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p300 and CBP are required for AMPK-stimulated glucose uptake in mouse skeletal muscle

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by

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The Thesis of Christina Ha is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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DEDICATION

I dedicate this to my parents and my older sister who have continuously shown their support for everything I do. Thank you for showering me with your unconditional love. I also dedicate this to BTS who has been there with me every step of the way. Thank you for blessing us with your melodic, yet impactful messages.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ZMP	AICAR monophosphate
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
AMPK	5’ adenosine monophosphate (AMP)-activated protein kinase
AMP	Adenosine monophosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
TBC1D1	TBC1 domain family member 1
TBC1D4	TBC1 domain family member 4
Thr^{172}	Threonine 172
Ser^{237}	Serine 237
Thr^{596}	Threonine 596
Ser^{660}	Serine 660
Ser^{700}	Serine 700
RabGAP	RabGTP-ase activating protein
EDL	Extensor digitorum longus
SOL	Soleus
HAT	Histone acetyltransferase
KHB	Krebs-Henseleit buffer

2DOG	2-deoxyglucose
2DOGU	2-deoxyglucose uptake
p300	EA1 binding protein p300
CBP	c-AMP response element-binding binding protein
T2D	Type 2 diabetes
WT	Wild type
i-mPCKO	p300/CBP double knockout
i-mPKO	p300 single knockout
i-mCKO	CBP single knockout
i-mPKO/CHZ	p300 single knockout and CBP heterozygous knockout
i-mCKO/PHZ	CBP single knockout and p300 heterozygous knockout

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ABSTRACT OF THE THESIS

p300 or CBP is required for AMPK-stimulated glucose uptake in mouse skeletal muscle

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The AMP-activated protein kinase (AMPK) is activated by exercise, and both pharmacological activation of AMPK and exercise robustly stimulate glucose uptake by skeletal muscle. Nevertheless, the molecular mechanisms underlying the increase in glucose uptake in response to these stimuli remain to be elucidated. Addressing this gap in knowledge, the goal of this study was to investigate the importance of the

acetyltransferases, EA1 binding protein p300 (p300) and c-AMP response element-binding binding protein (CBP), to AMPK-mediated glucose uptake by skeletal muscle. Specifically, we used a radioactive 2-deoxyglucose (2DOG) uptake (2DOGU) approach to measure *ex vivo* glucose uptake in unstimulated and AMPK-stimulated (using the pharmacological activators, 5-Aminoimidazole-4-carboxamide ribonucleotide [AICAR] and MK-8772) in the extensor digitorum longus (EDL) and soleus from ~12-16 week old male and female mice (n=261) with skeletal muscle-specific knockout of p300 and/or CBP and their wildtype (WT) littermates. As expected in WT mice, AICAR and MK-8772 significantly increased 2DOGU in soleus and EDL, regardless of sex. Remarkably, in mice with knockout of p300 *and* CBP (i-mPCKO), this effect of AICAR and MK-8722 was blocked in male and female mice. Equally remarkable, AICAR-stimulated 2DOGU in mice with individual knockout of p300 or CBP, or AICAR- and MK8772-stimulated 2DOGU in mice with just one allele of p300 or CBP, was normal and not different from WT littermates. Taken together, we highlight a critical role for p300 and CBP in the regulation of AMPK-stimulated glucose transport in skeletal muscle.

INTRODUCTION

1.1. Exercise, glucose uptake, and type 2 diabetes

Type 2 diabetes (T2D) is characterized by pancreatic beta cell failure and fasting and post-prandial hyperglycemia (Bogardus & Tataranni, 2002; DeFronzo, 1987). Key contributors to hyperglycemia in T2D are insufficient insulin secretion and an inability of the liver and skeletal muscle to respond in a normal manner to the physiological actions of insulin (Bogardus & Tataranni, 2002; Lillioja *et al.*, 1988). In the liver a normal response to insulin would be to decrease endogenous glucose production, whilst in skeletal muscle, it is to increase glucose uptake. This insulin 'resistance' is particularly important in skeletal muscle because in non-diabetic individuals, skeletal muscle accounts for as much as 90% of peripheral glucose uptake after a meal. As such, skeletal muscle is critical to post-prandial glycemic control, and in the case of T2D, it is a critical contributor to clinical hyperglycemia (Bogardus & Tataranni, 2002; DeFronzo, 1987; Lillioja *et al.*, 1993). It is important to note that the consequence of chronic hyperglycemia and T2D are substantial and increase the risk of coronary heart disease, ischaemic stroke, retinopathy, and leg amputation (Chatterjee *et al.*, 2017; Facchini *et al.*, 2001; Harris *et al.*, 1992; Ratliff *et al.*, 2021; The Emerging Risk Factors Collaboration, 2010). In the United States alone, about 11.3% of the total population (~37.3 million people) suffer from T2D as of late (CDC, 2022). There are about 462 million cases of T2D worldwide, which corresponds to 6.28% of the global population (Khan *et al.*, 2020). This number is projected to rise to 642 million cases by 2040 (Sapra & Bhandari, 2022). The continuous increase of individuals with T2D is an

alarming global issue, and it is imperative that we find effective methods to prevent and treat the development of T2D.

Managing clinical hyperglycemia in T2D through exercise. Diet, pharmacological approaches, and exercise are cornerstones to treating T2D (Chatterjee *et al.*, 2017; Diabetes Prevention Program Research Group, 2015; Ivy *et al.*, 1999; Merlotti *et al.*, 2014), with regular exercise robustly improving insulin sensitivity and glycemic control in individuals with T2D (Boulé *et al.*, 2005; Eriksson, 1999; Kirwan *et al.*, 2017; Zanuso *et al.*, 2010). The major explanation for this is that exercise, or more specifically contraction, stimulates glucose uptake by the active skeletal muscle (Richter & Hargreaves, 2013; Sapra & Bhandari, 2022; Verbrugge *et al.*, 2022), which lowers blood glucose concentration and improves glycemic control (Hamdy *et al.*, 2001a) in healthy as well as diabetic individuals (Betts *et al.*, 1993; Ivy *et al.*, 1999; Kennedy *et al.*, 1999; Kusunoki *et al.*, 2022; Wallberg-Henriksson & Holloszy, 1984). Thus, regular exercise is an effective prevention and treatment approach for clinical hyperglycemia in T2D patients.

Contraction-stimulated glucose uptake. In the context of exercise and glucose uptake, physical activity has been shown to increase whole-body glucose disposal, as well as increase insulin sensitivity in skeletal muscle (Goodyear & Kahn, 1998; Han *et al.*, 1995; Koistinen & Zierath, 2002; Rose & Richter, 2005; Ryder *et al.*, 2001). Contraction-stimulated glucose uptake occurs independent of insulin signaling molecules in skeletal muscle (Colberg *et al.*, 2016; Gao *et al.*, 1994; Lund *et al.*, 1995; Sylow *et al.*, 2017). This phenomenon was shown in the 1950's, with some of the first reliable studies on rat, frog, and dog muscle confirming contraction-stimulated glucose

uptake (Goldstein *et al.*, 1953; J. 0 Holloszy & Narahara, 1965; J. O. Holloszy & Narahara, 1967; Huycke & Kruhoffer, 1955; Richter & Hargreaves, 2013). Two decades later, more quantitative studies examining the process of contraction-stimulated glucose uptake were performed on human subjects through the use of radioactive glucose tracers (Jorfeldt & Wahren, 1970; Reichard *et al.*, 1961; Richter & Hargreaves, 2013; Wahren *et al.*, 1971). For example, Jorfeldt and Wahren found that 10 minutes post exercise, glucose uptake in human forearm muscle increased about 15 times more than resting level (Jorfeldt & Wahren, 1970), and that glucose uptake is increased about 10 to 20-fold more during moderate or intense exercise (Wahren *et al.*, 1971). Following these discoveries, many researchers over the past few decades have consistently demonstrated that exercise can increase glucose uptake in human (Koistinen & Zierath, 2002; McConell *et al.*, 1994) and murine skeletal muscle (Andersen *et al.*, 2019; Pataky *et al.*, 2020).

