PATHWAYS INVOLVED IN SKELETAL MUSCLE GLUCOSE UPTAKE INDUCED BY CONTRACTION AND INSULIN: THE ROLE OF ATYPICAL PROTEIN KINASE C

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Key Words: Wortmannin, GLUT4, GLUT1, *soleus* muscle, aPKC, diabetes, Wistar rats.

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie, 1867- 1934

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ABSTRACT

Muscle contraction acutely increases glucose uptake in skeletal muscle in both, healthy and type 2 diabetes individuals. Since enhancement on glucose uptake in the absence of insulin has been observed in contracted skeletal muscle, the existence of an insulin independent pathway was suggested to explain this phenomenon. However, the exact mechanism behind the increase on glucose transporter (GLUT4) translocation through the sarcolemma during muscle contraction is not completely identified. Meanwhile, studies reported that insulin cascade components, as atypical protein kinase C (aPKC), become more active after muscle contraction; thus the existence of convergence points between the insulin and contraction cascades is hypothesised in the present dissertation. For this purpose, in this document three studies comprising distinct experimental protocol involving the contraction of isolated *soleus* muscle from Wistar rats, were proceeded in order to analyse the effect muscle contraction on enhancement of glucose uptake on skeletal muscle and the role of aPKC on this process.

The first and the second study were undertaken to improve the experimental protocol. Therefore, in order to design a suitable electrical stimulation protocol to evoke muscle contraction in *soleus* muscle, two frequencies, high (100Hz) and low (10Hz), were tested. Glucose uptake, GLUT4 location and muscle integrity markers were assessed. The second study aimed to analyse the influence of age on glucose uptake, at basal and during contraction conditions and to associate it with the expression and the location of GLUTs isoforms. Therefore, glucose uptake, GLUT1 and GLUT4 (location and expression) were observed in rats with different ages: 4, 10, 22 and 42 weeks. Finally, the aim of the third study was to verify the role of aPKC on the enhancement of glucose uptake induced by muscle contraction. For this purpose *soleus* muscle of adult rats was incubated in presence and in absence of insulin and Wortmannin, an inhibitor of an aPKC insulin-upstream regulator, before muscle contraction protocol. At the end of the *in vitro* protocol glucose uptake, GLUT4 location, aPKC and phospho-aPKC expression was assessed.

The first study demonstrated that muscle contraction triggered at 100Hz induces higher rates of glucose uptake than in muscle stimulated at 10Hz. However, the increase on damaged fiber in muscle stimulation at 100Hz and the similar GLUT4 distribution in both groups suggests non-physiological mechanisms behind this enhancement on glucose uptake. Therefore 10Hz was the frequency of electrical stimulation choosen for further studies in this dissertation. In the second study it was observed an age related decline on GLUT4 expression into sarcolemma. This decline contributes for a decrease on the ability of skeletal muscle to take up glucose at basal and during contraction conditions. No difference was observed between 10 and 22 weeks, thus the sample of the last study was composed by *soleus* muscle of rats between these ages. In the third study it was observed that insulin and muscle contraction enhance glucose uptake without additive effect. Phospho-aPKC expression parallels with the enhancement on glucose uptake triggered by insulin and by contraction. In addition Wortmannin inhibited only insulin effects.

As the main conclusion, this dissertation presents evidences that aPKC is a convergence point between the cascades which muscle contraction and insulin enhance glucose uptake in *soleus* muscle of adult Wistars rats confirming the initial hypothesis. These findings did not support the concept about the existence of two distinct and separated cascades to increase glucose uptake in skeletal muscle.

RESUMO

A contração muscular de forma aguda aumenta o transporte de glicose para o músculo esquelético, tanto em indivíduos saudáveis quanto em indivíduos com diabetes tipo 2. Desde que o aumento do transporte de glicose foi observado durante a contração muscular na ausência de insulina, foi sugerido a existência de uma via independente desse hormônio para explicar esse fenômeno. No entanto, o mecanismo exato que promove o aumento da translocação do transportador de glicose (GLUT4) através do sarcolema durante a contração muscular ainda não é completamente identificado. Recentes estudos constataram que componentes da via de sinalização ativada pela insulina, como a atípica proteína quinase C (aPKC), tornam-se mais ativos após a contração muscular. Assim, a presente dissertação sugere pontos de convergência entre o mecanismo desencadeado pela insulina e pela contração muscular. Para este propósito foram executados três estudos com protocolos experimentais distintos envolvendo a contração do músculo *soleus* isolado de ratos Wistar, com o intuito de analisar o efeito da contração muscular no aumento da captação de glicose no músculo esquelético e o papel da aPKC sobre este processo.

