *ex vivo*. *American Society for Bone and Mineral Research Annual Meeting*, Denver, Colorado, USA (2017).

Declaration of co-authorship and co-contribution

1. PUBLICATION DETAILS

Title of paper:	Recombinant uncarboxylated osteocalcin <i>per se</i> enhances mouse skeletal muscle glucose uptake in both extensor digitorum longus and soleus muscles		
Name of journal:	Front Endocrinol		
Surname:	Lin	First name:	Xuzhu
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Status:			
Accepted and in press:		Date:	
Published: ✓		Date:	11/2017

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

	6/3/2018
Signature	Date

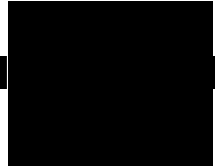
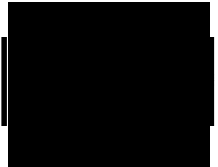
3. CO-AUTHOR(S) DECLARATION

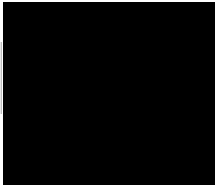
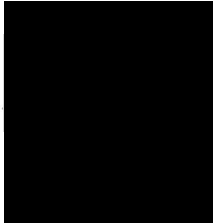
In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

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Lewan Parker	Analysed and interpreted the data; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Emma McLennan	Collected samples; Analysed and interpreted the data; Approved the final version of the paper.		6/3/2018
Xinmei Zhang	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Alan Hayes	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018

Glenn McConell	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Tara Brennan-Speranza	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Itamar Levinger	Designed the study; Analysed and interpreted the data; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018

4.0 General introduction

In Study 1, we reported that ucOC has limited effects on glucose uptake in resting mouse EDL. However, ucOC treatment exerted much greater effects on glucose uptake in cultured mouse myotubes. Thus, we hypothesised that the lack of effect of ucOC stimulation in Study 1 is attributed to the use of intact isolated muscles, as intact muscles are closely wrapped by connective tissue layers which could limit the contact of ucOC with myotubes [36].

Therefore, in Study 2 we performed ucOC stimulation in longitudinally divided/split muscle, a method that has been used by many other researchers for *ex vivo* stimulations in muscle tissue [233-235]. With this modification of methodology, we aimed to test the hypothesis that ucOC *per se* increases glucose uptake independent of insulin in both mouse EDL and Soleus (without *ex vivo* contraction), and investigating the involved signalling pathway(s).

4.1 Abstract

Emerging evidence suggests that ucOC improves muscle glucose uptake in rodents. However, whether ucOC can directly increase glucose uptake in both glycolytic and oxidative muscles and the possible mechanisms of action still need further exploration. We tested the hypothesis that ucOC *per se* stimulates muscle glucose uptake via ERK, AMPK, and/or mTORC2/Akt/AS160 signalling cascade. EDL and Soleus from male C57BL/6 mice were isolated, divided into halves and then incubated with ucOC with or without the pretreatment of ERK inhibitor U0126. ucOC increased muscle glucose uptake in both EDL and Soleus. It also enhanced phosphorylation of ERK2 (Thr202/Tyr204) and AS160 (Thr642) in both muscle types, and increased mTOR phosphorylation (Ser2481) in EDL only. ucOC had no significant effect on the phosphorylation of AMPK α (Thr172). The inhibition of ucOC-induced ERK phosphorylation had limited effect on ucOC-stimulated glucose uptake and AS160 phosphorylation in both muscle types, but appeared to inhibit the elevation in Akt phosphorylation only in EDL. Taken together, ucOC at the physiological range directly increased glucose uptake in both EDL and Soleus in mice. The molecular mechanisms behind this ucOC effect on muscle glucose uptake seem to be muscle type-specific, involving enhanced phosphorylation of AS160 but limitedly modulated by ERK phosphorylation. Our study suggests that, since ucOC increases muscle glucose uptake without insulin, it could be considered as a potential agent to improve muscle glucose uptake in insulin-resistant conditions.

4.2 Introduction

The skeleton is an endocrine organ that has been shown, at least in mice, to modulate glucose metabolism [22, 57, 236]. One bone-specific hormone that plays a role in this energy regulation is osteocalcin [60, 237]. ucOC, the biologically active form of OC, regulates glucose metabolism by targeting the pancreas and perhaps several

insulin-sensitive organs, including skeletal muscle [24, 25, 56]. The effect of ucOC on skeletal muscle may have important clinical implications for whole-body glycaemic control as it is the major site for glucose disposal and storage [30, 238]. It has been reported that ucOC increases insulin sensitivity in rodent skeletal muscle [23, 29, 72, 82, 239]. Recent evidence also suggests that ucOC may enhance muscle glucose uptake in the absence of insulin. For example, it has been shown that 10 ng/mL ucOC increases glucose uptake in C2C12 myotubes, and to a lesser extent in *ex vivo* Soleus which mainly relies on oxidative metabolism for energy production, but not in *ex vivo* EDL which largely utilise glycolytic metabolism as the energy source [23]. Similarly, in our previous study we did not observe any effect of ucOC on glucose uptake of non-contracted EDL *ex vivo* [239]. However, since GPRC6A, the presumable receptor for ucOC, is expressed in both EDL and Soleus [23, 239], the regulation on muscle glucose uptake by ucOC in both muscle types is still possible. We hypothesise that the limited direct effects of ucOC that was previously observed on EDL were likely due to the inadequateness of ucOC to access the internal area of intact muscle *in vitro*. Therefore, it is possible that a methodological limitation affected the results and improved techniques such as the application of muscle strips, which was previously performed by Cartee et al. [235] and others [233, 234], need to be introduced.

Furthermore, the potential mechanisms behind ucOC *per se* effect on skeletal muscle glucose uptake are still largely unknown. Our previous report exhibited enhanced insulin-stimulated glucose uptake and AS160 phosphorylation at Thr642 by ucOC treatment in EDL post-*ex vivo* contraction [239]. Insulin-induced phosphorylation of AS160, and subsequent increases in glucose uptake, requires fully activated Akt via the activation of mTORC2, which can be indicated by the phosphorylation of Akt at Ser473 and the phosphorylation of mTOR at Ser2481 [145, 240-242]. The mTORC2/Akt/AS160 signalling cascade can be stimulated not only by insulin, but also other growth factors and stimuli [243-245]. Recent findings indicate that ucOC may also be able to trigger this signalling pathway. In vascular smooth muscle cells, the phosphorylation of Akt was enhanced by the treatment of purified

bovine OC [246]. Furthermore, the phosphorylation of Akt at Ser473 was elevated following OC treatment in descending thoracic aortic strips of Apolipoprotein E-deficient (ApoE-KO) mice [247]. In addition, the phosphorylation of Akt (Ser473) was increased in C2C12 myotubes with ucOC exposure during cell differentiation [29]. Nevertheless, the upstream pathways that result in the phosphorylation of Akt and AS160 by ucOC are still unclear. It is possible that two previously identified downstream targets of ucOC, ERK and AMPK, may be involved [248, 249]. Indeed, in atrophic rat muscles lower serum ucOC levels were associated with lower phosphorylation levels of ERK (Thr202/Tyr204) and AMPK (Thr172), and the phosphorylation levels of ERK positively correlated with the phosphorylation levels of Akt (Ser473) in EDL [250]. In C2C12 myotubes, ucOC stimulated ERK phosphorylation (Thr202/Tyr204) likely contributed to the increase of Akt phosphorylation at Ser473 [29]. Furthermore, exercise-induced p-AMPK (Thr172) was augmented by ucOC injection in mice tibialis muscle, which could be responsible for ucOC-enhanced exercise-stimulated muscle glucose uptake [23].

Therefore, the aims of this study were to (a) test the hypothesis that physiological levels of ucOC *per se* increases glucose uptake in both EDL and Soleus; and (b) explore the mechanisms underlying the effects of ucOC on muscle glucose uptake.

4.3 Materials and methods

4.3.1 Animals

8 wk-old male C57BL/6J mice ($N = 55$) were purchased from Animal Resources Centre (ARC, Western Australia, Australia). All mice were group housed with a 12-hr light/12-hr dark cycle and fed standard laboratory chow (Specialty Feeds mouse food cubes (Glen Forrest, Western Australia, Australia) containing 20 % protein, 4.8 % fat and the rest carbohydrate and fibre) and water *ad libitum* until 9 – 12 wk old. The study was approved by the Animal Experimentation Ethics Committee of Victoria

University (AEC14/009) and conformed to the Australian National Code of Practice for the Care and Use of Animals for Scientific Purposes. The mice for each group in this study were randomly allocated.

4.3.2 Muscle dissection

Mice were fasted for 4 hr before deep anaesthetisation with 60 mg/kg intraperitoneal pentobarbital. Left and right EDL and Soleus were excised within 30 min of anaesthesia. Isolated muscles were bathed in carbogenated KHB (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, pH 7.4) and evenly divided into halves longitudinally. After muscle dissection, mice were euthanised via cervical dislocation under anaesthesia.

4.3.3 ucOC stimulation

Muscles were evenly divided longitudinally into halves to improve the infusion of ucOC into muscle fibre *ex vivo*, similar to what has been performed in rat muscle in previous studies [233-235]. The whole ucOC stimulation process is shown in **Figure 4.1**. In experiments without the ERK inhibitor U0126, muscle samples were pre-incubated in 30 °C baths containing carbogenated KHB for 1 hr. In experiments with U0126 ($N = 5$), after 30 min pre-incubation, muscle samples were exposed to the ERK inhibitor U0126 (1 μ M) (Cell Signaling, Massachusetts, United States) or dimethyl sulfoxide (DMSO) vehicle (Sigma Aldrich, Missouri, United States) for 30 min. Then muscle samples were stimulated for 90 min with increasing doses (0 ng/mL ($N = 6$), 0.3 ng/mL ($N = 10$), 3 ng/mL ($N = 10$), 10 ng/mL ($N = 14$), or 30 ng/mL ($N = 10$)) of recombinant ucOC (Bachem, Bubendorf, Switzerland). These doses of ucOC were chosen because they are within the physiological range in mice [54, 56]. In experiments without U0126, muscle halves from the same mouse were treated with KHB control or ucOC. In experiments with U0126, muscle halves from the same

mouse were treated with DMSO, DMSO with ucOC, U0126, and U0126 with ucOC, respectively.

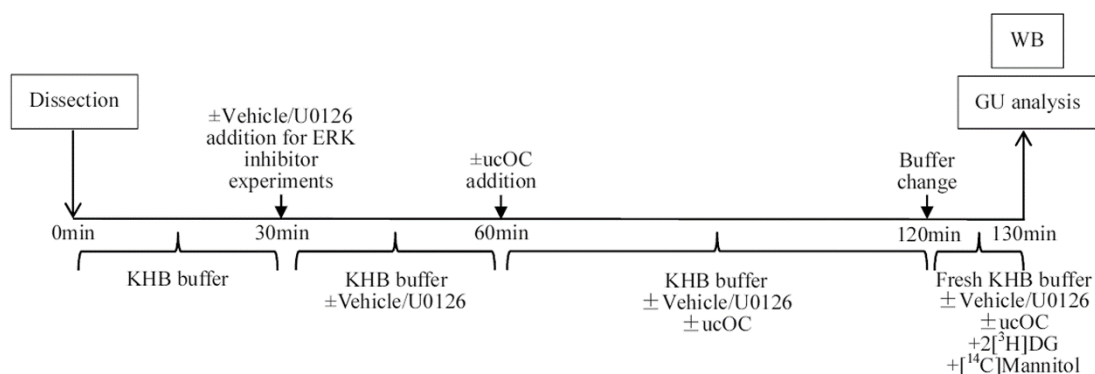


Figure 4.1. The flowchart of experimental procedure in Study 2.

4.3.4 2-DG uptake measurement and sample homogenisation

The method to assess 2-DG uptake has been described previously [239]. Briefly, after the 90 min ucOC treatment, muscles were transferred to chambers containing KHB + 0.1 % bovine serum albumin (BSA) (Sigma Aldrich) + 2 mM 2-Deoxy-D-[1,2-³H]-glucose (PerkinElmer, Massachusetts, United States) and 16 mM D-[1-¹⁴C] mannitol (PerkinElmer) with or without U0126/Vehicle or ucOC. After 10 min, muscles were rapidly rinsed with ice cold KHB, then immediately frozen in liquid nitrogen. On the day of sample processing, muscle samples were lysed in ice-cold RIPA buffer (60 μ L RIPA for 1mg sample) (Cell Signaling) with Inhibitor Cocktail (Cell Signaling) and 100mM dithiothreitol (Sigma Aldrich) using TissueLyser II (QIAGEN, Hilden, Germany) followed by gentle rocking at 4 °C for 1 hr. Half of the lysate was pipetted into vials with scintillation cocktail for scintillation counting (β -counter) with Tri-Carb 2910TR Liquid Scintillation Analyzer (PerkinElmer) and the other half was used in western blotting.

4.3.5 Western blotting

After muscle samples were homogenised using RIPA buffer, protein concentrations in the lysate were determined by Bio-Rad Protein Assay (Bio-Rad, California, United States). Equal amounts of protein were subjected to electrophoresis on Criterion Stain-Free precast gels (10 %; Bio-Rad) and then transferred electrophoretically using Trans-Blot Turbo Transfer System (Bio-Rad) onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Then a stain-free blot image was taken using ChemiDoc Imaging System (Bio-Rad) for total protein measurement in each sample lane. Immunoblotting was performed at optimum conditions for each antibody. Bands were identified using ChemiDoc Imaging System, using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Massachusetts, United States). Band densities of both stain-free blot and immunoblotting were measured using Image Lab Software (Bio-Rad). Values of immunoblotting bands were normalised using total protein values. p-ERK (Thr202/Tyr204), ERK, p-AMPK α (Thr172), AMPK α , p-mTOR (Ser2481), mTOR, p-Akt (Ser473), Akt, p-AS160 (Thr642), AS160, and p-PKC δ/θ (Ser643/676) antibodies were purchased from Cell Signaling.

Two data points for AMPK α phosphorylation assessment were excluded due to western blot imaging artefacts. However, their exclusion did not alter the statistical outcome, interpretation or conclusions of the results.

4.3.6 Statistical analysis

Fold-changes for western blotting data were calculated by normalisation to control groups within the same animals. 3 ng/mL group and 30 ng/mL group were chosen for western blotting and correlation analysis as representatives of low and high doses of ucOC.

Paired t-tests were used to analyse the effects of ucOC, for each individual concentration, on muscle glucose uptake, protein phosphorylation, protein abundance,

and phospho/total ratio compared to paired control samples. This paired comparison was used to exclude individual variances.

To analyse the dose-response effects of ucOC on muscle glucose uptake, basal glucose uptake data from all groups were combined, then one-way ANOVA with Tukey post hoc test was applied.

Spearman's correlation was performed between the variables from 30 ng/mL ucOC treatment group. Rule of thumb for interpreting the size of a correlation coefficient will be applied to measure the strength of correlation between two variables [251]. According to standard practice thresholds, the r ranges for negligible positive, low positive, moderate positive, high positive, and very high positive correlations are defined as $0.00 < r < 0.30$, $0.30 \leq r < 0.50$, $0.50 \leq r < 0.70$, $0.70 \leq r < 0.90$, and $0.90 \leq r \leq 1.00$, respectively.

All figures and analyses were performed using GraphPad 6 (GraphPad Software, La Jolla, CA, USA).

All data are reported as mean $\pm$ SEM.

4.4 Results

4.4.1 ucOC increased glucose uptake in both EDL and Soleus

Compared with paired controls, muscle glucose uptake was significantly higher following the treatment of ucOC at doses of 10 ng/mL ($P < 0.05$) and 30 ng/mL ($P < 0.01$) in EDL, and 0.3 ng/mL ($P < 0.01$) and 30 ng/mL ($P < 0.01$) in Soleus (**Figure 4.2A** and **4.2B**). When data were analysed for ucOC dose-response effects, ucOC significantly enhanced glucose uptake at doses equal or larger than 3 ng/mL in EDL and at a dose of 30 ng/mL in Soleus (**Figure 4.2C** and **4.2D**; $P < 0.05$, ANOVA $P < 0.01$), from 2.91 $\mu\text{mol/g/hr}$ to 4.32 $\mu\text{mol/g/hr}$ and from 3.12 $\mu\text{mol/g/hr}$ to 4.16 $\mu\text{mol/g/hr}$, respectively.

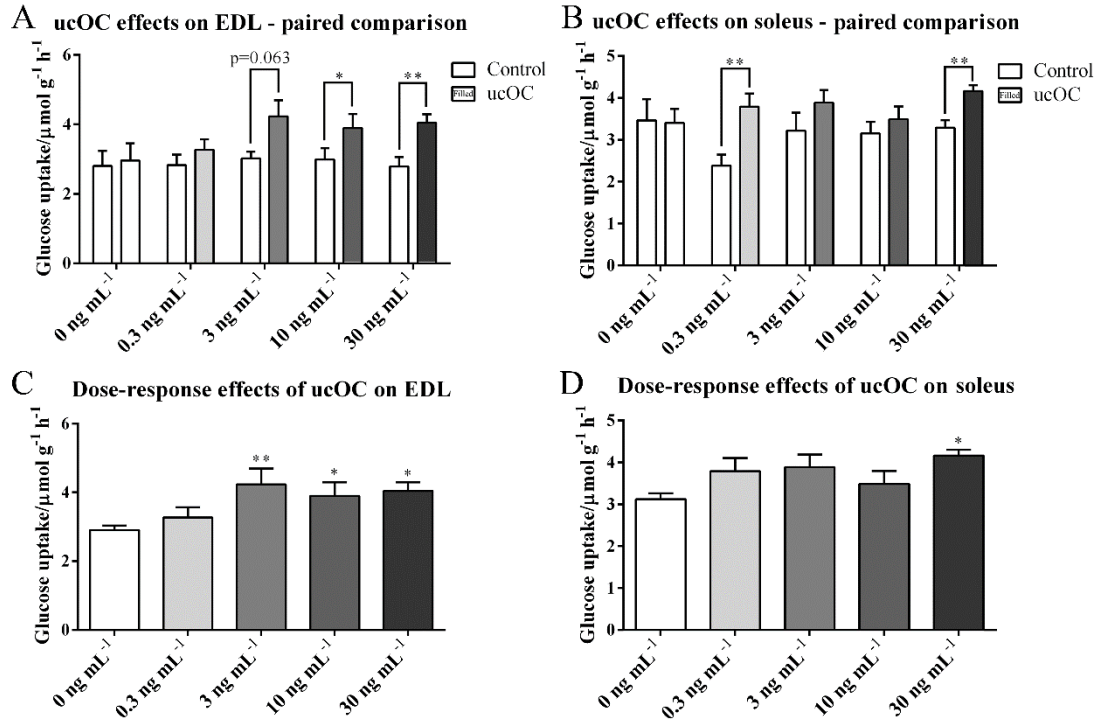


Figure 4.2. ucOC effects on and insulin-stimulated glucose uptake in EDL and Soleus. (A, B) glucose uptake of EDL and Soleus samples treated with KHB control and ucOC (0 ng/mL (mice $N = 6$), 0.3 ng/mL (mice $N = 10$), 3 ng/mL (mice $N = 10$), 10 ng/mL (mice $N = 14$), or 30 ng/mL (mice $N = 10$)) was detected. $*P \leq 0.05$ and $**P \leq 0.01$ between paired samples (paired t-test); (C, D) glucose uptake of EDL and Soleus samples was analysed for dose-response effect of ucOC with combined basal levels. Samples sizes for groups treated with 0 ng/mL, 0.3 ng/mL, 3 ng/mL, 10 ng/mL, or 30 ng/mL ucOC are 56, 10, 10, 14, or 10, respectively. $*P \leq 0.05$ and $**P \leq 0.01$ in Tukey's post-hoc test (compared with 0 ng/mL ucOC samples) of one-way ANOVA analysis.

4.4.2 ucOC stimulated the phosphorylation of mTOR, Akt, and AS160

In EDL, ucOC treatment at 30 ng/mL significantly increased p-mTOR (1.37-fold, $P < 0.05$, **Figure 4.3A**) and p-mTOR/tmTOR ratio (1.40-fold, $P < 0.05$, **Figure 4.3A**), and only tended to increase p-Akt (1.25-fold, $P = 0.074$, **Figure 4.3C**) but not p-Akt/tAkt ratio. Neither of these signalling molecules was affected in Soleus (**Figure 4.3B** and **4.3D**). In both EDL and Soleus, both p-AS160 and p-AS160/tAS160 ratio were considerably elevated 1.4-fold to 1.8-fold following ucOC treatments at 3 ng/mL ($P < 0.05$ and $P < 0.05$, **Figure 4.3E**; $P = 0.059$ and $P = 0.056$, **Figure 4.3F**) and 30

ng/mL ($P < 0.01$ and $P < 0.05$, **Figure 4.3E**; $P < 0.01$ and $P < 0.001$, **Figure 4.3F**). Total AS160 expression was also increased by ng/mL ucOC in EDL (1.13-fold, $P < 0.05$, **Figure 4.3E**).

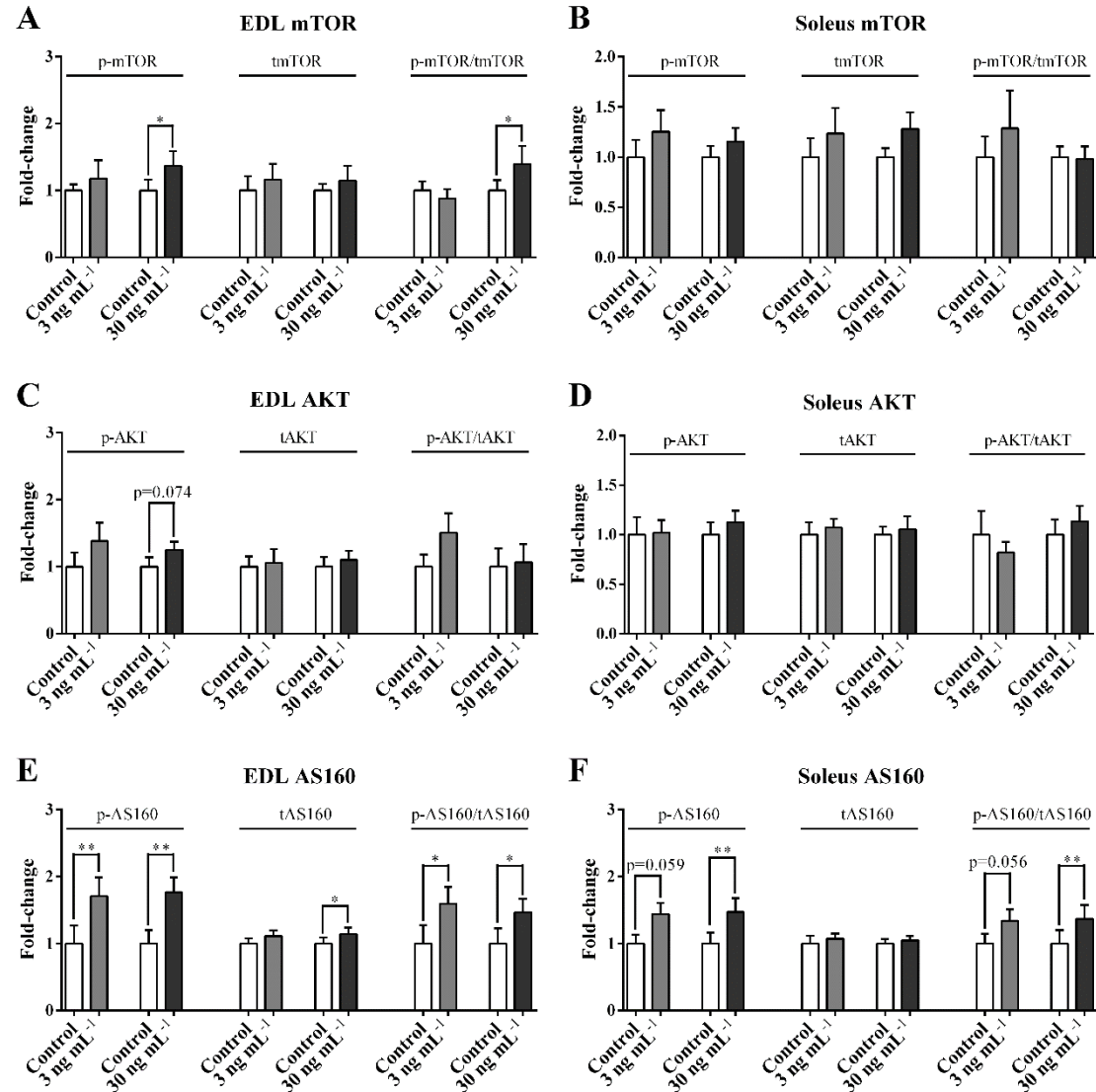


Figure 4.3. ucOC effects on the phosphorylation of mTOR, Akt, and AS160. The phosphorylation levels, total expression levels, and phospho/total ratio levels of mTOR (**A, B**), Akt (**C, D**), and AS160 (**E, F**) of EDL and Soleus samples treated with KHB control and ucOC (3 ng/mL and 30 ng/mL, $N = 10$ for each dose) were examined. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ paired samples from the same animal (t-test).

4.4.3 ucOC stimulated the phosphorylation of ERK but not AMPK

p-ERK2, but not p-ERK2/tERK2 ratio, was increased by the treatment of 30 ng/mL ucOC (1.14-fold, $P < 0.05$, **Figure 4.4A**) in EDL, and by both 3 ng/mL and 30 ng/mL

of ucOC in Soleus (1.24-fold and 1.17-fold, $P < 0.05$ and $P < 0.01$, **Figure 4.4B**). ucOC at 3 ng/mL or 30 ng/mL had limited effects on AMPK α phosphorylation in both EDL and Soleus. (**Figure 4.4C** and **4.4D**). However, Soleus total AMPK α levels were increased by the treatment of 30 ng/mL ucOC (1.30-fold, $P < 0.01$, **Figure 4.4D**).

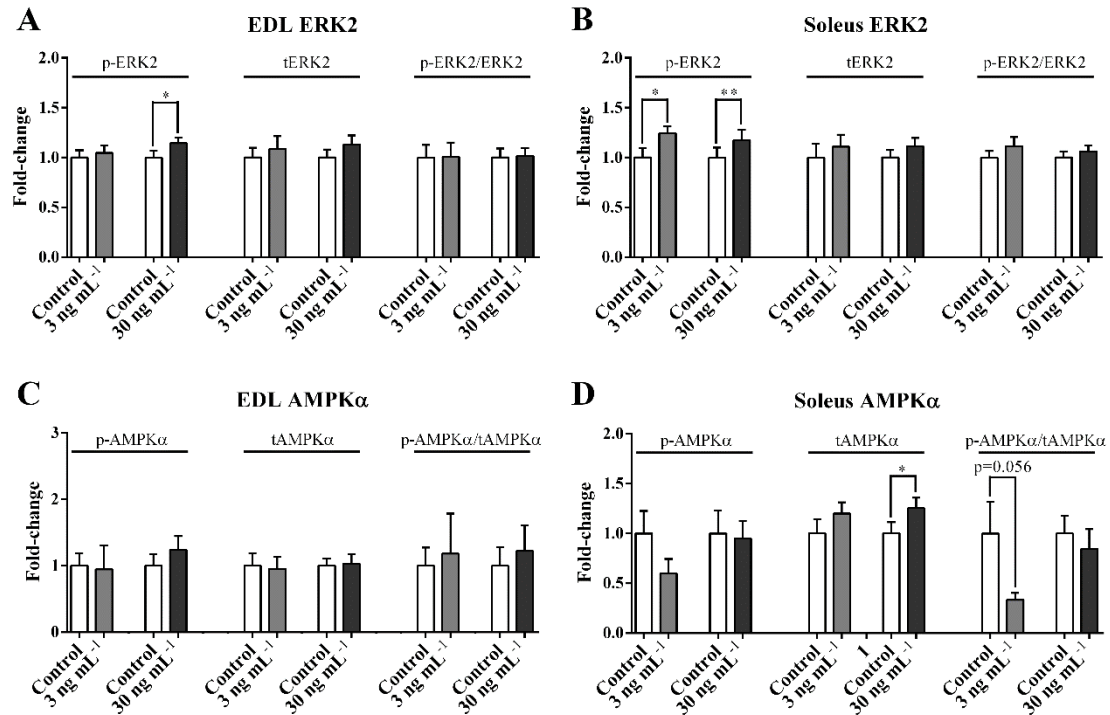


Figure 4.4. ucOC effects on the phosphorylation of ERK2 and AMPK α . The phosphorylation levels, total expression levels, and phospho/total ratio levels of ERK2 (**A**, **B**) and AMPK α (**C**, **D**) of EDL and Soleus samples treated with KHB control and ucOC (3 ng/mL and 30 ng/mL, $N = 9 - 10$ for each dose) were examined. * $P \leq 0.05$ and ** $P \leq 0.01$ paired samples from the same animal (t-test).

Treatment with 30 ng/mL ucOC had limited effects on phosphorylated protein kinase C δ/θ (PKC δ/θ) in both EDL and Soleus (**Figure 4.5**).

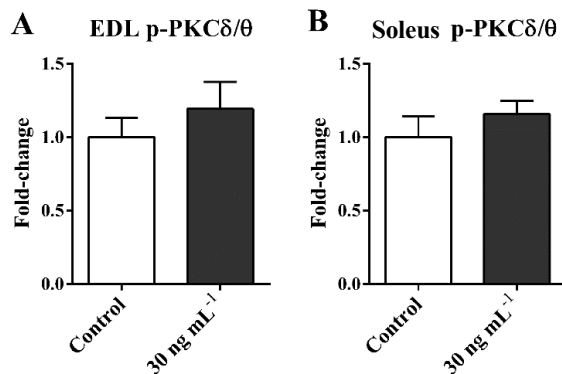


Figure 4.5. ucOC effects on the phosphorylation of PKC δ/θ . The phosphorylation levels of PKC δ/θ in EDL (**A**) and Soleus (**B**) samples treated with KHB control and 30 ng/mL ucOC were examined ($N = 10$).

4.4.4 The phosphorylation levels of ERK2 correlated with the phosphorylation levels of Akt and AS160

p-ERK2 levels were not associated with glucose uptake levels (**Figure 4.6A** and **4.6B**) or p-mTOR levels (**Figure 4.6C** and **4.6D**), in either EDL or Soleus. Higher levels of p-ERK2 were associated with higher levels of p-Akt in EDL ($P < 0.05$, **Figure 4.6E**) with a low positive correlation ($r = 0.48$), but not in Soleus (**Figure 4.6F**). In both EDL ($P < 0.05$, **Figure 4.6G**) and Soleus ($P < 0.001$, **Figure 4.6H**), higher p-ERK2 levels were associated with higher levels of p-AS160, with a low positive correlation ($r = 0.48$) and a high positive correlation ($r = 0.85$), respectively.

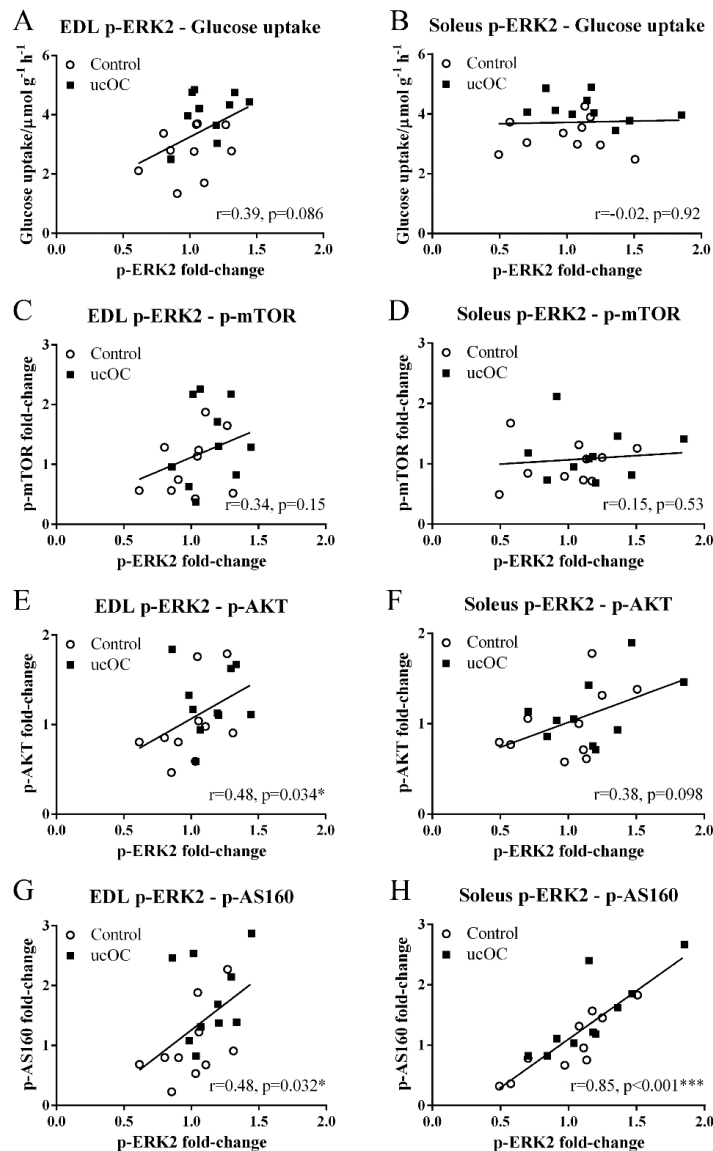


Figure 4.6. The correlations between the levels of p-ERK2 and the levels of glucose uptake, p-mTOR, p-Akt, and p-AS160. In EDL and Soleus samples, the correlations between the levels p-ERK2 fold-change and the levels of glucose uptake (**A, B**), p-mTOR fold-change (**C, D**), p-Akt fold-change (**E, F**), and p-AS160 fold-change (**G, H**) were analysed among samples from 30 ng/mL ucOC treatment group. *, and *** represent $P \leq 0.05$, and $P \leq 0.001$.

p-AMPK α levels were not associated with glucose uptake or any signalling protein phosphorylation levels in either muscle type (data not shown).

