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DOCTORAL THESIS

Extracellular matrix and integrin signalling proteins are novel therapeutic targets for diet-induced insulin resistance

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Extracellular matrix and integrin signalling proteins are novel therapeutic targets for diet-induced insulin resistance.

by

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General Introduction

Obesity, insulin resistance and glucose homeostasis

In the past half-century, the prevalence of obesity has surged dramatically, becoming a worldwide issue, particularly pronounced in high-income countries (Blüher, 2019; González-Muniesa et al., 2017; Marques et al., 2021). This trend is attributable to rapid changes in human behavior, lifestyle, global food systems, and frequency of physical activity (Blüher, 2019; Marques et al., 2021; Swinburn et al., 2011). One of the most notable shifts has been the increased availability of energy-dense foods, which tend to be more palatable and highly processed (Swinburn et al., 2011, Marques et al., 2021). Concurrently, urbanization and technological advancements have fostered more sedentary behavior, especially in affluent societies (Ekelund et al., 2016; Marques et al., 2021; Swinburn et al., 2011). Factors such as work typology, transportation, and leisure (Marques et al., 2021; Owen et al., 2011), which nowadays encourage sitting or low energy expenditure activities (e.g., office work, driving, watching television), play a significant role in promoting sedentary lifestyles (Dempsey et al., 2020; Marques et al., 2021). Additionally, the quantity of regular physical activity, particularly moderate to vigorous exercise, has declined among various global populations (Dempsey et al., 2020, Marques et al., 2021). Data from the World Health Organization reveals that 1 in 4 adults fail to meet the recommended levels of physical exercise globally, which entail at least 150–300 minutes of moderate-intensity aerobic physical activity or at least 75–150 minutes of vigorous-intensity aerobic physical activity (Organization WH, 2021, Marques et al., 2021). Sleep duration and quality have also suffered due to contemporary lifestyle adjustments within a 24/7 society, leading to health issues and predisposition to weight gain (Besedovsky et al., 2019; Cooper et al., 2018; Itani et al., 2017; Marques et al., 2021; Reddy & O'Neill, 2010), consequently correlating with obesity (Itani et al., 2017; Marques et al., 2021; Patel & Hu, 2008; Wu et al., 2014).

Obesity is characterized by the excessive accumulation of adipose tissue and is linked with low-grade chronic systemic inflammation (LGCSI) (González-Muniesa et al., 2017; Marques et al., 2021; Schwartz et al., 2017). Both obesity and chronic inflammation are pivotal risk factors for various chronic diseases, such as specific types of cancer, cardiovascular diseases, musculoskeletal disorders, and type 2 diabetes (T2D), thereby decreasing life expectancy (Gonzalez-Muniesa et al., 2017, Blüher, 2019, Marques et al., 2021). Obesity is intrinsically tied to T2D, primarily due to obesity's propensity to engender insulin resistance (IR), a fundamental pathophysiological feature of T2D (Zatterale et al., 2020; Kahn and Flier, 2000). In insulin-resistant individuals, the three primary insulin-sensitive tissues, namely skeletal muscle, liver, and adipose tissue (AT), display a diminished response to insulin action (Zatterale et al., 2020), causing significant disturbances in glucose uptake, glycogen synthesis, and glucose oxidation (Ormazabal et al., 2018). To compensate, pancreatic β-cells increase insulin secretion to normalize blood glucose concentrations. However, further escalation in IR leads to β-cell exhaustion, resulting in persistent hyperglycemia and T2D (Shulman, 2000; Zatterale et al., 2020). The number of T2D individuals has increased dramatically over the past three decades, and it is projected that over 300 million people may develop T2D as a complication of obesity by 2025 (Zatterale et al., 2020; Zhou et al., 2016). Hence, comprehending the mechanisms linking obesity to IR can enhance our understanding of T2D pathogenesis and our capacity to manage obesity-related chronic disorders (You et al., 2017; Zatterale et al., 2020).

Numerous studies using transgenic animal and human models have explored the relationship between dietary excess and the activation of the innate and adaptive immune systems in organs primarily responsible for systemic energy homeostasis (Lackey & Olefsky, 2016; Lumeng et al., 2007; Lumeng & Saltiel, 2011; Zatterale et al., 2020). Glucose homeostasis entails a complex interaction and cross-talk between pancreatic ß-cells and insulin-sensitive tissues such as skeletal muscle, adipose tissue, and the liver. This interaction becomes defective in Type 2 diabetes (T2D) due to a combination of β-cell dysfunction, reduced insulin sensitivity, and elevated endogenous glucose production (EGP) (Stumvoll et al., 2005). Reduced insulin sensitivity is considered the initial

trigger for T2D development, with β-cell dysfunction appearing in the later stages of the progression from pre-diabetes to full-blown T2D (Stumvoll et al., 2005).

The glucose uptake pathways in skeletal muscle

Glucose uptake in skeletal muscle is primarily mediated through two routes: contraction- and insulin-stimulated pathways, both of which have considerable overlap (Holloszy, 2003; Kjøbsted et al., 2019; Lundell & Krook, 2013; Cortright & Dohm, 1997; Wojtaszewski et al., 1996; Ihlemann et al., 2000; Kim et al., 2021; Richter et al., 2021). The role of skeletal muscle in maintaining normoglycemia is essential, as it assists in systemic glucose depletion under postprandial conditions (Defronzo et al., 1981; Zorzano et al., 2005). Insulin swiftly triggers glucose uptake and glycogen synthesis, via a protein synthesis-independent mechanism, by facilitating glucose's transmembrane movement into myocytes (Pereira & Lancha, 2004; Zorzano et al., 2005; Henriksen, 2010). Glycogen stores are vital for meeting the energetic demands of skeletal muscle. During muscle contraction in hypoxic conditions, both glycogenolysis and glucose uptake are elevated to supply sufficient glucose for glycolytic consumption (Zorzano et al., 2005). Furthermore, the muscle holds large reserves of triglyceride lipids, sustained by the uptake of free fatty acids. These lipids meet the energy demands during rest and oxidative conditions (Hegarty et al., 2003; Zorzano et al., 2005).

Skeletal muscle glucose uptake occurs via two glucose transporters, GLUT1 and GLUT4 (Zorzano, et al., 1996). In rat skeletal muscle, GLUT4 is responsible for 90-95% of total glucose carriers, whereas GLUT1 accounts for only 5-10% (Marette et al., 1992; Zorzano et al., 2005). Studies using the GLUT4 inhibitor indinavir estimated that around 60-70% of basal glucose transport in mouse skeletal muscle models is GLUT4-mediated (Rudich et al., 2003; Zorzano et al., 2005). The unique plasma membrane (PM) of the skeletal muscle fibre, which consists of the sarcolemma and the transverse tubules (T-tubules), transports glucose primarily via GLUT1 at rest and in the absence of insulin (Marette et al., 1992; Rudich et al., 2003). It is suggested that T-tubules are likely a significant

site for glucose transport in skeletal muscle, given that most GLUT4 mobilized in response to insulin inserts into the T-tubule membranes and only a smaller fraction into the sarcolemma (Henríquez-Olguin et al., 2019; Knudsen et al., 2020; Lauritzen, 2013; Marette et al., 1992). When stimulated by insulin or contraction, GLUT4 translocates from cytoplasmic GLUT storage vesicles (GSV) to the cell surface to enable glucose uptake across the sarcolemma (Sylow et al., 2021). Insulin binds to its receptor leading to insulin receptor substrate (IRS)-1 phosphorylation and subsequent activation of phosphatidylinositol 3-kinase (PI3K) (Sylow et al., 2021). This triggers two parallel signalling cascades for GLUT4 translocation, one involving the serine-threonine kinase Akt1/2 (Brozinick & Birnbaum, 1998; Lund et al., 1998; Wang et al.,1999) and the other, the Rho family GTPase Rac1 (JeBailey et al., 2007; Jensen et al., 2014; Sylow et al., 2013a, 2021; Ueda et al., 2008). Subsequent to Akt, the phosphorylation of TBC1 domain family member 4 (TBC1D4 or AS160) results in TBC1D4 inhibition and the release of the intracellular GSVs. These pathways, along with Rac1-mediated cytoskeletal reorganization, encourage GLUT4 translocation and subsequent glucose uptake in response to insulin (Sylow et al., 2021).

Skeletal muscle contraction also facilitates GLUT4 translocation and glucose uptake in an insulin-independent manner. This process partially requires AMP-activated protein kinase (AMPK) and Rac1 (Hardie & Lin, 2017; Kjøbsted et al., 2016, 2019; Sylow et al., 2016). Elevations in Ca2+ and AMP:ATP ratios activate AMPK in response to changes in cellular energy status and increased excitation-contraction (Jensen et al., 2007, 2014; Rose & Richter, 2005). This leads to TBC1D4Ser704 phosphorylation via the AMPK γ3-containing complexes (Blair et al., 2009; Chen et al., 2008, 2011; Eickelschulte et al., 2021; Cheung et al., 2000; Hardie & Carling, 1997), allowing for GLUT4 translocation to the cell membrane (Kjøbsted et al., 2019). Exercise is known to stimulate over 1,000 phosphorylation sites in human skeletal muscle, indicating a multitude of regulators yet to be discovered (Sylow et al., 2016).

A recent study identified a third, tension-mediated pathway that also facilitates glucose transport in skeletal muscle (Chambers et al., 2009; Jensen et al., 2014; Sylow et al., 2015). This pathway is hypothesized to need a structurally stable linkage between the extracellular matrix (ECM) and the actin cytoskeleton for normal nutrient uptake. The ECM, a dynamic structure comprised of a protein network, modulates various biological processes including insulin-stimulated glucose transport, cell migration, differentiation, development, and repair (Schuppan, 1990; Williams et al., 2015). Communication within the ECM is mediated by transmembrane cell surface receptors known as integrins (Mayer, 2003). These integrins form complexes with downstream proteins involved in both IGF and IRS signalling, such as integrin-linked kinase (ILK), focal adhesion kinase (FAK), serine/threonine-specific protein kinase (Akt), Rho GTPase Rac-1, and glycogen synthase kinase 3β (GSK-3β) (Williams et al., 2015). These complexes are believed to play a role in muscle protein synthesis (Byun et al., 2012; Clemente et al., 2012), insulin-mediated glucose uptake (Bisht et al., 2007; Bisht & Dey, 2008; Huang et al., 2006), and cytoskeletal stabilization (Williams et al., 2015a). Notably, integrins form a structural connection with the actin cytoskeleton, including the actin-binding protein talin, which links to the β subunit of integrin and contributes to force development within the actin cytoskeleton (Gheyara et al., 2007).

The role of extracellular matrix (ECM) and the integrin-signalling pathway in insulin resistance

Despite an extensive body of research exploring molecular defects associated with insulin signalling to devise novel treatments for conditions such as Type 2 Diabetes (T2D), there are still no available drugs that target insulin resistance in skeletal muscle (Williams et al., 2015). Given that the canonical insulin-mediated pathway has not yielded optimal targets, less traditional pathways, including the ECM, actin cytoskeleton, and focal adhesions, may present new opportunities for therapeutic intervention (Williams et al., 2015). The ECM is a complex network consisting of over 300 proteins, the major elements being collagens, proteoglycans, elastin, cell-binding glycoproteins, laminin, and integrins, each possessing distinct biochemical and physical properties (Hall & Sanest, 1993; Hynes & Naba, 2012; Williams et al., 2015). In response to a high-fat diet (HFD), specific proteins such as

collagen I, III, and IV, and integrin α2β1, are known to exhibit several-fold increase (Williams et al., 2015).

Research has shown that skeletal muscles of insulin-resistant obese individuals and those with T2D demonstrate increased collagen deposition (Berria et al., 2006; Richardson et al., 2005; Thomas et al., 2015). Rapid weight gain in healthy young males also led to impairments in insulin sensitivity, accompanied by upregulation of several muscle ECM genes (Tam et al., 2014; Williams et al., 2015). Interestingly, despite weight gain, there was no evidence of local adipose tissue or systemic inflammation, suggesting a critical role for muscle ECM in the regulation of glucose homeostasis, independent of secondary effects resulting from adipose tissue inflammation (Williams et al., 2015). Kang et al. (2011) studied the association between ECM remodeling, inflammation, and insulin resistance. They observed that 20 weeks of HFD in mice led to increased muscle collagen content, associated with enhanced gene expression of proinflammatory marker tumor necrosis factor (TNFa) and the macrophage marker F4/80. When insulin sensitivity improved, the expression of these inflammatory markers decreased, correlating with reduced collagen deposition (Kang et al., 2011; Williams et al., 2015). This suggests a connection between ECM remodeling in skeletal muscle and inflammation (Kang et al., 2011; Williams et al., 2015).

There exist several hypotheses explaining how increased ECM deposition in skeletal muscle during an HFD-fed state might contribute to insulin resistance. One hypothesis posits that the ECM acts as a physical barrier, impeding nutrient and hormone diffusion, thus restricting both glucose and insulin diffusion (Williams et al., 2015). ECM proteins accumulate in the interstitial space, increasing the diffusion distance and limiting substrate delivery to the muscle. A second hypothesis proposes that increased ECM deposition, in close contact with the endothelium, hampers vascular function and neovascular growth (Williams et al., 2015). As capillary recruitment and blood flow are essential for adequate insulin and glucose delivery to the muscle, reduced capillary density and vascular dysfunction have long been linked to the onset of insulin resistance and T2D (Jansson, 2007; Williams et al., 2015). Kang et al. demonstrated that increased capillaries in the muscle of HFD-fed

mice improved muscle insulin sensitivity (Kang et al., 2011; Kang et al., 2014; Williams et al., 2015), while decreased muscle capillaries exacerbated insulin resistance (Kang et al., 2014; Williams et al., 2015). Finally, the ECM might modulate insulin action through muscle integrins (Williams et al., 2015). Many studies have linked alterations in integrin signaling and ECM remodeling in the diet-induced obese (DIO) state to insulin resistance (Richardson et al., 2005, Berria et al., 2006, Kang et al., 2011; Wada et al., 2013; Dixon et al., 2013; Williams et al., 2015; Huber et al., 2007;Inoue et al., 2013; Kang et al., 2014; Kang et al., 2013).

So far, few studies have investigated the role of muscle integrin signaling in muscle insulin resistance *in vivo*. Deletion of muscle-specific integrin β1 in a chow-fed mouse model resulted in reduced whole-body insulin sensitivity and decreased insulin-stimulated glucose uptake in skeletal muscle during a hyperinsulinemic–euglycemic clamp (Williams et al., 2015; Zong et al., 2009). This deletion did not affect glucose metabolism in adipose tissue or liver. The reduction in insulin-stimulated glucose uptake in muscle was associated with a decrease in muscle glycogen synthesis and Akt^{Ser473} phosphorylation. Furthermore, whole-body deletion of integrin α2 in obese, HFD-fed mouse models, partially reversed diet-induced insulin resistance by increasing glucose uptake during a hyperinsulinemic–euglycemic clamp, and insulin signaling (Kang et al., 2011, Williams et al., 2015). Collectively, these data suggest that integrin signaling may be a mechanistic link between ECM and insulin resistance (Williams et al., 2015).

Metabolic disturbances in kidney failure patients

Individuals with renal failure undergoing maintenance haemodialysis (MHD) exhibit numerous metabolic and phenotypic changes, including muscle wasting (van Vliet et al., 2018; Workeneh & Mitch, 2010). This is attributed to the elevated degradation of muscle proteins via the ubiquitin-proteasome (Ub-P'some) system (Workeneh & Mitch, 2010). These patients lose between 1 to 3 kg of muscle mass annually (van Vliet et al., 2018), leading to decreased motor function, diminished

muscle glucose storage capacity, and an elevated risk of morbidity and mortality (Johansen et al., 2003; van Vliet et al., 2018). The mechanisms underlying skeletal muscle wasting in MHD patients remain elusive but seem to involve several factors, including inflammation, metabolic acidosis, reduced protein intake, hormonal abnormalities, increased substrate oxidation, and loss of amino acids and nutrients during dialysis (van Vliet et al., 2018; Wang & Mitch, 2014). Multiple studies have also revealed insulin resistance in patients with renal failure (Defronzo et al., 1981; Kobayashi et al., 2005; Bailey et al., 2006; Thomas et al., 2015; Turcotte & Fisher, 2008).

The dialysis treatment amplifies the rates of forearm phenylalanine uptake and release (Ikizler et al., 2002; Raj et al., 2004; van Vliet et al., 2018), leading to an overall negative forearm phenylalanine balance (Ikizler et al., 2002, van Vliet et al., 2018). The procedure itself is catabolic and induces a catabolic carryover that lasts for hours after dialysis (Ikizler et al., 2002), potentially disrupting skeletal muscle and whole-body protein metabolism in MHD subjects. Moreover, dialysis treatment results in a 20% loss of circulating amino acids in the dialysate (Ikizler et al., 1994; van Vliet et al., 2018), necessitating the replacement of amino acids through protein consumption during or after dialysis (van Vliet et al., 2018).

Considering that patients with kidney failure manifest signs of muscle atrophy (van Vliet et al., 2018; Workeneh & Mitch, 2010), and that muscle atrophy is associated with insulin resistance and reduced muscle integrin-associated protein content (Pattison et al., 2004; Peter et al., 2011; Postel et al., 2008; H. V. Wang et al., 2008), MHD subjects serve as excellent models to examine defects in extracellular matrix (ECM) mediated glucose transport. Decreased integrin-associated protein expression is also observed in insulin resistance, along with diminished Akt phosphorylation, which suggests a reduction in insulin-mediated glucose uptake (Urso et al., 2006).

Aims and Hypotheses

My doctoral research aimed to determine if MHD patients exhibit reduced integrin-associated protein content in skeletal muscle compared to non-MHD individuals. Given the accelerated atrophy in MHD skeletal muscle, we hypothesized that integrin-signalling proteins would be diminished in this population, potentially leading to impaired regulation of protein metabolism and nutrient flux (assessed using two-compartment whole-body measures of phenylalanine kinetics). We also examined, in a Rac1 KO insulin-resistant rodent model (Raun et al., 2018; Sylow et al., 2013), the potential role of Rac1, a critical actin-cytoskeleton regulatory protein, in upstream integrin-associated signaling in skeletal muscle. We hypothesized that integrin-associated protein expression would be reduced in both MHD patients and Rac1 KO rodents. Our research aimed to address the current literature gap regarding whether the loss of integrin-associated protein structure would facilitate or hinder amino acid metabolism and/or insulin sensitivity (i.e., nutrient flux) in MHD patients. However, this work was limited as no glucose tracer was used, thereby preventing further analysis of the effects of disrupted integrin signaling on glucose metabolism. Therefore, to further probe the implications of disrupted integrin-associated proteins in the development of diet-induced insulin resistance and reduced glucose clearance, we also examined skeletal muscles in a high-fat diet (HFD) mouse model. We hypothesized that under an HFD, there would be an increase in ECM-associated proteins, while the integrin-associated proteins would be downregulated, contributing to diet-induced insulin resistance and reduced whole-body glucose metabolism.

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Chapter 1: Involvement of the extracellular matrix and integrin signalling proteins in skeletal muscle glucose uptake

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Abstract

Whole-body euglycaemia is partly maintained by two cellular processes that encourage glucose uptake in skeletal muscle; *1)* the insulin- and *2)* contraction-stimulated pathways, with research suggesting convergence between these two previously separate processes. The normal structural integrity of the skeletal muscle requires an intact actin cytoskeleton as well as integrin-associated proteins, thus those structures are likely fundamental for effective glucose uptake in skeletal muscle. In contrast, excessive extracellular matrix (ECM) remodelling and integrin expression in skeletal muscle may contribute to insulin resistance owing to an increased physical barrier causing reduced nutrient and hormonal flux. This review paper explores the role of the ECM and the actin cytoskeleton in insulin- and contraction-mediated glucose uptake in skeletal muscle. This is a clinically important area of research given that defects in the structural integrity of the ECM and integrin-associated proteins may contribute to loss of muscle function and decreased glucose uptake in type 2 diabetes.

key words: integrin, ILK, Rac1, ECM, actin cytoskeleton, muscle contraction, insulin, insulin resistance.

General Introduction

Glucose homeostasis involves a complex interplay and cross-talk between pancreatic ß-cells and insulin-sensitive tissues such as skeletal muscle, adipose tissue, and the liver. This cross-talk is defective in Type 2 diabetes (T2D) due to a combination of failing β-cell function, reduced insulin sensitivity, and elevated endogenous glucose production (EGP) (Stumvoll *et al.*, 2005). Reduced insulin sensitivity is considered the starting point for the development of T2D, with β-cell dysfunction presenting at the latter stages of the progression from pre-diabetes to overt T2D (Stumvoll *et al.*, 2005). Human studies show that skeletal muscle is the primary site for insulin-mediated glucose disposal (DeFronzo *et al.*, 1985; Baron *et al.*, 1991a; Bouché *et al.*, 2004). Thus, a substantial amount of research has been directed towards understanding the molecular defects associated with insulin signalling in skeletal muscle to develop novel treatment strategies for conditions such as T2D. Yet, to date there are no drugs available that target skeletal muscle insulin resistance and therefore, it is key to clarify the underlying molecular mechanisms. As canonical insulin signalling has not presented optimal targets, lesser studied pathways, such as the actin cytoskeleton, extracellular matrix, and focal adhesions might present new opportunities.

The causes of insulin resistance and reduced insulin-stimulated glucose uptake in skeletal muscle are complex and likely involve disruptions to ***1)*** glucose delivery, ***2)*** transport across the sarcolemma and ***3)*** glucose metabolism with defects in the intracellular protein networks contributing to failure of the second and third of these processes (Zick, 2001; Karlsson *et al.*, 2005; Mackenzie & Elliott, 2014). Importantly, and related to the three points above, mounting evidence in rodents and humans supports the notion that the actin cytoskeleton and extracellular matrix may play an important role in muscle glucose uptake and GLUT4 transport (Richardson *et al.*, 2005; Berria *et al.*, 2006; Huber *et al.*, 2006; Kang *et al.*, 2011, 2013, 2014; Dixon *et al.*, 2013; Inoue *et al.*, 2013; Wada *et al.*, 2013; Williams *et al.*, 2015). Moreover, the major extracellular matrix (ECM) surface receptors integrins, and their downstream effectors, are emerging as potential players in skeletal muscle insulin action

and glucose uptake (Williams *et al.*, 2015). Therefore, understanding the role that integrin and its associated structural proteins plays in insulin action and glucose transport in skeletal muscle may be fundamental to identify new therapeutic targets for the treatment of insulin resistance and T2D. This review paper will outline and discuss the emerging evidence for the ECM and its associated integrin proteins playing a critical role in skeletal muscle glucose uptake.

The Canonical pathways responsible for facilitating glucose uptake in skeletal muscle

Skeletal muscle provides a large disposal site for both the oxidation and storage of glucose, making it a key tissue in the management of diseases such as T2D (DeFronzo *et al.*, 1982). The plasma membrane (PM) of the skeletal muscle fibre is unique in that it consists of the sarcolemma and the transverse tubules (T-tubules). In the absence of insulin, and at rest, muscle glucose transport likely occurs via glucose transporter (GLUT) 1 (Marette *et al.*, 1992*b*; Rudich *et al.*, 2003), localised constitutively at the sarcolemma.

In skeletal muscle, two key stimuli facilitate glucose uptake: ***1)*** insulin and ***2)*** contraction (Richter *et al.*, 1985; Wallberg-Henriksson & Holloszy, 1985; Lundell & Krook, 2013; Kjøbsted *et al.*, 2019; Holloszy, 2003). Yet rapidly evolving research reports significant overlap between contraction- and insulin-stimulated glucose transport, suggesting more convergence between these pathways (Wojtaszewski et al., 1996; Cortright & Dohm, 1997; Kim *et al.*, 2021; Richter *et al.*, 2021; Ihlemann *et al.*, 2000). In addition, exercise is known to stimulate over 1,000 phosphorylation sites in human skeletal muscle, suggesting that there are a great number of regulators yet to be identified (Sylow *et al.*, 2016). The T-tubules likely represent a major site of glucose transport in skeletal muscle, as the majority of GLUT4 mobilised in response to insulin inserts into the T-tubule membranes and only a smaller fraction into the sarcolemma in human and rodent muscle (Marette *et al.*, 1992*a*; Lauritzen,

2013; Knudsen *et al.*, 2020; Henríquez-Olguin *et al.*, 2019). Upon stimulation, GLUT4s translocate from GLUT storage vesicles (GSV) in the cytoplasm to the cell surface to facilitate glucose uptake across the sarcolemma (Sylow *et al.*, 2021). The processes involved in GLUT4 trafficking have received a great deal of attention and have been reviewed elsewhere (Richter & Hargreaves, 2013, McConell *et al.*, 2020, Tunduguru and Thurmond, 2017; Sylow *et al.*, 2017, 2021; Richter, 2021; Merz *et al.*, 2022); therefore, they will only be briefly outlined within this review.