Glucose, GLUT4, and glucose uptake. Skeletal muscle glucose uptake is regulated through three key steps: delivery, transport, and phosphorylation (Flores-Opazo *et al.*, 2020; Richter & Hargreaves, 2013; Rose & Richter, 2005). The translocation of these glucose transporters regulates the process of bringing glucose into the cell. Subsequently, glucose is used as an energy source and is ultimately converted into adenosine triphosphate (ATP) to fuel other cellular processes. Glucose is taken up by and enters the muscle cell (i.e. myocyte) through a process known as facilitated diffusion, which is regulated by two important proteins: glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) (Buse *et al.*, 1996; Hamdy *et al.*, 2001; Richter & Hargreaves, 2013). GLUT1 is primarily responsible for basal glucose transport in

skeletal muscle and will not be discussed further (Buse *et al.*, 1996). GLUT4, on the other hand, is highly expressed in insulin-responsive cells, including those critical to the regulation of systemic glycemia, such as cardiac muscle, skeletal muscle, and adipocytes (Buse *et al.*, 1996). As such, GLUT4 translocation is the mechanism by which contraction-stimulated glucose uptake can occur. Contraction robustly increases glucose uptake in skeletal muscle through GLUT4 translocation to the plasma membrane and transverse tubules, aiding in ATP production and muscle contractile function upon exercise (Fujimoto *et al.*, 2003; Kristiansen *et al.*, 2000; Richter & Hargreaves, 2013). The fundamental importance of GLUT4 to "stimulated" glucose uptake has been demonstrated in mice with knockout of GLUT4 in skeletal muscle, such that both insulin- and contraction-stimulated glucose uptake blocked in these mice (Carvalho *et al.*, 2005; Fueger *et al.*, 2007; Howlett *et al.*, 2013; Ryder *et al.*, 1999; Zisman *et al.*, 2000).

Summary. Muscle contraction plays a crucial role in glycemic control through increasing GLUT4 plasma membrane translocation and glucose uptake metabolism in skeletal muscle. Importantly, this occurs in the absence of insulin, making exercise a cornerstone for treating clinical hyperglycemia. In the next section, I will discuss a potential signaling pathway that is commonly associated with contracted-stimulated glucose uptake and discuss its relevance to my Thesis.

1.2. 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK)

AMPK is widely recognized as an important regulator for cellular energy homeostasis (Hardie *et al.*, 1998; Rose & Richter, 2005). The goal for the AMPK-

signaling pathway is to maximize ATP production by promoting catabolic processes that produce ATP, while simultaneously minimizing anabolic processes that consume ATP (Corton *et al.*, 1994; Fujii *et al.*, 2000; Hingst *et al.*, 2020). This serine/threonine protein kinase pathway is regulated by the adenosine monophosphate to adenosine triphosphate (AMP:ATP) ratio and free AMP availability (Corton *et al.*, 1994; Zhang *et al.*, 2006). The process to activate AMPK signaling involves adenylate kinase, which is an enzyme that converts two adenosine diphosphate (ADP) molecules into one ATP and one AMP (Boyer, 1973; Corton *et al.*, 1994; Hardie, 2018; Thomson, 2018). In one of the first papers demonstrating the interrelationship between the ATP:ADP ratio and AMPK activation, Corton and colleagues (Corton *et al.*, 1994) subjected rat hepatocytes to activate cellular stress through heat shock or arsenite exposure, both of which consume ATP, perturbate the ATP:ADP ratio, and increase AMP concentration. Heat shock and arsenite exposure at 45°C for 60 minutes caused a 5.2-fold increase in AMP levels and a 2.5-fold increase of ADP levels while decreasing ATP levels by 2.5-fold. The overall AMP:ATP ratio was changed by 13-fold compared to control cells (Corton *et al.*, 1994). In concert with these changes, there was a corresponding and robust increase in AMPK enzyme activity (Corton *et al.*, 1994). Altogether, this study and many others (Carling, 2017; Garcia & Shaw, 2017; Herzig & Shaw, 2018; Lin & Hardie, 2018; Yan *et al.*, 2018) demonstrate that AMPK is sensitive to perturbations in AMP and the ATP:ADP ratio. This is relevant to our discussion on exercise and glucose uptake, as the AMP concentration and the AMP:ADP ratio are increased and decreased, respectively, in contracting skeletal muscle in an intensity-dependent manner (Beard *et al.*, 2008; Dash *et al.*, 2007, 2008; Phillips *et al.*, 1996). In line with this, AMPK is

activated in an intensity-dependent manner during exercise (Chen *et al.*, 2003; Wadley *et al.*, 2006; Wojtaszewski *et al.*, 2003). As such, AMPK has long been proposed as a critical signaling molecule in contraction/exercise-stimulated glucose uptake (Hardman *et al.*, 2014; Kjøbsted *et al.*, 2019; Mu *et al.*, 2001; Sylow *et al.*, 2017).

AMPK structure and signaling. AMPK is mainly expressed in the liver, brain, and skeletal muscle (Hardie, 2018). The protein contains heterotrimeric complexes with α-subunits (catalytic) and β- and γ-subunits (regulatory) (Hardie, 2018). AMPKα and AMPKβ contain two isoforms (α1, α2, β1, β2), and AMPKγ is comprised of three isoforms (γ1, γ2, γ3), which are present in both human and rodents (Hardie, 2008). All of these isoforms interact in a 1:1:1 ratio of α:β:γ to result in 12 different AMPK complexes (Ross *et al.*, 2016; Trefts & Shaw, 2021; Yan *et al.*, 2018). Contrary to AMPKα1, which is expressed in all muscles, AMPKα2 is only expressed in skeletal and cardiac muscle; however, both isoforms have a threonine residue (Thr^{172}), and the phosphorylation of this amino acid by upstream kinases is required for AMPK activity (Hardie, 2008; Herzig & Shaw, 2018).

AMPK activation and glucose uptake. Activation of AMPK robustly stimulates glucose uptake by skeletal muscle (Friedrichsen *et al.*, 2013; Kjøbsted *et al.*, 2019; Spaulding & Yan, 2022). This effect is present across mammalian skeletal muscles, be it mouse (Cokorinos *et al.*, 2017; S. B. Jørgensen *et al.*, 2004), rat (Bergeron *et al.*, 1999; Zhou *et al.*, 2001), or human (Fujii *et al.*, 2000; Fujimoto *et al.*, 2003; Kristiansen *et al.*, 2000). The first paper to demonstrate the effect of AMPK activation on glucose uptake in muscle was by Merrill *et al.* (Merrill *et al.*, 1997), in which they demonstrated that AMPK activation with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)

for 45 minutes led to a 2.8 fold increase in fatty acid oxidation and a robust increase in glucose uptake in rat skeletal muscle. Following this discovery, other researchers began studying AMPK activators such as AMP, liver kinase B1, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), MK-8722, metformin, and exercise (Carling, 2017; Feng *et al.*, 2018; Kim *et al.*, 2016; Thomson, 2018). As it pertains to this Thesis, I will focus on two highly studied and validated AMPK activators, AICAR (Corton *et al.*, 1995; Hardman *et al.*, 2014; Merrill *et al.*, 1997) and MK-8722 (Feng *et al.*, 2018; Myers *et al.*, 2017; Olivier *et al.*, 2018; Thomson, 2018). Indeed, AMPK activation and activators (which are discussed below), are commonly used to mimic the effects of exercise on glucose uptake. It should be noted, however, that recent studies in AMPK deficient mice suggest that AMPK is dispensable for, or only plays a partial role in, contraction-stimulated glucose uptake (Hingst *et al.*, 2020; S. B. Jørgensen *et al.*, 2004; Mu *et al.*, 2001). Regardless, it is clear that AMPK activation can be used as a tool to probe the mechanisms of GLUT4-mediated glucose uptake in skeletal muscle.

AICAR. AICAR has been used for over 25 years to activate AMPK in skeletal muscle (Corton *et al.*, 1995; Hardman *et al.*, 2014; Merrill *et al.*, 1997), including the role of AMPK in the regulation of skeletal muscle glucose uptake (Hardie, 2008; N. Jørgensen *et al.*, 2018; Oki *et al.*, 2018). When AICAR is administered to muscle, it is converted to an AMP mimetic known as AICAR monophosphate (ZMP) and activates AMPK without changing any adenine nucleotide levels in the cell (Thomson, 2018; Zhang *et al.*, 2006).