4.4.5 The prevention of ucOC-induced ERK phosphorylation had limited effect on ucOC-stimulated muscle glucose uptake

Pre-incubation with 1 μ M U0126 blocked ucOC (30 ng/mL)-induced increases in ERK2 phosphorylation in both EDL and Soleus (**Figure 4.7A and 4.7B**). However, the addition of inhibitor did not significantly affect ucOC-stimulated muscle glucose uptake (**Figure 4.7C and 4.7D**).

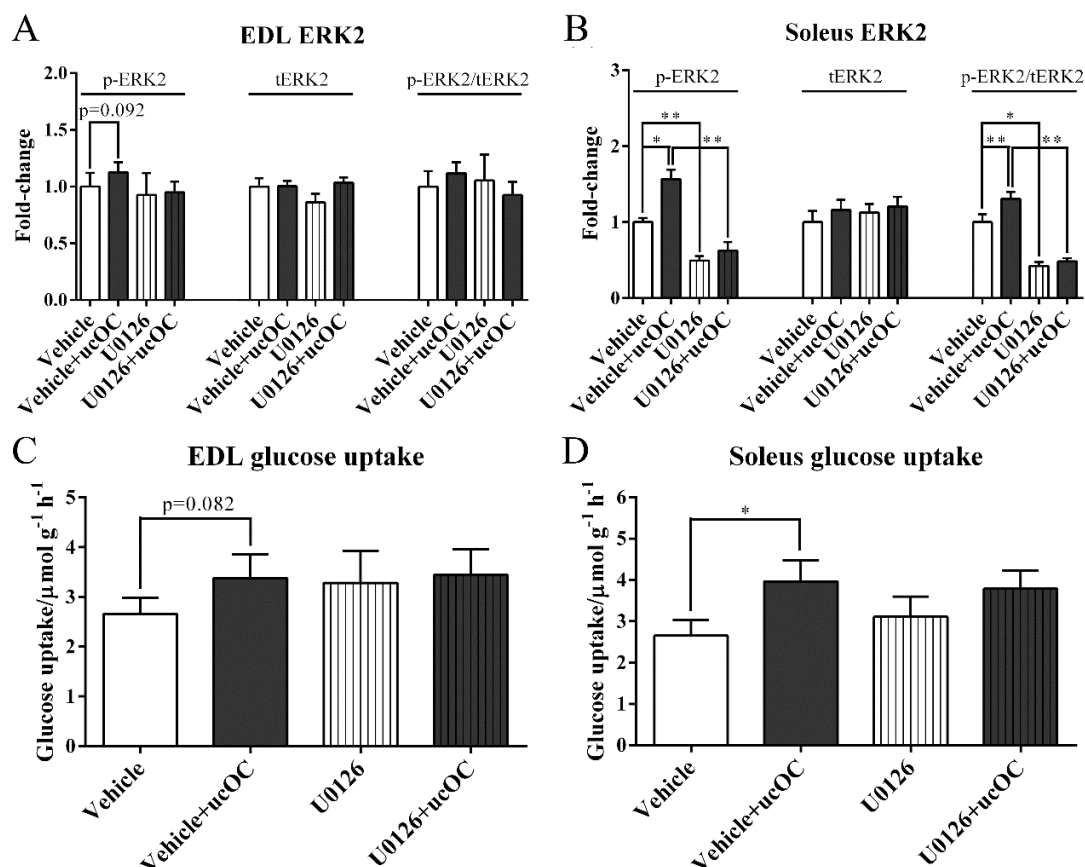


Figure 4.7. The effects of the removal of p-ERK2 enhancement by U0126 on ucOC-stimulated muscle glucose uptake. (A, B) The phosphorylation levels, total expression levels, and phospho/total ratio levels of ERK2 in samples treated with DMSO vehicle, vehicle plus 30 ng/mL ucOC, U0126 (1 μ M), and U0126 plus ucOC were assessed in EDL and Soleus; (C, D) The glucose uptake of samples treated with DMSO vehicle, vehicle plus ucOC (30 ng/mL), U0126 (1 μ M), or U0126 plus ucOC were examined in EDL and Soleus. * $P \leq 0.05$ and ** $P \leq 0.01$ between paired samples (t-test).

4.4.6 The removal of ucOC-induced ERK phosphorylation prevents ucOC-stimulated Akt phosphorylation in EDL, but has limited effect on the phosphorylation of mTOR and AS160 in both muscle types

U0126 (1 μ M) had limited effect on p-mTOR following ucOC treatment in either EDL or Soleus (**Figure 4.8A** and **4.8B**). However, it somewhat prevented the ucOC-mediated Akt activation in EDL with a change close to significant observed in phosphorylation levels ($P = 0.06$), but not in Soleus (**Figure 4.8C** and **4.8D**). Although AS160 phosphorylation shared similar patterns of modulation with those of Akt following the treatments, ucOC-stimulated AS160 phosphorylation levels were only marginally decreased by U0126 addition in both muscle types ($P > 0.1$; **Figure 4.8E** and **4.8F**).

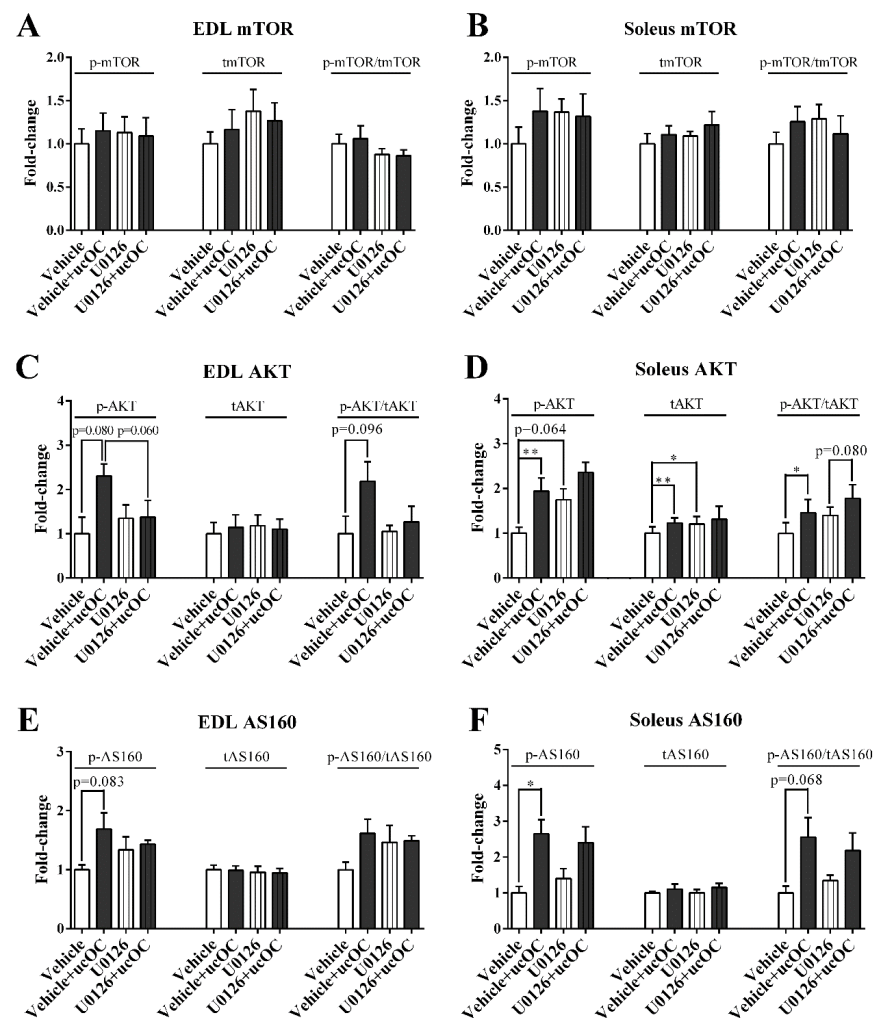


Figure 4.8. The effects of the removal of p-ERK2 enhancement by U0126 on ucOC-stimulated p-mTOR, p-Akt, and p-AS160. The phosphorylation levels, total expression levels, and phospho/total ratio levels of mTOR (**A**, **B**), Akt (**C**, **D**) and AS160 (**E**, **F**) of samples treated with DMSO vehicle, vehicle plus 30 ng/mL ucOC, 1 μ M U0126, and U0126 plus ucOC

were assessed in EDL and Soleus ($N = 5$). * $P \leq 0.05$ and ** $P \leq 0.01$ between paired samples from the same animal (t-test).

4.5 Discussion

We report that physiological levels of ucOC *per se* increased muscle glucose uptake *ex vivo* in both EDL (glycolytic muscle) and Soleus (oxidative muscle). Furthermore, ucOC increased the phosphorylation of ERK2, mTOR, and AS160 in EDL and enhanced the phosphorylation of ERK2 and AS160 in Soleus. It appears that ERK phosphorylation was not directly involved in ucOC-stimulated glucose uptake and AS160 phosphorylation in both muscle types.

We, and others, have previously reported that ucOC had no significant effect on resting EDL muscle glucose uptake, indicating that ucOC *per se* probably only up-regulates muscle glucose metabolism in oxidative muscle fibres [23, 239]. However, since the expression of GPRC6A, which is reported as the plausible receptor of ucOC, has been found in both muscle types [23, 239], we suggested that the results of these studies were affected by a potential methodological limitation that the usage of intact whole muscles may prevent adequate ucOC exposure to all muscle fibres. *In vivo*, muscle fibres are closely fed by capillaries that penetrate the epimysium and bifurcate throughout the muscle, primarily within the perimysium [36]. Since both epimysium and perimysium belong to robust collagenous connective tissue networks, without the help of blood vessels, ucOC in external solution may have limited direct contact with fibres of intact muscles during *ex vivo* incubation. By utilising the method of splitting muscles longitudinally into halves [233-235], in order to increase the ucOC saturation during treatment, we report that ucOC can increase muscle glucose uptake in the absence of insulin in both glycolytic (EDL) and oxidative (Soleus) muscles, suggesting the effect of ucOC on skeletal muscle glucose uptake is likely universal rather than muscle-type specific. It also seems that, compared with EDL, higher doses of ucOC are required for observing this effect on the glucose uptake of Soleus (**Figure 4.2C** and **4.2D**). Although there was a significant increase in Soleus treated with 0.3 ng/mL ucOC using paired comparison method (**Figure 4.2B**), this increase could not be observed when data were analysed

using one-way ANOVA. Thus, it was likely that this increase was merely resulted from an abnormally low control levels in that specific group. Since skeletal muscle is the major site of glucose disposal and utilisation in the postprandial state [238], these findings implicate ucOC as a possible therapeutic agent to improve muscle glucose transport even without insulin.

However, it should be noted that even we introduced muscle split in this study to enhance the interaction between ucOC to and interior muscle myotubes beneath muscle surface, some limitations which might result in enhanced biological variations, such as different ucOC saturation percentages due to different muscle dimensions, and different basal glucose uptake levels of different individuals, still cannot be ruled out. Therefore, future studies should explore the effect of ucOC in primary myotubes from animals and human, to avoid these limitations.

We report that ucOC treatment activated the mTORC2-Akt-AS160 signalling cascade in skeletal muscle, in a muscle type-specific manner. Importantly, ucOC elicited significant increases in AS160 phosphorylation (Thr642) despite relatively modest increases in Akt phosphorylation (Ser473) ($P = 0.074$). Therefore, ucOC may enhance AS160 phosphorylation via Akt independent mechanisms, which may be the major mechanisms underlying the ucOC-induced enhancement of glucose uptake and AS160 phosphorylation. Indeed, several other signalling proteins are also able to increase AS160 phosphorylation, including AMPK and c/nPKC [252].

Along with ERK, previous studies have suggested that AMPK is a potential downstream target of the ucOC signalling in skeletal muscle [23, 29]. Our data shows that following 30 ng/mL ucOC treatment, there is a significant increase in phosphorylated ERK2 (Thr202/Yyr204) in both EDL and Soleus. However, there was limited change in AMPK α phosphorylation at Thr172, a phosphorylation site that has widely been reported as an indicator of AMPK activity [253-255]. Similarly, it has been reported that AMPK α Thr172 phosphorylation was not increased following ucOC treatment in C2C12 cells [29]. A recent paper reported that intraperitoneal

injection of osteocalcin increased AMPK phosphorylation in mouse muscles during exercise [23]. Given that AMPK Thr172 phosphorylation is increased in skeletal muscle after acute exercise alone, without ucOC [256, 257], it is possible that ucOC treatment merely has an additive effect on exercise-enhanced phosphorylation of AMPK, but is unable to increase its phosphorylation level *per se*. However, it should be noted that until now no studies have shown ucOC-induced change of AMPK phosphorylation over the course of time. Thus, a transient increase of AMPK phosphorylation after ucOC treatment still cannot be ruled out.

We neither observed any significant increases in the phosphorylation in PKC δ/θ , two important members in novel PKC family, in both muscle types (**Figure 4.5**). This finding suggests a limited role of PKC δ/θ in the modulation of muscle glucose uptake by ucOC. However, the involvement of other types of PKC in the mechanisms behind this ucOC effect is still possible, which warrants further investigation.

We reported that p-Akt in EDL and p-ERK2 in both EDL and Soleus were enhanced following ucOC treatment with no significant changes in phospho/total ratio (**Figure 4.3C, 4.4A, and 4.4B**). This discrepancy may be attributed to an ucOC-induced modest increase in total protein expression (**Figure 4.3E and 4.4D**), in addition to its effect on protein phosphorylation. Consistent with our finding, protein synthesis has recently been reported to increase in mouse myotubes following 1 - 2 hr of ucOC treatment [26, 27]. It is possible that ucOC regulates kinase activity by both enhancing protein phosphorylation, and, to a lesser extent, increasing protein abundance.

We report a low positive correlation between p-ERK2 levels and p-Akt levels as well as a high positive correlation between p-ERK2 levels and p-AS160 levels in EDL, which was consistent with our previous findings showing that lower p-ERK levels were associated with lower p-Akt levels in rat EDL [250]. In Soleus, p-ERK2 levels were associated with p-AS160 levels, with a low positive correlation. As such,

we investigated whether the removal of ucOC-mediated ERK phosphorylation leads to the suppression of the effect of ucOC on skeletal muscles. Pre-treatment with 1 μ M U0126 blocked ucOC-stimulated increases in ERK phosphorylation in both muscle types. In EDL, it seems that the inhibition of p-ERK2 blocked ucOC-stimulated Akt phosphorylation ($P = 0.06$). Consistently, a previous finding also suggested that partial ERK inhibition dampened ucOC-stimulated Akt phosphorylation in C2C12 myotubes [29]. In contrast, in response to the loss of ucOC-induced ERK phosphorylation, ucOC-stimulated glucose uptake and AS160 phosphorylation was not compromised. Similarly, in Soleus the inhibition of p-ERK2 had limited effects on ucOC-stimulated glucose uptake and the phosphorylation of signalling proteins. These findings suggest that mechanisms underlying ucOC stimulation in skeletal muscle are probably muscle type-specific, but converging at AS160 phosphorylation, both resulting in the enhancement of glucose uptake. The ucOC-stimulated mTOR phosphorylation was also not modulated by U0126 pre-treatment in both muscle types. Thus, whether ERK signalling modulates Akt phosphorylation through mTORC2 needs further investigations.

The administration of U0126 as an ERK inhibitor has limitations. One limitation derives from the influence of vehicle DMSO, which is widely used as the solvent for U0126. DMSO has been shown to exert some impact on skeletal muscle, such as depressing muscle contractility and accelerating muscle injury [258, 259]. In our study, by comparing the results shown in **Figure 4.3** with those in **Figure 4.8**, it is suggested that even the presence of a low concentration of DMSO (0.1 %) may have slightly altered the ucOC effect on the phosphorylation of mTOR in EDL and Akt in Soleus. However, since DMSO was universally added to all samples in experiments involving the prevention of ucOC-induced ERK phosphorylation, the conclusions drawn from comparisons between these samples are unlikely to be affected by DMSO addition. The other limitation of U0126 administration in this study is the capability of U0126 to enhance glucose uptake and Akt phosphorylation by itself [260-262]. It has been suggested that this effect is due to the elevation of AMPK activity that is

independent of ERK inhibition [263]. In the current study, the application of low dose (1 μ M) of U0126 had a limited effect on AMPK phosphorylation in both muscle types (**Figure 4.9**), and glucose uptake and phosphorylation of most other signalling proteins were also minimally or not at all affected. Nevertheless, other inhibitors or methodologies for ERK inhibition should be investigated in future studies to confirm the involvement of ERK in the regulation of Akt phosphorylation.

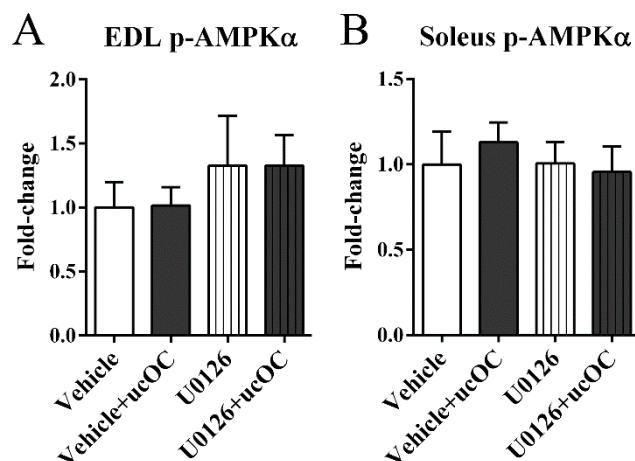


Figure 4.9. The effects of the removal of p-ERK2 enhancement by U0126 on ucOC-stimulated p-AMPK α . The phosphorylation levels of AMPK α at Thr172 in EDL (A) and Soleus (B) samples treated with treated with DMSO vehicle, vehicle plus 30 ng/mL ucOC, 1 μ M U0126, and U0126 plus ucOC were examined ($N = 5$).

In conclusion, ucOC increases glucose uptake in both glycolytic and oxidative muscles in the absence of insulin, via mechanisms involving enhanced AS160 phosphorylation. Therefore, ucOC should be considered as a potential agent to improve muscle glucose uptake in insulin-resistant conditions including T2DM.

Chapter 5. Study 3: ucOC enhances glucose uptake *ex vivo* in insulin-stimulated mouse oxidative but not glycolytic muscle

This study has been published as follows:

Lin X, Parker L, McLennan E, Zhang X, Hayes A, McConell G, Brennan-Speranza T.C, Levinger I. 2018. Uncarboxylated osteocalcin enhances glucose uptake *ex vivo* in insulin-stimulated mouse oxidative but not glycolytic muscle. *Calcif Tissue Int*, 9; 1-8. doi: 10.1007/s00223-018-0400-x.

This study has been presented at these conferences:

Lin X, McLennan E, Parker L, McConell G, Zhang X, Hayes A, Brennan-Speranza T.C, Levinger I. Undercarboxylated osteocalcin enhances basal and insulin-stimulated glucose uptake in isolated mouse EDL and soleus muscles. *Metabolic Diseases: Breakthrough Discoveries in Diabetes and Obesity*, St Kilda, Victoria, Australia (2016).

Lin X, McLennan E, Parker L, McConell G, Zhang X, Hayes A, Brennan-Speranza T.C, Levinger I. Undercarboxylated osteocalcin enhances basal and insulin-stimulated glucose uptake in isolated mouse muscles *ex vivo*. *Australian and New Zealand Bone and Mineral Society Annual Meeting*, Brisbane, Queensland, Australia (2017).

Lin X, McLennan E, Parker L, McConell G, Zhang X, Hayes A, Brennan-Speranza T.C, Levinger I. Undercarboxylated osteocalcin enhances basal and insulin-stimulated glucose uptake in isolated mouse muscles *ex vivo*. *American Society for Bone and Mineral Research Annual Meeting*, Denver, Colorado, USA (2017).

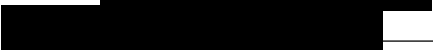
Declaration of co-authorship and co-contribution

1. PUBLICATION DETAILS

Title of paper:	Uncarboxylated osteocalcin enhances glucose uptake ex vivo in insulin-stimulated mouse oxidative but not glycolytic muscle		
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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

	6/3/2018
Signature	Date

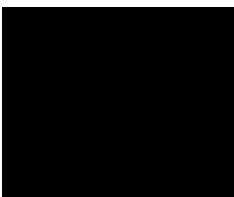
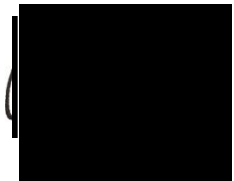
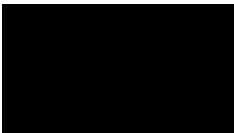
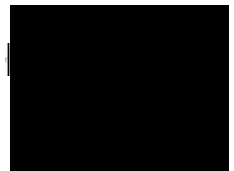
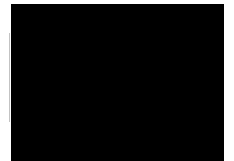
3. CO-AUTHOR(S) DECLARATION

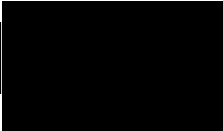
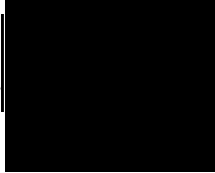
In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

Xuzhu Lin, Lewan Parker, Emma McLennan, Xinmei Zhang, Alan Hayes, Glenn McConnell, Tara Brennan-Speranza, Itamar Levinger

Name(s) of co-Author(s)	Nature of contribution	Signature	Date
Xuzhu Lin	Designed the study; Collected samples; Analysed and interpreted the data; Wrote the manuscript; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Lewan Parker	Analysed and interpreted the data; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Emma McLennan	Collected samples; Analysed and interpreted the data; Approved the final version of the paper.		6/3/2018
Xinmei Zhang	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Alan Hayes	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018

Glenn McConell	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Tara Brennan-Speranza	Designed the study; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018
Itamar Levinger	Designed the study; Analysed and interpreted the data; Undertook critical revision for important intellectual content; Approved the final version of the paper.		6/3/2018

5.0 General introduction

In Study 2, we reported that ucOC triggers non-insulin-stimulated glucose uptake in both resting EDL and Soleus. Thus, we then examined whether ucOC can also increase glucose uptake in insulin-stimulated muscles at rest, using the same methodology as performed in Study 2.

In resting state, insulin-stimulated glucose uptake occurs primarily in oxidative muscles [14]. The comparison of muscle type differences in glucose uptake, using different insulin stimulation models, has implied that humoral factors may play a considerable role in this muscle type specificity (**Section 2.3.1.1**). Thus, it is possible that ucOC belongs to these factors, by modulating glucose uptake in insulin-stimulated resting muscles in a muscle type-specific manner.

Therefore, with the same method of ucOC stimulation described in Study 2, we aimed to test the hypothesis that ucOC increases glucose uptake in insulin-stimulated mouse Soleus, but not in EDL, in Study 3 (without *ex vivo* contraction), and investigating the involved signalling pathway(s).

5.1 Abstract

ucOC stimulates muscle glucose uptake in mice EDL and Soleus. However, whether ucOC also exerts a similar effect in insulin-stimulated muscles in a muscle type-specific manner is currently unclear. We aimed to test the hypothesis that, with insulin stimulation, ucOC *per se* has a greater effect on oxidative muscle compared with glycolytic muscle, and to explore the underlying mechanisms. Mouse (C57BL6, male 9 – 12 wk) EDL and Soleus were isolated and longitudinally split into halves. Muscle samples were treated with varying doses of recombinant ucOC (0, 0.3, 1, 3, 30 ng/mL), followed by insulin addition. Muscle glucose uptake, protein phosphorylation and total Akt, AS160, ERK2, and AMPK α were assessed. ucOC treatment at 30 ng/ml enhanced muscle glucose uptake in insulin-stimulated Soleus, a mainly oxidative muscle (17.5 %, $P < 0.05$), but not in EDL - a mostly glycolytic muscle. In insulin-stimulated Soleus only, ucOC treatment (3 and 30 ng/mL) increased phosphorylation of AS160 and ERK2, but not Akt or AMPK α . The ucOC-induced increase in ERK2 phosphorylation in Soleus was not associated with the increase in glucose uptake or AS160 phosphorylation. In conclusion, ucOC enhances glucose uptake and AS160 phosphorylation in insulin-stimulated oxidative but not glycolytic muscle, via upstream mechanisms which appear to be independent of ERK or AMPK.

5.2 Introduction

OC is a multifunctional bone-secreted hormone that has been widely reported as a regulator of glucose metabolism in mice [25, 56, 57]. This function of OC is mostly modulated by its biologically active form, ucOC, which contributes to increased insulin secretion in mouse pancreatic β -cells as well as enhanced insulin sensitivity in mouse peripheral tissues including skeletal muscle [237, 264]. *In vivo* studies in mice showed that chronic ucOC administration led to stronger expression of insulin

sensitivity marker genes, greater mitochondrial function, and improved insulin signalling activation in the skeletal muscle of obese mice [24, 25, 72]. *In vitro*, relatively limited evidence showed a direct favorable effect of ucOC on glucose uptake of insulin-stimulated C2C12 myotubes [29, 239] and insulin-stimulated mouse EDL following *ex vivo* contraction [239]. However, the detail and mechanism of this effect are not fully elucidated. We recently showed that ucOC could induce basal muscle glucose uptake in both mice EDL and Soleus, mediated by a mechanism that was likely muscle type-specific [265]. As such, ucOC might also exert a similar effect on insulin-stimulated EDL and Soleus in a muscle type-specific manner, which will be explored in this study.

Skeletal muscle is a heterogeneous tissue consisting of different fibre types that possess different metabolic traits [42, 43]. In general, glycolytic muscles like EDL, that consist predominantly of type II fibres, rely on glycolytic metabolism as the major energy source, while oxidative muscles, like Soleus, which are enriched with type I fibres, mainly use oxidative metabolism for energy production [11]. To fulfil the muscle energy requirement with high efficiency, insulin-stimulated glucose uptake in the sedentary state in oxidative muscles is higher than that in glycolytic muscles [198, 266]. Therefore, it is possible that ucOC treatment exerts a larger effect on insulin-stimulated oxidative muscles (such as Soleus) than glycolytic muscles (such as EDL), contributing to the conditional regulation of glucose transport with insulin stimulation.

Previous studies have implicated that the effect of ucOC on insulin-stimulated skeletal muscle might be, at least in part, due to the enhancement of insulin-induced activation of the Akt-AS160 signalling cascade. For instance, pretreatment of 5 ng/mL ucOC resulted in a significant enhancement in insulin-stimulated Akt phosphorylation in C2C12 myotubes [29]. Furthermore, insulin-stimulated AS160 phosphorylation in EDL post contraction was enhanced by ucOC incubation, compared to insulin alone-treated EDL post contraction [239]. Several signalling proteins that can be

activated by ucOC have been suggested to enhance Akt-AS160 signalling cascade in skeletal muscle. Recent findings propose ERK as one of these signalling proteins since the suppression of ERK activation attenuated the ucOC effect on insulin-stimulated Akt phosphorylation in C2C12 myotubes [29]. In addition, AMPK activation promotes muscle glucose uptake and insulin sensitivity [12, 267], and exercise-induced AMPK phosphorylation was enhanced by ucOC injection in mouse muscle [23]. As such, ucOC mediated increases in insulin-stimulated glucose uptake and the Akt-AS160 signalling axis may occur via activated AMPK. However, whether the effect of ucOC on Akt, AS160, ERK, and AMPK in insulin-stimulated muscle is muscle type-specific is currently unclear.

Therefore, the aims of the current study were (1) to test the hypothesis that ucOC directly enhances glucose uptake in insulin-stimulated oxidative muscle (Soleus), and to a lesser degree in glycolytic muscle (EDL); and (2) to explore the potential signalling pathway(s) involved.

5.3 Materials and Methods

5.3.1 Animals

Eight wk-old male C57BL/6J mice (Animal Resources Centre, Perth, WA, Australia) were housed with a 12-hr light/12-hr dark cycle and fed standard laboratory chow (Glen Forrest, Western Australia, Australia) and water *ad libitum* until 9 – 12 wk old. The study was approved by the Animal Experimentation Ethics Committee of Victoria University and abided by the Australian National Code of Practice for the Care and Use of Animals for Scientific Purposes.

5.3.2 Muscle dissection

Mice were fasted for 4 hr before deep anaesthetisation with 60 mg/kg intraperitoneal

pentobarbital. EDL and Soleus of both legs were excised and evenly divided into halves longitudinally. After muscle isolation, euthanasia was performed via cervical dislocation under anaesthesia.

5.3.3 ucOC and insulin stimulation

Muscle samples were pre-incubated in 30 °C baths containing carbogenated KHB for 1 hr. Then they were stimulated with recombinant ucOC (0 ng/mL, 0.3 ng/mL, 3 ng/mL, 10 ng/mL, 30 ng/mL; Bachem, Bubendorf, Switzerland). After 60 min, insulin (60 µU/mL; Sigma Aldrich, Missouri, United States) was added to the sample baths for 30 min.

5.3.4 2-DG uptake measurement and sample homogenisation

The method of 2-DG uptake assessment was described previously [8]. Briefly, after the treatment, muscles were transferred to baths containing KHB + 0.1 % BSA + 2 mM 2-Deoxy-D-[1,2-³H]-glucose and 16 mM D-[1-¹⁴C] mannitol with ucOC and insulin. After 10 min, muscles were rapidly rinsed with ice cold KHB, followed with snap freezing with liquid nitrogen. On the processing day, muscle samples were lysed in ice-cold RIPA buffer (Cell Signaling, Massachusetts, United States) with Inhibitor Cocktail (Cell Signaling) and 100mM DTT (Sigma Aldrich) using TissueLyser II (QIAGEN, Hilden, Germany). Half of the lysate was pipetted into vials with scintillation cocktail for scintillation counting (β-counter) with Tri-Carb 2910TR Liquid Scintillation Analyzer (Perkin Elmer, Australia) and the other half was used in Western blotting.

5.3.5 Western blotting

The methods of protein assay, western blotting, and blot quantification were described

previously [19]. For the quantification of Glut4 blots, both major bands were analysed using Image Lab Software (Bio-Rad Laboratories, California, United States). Antibodies for p-Akt (Ser473; #9271), Akt (#9272), p-AS160 (Thr642; #8881), AS160 (#2670), p-ERK (Thr202/Tyr204; #4370), ERK (#4695), p-AMPK α (Thr172; #2535), AMPK α (#2603) were purchased from Cell Signaling. Glut4 antibody was bought from Santa Cruz Biotechnology (#sc-7938; Texas, United States).

5.3.6 Statistical analysis

Normalised density used for western blotting results were generated via normalisation to the total protein (stain-free loading control) of each sample lane and to an internal control (pooled sample). 3 ng/mL group and 30 ng/mL group were chosen for western blotting as representatives of low and high doses of ucOC.

Paired t-test was used to analyse the effects of insulin on muscle glucose uptake, protein phosphorylation, protein abundance, and phospho/total ratio, compared to paired control samples of the same mice.

Paired t-test was also used to analyse the effect of different doses of ucOC on insulin-stimulated muscle glucose uptake, protein phosphorylation, protein abundance, and phospho/total ratio, compared to paired insulin alone samples of the same mice.

Percentage increase in glucose uptake and protein phosphorylation induced by ucOC treatment in insulin-stimulated samples was calculated via $\frac{\text{ucOC} + \text{insulin level} - \text{insulin level}}{\text{insulin level}} \times 100 \%$. Pearson's correlation was performed between the percentage increases of glucose uptake, AS160 phosphorylation, and ERK2 phosphorylation from the 3 ng/mL ucOC treatment group, the 30 ng/mL ucOC treatment group, or both groups. All figures and analyses were performed using GraphPad 6 (GraphPad Software, La Jolla, CA, USA).

All data are reported as mean $\pm$ SEM.

5.4 Results

5.4.1 ucOC enhanced glucose uptake in insulin-stimulated Soleus but not EDL

In EDL, insulin alone increased muscle glucose uptake (31.9 %, $P < 0.001$, **Figure 5.1A**). ucOC treatment, had minimal effect on insulin-stimulated glucose uptake ($P > 0.1$, **Figure 5.1C**).

In Soleus, insulin alone increased glucose uptake compared to control (28.4 %, $P < 0.001$, **Figure 5.1B**). ucOC treatment (10 ng/mL) tended to increase insulin-stimulated glucose uptake (20.2 %, $P = 0.086$), while 30 ng/mL ucOC augmented insulin-stimulated muscle glucose uptake by 17.5 % ($P < 0.05$) (**Figure 5.1D**).