Upon binding to its receptor, insulin initiates a cascade of events at the muscle cell membrane that results in the phosphorylation of insulin receptor substrate (IRS)-1 and the subsequent activation of phosphatidylinositol 3-kinase (PI3K) (Sylow *et al.*, 2021). At this intersection, PI3K offers an important bifurcation point leading to two parallel signalling cascades required for GLUT4 translocation, one requiring the serine-threonine kinase Akt1/2 (Brozinick & Birnmbaum 1998; Lund *et al.*, 1998; Wang *et al.*, 1999) and the other, the Rho family GTPase Rac1 (JeBailey *et al.*, 2007; Sylow et al., 2013; Sylow *et al.*, 2014; Ueda *et al.*, 2008; Sylow *et al.*, 2021). Downstream of Akt, the phosphorylation of TBC1 domain family member 4 (TBC1D4 or AS160) leads to TBC1D4 inhibition, and the subsequent release of the intracellular GSVs. Together with Rac1-mediated cytoskeletal reorganisation, these signalling pathways promote GLUT4 translocation and increased glucose uptake in response to insulin. The reader is referred to recent reviews (Sylow *et al.*, 2021).

Contraction of skeletal muscle also provides a potent mechanism for GLUT4 translocation and glucose uptake. This process is independent of insulin and seems to require, in part, AMP-activated protein kinase (AMPK) and Rac1 (Kjøbsted *et al.*, 2017; Sylow *et al.*, 2016; Kjøbsted *et al.*, 2019; Yue *et al.*, 2020; Hardie & Lin, 2017; Ito *et al.*, 2005). With cellular changes in energy status, and increased excitation-contraction coupling, both elevated Ca2+ and AMP:ATP ratios are known activators of AMPK (Rose & Richter, 2005; Jensen *et al.*, 2007; Hawley *et al.*, 1996; Hayashi *et al.*, 1998; Jensen *et al.*, 2014) resulting in TBC1D4Ser704 phosphorylation via the AMPK γ3-containing complexes (Eickelschulte *et al.*, 2021; Blair *et al.*, 2009; Chen *et al.*, 2008; Chen *et al.*, 2011; Cheung

et al., 2000; Hardie & Carling, 1997), allowing for GLUT4 translocation to the cell membrane (Kjøbsted *et al.*, 2019). The reader is referred to recent reviews (Sylow *et al.*, 2017; Richter *et al.*, 2021). Emerging evidence suggests substantial cross-talk between these canonical pathways and the ECM in facilitating glucose transport in skeletal muscle, which will be outlined in the following sections.

Extracellular matrix (ECM) and Transmembrane Protein Structure

The extracellular matrix (ECM) network on the plasma membrane is linked to the intracellular cortical actin cytoskeleton in the cytoplasm through a nexus of proteins. These proteins include integrins, talin, vinculin, FAK, Arp 2/3, and Rac1 (DeMali *et al.*, 2002; Vicente-Manzanares *et al.*, 2009; Hsiao *et al.*, 2015; Csapo *et al.*, 2020; Delon & Brown, 2007), which are located downstream of the ECM and interact with the transmembrane integrins (**Figure 1**). The ECM is a dynamic structure, which consists of a network of proteins that modulate biological processes including insulin-stimulated glucose transport, cell migration, differentiation, development, and repair (Schuppan, 1990; Andez & Amenta, 1995; Haynes, 2009; Williams *et al.*, 2015). Moreover, the ECM is fundamental for cell-to-cell interaction as well as function and maintenance of all tissue (Mayer, 2003; Williams *et al.*, 2015). The fabrication of the ECM consists of over 300 proteins, with collagens, proteoglycans, laminin, integrins, elastin, and cell-binding glycoproteins forming the major elements of the ECM, each with distinct physical and biochemical properties (Hynes & Naba, 2011; Hall & Sanes, 1993).

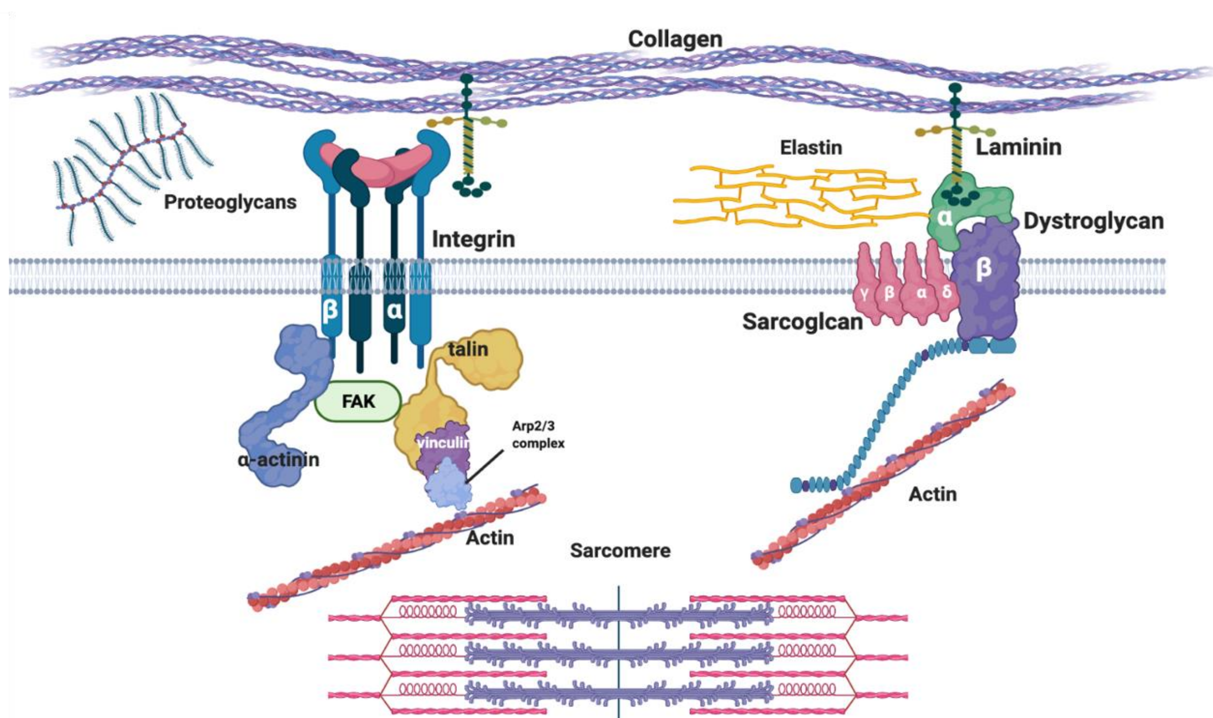


Figure 1. Key components of the extracellular matrix (ECM) of skeletal muscle, demonstrate the linkage between the ECM-associated proteins structures and the contractile filaments of muscle. Abbreviations: FAK, focal adhesion kinase; Arp2/3complex, actin-related protein 2/3 complex.

Collagen (Col) is the most abundant ECM structural component and is essential for cell adhesion, migration, differentiation, wound healing, and morphogenesis (Aumailley & Gayraud, 1998; Kang *et al.*, 2011; Zutter & McCall-Culbreath, 2008). Moreover, collagens are required for tissue support and structural integrity (Aumailley & Gayraud, 1998; Kang *et al.*, 2011). Collagen I, III, and IV are the most abundantly expressed isoforms in mammalian skeletal muscle with the latter found mostly in the basement membrane (Yurchenco & Patton, 2009; Kang *et al.*, 2011).

Proteoglycans are protein structures that are heavily glycosylated, formed of a core protein with one or more glycosaminoglycan (GAG) side chains attached, and are discussed elsewhere (Yue, 2014). Importantly for skeletal muscle, proteoglycans form a network with growth factors and growth factor

receptors, including the insulin receptor (Ussar *et al.*, 2012; Morcavallo *et al.*, 2014; Ohta *et al.*, 2018) and are associated in cell signalling and biological processes, including angiogenesis (Yue, 2014), which may provide a role in promoting glucose transport and diffusion.

The Dystrophin-glycoprotein complex (DGC) is a large multicomponent membrane-spanning complex that binds extracellular proteins with intracellular non-contractile proteins (Langenbach & Rando, 2002; Barresi & Campbell, 2006), thus providing a physical structure between the basement membrane and the subsarcolemmal cytoskeleton (**Figure 1**). The central component of the DGC complex is the dystroglycan protein, consisting of two glycoprotein subunits, α and β (Ibraghimov-Beskrovnaya *et al.*, 1992; Holt *et al.*, 2000; Langenbach & Rando, 2002), with each playing a distinct role in muscle structure and stability, as already described elsewhere (Ervasti & Campbell, 1991; Ibraghimov-Beskrovnaya *et al.*, 1992). The α-dystroglycan , binds to the ECM via various laminin isoforms (laminin 1, 2, and 4) on one side, and the organisation of the cortical actin cytoskeleton on the other side, through β-dystroglycan (Ibraghimov-Beskrovnaya *et al.*, 1992; Holt *et al.*, 2000; Langenbach & Rando, 2002). β-dystroglycan is transmembrane protein bound to ϒ-actin via the large non-contractile protein dystrophin, located intracellularly (Ervasti & Campbell, 1991; Rybakova *et al.*, 2000; Langenbach & Rando, 2002; Michele & Campbell, 2003). Interestingly, the DGC plays a protective role in preventing actin depolymerisation (Ervasti, 2013). This is an important point given that actin depolymerisation and optimal actin reorganisation are needed for insulin- (Sylow *et al.*, 2014; Brozinick *et al.*, 2004; JeBailey *et al.*, 2004; JeBailey *et al.*, 2007; Sylow *et al.*, 2013) and contraction- (Sylow *et al.*, 2012) mediated glucose uptake in skeletal muscle.

Integrins - The ECM communicates using transmembrane cell surface receptors known as integrins (Mayer, 2003). Laminin acts as a ligand for the integrin receptor (Grounds *et al.*, 2005), positioned at costameres and aligned with the Z-disks of the myofibrils (Csapo *et al.*, 2020). In forming a link between the ECM and the cytoplasm, integrins transduce signals across the plasma membrane and activates intracellular signalling including several downstream kinases that appear to be implicated in nutrient uptake and glucose metabolism (Williams *et al.*, 2015). Integrins are heterodimeric,

transmembrane glycoproteins with an α and a β chain non-covalently associated (Mayer, 2003; Rahimov & Kunkel, 2013). Thus far, 18 α and 8 β chains have been identified, which combine to form at least 24 different dimers (Van der Flier & Sonnenberg, 2001; Mayer, 2003). The diversity of these proteins is increased by the expression of splice variants that generates sub-chains. Seven α subunits are expressed in skeletal muscle; α1, α3, α4, α5, α6, α7, and αV, all of which are linked to the β1 integrin subunit (Williams *et al.*, 2015). Integrins are known to provide a bidirectional linkage between the ECM and the cytoskeleton, transferring external stimuli to regulate cellular processes while also allowing intracellular signalling proteins to contribute to external adhesion (Boppart & Mahmassani, 2019; Csapo *et al.*, 2020). Integrins lack endogenous enzymatic activity, so they are believed to form focal adhesions (FAs), composed of complex groups of downstream signalling molecules and proteins to provide a link to actin and microtubule cytoskeletons (Hynes, 2002; Schober *et al.*, 2007; Clemente *et al.*, 2012). Integrins form a structural linkage with the actin cytoskeleton including the actin-binding protein talin, which links to the β subunit of integrin, contributing to force development within the actin cytoskeleton (Gheyara *et al.*, 2007).

In the context of this review, it seems prudent to highlight the positioning of Rac1 within ECM-actin cytoskeleton arrangements given its central role in actin cytoskeleton remodelling and GLUT4 translocation. In response to stimuli that generate membrane expansion, the actin-related protein 2/3 (Arp 2/3) complex binds to vinculin, an interaction regulated by phosphatidylinositol-4,5-bisphosphate (PIP_2) and Rac1 (DeMali *et al.*, 2002). The activation of the Arp 2/3 complex by Rac1 and the Scar/WAVE proteins (Machesky & Insall, 1998; Miki *et al.*, 1998) results in the complex binding to actin (DeMali *et al.*, 2002). Importantly, the involvement of Rac1 in the linkage between integrin-talin-vinculin offers a clear network between the structural complements of skeletal muscle and the cellular processes involved in glucose transport activity.

Integrin-associated proteins and the actin cytoskeleton – implications for glucose uptake

Although the role of integrins and ECM-associated proteins in ECM remodelling and muscle cell integrity has been extensively characterised (Legate & Fässler, 2009; Wickström *et al.*, 2010; Draicchio *et al.*, 2020), little is known about the specific functions of the intracellular protein complexes within the context of muscle structural stability, and the regulation of insulin-stimulated cytoskeleton remodelling and glucose transport (Gheyara *et al.*, 2007; Draicchio *et al.*, 2020). It has been challenging to study the physiological role of integrins because it is difficult to recreate the complex ECM and cell-cell-junction environment *in vitro*, thus good model systems are still needed. Moreover, several of the key proteins involved in muscle structural stability seem to not have a catalytic activity (e.g., integrin b1 subunit, ILK, NCK).

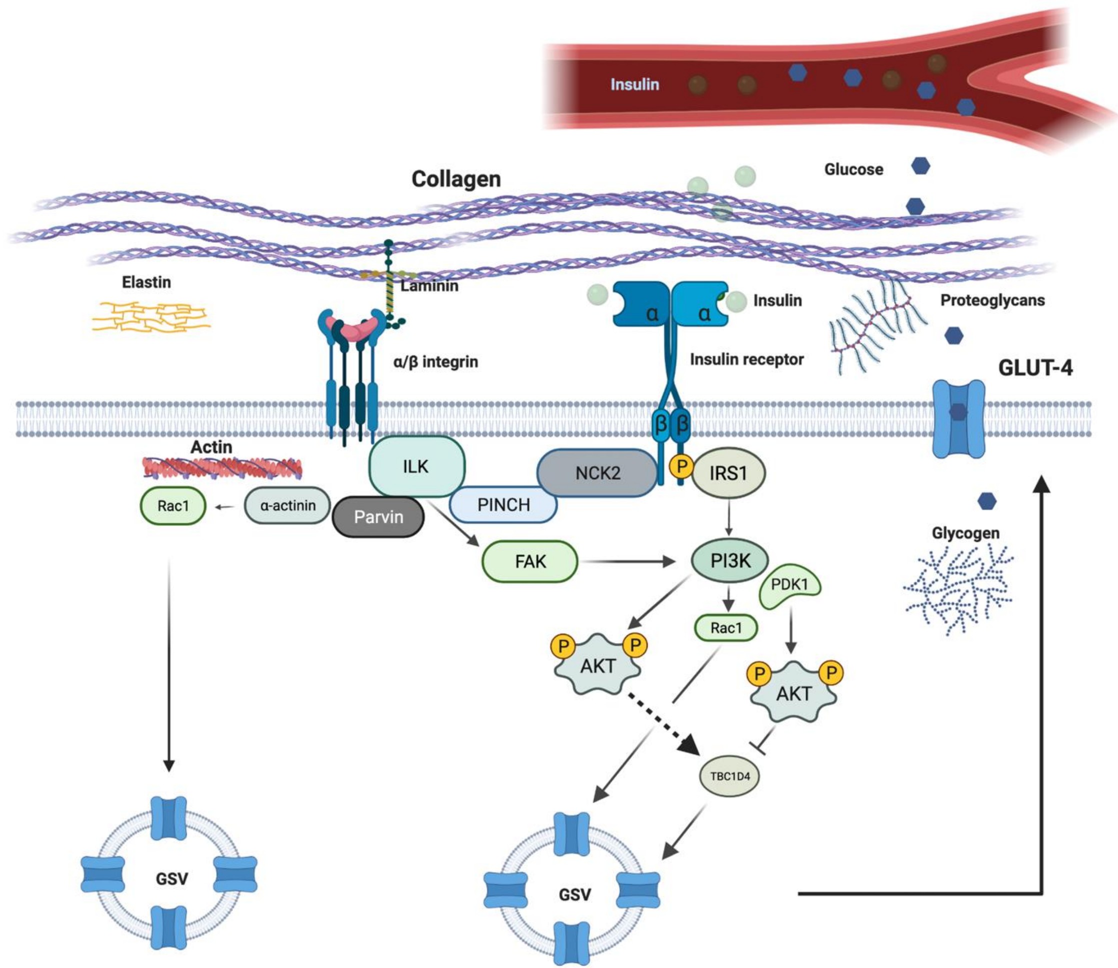
Skeletal muscle actin cytoskeleton consists of β- and γ-actin. The actin cytoskeleton undergoes substantial reorganisation in response to insulin stimulation (Dugina et al., 2009; Kee et al., 2009), in a Rac1-dependent manner via cofilin and Arp (Chiu et al., 2010; JeBailey *et al.*, 2007; Sylow et al., 2014), a process that mediates the very last steps of GLUT4 fusion with the PM (Brozinick et al., 2004; Brozinick et al., 2007). Accordingly, Rac1 is activated in response to insulin stimulation in skeletal muscle. Rac1 and the actin cytoskeleton likely act in parallel to Akt since individual inhibition of Rac1 or Akt partially decreased insulin-stimulated glucose transport by ~40% and ~60%. Yet, their simultaneous inhibition completely blocked insulin-stimulated glucose transport. Accordingly, the actin cytoskeleton depolymerising agent, LatrunculinB plus Akt inhibition blocked insulin-stimulated glucose uptake, while LatrunculinB had no additive effect on Rac1 inhibition.

Emerging evidence suggests that integrin and its associated downstream targets may play a fundamental role in glucose metabolism in skeletal muscle (Bisht *et al.*, 2008; Zong *et al.*, 2009;

Graae *et al*., 2019). This hypothesis is based on the following observations: *1)* integrin-associated proteins and Rac1 seem to play a pivotal role in promoting actin remodelling that contributes to glucose uptake in skeletal muscle; *2)* integrin-associated proteins provide an essential linkage between the ECM, the sarcolemma, and the actin cytoskeleton (Gheyara *et al*., 2007; Draicchio *et al*., 2020), therefore contributing to ECM structure stability, which may be necessary for normal nutrient transport in this tissue type (Gheyara *et al*., 2007; Draicchio *et al*., 2020).

Downstream integrin substrates include integrin-linked kinase (ILK), focal adhesion kinase (FAK) (Hynes, 2002; Schober *et al*., 2007; Draicchio *et al*., 2020), Akt, and Rac1 (Williams *et al*., 2015; Draicchio *et al*., 2020). The serine/threonine kinase ILK might be implicated in insulin-stimulated glucose transport. ILK complexes with PINCH and parvin forming the ILK-PINCH-parvin (IPP) complex, which functions at the earliest steps of integrin signalling (Wu, 1999; Stanchi *et al*., 2009; Draicchio *et al*., 2020). This complex links integrins to the actin cytoskeleton through a number of insulin-sensitive downstream effectors, including parvin, α-actinin, talin, Arp2/3 and the PI3K-Akt-Rac1 pathway (**Figure 2A**) (Qian et al., 2005; Gheyara *et al*., 2007). ILK is ubiquitously expressed in mammalian tissues and has three distinct domains: *1)* an N-terminus domain, *2)* a pleckstrin homology (PH)-like domain, and *3)* a C-terminal kinase-like domain (Stanchi *et al*., 2009; Wickström *et al*., 2010). ILK mediates its interactions with PINCH through the N-terminus domain, and parvin through the C-terminus domain (Legate *et al*., 2006; Stanchi *et al*., 2009; Wickström *et al*., 2010).

(A)



(B)

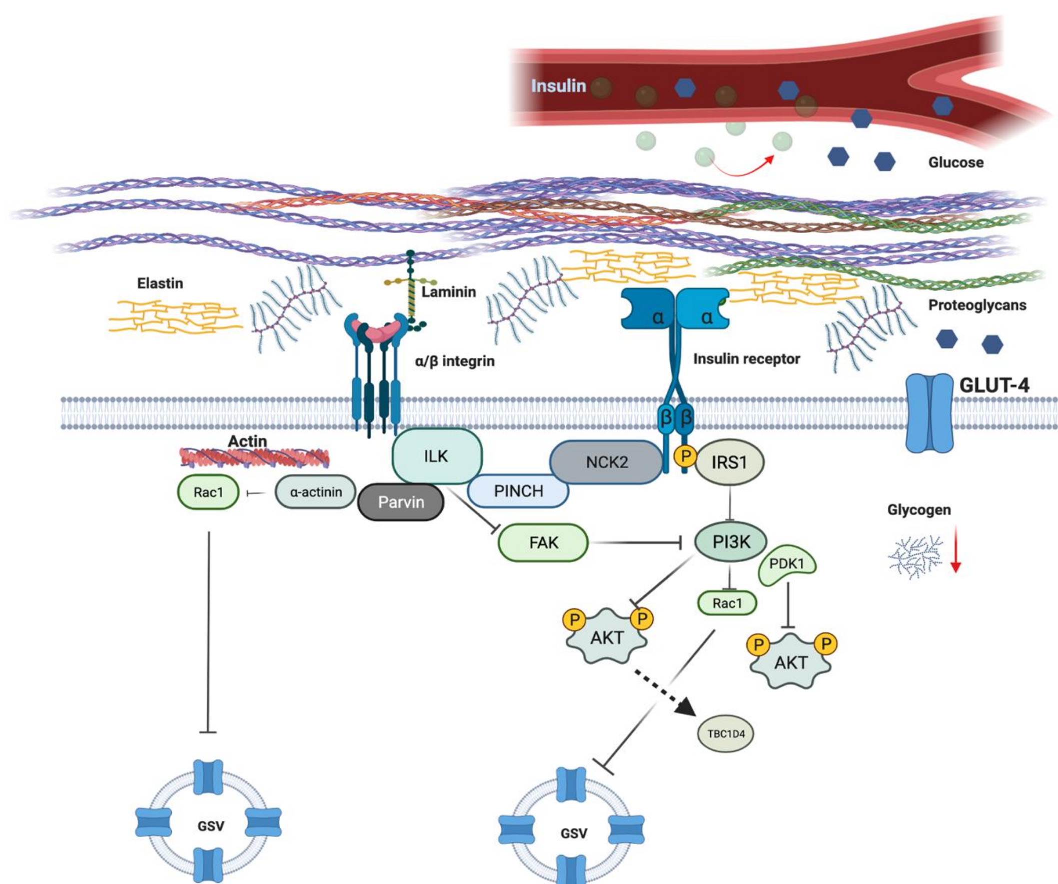


Figure 2A. Shows potential pathway linking integrins and their associated proteins in the regulation of glucose metabolism in muscle. **Figure 2B.** Shows the proposed disruptions to insulin-signalling and glucose transport due to excessive ECM remodelling. Abbreviations: ILK, integrin-linked kinase; FAK, focal adhesion kinase; Akt, protein kinase B; GSV, Glucose Storage Vesicles; IRS1, insulin receptor substrate 1; Rac1, Ras-related C3 botulinum toxin substrate 1; PINCH, particularly interesting new cysteine–histidine-rich protein; TBC1D4, TBC1 domain family member 4.

ILK interacts directly with the β-integrin subunits and recruits downstream targets implicated in the insulin-stimulated glucose uptake pathway, via IRS-1/Akt as well as the actin cytoskeleton remodelling via Rac1 (Gheyara *et al.*, 2007; Draicchio *et al.*, 2020). Specifically, ILK seems to activate PDK1 and GSK-3β through PINCH, therefore acting as an upstream regulator of Akt in skeletal and smooth muscle as well as other cell types (Wu & Dedhar, 2001; Qian *et al.*, 2005; Tang

et al., 2007; Williams *et al.*, 2015). In addition, ILK recruits the downstream effectors α-actinin and Rac1 through Parvin (Williams *et al.*, 2015; Draicchio *et al.*, 2020) (**Figure 2A**). This is likely implicated in promoting glucose uptake via cytoskeletal rearrangements because siRNA–mediated knockdown of ILK reduces Rac1 activation and cytoskeleton reorganisation in epithelial cells (Filipenko *et al.*, 2005).