MK-8722. MK-8722 is a small molecule AMPK activator that potently activates AMPK in skeletal muscle (Feng *et al.*, 2018; Myers *et al.*, 2017; Olivier *et al.*, 2018;

Thomson, 2018). This potent pan-AMPK activator binds to the ADaM site, activating the β1 and β2 complexes, which have been previously found to be crucial regulators for whole-body energy homeostasis (Hoffman *et al.*, 2020). MK-8722 can activate all 12 AMPK isoforms in mammals, as well as improve glucose homeostasis, insulin resistance, and hyperglycemia (Cokorinos *et al.*, 2017; Myers *et al.*, 2017).

Signaling mechanism(s) by which AMPK activation increases glucose uptake. Mechanistically, AMPK activation triggers a series of phosphorylation events that are important to skeletal muscle glucose uptake (de Wendt *et al.*, 2021). There are two downstream effectors involved in AMPK signaling: RabGTPase-activating proteins (RabGAPs) TBC1D1 and TBC1D4 (de Wendt *et al.*, 2021; Fontanesi & Bertolini, 2013). The mechanism underlying these RabGAPs is not fully understood, but these proteins are believed to exert an inhibitory effect on GLUT4 translocation to the plasma membrane, which is released when they are phosphorylated by AMPK at their specific AMPK-regulated residues (Espelage *et al.*, 2020; Mafakheri *et al.*, 2018). For example, previous studies demonstrated that TBC1D1 is the predominant protein in glycolytic skeletal muscle and mainly associated with contraction-stimulated glucose uptake, whereas TBC1D4 is more abundant in oxidative skeletal muscle and associated with insulin-stimulated glucose uptake (Szekeres *et al.*, 2012); Fontanesi and Bertolini, 2013; Wendt *et al.*, 2021). TBC1D1 phosphorylation at Ser^{237}, Thr^{596}, Ser^{660}, and Ser^{700} are all thought to be the main sites that increase with exercise stimulation in skeletal muscle (Espelage *et al.*, 2020; Treebak *et al.*, 2014). There is a plethora of studies that have demonstrated that phosphorylation of TBC1D1 in response to *ex vivo* skeletal muscle contraction is AMPK-dependent (Frøsig *et al.*, 2010; Jessen *et al.*, 2011; Pehmøller *et*

al., 2009; Treebak *et al.*, 2014). Similarly to TBC1D1, Ras-related C3 botulinum toxin substrate 1 (Rac1) and β-catenin are speculated to have a regulatory role in AMPK-activated glucose uptake. However, a critical gap in knowledge is that studies done in transgenic rodents or using chemical inhibitors of Rac1 (Sylow *et al.*, 2013, 2016; 2017 or TBC1D1 (An *et al.*, 2010; Cartee, 2015; Kjøbsted *et al.*, 2019; Stöckli *et al.*, 2015; Whitfield *et al.*, 2017) demonstrate that contraction- or AMPK-mediated glucose uptake in only slightly reduced (20-40% or less), but there is not complete inhibition of contraction-stimulated glucose uptake. Thus, while these proteins may contribute to contraction- or AMPK-mediated glucose uptake, they are not 'fundamental' and critical regulators, suggesting that other signaling molecules must be important.

Summary. AMPK activation robustly increases glucose uptake by skeletal muscle, making AMPK activators a useful tool for probing the regulation of GLUT4-mediated glucose transport by skeletal muscle. Nevertheless, the signaling events that underlie AMPK-mediated glucose uptake remain to be fully elucidated.

1.3. p300 and CBP: A potential role for acetylation in muscle glucose uptake

A key focus of our laboratory is to understand the fundamental mechanisms that control 'stimulated' (be it contraction or insulin) glucose uptake by skeletal muscle. Recently, we have focused on a potential role for the protein acetyltransferases, E1A binding protein p300 (p300) and c-AMP response element-binding binding protein (CBP). p300/CBP are orthologs that share ~90% conserved sequence identity in the evolutionary-conserved acetyltransferase domain (and ~65% across the length of the protein) (Wang *et al.*, 2008). Traditionally, p300 and CBP have been considered to

regulate cellular function and homeostasis through their well-known roles as histone acetyltransferases and subsequent effects on gene transcription (Dancy & Cole, 2015; Wang *et al.,* 2008). However, in recent years, their contributions to cellular homeostasis have been shown to extend beyond the nucleus, to many cytosolic (and non-nuclear) proteins. For example, in platelets, which are anuclear, many (cytosolic) proteins were identified to be regulated by p300, including proteins that are important to GLUT4 translocation (although the role in GLUT4 biology and glucose uptake was not measured) (Aslan *et al.*, 2015). With this in mind, our laboratory has focused on the potential role of p300/CBP in the regulation of various aspects of muscle biology, including glucose transport.

***p300/CBP and skeletal muscle*.** Our approach to study the role of p300/CBP in skeletal muscle biology is to generate and study mice with skeletal muscle-specific knockout (KO) of p300 and/or CBP. For instance, Martins *et al.*(Martins *et al.*, 2022) demonstrated that inhibiting mice with skeletal muscle-specific knockout of p300 and CBP (which was induced in adulthood) completely **blocked** insulin-stimulated glucose uptake (Martins *et al.*, 2022). Remarkably, however, in mice with just one allele of p300 or CBP (Martins *et al.*, 2022), or just p300 or CBP (Martins *et al.*, 2019), insulin-stimulated glucose uptake was normal, and not different from wildtype littermate. Further studies also demonstrated that these regulatory actions of p300/CBP were via a direct effect on GLUT4 translocation to the plasma membrane in response to insulin (Martins *et al.*, 2022). Together, these data suggest a potentially fundamental role for p300/CBP in the regulation of GLUT4-mediated glucose transport. To this end, since

AMPK activation can occur independently of insulin, a primary goal of this Thesis is to determine whether p300/CBP are required for AMPK-mediated glucose uptake.

Summary. P300/CBP HAT function, temporal effects of the inducible knockout, muscle fiber type, and sex-based differences are all important parameters to consider when analyzing the effects of AMPK-activated glucose uptake in skeletal muscle. All these factors can give insight to how glucose uptake can be mediated by AMPK.

1.4. Gaps in knowledge

It has been over 25 years since the initial study by Corton *et al.* demonstrating that AICAR, and presumably other AMPK-activating molecules, increases glucose uptake in skeletal muscle (Corton *et al.*, 1995). Since then, many studies have examined the effects of AMPK-activators on glucose homeostasis and muscle metabolism, although the mechanism by which AMPK activation leads to glucose uptake remains to be fully elucidated. As previously stated, p300/CBP are fundamental regulators of insulin-stimulated glucose uptake (Martins *et al.*, 2022; Svensson *et al.*, 2020), although whether this role of p300/CBP is common to other stimuli that increase muscle glucose uptake is unknown.

1.5. Research objective and hypothesis of this Thesis

The primary objective of this Thesis is to determine whether p300 and/or CBP are required for AMPK-activated glucose uptake in mouse skeletal muscle. To address this objective, I will study ***five*** different novel mouse models, in which we are able temporally (via a tamoxifen-inducible promoter) knockout p300 and/or CBP in skeletal

muscle; these mice were generated using Cre/LoxP methodology, and Cre negative littermates (referred to as “wildtype” [WT]) will be the experimental control. We will measure skeletal muscle glucose uptake using an *ex vivo* 2-deoxyglucose (2DOG) uptake approach; this dual tracer approach includes using ^{3}H-2-deoxyglucose and ^{14}C-mannitol. P300/CBP tamoxifen-inducible knockout mouse models were chosen to study the effects of genotype. The main muscles that will be studied are the soleus (which is primarily comprised of slow-twitch, oxidative fibers) and the extensor digitorum longus (EDL; which is primarily comprised of fast-twitch, glycolytic fibers). These muscles will be dissected from each leg, with one side serving as the “basal” (i.e. no AICAR/MK-8722) and the contralateral side being "AMPK activated" (i.e. incubated with 2 mM AICAR or 10 μM MK-8722).