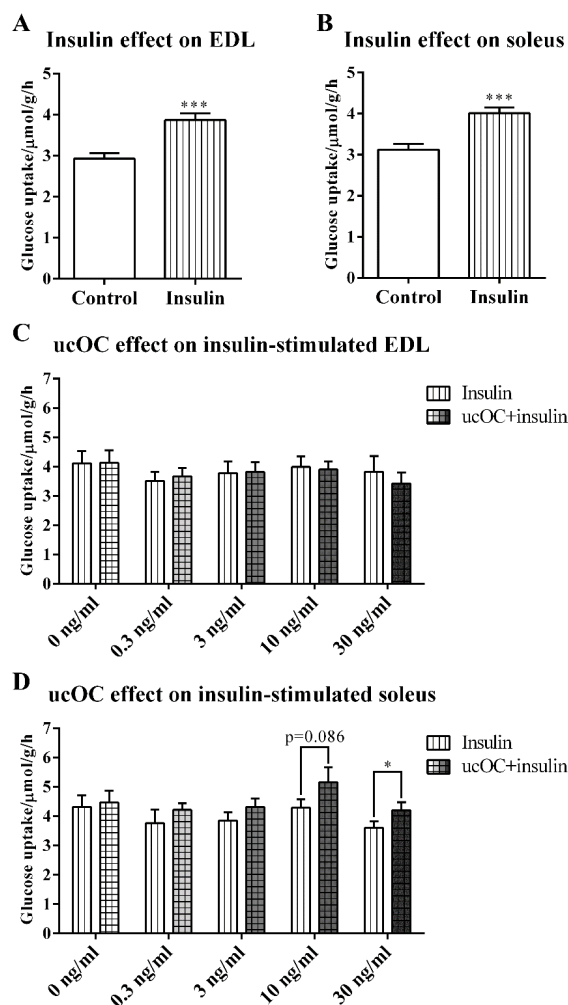


Figure 5.1. Direct ucOC effect on insulin-stimulated glucose uptake on ex vivo EDL and Soleus. Glucose uptake of EDL (**A**) and Soleus (**B**) samples treated with KHB control and insulin (60 μ U/mL) was detected ($N = 55$). Glucose uptake of EDL (**C**) and Soleus (**D**) samples treated with insulin (60 μ U/mL) and various doses of ucOC plus insulin was detected ($N = 6 - 13$ for each dose). * and *** represent $P \leq 0.05$ and $P \leq 0.001$ between paired samples (paired t-test).

5.4.2 ucOC increased phosphorylation of AS160 and ERK2 in insulin-stimulated Soleus

In EDL, insulin increased the phosphorylation of Akt and AS160 (Figure 5.2A and 5.2C). ucOC treatment at 3 ng/mL or 30 ng/mL plus insulin did not significantly affect phosphorylation, total expression, or phospho/total ratio of Akt (Figure 5.2A and 5.2B), AS160 (Figure 5.2C and 5.2D), ERK2 (Figure 5.2E and 5.2F), or AMPK α (Figure 5.2G and 5.2H), with the exception of p-AMPK α being increased by 30 ng/mL ucOC (24.8 %, $P < 0.05$, Figure 5.2G), compared to samples that were treated with insulin alone.

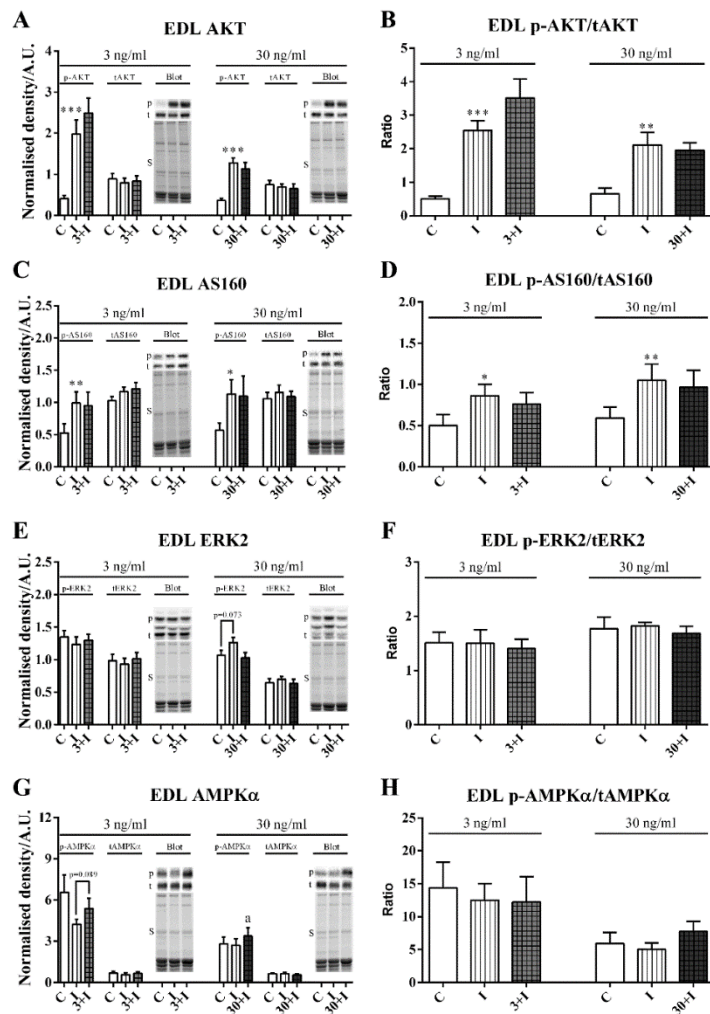
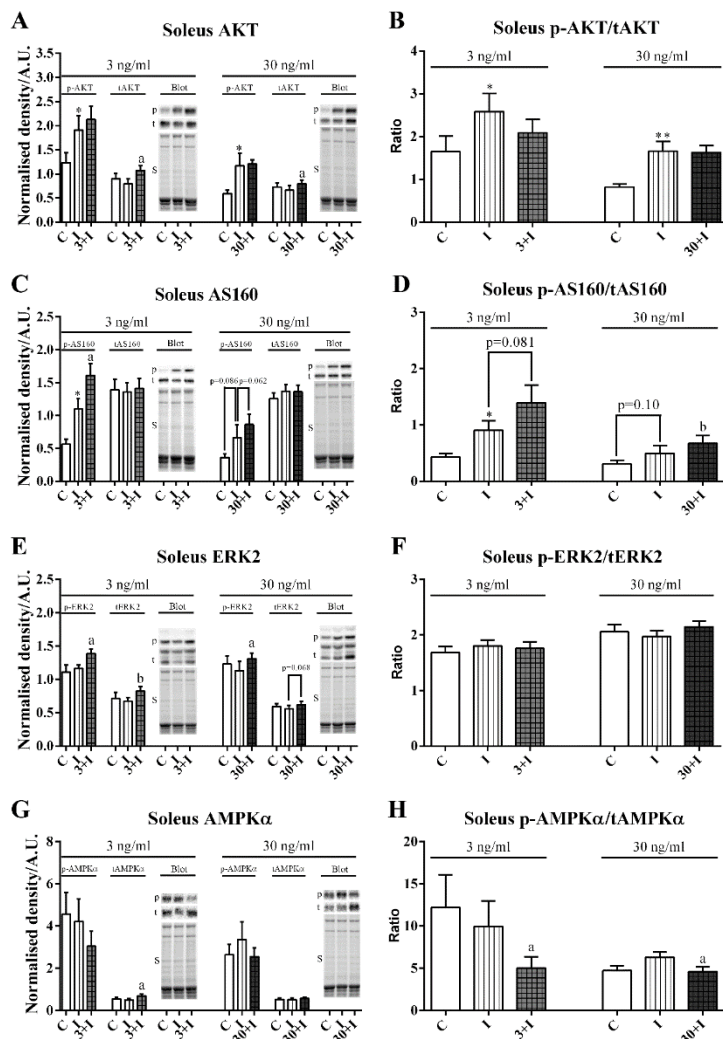


Figure 5.2. ucOC effects on signalling proteins in insulin-stimulated EDL. Phosphorylation levels, total protein levels, and representative blots (p: phosphorylated protein blots, t: total protein blots, S: stain-free blots) of Akt (phosphorylation site Ser473) (A), AS160 (phosphorylation site Thr642) (C), ERK2 (phosphorylation site Thr202/Tyr204) (E), and AMPK α (phosphorylation site Thr172) (G) of EDL samples treated with KHB control, insulin (60 μ U/mL), and ucOC (3 ng/mL and 30 ng/mL) were shown. Phospho/total ratios of Akt (B), AS160 (D), ERK2 (F), and AMPK α (H) of EDL samples treated with KHB control, insulin (60 μ U/mL), and ucOC (3 ng/mL and 30 ng/mL) were calculated. $N = 10$ for each dose group. *, **, and *** represent $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ compared with paired control samples (t-test). a represents $P \leq 0.05$ compared with paired insulin samples (t-test). A.U. stands for arbitrary unit.

and ucOC (3 ng/mL and 30 ng/mL) were calculated. $N = 10$ for each dose group. *, **, and *** represent $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ compared with paired control samples (t-test). a represents $P \leq 0.05$ compared with paired insulin samples (t-test). A.U. stands for arbitrary unit.

In Soleus, insulin enhanced the phosphorylation of Akt and AS160 (**Figure 5.3A** and **5.3C**). ucOC (3 ng/mL and 30 ng/mL) with insulin increased total protein levels of Akt (34.6 % and 19.6 %, $P < 0.05$) but not phosphorylation levels, compared with insulin stimulation alone (**Figure 5.3A** and **5.3B**). In contrast, ucOC increased phosphorylation (3 ng/mL, 46.1 %, $P < 0.05$) and phospho/total ratio (30 ng/mL, 36.0 %, $P < 0.01$) of AS160 without any changes in total protein levels in insulin-stimulated muscle (**Figure 5.3C** and **5.3D**). The phosphorylation levels of ERK2 were enhanced (19.0 % and 15.9 %, $P < 0.05$) by ucOC treatment (3 ng/mL and 30 ng/mL) on top of insulin stimulation, likely due to the increase in total protein levels of ERK2 (21.7 % by 3 ng/mL, $P < 0.01$, **Figure 5.3E** and **5.3F**). Compared to insulin alone samples, 3 ng/ml ucOC treatment increased total expression of AMPK α



(38.0 %, $P < 0.05$) and the treatment of ucOC at both doses decreased the phospho/total ratio levels (49.8 %, $P < 0.05$ for 3 ng/mL; 27.0 %, $P < 0.05$ for 30 ng/mL), however phosphorylated AMPK α remained unchanged (**Figure 5.3G** and **5.3H**).

Figure 5.3. ucOC effects on signalling proteins in insulin-stimulated Soleus. Phosphorylation levels, total protein levels, and representative blots (p: phosphorylated protein blots, t: total protein blots, S: stain-free blots) of Akt (phosphorylation site Ser473) (A), AS160 (phosphorylation

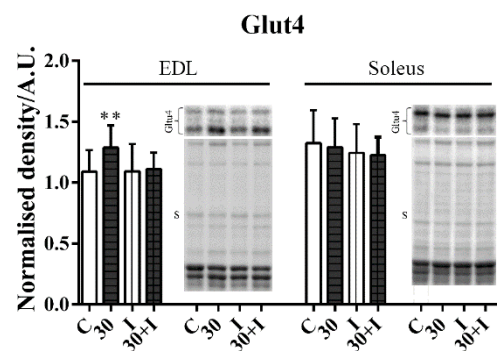
site Thr642) (C), ERK2 (phosphorylation site Thr202/Tyr204) (E), and AMPK α (phosphorylation site Thr172) (G) of Soleus samples treated with KHB control, insulin (60 μ U/mL), and ucOC (3 ng/mL and 30ng/mL) were shown. Phospho/total ratios of Akt (B), AS160 (D), ERK2 (F), and AMPK α (H) of Soleus samples treated with KHB control, insulin (60 μ U/mL), and ucOC (3 ng/mL and 30 ng/mL) were calculated. $N = 10$ for each dose group. *, **, and *** represent $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ compared with paired control samples (t-test). a and b represent $P \leq 0.05$ and $P \leq 0.01$ compared with paired insulin samples (t-test). A.U. stands for arbitrary unit.

5.4.3 ucOC-induced increase in p-ERK2 did not correlate with the increase in glucose uptake or the increase in p-AS160 in Soleus with insulin stimulation

In Soleus samples with insulin stimulation, 30 ng/mL ucOC-stimulated percentage increase in p-AS160 correlated with the percentage increase in glucose uptake ($r = 0.70$, $P = 0.035$). 30 ng/mL ucOC-induced percentage increase in p-ERK2 did not correlate with the percentage increase in glucose uptake ($r = -0.050$, $P = 0.89$). The percentage increase induced by ucOC (3 ng/mL and 30 ng/mL) in p-ERK2 did not associate with the percentage increase in p-AS160 ($r = 0.25$, $P = 0.51$).

5.4.4 ucOC did not increase Glut4 expression in insulin-stimulated muscles

30 ng/mL ucOC treatment did not alter total Glut4 expression in insulin-stimulated EDL or Soleus, but it elevated total Glut4 expression in EDL without insulin



stimulation (18.1 %, $P < 0.01$) (Figure 5.4).

Figure 5.4. ucOC effects on Glut4 expression in EDL and Soleus. Total expression levels and representative blots of Glut4 in EDL and Soleus samples treated with KHB control, ucOC (30 ng/mL), insulin (60 μ U/mL), and ucOC (30 ng/mL) plus insulin (60 μ U/mL) were shown. $N = 3$. ** represents $P \leq 0.01$ compared with paired control samples (t-test). A.U. stands for arbitrary unit.

5.5 Discussion

We report that ucOC *per se* enhanced glucose uptake in insulin-stimulated mouse Soleus but not EDL. Furthermore, in insulin-stimulated Soleus only, ucOC treatment enhanced phosphorylation of AS160 and ERK2, but not Akt or AMPK α . However, the ucOC-induced increase in ERK2 phosphorylation was not linked to the increase in either glucose uptake or AS160 phosphorylation, suggesting that ERK activation is not involved in the mechanism underlying ucOC-induced enhancement of muscle glucose uptake.

Emerging evidence indicates that ucOC may favor muscle insulin sensitivity in mice [29, 72, 83]. Our data appear to support this hypothesis, at least in oxidative muscle. However, it was recently shown that 30 ng/mL ucOC alone enhanced muscle glucose uptake in both EDL and Soleus by 45 % and 26 %, respectively [265]. It is therefore possible that the ucOC-mediated enhancement of glucose uptake in insulin-stimulated Soleus in the current study could be largely through the effect of ucOC treatment alone, with a limited further enhancement in insulin action. Nevertheless, our evidence also suggests that, although no ucOC effect was observed in insulin-stimulated EDL at rest (shown in **Figure 5.1C**), an insulin sensitisation mediated by ucOC treatment likely occurred in intact EDL following *ex vivo* contraction [239]. Thus, whether ucOC exerts insulin-sensitising effect in Soleus or not should be addressed in further investigations, with a means to suppress ucOC alone action on muscle glucose uptake. Consistent with the effect of ucOC on muscle glucose uptake, ucOC treatment enhanced AS160 phosphorylation in insulin-stimulated Soleus only, however did not affect Akt phosphorylation, indicating a possible convergence of ucOC cascade and insulin signalling at the level of AS160 phosphorylation. These results are similar to our previous observations that AS160 phosphorylation, but not Akt phosphorylation (Thr308), in insulin-stimulated EDL post *ex vivo* contraction was enhanced by ucOC [239], and similar to others who reported that 4 hr pretreatment of ucOC did not change phosphorylation of Akt in

insulin-stimulated L6 or C2C12 myotubes [72, 83]. It is worth noting that in these studies and the current study the treatment of ucOC was relatively short, while in another study 72 hr treatment of ucOC, but not 20 min treatment, led to elevated Akt phosphorylation in insulin-stimulated myotubes [29]. Therefore, the effect of ucOC on insulin-stimulated Akt needs further investigation involving assessments at multiple timepoints.

The discrepancy between alterations of p-Akt and p-AS160 indicate a non-Akt-dependent mechanism underlying ucOC-induced enhancement in AS160 phosphorylation. Since ERK activation was shown to participate in ucOC-induced glucose uptake in insulin-stimulated C2C12 myotubes [29], we assessed the phosphorylation of ERK2 in the current study. Results showed that although ucOC treatment increased p-ERK2 levels in insulin-stimulated Soleus, no correlation was found between the increase of p-ERK2 and the increase of insulin-stimulated glucose uptake or p-AS160, implying that the activation of ERK may not be a linking mechanism.

AMPK, another plausible downstream target of ucOC, has been characterised as a central player in both exercise-induced AS160 activation and enhanced insulin-stimulated AS160 activation following exercise [268, 269], thus it is possible that AMPK is also involved in ucOC-induced p-AS160 enhancement in insulin-stimulated Soleus. However, in the current study it appears that AMPK α phosphorylation occurred independently of AS160 phosphorylation, since AMPK α phosphorylation was increased by ucOC treatment in insulin-stimulated EDL (shown in **Figure 5.2G**) while ucOC reduced the phospho/total ratio of AMPK α in insulin-stimulated Soleus (shown in **Figure 5.3H**). This finding is consistent with a previous report that AMPK may not be the main regulator for ucOC-induced glucose uptake [23].

Therefore, the mechanisms behind ucOC-induced p-AS160 increase in insulin-stimulated Soleus may involve proteins other than ERK and AMPK, such as

members from PKC family which have also been linked with non-Akt-dependent AS160 phosphorylation [116, 270]. Thus, the activation changes of PKC members with ucOC treatment should be assessed in insulin-stimulated muscle cells in future investigations.

Interestingly, in this study we found that the total expression of ERK2, AMPK α , and Akt in insulin-stimulated Soleus was enhanced by ucOC treatment. This phenomenon is similar to what we previously reported in ucOC-stimulated muscle [265], and also consistent with ucOC-induced increased protein expression and synthesis rate in cultured muscle myotubes [26, 239]. Therefore, ucOC treatment may regulate muscle glucose uptake via the modulation of both signalling protein phosphorylation and signalling protein abundance. We also tested whether ucOC can enhance muscle glucose uptake via the increase of Glut4 abundance. There was no effect of ucOC on total Glut4 expression in insulin-stimulated muscles (**Figure 5.4**). However, we observed an increase in Glut4 expression in EDL, but not Soleus, induced by ucOC alone. As we previously showed that ucOC *per se* triggered glucose uptake in both EDL and Soleus [265], the results from the current study indicate that ucOC-induced enhancement of Glut4 expression may contribute, at least in part, to ucOC-induced increase of glucose uptake in EDL, but not in Soleus. However, this hypothesis needs to be further explored in future studies.

To clarify the mechanism underlying ucOC-induced enhancement of glucose uptake in insulin-stimulated Soleus, it could be helpful to compare the ucOC effects on insulin-stimulated glucose uptake between submaximal insulin doses and maximal doses. If ucOC treatment was found to increase glucose uptake with a maximal dose of insulin, similar to ucOC effects with submaximal doses, it would suggest that the ucOC action involves mechanisms beyond insulin-stimulated Glut4 translocation. Under these circumstances, the ucOC effect might be mediated by an increase of Glut4 abundance [26, 271], or an increase of expression of other glucose transporters [272, 273]. However, in the current study we only used a submaximal dose of insulin

(60 μ U/mL). Therefore, the hypothesis should be tested in future investigations, which involve both submaximal and maximal insulin doses.

In conclusion, ucOC increases glucose uptake in insulin-stimulated oxidative muscle (Soleus), but not in glycolytic muscle (EDL), which likely occurs through mechanisms independent of ERK or AMPK signalling. These findings highlight ucOC as a potential therapeutic method for improving postprandial glycaemic control.

Chapter 6. Study 4: Hindlimb immobilisation, but not castration, induces reduction of ucOC associated with muscle atrophy in rats

This study has been published as follows:

Lin X, Hanson E, Betik A.C, Brennan-Speranza, T.C, Hayes A, Levinger I. 2016. Hind limb immobilisation, but not castration, induces reduction of undercarboxylated osteocalcin associated with muscle atrophy in rats. J Bone Miner Res, 31; 1967-1978. doi: 10.1002/jbmr.2884.

This study has been presented at these conferences:

Lin X, Hanson E, Betik A.C, Brennan-Speranza T.C, Hayes A, Levinger I. Hindlimb immobilization, but not castration, induces reduction of undercarboxylated osteocalcin associated with muscle atrophy in rats. Australian and New Zealand Bone and Mineral Society Annual Meeting, Gold Coast, Queensland, Australia (2016). (Selected as a plenary poster)

Lin X, Hanson E, Betik A.C, Brennan-Speranza T.C, Hayes A, Levinger I. Hindlimb immobilization, but not castration, induces reduction of undercarboxylated osteocalcin associated with muscle atrophy in rats. American Society for Bone and Mineral Research Annual Meeting, Atlanta, Georgia, USA (2016). (Selected as a plenary poster)

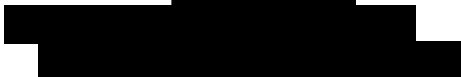
Declaration of co-authorship and co-contribution

1. PUBLICATION DETAILS

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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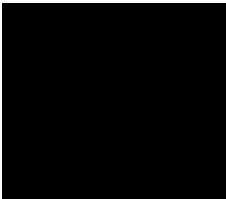
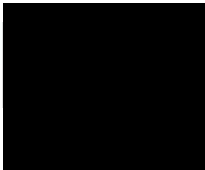
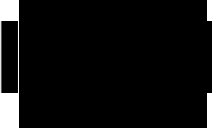
3. CO-AUTHOR(S) DECLARATION

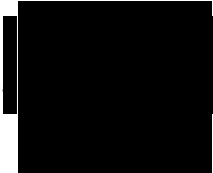
In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

Xuzhu Lin, Erik Hanson, Andrew Betik, Tara Brennan-Speranza, Alan Hayes, Itamar Levinger

Name(s) of Co-Author(s)	Nature of Contribution	Signature	Date
Xuzhu Lin	Designed the study; Collected samples; Analysed and interpreted the data; Wrote the manuscript; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
Erik Hanson	Designed the study; Collected samples; Analysed and interpreted the data; Undertook critical revisions for important intellectual content; Approved the final version of the paper.		6/3/2018
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6.0 General introduction

Study 1 – 3 of the current thesis have shown that ucOC regulates muscle glucose uptake, as well as explored the underlying metabolisms. It has been suggested by several other recent studies that, in addition to the regulation in muscle energy metabolism, ucOC also upregulates muscle mass growth by increasing protein synthesis, inhibiting protein degradation, and promoting myotube formation [26, 27, 32].

Testosterone depletion and muscle disuse, which are well known to induce muscle atrophy [125, 128], also exert detrimental effects on bone formation [126, 128]. Thus, it is possible that both lead to the loss of muscle mass by affecting the secretion of bone-derived factors, including osteocalcin. However, whether the loss of ucOC actions in muscle is associated with muscle loss in atrophic conditions, induced by testosterone depletion and muscle disuse, still remains unknown. Furthermore, disuse atrophy occurs primarily in oxidative muscles rather than glycolytic muscles [18, 19]. Thus, it is possible that this muscle type specificity may be linked with the muscle type-selective loss of ucOC actions.

Therefore, the aim of Study 4 was to provide further evidence to the role of ucOC effects on muscle mass growth, by testing the hypothesis that the loss of ucOC actions is associated with the muscle wasting, induced by castration and hindlimb immobilisation, in a muscle type-specific manner in male rats.

6.1 Abstract

ucOC has been implicated in skeletal muscle insulin sensitivity and function. However, whether muscle mass and strength loss in atrophic conditions is related to a reduction in ucOC is not clear. We hypothesised that both immobilisation and testosterone depletion would lead to reductions in ucOC, associated with not only the degree of muscle atrophy but also changes to atrophy signalling pathway/s in male rats. We subjected 8 wk old Male Fischer (F344) rats to 7 d Hindlimb immobilisation, 10 d after castration surgery. Hindlimb immobilisation, but not castration, resulted in a significant reduction in ucOC (30 %) and lower ucOC was correlated with the degree of muscle loss and muscle weakness. ucOC levels, the expression of ucOC-sensitive receptor GPRC6A, as well as the activity of ERK and AMPK were associated with the expression and activity of a number of proteins in the mTORC1 and FOXO signalling pathways in a muscle type-specific manner. These data suggest that ucOC may have other effects on skeletal muscle in addition to its insulin-sensitising effect.

6.2 Introduction

Bone is an endocrine organ involved in energy metabolism and possibly male fertility [274]. These functions are achieved by ucOC [25, 58]. Osteocalcin may play a role in cell growth and muscle fibre strength in skeletal muscles [28, 237, 275]. Indeed, there is some evidence suggesting that osteocalcin-deficient mice are characterised by lower muscle mass and weaker muscle strength [276]. In humans, lower ucOC/OC is associated with lower hip flexor, hip abductor and quadriceps muscle strength [133]. Furthermore, ucOC treatment promoted EDL cross-sectional area and grip strength in mice and myotube formation in C2C12 myoblast cultures *in vitro*. [28]

Muscle atrophy is a consequence of homeostatic imbalance between protein synthesis and protein degradation [277]. It is possible therefore, that the loss of muscle mass and strength during atrophic conditions might be, at least in part,

attributed to lower circulating ucOC. However, it is not clear whether atrophic conditions, as seen following limb immobilisation, has an impact on serum ucOC levels.

Testosterone is an anabolic hormone produced by the Leydig cells of the testes and plays a major role in the regulation of muscle mass in males [278, 279]. Loss of testosterone, as a consequence of aging or castration surgery, leads to muscle atrophy and muscle weakness [280, 281]. Testosterone modulates muscle anabolism not only via signalling networks that are directly orchestrated by itself, but also indirectly via other hormones such as follicle-stimulating hormone, luteinising hormone, and growth hormone [282, 283] due to widely expressed androgen receptors in endocrine organs, including bone [68, 284, 285]. Indeed, there are suggestions for the existence of a reciprocal interaction between bone and gonads [286, 287]. ucOC promotes testosterone secretion in testes [229]. In turn, testosterone is implicated in bone growth, maturation, and maintenance [287]. However, whether testosterone loss has an impact on circulating ucOC level is unknown. As such, it is not clear whether reductions in testosterone level indirectly influence skeletal muscle mass via decreases in ucOC level.

The beneficial effects of ucOC on skeletal muscle are likely mediated via its putative receptor GPRC6A [140]. Within the ucOC/GPRC6A cascade, ERK and AMPK are proposed downstream kinases [29, 276] that are also known to be involved in the proliferation, growth and lifespan of muscle cells [162, 288, 289].

Under atrophic conditions, there are several signalling pathways actively responsible for muscle loss. mTORC1 cascade plays a vital role in cell proliferation and growth [290]. Its dysfunction has been suggested as one of the main mechanisms underlying muscle atrophy [125, 291]. The phosphorylation of mTOR as well as the phosphorylation of P70S6K, one of the major mTORC1 downstream targets, are indicative of mTORC1 activation [292, 293]. Another important target of mTORC1 is ULK1. Phosphorylation of ULK1 at ser757 by mTORC1 blocks its pro-autophagy functions [294]. FOXO family proteins are also critical in the process of muscle

atrophy [121]. Phosphorylation of FOXO family members inhibit their translocation into the nucleus, thereby inhibiting the transcription of Muscle-specific RING Finger protein1 MuRF1 and MAFbx/Fbx32, the two important skeletal muscle-specific ubiquitin E3 ligases that tag proteins for proteolysis via the proteasome [121]. Furthermore, Akt functions as a critical convergent point, regulating both mTORC1 and FOXO signalling pathways via phosphorylation [125]. The upstream signalling partner of Akt, IRS-1, directly activated by the insulin receptor, plays a role in these pathways, and can be down-regulated via negative feedback in atrophic muscles [295]. Nevertheless, it is not clear how these signalling proteins interact with the ucOC/GPRC6A cascade under conditions of muscle atrophy.

The aims of this study were to test the hypotheses that (a) hindlimb immobilisation and castration surgery lead to reductions in ucOC levels and (b) the reductions in ucOC correlate with muscle loss and related muscle signalling proteins. We also hypothesised that the expression/activity of downstream proteins in ucOC signalling cascade will be correlated with the muscle atrophy signalling network. Given that different types of muscles respond differently during immobilisation-induced atrophy [296, 297], these correlations between signalling proteins would also be in a muscle type-specific manner.

6.3 Materials and Methods

6.3.1 Animals

Male Fischer (F344) rats ($N = 31$, body weight = 224.1 ± 3.1 g) were purchased ~8 wk of age (Animal Resource Centre, Canning Vale, WA, Australia) and housed in pairs in a light- and climate-controlled room (12:12 hr of light and dark, 20 – 22 °C) with *ad libitum* access to water and standard animal chow (AIN93G, Specialty Feeds, Glenn Forrest, WA, Australia). All experiments and procedures were approved by the Animal Ethics Committee at Victoria University and in accordance with the

6.3.2 Castration surgery and hindlimb immobilisation

After one wk of acclimatisation, animals were randomly allocated into castration or sham groups. Testosterone levels were manipulated by performing a bilateral orchiectomy or sham surgery via a scrotal incision under sterile conditions. Pain relief was administered via intraperitoneal injection 30 min prior to surgery (0.5 mg/kg, Meloxicam, Therapon, Burwood, VIC, Australia). Animals were allowed to recover for one wk at which time they underwent unilateral immobilisation of the right hindlimb (or free movement as Non-immobilised controls) for 10 d to induce muscle atrophy (7 rats for Sham + Non-immobilisation, 8 rats for Sham + Immobilisation, 7 rats for Castration + Non-immobilisation, 9 rats for Castration + Immobilisation). Hindlimb immobilisation was conducted under 2 – 4 % isoflurane anaesthesia, tape stirrups were attached to the top and bottom of the foot and the leg was then wrapped in cast padding and compression tape. The leg was immobilised by a thermoplastic splint (3' Vet-lite casting material, Therapon, Burwood, VIC, Australia) attached to the outside of the leg. The splint was secured in place using strapping tape with the foot in a neutral position. The splint was inspected and repaired daily, as required. Animals were also inspected daily by laboratory and veterinary staff, and their general level of activity, responsiveness, and appearance was assessed. After the immobilisation/non-immobilisation period, animals were killed in blood collection via heart puncture after ex vivo muscle contraction (see below).

6.3.3 *Ex vivo* muscle contraction and sample collection

After the 10-d immobilisation/non-immobilisation period, animals were deeply anaesthetised with sodium pentobarbital (60 mg/kg, Therapon, Burwood, VIC, Australia) via intraperitoneal injection. The EDL and Soleus from both legs were

excised tendon to tendon, then blood samples were collected via heart puncture and left on ice for 30 min at which time they were spun in a centrifuge at 16,000 g at 4 °C for 10 min for serum samples. *Ex vivo* muscle contraction was conducted as previously described [298]. After the completion of *ex vivo* contraction experiments, the EDL and Soleus were removed from the bath, blotted dried, cut free of tendon and weighed. At last, the serum and muscle samples were snap frozen in liquid nitrogen and stored at -80 °C until analysis.

6.3.4 Measurement of the level of serum hormones

Total osteocalcin was measured using ELISA kit purchased from Immunotopics (San Clemente, CA, USA) according to manufacturer's instructions. ucOC level was detected following hydroxyapatite binding as described previously [299] using the same ELISA kit. Serum testosterone and insulin were measured using ELISA kits (in duplicate) purchased from Crystal Chem (Downers Grove, IL, USA) based on kit instructions.

6.3.5 Western blotting

Muscle samples (~15 mg) were lysed in ice-cold RIPA buffer (Cell Signaling, Danvers, MA, USA) with Inhibitor Cocktail (Cell Signaling) and 25 mM DTT (Sigma Aldrich, St. Louis, MO, USA) using TissueLyser II (QIAGEN, Hilden, Germany) followed by gentle rocking at 4 °C for 1 hr. Protein homogenates were collected in the supernatant following centrifugation at 16,000 g at 4 °C for 15 min. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were subjected to electrophoresis on Criterion Stain-Free precast gels (10 %; Bio-Rad) and then transferred electrophoretically using Trans-Blot Turbo Transfer System (Bio-Rad) onto a PVDF membrane (Bio-Rad). Then a stain-free blot image was taken using ChemiDoc Imaging System (Bio-Rad)

for total protein measurement in each sample lane. Immunoblotting was performed at optimum conditions for each antibody. Bands were identified using ChemiDoc Imaging System, using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Waltham, MA, USA). Band densities of both stain-free blot and immunoblotting were measured by densitometry using Image Lab Software (Bio-Rad). Values of immunoblotting bands were normalised using total protein values before statistical analysis. p-mTOR (Ser2448), mTOR, IRS-1, p-ULK1 (Ser757), ULK1, p-FOXO3a (Ser253), FOXO3a, p-FOXO1 (Ser256), FOXO1, p-P70S6K (Thr389), P70S6K, p-AMPK (Thr172), AMPK, p-AKT (Ser473), AKT, p-ERK (Thr202/Tyr204), and ERK antibodies were purchased from Cell Signaling, GPRC6A antibody was provided by AVIVA (San Diego, CA, USA), Fbx32 and MuRF1 antibodies were purchased from Bioss (Woburn, MA, USA).

6.3.6 Statistical analysis

All values are expressed as the mean fold-change (normalised to non-immobilisation group) $\pm$ SEM. To analyse the effects of castration and immobilisation within non-immobilised animals and immobilised animals on serum hormone levels, 2-way ANOVA was applied. To analyse the effects of castration and immobilisation within non-immobilised animals and immobilised legs on the muscle mass/body weight ratio, muscle force, and expression/activity of signalling proteins, 2-way ANOVA was applied. To analyse the effects of immobilisation within immobilised legs and the contralateral legs on the muscle mass/body weight ratio, muscle force, and expression/activity of signalling proteins, 2-way ANOVA with repeated measure was applied. Spearman's Correlation was performed between the variables within all groups unless other ranges are described. All figures and analyses were performed using GraphPad 6 (GraphPad Software, La Jolla, CA, USA).