The focal adhesion kinase (FAK), together with ILK and the IPP complex, appears to be another molecule that exert integrins' function towards the actin cytoskeleton (Williams *et al.*, 2015; Draicchio et al., 2020). FAK is a non-receptor tyrosine kinase known to play an essential role in insulin signalling (Parsons, 2003; Huang *et al.*, 2006; Bisht *et al.*, 2007) via PI3K and its downstream substrates Akt (Bisht *et al.*, 2008) and TBC1D4 (AS160) (Rondas *et al.*, 2012; Deshmukh *et al.*, 2006; Geraghty *et al.*, 2007) (**Figure 2A**). Once integrins are engaged and bind to the ECM, FAK is activated and interacts with the β1 integrin subunits and other signalling and cytoskeletal molecules at the focal adhesion (FA) sites (Chen *et al.*, 2000; Schober *et al.*, 2007). In addition, reducing FAK expression in L6 myotubes reduced glucose uptake, GLUT4 translocation, p-FAK^{Tyr397}, and actin fibre rearrangements under maximal insulin stimulation (100 nmol/l) (Huang *et al.*, 2006). Under both control and maximal insulin treatment, glucose uptake and GLUT4 translocation were increased in C2C12 myocytes overexpressing FAK, implying that increased FAK protected C2FAK$^{wt/+}$ (FAK overexpressing) cells from hyperinsulinaemia-induced insulin resistance, whereas C2FAK$^{mut/+}$ (FAK tyrosine-397 mutated) cells developed insulin resistance. Furthermore, in the C2siRNAFAK^{wt}, (FAK siRNA cells) insulin-stimulated glucose uptake and GLUT4 translocation were significantly impaired under control and insulin treatment, supporting the notion that FAK elimination reduces glucose uptake and transport, likely as a consequence of impaired insulin signalling (Bisht *et al.*, 2007), perhaps via a reduction in FAK-mediated PI3K-Akt activation and TBC1D4 inhibition (Bisht *et al.*, 2008; Rondas *et al.*, 2012).

A potential role of extracellular matrix remodelling in diet-induced insulin resistance and reduced glucose uptake in muscle

Over the past decade, mounting evidence in humans (Berria *et al.*, 2006) and rodents (Kang *et al.*, 2011) suggests that increased ECM remodelling is associated with a number of pathological states, including insulin resistance in skeletal muscle. ECM proteins including fibronectin, proteoglycans, and connective tissue growth factors increase several fold in human (Richardson *et al.*, 2005; Berria *et al.*, 2006) as well as mouse skeletal muscles (Huber *et al.*, 2007; Kang *et al.*, 2011, 2013 and 2014), adipose tissue (Inoue *et al.*, 2013) and liver (Wada *et al.*, 2013; Dixon *et al.*, 2013), in response to diet-induced obesity (DIO). High-fat diets are associated with chronic systemic inflammation (Tan & Norhaizan, 2019; Duan *et al.*, 2018), which increases ECM protein synthesis as well as decreasing ECM protein degradation, resulting in increased deposition and remodelling of ECM (Ruiz-Ojeda *et al.*, 2019) (**Figure 2B**).

There are two working hypotheses that may explain the mechanisms underlying ECM-associated insulin resistance in DIO. The first is that the increased protein expression within the ECM creates a physical barrier preventing normal insulin action and glucose diffusion across the sarcolemma (Kang *et al.*, 2013; Williams *et al.*, 2015) (**Figure 2B**). The second hypothesis suggests that muscle ECM may expand to impair vascular function and neovascular growth, given the close contact between the ECM and the endothelium (Williams *et al.*, 2015). The first of these hypotheses suggests that collagen, fibronectin and proteoglycan proteins accumulate in the interstitial space increasing diffusion distance and impeding substrate and hormonal delivery (Berria *et al.*, 2006; Williams *et al.*, 2015). In support of this hypothesis, Kang et al. showed that hyaluronan (HA; a major ECM component) in skeletal muscle was significantly increased in the insulin-resistant DIO mice when

compared to chow-fed mice (Kang *et al.*, 2013). Interestingly, the same authors also showed that treatment with long-acting pegylated human recombinant PH20 hyaluronidase (PEGPH20) caused a dose-dependent reduction in muscle HA content and improved skeletal muscle insulin resistance in DIO mice (Kang *et al.*, 2013). These results show that whole-body depletion of an ECM polysaccharide improves muscle insulin sensitivity in obese mice, whereas ECM protein accumulation seems to exacerbate insulin resistance (Kang *et al.*, 2013).

The second hypothesis that may explain the role the ECM's plays in insulin resistance centres on the notion that nutrient delivery to the contracting muscle requires functional blood flow to ensure sufficient glucose (during exercise) and insulin (post-exercise) availability to facilitate glucose uptake and glycogen resynthesis, respectively. Thus, ECM vascular dysfunction and capillary rarefaction have been linked to insulin resistance and T2D (Jansson, 2007; Williams *et al.*, 2015). In muscle, nutrient blood flow is enhanced by unperfused capillaries recruited by insulin (Bonner *et al.*, 2013). Research shows that 40% of insulin-stimulated glucose uptake is attributed to augmented muscle perfusion; with this haemodynamic response known to be absent in insulin-resistant T2D patients (Laakso *et al.*, 1990; Baron *et al.*, 1991b, 2000; Vincent *et al.*, 2003; Ellmerer *et al.*, 2006; Kim *et al.*, 2008; Kubota *et al.*, 2011; Bonner *et al.*, 2013). Given that insulin-resistant rodents and humans show capillarity rarefaction, this highlights the importance of sufficient muscle capillarisation for insulin-mediated glucose disposal (Lillioja *et al.*, 1987; Mårin *et al.*, 1994; Gavin *et al.*, 2005; Chung *et al.*, 2006; Guo *et al.*, 2012; Bonner *et al.*, 2013). Furthermore, the angiotensin II receptor blocker Losartan (Guo *et al.*, 2012), which enhances vasculature-induced tissue perfusion, also improves insulin sensitivity and increases microvascular density in skeletal muscles (Kang *et al.*, 2011; Guo *et al.*, 2012; Chai *et al.*, 2011; Kang *et al.*, 2011; Guo *et al.,* 2011; Bonner *et al.*, 2013).

Using *mVEGF*$^{-/-}$ mice, Bonner *et al.* (2013) showed a ~60% decrease in capillary density in skeletal muscle. Moreover, KO mice lacking VEGF present with reduced insulin-mediated glucose disposal (Bonner *et al.*, 2013). Importantly, Bonner and colleagues observed that this reduction in insulin-

stimulated glucose uptake in skeletal muscle was not associated with a reduction in intracellular insulin signalling (IRS-1, p85, and p/t Akt), suggesting that reduced insulin-stimulated muscle glucose uptake was caused by poor muscle perfusion (Bonner *et al.*, 2013). Thus, it is hard to draw a complete picture of insulin signalling in skeletal muscle as it is possible that integrin-associated signalling could have been negatively affected in this *mVEGF*$^{-/-}$ rodent model (Bonner *et al.*, 2013). Taken together, the studies above suggest that muscle capillary rarefaction and endothelial dysfunction are two avenues through which the remodelling of the ECM may contribute to insulin resistance in skeletal muscles (Williams *et al.*, 2015).

Integrins: important players in diet-induced insulin resistance

It seems clear that excessive ECM protein remodelling harms insulin sensitivity in skeletal muscle. However, it also appears possible that loss of function in key receptor proteins may also be implicated in insulin resistance. Of the two main integrin-mediated adhesion molecules, integrin α2β1 is of particular interest as under stress conditions such as high-fat diets (HFD), it reveals an antiangiogenic and profibrotic nature, promoting increased collagen expression and ROS production (Langholz *et al.*, 1995; Kang *et al.*, 2011; Bedard & Krause, 2007). Integrin α2β1 deletion in mouse skeletal muscle leads to angiogenesis and cell proliferation *in vivo* (Zhang *et al.*, 2008; Kang *et al.*, 2011). In contrast, the collagen receptor integrin α1β1 is proangiogenic and antifibrotic with integrin α1-null mesangial cells displaying increased collagen synthesis (Chen *et al.*, 2004, 2007, Borza *et al.*, 2012). Moreover, integrin α1β1 deletion causes a reduction in angiogenesis and endothelial cell proliferation *in vivo* (Pozzi *et al.*, 2000; Abair *et al.*, 2008).

Zong and colleagues (2009) showed that deletion of muscle-specific integrin β1 in chow-fed mice led to a reduction in whole-body insulin-stimulated glucose uptake during a hyperinsulinaemic-euglycaemic clamp; a finding that was accompanied by a decrease in muscle glycogen synthesis and p-Akt^{Ser473} (Zong *et al.*, 2009). A notion demonstrated schematically in **Figure 2B**.

Using mouse models lacking integrin α2β1 (*itgα2$^{-/-}$*) and integrin α1β1 (*itgα1$^{-/-}$*), Kang *et al.* showed that a HFD caused insulin resistance in both controls and integrin-null mice (Kang *et al.*, 2011), yet glucose infusion rates (GIRs) were reduced in itga1$^{-/-}$ compared with control (*itgα1$^{+/+}$*) mice in response to HFD treatment. In addition, GIRs were higher in the HF-fed *itgα2$^{-/-}$* mice when compared with HF-fed *itgα2$^{+/+}$* mice. Moreover, HF-fed *itgα2$^{-/-}$* skeletal muscles had increased IRS-1 and p-Akt expression compared to controls (Kang *et al.*, 2011). Taken together, these data imply that HFD mice lacking integrin α2β1 have improved insulin sensitivity, suggesting that integrin α2β1 may play a role in the development of insulin resistance.

Interestingly, the HF-fed *itgα2$^{-/-}$* mice seemed to be protected against HFD induced insulin resistance, associated withthe presence of a normal collagen protein structure (Kang *et al.*, 2011). Moreover, the decreased insulin resistance observed in integrin α2β1 null rodent may be explained by an enhanced vascularisation in HF-fed *itgα2$^{-/-}$* mice compared to controls as shown using immunostaining techniques (Kang *et al.*, 2011), providing further evidence of the anti-angiogenic nature of integrin α2β1.

The study by Kang *et al.* (2011) provides mechanistic insight into the potential link between muscle insulin resistance, decreased glucose disposal, and increased collagen structure, which seems to be reversed in the absence of integrin α2β1. Importantly, HF-fed rodents in whom integrin α2β1 was deleted had significantly greater insulin-stimulated glucose uptake compared with chow-fed *itgα2$^{+/+}$* mice. This study proposes a key role of integrins in insulin resistance and glucose uptake, however it does not explain how integrins, at a mechanistic level, connect to the cytoskeleton and exert their functions. The next section will review the key integrin-associated proteins along with the potential role these proteins play in ECM-cytoskeleton stabilisation and glucose uptake in skeletal muscle.

ILK-deficient mice under HFD show improved insulin sensitivity and muscle capillarisation

Kang *et al.* (2016) used muscle-specific ILK-deficient ($ILK^{lox/lox}$HSAcre) mice to study ILK under diet-induced muscle insulin resistance. $ILK^{lox/lox}$HSAcre and WT mice ($ILK^{lox/lox}$) were fed HFD or chow diet for 16 wk (Kang *et al.,* 2016), with validated insulin clamps being performed at 0 and 16 wk. ILK deficiency did not alter body mass or body fat in the $ILK^{lox/lox}$HSAcre mice. In addition, glucose infusion rates and glucose disappearance (R_d) rates were not different between chow-fed groups, whereas both parameters were substantially higher in HF-fed $ILK^{lox/lox}$HSAcre compared with HF-fed WT, suggesting greater insulin-mediated glucose response in the ILK-deficient mice (Kang *et al.*, 2016). Glucose metabolic index (R_g) was greater in HF-fed WT compared with chow-fed WT, meaning the mice developed HFD-induced insulin resistance; however, the HFD-induced glucose metabolism impairment was absent in the HF-fed $ILK^{lox/lox}$HSAcre mice (Kang *et al.*, 2016). Moreover, the same authors found that R_g was not different between WT and ILK-deficient mice in adipose tissue, regardless of diet. These results suggest that, independently of adiposity, muscle-specific ILK deletion improves glucose intolerance despite diet-induced insulin resistance (Kang *et al.*, 2016).

Interestingly, Wasserman's group found that improved insulin resistance was associated with an increase in CD31, a vascular/endothelial marker protein found in proximity of capillaries, implying increased signalling for local capillarisation in the HFD ILK-deficient mice (Kang *et al.*, 2016). The increase of CD31 in HF-fed $ILK^{lox/lox}$HSAcre mice was also associated with a decrease in JNK, P38 and ERK1/2, all of which are known to inhibit endothelial function and capillary proliferation, with an increase in insulin-dependant Akt phosphorylation (Kang *et al.*, 2016). In sum, muscle-specific ILK-deficient mice showed increased muscle capillarization and increased Akt activity; highlighting the importance of ILK in insulin perfusion and insulin signalling in skeletal muscle.

ILK depletion impairs glucose uptake in muscles of adult mice in a non-pathological context

Elsewhere, a study by Hatem-Vaquero *et al.* (2017) used whole-body conditional ILK knockdown (cKD-ILK) mice to make comparisons with WT counterparts in response to a typical chow-fed diet with comparisons for glucose control measured by glucose (GTT) and insulin tolerance test (ITT). These data showed elevated glycaemia and insulinaemia in cKD-ILK mice and increased homeostasis model assessment of insulin resistance (HOMA-IR; Hatem-Vaquero *et al.*, 2017). In a parallel *in vitro* experiment showed that GLUT4 expression and p-Akt^{Ser473} were reduced in cKD-ILK tissues (Hatem-Vaquero *et al.*, 2017). Taken together, these results suggest that ILK depletion in adult mice in a non-pathological context, impairs GLUT4 expression and membrane presence, with subsequent reduction in peripheral glucose uptake and insulin sensitivity (Hatem-Vaquero *et al.*, 2017). It is worth noting that these authors intended to perform this research on healthy, basal-fed adult mice, to avoid any upstream ILK pathological changes linked to ECM-associated proteins content, a phenomenon typically seen in type 2 diabetes (Berria *et al.*, 2006; Pasarica *et al.*, 2009; Kang *et al.*, 2011; Williams *et al.*, 2015; Hatem-Vaquero *et al.*, 2017). Overall, Hatem-Vaquero and co-workers' (2017) results show differences in glucose homeostasis between the cCK ILK and the WT mice. This is contrary to the observations of Kang *et al.* (2016), where the non inducible skeletal muscle-specific ILK KO mice showed no differences in glucose homeostasis compared to WT under basal chow-fed diet. This discrepancy may be explained by the different origin and settings of the mice model used; Kang *et al.*'s (2016) KO mice were developed through a muscle-specific ILK deletion at birth, whereas in the Hatem-Vaquero *el al.*'s (2017) models of ILK depletion were induced in adulthood. That said, what both these studies seem to confirm is that ILK is an important effector in the integrin nexus that acts as a downstream regulator in diet-induced insulin resistance.

The ILK/Rac1/cytoskeleton pathway is involved in insulin resistance

Insulin-resistant skeletal muscle and muscle cells in culture displays altered actin remodelling. Sylow *et al.* (2013*a*) demonstrated decreased Rac1 expression by 20% in soleus and 15% in EDL, along with reduced glucose uptake in mice feed a HFD for 12 weeks. Elsewhere Raun *et al.* (2018) showed that HFD Rac1 muscle-specific knockout (mKO) mice display reduced insulin-stimulated glucose transport in triceps, gastrocnemius and soleus muscles, suggesting that Rac1 ablation and HFD treatment combined negatively effects insulin-dependent glucose transport in muscle. These mouse studies are corroborated in human based experimental trials showing that Rac1 signalling is impaired in insulin-resistant patients with T2D and in obese subjects (Sylow *et al.*, 2013a). ILK has been also shown to have a role in regulating actin filaments' rearrangement via PI3K-Rac1 (Qian *et al.*, 2005; Gheyara *et al.*, 2007). Indeed, ILK overexpression in CEF cells increased Rac1 activation, a finding completely reversed with the administration of the PI3K inhibitor LY294002 (10-20 μM), suggesting that Rac1 is implicated in ILK signalling in a PI3K-dependent manner (Qian *et al.*, 2005). In addition, the same authors demonstrated that Rac1 inhibition in CEF cells inhibited the effects of ILK on active filament and cell migration (Qian *et al.*, 2005). A similar link between ILK and Rac1 could be therefore speculated in skeletal muscle as well.

ILK depletion in *Caenorhabditis elegans* and *Drosophila melanogaster* leads to muscle detachment of focal adhesion sites (Zervas *et al.*, 2001; Mackinnon *et al.*, 2002; Gheyara *et al.*, 2007; Alessi *et al.*, 1997), while ILK KO mice presented severe muscular dystrophy and actin cytoskeleton restructuring and displacements of focal adhesion-related proteins, such as dystrophin and FAK (Gheyara *et al.*, 2007; Boccafoschi *et al.*, 2011; Hodges *et al.*, 1997). Taken together, these findings demonstrate that ILK is a possible actin cytoskeleton regulator sitting upstream of Rac1. However, Gheyara and co-workers (2007) did not investigate the metabolic consequences of ILK depletion. Thus, we speculate that glucose uptake may have decreased in the ILK KO model, given that ILK

depleted mice also showed a reduction in skeletal muscle mass, a tissue that accounts for 80% of the total glucose metabolism under insulin stimulation (Bisht & Dey, 2008). The hypothesis is supported elsewhere (Kang *et al.*, 2016; Hatem-Vaquero *et al.*, 2017).

Conclusion

The integrins and the actin cytoskeleton are suggested to have an important role in contraction- and insulin-stimulated glucose uptake in muscle. This functional link involves the ECM-associated integrin network, which might be required for all stimuli to be effective and inducing glucose transport in skeletal muscle. It is worth noting that the tension-mediated pathway seems to operate alongside both contraction- and insulin-stimulated glucose uptake, rather than separately. While the interacting proteins involved in this regulatory process need further investigation, integrin-associated proteins and the actin cytoskeleton have potential roles. ECM remodeling occurs in mouse and human models of obesity, insulin resistant and type 2 diabetes that are linked, in part, to elevated systemic inflammation, which is in turn associated with pro-fibrotic mechanisms. The ECM involvement in obesity-induced insulin resistance may be explained by the evidence that ECM remodeling contributes to increased collagen formation, a process responsible for generating a physical barrier for insulin fusion and glucose transport. Moreover, ECM remodeling causes distinct compositional changes in integrin signaling within the matrix, again a process known to contribute to reduced insulin and nutrient uptake in skeletal muscle. The research discussed in this review suggest that a stable linkage between the ECM and the cytoplasmic actin cytoskeleton is critical for effective glucose uptake in skeletal muscle in response to insulin-, contraction- and tension-mediated pathways. However, this research area is still in its infancy with a number of key question unanswered.

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Chapter 2 - Integrin-associated ILK and PINCH1 protein content are reduced in skeletal muscle of maintenance hemodialysis patients

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Key Points

- Patients with renal failure undergoing maintenance hemodialysis are associated with insulin resistance and protein metabolism dysfunction.
- Novel research suggests that disruption to the transmembrane protein linkage between the cytoskeleton and the extracellular matrix in skeletal muscle may contribute to reduced amino acid metabolism and insulin resistance in hemodialysis.
- ILK, PINCH1 and $pFAK^{Tyr397}$ were significantly decreased in hemodialysis compared to controls, whereas Rac1 and Akt2 showed no different between groups.
- Rac1 deletion in the Rac1 knockout model did not alter the expression of integrin-associated proteins.

- Phenylalanine kinetics were reduced in the hemodialysis group at 30 and 60 minutes after meal ingestion compared to controls; both groups showed similar levels of insulin sensitivity and β-cell function.
- Key proteins in the integrin-cytoskeleton linkage are reduced in hemodialysis patients, suggesting for the first time that integrin-associated proteins dysfunction may contribute to reduced phenylalanine flux without affecting insulin resistance in hemodialysis patients.

Abstract

Background: Muscle atrophy, insulin resistance and reduced muscle PI3K-Akt signaling are common characteristics of patients undergoing maintenance hemodialysis (MHD). Disruption to the transmembrane protein linkage between the cytoskeleton and the extracellular matrix (ECM) in skeletal muscle may contribute to reduced amino acid metabolism and insulin resistance in MHD patients. **Study Design**: Eight MHD patients (age: 56±5 y: BMI: 32±2 kg/m^2) and non-diseased controls (age: 50±2 y: BMI: 31±1 kg/m^2) received primed continuous L-[*ring*-$^2H_5$]phenylalanine before consuming a mixed meal. Phenylalanine metabolism was determined using two-compartment modelling. Muscle biopsies were collected prior to and at 300 minutes postprandial. In a separate experiment, skeletal muscle tissue from muscle-specific Rac1 knockout (Rac1 mKO) was harvested to investigate whether Rac1 depletion disrupted the cytoskeleton-integrin linkage, allowing for cross-model examination of proteins of interest. **Results:** ILK, PINCH1 and $pFAK^{Tyr397}$ were significantly lower in MHD ($P<0.01$). Rac1 and Akt showed no different between groups for the human trial. Rac1 deletion in the Rac1 mKO model did not alter the expression of integrin-associated proteins. Phenylalanine rates of appearance (R_a), disappearance (R_d) and metabolic clearance rates (MCR) were lower in the MHD group at 30 and 60 minutes after meal ingestion compared to controls ($P<0.05$). Both groups showed similar levels of insulin sensitivity and β-cell function. **Conclusions:** Key proteins in the integrin-cytoskeleton linkage are reduced in MHD patients, suggesting for the

first time that integrin-associated proteins dysfunction may contribute to reduced phenylalanine flux without affecting insulin resistance in hemodialysis patients.

Keywords: hemodialysis; insulin; phenylalanine; metabolism; integrins; cytoskeleton; ILK; PINCH; Rac1.

Introduction

Patients with renal failure undergoing maintenance hemodialysis (MHD) demonstrate several metabolic and phenotypic alterations including muscle wasting (Workeneh & Mitch, 2010; van Vliet *et al.*, 2018) as a result of increased degradation of muscle proteins through activation of the ubiquitin-proteasome (Ub-P'some) system (Workeneh & Mitch, 2010). Decreased muscle mass results in decreased motor function and glucose storage capacity (Johansen *et al.*, 2003). Using the 'gold standard' euglycemic-hyperinsulinemic clamp DeFronzo *et al.*, (DeFronzo *et al.*, 1981) showed that patients with renal failure demonstrated insulin resistance, a finding supported elsewhere (Kobayashi *et al.*, 2005; Bailey *et al.*, 2006; Semenkovich, 2006; Turcotte & Fisher, 2008; Thomas *et al.*, 2015).

Glucose transport into skeletal muscle is known to occur via two distinct pathways: 1) insulin-mediated GLUT4 translocation, partly requiring functional IRS-1-Akt/AS160 signaling (Deshmukh, 2016) and 2) contraction-stimulated GLUT4 movement via Ca^{+2}-dependent AMPK/AS160 signaling (Wright *et al.*, 2005; Kramer *et al.*, 2006; Mackenzie & Elliott, 2014; Sylow *et al.*, 2017). Of note, it is widely accepted that it is this first pathway that is defective in insulin resistant skeletal muscle. More recently, a third pathway that facilities glucose transport has been identified, termed the tension-mediated pathway (Chambers *et al.*, 2009; Jensen *et al.*, 2014; Sylow *et al.*, 2015). It is hypothesized that this latter pathway requires a structurally stable linkage between the extracellular matrix and the actin-cytoskeleton to encourage normal nutrient uptake.

The dystroglycan complex and integrins are major surface receptors of the extracellular matrix (ECM) in skeletal muscles (Von der Mark *et al.*, 1991; Hynes, 2002; Postel *et al.*, 2008; Williams *et al.*, 2015). Integrins are transmembrane proteins formed by α and β subunits, which mediate interactions between the ECM and the cytoplasmic cytoskeleton (Postel *et al.*, 2008), and they are responsible for transducing signals across the membrane to the intracellular integrin-binding proteins (Williams *et al.*, 2015; Kang *et al.*, 2016). Whereas the role of integrins in ECM and muscle cell integrity has been extensively characterized (Legate & Fässler, 2009; Wickström *et al.*, 2010), little is known about the function of the integrin-binding intracellular complexes in both mechanical stability (Postel *et al.*, 2008; Wickström *et al.*, 2010) and normal nutrient metabolism in skeletal muscle. Once activated, the integrin complex stimulates downstream targets implicated in both IGF and IRS signaling, including integrin-linked kinase (ILK), focal adhesion kinase (FAK), serine/threonine-specific protein kinase (Akt), Rho GTPase Rac-1 and glycogen synthase kinase 3β (GSK-3β) (Williams *et al.*, 2015). Thus, integrin and its associated target kinases appear to be implicated in muscle protein synthesis (Byun *et al.*, 2012; Clemente *et al.*, 2012), insulin-mediated glucose uptake (Huang *et al.*, 2006; Bisht *et al.*, 2007; Bisht & Dey, 2008) and cytoskeletal stabilization (Williams *et al.*, 2015).