O primeiro e o segundo estudos foram realizados com o objetivo de refinar o protocolo experimental. Com o intuito de estabelecer protocolo adequado para induzir a contração muscular através de estimulação eletrica no músculo *soleus*, duas freqüências, alta (100Hz) e baixa (10Hz), foram testadas no primeiro estudo. Transporte de glicose, localização GLUT4 e marcadores integridade muscular foram avaliados. O segundo estudo teve como objetivo analisar a influência da idade sobre o transporte de glicose e associar esse fenômeno com a expressão e a localização das isoformas de transportadores de glicose. Portanto, transporte de glicose, GLUT1 e GLUT4 (localização e expressão) foram observados em ratos com diferentes idades: 4, 10, 22 e 42 semanas. Finalmente, o objetivo do terceiro estudo foi verificar o papel da aPKC no aumento do transporte de glicose induzido pela contração muscular. Assim, músculos de ratos adultos foram incubados na presença e na ausência de insulina e de Wortmannin (inibidor de uma proteína activada pela insulina), antes da contração muscular. No final do protocolo *in vitro* foram verificados transporte de glicose, localização GLUT4, isoforma fosforilada (p-aPKC) e não fosforilada de aPKC.

O primeiro estudo demonstrou que a contração muscular estimulada numa frequência de 100Hz promove maior transporte de glicose do que em músculo estimulado à 10Hz. No entanto, o aumento de fibras musculares danificadas com o estímulo de 100Hz e a semelhança na distribuição GLUT4 entre ao dois grupos sugerem mecanismos não-fisiológicos associados a esse aumento. Portanto, 10Hz foi a frequência de estimulação aplicado nos demais estudos desta dissertação. No segundo estudo, foi observado um declínio na expressão de GLUT4 no musculo esquelético relacionado idade o que parece contribuir para a redução da capacidade do músculo de transportar glicose em condições basais e estimulado pela contração. Nenhuma diferença foi observada nos ratos entre 10 e 22 semanas. Assim, a amostra do terceiro estudo foi a composta por músculo *soleus* de ratos entre estas idades. No terceiro estudo, foi observado que tanto a contração muscular como a insulina promovem o aumento de transporte de glicose e que ambos não apresentam efeitos aditivos. A expressão de p-aPKC parece relacionar-se com o aumento na captação de glicose desencadeado por esses estímulos. De forma adicional a Wortmannin inibiu somente o efeito da insulina sobre a p-aPKC e o transporte de glicose.

Como principal conclusão, esta dissertação apresenta evidências de que aPKC é um ponto de convergência entre as vias que promovem o aumento do transporte de glicose

ativadas pela contração muscular e pela insulina em músculo *soleus* de ratos adultos confirmando a hipótese inicial. Estes resultados sugerem a não existência de vias completamente distintas a ativada pela insulina que justifique o aumento do transporte de glicose no músculo esquelético durante contração muscular.

RÉSUME

La contraction musculaire aiguë augmente la captation du glucose dans le muscle squelettique, autant les individus en bonne santé comme les diabètes de type 2. Depuis amélioration sur la captation du glucose en l'absence d'insuline a été observée dans le muscle squelettique contracté, l'existence d'une voie indépendante d'insuline a été proposé pour expliquer ce phénomène. Cependant, le mécanisme exact derrière la hausse sur le transporteur de glucose (GLUT4) translocation à travers le sarcolemme lors de la contraction musculaire n'est pas terminée identifier. Parallèlement, des études ont indiqué que les composants en cascade de l'insuline, comme atypique protéine kinase C (aPKC), à devenir plus actifs après la contraction musculaire; ainsi des points de convergence entre l'insuline et des cascades de contraction donc l'existence non une insuline voie indépendante pour améliorer la captation du glucose dans le muscle squelettique, suggèrent que les précédentes, est l'hypothèse dans la dissertation de présents. A cet effet, dans ce document de trois études comprenant protocole expérimental distinctes impliquant la contraction du muscle *soleus* isolé de rats Wistar, ont procédé dans le but d'analyser l'effet sur la contraction musculaire amélioré la captation du glucose au niveau du muscle squelettique et le rôle de l'aPKC sur ce processus.