6.4 Results

6.4.1 Impact of immobilisation and castration on circulating hormones

Both immobilisation and castration had no significant effect on the level of total OC (Figure 6.1A). ucOC was reduced by ~30 % ($P < 0.05$) after immobilisation but not changed after castration (Figure 6.1B). Testosterone was lower by more than 90 % ($P < 0.001$) following castration (Figure 6.1C) and -40 % ($P < 0.001$) following limb immobilisation. Circulating serum insulin was ~50 % higher ($P < 0.05$) in the immobilisation group compared to non-immobilised animals. In contrast, compared with sham surgery, insulin level was significantly lower (~50%) after castration (Figure 6.1D). Circulating testosterone did not correlate with either OC or ucOC (Figure 6.1E and 6.1F).

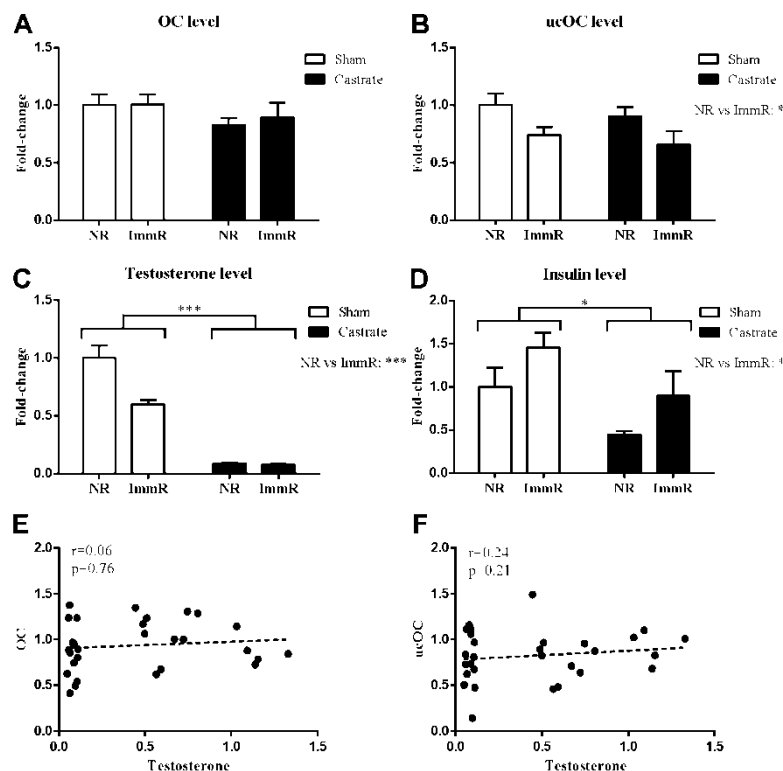


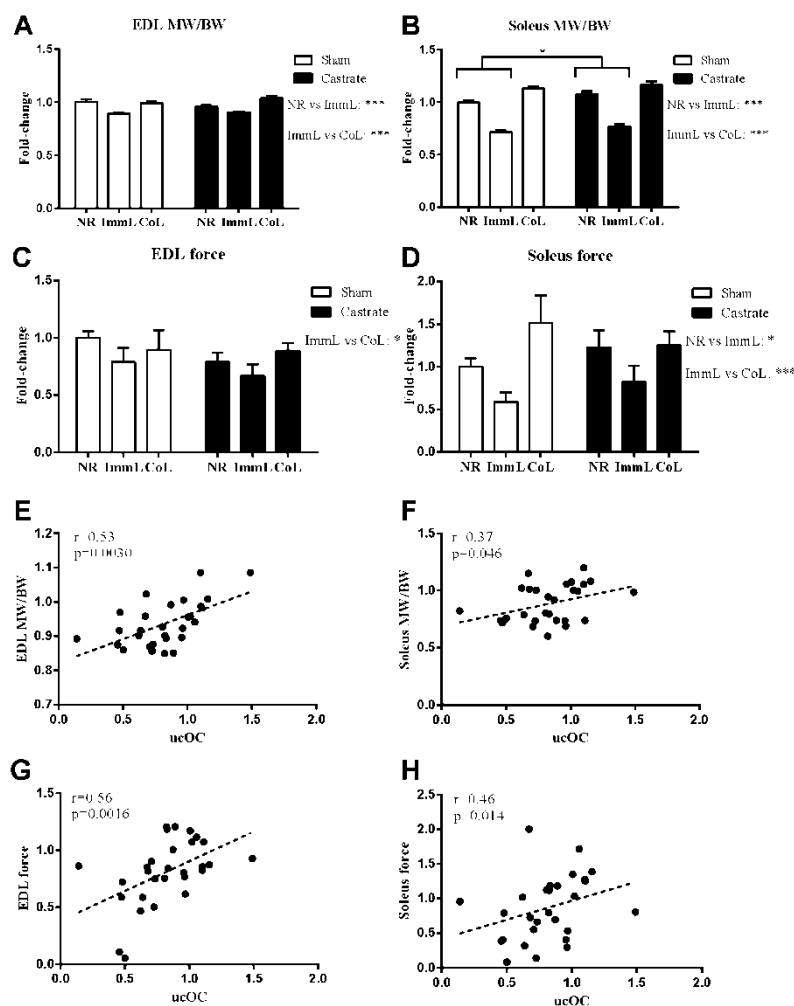
Figure 6.1. Impact of immobilisation and castration on circulating OC, ucOC, testosterone and insulin levels. Serum levels of total OC (A), ucOC (B), testosterone (C), and insulin (D) were detected in non-immobilised rats (NR)/Immobilised rats (ImmR) with sham/castration surgery. *, **, and *** stand for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ in 2-way ANOVA analysis.

Symbols of P value of Castration main effect are illustrated on top of columns while symbols of P value of Immobilisation main effects (named NR vs ImmL and ImmL vs CoL) are illustrated on the right side of each panel.

6.4.2 Impact of immobilisation and castration on EDL and Soleus mass and strength

In the EDL, hindlimb immobilisation but not castration led to a small, but significant reduction in muscle mass (**Figure 6.2A**). Immobilisation resulted in lower muscle force compared with the non-immobilised contralateral leg ($P < 0.05$, **Figure 6.2C**). Immobilisation resulted in a profound reduction in Soleus muscle mass and force in comparison with either non-immobilised animals ($\sim 30\%$; $P < 0.001$, $P < 0.05$) or the contralateral leg ($\sim 50\%$; $P < 0.001$) (**Figure 6.2B** and **6.2D**). Castration had no significant effect on either mass or strength in either muscle.

Higher ucOC level was associated with a higher EDL and Soleus mass (**Figure 6.2E** and **6.2F**, respectively) and also higher strength (**Figure 6.2G** and **6.2H**,



respectively).

Higher insulin level was associated with lower muscle mass in Soleus among all animals ($r = -0.46$, $P < 0.01$).

Figure 6.2. Impact of immobilisation and castration on EDL and Soleus mass and strength. EDL muscle weight/body weight (MW/BW) (**A**), Soleus MW/BW (**B**), EDL muscle force (**C**), and Soleus muscle force (**D**)

were examined for muscles isolated from non-immobilised rats (NR)/immobilised leg (ImmL) and the contralateral leg (CoL) of immobilised rats with sham/castration surgery. *, **, and *** stand for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ in 2-way ANOVA analysis. The correlation between ucOC and EDL MW/BW (**E**), EDL force (**F**), Soleus MW/BW (**G**), or Soleus force (**H**) was detected among all six groups. Symbols of P value of Castration main effect are illustrated on top of columns while symbols of P value of Immobilisation main effects (named NR vs ImmL and ImmL vs CoL) are illustrated on the right side of each panel.

6.4.3 Impact of immobilisation and castration on postulated ucOC signalling proteins

There was no alteration of GPRC6A protein expression in the EDL (**Figure 6.3A**). In contrast, GPRC6A expression in the Soleus was significantly lower following immobilisation (**Figure 6.3B**).

p-AMPK α levels were lower (~50 %, $P < 0.001$), in both EDL and Soleus following immobilisation compared immobilisation to non-immobilised rats (**Figure 6.3C** and **6.3D**). p-AMPK α as well as phospho/total ratio of AMPK α in EDL was lower in castrated animals compared to sham counterparts ($P < 0.05$) whereas its total expression was higher (**Figure 6.3C**).

ERK1/2 phosphorylation and phospho/total ratio of ERK1/2 were lower than non-immobilised animals following immobilisation in EDL (**Figure 6.3E**). In Soleus, higher phospho/total ratio of ERK1/2 was observed in immobilised leg ($P < 0.05$) (**Figure 6.3F**).

There was no significant correlation between ucOC level and the expression of GPRC6A in either muscle type ($P > 0.1$). In Soleus but not EDL, GPRC6A was correlated with muscle mass ($r = 0.51$, $P < 0.001$). ucOC level was correlated with p-ERK1/2 ($r = 0.49$, $P < 0.01$) and p-AMPK α ($r = 0.42$, $P < 0.05$) in EDL. In Soleus, ucOC tended to be correlated with p-AMPK α ($r = 0.34$, $P < 0.072$). Higher Soleus GPRC6A expression was associated with higher Soleus p-AMPK α ($r = 0.32$, $P < 0.05$). Higher insulin levels were correlated with lower Soleus p-AMPK α ($r = -0.48$, $P < 0.01$).

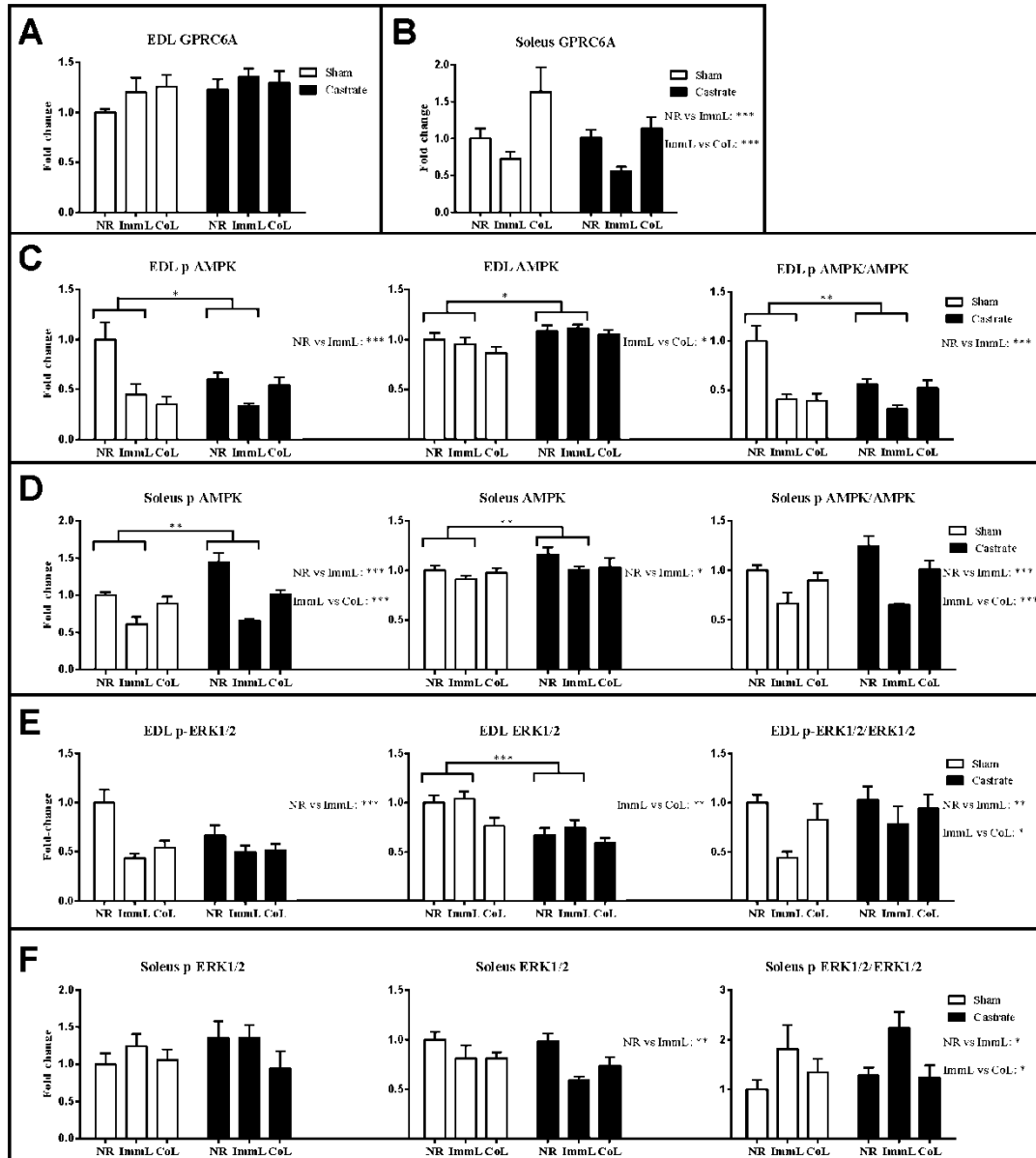


Figure 6.3. Impact of immobilisation and castration on postulated ucOC signalling proteins. GPRC6A expression (A, B), p-AMPK α , AMPK α , and p-AMPK α /AMPK α levels (C, D), as well as p-ERK1/2, ERK1/2, and p-ERK1/2/ERK1/2 levels (E, F) of EDL and Soleus isolated from non-immobilised rats (NR)/immobilised leg (ImmL) and the contralateral leg (CoL) of immobilised rats with sham/castration surgery were examined. *, **, and *** stand for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ in 2-way ANOVA analysis. Symbols of P value of Castration main effect are illustrated on top of columns while symbols of P value of Immobilisation main effects (named NR vs ImmL and ImmL vs CoL) are illustrated on the right side of each panel.

6.4.4 Impact of immobilisation and castration on IRS-1 and Akt

IRS-1 levels following immobilisation were significantly lower compared to either non-immobilised animals or the contralateral leg in both EDL and Soleus (**Figure 6.4A** and **6.4B**, respectively). In EDL, castration led to lower p-Akt/Akt ratio compared to sham control mainly due to an increased total Akt expression (**Figure 6.4C**). In Soleus, p-Akt/Akt was significantly lower following immobilisation compared to the contralateral leg mainly due to a higher total protein levels. Soleus Akt expression was higher following castration in comparison to sham animals (**Figure 6.4D**).

ucOC level were correlated with IRS-1 levels in EDL ($r = 0.48$, $P < 0.01$). EDL IRS-1 was also correlated with p-ERK ($r = 0.30$, $P < 0.04$) and p-AMPK α ($r = 0.54$, $P < 0.001$). Similarly, IRS-1 expression in Soleus correlated with GPRC6A ($r = 0.41$, $P < 0.01$), and p-AMPK α ($r = 0.54$, $P < 0.001$). No correlations between IRS-1 and p-Akt were observed in both types of muscle. EDL p-Akt was correlated with p-ERK in EDL ($r = 0.34$, $P < 0.05$).

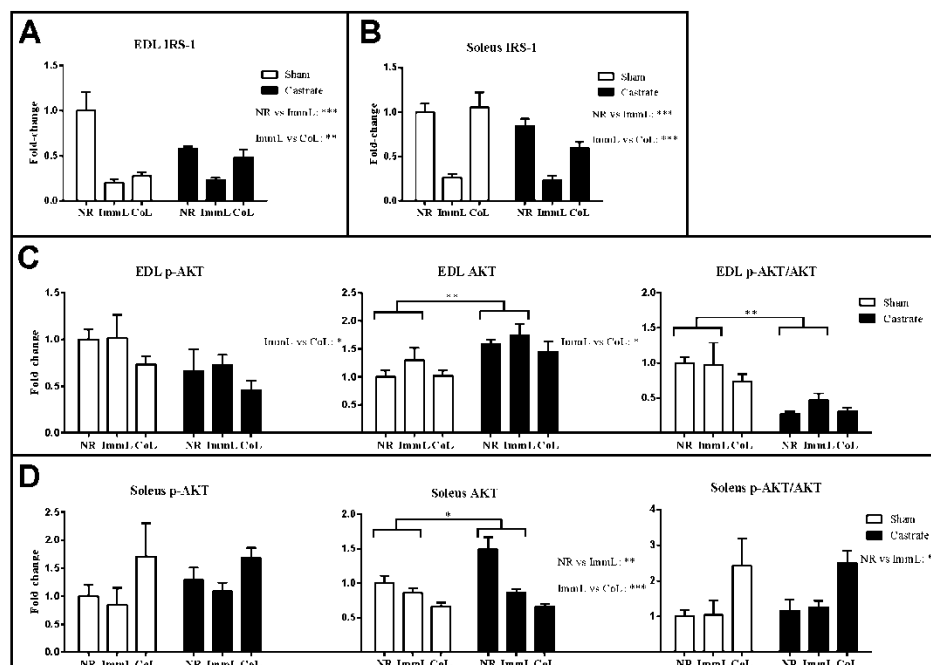


Figure 6.4. Impact of immobilisation and castration on IRS-1 and Akt. IRS-1 expression (A, B), p-Akt, Akt, and p-Akt/Akt levels (C, D) of EDL and Soleus isolated from

non-immobilised rats (NR)/immobilised leg (ImmL) and the contralateral leg (CoL) of immobilised rats with sham/castration surgery were examined. *, **, and *** stand for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ in 2-way ANOVA analysis. Symbols of P value of Castration main effect are illustrated on top of columns while symbols of P value of Immobilisation main effects (named NR vs ImmL and ImmL vs CoL) are illustrated on the right side of each panel.

6.4.5 Impact of immobilisation and castration on mTORC1 signalling proteins

In the EDL, p-mTOR/mTOR ratio was lower following immobilisation compared with both non-immobilised animals ($P < 0.001$) and the contralateral legs ($P < 0.05$) (**Figure 6.5A**). Castration led to enhanced total mTOR, but attenuated p-mTOR and p-mTOR/mTOR ratio in EDL. mTOR expression in immobilised Soleus was significantly lower than in non-immobilised rats (**Figure 6.5B**). In Soleus, p-mTOR and expression was lower in castrated animals compared to sham controls (**Figure 6.5B**).

The mTORC1 substrates P70S6K, both phosphorylation and expression, were lower ($P < 0.05$) in immobilised EDL compared to the contralateral muscle (**Figure 6.5C**). In immobilised Soleus, p-P70S6K and phospho/total ratio of P70S6K were higher than the contralateral leg while total P70S6K level was markedly lower than either control (**Figure 6.5D**). p-ULK1 was significantly lower following immobilisation compared to non-immobilisation rats in both the EDL (~70 %, **Figure 6.5E**) and the Soleus (~50 %, $P < 0.001$, **Figure 6.5F**).

In EDL, ucOC level was correlated with p-ULK1 ($r = 0.54$, $P < 0.01$). p-ERK had correlations with p-mTOR ($r = 0.28$, $P < 0.05$), and p-ULK1 ($r = 0.38$, $P < 0.01$). Lower EDL p-AMPK α was associated with lower EDL p-mTOR ($r = 0.31$, $P < 0.05$), and EDL p-ULK1 ($r = 0.53$, $P < 0.001$). EDL p-mTOR was associated with EDL p-ULK1 ($r = 0.50$, $P < 0.001$). EDL p-Akt was correlated with EDL p-mTOR ($r = 0.33$, $P < 0.05$), and p-ULK1 ($r = 0.44$, $P < 0.01$). In Soleus, higher Soleus p-AMPK α had an association with higher Soleus p-ULK1 ($r = 0.46$, $P = 0.001$).

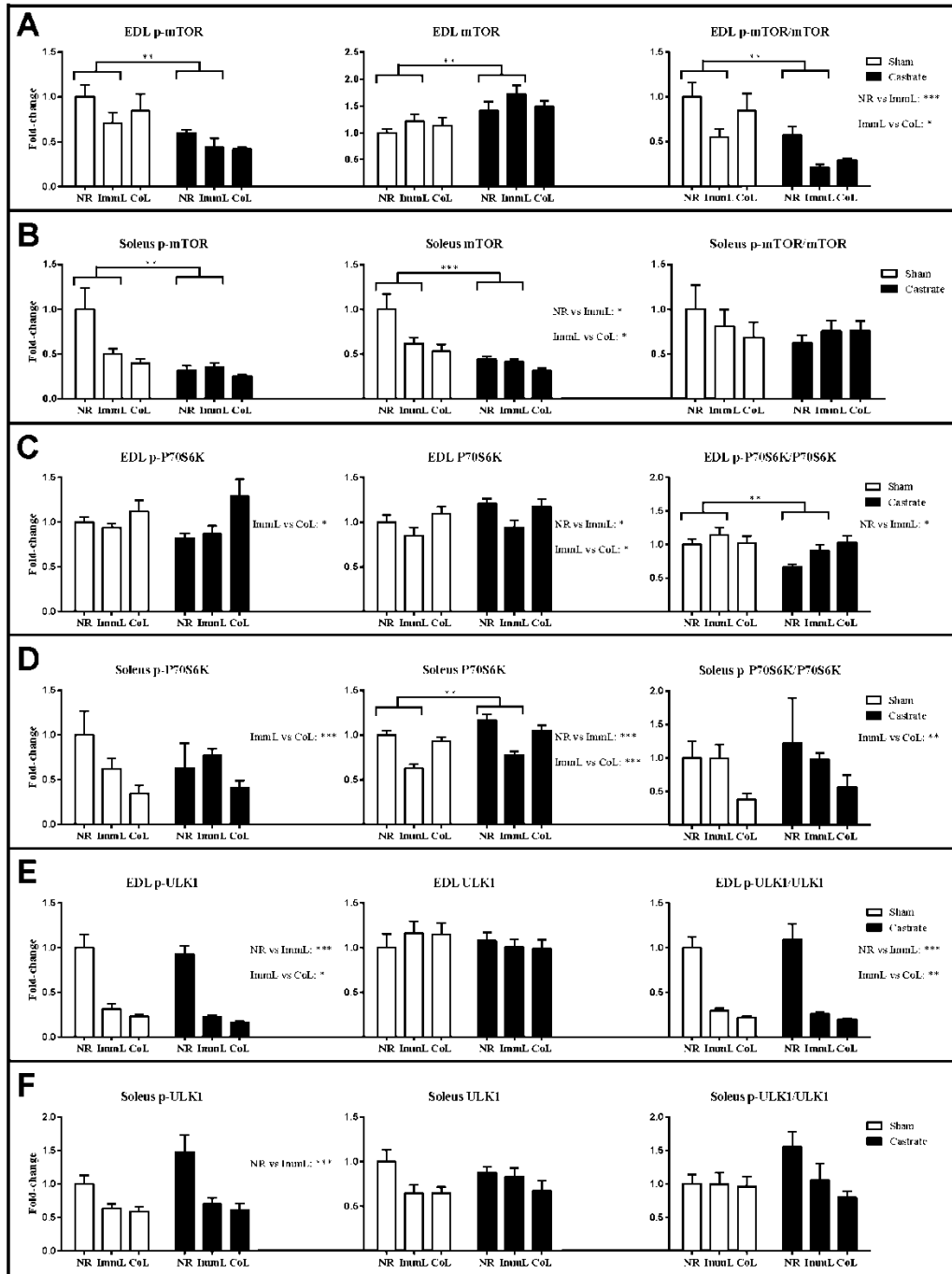


Figure 6.5. Impact of immobilisation and castration on mTORC1 signalling proteins. p-mTOR, mTOR and p-mTOR/mTOR levels (A, B), p-P70S6K, P70S6K, and p-P70S6K/P70S6K levels (C, D), and p-ULK1, ULK1 and p-ULK1/ULK1 levels (E, F) of EDL and Soleus isolated from non-immobilised rats (NR)/immobilised leg (ImmL) and the contralateral leg (CoL) of immobilised rats with sham/castration surgery were examined. *, **, and *** stand for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ in 2-way ANOVA analysis. Symbols of P value of Castration main effect are illustrated on top of columns while symbols of P value of Immobilisation main effects (named NR vs ImmL and ImmL vs CoL) are illustrated on the right side of each panel.

6.4.6 Impact of immobilisation and castration on FOXO signalling proteins

p-FOXO3a levels were significantly lower following immobilisation than the contralateral legs in EDL (**Figure 6.6A**). In castrated animals, total FOXO3a in EDL was higher than sham controls (**Figure 6.6A**). Castrated rats also had higher levels of total FOXO3a in Soleus (**Figure 6.6B**).

Both phosphorylation and expression of FOXO1 were unchanged in EDL after immobilisation or castration (**Figure 6.6C**). There was a marked reduction in the phosphorylation level of FOXO1 following immobilisation compared to non-immobilised animals in Soleus (**Figure 6.6D**). Castration led to an increase in total FOXO1 but a reduction in p-FOXO1/FOXO1 in Soleus (**Figure 6.6D**).

Limited alterations were observed in the expression of E3 ligase Fbx32 in EDL after both immobilisation and castration while immobilised leg showed significant increase of Fbx32 in Soleus comparing with non-immobilisation rats (**Figure 6.6E** and **6.6F**). Following immobilisation, MuRF1 was significantly lower in EDL but higher in Soleus compared to non-immobilisation animals (**Figure 6.6G** and **6.6H**). There was a higher (~40 %, $P < 0.01$) MuRF1 in Soleus in the castration group.

In Soleus, a higher GPRC6A expression was correlated with lower Fbx32 ($r = -0.29$, $P < 0.05$). Higher Soleus p-AMPK α was associated with higher Soleus p-FOXO1 ($r = 0.38$, $P < 0.01$). A higher Soleus p-AMPK α / AMPK α ratio was correlated with a lower Fbx32 ($r = -0.31$, $P < 0.05$) and was tended to be correlated with a lower MuRF1 ($r = -0.26$, $P = 0.07$). Furthermore, Soleus p-FOXO1 ($r = 0.33$, $P < 0.05$) and Soleus p-FOXO3a ($r = 0.32$, $P < 0.05$) was associated with p-ULK1. Lower p-FOXO1/FOXO1 ratio was tended to be associated with higher Fbx32 ($r = -0.27$, $P = 0.062$) and was associated with higher MuRF1 ($r = -0.48$, $P < 0.001$).

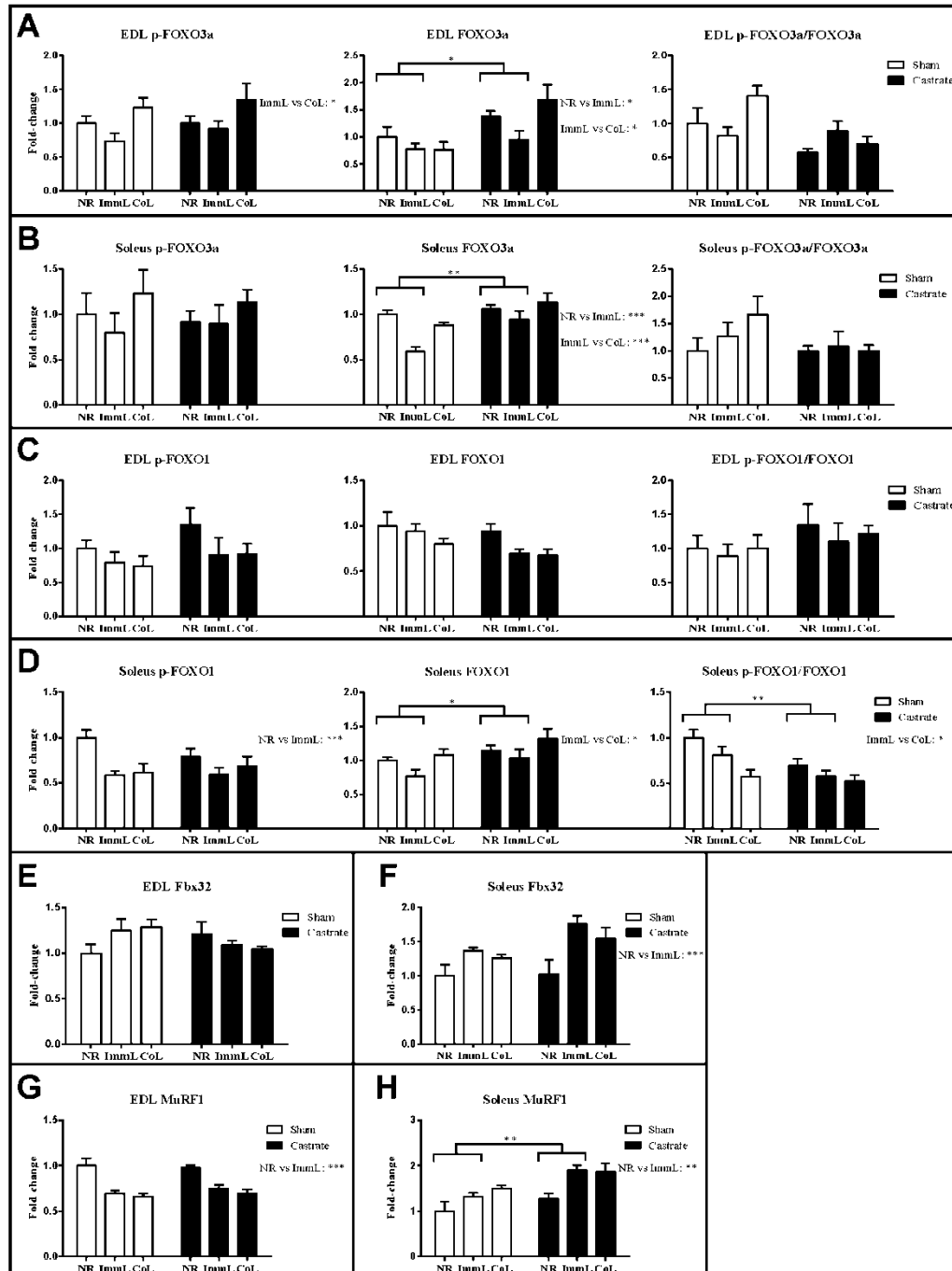


Figure 6.6. Impact of immobilisation and castration on the expression and activity of FOXO signalling proteins. p-FOXO3a, FOXO3a, and p-FOXO3a/FOXO3a levels (A, B), p-FOXO1, FOXO1, and p-FOXO1/FOXO1 levels (C, D), Fbx32 expressions (E, F), and MuRF1 expressions (G, H) of EDL and Soleus isolated from non-immobilised rats (NR)/immobilised leg (ImmL) and the contralateral leg (CoL) of immobilised rats with sham/castration surgery were examined. *, **, and *** stand for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ in 2-way ANOVA analysis. Symbols of P value of Castration main effect are illustrated on top of columns while symbols of P value of Immobilisation main effects (named NR vs ImmL and ImmL vs CoL) are illustrated on the right side of each panel.

6.5 Discussion

We report lower circulating ucOC following hindlimb immobilisation, but not testosterone depletion (via castration), was associated with lower muscle mass and force in both fast-twitch (EDL) and slow twitch (Soleus) muscles (**Figure 2E – H**). This association was not evident following castration. Finally, a higher serum ucOC and a higher GPRC6A expression were related to higher ERK1/2 and AMPK α phosphorylation in a muscle type-specific manner. The reduced activity of these putative ucOC signalling pathways was also related to decreased activity of mTORC1 activity in EDL and activation of FOXO pathway in Soleus following immobilisation.

It appears that skeletal muscle is one of the target organs of ucOC [28, 29, 133, 276, 298]. Previous studies mainly focused on the insulin-sensitising effects of ucOC in skeletal muscles [24, 57, 298]. But recently, it has been suggested that ucOC may play a role in muscle mass maintenance and physiological functions [28, 276, 298]. Here we report that lower circulating levels of ucOC correlates with lower muscle mass and strength in both EDL and Soleus following immobilisation, suggesting a link between ucOC and muscle mass and function.

ucOC promotes glucose metabolism by increasing the secretion of insulin and improving insulin sensitivity [57, 237]. Interestingly, insulin signalling in osteoblasts stimulates ucOC production in bone [66], indicating a positive feedback between ucOC and insulin that is likely to affect whole body glucose metabolism. Since ucOC was reported to contribute to male reproductive capacity by enhancing testosterone production in testis [58] and testosterone has been long known to be involved in bone remodelling and function [286], we hypothesised that reducing testosterone would lead to a reduction in ucOC. In contrast to our hypothesis, serum ucOC levels were not different in castrated rats compared to sham controls. In addition, ucOC did not correlate with testosterone levels. We also observed that testosterone depletion following castration surgery had limited effects on muscle mass and muscle strength. A potential limitation of the current study is the relative short duration of the

intervention of testosterone depletion via castration. Indeed, in other rat studies, a moderate decrease in Soleus mass/body weight ratio was found in animals following 5 wk of castration surgery [300, 301]. In a mice study, 10 wk of castration time was needed to have moderate decrease in Soleus mass/body weight ratio but not in fast twitch muscles [302]. It is thus possible that a longer duration post-castration is required to observe significant effects on circulating ucOC levels, muscle mass and muscle strength. Lower phosphorylation of AMPK, Akt, and mTOR in EDL as well as lower phosphorylation of mTOR, FOXO1 but higher expression of MuRF1 in Soleus was observed, in response to testosterone deprivation, which was similar to what was previously reported [303]. As such, it is likely that, in the short time frame of castration intervention in our study, molecular changes that favor muscle protein loss were already underway. However, these changes had not yet led to any apparent reductions in muscle mass and force. Indeed, in mice with long-term testosterone depletion (7 wk post-castration surgery) both molecular and functional changes, suggesting muscle atrophy, were reported [303]. It is also plausible that the testosterone effect on skeletal muscle is muscle type-specific as higher testosterone levels correlated with higher Soleus muscle weight within sham controls. This indicates testosterone might play a role in maintaining muscle mass more predominantly in slow twitch muscles.