Muscle atrophy, a recognized characteristic of MHD (van Vliet *et al.*, 2018), has been linked with reduced muscle integrin-associated protein content (Pattison *et al.*, 2004; Postel *et al.*, 2008; Wang *et al.*, 2008; Peter *et al.*, 2011). In addition, reduced integrin-associated proteins' expression is also noted in insulin resistance with decreased Akt phosphorylation, a candidate for reduced insulin-mediated glucose (Urso *et al.*, 2006). Given that kidney failure patients display signs of muscle atrophy (Workeneh & Mitch, 2010; van Vliet *et al.*, 2018), we hypothesized that MHD patients would demonstrate reduced integrin-associated protein content in skeletal muscle when compared to non-MHD individuals. We further hypothesized that the structural and signaling disruption might lead to impaired regulation of protein metabolism and nutrient flux. The current work used two compartment whole-body measures of phenylalanine kinetics and surrogate measures of insulin resistance to partly test this hypothesis.

Rac1 is a well characterized member of Rho GTPase family and is implicated in cytoskeletal reorganization, transcriptional regulation, cell migration and nutrient uptake (Burridge & Wennerberg, 2004; Ueda *et al.*, 2008). Rac1 is known to promote GLUT4 translocation and glucose uptake via mechanisms requiring actin remodeling in skeletal muscle (Török *et al.*, 2004; JeBailey *et al.*, 2007; Sylow *et al.*, 2013*b*). Thus, proteins involved in vesicle trafficking may only operate fully in the presence of a functional cytoskeleton (Ueda *et al.*, 2008). The ILK PINCH parvin (IPP) complex functions at one of the early steps of the integrin signaling cascade (Wu, 1999; Stanchi *et al.*, 2009). The pseudokinase integrin-linked kinase ILK recruits downstream targets implicated in insulin-mediated glucose uptake and links integrins to the actin cytoskeleton (Gheyara *et al.*, 2007); among its downstream targets, there are PDK1 and GSK-3β (through PINCH1) as well as α-actinin and Rac1 (through parvin) (Williams *et al.*, 2015). ILK directly interacts with the β-integrin subunits and is stimulated by both integrin activation and growth factors and seems to act as an upstream regulator of Akt (Wu & Dedhar, 2001; Tang *et al.*, 2007). Therefore, this integrin signaling pathway is suggested to facilitate glucose uptake via both insulin-dependent and -independent mechanisms in a cross-sarcolemma fashion.

We therefore probed skeletal muscle from insulin resistant, but otherwise healthy controls, and made comparisons to MHD patients in both a fasted and postprandial state for integrin -associated protein signaling. Using an established muscle-specific Rac-1 knock out rodent model (Sylow *et al.*, 2013*a*; Raun *et al.*, 2018), a secondary aim was to investigate the potential linkage between actin cytoskeleton and integrin-associated signaling.

Materials and Methods

Ethical Approval

Ethics for human experiments was approved by the local Ethics Board (University of Illinois at Urbana-Champaign) and met all conditions outlined in the seventh revision of the Declaration of

Helsinki for use of human volunteers. Clinical Trial Registration Number: NCT03478722 (www.clinicaltrials.gov).

All experiments regarding the mouse models were approved by the Danish Animal Experimental Inspectorate (License: 2016-15-0201-01043).

Human Experiment

Eight maintenance hemodialysis (MHD) patients [mean (SEM) age 56 (5) y, male sex 6 (75%), BMI 32 (2), body fat 31 (3)%, Homeostatic Model Assessment of Insulin Resistance ($HOMA_{IR}$) 3.9 (0.9) and $HOMA_{\beta\text{-Cell}}$ 4.3 (1.24)] and eight matched insulin resistant controls [age 50±2 y, male sex 6 (75%), BMI 31±1, body fat 29 (2)%, $HOMA_{IR}$ 4.0 (0.6) and $HOMA_{\beta\text{-Cell}}$ 4.8 (0.65)] were recruited for this study. These participants were part of a larger investigation being conducted in our laboratory. $HOMA_{IR}$; fasting insulin (µU/ml) x fasting glucose (mmol/L) / 22.5] and HOMA of β-cell function [$HOMA_{\beta\text{-Cell}}$; 20 x fasting insulin (µU/ml) / fasting glucose – 3.5 (mmol/L)] were calculated using validated equations (Uwaifo *et al.*, 2002). Volunteers received written and verbal details on the experimental design, the study aims and potential risks before giving written consent. For MHD patients, we requested physician clearance from their nephrologist to further ensure it was safe for the patient to participate in this study.

A full description of the pre-screening and experimental procedures have been reported elsewhere (van Vliet *et al.*, 2018). The MHD patients analyzed in this study have been in dialysis treatment for about five years and their current nutritional status comprised ingestion of protein-rich meals to achieve the recommended protein intakes (set at 1.2 g/kg body weight per day) to limit muscle protein losses observed in end-stage kidney disease.

MHD patients were studied ~24 hours after a dialysis treatment session (i.e., non-dialysis day). Volunteers ingested an identical meal (320 kcal; 22 g protein, 43 g carbohydrates, 7 g fat) at least 12 hours prior to trials and arrived in a fasted state to the laboratory at 07:00 h. An 18-gauge cannula

was positioned into a dorsal hand vein to allow frequent sampling of arterialized blood (every 30-60 min), with a thermoregulated hot box (~65°C). A second 18-gauge cannula was placed into an antecubital vein for the primed constant infusion of L-[*ring*-$^{2}H_{5}$]phenylalanine (prime; 2.0 μmol·kg^{-1} FFM, infusion rate; 0.05 μmol·kg^{-1} FFM·min^{-1}). A baseline blood sample was collected at *t*=-180 min immediately prior to the start of the infusion protocol. Muscle biopsies were collected at *t*=-120 and -0 min of the infusion reflecting a postabsorptive state.

Subsequently, volunteer ingested a mixed meal (546 kcal; 20 g protein, 59 g carbohydrates, 26 g fat), the completion of which marked the start of the postprandial phase (*t*=0 min). An additional muscle biopsy was collected at 300 min. Biopsies were sampled from the middle region of the *vastus lateralis* (15 cm above the patella) with a Bergström needle modified for suction under local anesthesia (2% lidocaine) (van Vliet *et al.*, 2018). The postabsorptive muscle biopsies were sampled from the same incision with the needle pointed in the distal and proximal directions, respectively (van Vliet *et al.*, 2018). Muscle samples were immediately frozen in liquid nitrogen and stored at -80˚C until subsequent analysis. Blood samples were centrifuged at 3000 × g for 10 min at 4°C and plasma was stored at -80°C for later analysis.

Mouse Models

Considering the importance of an intact cytoskeleton for normal glucose uptake in skeletal muscles, we aimed to investigate the potential role of the major actin regulators, Rac1, in the integrin signaling pathway. Thus, nine muscle specific tetracycline-inducible knockout (mKO) mice and seven control mice were analyzed in this study. A full description of the Rac1 muscle specific tetracycline-inducible knockout (mKO) mouse model used in this experiment has been previously described elsewhere (Sylow *et al.*, 2013*b*, 2013*a*). Skeletal muscle from Rac1 mKO mice were analyzed when the mice were 31-33 weeks of age. Rac1 mKO was induced at 12–16 weeks of age by adding doxycycline (a tetracycline analogue) in the drinking water (1 g L−1; Sigma Aldrich, Copenhagen, Denmark) for 3 weeks to deplete Rac1 specifically in skeletal muscle (PMID: 29749029). This treatment was repeated at week 10 of the intervention period to ensure continuous Rac1 knockout throughout the entire intervention period. An 18 weeks diet intervention was started at 14–18 weeks of age and mice received either a standard

rodent chow diet (Altromin no. 1324; Brogaarden, Horsholm, Denmark), or a 60% high-fat diet (HFD) (no. D12492; Brogaarden, Denmark) and water ad libitum. Body weight was assessed every week. In this study we investigated skeletal muscles of chow-fed mice. All animals were maintained on a 12 h:12 h light–dark cycle, and group housed at 20–21°C. For muscle tissue sampling, mice were fasted for 3–5 h from 07:00 h and anaesthetized (intraperitoneal injection of 7.5/9.5 mg (chow/HFD) pentobarbital sodium 100 g−1 body weight). After 25 min skeletal muscle were excised and quickly frozen in liquid nitrogen and stored at −80°C until processing.

Immunoblotting

Relative total muscle protein content and phosphorylation levels of relevant proteins were determined by standard immunoblotting techniques loading equal amounts of protein. The primary antibodies used were Anti-Integrin linked ILK antibody (ab227154, Abcam), Anti-PINCH1 antibody [EP1943Y] (ab76112, Abcam), α-Parvin (D7F9) XP® Rabbit mAb (8190S, Cell Signalling) (37), $pFAK^{Tyr397}$ (D20B1) Rabbit mAb (8556S, Cell Signalling) (Tuguzbaeva *et al.*, 2019), anti-total FAK (ab40794, Abcam) (Bian *et al.*, 2019), anti-Rac1 (ARC03, Cytoskeleton Inc.) (Raun *et al.*, 2018), anti-total Akt2 (no. 3063 Cell Signalling Technology) (Raun *et al.*, 2018), $pAkt^{S473}$ (no. 9271, Cell Signalling Technology) (Karusheva *et al.*, 2019), $pAkt^{T308}$ (no. 9275, Cell Signalling Technology) (Raun *et al.*, 2018), Anti-IP6K1 antibody (ab129595, Abcam) and anti-dystrophin (ab15277, Abcam) (Jelinkova *et al.*, 2019).

Polyvinylidene difluoride membranes (Immobilon TransferMembrane;Millipore) were blocked in Tris-buffered saline (TBS)-Tween 20 containing 2% skimmed milk or 5% BSA protein for 30–60 min at room temperature. Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with rabbit or mouse peroxidase-conjugated secondary antibody for 30 min at room temperature. Coomassie brilliant blue staining was used as a loading control (Welinder & Ekblad, 2011). Bands were visualized using the Bio-Rad ChemiDoc MP Imaging System. Raw data for all the above protein analyzed are openly available in figshare at http://doi.org/ 10.6084/m9.figshare.12886943 (Draicchio *et al.*, 2020).

Blood analyses

Blood metabolites were determined using a point-of-care chemistry analyzer (Piccolo Xpress Chemistry Analyzer, Abaxis, Union City, CA). Plasma insulin concentrations were determined using a commercially available enzyme-linked immunosorbent assays (Alpco Diagnostics; Salem, NH). Plasma phenylalanine concentrations (unlabeled) and phenylalanine _enrichments (labeled) were determined by GC-MS analysis (Agilent 7890A GC/5975C; MSD, Little Falls, DE) as described in our previous work (van Vliet *et al.*, 2016). Briefly, plasma samples were prepared for amino acid analysis using a mixture of isopropanol:acetonitrile:water (3:3:2, v/v) and centrifuged at 20000 × g for 10 min at 4ºC. Subsequently, the supernatant was dried, and the amino acids converted into tert-butyldimethylsilyl (*t*-BDMS) derivatives prior to GC-MS analysis. L-[*ring*-$^{2}H_5$]phenylalanine, *m/z* 336 (*m*+0) and 341 (*m*+5) were monitored for unlabeled and labelled phenylalanine, respectively.

Data Modelling

Plasma insulin and endogenous glucose concentrations were used to model the following measures of insulin sensitivity (ISI) (Matsuda Index) (Matsuda & Defronzo, 1999), acute insulin response to glucose (AIR_g), 2nd phase insulin response ($\Delta I_{60-120}/\Delta G_{60-120}$) (Lorenzo *et al.*, 2013) and disposition index ($DI = S_I \times AIR_g$) (Utzschneider *et al.*, 2009).

Whole body phenylalanine rate of disappearance (R_d), rate of appearance (R_a) and metabolic clearance rates (MCRs) were calculated as previously described (Bergman *et al.*, 1989; Gastaldelli *et al.*, 1999; Barclay *et al.*, 2020). Raw data for insulin and phenylalanine metabolism are openly available in figshare at http://doi.org/ 10.6084/m9.figshare.12886943 (Draicchio *et al.*, 2020).

Statistical analyses

R software and *Lme4* package (Bates *et al.*, 2015) were used to perform linear mixed effects analyses of the relationship between insulin, phenylalanine or protein values, and time and condition (controls and MHD). Condition interacting with time was entered as fixed effect into the model,

whereas the main effects of subjects and time as random effects. If the null hypothesis was rejected, post hoc tests were performed to determine which groups differed from each other. P<0.05 was considered statistically significant.

Results

Insulin sensitivity

$HOMA_{IR}$ and $HOMA_{\beta\text{-Cell}}$ (P>0.05) were comparable between groups, suggesting no difference metabolic dysfunction between MHD and non-MHD individuals (data not shown). Estimations of insulin sensitivity and β-cell function based on the 59g oral carbohydrate load are shown in **Figure 1.** These data show that there were no significant differences for insulin sensitivity (ISI; P=0.3948; A). β-Cell function was also similar between groups with AIR_g (P=0.2006; B), 2nd phase insulin response to oral glucose (P=0.8286; C) or DI (P=0.5605; D) showing no difference at 2 hours' post feeding.

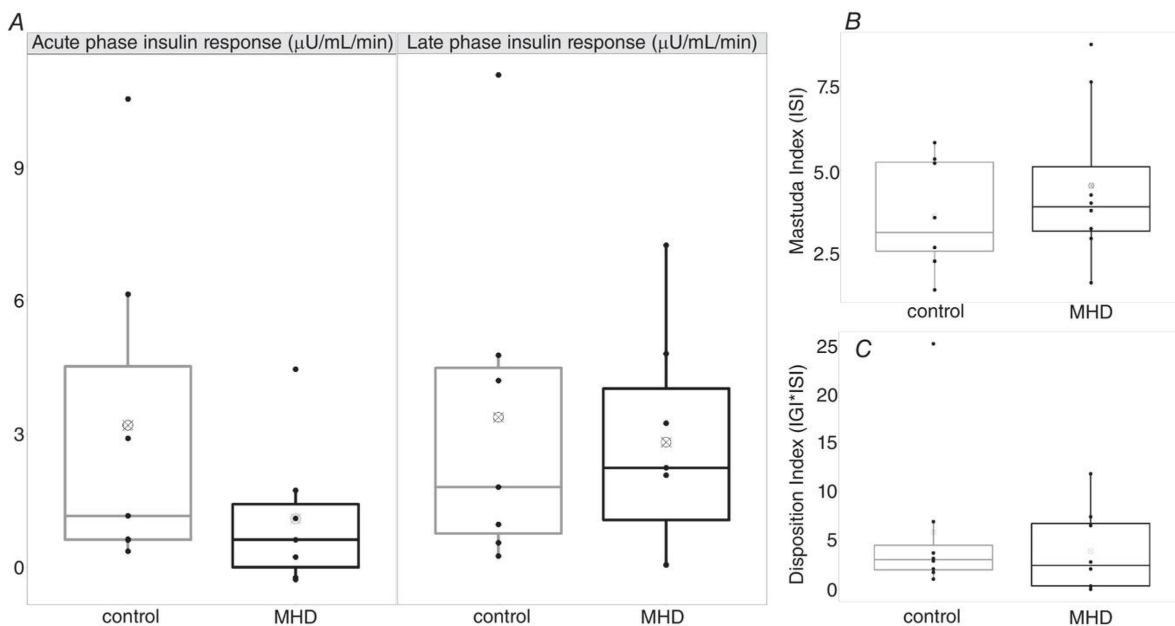

Figure 1. Acute insulin response to glucose (AIR_g, on the left) and 2nd (late) phase insulin response (on the right) (A), Matsuda Index (ISI) (B), and Disposition Index, (DI) (C), after mixed meal feeding in maintenance hemodialysis (MHD, n=8) patients and control (n=8) participants. No statistically significant differences were observed (P>0.05). Quartiles, means and individual data points are shown.

Whole-body protein metabolism was estimated using L-[*ring*-$^2H_5$]phenylalanine to allow for the calculations of phenylalanine R_a, R_d and MCR (**Figure 2**) from time 0 to 300 min of the postprandial period. The MHD group showed a significant decrease in R_a, (P=0.0451 at 30 min, P=0.0131 at 60 min; P=0.0002 at 90 min and P=0.0015 at 300 min, **Figure 2A and B**), R_d (P=0.0007 at 30 min, P=0.0022 at 60 min; P=0.0003 at 90 min and P=0.00141 at 300 min, **Figure 2C and D**) and MCR (P=0.0021 at 30 min; P=0.0025 at 60 min; P=0.0001 at 90 min and P=0.0003 at 300 min, **Figure 2E and F**) in the 300 min post feeding that, overall, was not apparent for the Control group. In the Controls, R_a, (P=0.9664 at 30 min; P=0.9933 at 60 min; P=0.1129 at 90 min and P=0.2512 at 300, **Figure 2A and B**) and R_d (P=0.6071 at 30 min; P=0.3175 at 60 min; P=0.1523 at 90 min and P=0.903 at 300 min, **Figure 2C and D**) did not show relevant changes post feeding, whereas MCR (**Figure 2E and F**) only showed a decrease at 90 minutes (P=0.0247) and 300 minutes (P=0.0252), but not at 30 minutes (P=0.7865) or 60 minutes (P=0.6762).There were significantly differences at 30 min for R_d (P=0.0476) and MCR (P=0.0345), but not for R_a (P=0.1595), between groups.

Both R_a (P=0.0501) and R_d (P=0.0402) were significantly different at 60 minutes while MCR expressed a tendency to be different (P=0.0695). Similar trends between groups were observed at 90 minutes (R_a, P=0.2539; R_d, P=0.4363; MCR, P=0.1637) and 300 minutes (R_a, P=0.2977; R_d, P=0.415; MCR, P=0.4043).

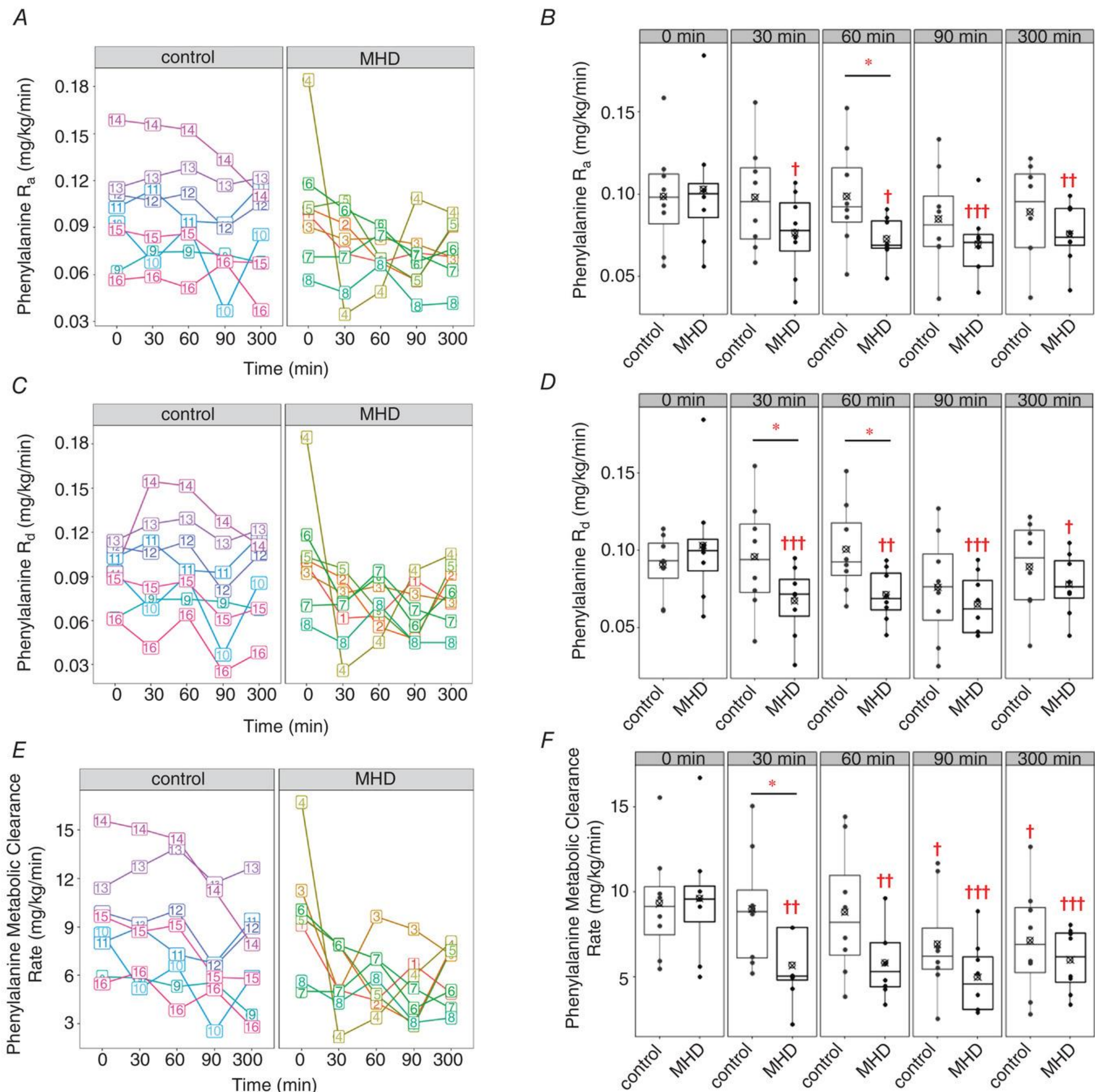

Figure 2. Whole-body phenylalanine kinetics at baseline and postprandial for both MHD and controls (MHD, n=8 & controls, n=8). Phenylalanine R_a (A-B), phenylalanine R_d (C-D) and phenylalanine Metabolic Clearance Rates (MCR) (E-F) at baseline, 30, 60, 90- and 300-minutes post meal ingestion. (A), (C) and (E) show the phenylalanine trend over time of each individual. (B), (D) and (F) show individual data points, quartiles and means. *Denotes significant difference between MHD and non-MHD (P<0.05). † Denotes significant differences from time 0. * P<0.05, ** P<0.01, *** P<0.001. † P<0.05, †† P<0.01, ††† P<0.001.

Human skeletal muscle analysis

We investigated total and phosphorylated states of proteins implicated in the integrin signaling cascade, as well as those involved in the insulin-dependent pathway in eight MHD patients and eight controls. From western blot analyses, ILK (**Figure 3A**) and PINCH1 protein content (**Figure 3B**), proxy indicators of integrin signaling activity, were significantly reduced in the MHD patients compared to the controls, in both fasted (P=0.0133 and P=0.0001, respectively) and post prandial (P=0.0001 and P=0.0001, respectively) state. $pFAK^{Tyr397}$/tFAK (**Figure 3D**) was also reduced in MHD; however, statistical analyses revealed a significant decrease in the fasted state (P=0.0026), but not in the post prandial (P=0.1164) state. The actin cytoskeleton regulating protein Rac1 was not different in MHD compared to control (P=0.5783 and P=0.3024 in the fasted and post prandial states, respectively, **Figure 3E**). Representative blots are shown in **Figure 3H**. No difference was noted for either Akt2 (P=0.8552 and P=0.2496 in the fasted and post prandial states, respectively, **Figure 4A**) or IP6K1 (P=0.4544 and P=0.2759 in the fasted and post prandial states, respectively, **Figure 4B**) between groups. Representative blots are shown in **Figure 4C**. Parvin (P=0.6778 fasted and P=0.2886 post prandial, **Figure 4C**), total FAK (P=0.3963 fasted and P=0.6549 post prandial, **Figure 4E**) and dystrophin (P=0.3091 fasted and P=0.437 post prandial, **Figure 4G**) were similar between groups. No bands were detected for phosphorylation targets at Akt^{S473} or Akt^{T308} in control or in MHD skeletal muscles (Data not shown). No significant differences were observed in protein content between fasted and post prandial state for ILK (P=0.1742 for MHD and P= 0.5391 for controls, **Figure 3A**), PINCH1 (P=0.5384 for MHD and P= 0.2276 for controls, **Figure 3B**), parvin (P=0.6804 for MHD and P= 0.4003 for controls, **Figure 3C**), $pFAK^{Tyr397}$(P=0.9352 for MHD and P= 0.2554 for controls, **Figure 3D**), dystrophin (P=0.1533 for MHD and P=0.1324 for controls, **Figure 3G**) or IP6K1 (P=0.7282 for MHD and P= 0.0783 for controls, **Figure 4B**), except for total FAK and Rac1, which resulted significantly decrease after meal ingestion in controls (P=0.0342, **Figure 3E** and P=0.033, **Figure 3F**, respectively), but not in the MHD (P=0.0551 and P=0.052, respectively), and total Akt2 that resulted reduced after meal in the MHD group (P=0.0051, **Figure 4A**), but not in the controls (P=0.0693).