La première et la deuxième étude ont été entreprises pour améliorer le protocole expérimental. Par conséquent, afin de concevoir un protocole approprié électro stimulation pour évoquer la contraction musculaire dans le muscle *soleus*, deux fréquences élevées (100 Hz) et basse (10 Hz), étaient d'essai. La captation du glucose, l'emplacement GLUT4 l'intégrité musculaire et des marqueurs ont été évalués. La seconde étude visait à analyser l'influence du vieillissement sur la captation du glucose, à associer la base et dans des conditions et de la contraction de l'expression et la localisation des isoformes de GLUT. Par conséquent, l'absorption de glucose, GLUT1 et l'emplacement de GLUT4 et d'expression ont été observés chez des rats avec des âges différents: 4, 10, 22 et 42 semaines. Enfin, l'objectif de la troisième étude était de vérifier le rôle de l'aPKC sur le renforcement de l'absorption du glucose induite par la contraction musculaire. Pour ce fins muscle *soleus* de rats adultes aient été incubés en présence et en absence d'insuline et Wortmannin, un inhibiteur d'une insuline à aPKC régulateur en amont, avant protocole de la contraction musculaire. À la fin de l'expression de l'absorption du glucose protocole *in vitro*, l'emplacement GLUT4, aPKC et phospho-aPKC (a-PKC) a été évaluée.

La première étude a démontré que la contraction musculaire déclenchée à 100Hz induit des taux plus élevés que la captation du glucose dans le muscle stimulé à 10Hz. Toutefois, l'augmentation des fibres musculaires endommagées dans la stimulation à 100 Hz et la distribution de GLUT4 similaire dans les deux groupes révèle des mécanismes non-physiologiques de cette amélioration sur l'absorption du glucose. Par conséquent, 10Hz est la fréquence de stimulation électrique choisit de poursuivre les études dans cette thèse. Dans la deuxième étude, on a observé une baisse liée à l'âge sur l'expression de GLUT4 dans sarcolemme. Cette baisse contribue à une diminution de la capacité du muscle squelettique d'absorber le glucose à la base et dans des conditions de contraction. Aucune différence n'a été observée entre rats de 10 et 22 semaines, donc l'échantillon de la dernière étude a été composé par le muscle *soleus* de rats entre ces âges. Dans la troisième étude on a constaté que l'insuline et la contraction musculaire améliorer la captation du glucose sans effet additif. Phospho-aPKC Parallels expression avec la mise en valeur sur l'absorption du glucose par l'insuline et déclenché par contraction. En Wortmannin plus inhibé que des effets d'insuline.

Comme la principale conclusion, ce mémoire présente des preuves que aPKC est un point de convergence entre les cascades qui la contraction musculaire et l'insuline d'améliorer la captation du glucose dans le muscle *soleus* de rats adultes Wistars confirmant l'hypothèse initiale. Ces constatations ne confirment pas l'existence de deux cascades distinctes pour augmenter l'absorption du glucose dans le muscle squelettique.

ABBREVIATION LIST

AMP	Adenosine-5´-monophosphate
AICAR	5-aminoimidazole-4-carboxamide-riboside
AMPK	AMP- activated protein kinase
aPKC	Atypical protein kinase C
AS160	Protein kinase B substrate
ATP	Adenosine Triphosphate
CaMK	Ca/calmodulin-dependent protein kinase
CaMKK	CaMK kinase
cPKC	Conventional protein kinase C
DG	Diacylglycerols
DMT2	Diabetes mellitus type 2
EDL	*Extensor digitorum longus*
ERK	Extracellular signal-regulated kinases
FFA	Free fatty acid
GTP	Guanosine triphosphate
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
Hz	Hertz
IKKβ	Inhibitor of kappa β kinase
IR	Insulin receptors
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal Kinase
KIF2	Kinesin II