Following hindlimb immobilisation, muscle mass/body weight ratio of Soleus, but not EDL, from the non-immobilised legs was significantly higher (11 %, $P < 0.05$) compared with non-immobilised rats. This finding is similar to a previous studies that reported that Soleus mass/body weight ratio in the non-immobilised leg gradually increased during a 7-d hindlimb immobilisation in rats while the ratio of non-immobilised gastrocnemius (consist mostly of glycolytic fibres) remained unchanged [304]. Furthermore, it was also reported that compared with non-immobilised rats, the rate of protein synthesis in non-immobilised Soleus was moderately higher after 3 d immobilisation. As such, it appears that oxidative muscles, but not glycolytic muscles, in the non-immobilised leg may exhibit some 'mild

overload' that resulted in increased mass.

To date, the down-stream targets of ucOC-GPRC6A cascade in skeletal muscle cells have not yet been identified. However, it is possible that they involve ERK and AMPK, as ucOC treatment in C2C12 myotubes led to ERK1/2 phosphorylation, which can be inhibited by the administration of the inhibitor of the upstream kinase of ERK [29]. Moreover, in primary myotubes, ucOC activated AMPK/mTOR/P70S6 kinase axis via GPRC6A, implicating AMPK as a key protein responsible for the anabolic effects of ucOC in skeletal muscle [276]. Our results demonstrate that lower ucOC level was associated with lower ERK1/2 phosphorylation in EDL. In contrast, Soleus ERK1/2 phospho-/total ratio was elevated post immobilisation compared with both non-immobilised rats and non-immobilised legs, despite more profound muscle wasting. It is possible that this increase in ERK activity in the Soleus is a compensatory response to the greater muscle loss as was reported previously [305]. Hence, our results may indicate that ERK involvement in the ucOC cascade in skeletal muscle is muscle type-specific.

Although lower ucOC was associated with lower p-AMPK α in both EDL and Soleus, only in Soleus did lower p-AMPK α correlate with lower GPRC6A expression compared to both non-immobilised rats and the contralateral leg. This lower p-AMPK α in atrophic oxidative muscle is consistent with a number of other studies. For instance, 10-d limb immobilisation in rats led to a significant reduction in p-AMPK expression in red gastrocnemius muscle compared to the contralateral leg [171]. Furthermore, 2-wk tail suspension led to a significant reduction in p-AMPK mainly in Soleus and to a lesser degree in EDL in rats [173]. In humans, patients with critical myopathy showed decreased AMPK activity in skeletal muscles compared with healthy controls [306]. Accordingly, the attenuation of the ucOC signalling cascade may be related to Soleus muscle wasting attributed to lower AMPK activity. However, unchanged or even a higher AMPK activity during muscle atrophy has also been reported [304, 307]. These incompatible results fit the contradictory role of

AMPK in cell fate in skeletal muscles. On one hand, high activity of AMPK induces membrane Glut4 expression as well as mitochondrial biogenesis, benefiting muscle glucose metabolism and cell growth.[308, 309] On the other hand, AMPK activation leads to cell autophagy via enhancing the activity of cell autophagy pathways [120, 169]. Notably, the majority of findings showing reduced AMPK activity in atrophic muscle were obtained either in animals subject to inactive conditions for at least 10 d or in inactive patients, while short time activation or inhibition of AMPK was mainly employed in studies investigating its role in protein degradation. Therefore, impaired cellular glucose metabolism and energy production in muscle cells due to low AMPK activity can only be observed after a relatively long duration. Indeed, people with short-term leg immobilisation and patients with myopathy are characterised by lower insulin sensitivity [306, 310]. Intriguingly, patients with T2DM have lower circulating ucOC [31, 311], which is of interest as low serum ucOC is also linked with impaired insulin sensitivity in animals [56]. Moreover, we observed that hyperinsulinemia induced by limb immobilisation (**Figure 6.1**) was associated with loss of muscle mass and lower p-AMPK α in the Soleus. Given that patients with T2DM are at a high risk for muscle atrophy [291, 312], glucose dysmetabolism due to low AMPK activity in slow twitch muscle during disuse atrophy may be a plausible scenario. However, this hypothesis should be tested in future studies.

We observed decreased expressions of IRS-1 in both muscles following immobilisation, and these reductions of IRS-1 expression were associated with not only muscle mass loss but also alterations of several signalling proteins. However, we did not find any significant correlations between IRS-1 level and the phosphorylation of its downstream kinase Akt. These results might indicate that the reductions of IRS-1 level were likely the consequence of protein degradation, but were not able to exert significant impact on insulin signalling pathway. Although phosphorylated Akt did not exhibit apparent decrease in EDL, its level was correlated with p-ERK, p-mTOR, and p-ULK1. These correlations were not observed in Soleus. Recently, it has been reported that in C2C12 myotubes, the enhancement of Akt phosphorylation

induced by osteocalcin treatment was abolished by U0126, an inhibitor of ERK kinase (MEK) [29]. As such, in immobilised EDL, Akt phosphorylation could be affected by decreased activity of ucOC/ERK cascade, thus leading to reduced mTORC1 activity.

Higher ULK1 activity was correlated with lower p-mTOR in EDL only. In Soleus, p-ULK1 was associated with p-FOXO1 and p-FOXO3a, which is consistent with the results that FOXO proteins can lead to muscle autophagy by interacting with ULK1 [169]. Ubiquitin E3 ligases Fbx32 and MuRF1 were higher only in Soleus compared with non-immobilised rats following immobilisation, and they were also correlated with muscle loss, GPRC6A level, and/or p-FOXO1/FOXO1 ratio. This suggests that protein degradation caused by FOXO/Fbx32 (MuRF1) is, at least partly, responsible for Soleus muscle loss.

Previous studies have shown that muscle atrophy caused by immobilisation or suspension occurs in a muscle type-specific manner, with muscle mass loss predominantly found in oxidative slow twitch muscles and to a lesser extent in glycolytic fast twitch muscles [173, 297, 313]. However, even though signalling pathways involved in muscle atrophy have been widely reviewed [120, 121, 125, 291], the molecular mechanism for the selectivity of muscle type atrophy still remains unresolved. Our data not only confirmed that hindlimb immobilisation does lead to more severe muscle atrophy and weakness in Soleus compared with EDL, but also highlighted a potential molecular mechanisms. The potential mechanisms are illustrated in **Figure 6.7**. We propose that lower circulating ucOC and an attenuated ucOC signalling, caused by immobilisation, reduces muscle mass in a muscle type-specific manner. In EDL, it leads to attenuated activity of AMPK and ERK, resulting in increased ULK1 activity and reductions in Akt activity and mTOR activity leading to muscle atrophy. In Soleus, reduced ucOC and decreased GPRC6A expression results in larger reductions in AMPK activity, leading to more profound muscle atrophy via enhanced activity of ULK1 and increased expression of Fbx32 and MuRF1 through amplified activity of FOXO proteins, and potentially glucose

dysmetabolism. These hypotheses need to be fully explored in future studies.

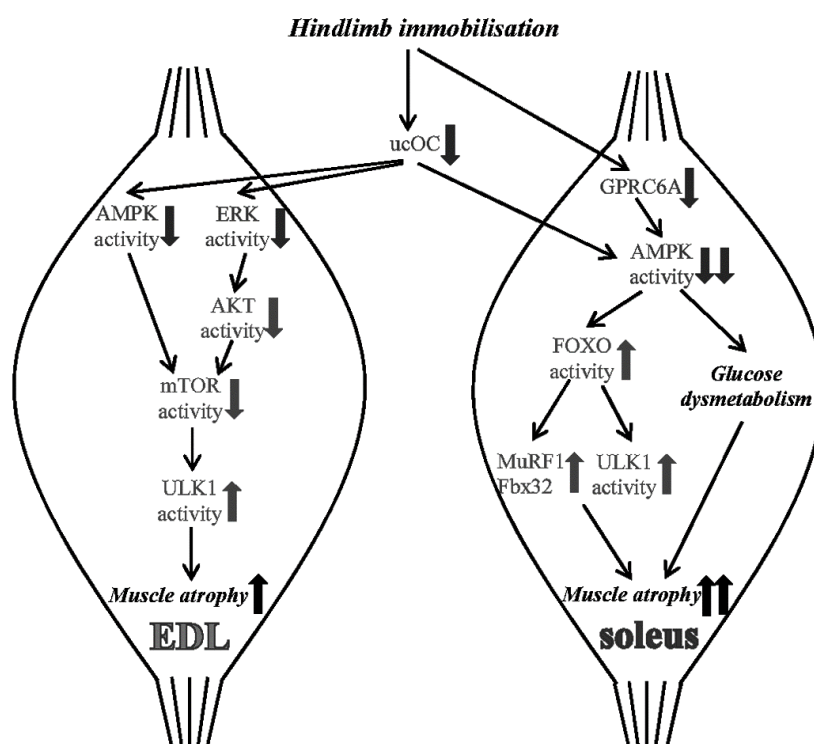


Figure 6.7. Reduced level of circulating ucOC and GPRC6A expression caused by hindlimb immobilisation might lead to enhanced muscle atrophy in a muscle type-specific manner.

In conclusion, hindlimb immobilisation, but not testosterone depletion via castration, leads to a significant reduction in ucOC and lower ucOC was correlated with lower muscle mass and force in both fast-twitch (EDL) and slow twitch (S*oleus) muscles. In addition, the putative ucOC/GPRC6A/ERK (AMPK) signalling cascade was affected by immobilisation, and the expression and activity of proteins in ucOC signalling pathway were also associated with the expression and activity of a number of proteins in the mTORC1 and FOXO signalling pathways in a muscle type-specific manner. However, whether ucOC and its putative signalling pathway play a role in muscle anabolism in addition to its insulin-sensitising effects will need to be explored in detail in future studies involving methods of ucOC deprivation and restoration. Additional investigations are also required to explore the role of ucOC in both muscle gene expression, as well as whether the alteration of gene expression is responsible for the reduced mass and strength following castration and immobilisation.

Chapter 7. General discussion

7.1 Major findings

Maintaining normal muscle glucose uptake and muscle mass is important for general health and quality of life. The results presented in this thesis provide novel and important knowledge supporting the hypothesis that ucOC, a hormone secreted from bone, contributes to the regulations of muscle glucose uptake and mass in a muscle type-specific manner, at least in rodents.

The major findings of this thesis are:

1. ucOC has a muscle type-specific effect on insulin-stimulated muscle glucose uptake at rest and following *ex vivo* contraction (**Chapter 3 and 5**). In addition, the link between the loss of ucOC/GPRC6A cascade and muscle loss during hindlimb immobilisation is muscle type-specific (**Chapter 6**).
2. ucOC *per se* increases muscle glucose uptake in both glycolytic (EDL) and oxidative (Soleus) muscles independent of insulin (**Chapter 4**).
3. Molecular mechanisms involved in ucOC effects on muscle glucose uptake include the enhancement of AS160 phosphorylation under various conditions, likely independent of Akt mediation (**Chapter 3, 4, and 5**).
4. Reduced expression/activity of ucOC, GPRC6A, ERK and AMPK is associated with reduced activity of signalling proteins in anabolic pathway, and associated with enhanced activity of signalling proteins in catabolic pathway, in a muscle type-specific manner (**Chapter 6**).

7.2 ucOC is connected with muscle type specificities in insulin-stimulated glucose uptake and disuse atrophy

Insulin-stimulated muscle glucose uptake is muscle-type specific and this specificity

is also condition-dependent, for instance it varies between resting and post-exercise states (**Section 2.3.1**). In resting muscles, insulin-stimulated glucose uptake predominantly occurs in oxidative muscles (**Section 2.3.1.1**) while following acute exercise the insulin-sensitising effect may occur mainly in glycolytic muscles (**Section 2.3.1.2**). By selectively transporting glucose to specific type of muscles in different conditions, the body is able to supply the nutrient for skeletal muscle with optimum energy efficiency. The identification of underlying mechanisms involved in this muscle type-selective muscle glucose uptake can lead to a better understanding of muscle physiology and to the design of therapeutic interventions appropriate for the specific muscle types. Nevertheless, currently the mechanisms are not clear.

Previous studies implied that circulating factors may play a major role in this condition-dependent muscle type-selective regulation in insulin-stimulated muscle glucose uptake (**Section 2.2.3.2.1.3** and **2.3.1**). This thesis demonstrates that ucOC is one such factor. ucOC enhanced muscle glucose uptake in insulin-stimulated Soleus but not insulin-stimulated EDL in the resting state (**Chapter 5**), while following *ex vivo* contraction ucOC increased insulin-stimulated glucose uptake in EDL but not in Soleus (**Chapter 3**). As the experiments were done in isolated skeletal muscles in a bathing solution, the effects can be attributed to ucOC as other potential circulating factors are not present.

However, the regulation of muscle glucose uptake by ucOC is a complex process that is influenced not only by muscle state (at rest or following contraction), but also by other conditions including the presence, or absence, of insulin. ucOC was recently reported to trigger non-insulin-stimulated glucose uptake only in oxidative muscles, which is contradictory to its possible role in exercise-induced muscle glucose uptake (**Section 2.3.1.3**). In **Chapter 4**, using muscle split instead of intact muscles, we have shown that ucOC enhanced glucose uptake in both EDL and Soleus, indicating that ucOC-triggered glucose uptake occurs in both muscle types, similar to the non-insulin-stimulated muscle glucose uptake induced by other stimuli (contraction,

stretching, and hypoxia) [17, 207-209]. This result also suggests that the ucOC-induced enhancement of glucose uptake in insulin-stimulated Soleus, shown in **Chapter 5**, is likely majorly attributed to the ucOC alone effect on glucose uptake. In addition, it is also possible that the lack of ucOC effect on glucose uptake in insulin-stimulated EDL is due to an inhibitory effect of insulin on the effect of ucOC *per se*.

Overall, the findings from the current thesis suggest that there may be a complex interaction between ucOC and insulin, which is behind the muscle type-specific ucOC effect on muscle glucose uptake in various conditions. This conclusion should be explored in future studies.

The connection of ucOC with muscle type specificity may not be restricted to insulin-stimulated glucose uptake, but may also be the case in muscle atrophy. Disuse-induced muscle atrophy predominantly occurs in oxidative muscles rather than glycolytic muscles (**Section 2.3.2**). **Chapter 6** confirmed this observation by demonstrating a greater loss of muscle mass and muscle strength in Soleus than EDL after hindlimb immobilisation. However, the exact mechanisms behind this susceptibility of oxidative muscles remain largely unknown. In **Chapter 6**, we showed that hindlimb immobilisation caused a decrease in the postulated receptor of ucOC, GPRC6A, in Soleus but not EDL, which was closely associated with reduced muscle mass. Accordingly, these findings suggest that a muscle type-specific loss of ucOC/GPRC6A signalling may account for the greater hindlimb immobilisation-induced muscle wasting in oxidative muscles. However, the cause-effect relationship between the ucOC/GPRC6A cascade and muscle mass need to be studied directly using GPRC6A knockout animals with hindlimb immobilisation.

Taken together, the muscle type variance in insulin-stimulated muscle glucose uptake may be linked with the condition-dependent muscle type-specific ucOC regulation, and the muscle type specificity of hindlimb immobilisation-induced

muscle atrophy may be due to the muscle type-specific loss of ucOC/GPRC6A cascade.

7.3 Signalling pathways underlying ucOC effects on skeletal muscle

The findings from this PhD indicate that ucOC/GPRC6A-mediated downstream proteins likely include ERK/Akt, AMPK, AS160, mTORC1, MuRF1, Fbx32, and/or ULK1. The signalling pathways are muscle type-specific in different conditions (with the presence/absence of insulin, at rest/post contraction), which may have implications for the identification of future drug targets for insulin resistance/T2DM and muscle atrophy.

7.3.1 Signalling pathways underlying ucOC effects in EDL

In resting EDL, ucOC alone triggers glucose uptake via AS160 phosphorylation. Although ucOC/GPRC6A cascade enhances Akt phosphorylation via ERK activation, the ERK/Akt cascade may only play a small role in ucOC-triggered AS160 phosphorylation (**Figure 7.1**). Therefore, this AS160 phosphorylation is probably due to ucOC-induced activation of other signalling proteins. These proteins may include CREB as recently reported [23], but whether AMPK or PKC is involved or not needs more evidence, as described in **Chapter 4**. However, in the presence of insulin this ucOC action disappears in EDL. Therefore, there could be an inhibitory effect of insulin action on the ucOC/GPRC6A cascade in EDL, which needs to be investigated in the future.

Nevertheless, the regulation of ERK/Akt via ucOC in resting EDL (without insulin) results in enhanced activity of mTORC1, leading to increased protein synthesis mediated by P70S6K [129, 152] and decreased autophagy via the inhibition of ULK1 [153] (**Figure 7.1**). During hindlimb immobilisation, the effect of possible

ERK/Akt signalling on muscle growth is attenuated probably due to decreased serum ucOC levels.

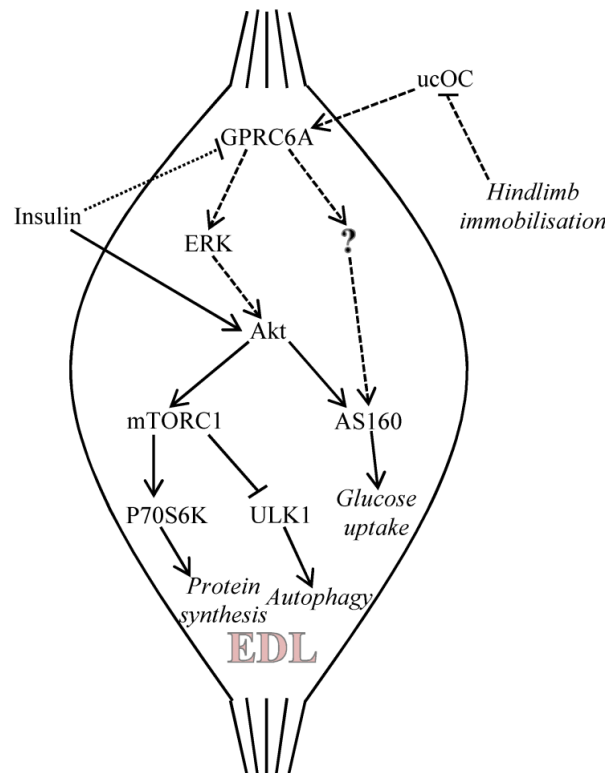


Figure 7.1. Hypothesised mechanisms underlying ucOC actions on glucose uptake and muscle mass regulation in resting EDL. In resting EDL, the plausible ucOC/GPRC6A cascade triggers non-insulin-stimulated AS160 phosphorylation and glucose uptake majorly via unknown mechanisms, and to a much lesser extent via the ERK/Akt cascade. The activation of ERK/Akt cascade favours muscle mass growth by increasing protein synthesis via the mTORC1/P70S6K axis, and by attenuating autophagy via the inhibition of ULK1. The subsequent insulin stimulation may inhibit the ucOC-induced glucose uptake, by inhibiting the activation of ucOC/GPRC6A cascade, in resting EDL. Hindlimb immobilisation possibly attenuates ucOC actions in EDL by reducing circulating ucOC levels.

ucOC: undercarboxylated osteocalcin; GPRC6A: G protein-coupled receptor family C group 6 member A; ERK: extracellular signal-regulated kinase; Akt: protein kinase B; AS160: Akt substrate of 160 kDa; mTORC1: mammalian target of rapamycin complex 1; P70S6K: ribosomal protein S6 kinase; ULK1: UNC-51-like kinase; EDL: extensor digitorum longus muscle.

—————> : activation or increase;

—————| : inhibition;

-----> : suggested activation or increase which is evidenced by recent studies or the current thesis;

-----| : suggested inhibition which is evidenced by recent studies or the current thesis;

.....| : hypothesised inhibition which is not evidenced by current literature or the current thesis;

Italic words: process or event.

In EDL post *ex vivo* muscle contraction (**Figure 7.2**), ucOC likely has an insulin-sensitising effect via the enhancement of insulin-stimulated AS160 phosphorylation, without the involvement of Akt. However, mechanisms mediating this enhancement are unknown, may include a GPRC6A-mediated activation of AMPK α 2 β 2 γ 3 which is more abundantly expressed in mice EDL, compared with Soleus [12]. Since the percentage of AMPK α 2 β 2 γ 3 among total AMPK complexes in human quadriceps muscle is similar to mice EDL (~20 %) [162], this ucOC-induced insulin-sensitising effect may also occur in human muscles after acute exercise.

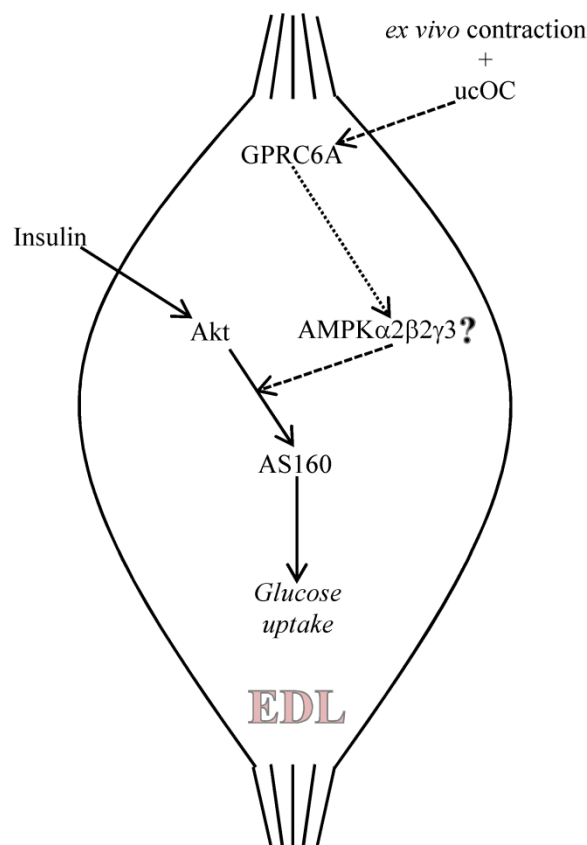


Figure 7.2. Hypothesised mechanisms underlying ucOC action on insulin-stimulated glucose uptake in EDL post-ex vivo contraction. In EDL after *ex vivo* contraction, the activation of plausible ucOC/GPRC6A cascade may induce persistent activation of AMPK α 2 β 2 γ 3, which enhances a latter insulin-stimulated activation of AS160 phosphorylation and glucose uptake.

ucOC: undercarboxylated osteocalcin; GPRC6A: G protein-coupled receptor family C group 6 member A; AMPK α 2 β 2 γ 3: 5' AMP-activated protein kinase consisting of α 2, β 2, and γ 3 subunits; Akt: protein kinase B; AS160: Akt substrate of 160 kDa; EDL: extensor digitorum longus muscle.

—————> : activation or increase;

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increase which is evidenced by recent studies or the current thesis;

.....> : hypothesised activation which is not evidenced by current literature or the current thesis;

Italic words: process or event.

Taken together, findings from this thesis suggest an important role of the enhancement of AS160 phosphorylation in the ucOC effect on glucose uptake in EDL. Furthermore, it is also implied that ucOC/GPRC6A cascade contributes to EDL muscle mass via the signalling of ERK/Akt/mTORC1.

7.3.2 Signalling pathways underlying ucOC effects in Soleus

Similar to EDL, in resting Soleus ucOC *per se* also elicits AS160 phosphorylation, resulting in increased glucose uptake (**Figure 7.3**). This ucOC effect occurs regardless of the presence of insulin, as such ucOC favors AS160 phosphorylation in both non-insulin-stimulated and insulin-stimulated Soleus, probably in an Akt-independent manner (**Chapter 4 and 5**). Although ERK is activated by ucOC, it is likely that the ERK activation was not linked with the activation of AS160 (**Chapter 4 and 5**). The exact mechanisms involved in this ucOC-induced AS160 phosphorylation need more evidence from future studies.

Low AMPK activity may cause muscle wasting via the HDAC4/FOXO axis and impaired cellular energy metabolism in atrophic conditions caused by hindlimb immobilisation and tail suspension (**Section 2.2.5.4.1**). Accordingly, our results in **Chapter 6** suggest that the loss of regulation of ucOC/GPRC6A on AMPK activity may contribute to muscle wasting in Soleus during hindlimb immobilisation. Firstly, the loss of ucOC/GPRC6A activity is linked to increased expression of MuRF1 and Fbx32 and enhanced activity of ULK1. Consequently, the processes of proteolysis and autophagy are enhanced, respectively. Furthermore, the loss of ucOC/GPRC6A activity compromises energy metabolism due to low AMPK activity, failing to provide enough energy for protein synthesis.

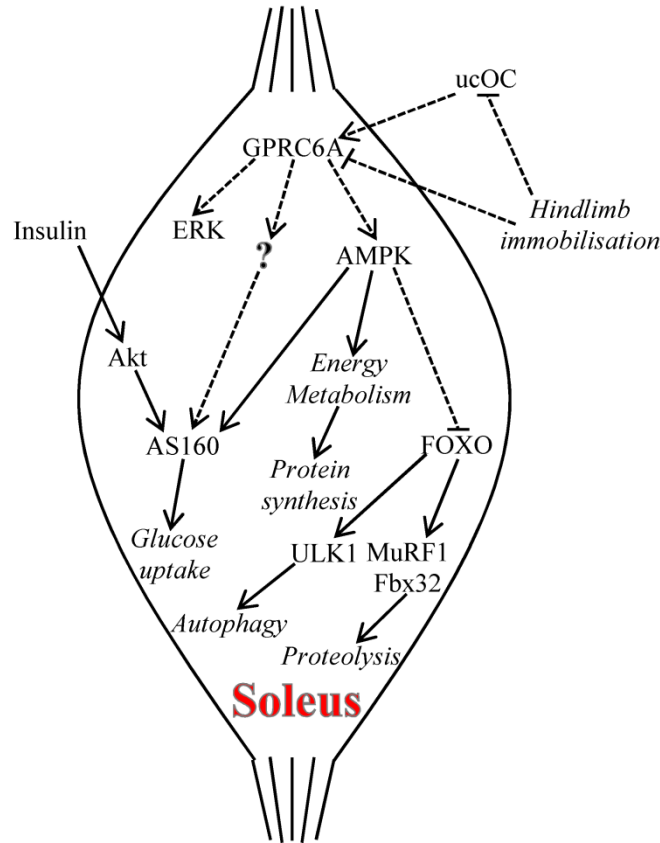


Figure 7.3. Hypothesised mechanisms underlying ucOC actions on glucose uptake and muscle mass regulation in resting Soleus. In Soleus, the activation of plausible ucOC/GPRC6A cascade increases AS160 phosphorylation, independent of insulin, via unknown mechanisms that may include AMPK activation. Furthermore, the ucOC/GPRC6A/AMPK cascade probably upregulates muscle mass growth by attenuating proteolysis and autophagy via inhibiting FOXO activity, as well as by favouring protein synthesis via maintaining normal energy metabolism. Hindlimb immobilisation considerably compromises ucOC actions in Soleus probably by reducing levels of both circulating ucOC and GPRC6A expression.

ucOC: undercarboxylated osteocalcin; GPRC6A: G protein-coupled receptor family C group 6 member A; ERK: extracellular signal-regulated kinase; AMPK: 5' AMP-activated protein kinase; Akt: protein kinase B; AS160: Akt substrate of 160 kDa; FOXO: the class O of forkhead box transcription factor; MuRF1: muscle RING-finger protein-1; Fbx32: muscle atrophy F-box protein; ULK1: UNC-51-like kinase.

—————> : activation or increase;

—————| : inhibition;

- - - - -> : suggested activation or increase which is evidenced by recent studies or the current thesis;

Italic words: process or event.

In general, ucOC increases glucose uptake in both non-insulin-stimulated and insulin-stimulated Soleus, likely via the enhancement of AS160 phosphorylation. Furthermore, the downregulation of ucOC/GPRC6A/AMPK cascade may contribute to muscle loss in Soleus during hindlimb immobilisation.

7.4 Potential limitations

Specific limitations of each study are described in the relevant chapters (**Chapter 3, 4, 5, and 6**). More general limitations of the thesis are:

1. The dose of insulin and the time period of ucOC treatment were determined based on results from previous studies with *in vitro* treatments in skeletal muscle [201, 239]. Therefore, the time course of ucOC treatment and the dose effect of insulin stimulation were not assessed.
2. EDL and Soleus were examined as they are good examples of glycolytic and oxidative muscles. However, albeit EDL and Soleus predominantly consist of glycolytic and oxidative fibres, respectively, they also contain different types of fibres. Thus, the effects of ucOC on each fibre type were not analysed.
3. It is possible that ucOC exerts effects on skeletal muscle via affecting other signalling proteins or factors that were not assessed in this thesis.
4. The studies were conducted in rodents. Therefore, the findings in this thesis may not be transferable to humans.

7.5 General conclusion and suggestions for future research

In conclusion, the results of this PhD thesis suggest ucOC as an important regulator in skeletal muscle. Of particular, its muscle type-specific effect on glucose uptake in various conditions, and muscle type-specific signaling loss during hindlimb immobilisation, may contribute to the muscle type specificities observed in

insulin-stimulated glucose uptake and disuse atrophy, respectively. The major findings of this thesis provide not only an enrichment of knowledge in muscle physiology and pathology, but also imply a therapeutic potential of ucOC administration in preventing or alleviating the impairment of muscle glucose uptake, and the loss of muscle mass and strength, particularly in oxidative muscles which are the major sites of glucose disposal at rest and more severely affected by disuse (**Section 2.3.1.1** and **2.3.2**).

Future investigations are warranted to identify the full mechanisms underlying the ucOC effects on skeletal muscle, and test the therapeutic potential of ucOC administration. Specifically:

1. To further explore the role of ucOC in non-insulin-stimulated glucose uptake and insulin-sensitising effect in skeletal muscle, *ex vivo* studies with various ucOC treatment times and different insulin doses are needed.
2. To explore the fibre-type specific effects of ucOC, single fibre examination should be involved in future studies.
3. To delineate the underlying mechanisms of ucOC effects on skeletal muscle, studies with quantitative proteomics and phosphoproteomics are warranted.
4. Muscle disuse causes insulin resistance and muscle atrophy at the same time [23, 29, 314, 315]. To test the therapeutic potential of ucOC treatment in countering insulin resistance and mass loss simultaneously, pre-clinical studies with intermittent ucOC injections in mice with hindlimb immobilisation should be performed.
5. To explore whether ucOC exerts these effects in human muscle or not, mechanistic studies using human primary myotubes are needed.

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Appendices: Publications related to this thesis

The effects of muscle contraction and recombinant osteocalcin on insulin sensitivity ex vivo by L. Levinger, X. Lin, X. Zhang, T.C. Brennan-Speranza, B. Volpato, A. Hayes, G. Jerums, E. Seeman, G. McConell was published in the peer review journal, *Osteoporosis International*, 27, 653-663, 2016.

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Uncarboxylated Osteocalcin Enhances Glucose Uptake Ex Vivo in Insulin-Stimulated Mouse Oxidative But Not Glycolytic Muscle by X. Lin, L. Parker, E. McLennan, X. Zhang, A. Hayes, G. McConell, T. C. Brennan-Speranza, I. Levinger was published in the peer review journal, *Calcified Tissue International*, 103, 198-205, 2018

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Recombinant Uncarboxylated Osteocalcin *Per Se* Enhances Mouse Skeletal Muscle Glucose Uptake in both Extensor Digitorum Longus and Soleus Muscles

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Emerging evidence suggests that undercarboxylated osteocalcin (ucOC) improves muscle glucose uptake in rodents. However, whether ucOC can directly increase glucose uptake in both glycolytic and oxidative muscles and the possible mechanisms of action still need further exploration. We tested the hypothesis that ucOC *per se* stimulates muscle glucose uptake via extracellular signal-regulated kinase (ERK), adenosine monophosphate-activated protein kinase (AMPK), and/or the mechanistic target of rapamycin complex 2 (mTORC2)-protein kinase B (AKT)-AKT substrate of 160 kDa (AS160) signaling cascade. Extensor digitorum longus (EDL) and soleus muscles from male C57BL/6 mice were isolated, divided into halves, and then incubated with ucOC with or without the pretreatment of ERK inhibitor U0126. ucOC increased muscle glucose uptake in both EDL and soleus. It also enhanced phosphorylation of ERK2 (Thr202/Tyr204) and AS160 (Thr642) in both muscle types and increased mTOR phosphorylation (Ser2481) in EDL only. ucOC had no significant effect on the phosphorylation of AMPK α (Thr172). The inhibition of ucOC-induced ERK phosphorylation had limited effect on ucOC-stimulated glucose uptake and AS160 phosphorylation in both muscle types, but appeared to inhibit the elevation in AKT phosphorylation only in EDL. Taken together, ucOC at the physiological range directly increased glucose uptake in both EDL and soleus muscles in mouse. The molecular mechanisms behind this ucOC effect on muscle glucose uptake seem to be muscle type-specific, involving enhanced phosphorylation of AS160 but limitedly modulated by ERK phosphorylation. Our study suggests that, since ucOC increases muscle glucose uptake without insulin, it could be considered as a potential agent to improve muscle glucose uptake in insulin resistant conditions.