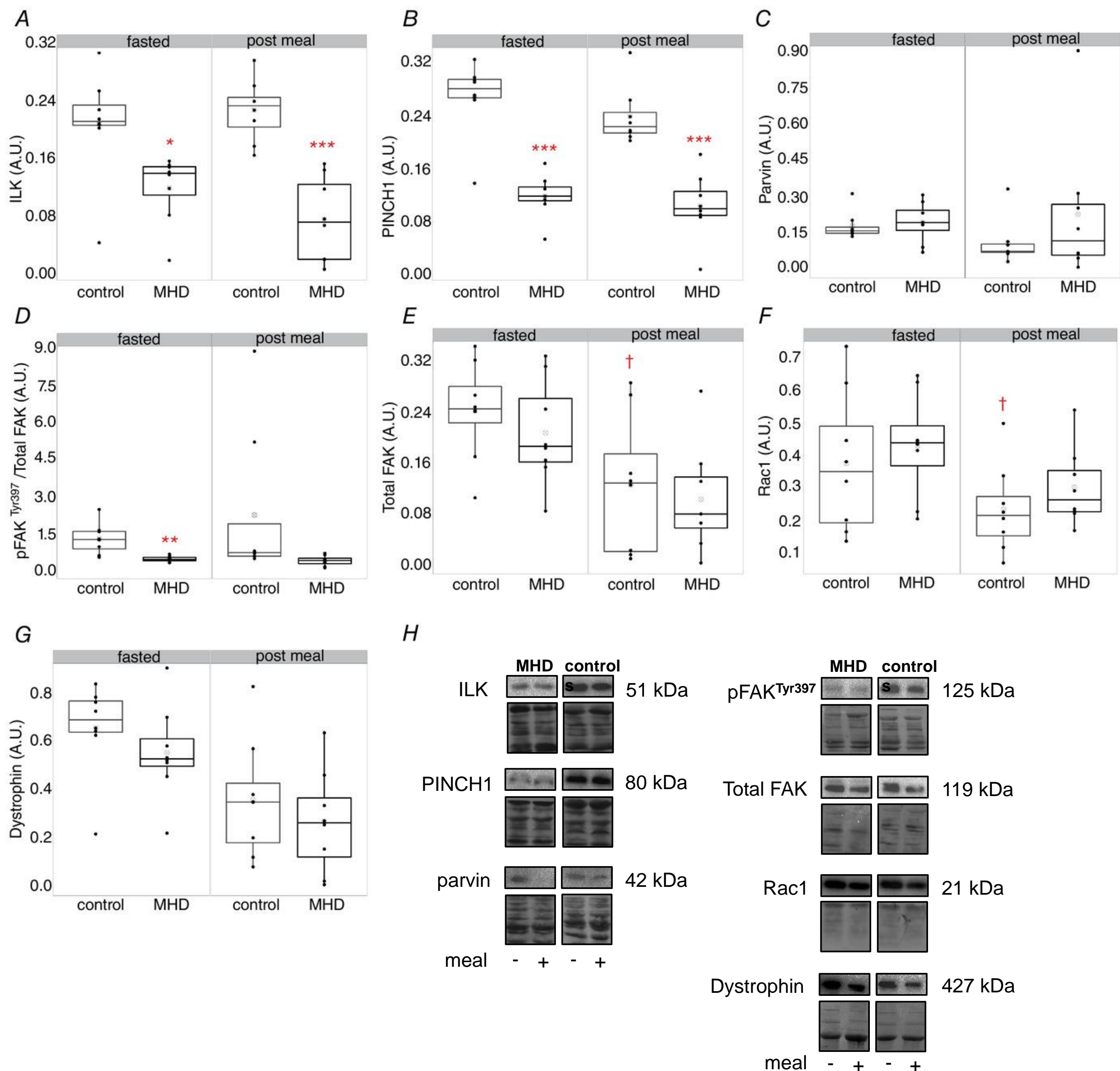

Figure 3. Skeletal muscle protein content for targets of interest in insulin resistant control and MHD patients. Content in the fasted state and after meal ingestion (MHD, n=8 & controls, n=8) of ILK (A), PINCH1 (B), Parvin (C), pFAK^{Tyr397}/Total FAK (D), Total FAK (E), Rac1 (F) and dystrophin (G). Representative western blots, including the corresponding Coomassie brilliant blue staining, of the proteins of interest (H). *Denotes significant difference between MHD and non-MHD. † Denotes significant differences between fasted and post meal within the same group. * P<0.05, ** P<0.01, *** P<0.001. † P<0.05, †† P<0.01, ††† P<0.001. Quartiles, means and individual data points are shown.

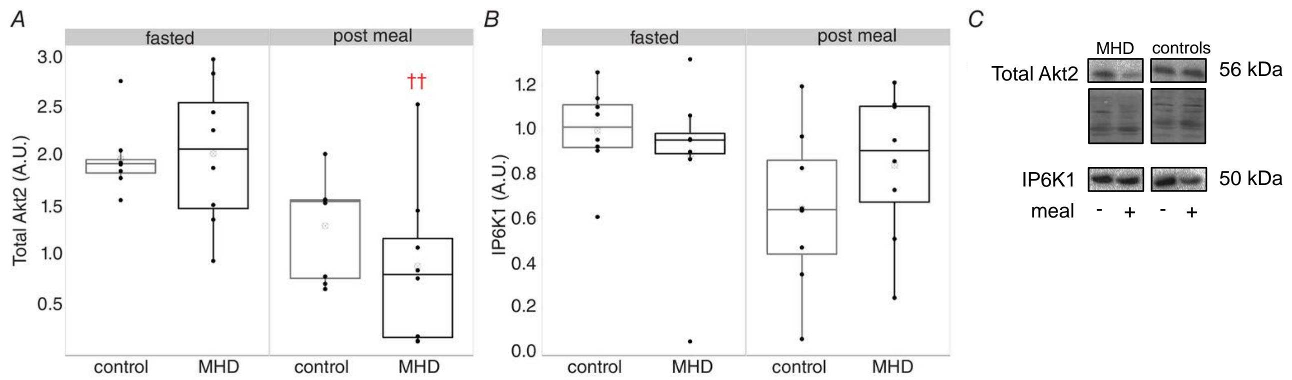

Figure 4. Skeletal muscle protein content for Akt2 (A) and IP6K1 (B) in the fasted state and after meal ingestion (MHD, n=8 & controls, n=8). Representative western blots of the proteins of interest, including the corresponding Coomassie brilliant blue staining, except for IP6K1 (C). No significant difference was noted (P>0.05). † Denotes significant differences between fasted and post meal within the same group. † P<0.05, †† P<0.01, ††† P<0.001. Quartiles, means and individual data points are shown.

Rac1 mKO mouse muscle analysis

In a separate experiment, skeletal muscle from nine muscle-specific Rac1 knockout (Rac1 mKO) and seven wild type were analyzed. Rac1 is a well-known regulator of the actin cytoskeleton (JeBailey *et al.*, 2007), but it is unknown if Rac1 regulates the protein expression of the integrin nexus and actin cytoskeleton proteins. Rac1 deletion did not alter the expression of integrin-associated proteins when compared with the wild type in skeletal muscle (ILK, P=0.774, **Figure 5A**; PINCH1, P=0.0589, **Figure 5B**; Parvin, P=0.0696, **Figure 5C**; pFAK^{Tyr397}, P=0.9629, **Figure 5D**; Total FAK, P=0.9784, **Figure 5E**) confirming the results obtained in the MHD human samples, where Rac1 protein expression was unaltered. Also, pAkt^{S473} (P=0.2952, **Figure 5G**), pAkt^{T308} (P=0.511, **Figure 5H**) and total Akt2 (P=0.857, **Figure 5I**) levels were not altered by the lack of Rac1 as also reported previously (Raun *et al.*, 2018). Furthermore, IP6K1 (P=0.3278, **Figure 5F**) and dystrophin

(P=0.2054, **Figure 5L**) expression was similar in both Rac1 mKO and wildtype. Blots in **Figures 5M and 5N** were cropped from the same membrane as shown in supplemental **Figure S2**.

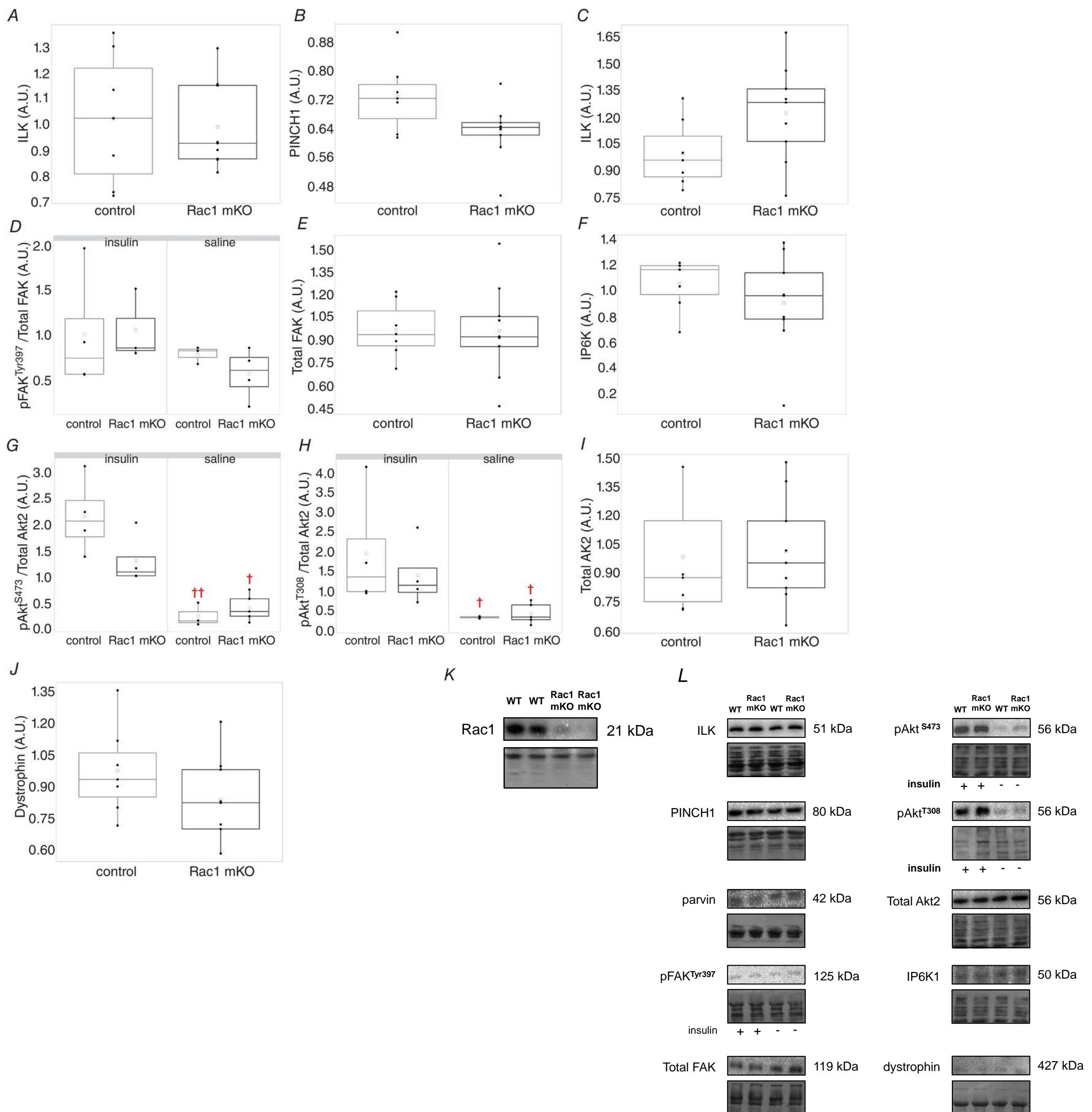

Figure 5. Skeletal muscle protein content of targets of interest in TA muscles of chow-fed WT (n=7) and Rac1 mKO mice (n=9). Insulin-stimulated and non insulin-stimulated (saline) samples were considered as separated groups for the phosphorylated proteins. Content of ILK (A), PINCH1 (B), parvin (C), pFAK ^{Tyr397}/Total FAK (D), Total FAK (E), IP6K1 (F), pAkt^{S473}/Total Akt2 (G), pAkt^{T308}/Total Akt2 (H), Total Akt2 (I) and dystrophin (L). Representative western blots of Rac1,

including the corresponding Coomassie brilliant blue staining, confirming effectiveness knock out (M). Representative blots, including the corresponding Coomassie brilliant blue staining (N). Analogous results were obtained in the *GA* mouse muscles. No significant difference was noted (P>0.05). † Denotes significant differences between saline and insulin stimulation within the same group. † P<0.05, †† P<0.01, ††† P<0.001. Quartiles, means and individual data points are shown.

Discussion

The current study investigated integrin-associated protein signaling in skeletal muscle of MHD patients. In addition, using a Rac1 KO insulin resistant rodent model, we also examined the potential role this important actin-cytoskeleton regulatory protein would play in upstream integrin-associated signaling in skeletal muscle. We hypothesized that integrin-associated protein expression would be reduced in both MHD patients and Rac1 KO rodents. Yet it was unclear from the literature whether loss of the integrin-associated protein structure would facilitate or hinder amino acid metabolism and / or insulin sensitivity (i.e. nutrient flux) in the MHD patients. Here we show, for the first time, that phenylalanine flux was reduced in the period immediately post meal ingestion (30 and 60 minutes) in MHD patients when compared to controls. In addition, we also noted a reduction in the integrin-associated protein expression (ILK and PINCH1) for the same comparison.

Evidence suggests that the over development of the ECM structure is linked to increased insulin resistance in mouse models (Kang *et al.*, 2011, 2013, 2014; Williams *et al.*, 2015), owing to a proposed increase in the physical barrier to hormonal and nutrient transport across the sarcolemma (Jansson, 2007; Williams *et al.*, 2015). In support of this, Williams *et al.* (Williams *et al.*, 2015) suggested that ECM protein accumulation within the interstitial space impedes substrate transport due to increased diffusion distance. Located directly downstream of the ECM structure, cytoplasmic integrin-associated proteins seem to have an important role as effectors in integrins signaling in

skeletal muscle, providing an essential linkage between the ECM, the sarcolemma and the actin-cytoskeleton (Gheyara *et al.*, 2007), therefore contributing to ECM structure stability. Integrin-deficient mice show progressive muscle dystrophy after birth (Mayer *et al.*, 1997) due to a decreased function of the myotendinous junction (MTJ), which gives structural stability between ECM and the associated myofibrils and their non-contractile structural proteins (Postel *et al.*, 2008). The central component of the IPP complex, ILK, is recruited to the MTJ, which requires the presence of the ECM protein laminin as well as integrin-α7 (itgα7) in the sarcolemma (Postel *et al.*, 2008), suggesting the involvement of ILK in ECM stability. In line with our hypothesis, our data showed a reduction in integrin-associated ILK and PINCH1, suggesting that MHD patients may present with reduced stability in the extracellular matrix owing to dysregulation of integrin-associated protein signaling.

Yet, the influence of a reduced integrin-associate protein nexus on nutrient uptake are unclear. The research suggests that the over development of the ECM and its transmembrane associated structures are linked to insulin resistance in mouse models (Kang *et al.*, 2011, 2013, 2014; Williams *et al.*, 2015). In support of this Williams et al. (Williams *et al.*, 2015) have shown that ECM protein accumulation within the interstitial space impedes substrate transport. Conversely, Kang et al. (Kang *et al.*, 2013) demonstrated that a reduction of ECM-associated glycosaminoglycan hyaluronan induced by intravenous injection of PEGylated recombinant hyaluronidase PH-20 (PEGPH20), led to a dose-dependent increase in glucose infusion rates and glucose clearance in mouse skeletal muscle during a hyperinsulinemic–euglycemic clamp. Taken together, these data may suggest that an increase in protein content within both the interstitial space and ECM is linked to insulin resistance and reduced nutrient transport, whereas a reduction in the protein content within these compartments may increase nutrient uptake owing to a reduction in the physical barrier between the ECM and the intracellular compartments. Therefore, we further postulated that a reduction in the integrin-associated nexus and a reduction in ECM structural protein content, may result in increased phenylalanine flux and improved glucose handling in MHD patients during the post-absorptive state (Williams *et al.*, 2015).

Total phenylalanine rate of appearance (R_a) is a measure of the appearance of dietary protein-derived phenylalanine and that from whole body protein breakdown into circulation (van Vliet *et al.*, 2018), whereas phenylalanine R_d and MCR reflect whole-body amino acid clearance and its subsequent utilization and storage to the different tissue compartments within the body (Matthews, 2007; Barclay *et al.*, 2020).

Here, we show that one-compartment models of Matsuda Index (ISI) and Disposition Index (DI) were similar between groups, while phenylalanine R_a, R_d and MCR rates decreased immediately after time 0 in MHD but not in controls, who maintained a more constant trend over time, suggesting that the hemodialysis group may suffer from decreased skeletal muscle amino acid uptake and utilization in the post-absorptive state. Moreover, phenylalanine R_a, R_d and MCR were also significantly lower in the period immediately post feeding (30 and 60 minutes) in the MHD group. Taken together, these results suggest that disruptions to the integrin-associate protein nexus may contribute to dysregulation in amino acid metabolism but not insulin sensitivity (data presented in **Figure 1**).

We have previously shown that MHD patients demonstrated an anabolic resistance of muscle protein synthesis rates after mixed meal ingestion, supporting the notion that MHD patients may present with dysregulation in amino acid flux and metabolism (van Vliet *et al.*, 2018). The findings from the current research suggests that this dysregulation in amino acid metabolism may be partly attributed to disruptions to the integrin-associate protein nexus in skeletal muscle. Decrease in the integrin-associated proteins may be explained by a separate finding that MHD patients display both increased basal myofibrillar protein synthesis and muscle caspase-3 protein content (van Vliet *et al.*, 2018). This evidence, together with increased basal muscle protein synthesis and degradation rates, may indicate that MHD muscle is over-stimulated and remains in a state of constant flux (van Vliet *et al.*, 2018). Yet it is not clear why phenylalanine flux is reduced in MHD patients given that amino acid requirements are likely to be increased, given the raised muscle protein synthesis and breakdown rates in this population. However, this raised turnover may be linked to an upregulation in the genes regulating the ubiquitin proteasome pathway (Hasselgren, 1999; Lecker *et al.*, 1999;

Mitch *et al.*, 1999; Bodine *et al.*, 2001; Ikemoto *et al.*, 2001; St-Amand *et al.*, 2001; Bey *et al.*, 2003; Stevenson *et al.*, 2003; Urso *et al.*, 2006). In addition, the reduction in phenylalanine flux after a meal, and thus proposed availability to cell demands, may partly explain why this population group presents with both reduced muscle mass (van Vliet *et al.*, 2018) and muscle atrophy (Johansen *et al.*, 2003). Interestingly, van Vliet *et al.* (van Vliet *et al.*, 2018) also showed that both basal and fed-state muscle LAT1 protein content are decreased in MHD patients. LAT1 is an amino acid transporter found in proximity of capillaries (Hodson *et al.*, 2018; van Vliet *et al.*, 2018). Blood flow and capillary recruitment play a fundamental role in nutrient and hormonal delivery to muscle (Mitch *et al.*, 1999; Williams *et al.*, 2015). Therefore, a decrease in this protein in MHD could be due to capillarity rarefaction in MHD skeletal muscles, which is linked to insulin resistance and poor nutrients perfusion (Mitch *et al.*, 1999; Solomon *et al.*, 2011; Bonner *et al.*, 2013; Williams *et al.*, 2015), a finding that may also have contributed to the reduced phenylalanine flux observed in our MHD sample.

Several studies suggest a role of the IPP complex in muscle degeneration (Gheyara *et al.*, 2007; Postel *et al.*, 2008). Postel et al. (Postel *et al.*, 2008) showed that zebrafish unable to express ILK developed mechanical instability in skeletal muscle. In addition, ILK-KO mice displayed progressive muscular dystrophy with ILK mutants showing displacement of FAK, dystrophin and α7β1D-integrin subunits (Gheyara *et al.*, 2007). These results are similar to those observed in mice and humans lacking α7-integrin subunit, suggesting that ILK may act as a cytoplasmic effector of α7β1-integrin in the pathogenesis of muscle degeneration (Gheyara *et al.*, 2007; Postel *et al.*, 2008). The adapter protein PINCH1 is known to bind with ILK and locates to integrin-mediated adhesion sites (Li *et al.*, 2005; Stanchi *et al.*, 2009; Karaköse *et al.*, 2015). Several studies suggest a crucial role of PINCH1 in promoting cell adhesion, ECM assembly, muscle attachment and Akt activity (Wu, 1999; Eke *et al.*, 2010; Vakaloglou & Zervas, 2012). Genetic ablation of PINCH1 in skeletal muscle results in cell death at embryonic stage in mice (Sakai *et al.*, 2003; Li *et al.*, 2005; Wickström *et al.*, 2010). Moreover, the interaction between ILK and PINCH1 is necessary to prevent degradation of IPP complex (Fukuda *et al.*, 2003; Li *et al.*, 2005), suggesting that defects in one of these proteins leads

to impairment of the associated kinases of the IPP complex, partly supported by our finding that showed significant reductions in the protein content of ILK and PINCH1 in MHD skeletal muscle.

The inositol hexakisphosphate (IP6) kinase1 (IP6K1) produces the diphosphoinositol pentakisphosphate (IP7), which competes with PIP3 to bind the pleckstrin homology (PH) domain of Akt, preventing Akt translocation to the cell membrane and its phosphorylation by PDK1, leading to decreased muscle insulin sensitivity (Naufahu *et al.*, 2018). IP6K1 can therefore represents a negative feedback mechanism in the insulin-dependent Akt pathway, which has been shown to be increased in insulin resistant models while Akt was reduced (Chakraborty *et al.*, 2010; Naufahu *et al.*, 2018).

We further hypothesized that disruptions to the integrin-actin cytoskeleton linkage, largely due to impairments in the Rho GTPase Rac1, would result in upstream dysregulation of integrin-associated protein and Akt signaling in skeletal muscle, showing a decrease in Akt activity and an increase in IP6K1 protein, which may then contribute to the reduced nutrient handling in MHD patients. In contrast, our results showed that Rac1 as well as Akt2 and IP6K1 were similar in MHD muscle compared to insulin-resistant matched controls despite differences in ILK and PINCH1.

These finding give rise two notions; ***1)*** Rac1 and Akt2 do not likely play a major role in the development of insulin resistance in MHD muscle, given that MHD and controls present with similar levels of insulin resistance and muscle protein content of Rac1 and Akt2 and ***2)*** Rac1 is not essential for upstream integrin-associated protein regulation. In a separate experiment, we used a Rac1 KO rodent model to investigate the role of this molecule on the integrin-associated protein signaling further. These experiments revealed that there was no difference in the IPP complex protein content in Rac1 mKO when compared to wild-type rodents. Rac1, together with Akt, are important regulators of insulin-stimulated glucose transport (JeBailey *et al.*, 2007; Ueda *et al.*, 2010; Sylow *et al.*, 2013*a*, 2014; Moller *et al.*, 2019). Several studies suggest that Akt2 and Rac1 bifurcate downstream of PI3K (Nozaki *et al.*, 2012; Satoh, 2014; Takenaka *et al.*, 2015) into two distinct parallel pathways, both promoting GLUT4 trafficking and muscle glucose uptake in an insulin-dependent manner (JeBailey *et al.*, 2007; Ueda *et al.*, 2008, 2010; Sylow *et al.*, 2014; Raun *et al.*, 2018; Moller *et al.*, 2019), which

is regulated via actin cytoskeleton organisation (Jaffe & Hall, 2005; Chiu *et al.*, 2011; Sit & Manser, 2011; Spiering & Hodgson, 2011; Moller *et al.*, 2019). Our data shows that Rac1 is not different between MHD and non-MHD individuals and that both display similar degrees of insulin sensitivity. In addition, our KO Rac1 mouse model showed no difference in integrin-associated proteins compared to the wild-type. Taken together, these data suggest that Rac1 may not be required for effective actin-integrin structural stability / remodeling or nutrient flux in MHD skeletal muscle. However, we cannot completely rule out a contribution of Rac1 in these processes. The authors recognize that whole-body phenylalanine kinetics and insulin sensitivity are not direct measures of local amino acid turnover or insulin action in skeletal muscle, yet it has been recognized that skeletal muscle is a major disposal site for amino acids (Chang & Goldberg, 1978) and glucose (Thiebaud *et al.*, 1982) in the postprandial state. We do acknowledge the limitations in our measurement and that the inclusion of multiple amino acid tracers and two-compartment models of glucose metabolism and insulin sensitivity would offer a more accurate measures nutrient kinetics in this population. We also acknowledge that we analyzed small sample size groups, therefore further research involving more participants is required to give more robustness to our findings.