KIF3	Kinesin III
KKAy-CETP	KKAy- cholesteryl ester transfer protein
LC-CoA	Long-chain fatty acid-Coenzyme A
LKB1	Serine/threonine kinase 11
MAPK	Mitogen activated protein kinases
nPKC	Novel protein kinase C
ob/ob	Obese mouse model of DMT2
PA	Phosphatidic acid
p-aPKC	Phospho-aPKC
p-AS160	Phosphorylated AS160
PDK	3-phosphoinositide-dependent protein kinase
PI3K	Phosphatidylinositol (PI) -3-kinase
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKB/AKT	Protein kinase B
PLD	Phospholipase D
ROS	Reactive oxygen species
TG	Triacylglycerol

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CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

The prevalence of diabetes has been progressively increasing during the last few decades; epidemiologic data estimate that worldwide at least 171 millions of people had diabetes in 2000, and it is expected that this number will reach 366 millions in 2030 (Wild et al. 2004). One main force driving this increasing prevalence is a staggering enhancement in obesity, which is considered an important contributor for the pathogenesis of diabetes mellitus type 2 (DMT2) that represents 90-95% of all diabetic cases. Additionally to obesity (particularly the accumulation of visceral fat), other environmental conditions, such as physical inactivity and stress, associated with age and genetic factors also appear to contribute to the epidemic character of DMT2 (Litherland et al. 2001; Moller 2001; 2006). All these factors will favour the development of an impaired glucose tolerance, which may result from pancreatic β cell dysfunction and/or from the increase of insulin resistance in target tissues, such as skeletal muscle and adipose tissue (Ostenson 2001).

The resulting chronic hyperglycemia, as the main characteristic of diabetes mellitus, may negatively influence the structure and function of many organs and systems, particularly the cardiovascular, nervous, and the renal system (Ostenson 2001). The state of insulin resistance, which is the initial abnormality for the development of DMT2, is also linked with the progress of atherosclerosis, hyperlipidemia, and hypertension. As a consequence, these patients have a higher risk of myocardial infarction, of cerebral and peripheral arterial diseases, and of limb amputation (Brownlee 2001; Williams and Pickup 2005).

The therapeutic approaches for DMT2 and its secondary complications consist of the daily control of hyperglycemia and of the improvement of insulin sensitivity (Moller 2001). The important goal behind these measures is the increase of glucose uptake in

skeletal muscle, which accounts for ~80% of glucose disposal under insulin-stimulated conditions in healthy subjects (Ryder et al. 2001). Over the past years, a considerable number of findings have revealed that an acute bout of physical exercise enhances skeletal muscle glucose uptake and that regular physical activity improves the ability of insulin to stimulate the glucose uptake at rest (Richter et al. 1982; Helmrich et al. 1991; Hayashi et al. 1997; Tomas et al. 2002). This increased skeletal muscle glucose transport and insulin sensitivity induced by acute and chronic exercise, respectively, may be a key mechanism to explain the strong epidemiological evidence that the practice of regular exercise prevents or delays the onset of DMT2 (Helmrich et al. 1991; Hayashi et al. 1997; Sigal et al. 2004; Jessen and Goodyear 2005). Although the improvement of glucose tolerance induced by exercise has already been fully demonstrated, the intracellular mechanism mediating this phenomenon is not yet well understood.

Therefore, in the past decades, several studies have used *in vitro* and *in situ* muscle contraction protocols in order to elucidate the intracellular mechanism leading to exercise-induced an enhance in glucose uptake (Goodyear et al. 1995; Wojtaszewski et al. 1996; Hayashi et al. 1999; Woods et al. 2005; Wright et al. 2005). However, most of the methodologies *in vitro* (or also referred as *ex vivo*) do not exactly mimic the *in vivo* muscle contraction conditions. A standard protocol regarding the frequency of stimulation, 100Hz, seems to be established on studies which evoke *in vitro* muscle contraction by electrical stimulation. This common protocol is employed for skeletal muscle despite the fiber composition; therefore studies have been applying high frequency of electrical stimulation even in slow-twitch muscles which in physiological condition contract at low frequencies roughly 10Hz (Hayashi et al. 1999; Ihlemann et al. 1999; Aslesen et al. 2001; Barnes et al. 2002; Wright et al. 2004). Compared to fast-twitch fibers, the phenotype of slow-twitch muscle fibers is mainly characterized by a

fragile machinery to sustain fast and abrupt increases of sarcoplasmic calcium concentrations triggered by high frequency of stimulation (Berchtold et al. 2000; Bassel-Duby and Olson 2006). However, it is important to highlight that this protocol of high frequency electrical stimulation has been applied even in the absence of studies regarding the possible damage on skeletal muscle structure triggered by the use of inappropriate frequency of electrical stimulation.