Keywords: undercarboxylated osteocalcin, skeletal muscle, glucose uptake, extracellular signal-regulated kinase, adenosine monophosphate-activated protein kinase, mechanistic target of rapamycin complex 2-AKT-AS160 signaling cascade

INTRODUCTION

The skeleton is an endocrine organ that has been shown, at least in mice, to modulate glucose metabolism (1–3). One bone-specific hormone that plays a role in this energy regulation is osteocalcin (OC) (4, 5). Undercarboxylated osteocalcin (ucOC), the biologically active form of OC, regulates glucose metabolism by targeting the pancreas and perhaps several insulin-sensitive organs, including skeletal muscle (6–8). The effect of ucOC on skeletal muscle may have important clinical implications for whole-body glycemic control as it is the major site for glucose disposal and storage (9, 10). It has been reported that ucOC increases insulin sensitivity in rodent skeletal muscle (11–15). Recent evidence also suggests that ucOC may enhance muscle glucose uptake in the absence of insulin. For example, it has been shown that 10 ng mL⁻¹ ucOC increases glucose uptake in C2C12 myotubes, and to a lesser extent in *ex vivo* soleus muscle which mainly relies on oxidative metabolism for energy production, but not in *ex vivo* extensor digitorum longus (EDL) muscle, which largely utilize glycolytic metabolism as the energy source (15). Similarly, in our previous study, we did not observe any effect of ucOC on glucose uptake of non-contracted EDL *ex vivo* (14). However, since GPRC6A, the presumable receptor for ucOC, is expressed in both EDL and soleus (14, 15), the regulation on muscle glucose uptake by ucOC in both muscle types is still possible. We hypothesize that the limited direct effects of ucOC that was previously observed on EDL were likely due to the inadequateness of ucOC to access the internal area of intact muscle *in vitro*. Therefore, it is possible that a methodological limitation affected the results and improved techniques such as the application of muscle strips, which was previously performed by Cartee et al. (16) and others (17, 18), need to be introduced.

Furthermore, the potential mechanisms behind ucOC *per se* effect on skeletal muscle glucose uptake are still largely unknown. Our previous report exhibited enhanced insulin-stimulated glucose uptake and AS160 phosphorylation at Thr642 by ucOC treatment in EDL muscle post *ex vivo* contraction (14). Insulin-induced phosphorylation of AS160, and subsequent increases in glucose uptake, requires fully activated AKT *via* the activation of mechanistic target of rapamycin complex 2 (mTORC2), which can be indicated by the phosphorylation of AKT at Ser473 and the phosphorylation of mTOR at Ser 2481 (19–22). The mTORC2-AKT-AS160 signaling cascade can be stimulated not only by insulin but also other growth factors and stimuli (23–25). Recent findings indicate that ucOC may also be able to trigger this signaling pathway. In vascular smooth muscle cells, the phosphorylation of AKT was enhanced by the treatment of purified bovine OC (26). Furthermore, the phosphorylation of AKT at Ser473 was elevated following OC treatment in descending thoracic aortic strips of ApoE-KO mice (27). In addition, the phosphorylation of AKT (Ser473) was increased in C2C12 myotubes with ucOC exposure during cell differentiation (13). Nevertheless, the upstream pathway/s that result in the phosphorylation of AKT and AS160 by ucOC are still unclear. It is possible that two previously identified downstream targets of ucOC, extracellular signal-regulated kinase (ERK) and adenosine monophosphate-activated protein kinase (AMPK),

may be involved (28, 29). Indeed, in atrophic rat muscles, lower serum ucOC levels were associated with lower phosphorylation levels of ERK (Thr202/Tyr204) and AMPK (Thr172), and the phosphorylation levels of ERK positively correlated with the phosphorylation levels of AKT (S473) in EDL muscle (30). In C2C12 myotubes, ucOC-stimulated ERK phosphorylation (Thr202/Tyr204) likely contributed to the increase of AKT phosphorylation at Ser473 (13). Furthermore, exercise-induced p-AMPK (Thr172) was augmented by ucOC injection in mice tibialis muscle, which could be responsible for ucOC-enhanced exercise-stimulated muscle glucose uptake (15).

Therefore, the aims of this study were to (a) test the hypothesis that physiological levels of ucOC *per se* increases glucose uptake in both EDL and soleus muscles and (b) explore the mechanisms underlying the effects of ucOC on muscle glucose uptake.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6J mice ($N = 55$) were purchased from Animal Resources Centre (WA, Australia). All mice were group housed with a 12-h light/12-h dark cycle and fed standard laboratory chow [Specialty Feeds mouse food cubes (Glen Forrest, WA, Australia) containing 20% protein, 4.8% fat, and the rest carbohydrate and fiber] and water *ad libitum* until 9–12 weeks old. The study was approved by the Animal Experimentation Ethics Committee of Victoria University (AEC14/009) and conformed to the Australian National Code of Practice for the Care and Use of Animals for Scientific Purposes. The mice for each group in this study were randomly allocated.

Muscle Dissection

Mice were fasted for 4 h before deep anaesthetization with 60 mg kg⁻¹ intraperitoneal pentobarbital. Left and right EDL and soleus muscles were excised within 30 min of anesthesia. Isolated muscles were bathed in carbogenated Krebs–Henseleit buffer (KHB) (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, pH 7.4) and evenly divided into halves longitudinally. After muscle dissection, mice were euthanized *via* cervical dislocation under anesthesia.

ucOC Stimulation

Muscles were evenly divided longitudinally into halves to improve the effusion of ucOC into muscle fiber *ex vivo*, similar to what has been performed in rat muscle in previous studies (16–18). The whole ucOC stimulation process is shown in Figure S1 in Supplementary Material. In experiments without the ERK inhibitor U0126, muscle samples were preincubated in 30°C baths containing carbogenated KHB buffer for 1 h. In experiments with U0126 ($N = 5$), after 30 min preincubation, muscle samples were exposed to the ERK inhibitor U0126 (1 μ M) (Cell Signaling, MA, USA) or dimethyl sulfoxide (DMSO) vehicle (Sigma-Aldrich, MO, USA) for 30 min. Then, muscle samples were stimulated for 90 min with increasing doses [0 ng mL⁻¹ ($N = 6$), 0.3 ng mL⁻¹ ($N = 10$), 3 ng mL⁻¹ ($N = 10$), 10 ng mL⁻¹ ($N = 14$), or 30 ng mL⁻¹ ($N = 10$)] of recombinant ucOC

(Bachem, Bubendorf, Switzerland). These doses of ucOC were chosen because they are within the physiological range in mice (7, 31). In experiments without U0126, muscle halves from the same mouse were treated with KHB buffer control or ucOC. In experiments with U0126, muscle halves from the same mouse were treated with DMSO, DMSO with ucOC, U0126, and U0126 with ucOC, respectively.

2-Deoxyglucose Uptake Measurement and Sample Homogenization

The method to assess 2-Deoxy-D-glucose (2-DG) uptake has been described previously (14). Briefly, after the 90 min ucOC treatment, muscles were transferred to chambers containing KHB + 0.1% bovine serum albumin (Sigma-Aldrich) + 2 mM 2-Deoxy-D-[1,2- ^3H]-glucose (PerkinElmer, MA, USA) and 16 mM D-[1- ^{14}C] mannitol (PerkinElmer) with or without U0126/Vehicle or ucOC. After 10 min, muscles were rapidly rinsed with ice cold KHB buffer, then immediately frozen in liquid nitrogen. On the day of sample processing, muscle samples were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (60 μL RIPA for 1 mg sample) (Cell Signaling) with Inhibitor Cocktail (Cell Signaling) and 100 mM dithiothreitol (Sigma-Aldrich) using TissueLyser II (QIAGEN, Hilden, Germany) followed by gentle rocking at 4°C for 1 h. Half of the lysate was pipetted into vials with scintillation cocktail for scintillation counting (β -counter) with Tri-Carb 2910TR Liquid Scintillation Analyzer (PerkinElmer) and the other half was used in western blotting.

Western Blotting

After muscle samples were homogenized using RIPA buffer, protein concentrations in the lysate were determined by Bio-Rad Protein Assay (Bio-Rad, CA, USA). Equal amounts of protein were subjected to electrophoresis on Criterion stain-free precast gels (10%; Bio-Rad) and then transferred electrophoretically using Trans-Blot Turbo Transfer System (Bio-Rad) onto a polyvinylidene fluoride membrane (Bio-Rad). Then, a stain-free blot image was taken using ChemiDoc Imaging System (Bio-Rad) for total protein measurement in each sample lane. Immunoblotting was performed at optimum conditions for each antibody. Bands were identified using ChemiDoc Imaging System, using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, MA, USA). Band densities of both stain-free blot and immunoblotting were measured using Image Lab Software (Bio-Rad). Values of immunoblotting bands were normalized using total protein values. p-ERK (Thr202/Tyr204), ERK, p-AMPK α (Thr172), AMPK α , p-mTOR (Ser2481), mTOR, p-AKT (Ser473), AKT, p-AS160 (Thr642), AS160, and p-PKC δ/θ (Ser643/676) antibodies were purchased from Cell Signaling.

Two data points for AMPK α phosphorylation assessment were excluded due to western blot imaging artifacts. However, their exclusion did not alter the statistical outcome, interpretation, or conclusions of the results.

Statistical Analysis

Fold-changes for western blotting data were calculated by normalization to control groups within the same animals. 3 ng mL $^{-1}$

group and 30 ng mL $^{-1}$ group were chosen for western blotting and correlation analysis as representatives of low and high doses of ucOC.

Paired *t*-tests were used to analyze the effects of ucOC, for each individual concentration, on muscle glucose uptake, protein phosphorylation, protein abundance, and phospho/total ratio compared to paired control samples. This paired comparison was used to exclude individual variances.

To analyze the dose-response effects of ucOC on muscle glucose uptake, basal glucose uptake data from all groups were combined, then one-way ANOVA with Tukey *post hoc* test was applied.

Spearman's correlation was performed between the variables from 30 ng mL $^{-1}$ ucOC treatment group. Rule of thumb for interpreting the size of a correlation coefficient will be applied to measure the strength of correlation between two variables (32). According to standard practice thresholds, the *r* ranges for negligible positive, low positive, moderate positive, high positive, and very high positive correlations are defined as $0.00 < r < 0.30$, $0.30 \leq r < 0.50$, $0.50 \leq r < 0.70$, $0.70 \leq r < 0.90$, and $0.90 \leq r \leq 1.00$, respectively.

All figures and analyses were performed using GraphPad 6 (GraphPad Software, La Jolla, CA, USA).

All data are reported as mean $\pm$ SEM.

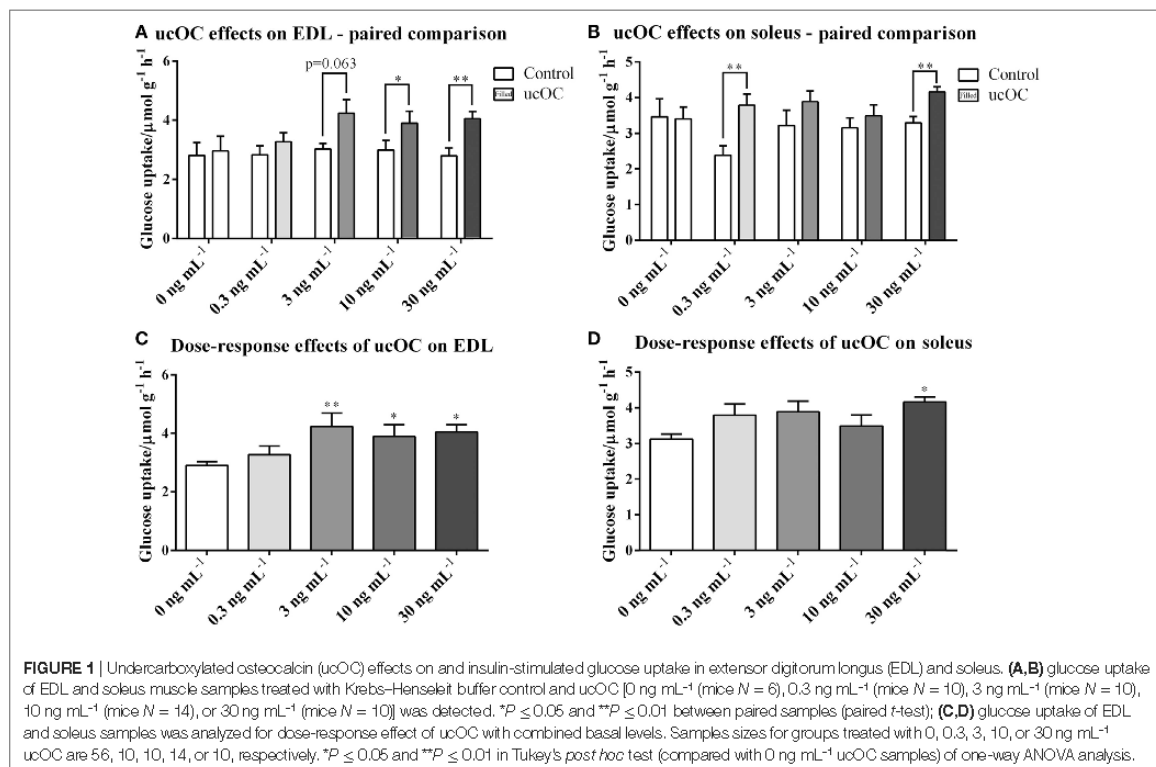
RESULTS

ucOC Increased Glucose Uptake in both EDL and Soleus Muscles

Compared with paired controls, muscle glucose uptake was significantly higher following the treatment of ucOC at doses of 10 ng mL $^{-1}$ ($P < 0.05$) and 30 ng mL $^{-1}$ ($P < 0.01$) in EDL, and 0.3 ng mL $^{-1}$ ($P < 0.01$) and 30 ng mL $^{-1}$ ($P < 0.01$) in soleus (Figures 1A,B). When data were analyzed for ucOC dose-response effects, ucOC significantly enhanced glucose uptake at doses equal or larger than 3 ng mL $^{-1}$ in EDL and at a dose of 30 ng mL $^{-1}$ in soleus (Figures 1C,D; $P < 0.05$, ANOVA $P < 0.01$), from 2.91 to 4.32 $\mu\text{mol g}^{-1} \text{h}^{-1}$ and from 3.12 to 4.16 $\mu\text{mol g}^{-1} \text{h}^{-1}$, respectively.

ucOC Stimulated the Phosphorylation of mTOR, AKT, and AS160

In EDL, ucOC treatment at 30 ng mL $^{-1}$ significantly increased p-mTOR (1.37-fold, $P < 0.05$, Figure 2A) and p-mTOR/tmTOR ratio (1.40-fold, $P < 0.05$, Figure 2A), and only tended to increase p-AKT (1.25-fold, $P = 0.074$, Figure 2C) but not p-AKT/tAKT ratio. Neither of these signaling molecules was affected in the soleus (Figures 2B,D). In both EDL and soleus, both p-AS160 and p-AS160/tAS160 ratio were considerably elevated 1.4-fold to 1.8-fold following ucOC treatments at 3 ng mL $^{-1}$ ($P < 0.05$ and $P < 0.05$, Figure 2E; $P = 0.059$ and $P = 0.056$, Figure 2F) and 30 ng mL $^{-1}$ ($P < 0.01$ and $P < 0.05$, Figure 2E; $P < 0.01$ and $P < 0.001$, Figure 2F). Total AS160 expression was also increased by 30 ng mL $^{-1}$ ucOC in EDL (1.13-fold, $P < 0.05$, Figure 2E). Blots of phosphorylated proteins and total expression of proteins are shown as Figure S2 in Supplementary Material.



ucOC Stimulated the Phosphorylation of ERK but Not AMPK

p-ERK2, but not p-ERK2/ERK2 ratio, was increased by the treatment of 30 ng mL⁻¹ ucOC (1.14-fold, *P* < 0.05, **Figure 3A**) in EDL, and by both 3 and 30 ng mL⁻¹ of ucOC in soleus (1.24-fold and 1.17-fold, *P* < 0.05 and *P* < 0.01, **Figure 3B**). ucOC at 3 or 30 ng mL⁻¹ had limited effects on AMPKα phosphorylation in both EDL and soleus (**Figures 3C,D**). However, soleus total AMPKα levels were increased by the treatment of 30 ng mL⁻¹ ucOC (1.30-fold, *P* < 0.01, **Figure 3D**). Blots of phosphorylated proteins and total expression of proteins are shown as Figure S3 in Supplementary Material.

Treatment with 30 ng mL⁻¹ ucOC had limited effects on phosphorylated protein kinase C δ/θ (PKCδ/θ) in both EDL and soleus muscles (Figure S4 in Supplementary Material).

The Phosphorylation Levels of ERK2 Correlated with the Phosphorylation Levels of AKT and AS160

p-ERK2 levels were not associated with glucose uptake levels (**Figures 4A,B**) or p-mTOR levels (**Figures 4C,D**), in either EDL or soleus. Higher levels of p-ERK2 were associated with higher levels of p-AKT in EDL (*P* < 0.05, **Figure 4E**) with a low positive correlation (*r* = 0.48), but not in soleus (**Figure 4F**). In both EDL (*P* < 0.05, **Figure 4G**) and soleus (*P* < 0.001, **Figure 4H**), higher

p-ERK2 levels were associated with higher levels of p-AS160, with a low positive correlation (*r* = 0.48) and a high positive correlation (*r* = 0.85), respectively.

p-AMPKα levels were not associated with glucose uptake or any signaling protein phosphorylation levels in either muscle type (data not shown).

The Prevention of ucOC-Induced ERK Phosphorylation Had Limited Effect on ucOC-Stimulated Muscle Glucose Uptake
Preincubation with 1 μM U0126 blocked ucOC (30 ng mL⁻¹)-induced increases in ERK2 phosphorylation in both EDL and soleus (**Figures 5A,B**). However, the addition of inhibitor did not significantly affect ucOC-stimulated muscle glucose uptake (**Figures 5C,D**).

The Removal of ucOC-Induced ERK Phosphorylation Prevents ucOC-Stimulated AKT Phosphorylation in EDL, but Has Limited Effect on the Phosphorylation of mTOR and AS160 in Both Muscle Types
U0126 (1 μM) had limited effect on p-mTOR following ucOC treatment in either EDL or soleus (**Figures 6A,B**). However,

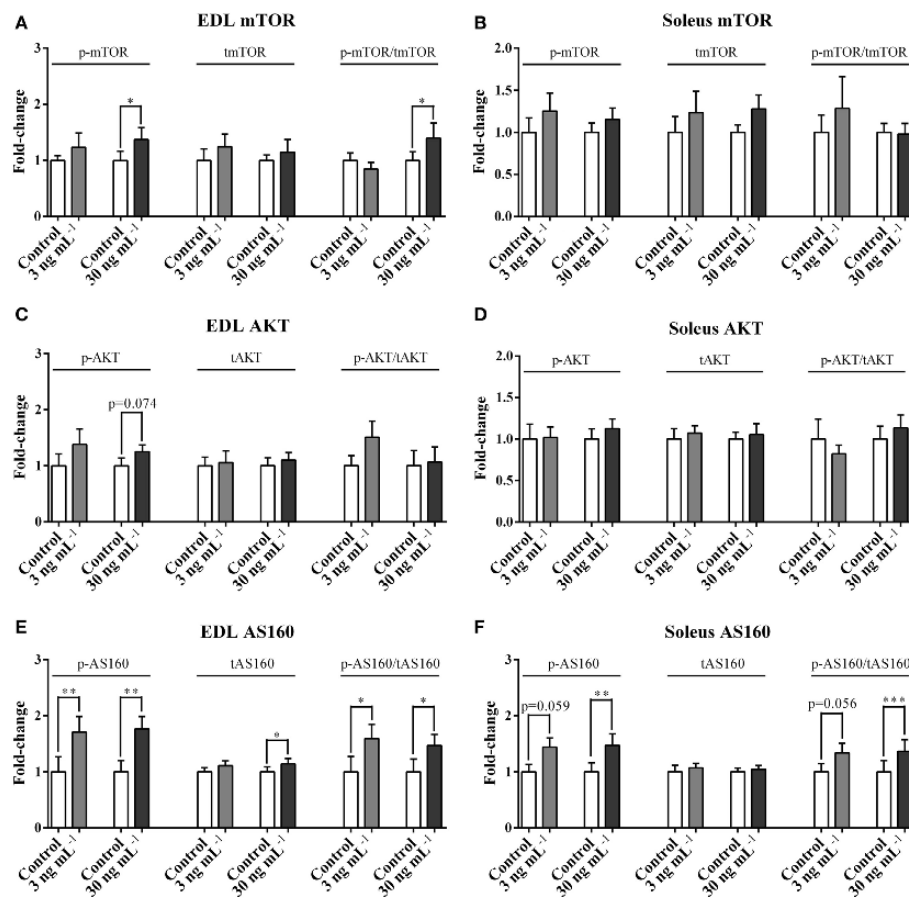


FIGURE 2 | Undercarboxylated osteocalcin (ucOC) effects on the phosphorylation of mTOR, AKT, and AS160. The phosphorylation levels, total expression levels, and phospho/total ratio levels of mTOR (A,B), AKT (C,D), and AS160 (E,F) of Extensor digitorum longus (EDL) and soleus samples treated with Krebs–Henseleit buffer control and ucOC (3 and 30 ng mL⁻¹, *N* = 10 for each dose) were examined. **P* ≤ 0.05, ***P* ≤ 0.01, and ****P* ≤ 0.001 paired samples from the same animal (*t*-test).

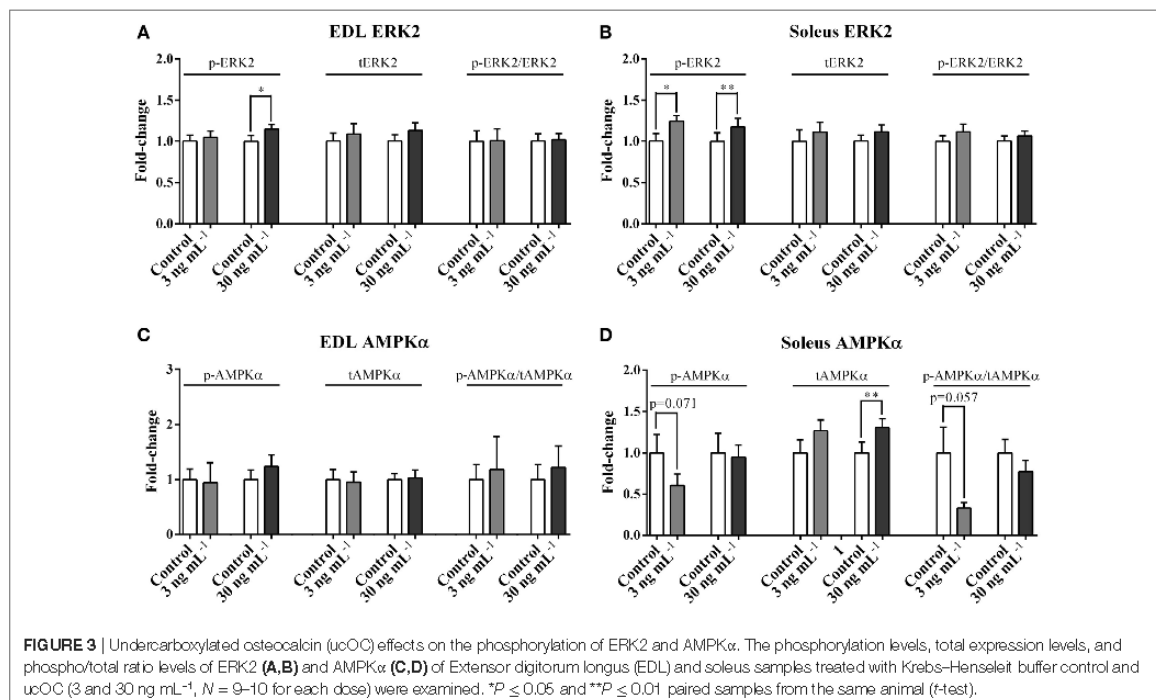
it somewhat prevented the ucOC-mediated AKT activation in EDL with a change close to significant observed in phosphorylation levels (*P* = 0.06), but not in soleus (Figures 6C,D). Although AS160 phosphorylation shared similar patterns of modulation with those of AKT following the treatments, ucOC-stimulated AS160 phosphorylation levels were only marginally decreased by U0126 addition in both muscle types (*P* > 0.1; Figures 6E,F).

DISCUSSION

We report that physiological levels of ucOC *per se* increased muscle glucose uptake *ex vivo* in both EDL (glycolytic muscle) and soleus (oxidative muscle) muscles. Furthermore, ucOC increased the phosphorylation of ERK2, mTOR, and AS160 in EDL and enhanced the phosphorylation of ERK2 and AS160

in soleus muscle. It appears that ERK phosphorylation was not directly involved in ucOC-stimulated glucose uptake and AS160 phosphorylation in both muscle types.

We, and others, have previously reported that ucOC had no significant effect on resting EDL muscle glucose uptake, indicating that ucOC *per se* probably only upregulates muscle glucose metabolism in oxidative muscle fibers (14, 15). However, since the expression of GPRC6A, which is reported as the plausible receptor of ucOC, has been found in both muscle types (14, 15), we suggested that the results of these studies were affected by a potential methodological limitation that the usage of intact whole muscles may prevent adequate ucOC exposure to all muscle fibers. *In vivo*, muscle fibers are closely fed by capillaries that penetrate the epimysium and bifurcate throughout the muscle, primarily within perimysium (33). Since both epimysium and perimysium belong to robust collagenous connective tissue



networks, without the help of blood vessels, ucOC in external solution may have limited direct contact with fibers of intact muscles during *ex vivo* incubation. By utilizing the method of splitting muscles longitudinally into halves (16–18), in order to increase the ucOC saturation during treatment, we report that ucOC can increase muscle glucose uptake in the absence of insulin in both glycolytic (EDL) and oxidative (soleus) muscles, suggesting the effect of ucOC on skeletal muscle glucose uptake is likely universal rather than muscle-type specific. It also seems that, compared with EDL, higher doses of ucOC are required for observing this effect on the glucose uptake of soleus (**Figures 1C,D**). Although there was a significant increase in soleus treated with 0.3 ng mL⁻¹ ucOC using paired comparison method (**Figure 1B**), this increase could not be observed when data were analyzed using one-way ANOVA. Thus, it was likely that this increase was merely resulted from an abnormally low control levels in that specific group. Since skeletal muscle is the major site of glucose disposal and utilization in the postprandial state (9), these findings implicate ucOC as a possible therapeutic agent to improve muscle glucose transport even without insulin.

However, it should be noted that even though we introduced muscle splits in this study to enhance the interaction between ucOC and interior muscle myotubes beneath muscle surface, some limitations, which might result in enhanced biological variations, such as different ucOC saturation percentages due to different muscle dimensions, and different basal glucose uptake levels of different individuals, still cannot be ruled out. Therefore,

future studies should explore the effect of ucOC in primary myotubes from animals and human, to avoid these limitations.

We report that ucOC treatment activated the mTORC2-AKT-AS160 signaling cascade in skeletal muscle, in a muscle type-specific manner. Importantly, ucOC elicited significant increases in AS160 phosphorylation (Thr642) despite relatively modest increases in AKT phosphorylation (Ser473) (*P* = 0.074). Therefore, ucOC may enhance AS160 phosphorylation *via* AKT independent mechanisms, which may be the major mechanisms underlying the ucOC-induced enhancement of glucose uptake and AS160 phosphorylation. Indeed, several other signaling proteins are also able to increase AS160 phosphorylation, including AMPK and conventional/novel (c/n) protein kinase C (PKC) (34).

Along with ERK, previous studies have suggested that AMPK is a potential downstream target of the ucOC signaling in skeletal muscle (13, 15). Our data shows that following 30 ng mL⁻¹ ucOC treatment, there is a significant increase in phosphorylated ERK2 (Thr202/Yr204) in both EDL and soleus muscles. However, there was limited change in AMPKα phosphorylation at Thr172, a phosphorylation site that has widely been reported as an indicator of AMPK activity (35–37). Similarly, it has been reported that AMPKα Thr172 phosphorylation was not increased following ucOC treatment in C2C12 cells (13). A recent paper reported that intraperitoneal injection of osteocalcin increased AMPK phosphorylation in mouse muscles during exercise (15). Given that AMPK Thr172 phosphorylation is increased in skeletal muscle after acute exercise alone, without ucOC (38, 39),

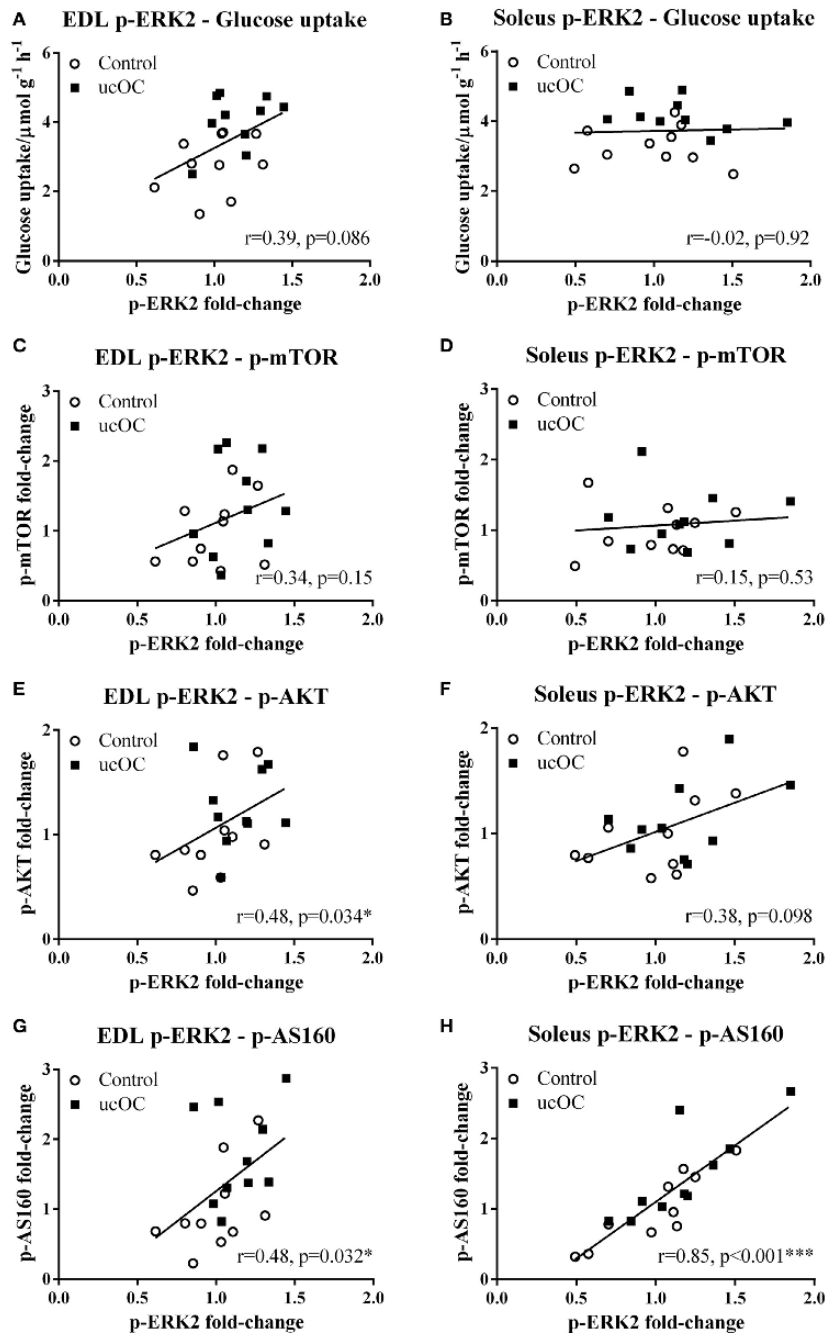
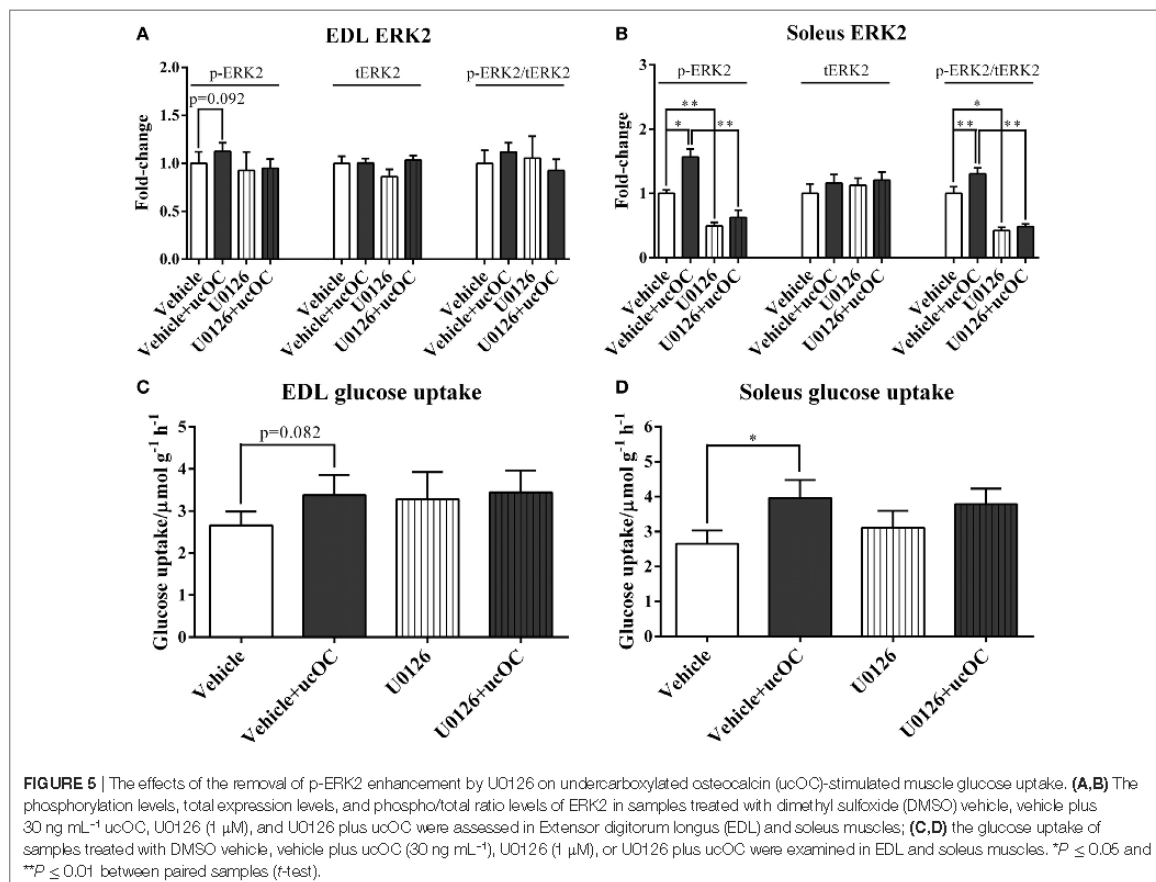


FIGURE 4 | The correlations between the levels of p-ERK2 and the levels of glucose uptake, p-mTOR, p-AKT, and p-AS160. In extensor digitorum longus (EDL) and soleus samples, the correlations between the levels p-ERK2 fold-change and the levels of glucose uptake (**A,B**), p-mTOR fold-change (**C,D**), p-AKT fold-change (**E,F**), and p-AS160 fold-change (**G,H**) were analyzed among samples from 30 ng mL^{-1} undercarboxylated osteocalcin (ucOC) treatment group; $^*P \leq 0.05$ and $^{***}P \leq 0.001$.