Conclusions

In conclusion, our results suggest that neither insulin sensitivity nor DI seem to be different between MHD patients and to controls in the postprandial state. This finding may be explained by the similar levels of Rac1 and Atk2, two protein kinases implicated in insulin-dependent glucose uptake. Phenylalanine metabolism was lower within the MHD group which may be linked to disruptions to ILK-PINCH1 nexus and LAT1 (van Vliet *et al.*, 2018), suggesting for the first time that the integrin-associated protein network may be required for effective nutrient uptake in skeletal muscle.

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Chapter 3: ILK-Rac1 skeletal muscle signalling and glucose clearance rates are reduced in response to a high fat diet in mice

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Abstract

Background: Excessive extracellular matrix (ECM) and actin-cytoskeleton remodelling alongside reduced integrin signalling are associated with diet-induced insulin resistance and type 2 diabetes (T2D). We therefore hypothesise that a high-fat diet (HFD) would increase ECM-associated proteins while downregulating integrin-associated and Rac1 signalling in skeletal muscle. **Study Design**: C57BL / 6J female mice aged 14-18 weeks underwent a 23-week high fat diet (HFD) or standard (chow) diet. After the 23-week diet intervention, a 10-minute intravenous insulin or saline treatment with 2-deoxy-glucose (2DG) was administrated to assess insulin-stimulated glucose clearance. In addition, the mice were evenly (±2) and randomly allocated to exercise vs sedentary groups (Raun et al., 2018), to assess if prior exercise training alleviated any adverse effects on glucose metabolism induced by HFD. Exercise groups had free access to running wheels throughout the intervention period. Phosphorylation and total protein content were determined using immunoblotting with differences assessed using linear mixed-effect model analyses. **Results**: Under insulin stimulation, the HFD sedentary (HFDSed+Ins) rodents showed reduced 2DG metabolic clearance rate (MCR) following 10 minutes of intravenous insulin administration when compared to the chow-fed sedentary saline-stimulated (CHOWSed) group, which was associated with an increase in laminin and total Akt2 and a decrease in ILK and Rac1 in the HFDSed+Ins mice. No differences between groups were observed for PINCH1, parvin, integrin α2, or IP6K1. Exercise did not show any significant effect on glucose metabolism or protein expression. **Conclusions:** Our results suggest that a high-fat diet leads causes a decrease muscle glucose uptake, which was associated with an increase in the ECM protein laminin, but not integrin, and a decrease in the integrin-associated protein ILK, suggesting a

potential role for ILK in skeletal muscle insulin resistance. Moreover, the HFD decreased Rac1, but not Akt2, suggesting that diet mainly affects Rac1-regulated glucose uptake in skeletal muscle, rather than Akt signalling.

Abbreviations: **HFD** = high-fat diet; **DIO** = diet-induced obesity; **ECM** = extracellular matrix; **2DG** = 2-deoxy-glucose; **MCR** = metabolic clearance rate; $\mathbf{R_d}$ = glucose disappearance; **HFDSed** = HFD-fed sedentary saline stimulated; **HFDSed+Ins** = HFD-fed sedentary insulin stimulated; **HFDEx** = HFD-fed exercise saline stimulated; **HFDEx+Ins** = HFD-fed exercise insulin stimulated; **CHOWSed** = chow-fed sedentary saline-stimulated; **CHOWSed+Ins** = chow-fed sedentary insulin-stimulated; **CHOWEx** = chow-fed exercise saline-stimulated; **CHOWEx+Ins** = chow-fed exercise insulin-stimulated

Introduction

The extracellular matrix (ECM) is a highly dynamic structure consisting of a network of diverse proteoglycans and proteins that modulate biological processes, including cell migration, differentiation, development, and repair (Schuppan, 1990; Andez & Amenta, 1995; Williams et al., 2015), while providing structural support and tissue-specific response to disease and injury (Martinez-Huenchullan et al., 2017; Ahmad et al., 2018). The ECM communicates using transmembrane cell surface receptors known as integrins (Mayer, 2003). ECM remodelling and integrin signaling have been associated with pathological states (Williams et al., 2015; Lark et al., 2016; Kang et al., 2016; Ahmad et al., 2018). Studies in humans and rodents have demonstrated that diet-induced obesity (DIO) negatively affects ECM-associated proteins (Williams et al., 2015), with collagen proteins I, III, and IV, and integrin α2β1 known to increase several-fold in response to a high-fat diet (HFD) (Williams et al., 2015). These findings support the notion that an increase in the physical barrier at the cell membrane, brought on by an increase in protein expression, contributes to fat-induced insulin resistance in skeletal muscle (Richardson et al., 2005; Berria et al.,

2006; Kang et al., 2011, 2013, 2014; Wada et al., 2013; Dixon et al., 2013; Williams et al., 2015b; Huber et al., 2007; Inoue et al., 2013; Williams et al., 2015, Kang et al., 2016, Lark et al., 2016).

Integrin α2β1 interacts with laminin, an important ECM surface receptor (Ahmad et al., 2018, Yurchenco et al., 2018) which connects the ECM to the cytoplasmic cytoskeleton through the dystroglycan complex. Laminin and dystroglycan mutations are associated with several congenital muscular dystrophies (Yurchenco et al., 2018), which are often accompanied by progressive increases in intramuscular lipid storage (Tyler, 2003; Groh et al., 2009) and local insulin resistance (Groh et al., 2009). Moreover, laminin seems to contribute to ECM expansion in diabetes and diet-induced insulin resistance in renal (Mariappan et al., 2007) and adipose (Vaicik et al., 2014) tissue. One hypothesis that may explain the mechanisms surrounding ECM-associated insulin resistance in response to a HFD is that increased protein expression within the ECM creates a physical barrier preventing normal insulin action and glucose diffusion across the sarcolemma (Kang *et al.*, 2013; Williams *et al.*, 2015).

A stable and functional ECM linkage with the actin-cytoskeleton may be required for promoting effective glucose uptake in skeletal muscle (Williams et al., 2015). Forming a link between the ECM and the cytoplasm, integrins transduce signals across the plasma membrane and activate intracellular signaling, including several downstream kinases that are implicated in insulin-stimulated glucose uptake (Williams et al., 2015). The most central of the integrin-binding proteins is the integrin-linked kinase (ILK), which is part of the ILK-PINCH-parvin (IPP) complex that functions as one of the early steps of the integrin signaling cascade (Wu, 1999; Stanchi et al. 2009, Draicchio et al., 2020). ILK interacts with the β1-integrin cytoplasmic subunit as well as with numerous cytoskeleton-associated proteins, including actin (Wickström et al., 2010; Legate et al., 2009; Williams et al., 2017). ILK is therefore suggested as a major mediator in many critical cellular cascades, including insulin signaling (Delcommenne et al., 1998; Troussard et al., 2003; Williams et al, 2017).

Our previous research showed that disruptions to this integrin-associated linkage in skeletal muscle is associated with impaired amino acid metabolism in maintenance hemodialysis (MHD) patients, who present with muscle degeneration, obesity, and insulin resistance (Draicchio et al., 2020). Our results showed that MHD muscles had reduced phenylalanine metabolic clearance rates (MRC) and rate of disappearance (R_d) compared to insulin resistant non-MHD controls (Draicchio et al., 2020). This finding was accompanied by marked decreases in skeletal muscle content of ILK, PINCH1, and $pFAK^{Tyr397}$. Together, our findings suggest a key role of these integrin-associated proteins in amino acid metabolism in skeletal muscles under pathological conditions. Yet this work was limited in that no glucose tracer was used, preventing further analysis on the effects of disrupted ILK, PINCH1, and FAK signaling on glucose metabolism.

Therefore, the current data set was analysed to explore the role of the integrin-associated proteins in the development of diet-induced insulin resistance and reduced glucose clearance in a high-fat mouse model. We hypothesised that integrin-associated protein signalling would be reduced under HFD conditions, leading to a reduction in insulin-mediated glucose uptake.

Materials and Methods

All experiments were approved by the Danish Animal Experimental Inspectorate (License: 2016-15-0201-0 1043) (Raun et al., 2018).

Mouse models

The mouse model used in the current study has been previously described elsewhere (Raun et al. 2018). This study harvested the tissue from WT C57BL/6J female mice. The C57BL/6J mouse model is widely used in cancer, immunology and degenerative diseases. The C57BL/6J mouse is susceptible to diet-induced obesity, type 2 diabetes and atherosclerosis. A 23-week diet intervention was started in 58 C57BL/6J female mice aged 14-18 weeks (Raun et al. 2018). The mice received either a standard rodent chow diet (Altromin no. 1324; Brogaarden, Horsholm, Denmark) or a 60% high-fat diet (HFD) (no. D12492; Brogaarden, Denmark) and water ad libitum. Mice body weight was measured every week. Animals were group-housed at 20–21°C and maintained on a 12 h:12 h light-dark cycle. The mice were evenly (±2) and randomly allocated to insulin (Ins) vs saline, HFD vs chow (CHOW) and exercise (Ex) vs sedentary (Sed) groups (Raun et al., 2018). Ex groups had free access to running wheels throughout the intervention period.

Immunoblotting

Relative total muscle protein content and phosphorylation levels of proteins of interest were determined by standard immunoblotting techniques loading equal amounts of protein (20 µg). Primary antibodies were anti-Integrin linked ILK antibody (ab227154; Abcam, Cambridge, MA, USA), anti-PINCH1 antibody [EP1943Y] (ab76112; Abcam), α-parvin (D7F9) XP Rabbit mAb (8190S; Cell Signaling Technology, Beverly, MA, USA) (37), anti-Rac1 Antibody (ab155938; Abcam, Cambridge, MA, USA), recombinant anti-integrin alpha 2 antibody [EPR5788] (ab133557; Abcam, Cambridge, MA, USA), anti-Laminin 1+2 antibody (ab7463; Abcam, Cambridge, MA, USA), anti-total Akt2 (no. 3063; Cell Signaling Technology) (Raun *et al.* 2018), $pAkt^{S473}$ (no. 9271; Cell Signaling

Technology) (Karusheva *et al.* 2019), pAkt^{T308} (no. 9275; Cell Signaling Technology) (Raun *et al.* 2018), IP6K1 polyclonal antibody (12057-2-AP; Proteintech; Rosemont; USA).

Samples (20 µg total protein) was loaded to 7.5% precast polyacrylamide gel. Membranes were blocked in Tris-buffered saline-Tween 20 containing 2% skimmed milk or 5% BSA protein for 30–60 min at room temperature and then incubated with primary antibodies overnight at 4°C, followed by incubation with rabbit secondary antibodies conjugated to IRDye® for one hour at room temperature. After incubation membranes were imaged using Li–Cor Fe and quantified using ImageStudio Pro. Band normalization was quantified by measurement of total protein stain (Bio Rad, USA).

***In vivo* 2-deoxy-glucose experiments**

2-deoxy-glucose (2DG) uptake was determined in muscle by injecting [^{3}H]2DG (Perkin Elmer) retro-orbitally in a bolus of saline containing 66.7 µCi mL^{-1} [^{3}H]2DG corresponding to ∼9 µCi/mouse (6 µL g^{-1} body weight) in WT control mice (Raun et al., 2018). The injectate also contained insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) at 0.5 U kg^{-1} body weight (maximal insulin stimulation) for chow or HFD mice, respectively, or a comparable volume of saline.

Before stimulation, mice were fasted for 3–5 h from 07:00 h and anesthetized (intraperitoneal injection of 7.5/9.5 mg (chow/HFD) pentobarbital sodium 100 g^{-1} body weight) for 15 min. Immediately before insulin or saline injection and after 5 and 10 min blood samples were collected from the tail vein, and analysed for glucose concentration using a glucometer (Bayer Contour; Bayer, Münchenbuchsee, Switzerland). At 10 min, blood plasma samples were also analyzed for insulin and [^{3}H]2DG tracer activity. After 10 min the soleus muscles were excised and flash frozen (liquid nitrogen) before being stored at −80°C until further analyses.

2DG uptake analysis has been described previously (Fueger *et al.* 2004; Sylow *et al.* 2016, Raun *et al.*, 2018); tissue-specific 2-DG-6-phosphate accumulation was measured by precipitation of phosphorylated 2DG using 0.1 m $Ba(OH)_2$ and 0.1 m ZnSO4. The activity of total tissue 2-[^{3}H]DG

tracer in 2-DG-6-phosphate was divided by the area under the curve of the mean specific activity at 10 min and 20 min and, for glucose, at 0, 10, and 20 min. ^{3}H labelled 2DG tracer was relative to muscle weight and the time to obtain the tissue-specific 2DG uptake (µmol g^{-1} h^{-1}), whereas calculation of percentage increase by exercise was obtained by adjusting the exercise-stimulated values to the basal control values for the specific genotype group (Sylow et al., 2016).

Statistical analysis

R software (R Foundation for Statistical Computing, Viena, Austria) and the Nlme and Emmeans packages were used to perform linear mixed-effect model analyses of the relationship between glucose, or protein values, and stimulation (insulin [Ins] and saline), diet (chow [CHOW] and HFD), and activity (sedentary [Sed] and exercise [Ex]).

Glucose or protein values were entered as dependent (response) variables, whereas stimulation, diet, and activity as independent (explanatory) variables. If the null hypothesis was rejected, post hoc tests were performed to determine which groups differed from each other. $P < 0.05$ was considered statistically significant.

Results

2DG Metabolic Clearance (MCR) and Disappearance Rates (R_d)

Blood glucose and plasma insulin in C57BL/6J mice have been previously described by Raun et al. (2018). Plasma insulin was analysed in mice on a chow or 60% HFD to investigate if lean and obese C57BL/6J mice show similar plasma insulin concentration under the same insulin dose. After 18 weeks of HFD, mice displayed an average of 9 g body weight increase. A glucose tolerance test at week 17 showed reduced glucose tolerance in HFD mice compared to chow littermates (Raun et al., 2018). Mice were anesthetized 15 minutes prior stimulation. The *in vivo* stimulation of saline, with or without 0.5 U kg^{-1} insulin was injected in the retro-orbital (r.o.) vein (Figure 1A). At 5 minutes post insulin injection, chow-fed mice showed a drop in blood glucose concentrations of 2 mM, whereas the HFD-fed group did not (Figure 1A). After 10 minutes of insulin injection, blood glucose was lowered by 4mM in chow-fed mice and by 2 mM in the HFD group (Raun et al., 2018). Plasma insulin concentration was increased 3-fold at 10 minutes post r.o. injection of 0.5 U kg^{-1} insulin in the chow-fed group. The same 0.5 U kg^{-1} insulin increased plasma insulin concentration by 7-fold in the HFD groups (Raun et al., 2018)

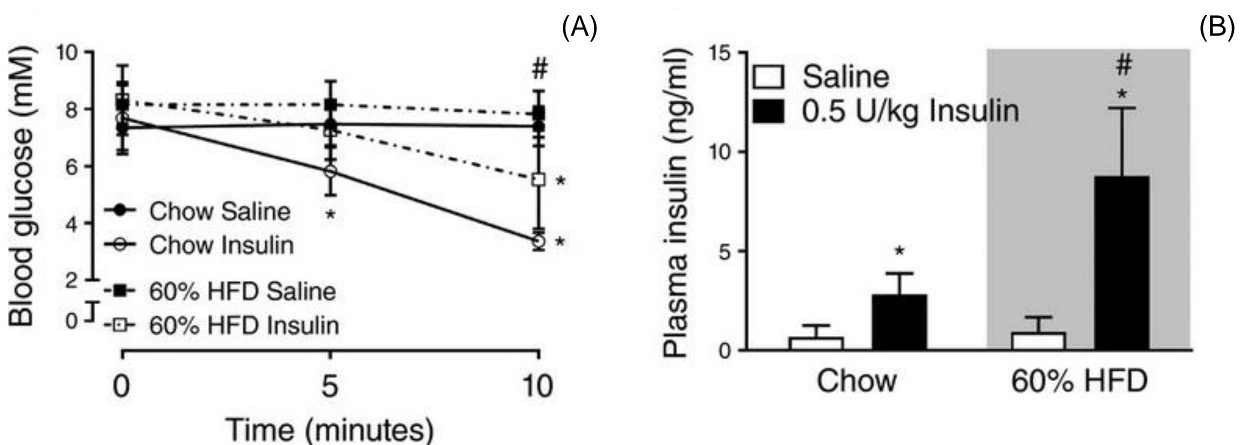
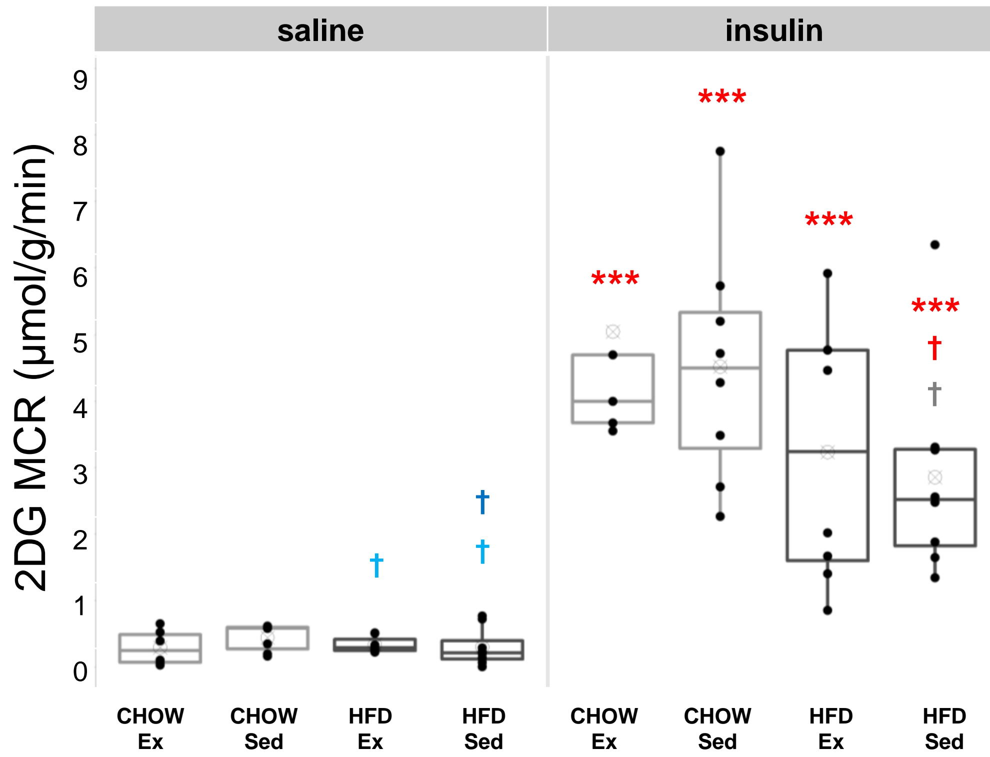


Figure 1. Stimulation protocol for *in vivo* analysis insulin stimulated glucose uptake following retro-orbital (r.o.) insulin injection (0.5 U kg^{-1}). Shaded area indicates HFD. * Denotes significant effect of insulin stimulation (P < 0.001). # Denotes significant effect of diet (P < 0.001) (Raun et al., 2018).

2DG MCR and glucose disappearance rates (R_d) were determined in C57BL/6J female mice from both groups. Both 2DG MCR (Figure 2A) and R_d (Figure 2B) were significantly increased in insulin-stimulated mice compared to rodents exposed to the sham saline infusion ($P < 0.0001$). Interestingly, although HFD did not seem to affect R_d values ($P=0.2055$), mice under HFD showed decreased 2DG clearance values when compared to the chow-fed groups ($P=0.0214$), suggesting that HFD reduces glucose metabolism. Specifically, under insulin stimulation, the HFDSed+Ins rodents showed a significant decrease in glucose MCR, compared to the CHOWSed ($P < 0.0001$) and CHOWEx ($P = 0.0003$) mice under saline injection (Figure 2A). On the other hand, the HFDEx+Ins mice did not show reduced 2DG MCR under insulin stimulation, compared to HFDSed+Ins ($P=0.6298$), suggesting that exercise may protect against the adverse effects of an HFD.

(A)



(B)

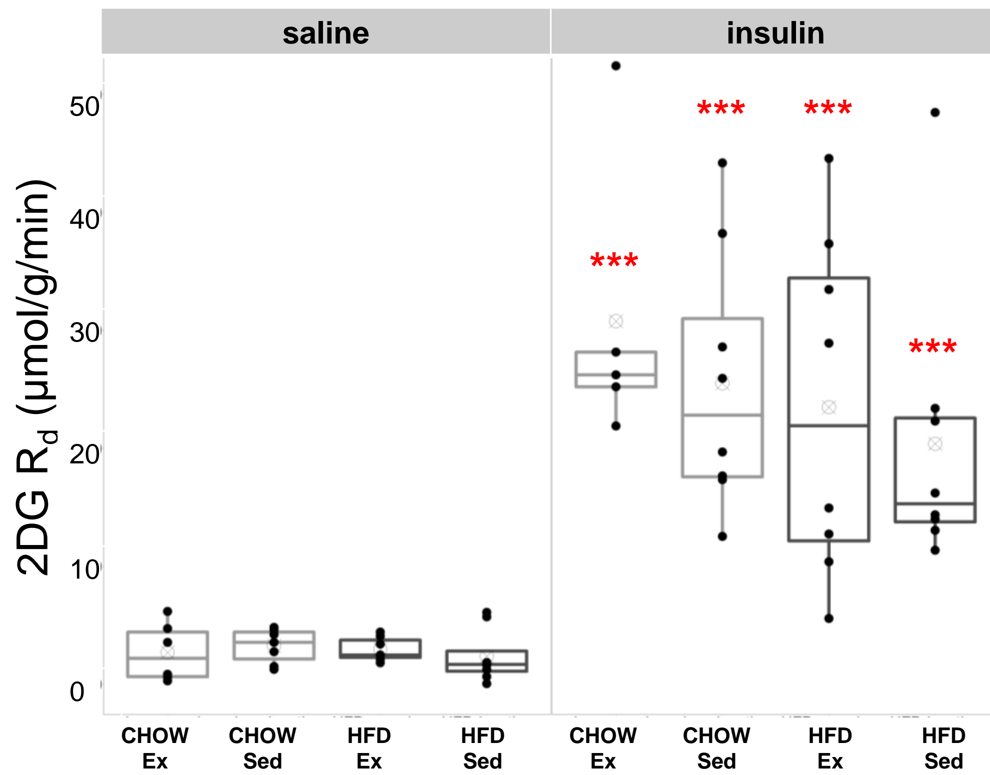


Figure 2. Glucose Clearance and uptake in the C57BL/6J female mice (n=58). 2DG MCR (A) and R_d (B) after 10 minutes of insulin injection. ***Significant differences between saline and insulin groups (P<0.001). † Significant differences from a CHOWEx group (P<0.05). † Significant differences from a CHOWSed group (P<0.05). † Significant differences from a CHOWSed+Ins group (P<0.05). † Significant differences from a CHOWEx+Ins group (P<0.05). Linear mixed-effect models were performed for statistical analysis.

Mouse Muscle Analyses

Proteins associated with the ECM, integrin, and insulin-stimulated signaling were determined in soleus muscle extracted from 58 C57BL/6J female mice. From the western blot analyses, the intravenous stimulation of saline or insulin was the main factor affecting ILK protein content (**Figure 3A**); ILK was reduced in CHOWSed+Ins group compared to CHOWSed mice (P=0.0325), as well as in HFDSed+Ins mice compared to HFDSed subjects (P=0320).