My hypotheses are as follows:

1) AICAR-stimulated and MK-8722-stimulated glucose uptake will both be greater than basal glucose uptake in the soleus and EDL muscles of WT mice.

2) AICAR-stimulated and MK-8722-stimulated glucose uptake will be lost in mice with knockout of p300 and CBP.

3) Just one allele of p300 or CBP will rescue AICAR-stimulated and MK-8722-stimulated glucose uptake back to levels seen in WT mice.

RESULTS

Individual knockout of p300 or CBP in mouse skeletal muscle does not impact AICAR-stimulated glucose uptake.

In soleus and EDL from WT littermates of i-mPKO **(Figure 1)** and i-mCKO **(Figure 2)** mice, 2DOG uptake in the presence of AICAR was significantly greater than Basal and resulted in robust "AICAR-stimulated" 2DOG uptake (i.e., AICAR 2DOG uptake minus Basal 2DOG uptake). Similarly, 2DOG uptake rate with AICAR and A-Stim 2DOG uptake was significantly increased in the soleus and EDL of i-mPKO and i-mCKO mice, with this increase being comparable to WT mice. There was no effect of genotype on basal 2DOG uptake in soleus or EDL.

Knockout of p300 ***and*** CBP blocks AICAR-stimulated glucose uptake in mouse skeletal muscle.

Given that individual loss of p300 or CBP does not impact A-Stim 2DOG uptake, next we studied the effect of loss of *both* p300 and CBP in skeletal muscle. In the soleus, 2DOG uptake in the presence of AICAR, and "AICAR-stimulated" 2DOG uptake (i.e., AICAR 2DOG uptake minus basal 2DOG uptake) were comparable between i-mPCKO and WT mice at Day 1 **(Figure 3, A & D)** and Day 3 **(Figure 3, B & E)**. However, AICAR-stimulated 2DOG uptake in the soleus was severely impaired at Day 5, such that it was ~67% lower in i-mPCKO mice compared with WT mice (Figure 3, F) and was not statistically different from basal 2DOG uptake **(Figure 3, C)**. These effects on 2DOG uptake were more rapid in the EDL with AICAR-stimulated 2DOG uptake being approximately 30% lower at D3 and not significantly different

from basal at D5 **(Figure 3, G–I)**. In both soleus and EDL, these effects on AICAR-stimulated 2DOG uptake in i-mPCKO mice were driven by an inability of muscle to respond to AICAR as basal 2DOG uptake was not different from WT mice at any time point.

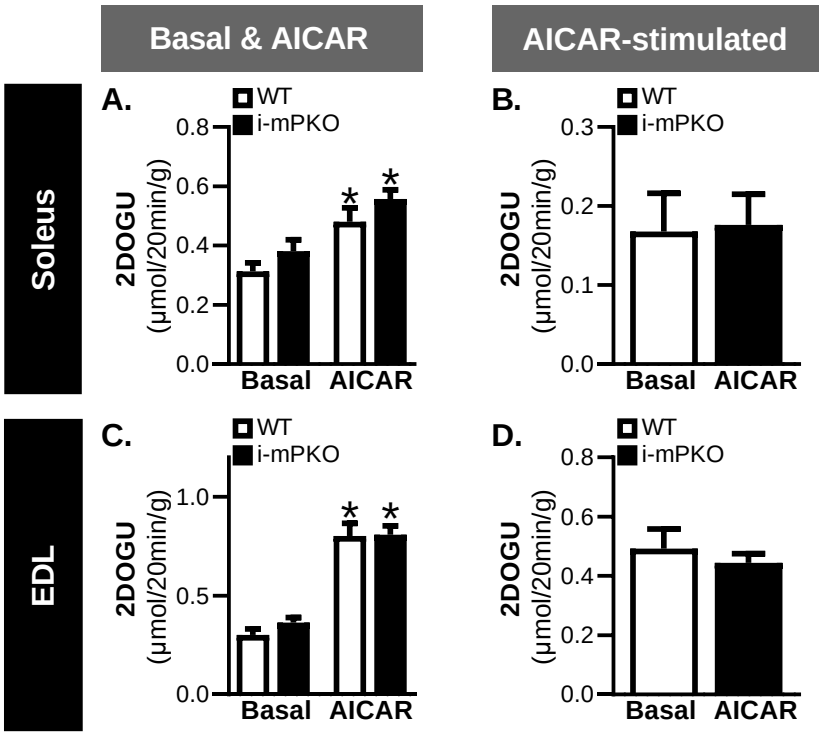
A single allele of p300 or CBP rescues the loss of AICAR-stimulated glucose uptake seen in PCKO skeletal muscle.

It is clear from these findings that loss of both p300 and CBP, but not individual loss of either one, impacts A-Stim 2DOG uptake. Thus, to determine dosage effects of p300 and CBP on 2DOG uptake in response to AICAR-stimulated glucose uptake, using a single-allele rescue approach, we studied mice with just 1 allele of p300 or CBP and knockout of the other protein (i.e., just 1 of 4 possible alleles) in skeletal muscle and their WT littermates; these mice are referred to as i-mCKO/PHZ and i-mPKO/CHZ, respectively. Remarkably, and comparable to our findings in i-mPKO and i-mCKO mice, 2DOG uptake rate with AICAR and A-Stim 2DOG uptake in soleus and EDL was comparable to WT mice in i-mCKO/PHZ **(Figure 4)** and i-mPKO/CHZ **(Figure 5)**. There was no effect of genotype on basal 2DOG uptake in soleus or EDL.

Stimulation of 2DOG uptake by the AMPK activator MK-8722 is also blocked by loss of the p300 and CBP and is rescued by a single allele of p300 or CBP.

To determine whether the role of p300 and CBP in AMPK-mediated glucose uptake is specific to AICAR or a general role of AMPK, we studied the effect of a separate and highly validated AMPK agonist, MK-8722 (Feng *et al.,* 2017; Myers *et al.,*

2017; Olivier *et al.,* 2018; Thomson, 2018) on 2DOG uptake in the EDL. For this, we studied i-mPCKO mice 5 days after initiating TMX treatment, as this is the timepoint at which AICAR-stimulated glucose uptake was most robustly inhibited. Comparable to our results with AICAR, 2DOG uptake with MK-8722 and MK-8722-stimulated glucose uptake (i.e., 2DOG uptake with MK-8722 minus Basal 2DOG uptake) was blocked and not different from basal in i-mPCKO mice **(Figure 6, A & D)**. Moreover, this effect on MK-8722-mediated 2DOG uptake was rescued in i-mPKO/CHZ **(Figure 6, B & E)** and i-mCKO/PHZ mice **(Figure 6, C & F)**. There was no effect of genotype on basal 2DOG uptake.



<u>Figure 1</u>. p300 is not required for AICAR-stimulated glucose uptake by skeletal muscle. Basal and AICAR-stimulated glucose uptake was assessed *ex vivo* in isolated soleus and EDL from WT and i-mPKO mice at Day 5 after initiating tamoxifen dosing. Mice were 12-16 weeks of age and both male and female mice were studied, with data collapsed together (not statistical differences in any parameters were noted between male and female mice). ***(A & C)*** Basal and AICAR (2mM) 2-deoxyglucose (2DOG) uptake (2DOGU) in soleus and EDL. ***(B & D)*** AICAR-stimulated 2DOG uptake (2DOGU) (calculated as AICAR 2DOG uptake – Basal 2DOG uptake) soleus and EDL. <u>Mouse numbers</u>: WT/i-mPKO, n = 15/17. <u>Statistics</u>: *Panels A & B* – 2-way ANOVA with Tukey multiple comparisons (P<0.05). *, versus basal within genotype. *Panels C & D* – unpaired t-test (P<0.05). *, versus WT. Data are presented as mean±SEM.