During the life span, several phenotype modifications occur in skeletal muscle compromising, between others, the ability of this tissue to uptake glucose in basal state and stimulated by insulin (Leighton et al. 1989; Houmard et al. 1995; Qiang et al. 2007). The underlying mechanisms leading to a decreased ability to uptake glucose by skeletal muscle throughout life are currently unknown. The expression of skeletal muscle glucose transporters isoforms (GLUT1 and GLUT4) have been associated with this phenomenon although studies do not present a consensual conclusion (Santalucia et al. 1992; Doria-Medina et al. 1993; Houmard et al. 1995; Gaster et al. 2000; Gaster et al. 2000; Beeson et al. 2003). Apart from insulin, scanty and contradictory studies have been proceeded in order to verify the influence of age on muscle contraction-induced glucose uptake (Gulve et al. 1993; Dolan et al. 1995).

There are many *in vitro* and *in situ* studies demonstrating that muscle contraction induces an increase on glucose uptake even in absence of insulin (Nesher et al. 1985; Dolan et al. 1993; Megeney et al. 1993; Dolan et al. 1995; Aslesen et al. 2001; Wright et al. 2004; Bruss et al. 2005; Hannukainen et al. 2005; Wright et al. 2005). Therefore, these results suggest the existence of an insulin independent pathway to enhance glucose uptake triggered by muscle contraction. Among several potential substances that have been responsible for this process, the role of AMP- activated protein kinase (AMPK) and Ca^{+2} activated proteins have been identified as main contributors (Winder and Hardie

1999; Tomas et al. 2002; Wright et al. 2004; Jessen and Goodyear 2005; Wright et al. 2005; Jensen et al. 2007; Jensen et al. 2007; Jensen et al. 2008). However, the isolated effect of these substances on glucose transporters signalling is lacking. For instance, it is not clear whether the increase in muscle glucose uptake in response to exercise *in vivo* requires or does not require the involvement of molecular components of the insulin cascade. Besides, recent studies reported that downstream regulators of insulin pathway, as atypical protein kinase C (aPKC) and protein kinase B substrate (AS160), become activated during exercise and AMPK pharmacologic activators also activate AS160 and aPKC (Chen et al. 2002; Farese 2002; Bruss et al. 2005; Kramer et al. 2006; Luna et al. 2006). Therefore, it may be speculated that this insulin-like effect induced by muscle contraction might be explained by molecular changes in muscle fibers with an enhancement of several metabolic substances that are simultaneously key elements in the insulin-signalling cascade.

Taken into account the above mentioned context the first aim of the present work is to verify, in *soleus* muscle, the effect of a high vs. low frequency *in vitro* electrical stimulation on glucose uptake and on several biochemical and histological markers of muscular damage closely associated with the loss of calcium homeostasis. Our second aim is to evaluate the possible effect of age on glucose uptake induced by muscle contraction and to associate this mechanism with the expression and localization of GLUT1 and GLUT4 in *soleus* muscle of rats, searching for a correlation between these parameters. Finally, the third and main aim of the present work, is to analyse the aPCK activity in *soleus* muscle induced by muscle contraction and insulin and/or to associate this activity with the changes on glucose uptake.

We hypothesized in the first study that the use of high frequency of electrical stimulation protocol applied to a slow twitch muscle, *as soleus*, induces cell damage

with loss of sarcolemmal permeability. Therefore, non-physiological mechanisms might contribute to the enhancement on the rate of glucose uptake reported in literature with high frequency of stimulation. In the second study we hypothesized that ageing affects the ability of skeletal muscle to uptake glucose at basal state and after muscle contraction being this process directly associated with the expression of GLUT4 and GLUT1. Finally, the hypothesis of the third study was that aPKC, which mediates the cascade of insulin-induced glucose uptake, may also be activated during muscle contraction even in the absence of this hormone. Therefore, it was mainly hypothesized the non existence of distinct pathways to increase glucose uptake in the skeletal muscle induced by contraction and by insulin.