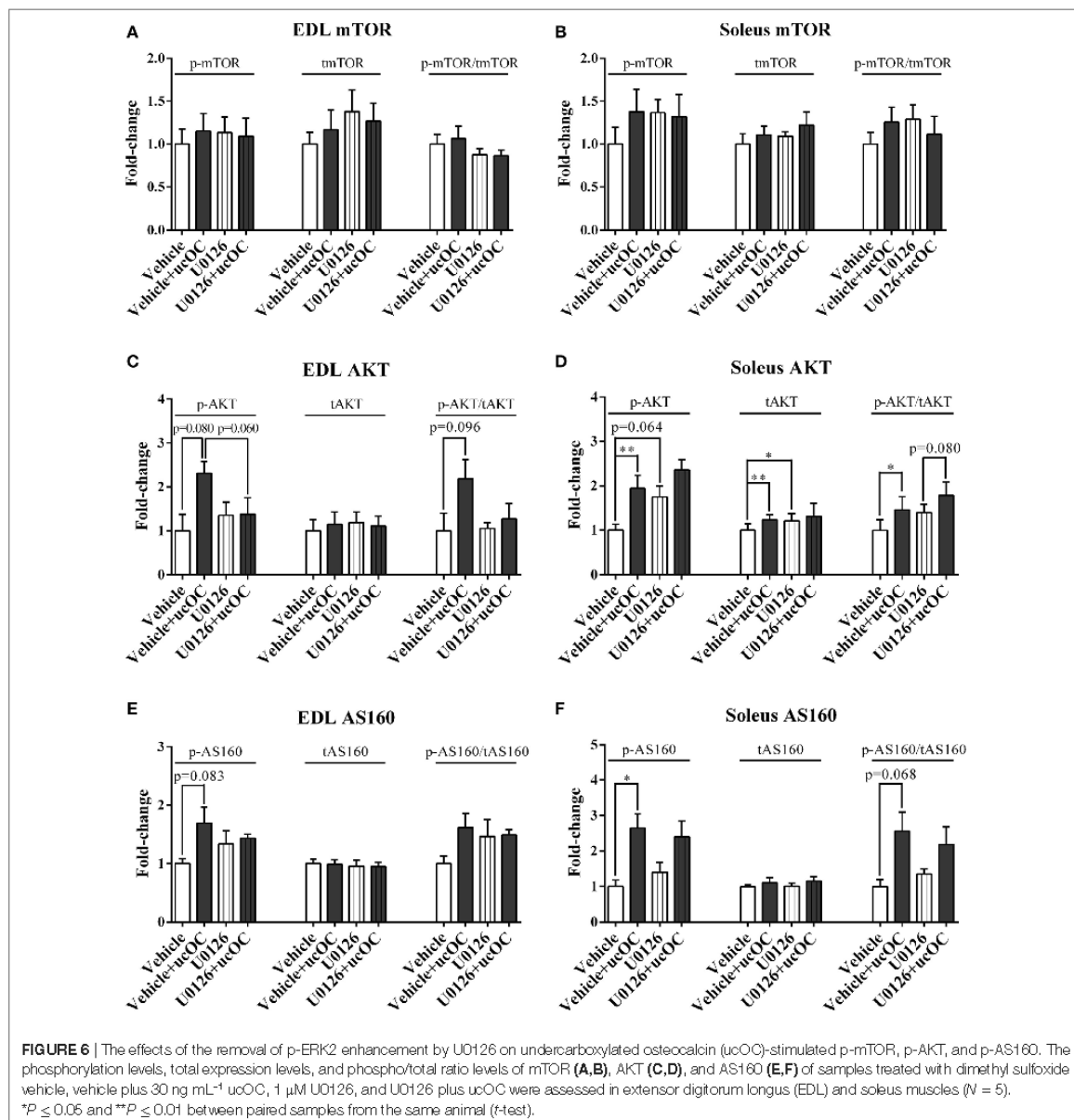
it is possible that ucOC treatment merely has an additive effect on exercise-enhanced phosphorylation of AMPK, but is unable to increase its phosphorylation level *per se*. However, it should be noted that until now no studies have shown ucOC-induced change of AMPK phosphorylation over the course of time. Thus, a transient increase of AMPK phosphorylation after ucOC treatment still cannot be ruled out.

We neither observed any significant increases in the phosphorylation in PKC δ/θ , two important members in novel PKC family, in both muscle types (Figure S4 in Supplementary Material). This finding suggests a limited role of PKC δ/θ in the modulation of muscle glucose uptake by ucOC. However, the involvement of other types of PKC in the mechanisms behind this ucOC effect is still possible, which warrants further investigation.

We reported that p-AKT in EDL and p-ERK2 in both EDL and soleus were enhanced following ucOC treatment with no significant changes in phospho/total ratio (Figures 2C and 3A,B). This discrepancy may be attributed to an ucOC-induced modest increase in total protein expression (Figures 2E and 3D), in addition to its effect on protein phosphorylation. Consistent with our finding, protein synthesis has recently been reported to increase in mouse myotubes following 1–2 h of ucOC treatment (40, 41).

It is possible that ucOC regulates kinase activity by both enhancing protein phosphorylation, and, to a lesser extent, increasing protein abundance.

We report a low positive correlation between p-ERK2 levels and p-AKT levels as well as a high positive correlation between p-ERK2 levels and p-AS160 levels in EDL, which was consistent with our previous findings showing that lower p-ERK levels were associated with lower p-AKT levels in rat EDL muscle (30). In soleus, p-ERK2 levels were associated with p-AS160 levels, with a low positive correlation. As such, we investigated whether the removal of ucOC-mediated ERK phosphorylation leads to the suppression of the effect of ucOC on skeletal muscles. Pretreatment with 1 μM U0126 blocked ucOC-stimulated increases in ERK phosphorylation in both muscle types. In EDL, it seems that the inhibition of p-ERK2 blocked ucOC-stimulated AKT phosphorylation ($P = 0.06$). Consistently, a previous finding also suggested that partial ERK inhibition dampened ucOC-stimulated AKT phosphorylation in C2C12 myotubes (13). In contrast, in response to the loss of ucOC-induced ERK phosphorylation, ucOC-stimulated glucose uptake and AS160 phosphorylation was not compromised. Similarly, in soleus, the inhibition of p-ERK2 had limited effects on ucOC-stimulated glucose uptake and the



phosphorylation of signaling proteins. These findings suggest that mechanisms underlying ucOC stimulation in skeletal muscle are probably muscle type-specific, but converging at AS160 phosphorylation, both resulting in the enhancement of glucose uptake. The ucOC-stimulated mTOR phosphorylation was also not modulated by U0126 pretreatment in both muscle types. Thus, whether ERK signaling modulates AKT phosphorylation through mTORC2 needs further investigations.

The administration of U0126 as an ERK inhibitor has limitations. One limitation derives from the influence of vehicle DMSO,

which is widely used as the solvent for U0126. DMSO has been shown to exert some impact on skeletal muscle, such as depressing muscle contractility and accelerating muscle injury (42, 43). In our study, by comparing the results shown in Figure 2 with those in Figure 6, it is suggested that even the presence of a low concentration of DMSO (0.1%) may have slightly altered the ucOC effect on the phosphorylation of mTOR in EDL and AKT in soleus. However, since DMSO was universally added to all samples in experiments involving the prevention of ucOC-induced ERK phosphorylation, the conclusions drawn from comparisons

between these samples are unlikely to be affected by DMSO addition. The other limitation of U0126 administration in this study is the capability of U0126 to enhance glucose uptake and AKT phosphorylation by itself (44–46). It has been suggested that this effect is due to the elevation of AMPK activity that is independent of ERK inhibition (47). In the current study, the application of low dose (1 μ M) of U0126 had a limited effect on AMPK phosphorylation in both muscle types (Figure S6 in Supplementary Material), and glucose uptake and phosphorylation of most other signaling proteins were also minimally or not at all affected. Nevertheless, other inhibitors or methodologies for ERK inhibition should be investigated in future studies to confirm the involvement of ERK in the regulation of AKT phosphorylation.

In conclusion, ucOC increases glucose uptake in both glycolytic and oxidative muscles in the absence of insulin, *via* mechanisms involving enhanced AS160 phosphorylation. Therefore, ucOC should be considered as a potential agent to improve muscle glucose uptake in insulin resistant conditions including type 2 diabetes.

ETHICS STATEMENT

The study was approved by the Animal Experimentation Ethics Committee of Victoria University (AEC14/009) and conformed to the Australian National Code of Practice for the Care and Use of Animals for Scientific Purposes.

AUTHOR CONTRIBUTIONS

XL, XZ, TB-S, and IL design the study. XL, LP, and EM performed experiments and data collection. XL and LP did the data analysis. XL wrote the paper. XL, LP, XZ, AH, GM, TB-S, and IL substantially contributed to manuscript revision and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/article/10.3389/fendo.2017.00330/full#supplementary-material>.

FIGURE S1 | Flow-chart of the protocol used in this study.

FIGURE S2 | Blots of all samples in western blot analysis for p-mTOR, p-AKT, and p-AS160. Blots of p-mTOR at Ser2481 in Extensor digitorum longus (EDL) (A) and soleus (B) samples, p-AKT at Ser473 in EDL (C) and soleus (D) samples, and p-AS160 at Thr642 in EDL (E) and soleus (F) samples from 3 ng mL⁻¹ (N = 10) and 30 ng mL⁻¹ (N = 10) groups are exhibited.

FIGURE S3 | Blots of all samples in western blot analysis for p-ERK2 and p-AMPK α . Blots of p-ERK2 at Thr202/Tyr204 in Extensor digitorum longus (EDL) (A) and soleus (B) samples as well as p-AMPK α at Thr172 in EDL (C) and soleus (D) samples from 3 ng mL⁻¹ (N = 9–10) and 30 ng mL⁻¹ (N = 9–10) groups are exhibited.

FIGURE S4 | The phosphorylation levels of protein kinase C (PKC) δ /9 in Extensor digitorum longus and soleus samples treated with Krebs–Henseleit buffer control and 30 ng mL⁻¹ ucOC were examined (N = 10).

FIGURE S5 | Blots of all samples in western blot analysis for p-ERK2, p-mTOR, p-AKT, and p-AS160 in ERK inhibition experiments. Blots of p-ERK2 at Thr202/Tyr204 in Extensor digitorum longus (EDL) (A) and soleus (B) samples, p-mTOR at Ser2481 in EDL (C) and soleus (D) samples, p-AKT at Ser473 in EDL (E) and soleus (F) samples, and p-AS160 at Thr642 in EDL (G) and soleus (H) samples in ERK inhibition experiments are exhibited (N = 5).

FIGURE S6 | The phosphorylation levels of AMPK α at Thr172 in Extensor digitorum longus (EDL) and soleus samples treated with dimethyl sulfoxide vehicle, vehicle plus 30 ng mL⁻¹ ucOC, 1 μ M U0126, and U0126 plus ucOC were examined (N = 5).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Hindlimb Immobilization, But Not Castration, Induces Reduction of Undercarboxylated Osteocalcin Associated With Muscle Atrophy in Rats

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ABSTRACT

Undercarboxylated osteocalcin (ucOC) has been implicated in skeletal muscle insulin sensitivity and function. However, whether muscle mass and strength loss in atrophic conditions is related to a reduction in ucOC is not clear. We hypothesized that both immobilization and testosterone depletion would lead to reductions in ucOC, associated with not only the degree of muscle atrophy but also changes to atrophy signaling pathway(s) in male rats. We subjected 8-week-old male Fischer (F344) rats to 7 days of hindlimb immobilization 10 days after castration surgery. Hindlimb immobilization, but not castration, resulted in a significant reduction in ucOC (30%) and lower ucOC was correlated with the degree of muscle loss and muscle weakness. ucOC levels, the expression of ucOC-sensitive receptor G protein-coupled receptor, class C, group 6, member A (GPRC6A), as well as the activity of extracellular signal-regulated kinase (ERK) and 5' adenosine monophosphate-activated protein kinase (AMPK) were associated with the expression and activity of a number of proteins in the mammalian target of rapamycin complex 1 (mTORC1) and Forkhead Box O (FOXO) signaling pathways in a muscle type-specific manner. These data suggest that ucOC may have other effects on skeletal muscle in addition to its insulin sensitizing effect. © 2016 American Society for Bone and Mineral Research.

KEY WORDS: UNDERCARBOXYLATED OSTEOCALCIN; MUSCLE ATROPHY; TESTOSTERONE; HINDLIMB IMMOBILIZATION; CASTRATION

Introduction

Bone is an endocrine organ involved in energy metabolism and possibly male fertility.⁽¹⁾ These functions are achieved by the undercarboxylated form of osteocalcin (ucOC).^(2,3) Osteocalcin may play a role in cell growth and muscle fiber strength in skeletal muscles.^(4–6) Indeed, there is some evidence suggesting that osteocalcin-deficient mice are characterized by lower muscle mass and weaker muscle strength.⁽⁷⁾ In humans, lower ucOC/OC is associated with lower hip flexor, hip abductor, and quadriceps muscle strength.⁽⁸⁾ Furthermore, ucOC treatment promoted increased extensor digitorum longus (EDL) muscle cross-sectional area and grip strength in mice and myotube formation in C2C12 myoblast cultures in vitro.⁽⁶⁾

Muscle atrophy is a consequence of homeostatic imbalance between protein synthesis and protein degradation.⁽⁹⁾ It is possible therefore, that the loss of muscle mass and strength during atrophic conditions might be, at least in part, attributed to lower circulating ucOC. However, it is not clear whether atrophic conditions, as seen following limb immobilization, has an impact on serum ucOC levels.

Testosterone is an anabolic hormone produced by the Leydig cells of the testes and plays a major role in the regulation of muscle mass in males.^(10,11) Loss of testosterone, as a consequence of aging or castration surgery, leads to muscle atrophy and muscle weakness.^(12,13) Testosterone modulates muscle anabolism not only via signaling networks that are directly orchestrated by itself, but also indirectly via other hormones such as follicle-stimulating hormone, luteinizing hormone, and growth hormone^(14,15) due to widely expressed androgen receptors in endocrine organs, including bone.^(16–18) Indeed, there are suggestions for the existence of a reciprocal interaction between bone and gonads.^(19,20) ucOC promotes testosterone secretion in testes.⁽²¹⁾ In turn, testosterone is implicated in bone growth, maturation, and maintenance.⁽²⁰⁾ However, whether testosterone loss has an impact on circulating ucOC level is unknown. As such, it is not clear whether reductions in testosterone level indirectly influence skeletal muscle mass via decreases in ucOC level.

The beneficial effects of ucOC on skeletal muscle are likely mediated via its putative receptor G protein-coupled receptor, class C, group 6, member A (GPRC6A).⁽²²⁾ Within the

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ucOC-GPRC6A cascade, extracellular signal-regulated kinase (ERK) and 5' adenosine monophosphate-activated protein kinase (AMPK) are proposed downstream kinases^(7,23) that are also known to be involved in the proliferation, growth, and lifespan of muscle cells.^(24–26)

Under atrophic conditions, there are several signaling pathways actively responsible for muscle loss. The mammalian target of rapamycin complex 1 (mTORC1) cascade plays a vital role in cell proliferation and growth.⁽²⁷⁾ Its dysfunction has been suggested as one of the main mechanisms underlying muscle atrophy.^(28,29) The phosphorylation of mTOR as well as the phosphorylation of 70-kDa ribosomal protein S6 kinase (P70S6K), one of the major mTORC1 downstream targets, are indicative of mTORC1 activation.^(30,31) Another important target of mTORC1 is unc-51-like autophagy activating kinase 1 (ULK1). Phosphorylation of ULK1 at ser757 by mTORC1 blocks its pro-autophagy functions.⁽³²⁾ Forkhead Box O (FOXO) family proteins are also critical in the process of muscle atrophy.⁽³³⁾ Phosphorylation of FOXO family members inhibit their translocation into the nucleus, thereby inhibiting the transcription of Muscle-specific RING Finger protein1 (MuRF1) and Muscle Atrophy F-box (MAFbx/Fbx32), the two important skeletal muscle-specific ubiquitin E3 ligases that tag proteins for proteolysis via the proteasome.⁽³³⁾ Furthermore, protein kinase B (AKT) functions as a critical convergent point, regulating both mTORC1 and FOXO signaling pathways via phosphorylation.⁽²⁸⁾ The upstream signaling partner of AKT, insulin receptor substrate 1 (IRS-1), directly activated by the insulin receptor, plays a role in these pathways, and can be downregulated via negative feedback in atrophic muscles.⁽³⁴⁾ Nevertheless, it is not clear how these signaling proteins interact with the ucOC-GPRC6A cascade under conditions of muscle atrophy.

The aims of this study were to test the hypotheses that (1) hindlimb immobilization and castration surgery lead to reductions in ucOC levels and (2) the reductions in ucOC correlate with muscle loss and related muscle signaling proteins. We also hypothesized that the expression/activity of downstream proteins in ucOC signaling cascade will be correlated with the muscle atrophy signaling network. Given that different types of muscles respond differently during immobilization-induced atrophy,^(35,36) these correlations between signaling proteins would also be in a muscle type-specific manner.

Materials and Methods

Animals

Male Fischer (F344) rats ($n = 31$, body weight = 224.1 ± 3.1 g) were purchased ~8 weeks of age (Animal Resource Centre, Canning Vale, WA, Australia) and housed in pairs in a light- and climate-controlled room (12:12 hours of light and dark, 20°C to 22°C) with ad libitum access to water and standard animal chow (AIN93G; Specialty Feeds, Glenn Forrest, WA, Australia). All experiments and procedures were approved by the Animal Ethics Committee at Victoria University and in accordance with the Australian code of practice for the care and use of animals in scientific research.

Castration surgery and hindlimb immobilization

After 1 week of acclimatization, animals were randomly allocated into castration or sham groups. Testosterone levels were manipulated by performing a bilateral orchiectomy or sham surgery via a scrotal incision under sterile conditions. Pain

relief was administered via intraperitoneal injection 30 min prior to surgery (0.5 mg/kg, Meloxicam; Therapon, Burwood, VIC, Australia). Animals were allowed to recover for 1 week, at which time they underwent unilateral immobilization of the right hindlimb (or free movement as non-immobilized controls) for 10 days to induce muscle atrophy (7 rats for Sham+Non-immobilization, 8 rats for Sham+Immobilization, 7 rats for Castration+Non-immobilization, 9 rats for Castration+Immobilization). Hindlimb immobilization was conducted under 2% to 4% isoflurane anesthesia, tape stirrups were attached to the top and bottom of the foot, and the leg was then wrapped in cast padding and compression tape. The leg was immobilized by a thermoplastic splint (3-inch Vet-lite casting material; Therapon, Burwood, VIC, Australia) attached to the outside of the leg. The splint was secured in place using strapping tape with the foot in a neutral position. The splint was inspected and repaired daily, as required. Animals were also inspected daily by laboratory and veterinary staff, and their general level of activity, responsiveness, and appearance was assessed. After the immobilization/non-immobilization period, animals were killed in blood collection via heart puncture after ex vivo muscle contraction (see below in Ex vivo muscle contraction and sample collection).

Ex vivo muscle contraction and sample collection

After the 10-day immobilization/non-immobilization period, animals were deeply anaesthetized with sodium pentobarbital (60 mg/kg; Therapon, Burwood, VIC, Australia) via intraperitoneal injection. The EDL and soleus muscles from both legs were excised tendon to tendon and ex vivo contraction was conducted as described.⁽³⁷⁾ After the completion of ex vivo contraction experiments, the EDL and soleus were removed from the bath, blotted dried, cut free of tendon, and weighed. The muscles were then snap-frozen in liquid nitrogen. Blood samples were then collected via heart puncture and left on ice for 30 min, at which time they were spun in a centrifuge at 16,000g at 4°C for 10 min for serum samples. Serum was stored at –80°C until analysis.

Measurement of the level of serum hormones

Total osteocalcin was measured using ELISA kit purchased from Immunotopics (San Clemente, CA, USA) according to the manufacturer's instructions. ucOC level was detected following hydroxyapatite binding as described⁽³⁸⁾ using the same ELISA kit. Serum testosterone and insulin were measured using ELISA kits (in duplicate) purchased from Crystal Chem (Downers Grove, IL, USA) based on kit instructions.

Western blotting

Muscle samples (~15 mg) were lysed in ice-cold radioimmuno-precipitation assay [RIPA] buffer (Cell Signaling, Danvers, MA, USA) with Inhibitor Cocktail (Cell Signaling) and 25 mM dithiothreitol (Sigma Aldrich, St. Louis, MO, USA) using TissueLyser II (QIAGEN, Hilden, Germany) followed by gentle rocking at 4°C for 1 hour. Protein homogenates were collected in the supernatant following centrifugation at 16,000g at 4°C for 15 min. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were subjected to electrophoresis on Criterion Stain-Free precast gels (10%; Bio-Rad) and then transferred electrophoretically using Trans-Blot Turbo Transfer System (Bio-Rad) onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad).

Then a stain-free blot image was taken using ChemiDoc Imaging System (Bio-Rad) for total protein measurement in each sample lane. Immunoblotting was performed at optimum conditions for each antibody. Bands were identified using ChemiDoc Imaging System, using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Waltham, MA, USA). Band densities of both stain-free blot and immunoblotting were measured by densitometry using Image Lab Software (Bio-Rad). Values of immunoblotting bands were normalized using total protein values before statistical analysis. p-mTOR (Ser2448), mTOR, IRS-1, p-ULK1 (Ser757), ULK1, p-FOXO3a (Ser253), FOXO3a, p-FOXO1 (Ser256), FOXO1, p-P70S6K (Thr389), P70S6K, p-AMPK (Thr172), AMPK, p-AKT (Ser473), AKT, p-ERK (Thr202/Tyr204), and ERK antibodies were purchased from Cell Signaling; GPRC6A antibody was provided by AVIVA (San Diego, CA, USA); and Fbx32 and MuRF1 antibodies were purchased from Bioss (Woburn, MA, USA).

Statistical analysis

All values are expressed as the mean fold-change (normalized to non-immobilization group) $\pm$ SE. To analyze the effects of castration and immobilization within non-immobilized animals and immobilized animals on serum hormone levels, two-way ANOVA was applied. To analyze the effects of castration and immobilization within non-immobilized animals and immobilized legs on the muscle mass/body weight ratio, muscle force, and expression/activity of signaling proteins, two-way ANOVA was applied. To analyze the effects of immobilization within immobilized legs and the contralateral legs on the muscle mass/body weight ratio, muscle force, and expression/activity of signaling proteins, two-way ANOVA with repeated measure was applied. Spearman's correlation was performed between the variables within all groups unless other ranges are described. All figures and analyses were performed using GraphPad 6 (GraphPad Software, La Jolla, CA, USA).

Results

Impact of immobilization and castration on circulating hormones

Both immobilization and castration had no significant effect on the level of total OC (Fig. 1A). ucOC was reduced by $\sim 30\%$ ($p < 0.05$) after immobilization but not changed after castration (Fig. 1B). Testosterone dropped by more than 90% ($p < 0.001$) following castration (Fig. 1C) and by 40% ($p < 0.001$) following limb immobilization. Circulating serum insulin was $\sim 50\%$ higher ($p < 0.05$) in the immobilization group compared to non-immobilized animals. In contrast, compared with sham surgery, insulin level was significantly lower ($\sim 50\%$) after castration (shown in Fig. 1D). Circulating testosterone did not correlate with either OC or ucOC (Fig. 1E, F).

Impact of immobilization and castration on EDL and soleus mass and strength

In the EDL, hindlimb immobilization but not castration led to a small, but significant reduction in muscle mass (Fig. 2A). Immobilization resulted in lower muscle force compared with the non-immobilized contralateral leg ($p < 0.05$, Fig. 2C). Immobilization resulted in a profound reduction in soleus muscle mass and force in comparison with either non-immobilized animals ($\sim 30\%$; $p < 0.001$, $p < 0.05$) or the contralateral leg ($\sim 50\%$;

$p < 0.001$) (Fig. 2B, D). Castration had no significant effect on either mass or strength in either muscle.

Higher ucOC level was associated with a higher EDL and soleus mass (Fig. 2E and Fig. 2F, respectively) and also higher strength (Fig. 2G and Fig. 2H, respectively). Higher insulin level was associated with lower muscle mass in soleus among all animals ($r = -0.46$, $p = 0.01$).

Impact of immobilization and castration on postulated ucOC signaling proteins

There was no alteration of GPRC6A protein expression in the EDL (Fig. 3A). In contrast, GPRC6A expression in the soleus was significantly lower following immobilization (Fig. 3B).

p-AMPK α levels were lower ($\sim 50\%$, $p < 0.001$), in both EDL and soleus following immobilization compared immobilization to non-immobilized rats (Fig. 3C, D). p-AMPK α as well as phospho/total ratio of AMPK α in EDL was lower in castrated animals compared to sham counterparts ($p < 0.05$) whereas its total expression was higher (Fig. 3C).

ERK1/2 phosphorylation and phospho/total ratio of ERK1/2 were lower than non-immobilized animals following immobilization in EDL (Fig. 3E). In soleus, higher phospho/total ratio of ERK1/2 was observed in immobilized leg ($p < 0.05$) (Fig. 3F).

There was no significant correlation between ucOC level and the expression of GPRC6A in either muscle type ($p > 0.05$). In soleus but not EDL, GPRC6A was correlated with muscle mass ($r = 0.51$, $p < 0.001$). ucOC level was correlated with p-ERK1/2 ($r = 0.49$, $p < 0.01$) and p-AMPK α ($r = 0.42$, $p < 0.05$) in EDL. In soleus, ucOC tended to be correlated with p-AMPK α ($r = 0.34$, $p = 0.072$). Higher soleus GPRC6A expression was associated with higher soleus p-AMPK α ($r = 0.32$, $p < 0.05$). Higher insulin levels were correlated with lower soleus p-AMPK α ($r = -0.48$, $p < 0.01$).

Impact of immobilization and castration on IRS-1 and AKT

IRS-1 levels following immobilization were significantly lower compared to either non-immobilized animals or the contralateral leg in both EDL and soleus (Fig. 4A and Fig. 4B, respectively). In EDL, castration led to lower p-AKT/AKT ratio compared to sham control mainly due to an increased total AKT expression (Fig. 4C). In soleus, p-AKT/AKT was significantly lower following immobilization compared to the contralateral leg mainly due to a higher total protein levels. Soleus AKT expression was higher following castration in comparison to sham animals (Fig. 4D).

ucOC level were correlated with IRS-1 levels in EDL ($r = 0.48$, $p < 0.01$). EDL IRS-1 was also correlated with p-ERK ($r = 0.30$, $p = 0.04$) and p-AMPK α ($r = 0.54$, $p < 0.001$). Similarly, IRS-1 expression in soleus muscle correlated with GPRC6A ($r = 0.41$, $p < 0.01$), and p-AMPK α ($r = 0.54$, $p < 0.001$). No correlations between IRS-1 and p-AKT were observed in both types of muscle. EDL p-AKT was correlated with p-ERK in EDL muscle ($r = 0.34$, $p < 0.05$).

Impact of immobilization and castration on mTORC1 signaling proteins

In the EDL, p-mTOR/mTOR ratio was lower following immobilization compared with both non-immobilized animals ($p < 0.001$) and the contralateral legs ($p < 0.05$) (Fig. 5A). Castration led to enhanced total mTOR, but attenuated p-mTOR and p-mTOR/mTOR ratio in EDL. mTOR expression in immobilized soleus was significantly lower than in

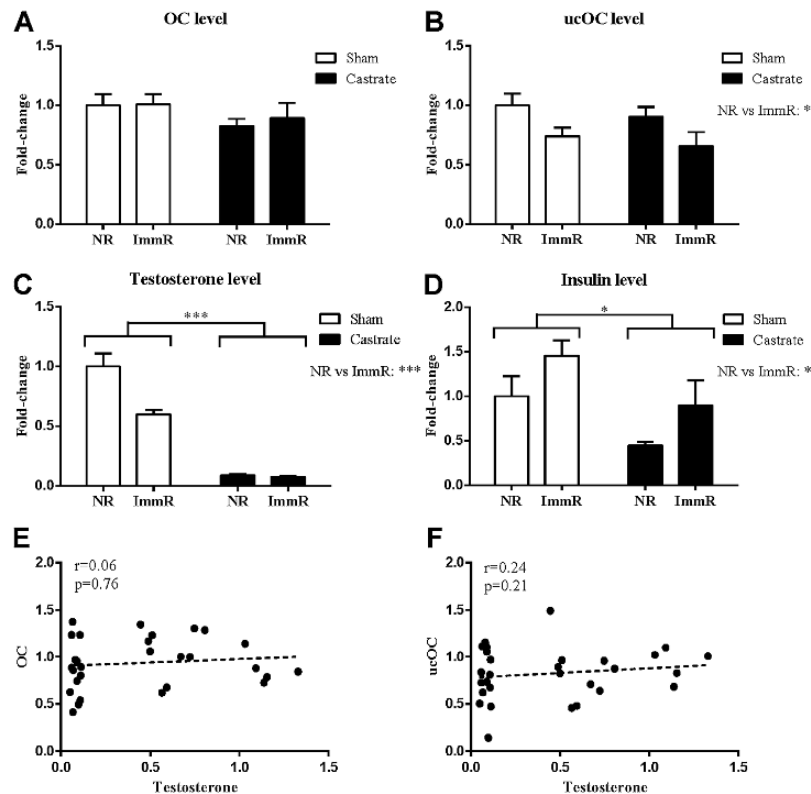


Fig. 1. Impact of immobilization and castration on circulating OC, ucOC, testosterone and insulin levels. Serum levels of total OC (A), ucOC (B), testosterone (C), and insulin (D) were detected in NR/ImmR with sham/castration surgery. The correlation between testosterone and OC (E) and ucOC (F) was analyzed among all animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in two-way ANOVA analysis. Symbols of p value of Castration main effect are illustrated on top of columns; symbols of p value of Immobilization main effects ("NR vs ImmR") are illustrated on the right side of each panel. OC = osteocalcin; ucOC = undercarboxylated osteocalcin; NR = non-immobilized rats; ImmR = immobilized rats.

non-immobilized rats (Fig. 5B). In soleus muscle, p-mTOR and expression was lower in castrated animals compared to sham controls (Fig. 5B).

The mTORC1 substrates P70S6K, both phosphorylation and expression, were lower ($p < 0.05$) in immobilized EDL compared to the contralateral muscle (Fig. 5C). In immobilized soleus, p-P70S6K and phospho/total ratio of P70S6K were higher than the contralateral leg whereas total P70S6K level was markedly lower than either control (Fig. 5D). p-ULK1 was significantly lower following immobilization compared to non-immobilization rats in both the EDL (~70%, Fig. 5E) and the soleus (~50%, $p < 0.001$, Fig. 5F) immobilization.

In EDL, ucOC level was correlated with p-ULK1 ($r = 0.54$, $p < 0.01$). p-ERK had correlations with p-mTOR ($r = 0.28$, $p = 0.05$), and p-ULK1 ($r = 0.38$, $p < 0.01$). Lower EDL p-AMPK α was associated with lower EDL p-mTOR ($r = 0.31$, $p < 0.05$), and EDL p-ULK1 ($r = 0.53$, $p < 0.001$). EDL p-mTOR was associated with EDL p-ULK1 ($r = 0.50$, $p < 0.001$). EDL p-AKT was correlated with EDL p-mTOR ($r = 0.33$, $p < 0.05$), and p-ULK1 ($r = 0.44$, $p < 0.01$). In soleus, higher soleus p-AMPK α had an association with higher soleus p-ULK1 ($r = 0.46$, $p = 0.001$).