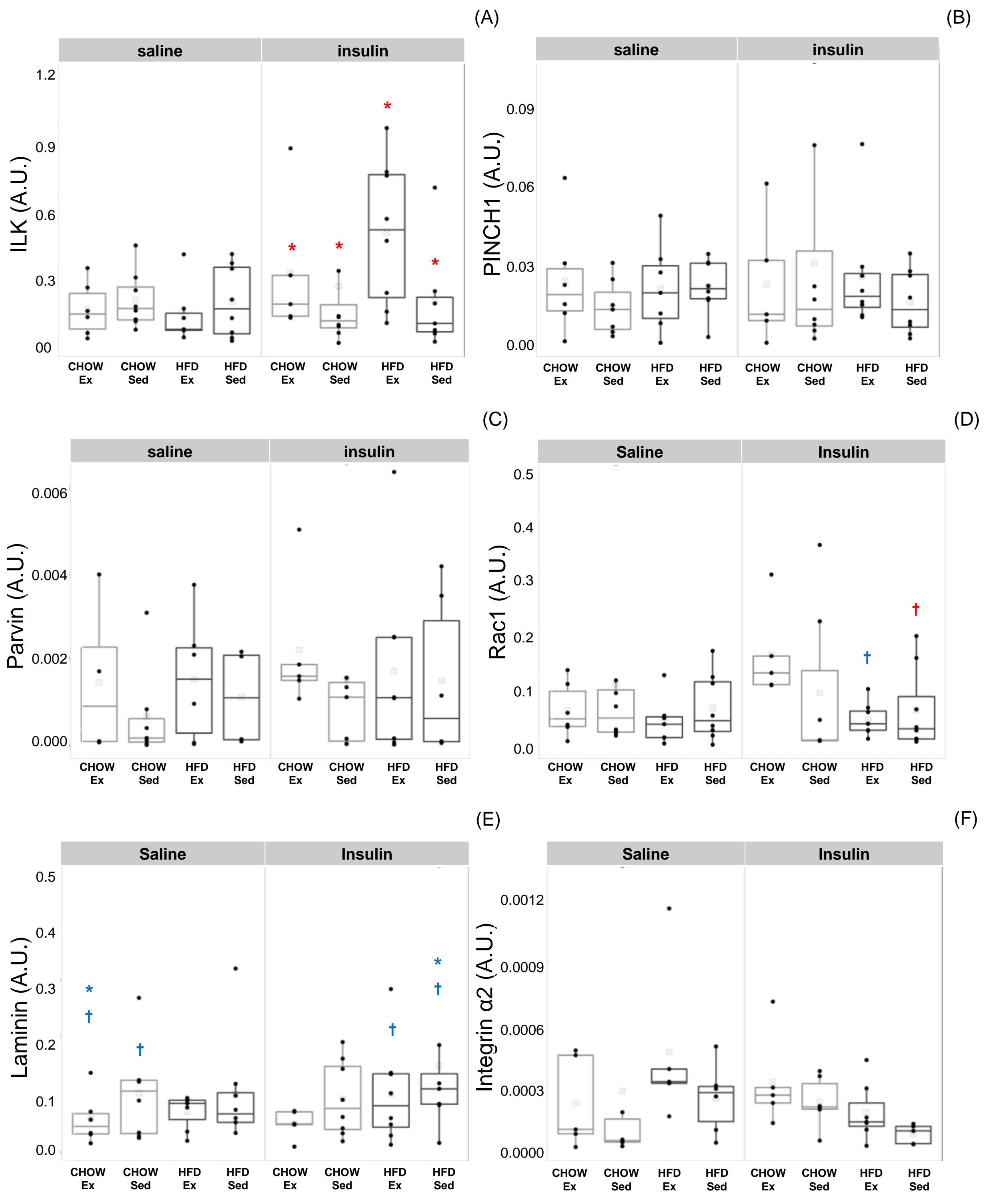
Interestingly, ILK content was increased in HFDEx+Ins compared to HFDEx (P=0.0330) as well as in CHOWEx+Ins compared to CHOWEx rodents (P=0.0325) (**Figure 3A**).

Rac1 content (**Figure 3D**) was reduced in HFDSed+Ins vs CHOWSed+Ins group (P= 0.0468) and in HFDEx+Ins vs CHOWEx+Ins group (P=0.0466). Diet or training status did not affect Rac1 in groups under saline stimulation (P>0.05).

Laminin was affected by exercise (**Figure 3E**). Laminin protein content was highest in HFDSed+Ins mice, and lowest in CHOWEx subjects (P = 0.0247). In the HFDSEd+Ins mice there was a significant decrease in laminin content compared to CHOWEx+Ins mice (P = 0.0319), while in HFDSed there was a decrease in laminin compared to CHOWEx mice (P = 0.0318).

Independent of exercise and diet, $pAkt^{S473}$ (**Figure 3G**) was significantly higher under insulin stimulation compared to saline-stimulated mice (P=0.0147), whereas $pAkt^{T308}$ (**Figure 3H**) had a tendency to increase under insulin stimulation (P=0.0768). Total Akt protein content seem to be affected by diet (**Figure 2I**), with overall higher levels in HFD mice compared to chow-fed littermates.

More specifically, total Akt was significantly higher in HFDEx subjects compared to CHOWEx mice (P=0.0248) and in HFDEx+Ins mice compared to CHOWEx+Ins groups (P=0.0238). Moreover, the HFDSed and HFDSed+Ins showed increased Akt levels than CHOWSed and CHOWSed+Ins mice (P=0.0248 and P=0.0240, respectively). No differences between groups were observed for PINCH1 (**Figure 3B**), parvin (**Figure 3C**), integrin α2 (**Figure 3F**), or IP6K1 (**Figure 3L**).


(A)
saline
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ILK (A.U.)
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(B)
PINCH1 (A.U.)
0.09
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0.00
(C)
Parvin (A.U.)
0.006
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(D)
Saline
Insulin
Rac1 (A.U.)
0.5
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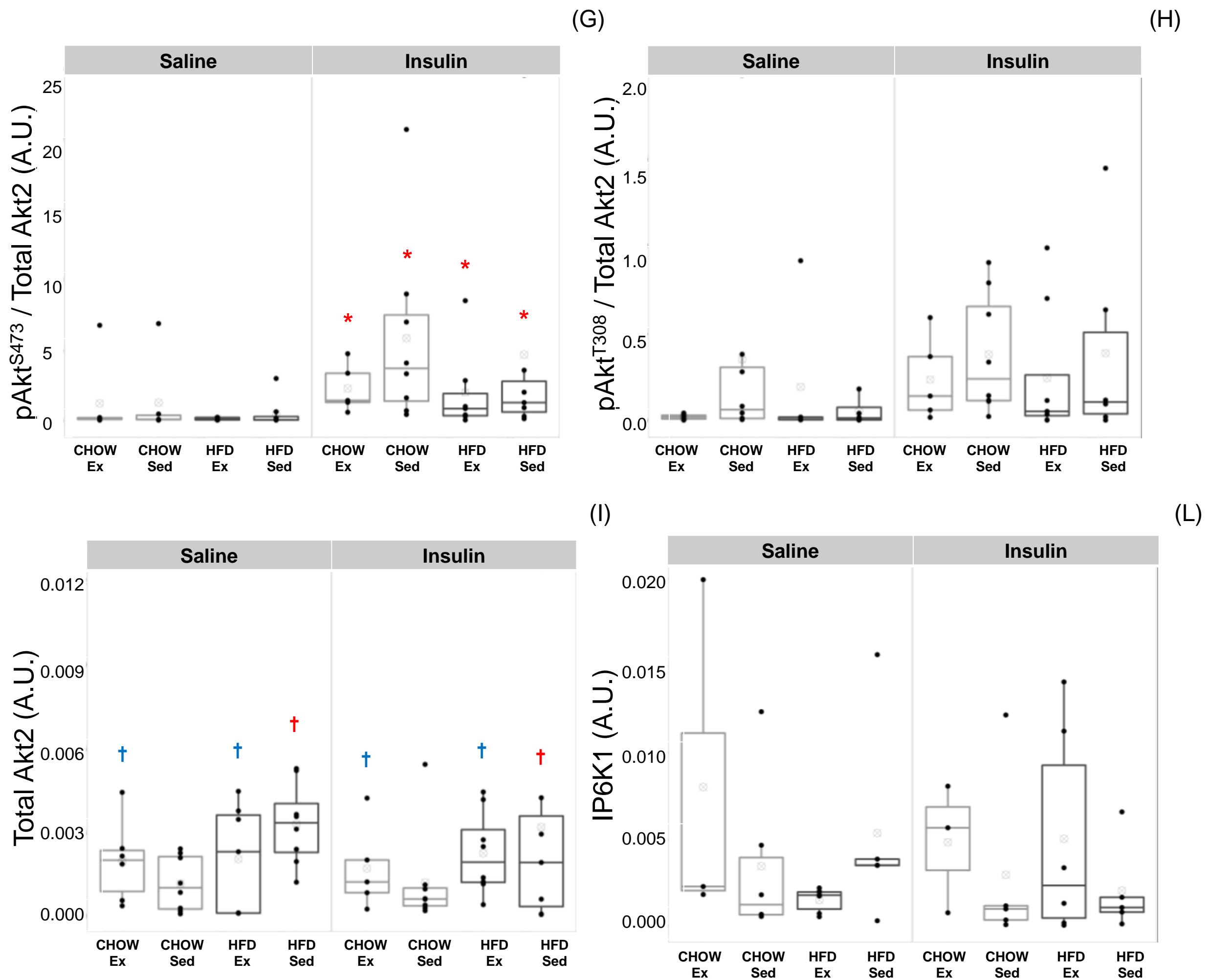
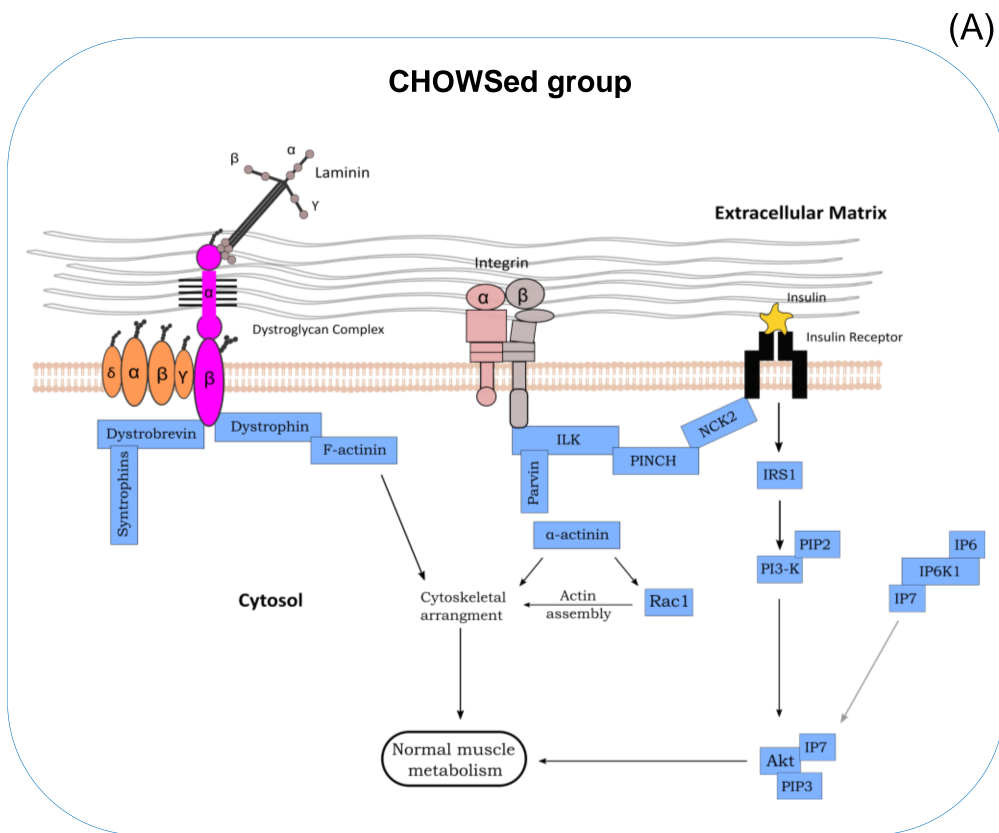


Figure 3. Skeletal muscle protein content in the C57BL/6J female mice (n=58). Content of ILK (A), PINCH1 (B), Parvin (C), Rac1 (D), Laminin (E), Integrin α2 (F), pAkt^{S473}/total Akt2 (*G*), pAkt^{T308}/total Akt2 (*H*), total Akt2 (*I*) and IP6K1 (L) in mice groups under different stimulation (saline and insulin [Ins]), diet (chow [CHOW] and HFD) and activity levels (sedentary [Sed] and exercise [Ex]). *Significant differences between saline and insulin groups. † Significant differences from the CHOWEx group. † Significant differences from the CHOWEx+Ins group (P<0.05). † Significant differences from the CHOWSed+Ins group (P<0.05). † Significant differences from the CHOWSed group (P<0.05). Linear mixed-effect models were performed for statistical analysis.

Discussion

This study investigated key ECM-associated and the integrin-associated proteins in mouse skeletal muscle following a 23 week HFD. We hypothesised that under HFD, there would be an expansion in ECM-associated proteins, whereas the integrin-associated proteins would be downregulated, contributing to diet-induced insulin resistance and reduced whole-body glucose metabolism. We also hypothesised that exercise would offer protection against the adverse effects of a HFD, partially repristinating the normal levels of the ECM and integrin protein signalling in active subjects, improving glucose clearance. Collectively our data show that the HFDSed+Ins rodents showed reduced glucose clearance following 10 minutes of intravenous insulin administration when compared to the CHOW saline-stimulated groups (Figure 2A), which was associated with an increase in laminin and total Akt2 and a decrease in ILK and Rac1 (Figure 4B). This suggests that impairments in glucose uptake, in diet-induced insulin resistant sedentary mice, may in part be caused by an increased ECM-associated proteins content (i.e. laminin) and a decrease in the integrin-associated ILK and Rac1.

However, the approach used in our study has limitations, as we related the whole-body glucose clearance's measures to skeletal muscle specific proteins, without considering other tissue types. However, our results are relevant as skeletal muscle accounts for most (80%) of the glucose disposal in the postprandial state, making it the key tissue is post eating glucose storage (DeFronzo & Tripathy, 2009).



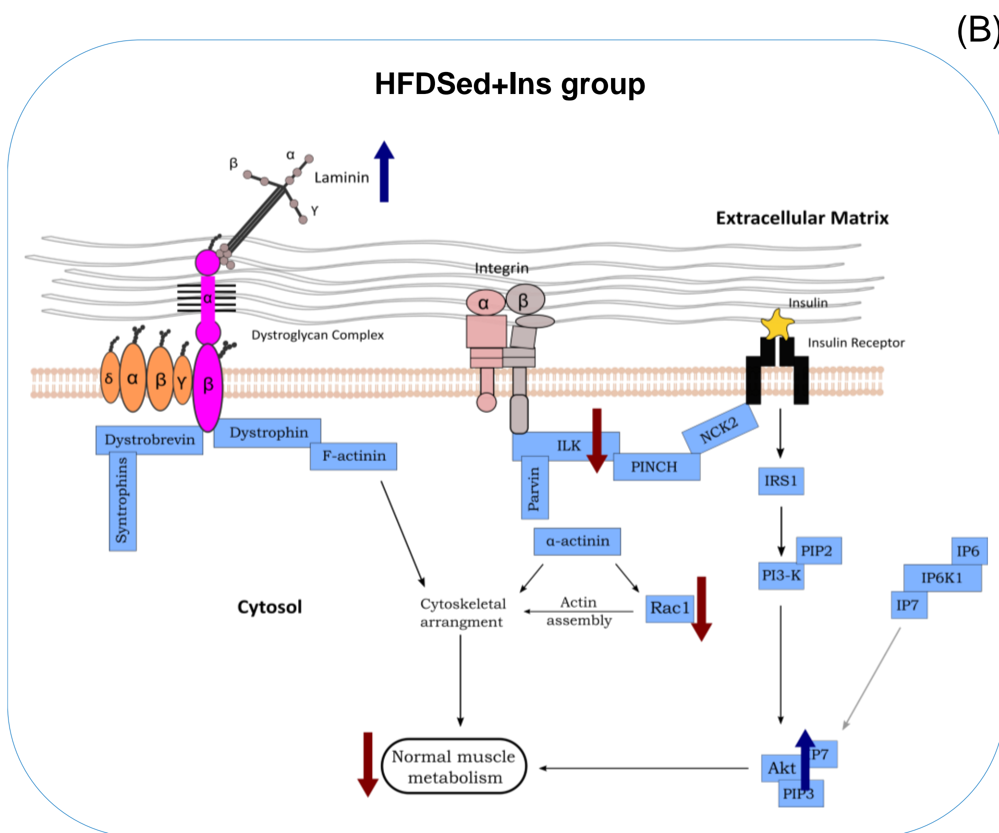


Figure 4. Protein profile in the CHOWSed (A) compared to the HFDSed+Ins (B). (A) Under normal muscle metabolism, the two main ECM surface receptors integrin and laminin-dystroglycan

complexes connect the ECM to the cytoplasmic cytoskeleton, both promoting cytoskeleton rearrangements, which preserve muscle structural stability and promote normal glucose uptake in skeletal muscle (Draicchio et al., 2022). The downstream integrin substrates include the ILK-PINCH1-parvin (IPP) complex, Akt and Rac1, while the laminin-dystroglycan complex mainly involves the downstream effectors dystrophin and F-actinin (Draicchio et al., 2022). (B) Under reduced muscle metabolism, the integrin-associated proteins ILK and Rac1 are downregulated, while laminin content is increased in response to insulin stimulation.

Under stress conditions, integrin α2β1 is of particular interest as it reveals an antiangiogenic and profibrotic nature, promoting reactive oxygen species (ROS) production and upregulated collagen expression (Langholz *et al.*, 1995; Kang *et al.*, 2011). In mouse skeletal muscle, deletion of integrin α2β1 leads to cell proliferation and angiogenesis (Zhang *et al.*, 2008; Kang *et al.*, 2011). Moreover, ECM expansion and increased expression of integrin α2β1 have been found to contribute to HF diet-induced insulin resistance in skeletal muscle (Kang et al., 2011, Kang et al., 2013, Kang, Mayes, et al., 2013, Kang et al., 2016). Laminin α2 binds to dystroglycan and interacts with integrin α2β1 (Ahmad et al., 2018, Yurchenco et al., 2018) and α7β1 (Ibraghimov-Beskrovnaya et al., 1992, Talts et al., 1999, von der Mark et al., 2002, Gawlik & Durbeej, 2011, Menezes de Oliveira et al., 2014). It is established that both dystroglycan and integrin pathways are fundamental for normal muscle metabolism and function, therefore laminin, like integrins, may have a role in maintaining muscle structural integrity (Han et al., 2009, Menezes de Oliveira et al., 2014). Laminin is a mosaic protein that has several key roles in proper basement membrane assembly and function (Holmberg & Durbeej, 2013). In congenital muscular dystrophy (MDC1A), laminin α2 is severely reduced or absent, and both dystroglycan and α7β1 are dysregulated (Vachon et al., 1997, Gawlik et al., 2006, Moll et al., 2001, Menezes de Oliveira et al., 2014), suggesting that the structural link between these proteins is hindered, due to interruption to the dystroglycan-cytoskeleton pathway. Laminin α2 chain–deficient muscle fibers undergo degeneration–regeneration cycles with progressive replacement of muscle tissue with connective tissue (Muntoni & Voit, 2004, Gawlik et al., 2011, Menezes de Oliveira

et al., 2014), leading to severe muscle wasting. Since muscle is fundemetnal tissue in relation to glucose metabolism and storage, its degeneration could cause impaired glucose metabolism and insulin resistance (Cerf, 2013).

Before analysis of our data, we hypothesised that both integrin and laminin would have been upregulated in response to a HFD, contributing to ECM expansion and reduced glucose metabolism. In line with our hypothesis, laminin was increased in sedentary groups under HFD; however, we did not observe any integrin dysregulation in the HFD associated groups, suggesting that laminin, but not integrins, may play a role in the development of diet-induced insulin resistance in C57BL/6J female mice. Moreover, our data showed a reduction in integrin-associated ILK in the HFDSed+Ins group, which presented reduced glucose MCR, suggesting that ILK may also contribute to diet-induced insulin resistance leading to decreased glucose uptake.

ILK is recruited to the myotendinous junction (MTJ), a process that requires the presence of both integrin α7 and laminin in the sarcolemma (Postel *et al.* 2008), suggesting the involvement of ILK in ECM stability. At a metabolic level, Kang and coworkers observed that muscle-specific ILK-deficient (ILK$^{lox/lox}$HSAcre) mice following 16 weeks of HFD showed higher glucose infusion and glucose disappearance (R_d) rates compared to HF-fed WT mice, implying improved insulin-mediated glucose uptake in the ILK deficient mice (Kang et al., 2016). Kang et al.' results suggest that ILK deficiency ameliorates insulin sensitivity and glucose metabolism. However, our findings disagree with Kang et al. (2016) since reduced ILK in our samples worsened, not improved, glucose uptake under diet-induced insulin resistance, as shown by higher insulin responses to IV administration in the HFDSed+Ins group suggesting reduced insulin sensitivity. More in line with our findings, Hatem-Vaquero et al. (2017) showed that conditional ILK knockdown (cKD-ILK) mice developed hyperglycaemia and hyperinsulinemia, suggesting insulin resistance, as confirmed by indexes of insulin resistance (homeostasis model assessment of insulin resistance [HOMA-IR] and quantitative insulin sensitivity check index [QUICKI) (Hatem-Vaquero et al., 2017). However, the cKD-ILK mice

used in Hatem-Vaquero et al. study were chow-fed and not under HFD or other pathological conditions (Hatem-Vaquero et al., 2017). Despite different findings and different models used, our data combined with the other findings (Kang et al., 2016; Hatem-Vaquero et al., 2017) may suggest ILK contribution to diet-induced insulin resistance and in maintaining normal glucose metabolism in skeletal muscles. Differences in the results of studies most likely occur due to different models used as well as different interventions (e.g. differences in diet, pathological status and / or activity level) and skeletal muscle examined, which have different type of fibers that may respond differently to stimuli. For example, there are fiber type-specific differences in exercise and HFD effects on insulin-stimulated glucose uptake in rat skeletal muscles (Pataky et al., 2019).

Although several ILKs functions in the physiological and pathological spaces are presently known (Gorska & Mazur, 2022), further studies will be needed to investigate the role of ILK in muscle glucose metabolism and weather a decrease in ILK exacerbates or improves muscle glucose uptake. However, we suggest that ILK downregulation contributed to impaired glucose metabolism, as seen in ILK deficient zebrafish with muscle degeneration (Postel et al., 2008) and mouse models (Gheyara et al., 2007), given that skeletal muscles is responsible for ~80% of whole-body glucose uptake (DeFronzo & Tripathy, 2009). Gheyara et al. (2007) reported that ILK KO mice developed severe muscular dystrophy at the myofascial junctions (MFJs) along with displacement of focal adhesion-related proteins, such as FAK, dystrophin, α7β1D-integrin subunits, and cytoskeleton restructuring (Gheyara et al., 2007, Draicchio et al., 2020). These results were similar to those observed in mice and humans lacking the α7-integrin subunit that develop severe muscle atrophy (Gawlik & Durbeej, 2015; Milner & Kaufman, 2007; Rooney et al., 2006; Liu et al., 2008). In addition, our previous study (Draicchio et al., 2020) showed a substantial reduction of ILK protein content in MHD skeletal muscle, a population that presents with both accelerated muscle degeneration (van Vliet et al., 2018), increased rates of muscle breakdown (Deger et al., (017) and insulin resistance (Draicchio et al., 2020). Collectively, these results suggest the mechanisms through which ILK may contribute to insulin resistance and glucose metabolism impairments.

Glucose uptake and GLUT4 translocation partially require dynamic changes in the actin cytoskeleton (JeBailey et al., 2007). The disruption of actin filament remodelling with cytochalasin D and latrunculin B inhibit GLUT4 translocation, leading to impaired glucose uptake in muscle (JeBailey et al., 2007). Here we hypothesised that Rac1, a key protein involved in actin remodelling would be dysregulated in response to a HFD resulting in impaired insulin-mediated glucose transport. In line with our hypothesis, the HFDSed mice presented with reduced glucose R_d and lower in Rac1 protein levels. A finding supported in work elsewhere (JeBailey et al., 2007; Ueda et al., 2008; Sylow et al., 2013a; Raun et al., 2018).

Raun et al. (2018) examined the effects of muscle-specific Rac1 ablation on insulin-stimulated glucose uptake and muscle metabolism in the context of a HFD. These data showed that insulin injection reduced blood glucose in chow-fed mice by 50%, and only 35% in mice fed a HFD after 10 minutes. Moreover, Rac1 mKO mice, under HFD, had higher blood glucose concentrations after 5 and 10 min of insulin injection, compared to the HFD WT group, due to a reduced insulin response in Rac1 KO (Raun et al., 2018). These results indicate that HFD Rac1 KO mice displayed reduced insulin stimulated glucose transport (Raun et al., 2018). Furthermore, insulin-stimulated glucose uptake in the chow-fed Rac1 mKO group was decreased by 15%, 45% and 25% in gastrocnemius, triceps and soleus, respectively, compared to control (Raun et al., 2018). Of relevance, HFD mice displayed reduced insulin-stimulated glucose uptake in all analysed muscles, which was significantly lower in all skeletal muscle of the HFD Rac1 mKO group compared to WT, except for soleus (Raun et al., 2018), most likely because in this muscle Rac1 was ablated by only 50%, while in the other muscles Rac1 ablation reached 85%.