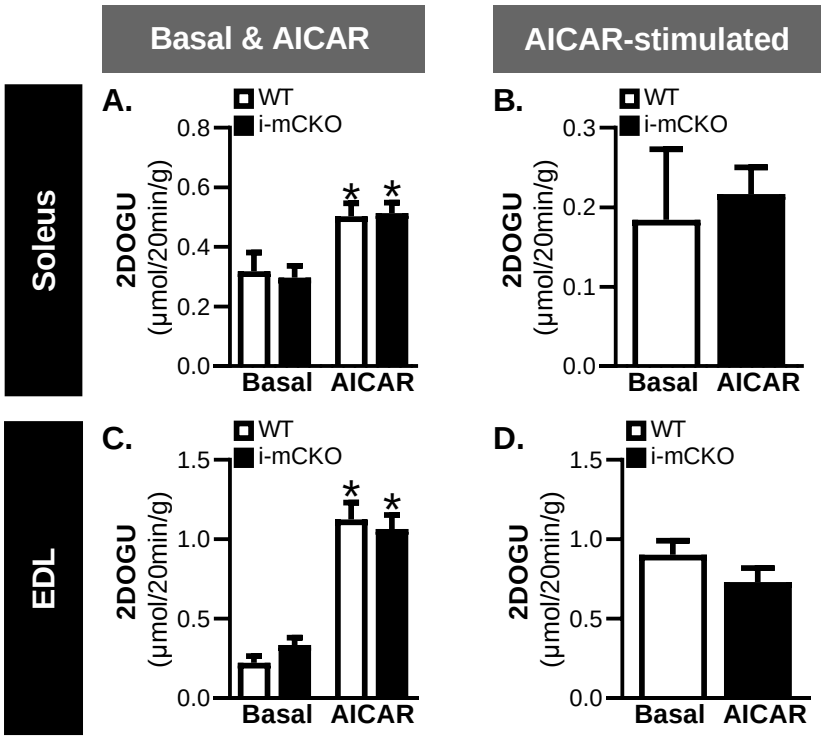
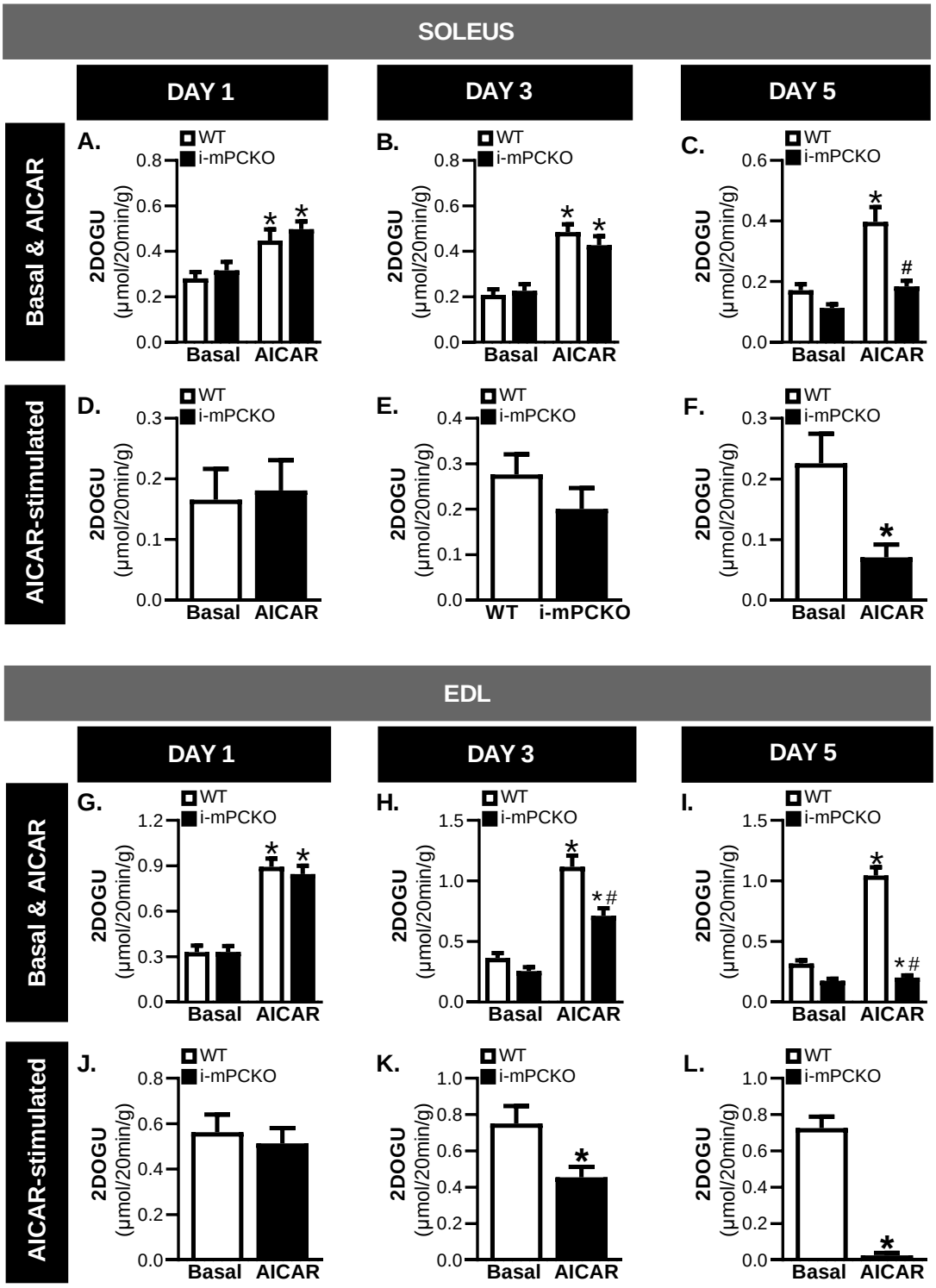


Figure 2. CBP is not required for AICAR-stimulated glucose uptake by skeletal muscle. Basal and AICAR-stimulated glucose uptake was assessed *ex vivo* in isolated soleus and EDL from WT and i-mCKO mice at Day 5 after initiating tamoxifen dosing. Mice were 12-16 weeks of age and both male and female mice were studied, with data collapsed together (not statistical differences in any parameters were noted between male and female mice). ***(A & C)*** Basal and AICAR (2mM) 2-deoxyglucose (2DOG) uptake (2DOGU) in soleus and EDL. ***(B & D)*** AICAR-stimulated 2DOG uptake (2DOGU) (calculated as AICAR 2DOG uptake – Basal 2DOG uptake) soleus and EDL. Mouse numbers: WT/i-mCKO, n = 16/19. Statistics: *Panels A & B* – 2-way ANOVA with Tukey multiple comparisons ($P<0.05$). *, versus basal within genotype. *Panels C & D* – unpaired t-test ($P<0.05$). *, versus WT. Data are presented as mean±SEM.

Figure 3. p300 and CBP are required for AICAR-stimulated glucose uptake by skeletal muscle. Basal and AICAR-stimulated glucose uptake was assessed *ex vivo* in isolated soleus and EDL from WT and i-mPCKO mice at Day 1, Day 3, or Day 5 after initiating tamoxifen dosing. Mice were 12-16 weeks of age and both male and female mice were studied, with data collapsed together (not statistical differences in any parameters were noted between male and female mice). ***(A–C & G-I)*** Basal and AICAR (2mM) 2-deoxyglucose (2DOG) uptake (2DOGU) in soleus and EDL. ***(D–F & J-L)*** AICAR-stimulated 2DOG uptake (2DOGU) (calculated as AICAR 2DOG uptake – Basal 2DOG uptake) soleus and EDL. Mouse numbers: Day 1 – WT/i-mPCKO, n = 13/14, Day 3 – WT/PCKO, n = 11/14, Day 5 – WT/PCKO, n = 16/18. Statistics: *Panels A-C & G-I* – 2-way ANOVA with Tukey multiple comparisons ($P<0.05$). *, versus basal within genotype; #, $P < 0.05$ versus WT within AICAR. *Panels D-F & J-L* – unpaired t-test ($P<0.05$). *, versus WT. Data are presented as mean±SEM.