Impact of immobilization and castration on FOXO signaling proteins

p-FOXO3a levels were significantly lower following immobilization than the contralateral legs in EDL (Fig. 6A). In castrated animals, total FOXO3a in EDL was higher than sham controls (Fig. 6A). Castrated rats also had higher levels of total FOXO3a in soleus (Fig. 6B).

Both phosphorylation and expression of FOXO1 were unchanged in EDL after immobilization or castration (Fig. 6C). There was a marked reduction in the phosphorylation level of FOXO1 following immobilization compared to non-immobilized animals in soleus (Fig. 6D). Castration led to an increase in total FOXO1 but a reduction in p-FOXO1/FOXO1 in soleus muscle (Fig. 6D).

Limited alterations were observed in the expression of E3 ligase Fbx32 in EDL after both immobilization and castration whereas the immobilized leg showed significant increase of Fbx32 in soleus comparing with non-immobilization rats (Fig. 6E, F). Following immobilization, Murf1 was significantly lower in EDL but higher in soleus compared to non-immobilization

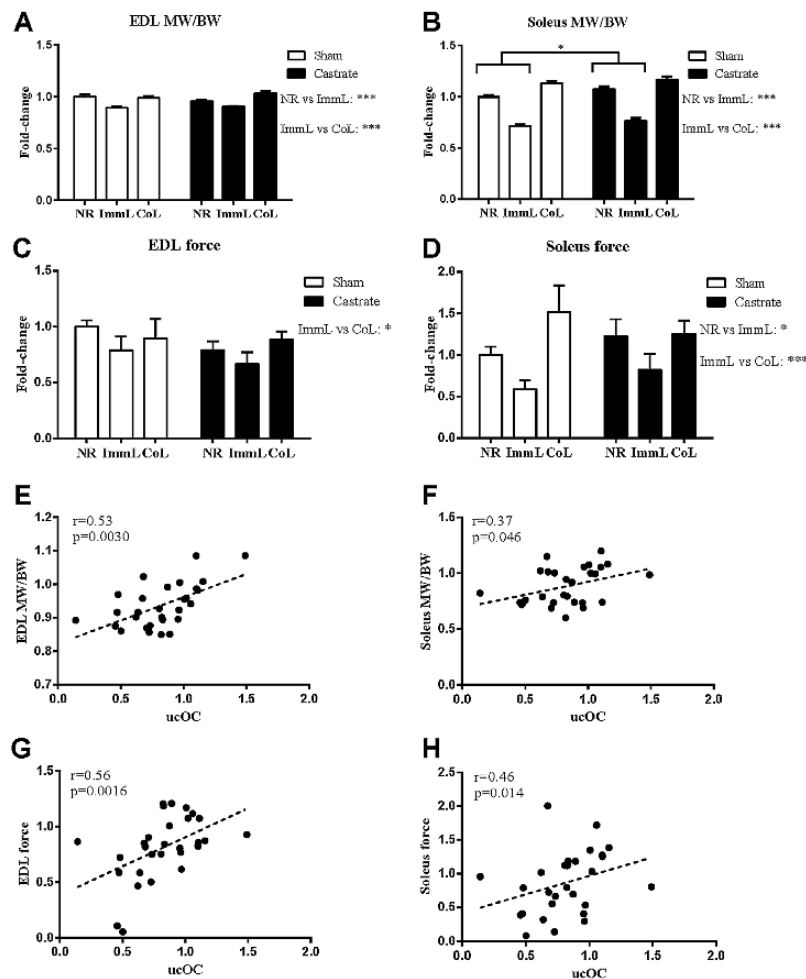


Fig. 2. Impact of immobilization and castration on EDL and soleus mass and strength. EDL MW/BW (A), soleus MW/BW (B), EDL muscle force (C), and soleus muscle force (D) were examined for muscles isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in two-way ANOVA analysis. The correlation between ucOC and EDL MW/BW (E), EDL force (F), soleus MW/BW (G), or soleus force (H) was detected among all six groups. Symbols of p value of Castration main effect are illustrated on top of columns; symbols of p value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. MW/BW = muscle weight/body weight; NR = non-immobilized rats; ImmR = immobilized rats; CoL = contralateral leg.

animals (Fig. 6G, H). There was a higher (~40%, $p < 0.01$) MuRF1 in soleus in the castration group.

In soleus, a higher GPRC6A expression was correlated with lower Fbx32 ($r = -0.29$, $p < 0.05$). Higher soleus p-AMPK α was associated with higher soleus p-FOXO1 ($r = 0.38$, $p < 0.01$). A higher soleus p-AMPK α /AMPK α ratio was correlated with a lower Fbx32 ($r = -0.31$, $p < 0.05$) and was tended to be correlated with a lower MuRF1 ($r = -0.26$, $p = 0.07$). Furthermore, soleus p-FOXO1 ($r = 0.33$, $p < 0.05$) and soleus p-FOXO3a ($r = 0.32$, $p < 0.05$) was associated with p-ULK1. Lower p-FOXO1/FOXO1 ratio was tended to be associated with higher Fbx32 ($r = -0.27$, $p = 0.062$) and was associated with higher MuRF1 ($r = -0.48$, $p < 0.001$).

Discussion

We report that lower circulating ucOC following hindlimb immobilization, but not testosterone depletion (via castration), was associated with lower muscle mass and force in both fast-twitch (EDL) and slow twitch (soleus) muscles (Fig. 2E–H). This association was not evident following castration. Finally, a higher serum ucOC and a higher GPRC6A expression were related to higher ERK1/2 and AMPK α phosphorylation in a muscle-specific manner. The reduced activity of these putative ucOC signaling pathways was also related to decreased activity of mTORC1 activity in EDL and activation of FOXO pathway in soleus following immobilization.

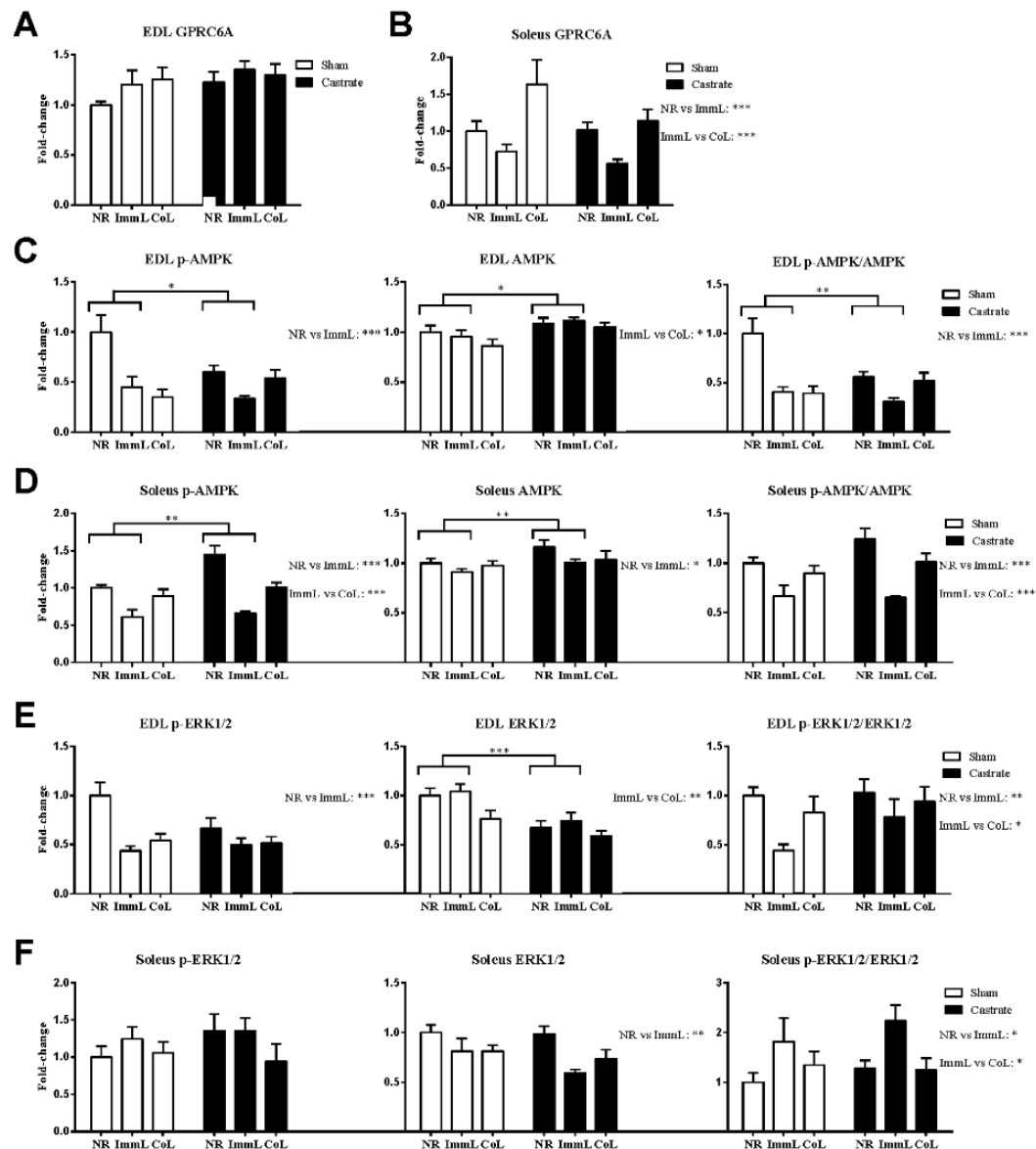


Fig. 3. Impact of immobilization and castration on postulated ucOC signaling proteins. GPRC6A expression (A, B), p-AMPK α /AMPK α levels (C, D), as well as p-ERK1/2, ERK1/2, and p-ERK1/2/ERK1/2 levels (E, F) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in two-way ANOVA analysis. Symbols of p value of Castration main effect are illustrated on top of columns; symbols of p value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. NR = non-immobilized rats; ImmL = immobilized rats; CoL = contralateral leg.

It appears that skeletal muscle is one of the target organs of ucOC.^(6-8,23,37) Previous studies mainly focused on the insulin-sensitizing effects of ucOC in skeletal muscles,^(37,39,40) but recently, it has been suggested that ucOC may play a role in muscle mass maintenance and physiological functions.^(6,7,37) Here we report that lower circulating levels of ucOC correlates with lower muscle mass and strength in both EDL and soleus

muscle following immobilization, suggesting a link between ucOC and muscle mass and function.

ucOC promotes glucose metabolism by increasing the secretion of insulin and improving insulin sensitivity.^(5,40) Interestingly, insulin signaling in osteoblasts stimulates ucOC production in bone,⁽⁴¹⁾ indicating a positive feedback between ucOC and insulin that is likely to affect whole-body glucose

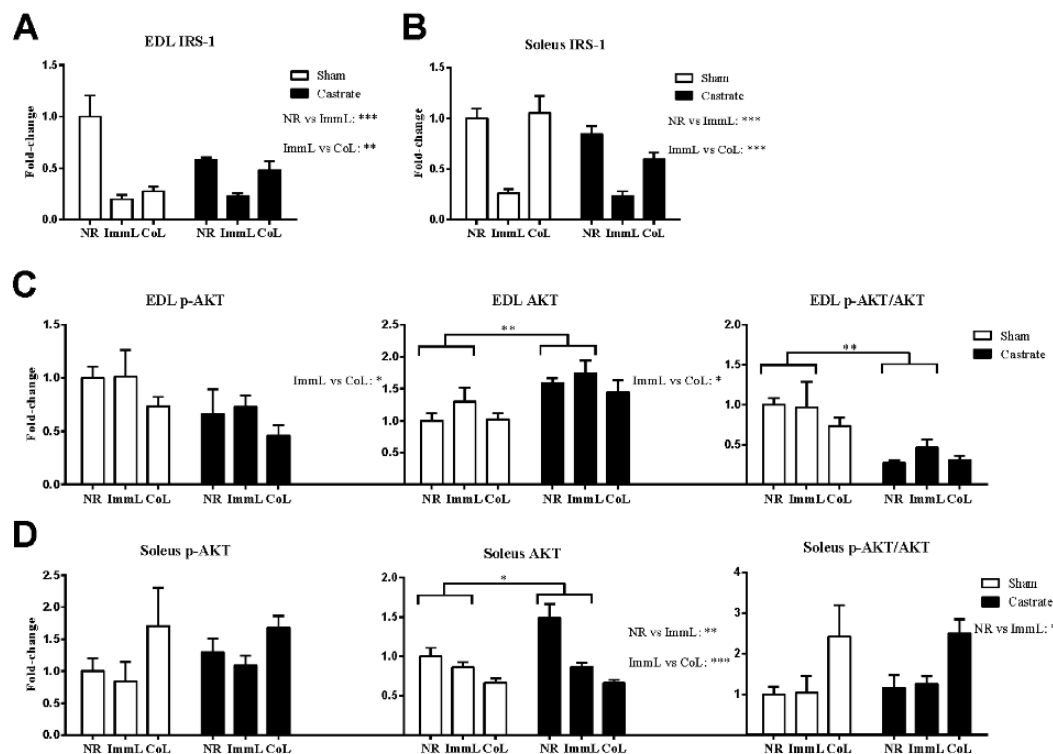


Fig. 4. Impact of immobilization and castration on IRS-1 and AKT. IRS-1 expression (A, B), p-AKT, AKT, and p-AKT/AKT levels (C, D) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in two-way ANOVA analysis. Symbols of p value of Castration main effect are illustrated on top of columns; symbols of p value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. NR = non-immobilized rats; ImmR = immobilized rats; CoL = contralateral leg.

metabolism. Because ucOC was reported to contribute to male reproductive capacity by enhancing testosterone production in testis⁽²⁾ and testosterone has long been known to be involved in bone remodeling and function,⁽¹⁹⁾ we hypothesized that reducing testosterone would lead to a reduction in ucOC. In contrast to our hypothesis, serum ucOC levels were not different in castrated rats compared to sham controls. In addition, ucOC did not correlate with testosterone levels. We also observed that testosterone depletion following castration surgery had limited effects on muscle mass and muscle strength. A potential limitation of the current study is the relative short duration of the intervention of testosterone depletion via castration. Indeed, in other rat studies, a moderate decrease in soleus mass/body weight ratio was found in animals following 5 weeks of castration surgery.^(42,43) In a mice study, 10 weeks of castration time was needed to have moderate decrease in soleus mass/body weight ratio but not in fast twitch muscles.⁽⁴⁴⁾ It is thus possible that a longer duration post-castration is required to observe significant effects on circulating ucOC levels, muscle mass, and muscle strength. Lower phosphorylation of AMPK, AKT, and mTOR in EDL as well as lower phosphorylation of mTOR and FOXO1 but higher expression of MuRF1 in soleus was observed, in response to testosterone deprivation, which was similar to what was previously reported.⁽⁴⁵⁾ As such, it is likely that, in the short time frame of castration intervention in our

study, molecular changes that favor muscle protein loss were already underway. However, these changes had not yet led to any apparent reductions in muscle mass and force. Indeed, in mice with long-term testosterone depletion (7 weeks post-castration surgery) both molecular and functional changes, suggesting muscle atrophy, were reported.⁽⁴⁵⁾ It is also plausible that the testosterone effect on skeletal muscle is muscle-specific because higher testosterone levels correlated with higher soleus muscle weight within sham controls. This indicates testosterone might play a role in maintaining muscle mass more predominantly in slow twitch muscles.

Following hindlimb immobilization, muscle mass/body weight ratio of soleus, but not EDL, from the non-immobilized legs was significantly higher (11%, $p < 0.05$) compared with non-immobilized rats. This finding is similar to a previous studies that reported that soleus mass/body weight ratio in the non-immobilized leg gradually increased during a 7-day hindlimb immobilization in rats while the ratio of non-immobilized gastrocnemius (consist mostly of glycolytic fibers) remained unchanged.⁽⁴⁶⁾ Furthermore, it was also reported that compared with non-immobilized rats, the rate of protein synthesis in non-immobilized soleus was moderately higher after 3-day immobilization. As such, it appears that oxidative muscles, but not glycolytic muscles, in the non-immobilized leg may exhibit some "mild overload" that resulted in increased mass.

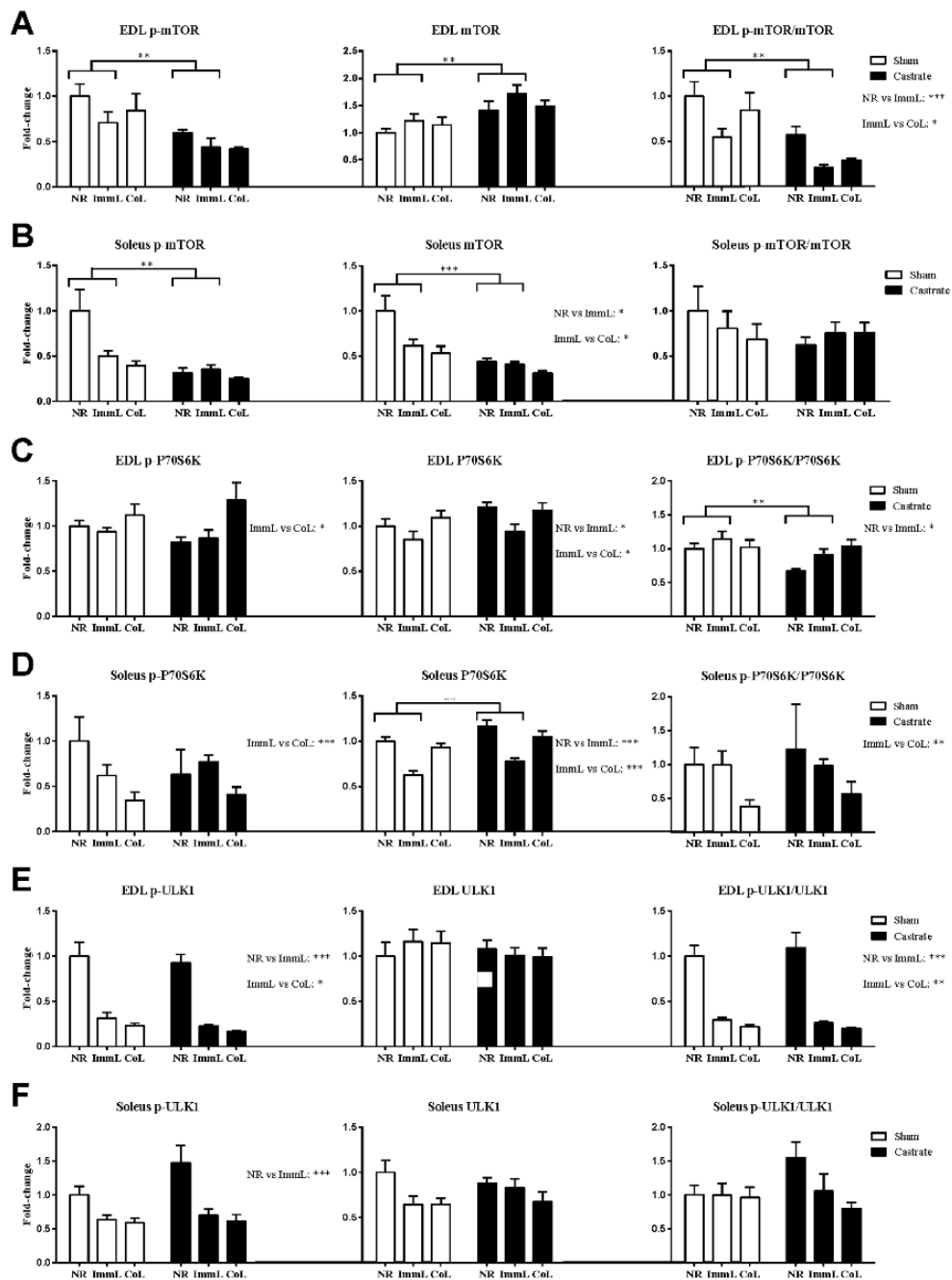


Fig. 5. Impact of immobilization and castration on mTORC1 signaling proteins. p-mTOR, mTOR and p-mTOR/mTOR levels (A, B), p-P70S6K, P70S6K, and p-P70S6K/P70S6K levels (C, D), and p-ULK1, ULK1 and p-ULK1/ULK1 levels (E, F) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in two-way ANOVA analysis. Symbols of p value of Castration main effect are illustrated on top of columns; symbols of p value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. NR = non-immobilized rats; ImmL = immobilized rats; CoL = contralateral leg.

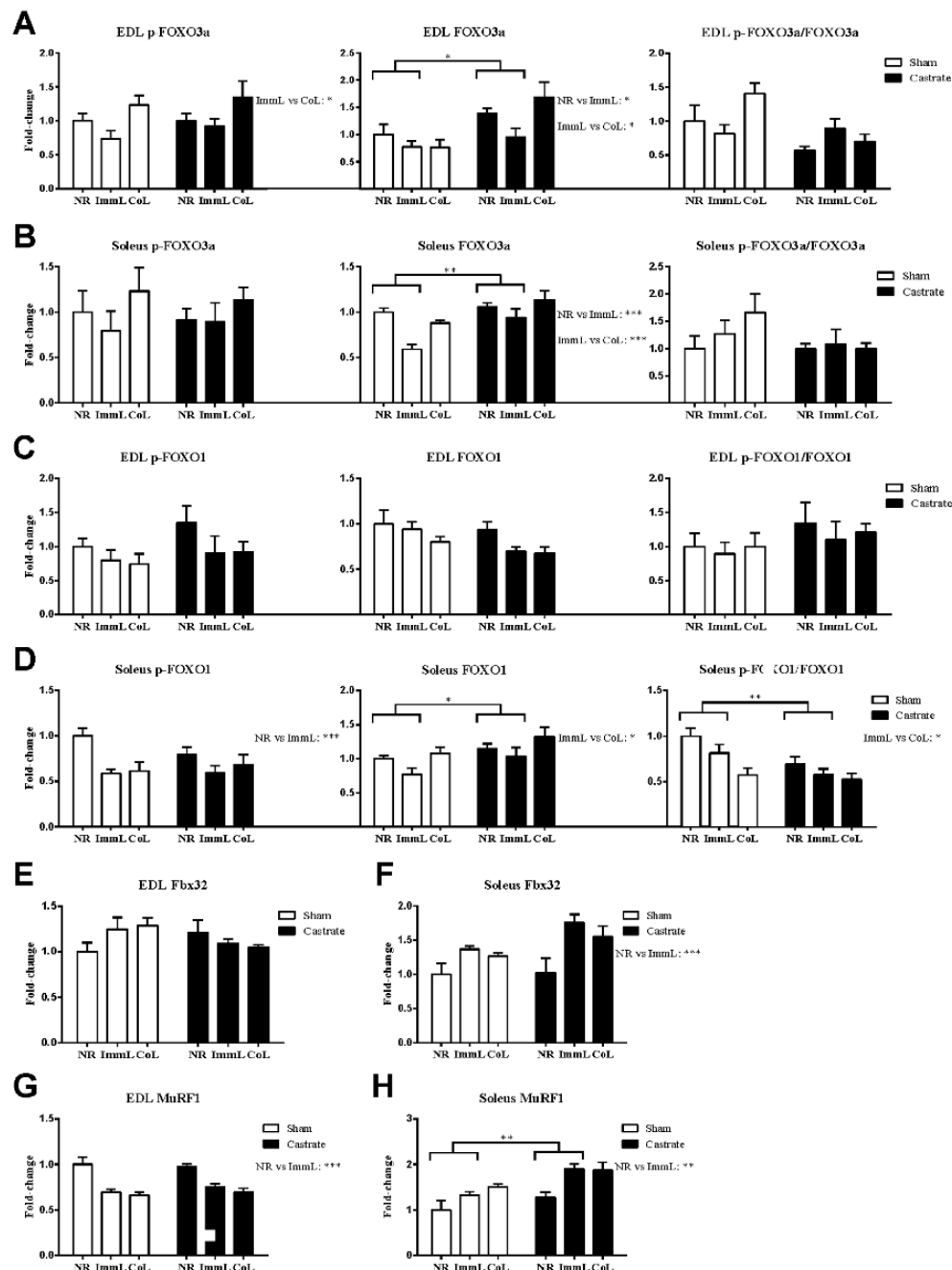


Fig. 6. Impact of immobilization and castration on the expression and activity of FOXO signaling proteins. p-FOXO3a, FOXO3a, and p-FOXO3a/FOXO3a levels (A, B), p-FOXO1, FOXO1, and p-FOXO1/FOXO1 levels (C, D), Fbx32 expressions (E, F), and MuRF1 expressions (G, H) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in two-way ANOVA analysis. Symbols of p value of Castration main effect are illustrated on top of columns; symbols of p value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. NR = non-immobilized rats; ImmL = immobilized rats; CoL = contralateral leg.

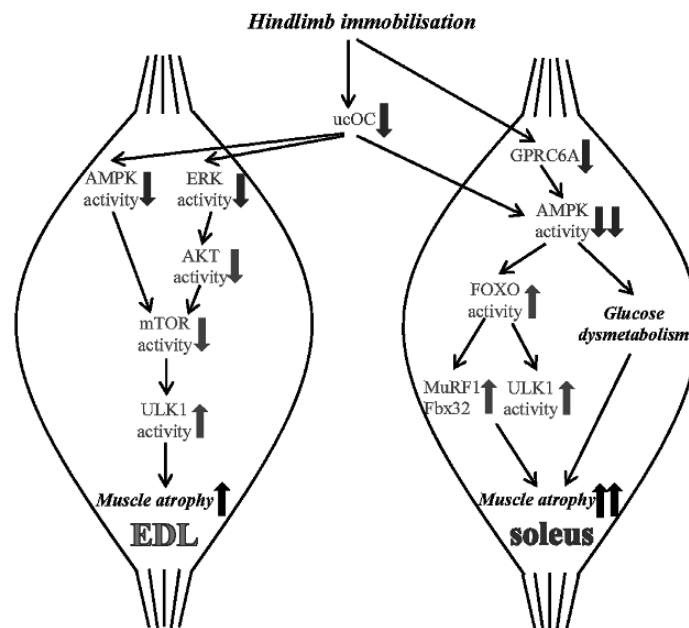


Fig. 7. Reduced level of circulating ucOC and GPRC6A expression caused by hindlimb immobilization might lead to enhanced muscle atrophy in a muscle type-specific manner.

To date, the downstream targets of ucOC-GPRC6A cascade in skeletal muscle cells have not yet been identified. However, it is possible that they involve ERK and AMPK, because ucOC treatment in C2C12 myotubes led to ERK1/2 phosphorylation, which can be inhibited by the administration of the inhibitor of the upstream kinase of ERK.⁽²³⁾ Moreover, in primary myotubes, ucOC activated AMPK/mTOR/P70S6 kinase axis via GPRC6A, implicating AMPK as a key protein responsible for the anabolic effects of ucOC in skeletal muscle.⁽⁷⁾ Our results demonstrate that lower ucOC level was associated with lower ERK1/2 phosphorylation in EDL. In contrast, soleus ERK1/2 phospho/total ratio was elevated post-immobilization compared with both non-immobilized rats and non-immobilized legs, despite more profound muscle wasting. It is possible that this increase in ERK activity in the soleus is a compensatory response to the greater muscle loss as was reported previously.⁽⁴⁷⁾ Hence, our results may indicate that ERK involvement in the ucOC cascade in skeletal muscle is muscle-specific.

Although lower ucOC was associated with lower p-AMPK α in both EDL and soleus muscle, only in soleus did lower p-AMPK α correlate with lower GPRC6A expression compared to both non-immobilized rats and the contralateral leg. This lower p-AMPK α in atrophic oxidative muscle is consistent with a number of other studies. For instance, 10-day limb immobilization in rats led to a significant reduction in p-AMPK expression in red gastrocnemius muscle compared to the contralateral leg.⁽⁴⁸⁾ Furthermore, 2-week tail suspension led to a significant reduction in p-AMPK mainly in soleus and to a lesser degree in EDL in rats.⁽⁴⁹⁾ In humans, patients with critical myopathy showed decreased AMPK activity in skeletal muscles compared with healthy controls.⁽⁵⁰⁾ Accordingly, the attenuation of the ucOC signaling cascade may be related to soleus muscle wasting attributed to lower AMPK activity. However,

unchanged or even a higher AMPK activity during muscle atrophy has also been reported.^(46,51) These incompatible results fit the contradictory role of AMPK in cell fate in skeletal muscles. On one hand, high activity of AMPK induces membrane GLUT4 expression as well as mitochondrial biogenesis, benefiting muscle glucose metabolism and cell growth.^(52,53) On the other hand, AMPK activation leads to cell autophagy via enhancing the activity of cell autophagy pathways.^(54,55) Notably, the majority of findings showing reduced AMPK activity in atrophic muscle were obtained either in animals subject to inactive conditions for at least 10 days or in inactive patients, whereas short time activation or inhibition of AMPK was mainly employed in studies investigating its role in protein degradation. Therefore, impaired cellular glucose metabolism and energy production in muscle cells due to low AMPK activity can only be observed after a relatively long duration. Indeed, people with short-term leg immobilization and patients with myopathy are characterized by lower insulin sensitivity.^(50,56) Intriguingly, patients with T2DM have lower circulating ucOC,^(57,58) which is of interest because low serum ucOC is also linked with impaired insulin sensitivity in animals.⁽⁵⁹⁾ Moreover, we observed that hyperinsulinemia induced by limb immobilization (Fig. 1) was associated with loss of muscle mass and lower p-AMPK α in the soleus. Given that patients with T2DM are at a high risk for muscle atrophy,^(29,60) glucose dysmetabolism due to low AMPK activity in slow twitch muscle during disuse atrophy may be a plausible scenario. However, this hypothesis should be tested in future studies.

We observed decreased expressions of IRS-1 in both muscles following immobilization, and these reductions of IRS-1 expression were associated not only with muscle mass loss but also with alterations of several signaling proteins. However, we did not find any significant correlations between IRS-1 level and the phosphorylation of its downstream kinase AKT. These

results might indicate that the reductions of IRS-1 level were likely the consequence of protein degradation, but were not able to exert significant impact on insulin signaling pathway. Although phosphorylated AKT did not exhibit apparent decrease in EDL, its level was correlated with p-ERK, p-mTOR, and p-ULK1. These correlations were not observed in soleus. Recently, it has been reported that in C2C12 myotubes, the enhancement of AKT phosphorylation induced by osteocalcin treatment was abolished by U0126, an inhibitor of ERK kinase (MEK).⁽²³⁾ As such, in immobilized EDL, AKT phosphorylation could be affected by decreased activity of ucOC/ERK cascade, thus leading to reduced mTORC1 activity.

Higher ULK1 activity was correlated with lower p-mTOR in EDL only. In soleus, p-ULK1 was associated with p-FOXO1 and p-FOXO3a, which is consistent with the results that FOXO proteins can lead to muscle autophagy by interacting with ULK1.⁽⁵⁴⁾ Ubiquitin E3 ligases Fbx32 and MuRF1 were higher only in soleus compared with non-immobilized rats following immobilization, and they were also correlated with muscle loss, GPRC6A level, and/or p-FOXO1/FOXO1 ratio. This suggests that protein degradation caused by FOXO/Fbx32 (MuRF1) is, at least partly, responsible for soleus muscle loss.

Previous studies have shown that muscle atrophy caused by immobilization or suspension occurs in a muscle-specific manner, with muscle mass loss predominantly found in oxidative slow twitch muscles and to a lesser extent in glycolytic fast twitch muscles.^(36,49,61) However, even though signaling pathways involved in muscle atrophy have been widely reviewed,^(28,29,33,55) the molecular mechanism for the selectivity of muscle type atrophy still remains unresolved. Our data not only confirmed that hindlimb-immobilization does lead to more severe muscle atrophy and weakness in soleus compared with EDL, but also highlighted a potential molecular mechanisms. The potential mechanisms are illustrated in Fig. 7. We propose that lower circulating ucOC and an attenuated ucOC signaling, caused by immobilization, reduces muscle mass in a muscle type-specific manner. In EDL, it leads to attenuated activity of AMPK and ERK, resulting in increased ULK1 activity and reductions in AKT activity and mTOR activity leading to muscle atrophy. In soleus, reduced ucOC and decreased GPRC6A expression results in larger reductions in AMPK activity, leading to more profound muscle atrophy via enhanced activity of ULK1 and increased expression of Fbx32 and MuRF1 through amplified activity of FOXO proteins, and potentially glucose dysmetabolism. These hypotheses need to be fully explored in future studies.

In conclusion, hindlimb immobilization, but not testosterone depletion via castration, leads to a significant reduction in ucOC, and lower ucOC was correlated with lower muscle mass and force in both fast-twitch (EDL) and slow-twitch (soleus) muscles. In addition, the putative ucOC/GPRC6A/ERK (AMPK) signaling cascade was affected by immobilization, and the expression and activity of proteins in ucOC signaling pathway were also associated with the expression and activity of a number of proteins in the mTORC1 and FOXO signaling pathways in a muscle type-specific manner. However, whether ucOC and its putative signaling pathway play a role in muscle anabolism in addition to its insulin sensitizing effects will need to be explored in detail in future studies involving methods of ucOC deprivation and restoration. Additional investigations are also required to explore the role of ucOC in both muscle gene expression, as well as whether the alteration of gene expression is responsible for the reduced mass and strength following castration and immobilization.

Disclosures

All authors state that they have no conflicts of interest.

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