Our results showed that while a HF diet reduced Rac1 protein content, the same intervention had no such effect on Akt phosphorylation, and it was associated with an increase in total Akt2 in the insulin-stimulated sedentary mice. Similarly, Raun et al. (2018) observed normal Akt phosphorylation during insulin stimulation in mouse skeletal muscle following HFD. Specifically, p-

Akt^{Thr308} and p-Akt^{Ser473} were equally stimulated by insulin in gastrocnemius muscle of WT and Rac1 mKO mice with no effect of diet. However, in contrast with our observations, in Raun et al.'s study, a HFD induced a reduction in Akt2 by ~30% (Raun et al., 2018). Despite the discrepancies in total Akt2 abundancy, in both our and Raun's studies Akt phosphorylation, which reflects the levels of insulin action in skeletal muscle, was unaffected, suggesting that Rac1 signalling and HFD act on glucose uptake independently of Akt signalling in muscle. In addition, JeBailey et al. (2007) investigated Rac1 contribution to insulin-dependent actin remodelling and GLUT4 translocation by silencing Rac1 expression using siRNA oligonucleotides. Their findings showed that insulin-dependent actin remodelling was markedly prevented, and this was associated with 48% inhibition in insulin-stimulated GLUT4 translocation, compared to control. Moreover, Rac1 knockdown did not alter Akt phosphorylation, suggesting that Rac1 signalling and its consequent acting remodelling are independent of Akt pathway (JeBailey et al., 2007).

Collectively, these studies suggest that, in the absence of Rac1, Akt-dependent pathway may compensate Rac1's absence and mediate insulin-stimulated glucose uptake in skeletal muscle (Raun et al., 2018). Our results, where we observed a reduction in Rac1 but not in Akt2 in our HF-fed, inactive mice, are therefore in line with previous studies and further support the hypothesis that HFD affects Rac1 signalling, leading to a decrease in insulin-stimulated glucose uptake regulated by Rac1 and actin cytoskeleton, rather than Akt, in skeletal muscles.

Conclusion

In conclusion, our results suggest that sedentary mice under HFD display reduced muscle glucose clearance, suggesting that high fat diets and sedentary behaviours impair glucose metabolism. Moreover, these mouse models show an increase in laminin, but not in integrin, suggesting that there is a partial expansion in the ECM-associated proteins that may contribute to reduced glucose uptake in skeletal muscle. Furthermore, the integrin-associated protein, ILK was

reduced in insulin-stimulated mice on a HFD, indicating a potential role of ILK in muscle glucose metabolism, in line with our previous research (Draicchio et al., 2020). Rac1, but not Akt, was reduced under HFD, indicating that diet impairs insulin-stimulated glucose uptake, partly, via by Rac1 signalling, which is independent of Akt signalling.

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General discussion

The maintenance of skeletal muscle's structural integrity appears to be dependent on an intact actin cytoskeleton and proteins associated with integrins. However, the effects of a diminished integrin-associated protein network on nutrient uptake remains ambiguous. Several studies indicate a connection between insulin resistance in mouse models and the overgrowth of the extracellular matrix (ECM) along with its associated transmembrane structures (Kang et al., 2011, 2013, 2014; Williams, Kang, & Wasserman, 2015). Supporting this notion, Williams et al. demonstrated that the accumulation of ECM proteins within the interstitial space impedes substrate transport (Williams, Kang, & Wasserman, 2015).

In Chapter 2, we delved into the investigation of integrin-associated protein signaling and Rac1, an essential protein in the actin cytoskeleton pathway, in the skeletal muscles of MHD patients (Draicchio et al., 2020). Prior literature did not provide a clear understanding of whether a loss in the integrin-associated protein structure would impede or facilitate amino acid metabolism and insulin-mediated glucose transport (i.e., nutrient flux) in these patients. We hypothesized that the expression levels of integrin-associated proteins (ILK, PINCH1, parvin, FAK) and Rac1 would be diminished in MHD patients (Chapter 2) as well as Rac1 KO rodents (Chapter 3). Our findings unveiled, for the first time, a reduction in phenylalanine flux during the initial period following protein intake (30 and 60 minutes) in MHD patients, compared to insulin-resistant non-MHD control volunteers. This was in line with the observed decrease in the expression of integrin-associated proteins (ILK, PINCH1, and $pFAK^{Tyr397}$), supporting our hypothesis.

The decreased signaling of ILK, PINCH1, and pFAKTyr397 implies that MHD patients might experience a loss in ECM stability due to the dysregulation of integrin-associated protein signaling. While one-compartment models of the Matsuda Index (ISI) and Disposition Index (DI) did not exhibit

any difference in insulin resistance between MHD patients and controls, phenylalanine Ra, Rd, and MCRs were found to decrease right after time 0 in MHD patients, but not in controls. This insinuates that MHD patients may experience a decline in skeletal muscle amino acid uptake in the post-absorptive state. Moreover, phenylalanine Ra, Rd, and MCR were significantly lower in MHD patients during the immediate period following feeding (30 and 60 minutes). In summary, our findings suggest that disturbances in the integrin-associated protein network could potentially lead to amino acid metabolism dysregulation, but not necessarily insulin resistance.

Further, we hypothesized that the interruptions in the integrin-actin cytoskeleton linkage might partly stem from impairments in the Rho GTPase Rac1, a pivotal agent in actin-cytoskeleton remodeling. Such Rac1 impairments might induce an upstream dysregulation of integrin-associated protein and Akt signaling in skeletal muscle, resulting in decreased Akt activity and increased IP6K1 protein, thereby contributing to diminished nutrient handling in MHD patients. It is noteworthy that IP6K1 synthesizes diphosphoinositol pentakisphosphate (IP7), which competes with PIP3 to bind the pleckstrin homology (PH) domain of Akt, thereby hindering Akt translocation to the cell membrane and its phosphorylation by PDK1, ultimately leading to reduced muscle insulin sensitivity (Naufahu et al., 2018). Thus, IP6K1 can act as a negative feedback mechanism in the insulin-dependent Akt pathway, which has been found to increase in insulin-resistant models while Akt levels decrease (Chakraborty et al., 2010; Naufahu et al., 2018). However, our findings revealed comparable levels of Rac1, Akt2, and IP6K1 protein content in MHD muscles despite reductions in ILK and PINCH1. This suggests that these proteins might not significantly contribute to the impairment of muscle amino acid metabolism in MHD, and Rac1 may not be a key factor in the upstream regulation of integrin-associated proteins. This conclusion was further reinforced by our observations in the Rac1 KO rodent model, which exhibited no changes in the IPP complex or FAK content in Rac1 mKO as compared to wild-type rodents (Chapter 2). Hence, Rac1 may not be necessary for efficient actin-integrin structural stability/remodeling or amino acid flux in MHD skeletal muscle.

Collectively, our research suggests that integrin-associated proteins might have a role in amino acid metabolism within skeletal muscles under pathological conditions (Draicchio et al., 2020). Nonetheless, this study had a limitation as no glucose tracer was employed, hence restricting further exploration of the effects of disrupted ILK, PINCH1, and FAK signaling on glucose clearance in skeletal muscle.

We subsequently sought to delve deeper (in Chapter 3) into the study of integrin-associated proteins and the principal ECM receptors, namely integrin and laminin, within the skeletal muscles of mouse models subjected to a high-fat diet (HFD). We hypothesized that there would be an increase in ECM-associated proteins, whereas critical integrin-associated proteins would be downregulated. This could contribute to diet-induced insulin resistance and a decrease in whole-body glucose metabolism. Additionally, we proposed that exercise could mitigate the adverse effects of HFD, partially re-establishing normal ECM and integrin protein signaling in physically active subjects, thereby enhancing glucose clearance. Our findings demonstrated that insulin-stimulated, sedentary mice on a HFD (HFDSed+Ins) exhibited diminished glucose clearance following ten minutes of intravenous insulin administration, compared to the CHOW saline-stimulated groups. This reduction in glucose clearance was linked with an increase in laminin and total Akt, and a decrease in ILK and Rac1. This suggests that an increase in ECM-associated protein content such as laminin, and a decrease in ILK and Rac1 protein content, may partially account for impairments in glucose uptake in diet-induced insulin-resistant, sedentary mice.

Our hypothesis postulated that both integrin and laminin would be upregulated under HFD, resulting in ECM expansion and diminished glucose MCR. Our data indicate that laminin, but not integrin, might play a role in the development of diet-induced insulin-mediated glucose transport in female C57BL/6J mice. Moreover, the observed reduction in integrin-associated protein ILK in the HFDSed+Ins group suggested that ILK may also contribute to diet-induced reductions in insulin-stimulated glucose uptake. As glucose uptake and GLUT4 translocation partially depend on changes

in actin cytoskeleton dynamics (JeBailey et al., 2007; Kanzaki & Pessin, 2001), we also hypothesized that Rac1, via actin remodeling regulation, would contribute to glucose uptake in skeletal muscle and would also be dysregulated under HFD. Consistent with our hypothesis, the HFDSed mice displaying reduced glucose Rd also demonstrated reductions in Rac1 protein content. Although a HFD decreased Rac1 protein content, it did not impact Akt phosphorylation and increased total Akt2 in the HFDSed+Ins group. Thus, our results suggest that HFD disrupts Rac1 signaling, leading to a decrease in insulin-stimulated glucose uptake regulated by Rac1 and actin cytoskeleton, rather than Akt, in skeletal muscle.

However, our study is not without limitations. Given that both the MHD patients and the control group were administered a primed constant infusion of stable isotope L-[ring-$_2H^5$]phenylalanine, we acknowledge that measuring a single amino acid tracer and its kinetics provides only an estimate of whole-body amino acid metabolism. Therefore, multiple essential amino acid tracers could have been employed in the MHD population for a more accurate measure of whole-body nutrient kinetics. Primed constant infusion of stable isotope-labeled amino acids has been routinely used to study whole-body and muscle metabolism in vivo in exercise and nutrition research (Phillips et al., 2011; Hirsch et al., 2022; Kim et al., 2016; Rennie et al., 1994; van Vliet et al., 2016; Watt et al., 1991). This method offers various advantages, including the use of labeled amino acids at true tracer doses (i.e., without metabolic disturbance) (Rennie et al., 1994), quick data collection (usually within 90 minutes from the start of infusion), and rapid tracer responses sensitive to perturbations associated with acute exercise or meal ingestion (Hirsch et al., 2022; Wolfe et al., 2021). Thus, the use of stable isotope-labeled amino acid infusion was an effective approach to determine the acute physiological response to meal intervention in the MHD patients, measuring the dietary protein-derived amino acids' entrance into circulation after the observed 5-hour postprandial phase.

While the primed constant amino acid infusion approach proves effective, it also presents certain limitations. For instance, its administration is restricted to a limited timeframe, meaning that findings

from acute scenarios may not consistently mirror chronic conditions (van Vliet et al., 2018; Wilkinson et al., 2017). In the case of MHD patients, L-[ring-$_2H^5$]phenylalanine flux measurements spanned a 5-hour postprandial period, rendering it impossible to ascertain whether the impaired amino acid kinetics in MHD patients persisted beyond the measured interval. However, executing extended studies in this field is challenging due to the invasive nature of primed constant amino acid infusion methods (Wilkinson et al., 2017). This necessitates the involvement of several trained personnel (e.g., nurses, researchers, pharmacists) to administer the tracers (Hirsch et al., 2022; Wilkinson et al., 2017), further limiting its practical application as it cannot be conducted outside of a lab or hospital setting. Moreover, the associated costs of formulating and testing tracers render this method both expensive and logistically demanding (Hirsch et al., 2022).

In Chapter 2, L-[ring-$_2H^5$]phenylalanine was utilized to ascertain nutrient metabolism in MHD muscle. The employment of a single, established primed essential amino acid tracer simplifies these studies and proves less costly than using multiple amino acid tracers. Phenylalanine exhibits particular characteristics that make it a useful marker of protein metabolism. For one, it is an essential amino acid, meaning that in the postabsorptive state, no amino acids enter from dietary sources; thus, phenylalanine flux in the body originates solely from the phenylalanine released from protein breakdown, balanced by phenylalanine removal through protein synthesis and metabolic disposal via conversion to tyrosine, providing an accurate representation of whole-body protein breakdown (Matthews 2007).

We connected whole-body phenylalanine flux to muscle nutrient flux in the MHD patients. Phenylalanine tracer has been consistently used to measure protein metabolism in tissues that do not hydroxylate this amino acid, such as muscle (Matthews, 2007). This indicates that phenylalanine is not subject to secondary metabolism within the muscle tissue (Biolo et al., 1992, 1994, 1995; Wilkinson et al., 2017), which could affect measurements of muscle protein kinetics. Since it is neither synthesized nor metabolized by muscle, its appearance (R_a) and disappearance (R_d) serve

as accurate representations of protein degradation and synthesis, respectively (Lim et al., 2005). Thus, our findings are significant as phenylalanine metabolism facilitated the determination of nutrient kinetics in the skeletal muscle of MHD patients, with phenylalanine appearance (R_a) indicating protein degradation and phenylalanine disappearance (R_d) signifying protein synthesis in the MHD muscle. Although one could argue that the whole-body phenylalanine kinetics measured in our study (Chapter 2) are not direct measures of local amino acid turnover in muscle, skeletal muscle is a tissue that significantly contributes to overall metabolic health (Garlick, 1969; Wilkinson, 2017). Ikizler et al. (2002) explored the effect of haemodialysis (HD) on whole-body protein kinetics. Their findings showed that HD resulted in a 10% whole-body increase and a 133% muscle forearm increase in protein proteolysis. Simultaneously, while whole-body protein synthesis remained unchanged, forearm muscle synthesis increased by 120%. The cumulative results comprised net whole-body protein loss (96%) and net forearm protein loss (164%) (Ikizler et al., 2002). Taken together, these data suggest that the imbalance in protein metabolism was significantly larger for local muscle than for whole-body measurements, underscoring the considerable contribution of skeletal muscle to overall protein metabolism. Therefore, our results in MHD patients, which tie whole-body amino acid flux to muscle amino acid metabolism, are both relevant and a representative portrayal of metabolic imbalances in MHD skeletal muscle.

An additional limitation to the experimental chapters in this thesis is that these studies connected whole-body glucose clearance with proteins of interest specifically expressed in skeletal muscle. Consequently, they did not entirely consider other tissue types. Even though our findings remain pertinent, as skeletal muscle is responsible for ~80% of glucose disposal in the postprandial state (DeFronzo & Tripathy, 2009), assessing ECM-associated and integrin-associated proteins in insulin-sensitive tissues (i.e., adipose and liver) would have provided a more comprehensive understanding of the effects of integrin, laminin, ILK, PINCH1, and Rac1 on whole-body glucose metabolism.

Obesity is typified by the expansion of adipose tissue (AT), a condition necessitating extracellular matrix (ECM) remodelling and reorganisation to accommodate adipocyte hypertrophy and adipogenesis (Ruiz-Ojeda et al., 2019; Schoettl et al., 2018). This process triggers immune cell infiltration, fibrosis (characterized by an excessive deposition of ECM components such as collagen, elastin, and fibronectin), and inflammation. This inflammation, in turn, induces local hypoxia and insulin resistance (Williams, Kang, & Wasserman, 2015; Ruiz-Ojeda et al., 2019). Furthermore, the increase in collagen deposition presents a physical impediment to adipocyte expansion during obesity, thus encouraging lipid infiltration into other tissues like liver and skeletal muscle (Williams, Kang, & Wasserman, 2015; Ruiz-Ojeda et al., 2019). In a study by Sun et al. (2014), the deletion of collagen VI in ob/ob mice led to enhanced glucose tolerance and insulin signalling in AT due to unhindered adipocyte expansion. A surge in collagen proteins in AT has been observed in obese mice on a high-fat diet (HFD) (Huber et al., 2007; Ruiz-Ojeda et al., 2019; Williams, Kang, & Wasserman, 2015) and obese humans (Pasarica et al., 2009; Ruiz-Ojeda et al., 2019), leading to diet-induced insulin resistance.

Like skeletal muscle and AT, the liver is a vital organ for whole-body insulin sensitivity, serving as a major site for insulin clearance and glucose metabolism (Williams et al., 2017; Williams, Kang, & Wasserman, 2015). Type 2 Diabetes (T2D) in humans is associated with hepatic ECM remodelling (Harrison, 2006; Jaskiewicz et al., 2008; Williams, Kang, & Wasserman, 2015), with T2D patients displaying increased immunostaining for hepatic collagen IV, alpha-smooth muscle actin (a-SMA), and laminin protein content (Harrison, 2006; Jaskiewicz et al., 2007).

It has been suggested that integrins β2 modulate glucose homeostasis under HFD, predominantly through actions on skeletal muscle and AT (Meakin et al., 2015). Although the role of integrins in AT is still somewhat unclear, some studies suggest their significant role in AT (Ruiz-Ojeda et al., 2019). For instance, Gao et al. (2019) demonstrated that adipose-specific loss of kindlin-2, which facilitates integrin activation, incites lipodystrophy and induces glucose intolerance and peripheral insulin resistance in kindlin-2 knockout (KO) mice fed with a 60% HFD for up to 18 weeks compared to wild

type (WT) mice on the same diet. This deregulation of glucose homeostasis in HFD KO mice was connected with increased inflammation of AT, which augmented the level of circulating lipids, leading to insulin resistance (Gao et al., 2019; Softic et al., 2016; Xia et al., 2017). Moreover, these circulating lipids also contributed to hepatic steatosis in the kindlin-2 KO mice, exacerbated by HFD, as depicted by the histology of liver sections (Gao et al., 2019). In aggregate, these findings suggest that maintaining appropriate AT mass is essential for glucose metabolism and insulin sensitivity, and that the kindlin-2/integrin pathway may play a critical role in preserving AT mass and, consequently, glucose metabolism.

In the liver, integrin α1β1 has been proposed to defend against the development of hepatic insulin resistance (Dixon et al., 2013; Kang et al., 2011; Williams, Kang, & Wasserman, 2015). Additionally, Williams and colleagues revealed that α1 integrin expression is heightened in hepatocytes isolated from mice on HFD compared to those on chow diet (Dixon et al., 2013; Williams, Kang, & Wasserman, 2015). Therefore, while our results (chapter 3) suggest that skeletal muscle integrin does not appear to influence whole-body glucose metabolism in the HFD Sedentary + Insulin (HFDSed+Ins) mice, a more comprehensive analysis on integrin expression in the adipose tissue and liver is required to understand the full impact of integrins on the impairment of whole-body glucose metabolism and diet-induced insulin resistance observed in our HFD, insulin-stimulated mice.

Shifting to the downstream effectors of integrins, in the AT, Focal Adhesion Kinase (FAK) phosphorylation is diminished in HFD mouse models (Williams, Kang, Zheng, et al., 2015). Additionally, FAK governs insulin sensitivity by regulating adipocyte survival (Luk et al., 2017; Ruiz-Ojeda et al., 2019), and it has been implicated in the regulation of insulin action and glucose homeostasis in the liver (Bisht et al., 2008; Cheung et al., 2000; Huang et al., 2006; Williams, Kang, & Wasserman, 2015). For instance, siRNA-mediated knockdown of FAK results in hyperinsulinemia and hyperglycaemia in chow-fed mice (Bisht et al., 2008). This indicates that a reduction in integrin

signalling through FAK may foster the development of hepatic insulin resistance (Williams, Kang, & Wasserman, 2015).

The AT Integrin-Linked Kinase (ILK) interfaces with the integrin cytoplasmic domains and several cytoskeleton-associated proteins (Ruiz-Ojeda et al., 2019). Bugler-Lamb and colleagues (2021) scrutinized the role of ILK in adipose function and insulin resistance in adipocyte-specific ILK-deficient mice (ILK$^{lox/lox}$*AdCre*). Remarkably, AT ILK deficiency improved glucose tolerance, reduced fat mass in the HF-fed ILK$^{lox/lox}$*AdCre*, and exhibited improved insulin resistance in AT during a hyperinsulinemic-euglycemic clamp (Bugler-Lamb et al., 2021). Notably, AT insulin sensitivity in lean mice remained unaffected by ILK deletion. Bugler-Lamb et al.'s (2021) results propose that adipocyte ILK plays a significant role in modulating HFD-mediated insulin resistance in AT.

Liver ILK expression is augmented in rodent models of liver fibrosis (Toole, 2004; Williams et al., 2017), and it has been shown to be implicated in regulating matrix-induced hepatocyte differentiation *in vitro* and *in vivo* (Dasu et al., 2010; Williams et al., 2017). Williams et al. (Williams et al., 2017) investigated in hepatocyte-specific ILK deletion (ILK$^{lox/lox}$Alb*cre*) mice whether the biomechanical link of ILK to insulin signalling contributes to hepatic metabolic dysregulation under HFD. Interestingly, ILK expression decreased while PINCH and α-parvin expression increased in hepatocytes isolated from HFD mice (Williams et al., 2017), suggesting that hepatic insulin action might be modulated by ILK downregulation in combination with PINCH and α-parvin upregulation under HFD, rather than solely ILK downregulation (Williams et al., 2017). Furthermore, no difference was observed in the glucose infusion rate (GIR), glucose R_d, or glucose R_a during the insulin clamp between the chow-fed ILK-deficient mice and the control group. In contrast, the GIR was reduced in HFD ILK$^{lox/lox}$Alb*cre* mice by 50% compared to the controls (Williams et al., 2017). Collectively, these data suggest a major contribution of ILK to hepatic insulin resistance in the HF-fed state, with potential contribution from PINCH and α-parvin as well.

In our research, muscle ILK, PINCH1, and FAK signalling were decreased in insulin-resistant MHD skeletal muscle, which were associated with reduced amino acid flux (chapter 2). Additionally, ILK was also reduced in skeletal muscle of diet-induced insulin-resistant mice, which exhibited decreased glucose clearance (chapter 3). Thus, our findings, along with the studies discussed above, suggest that a fully functional pathway of integrin-associated proteins, especially ILK signalling, might be critical to maintain healthy whole-body glucose metabolism in mouse models. However, further investigation in our HFDSed+Ins group will be needed to discern which tissue and its nexus of integrin-associated proteins contribute most to the development of impaired insulin-mediated glucose metabolism in the HFD state.

In sum, current evidence indicates a prominent role of integrins and the actin cytoskeleton in regulating insulin-stimulated glucose metabolism in muscle tissue. This association draws upon the involvement of the ECM-associated integrin network, which is likely necessary for all stimulatory factors to efficiently trigger glucose transport in skeletal muscle. The process of ECM remodelling is observed in both mouse and human models of obesity, insulin resistance, and type 2 diabetes, conditions that are partly associated with heightened systemic inflammation and resultant pro-fibrotic mechanisms. The role of ECM in obesity-induced insulin resistance can be rationalized by its contribution to increased collagen formation – a process which effectively creates a physical obstruction to insulin diffusion and glucose transport. Furthermore, ECM remodelling triggers distinct alterations in integrin signalling within the matrix itself, a phenomenon known to impede insulin and nutrient uptake in skeletal muscle.

Based on our research findings, we propose several crucial points:

1. Whole-body phenylalanine metabolism was found to be diminished within the MHD group, correlating with perturbations to ILK-PINCH1 and FAK signalling. This provides novel

evidence suggesting that the integrin-associated protein network may play a role for efficient amino acid uptake in skeletal muscle.

2. Sedentary mice subjected to a HFD exhibited decreased muscle glucose clearance, indicating that high-fat diets coupled with sedentary lifestyles can adversely affect glucose metabolism. Additionally, these mouse models demonstrated an increase in laminin without a corresponding increase in integrin, hinting at a partial expansion of ECM-associated proteins potentially contributing to diminished glucose uptake in skeletal muscle. Notably, the integrin-associated protein ILK was found to be reduced in insulin-stimulated mice on an HFD, suggesting a potential role of ILK in muscle glucose metabolism consistent with our previous research (Draicchio et al., 2020).
3. Rac1, in contrast to Akt, was observed to be reduced under HFD conditions, implying that diet might negatively impact insulin-stimulated glucose uptake, in part, through Rac1 signalling, independent of Akt signalling.

Our collective findings, when juxtaposed with the results from other studies, underscore the potential of ECM- and integrin-associated proteins as novel therapeutic targets for managing diet-induced insulin resistance and associated glucose dysregulations, particularly in the context of chronic metabolic disorders such as kidney disease and Type 2 Diabetes (T2D).

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