SOLEUS
DAY 1
DAY 3
DAY 5
Basal & AICAR
AICAR-stimulated
A.
B.
C.
D.
E.
F.
WT
i-mPCKO
2DOGU (µmol/20min/g)
Basal
AICAR
EDL
G.
H.
I.
J.
K.
L.

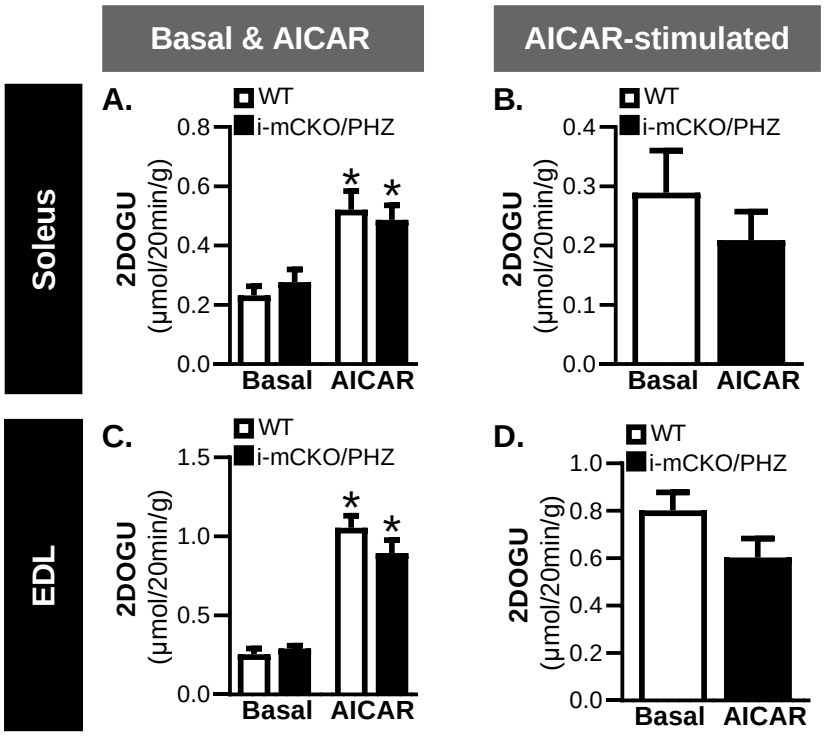


Figure 4. Mice with a single allele of p300 have normal AICAR-stimulated glucose uptake. Basal and AICAR-stimulated glucose uptake was assessed *ex vivo* in isolated soleus and EDL from WT and i-mCKO/PHZ mice at Day 5 after initiating tamoxifen dosing. Mice were 12-16 weeks of age and both male and female mice were studied, with data collapsed together (not statistical differences in any parameters were noted between male and female mice). ***(A & C)*** Basal and AICAR (2mM) 2-deoxyglucose (2DOG) uptake (2DOGU) in soleus and EDL. ***(B & D)*** AICAR-stimulated 2DOG uptake (2DOGU) (calculated as AICAR 2DOG uptake – Basal 2DOG uptake) soleus and EDL. Mouse numbers: WT/i-mCKO/PHZ, n = 12/14. Statistics: *Panels A & B* – 2-way ANOVA with Tukey multiple comparisons ($P<0.05$). *, versus basal within genotype. *Panels C & D* – unpaired t-test ($P<0.05$). *, versus WT. Data are presented as mean±SEM.

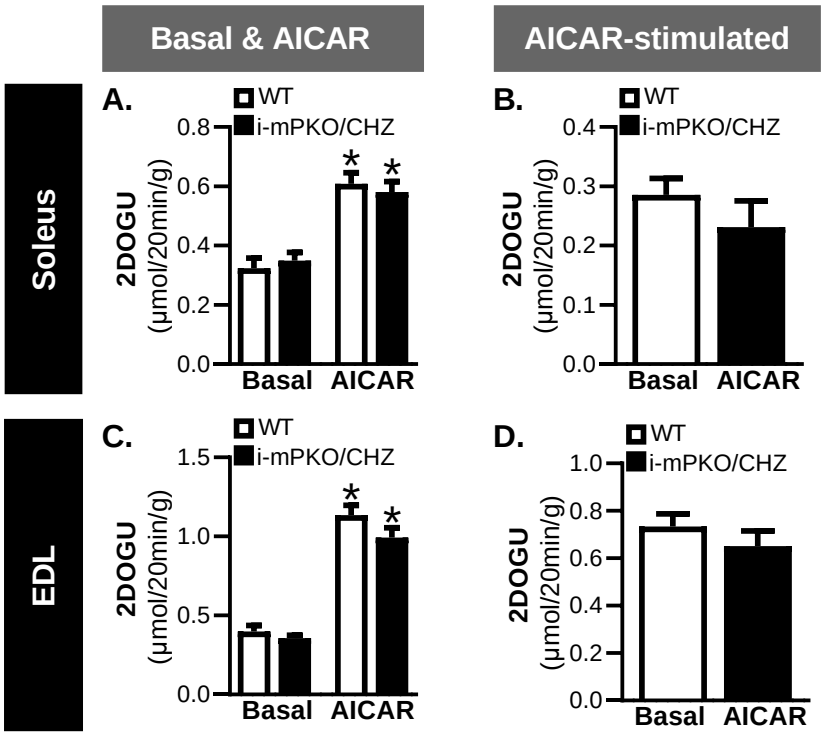
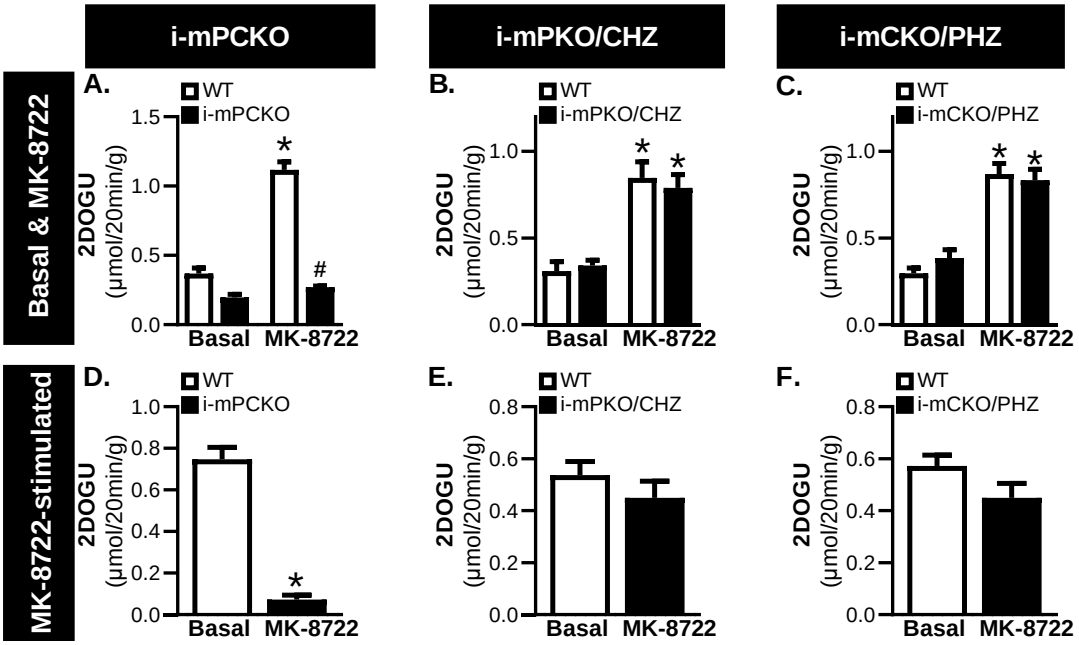


Figure 5. Mice with a single allele of CBP have normal AICAR-stimulated glucose uptake. Basal and AICAR-stimulated glucose uptake was assessed *ex vivo* in isolated soleus and EDL from WT and i-mPKO/CHZ mice at Day 5 after initiating tamoxifen dosing. Mice were 12-16 weeks of age and both male and female mice were studied, with data collapsed together (not statistical differences in any parameters were noted between male and female mice). ***(A & C)*** Basal and AICAR (2mM) 2-deoxyglucose (2DOG) uptake (2DOGU) in soleus and EDL. ***(B & D)*** AICAR-stimulated 2DOG uptake (2DOGU) (calculated as AICAR 2DOG uptake – Basal 2DOG uptake) soleus and EDL. Mouse numbers: WT/i-mPKO/CHZ, n = 13/20. Statistics: *Panels A & B* – 2-way ANOVA with Tukey multiple comparisons ($P<0.05$). *, versus basal within genotype. *Panels C & D* – unpaired t-test ($P<0.05$). *, versus WT. Data are presented as mean±SEM.



<u>Figure 6</u>. p300 and CBP are required for MK-8722-stimulated glucose uptake by skeletal muscle. Basal and MK-8722-stimulated glucose uptake was assessed *ex vivo* in isolated soleus and EDL from WT, i-mPCKO, i-mPKO/CHZ, and i-mCKO/PHZ mice at Day 5 after initiating tamoxifen dosing. Mice were 12-16 weeks of age and both male and female mice were studied, with data collapsed together (not statistical differences in any parameters were noted between male and female mice). ***(A–C)*** Basal and MK-8722 (10µM) 2-deoxyglucose (2DOG) uptake (2DOGU) in soleus and EDL. ***(D–F)*** MK-8722-stimulated 2DOG uptake (2DOGU) (calculated as MK-8722 2DOG uptake – Basal 2DOG uptake) soleus and EDL. <u>Mouse numbers</u>: Day 5 – WT/i-mPCKO, n = 9/7, WT/i-mPKO/CHZ, n = 5/4, WT/i-mCKO/PHZ, n = 11/13. <u>Statistics</u>: *Panels A-C & G-I* – 2-way ANOVA with Tukey multiple comparisons (P<0.05). *, versus basal within genotype; #, P < 0.05 versus WT within AICAR. *Panels D-F & J-L* – unpaired t-test (P<0.05). *, versus WT. Data are presented as mean±SEM.

DISCUSSION

Skeletal muscle, especially in response to insulin or contraction, plays a critical role in the regulation of systemic glucose concentration. As it relates to exercise, AMPK activators are commonly used to mimic the effects of contraction on GLUT4-mediated glucose transport, although surprisingly, the specific mechanisms underlying AMPK-mediated glucose transport in skeletal muscle remain to be defined. To this end, we investigated the role of p300 and CBP in the regulation of AMPK-mediated glucose transport. Similar to our findings with insulin (Martins *et al.,* 2022), our results demonstrate that AMPK-mediated glucose transport is blocked in skeletal muscle from mice with knockout of both p300 and CBP. Moreover, remarkably, this effect is reversed in mice with just allele of p300 or CBP, or that have knockout of only p300 or CBP. Together, our results demonstrate that p300 or CBP are required for AMPK-mediated glucose uptake in mouse skeletal muscle.

Activation of AMPK has long been known to increase glucose uptake in mouse skeletal muscle (Hardie *et al.,* 1998; Rose and Richter, 200; Friedrichsen *et al.,* 2013; Kjøbsted *et al.,* 2018; Spaulding and Yan, 2022). In recent years, the signaling mechanisms underlying this effect of AMPK has focused on TBC1D1 and Rac1 (Pehmøller *et al.,* 2009; Frøsig *et al.,* 2010; Jessen *et al.,* 2011; Treebak *et al.,* 2014; Sylow *et al.,* 2013; 2016; 2017). However, studies in transgenic rodents or using chemical inhibitors of Rac1 (Sylow *et al.,* 2013; 2016; 2017) or TBC1D1 (An *et al.,* 2010; Cartee *et al.,* 2015; Stockli *et al.,* 2015; Whitfield *et al.,* 2017; Kjobsted *et al.,* 2019) demonstrate that contraction- or AMPK-mediated glucose uptake in only slightly reduced (20-40% or less), but there is not complete inhibition of contraction-stimulated

glucose uptake; this suggests other signaling steps must be critical to AMPK-stimulated glucose uptake. Indeed, here we address this gap in knowledge by demonstrating that p300 or CBP are absolutely required for AMPK-mediated glucose uptake. Importantly, this role of p300 or CBP is not dependent on muscle type or sex, as our findings were identical in the soleus and EDL, and in male and female mice. Moreover, they were not dependent on how AMPK was activated, with our findings being the same in response to AICAR and MK-8722. Thus, similar to our findings with insulin-stimulated glucose uptake (Martins *et al.*, 2019) p300 and CBP are integral to AMPK-stimulated glucose uptake in mouse skeletal muscle.

In humans and rodents alike, skeletal muscle contains two major fiber types: type I (slow-twitch) and type II (fast-twitch). Both of these fiber types have different properties that allow the body to maintain structural support, motility, and contraction (Dave *et al.*, 2021; Qaisar *et al.*, 2016; Schiaffino & Reggiani, 2011). In this study, we found that the EDL (i.e., fast-twitch) had greater AMPK-stimulated glucose uptake compared to the soleus in both male and female mice regardless of p300/CBP single or heterozygous knockout. This finding was consistent with previous studies where AICAR and MK-8722 had an overall greater AMPK-stimulated glucose uptake in the EDL compared to the soleus (i.e., slow-twitch) in rodent models (N. O. Jørgensen *et al.*, 2021; Knudsen *et al.*, 2020; Rhein *et al.*, 2021). By studying the glucose uptake capacity in different fiber types, we can distinguish physiological differences between oxidative (i.e. soleus) and glycolytic (i.e. EDL) muscle fiber types. Specifically, our results demonstrate a clear main effect of fiber type, regardless of sex or p300/CBP knockout. We interpret this data as suggesting that a reason for the higher 2DOG uptake

in the EDL versus the soleus is due to the subunits present in AMPK. AMPK contains three subunits (α, β, γ) with different isoforms for each subunit (α1, α2, β1, β2, γ1, γ2, γ3) that all interact in a 1:1:1 ratio (Hardie, 2008; Ross *et al.*, 2016; Trefts & Shaw, 2021; Zhou *et al.*, 2001). In mouse skeletal muscle, γ2 and γ3 are predominantly expressed in the EDL compared to the soleus (Barnes *et al.*, 2004; Mahlapuu *et al.*, 2004; O'Neill, 2013). AMPK β1 and β2 also associate with α2 in the glycolytic EDL, but only β1 associates with α2 in the oxidative soleus in mouse muscle. In humans, α1 and β1 are expressed in oxidative muscles like the soleus, whereas γ3 is expressed in glycolytic muscles like the EDL (Mortensen *et al.*, 2009; Murphy, 2011; O'Neill, 2013). Additionally, TBC1D1, the main RabGAP associated with AMPK activation, is also more expressed in glycolytic muscle fibers (O'Neill, 2013; Taylor *et al.*, 2008). Taking these factors into account, it most likely explains the greater AMPK-mediated glucose uptake in the glycolytic EDL muscle compared to the oxidative soleus muscle.

To our knowledge, this is the first study to investigate the role of sex in the regulation of AMPK-mediated glucose uptake with tamoxifen-inducible, muscle-specific knockout of p300/CBP histone acetyltransferase activity. Most studies on skeletal muscle glucose uptake focus mainly on either male only or female mice, but rarely the two sexes together. By studying both sexes, we would be able to uncover any potential physiological distinctions between male and female mice in terms of skeletal muscle. Contrary to what we expected, there were no sex differences observed between male and female in all genotypes (i.e. WT, i-mPCKO, i-mPKO, i-mCKO, i-mPKO/CHZ, i-mCKO/PHZ). It is widely known that male and female skeletal muscle have differences in fiber type composition, contractile speed, and metabolism

(Glenmark *et al.*, 2004). Previous studies have also looked at the sex differences in relation to AMPK-stimulation through exercise and found that there are significant differences in protein expression or activation of AMPK between sexes, and many studies attributed this finding to the differences in fiber type composition (Brown *et al.*, 2020; Guadalupe-Grau *et al.*, 2016; Mortensen *et al.*, 2009; Roepstorff *et al.*, 2006; Tobias *et al.*, 2020). For instance, it was found that male human skeletal muscle displayed robustly greater AMPK activation (about 200% increase) compared to their female counterparts (Roespstorff *et al.*, 2006). Thereafter, these data suggests that p300/CBP may play an important role in AMPK by negating the sex differences seen in non-p300/CBP-deficient subjects, yet the mechanism is unclear.

Although this Thesis looked at many parameters (sex, genotype, muscle fiber, time), there are still a few limitations. Future directions for potential research include identifying proteins within the GLUT4 translocation pathway that are regulated by p300/CBP and that, by extension, could affect AMPK signaling in the context of glucose uptake. To date, there are a plethora of studies analyzing the effects of AICAR-stimulated glucose uptake *ex vivo* (Hingst *et al.*, 2020; Jørgensen *et al.*, 2018; Oki *et al.*, 2018), which allows for a controlled environment and stimulus. Therefore, it would be interesting to further study these mice *in vivo* to discern the effects of AMPK-stimulated glucose uptake in p300/CBP-deficient skeletal muscle.

In conclusion, we investigated whether p300 or CBP are required for AMPK-stimulated glucose uptake. Our results demonstrate that p300 or CBP are required for AMPK-stimulated glucose uptake in adult mouse skeletal muscle, regardless of sex or

muscle type. The goal of future work will be to investigate the molecular mechanisms by which p300/CBP regulate AMPK-stimulated glucose uptake.

MATERIALS AND METHODS

Mouse Models

Studies were conducted in 12-16 week old female and male mice. Mice with inducible, muscle-specific knockout of p300 and/or CBP were generated using Cre-LoxP methodology and have previously been described in detail (LaBarge *et al.*, 2016; Martins *et al.*, 2019, 2022; Svensson *et al.*, 2020). Briefly, p300 and CBP floxed mice, which have LoxP sites flanking exons 9 of the p300 (Kasper *et al.*, 2006) or CBP (Kang-Decker *et al.*, 2004) gene, were used; these mice were generated by others and are commercially available. Both the p300 and CBP floxed mice were initially bred with mice that have a tamoxifen-inducible Cre recombinase expressed under the human α-skeletal promoter (iHSA) (Martins *et al.*, 2019; 2022 Svensson *et al.*, 2020). Then, through judicious interbreeding five separate knockout (KO) mouse models were generated and studied. The experimental control for all mouse lines were “floxed” but Cre negative (Cre^{-}) littermates; these mice are collectively referred to as “wildtype” (WT). Table 1 provides a description of the different mouse lines. The breeding strategy for the i-mPKO, i-mCKO and i-PCKO was as follows: a “floxed” and Cre-positive (Cre^{+}, on one allele) mouse (i.e. i-mPKO, i-mCKO and i-PCKO) was bred with a "floxed” and Cre^{-} mouse; the genotype of the sire and dam was randomly chosen. For i-mPKO/CZ and i-mCKO/PZ, a “floxed” Cre^{+} i-mPCKO mouse was bred with a "floxed” and a Cre^{-} i-mPKO or i-mCKO mouse respectively. These breedings produced litters that contained Cre^{-} and Cre^{+} littermates. Mice were housed on a 12:12-hr (0600h-1800h) light-dark cycle at room temperature (~21°C) in a vivarium facility and mice had *ad libitum* access to chow (catalog no. 7912, irradiated; Envigo Teklad) and water.

At 98±0.3 days old, all mice were orally administered tamoxifen for 1, 3, or 5 consecutive days at 1200h. Procedures were carried out with the approval of, and in accordance with, the Animal Care Program and Institutional Animal Care and Use Committee at the University of California, San Diego.

Table 1. Description of mouse models. Legend: f, floxed; wt, wildtype; iHSA Cre, tamoxifen-inducible Cre recombinase expressed under the human α-skeletal promoter; +, positive; –, negative.

Name	Mouse model	p300 allele	CBP allele	iHSA Cre	
				KO	WT
i-mPKO	Inducible, skeletal muscle-specific knockout of p300	f/f	wt/wt	+	—
i-mCKO	Inducible, skeletal muscle-specific knockout of CBP	wt/wt	f/f	+	—
i-mPCKO	Inducible, skeletal muscle-specific knockout of p300 and CBP	f/f	f/f	+	—
i-mPKO/CZ	Inducible, skeletal muscle-specific knockout of p300 and heterozygous knockout of CBP	f/f	f/wt	+	—
i-mCKO/PZ	Inducible, skeletal muscle-specific knockout of CBP and heterozygous knockout of p300	f/wt	f/f	+	—

Tissue collection and fasting blood glucose

Mice were fasted at ~0800h for 4 h, and afterwards, the body weight and blood glucose concentration (tail vein; Ascensia Contour, Bayer HealthCare, Mishawaka, IL) were measured. Then, mice were anesthetized and the gastrocnemius and tibialis anterior were dissected from both legs and weighed (nearest 0.1 mg). The soleus and extensor digitorum longus (EDL) from both legs were dissected and for the 2-deoxyglucose (2DOG) uptake (2DOGU) assay.

***Ex vivo* 2DOG uptake**

Immediately after dissection, the soleus and EDL were transferred to individual vials containing oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit Buffer (KHB; 1.16 M sodium chloride, 0.05 M potassium chloride, 0.01 M potassium phosphate, 0.25 M sodium bicarbonate, 0.025 M calcium chloride dihydrate, 0.01 M magnesium sulfate

heptahydrate, 0.1% bovine serum albumin (BSA), containing 2 mM sodium pyruvate, and 6 mM mannitol for 1 h in a 35°C shallow form shaking water bath. Subsequently, muscles were transferred to a second vial containing KHB, 1 mM 2DOG, 9 mM mannitol, [^{3}H]-2DG (3 mCi/mmol; American Radiolabeled Chemical [ARC]) and [^{14}C]-mannitol (0.053 mCi/mmol; ARC) (Inc-KHB), with the soleus and EDL from one leg incubated in 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, 2 mM; Toronto Research Chemicals) or MK-8722 (10 μM; MedChemExpress) and the contralateral side incubated without AICAR or MK-8722. The duration of incubation in Inc-KHB was 20 minutes. After incubation, the muscles were rapidly blotted on filter paper, trimmed, flash-frozen in liquid nitrogen, and stored at -80°C. 2DOG uptake was calculated as previously described (Schenk *et al.*, 2011; Martins *et al.*, 2019).

Muscle Homogenization

Soleus and EDL were homogenized (Bullet Blender Tissue Homogenizer, Next Advance #BT24M) in 500 μL of ice cold homogenization buffer (50 mm Tris [pH 7.5], 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 1 mM $NaVO_2Na_2(PO_4)_2$, and 0.1% DTT) containing 1 M nicotinamide (MilliporeSigma #N0636), 1 mM Pefabloc SC PLUS (MilliporeSigma #11873601001), 1 mM trichostatin A (Cell Signaling #9950S), Complete (MilliporeSigma #11836170001), phosphatase inhibitor cocktail (PIC) 2 (MilliporeSigma #P5726), and PIC3 (MilliporeSigma #P0044). Homogenates were then rotated for 2 h at 4°C and centrifuged (12,000 rpm/14,167 g) for 20 minutes at 4°C. After centrifugation, the supernatant was collected and stored at -80°C for scintillation counting.

Statistics

Statistical analyses were performed using Prism 9 (GraphPad Software Incorporated, La Jolla, CA, USA). All data were analyzed using an unpaired Student's t-test or repeated measured 2-way ANOVA, as appropriate. For simplicity we have collapsed the data for male and female together; this was done because when we compared the AICAR-stimulated 2DOG uptake or MK-8722-stimulated glucose uptake in male versus female mice (within a given genotype), using an unpaired t-test, we did not see any statistical difference in these parameters (i.e. there were no sex-based differences in AMPK-stimulated glucose uptake. All data are expressed as mean±SEM.

This thesis, in whole, is currently being prepared for submission for publication of the material. Ha, Christina; Long, Laura M.; Casanova-Vallve, Nuria; Schenk, Simon. The thesis author was the primary investigator and author of this material.

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