1.1 Organization of the thesis

The present document is organized in seven chapters. The first chapter (General introduction) presents epidemiological data, general characteristics and consequences of diabetes type 2. In addition, in this chapter the clinical importance of the identification of mechanisms which promote enhancement on glucose uptake during exercise was highlighted. Finally, the chapter presents the purposes of this work and the hypothesis of this dissertation.

In the second chapter (state of the art) the well established pathway in which insulin induces glucose uptake was described. In addition, several proteins activated during muscle contraction that have been associated with glucose uptake induced by muscle contraction were also analysed. Moreover the data underlying the effect of acute and chronic alteration in skeletal muscle post-exercise responsible for the enhancement of insulin sensitivity was analysed.

The third chapter (methodology) was divided in three independent studies. In the first study it was analysed the effect of two protocols with different frequencies of electrical stimulation to evoke muscle contraction in *soleus* muscle. It was assessed glucose uptake, morphologic signals of muscle damage and expression and localization of cleaved caspase-3 in order to define the best protocol to induce muscle contraction. In the second study it was verified in *soleus* muscle of rats with different ages glucose uptake at basal state and after muscle contraction being this data associated with the expression and localization of glucose transporter isoforms (GLUT1 and GLUT4). Finally, in the third study, it was analysed in *soleus* muscle incubated with insulin and a PI3K inhibitor (Wortmannin) the effect of muscle contraction on glucose uptake, expression and the location of aPKC and the active form of aPKC (phospho-aPKC).

Chapter 4 (results) presents graphics, tables, figures and a description referent to the results of the three studies referred above. The finding of each study is presented independently.

In chapter 5 (discussion) all the results presented previously (chapter 4) were analysed and compared to different findings and conclusions published in literature until the present moment.

In chapter 6 (final conclusions) the main findings of these three studies as well as the main conclusion of this dissertation is presented.

Finally, the chapter 7 (references) contains the bibliography of the present work.

CHAPTER 2

STATE OF THE ART

2. STATE OF THE ART

2.1 Glucose uptake in skeletal muscle

Glucose is carried across the cell membrane by a family of specialized transporter proteins called glucose transporters (GLUT) (Williams and Pickup 2005; Watson and Pessin 2006). Skeletal muscle contains different isoforms of GLUT, of which GLUT4 is the major contributor for glucose transport (Henriksen et al. 1990; Hansen et al. 1998; Ai et al. 2002). These proteins are mainly located in the membrane of intracellular vesicles localized in the perinuclear compartment at basal state (unstimulated). The fast cellular translocation of these vesicles to the sarcolemma, and the consequent redistribution of GLUT4 through the plasma membrane (Figure 2.1), is stimulated by a complex intracellular cascade of molecular events (Saltiel and Kahn 2001; Karylowski et al. 2004; Santos et al. 2008).

Microtubules provide an intracellular structure so that GLUT4 vesicles move to the muscle membrane under the influence of motor proteins. The coordination of the direction and velocity of GLUT4 vesicles is dependent on specific motor proteins. Kinesin II (KIF2), KIF3 in mice, seems to be the type of motor protein responsible for the fusion of GLUT4 vesicles to the cell surface (Ai et al. 2003; Imamura et al. 2003).

Insulin maintains the glucose homeostasis by activating several substances which result on GLUT4 mobilization through the plasma membrane and a consequence increase in glucose uptake into skeletal muscle (Saltiel and Kahn 2001; Sano et al. 2003). Nevertheless, apart from insulin other factors like muscle contraction (Whitehead et al. 2000), hypoxia (Wright et al. 2005), pharmacologic agents (Zhou et al. 2001; Song et al. 2002), and K^+ depolarization (Wijesekara et al. 2006; Thong et al. 2007) may also increase the amount of GLUT4 on cell surface, and consequently, the glucose uptake.

In mammalian skeletal muscle, the major site for glucose disposal, the amount of GLUT4 is dependent on metabolic and dynamic characteristics of the fibers. In muscle composed predominantly by type I fibers, also called slow-twitch muscle, as *soleus*, the amount of GLUT4 is higher than in muscles mainly constituted by type II fibers (Henriksen et al. 1990). Thus, using intact muscles of rats, it was demonstrated that the rates of glucose uptake in muscle exposed to insulin directly correlate upon the relative abundance of GLUT4. Therefore, the capacity of slow-twitch muscles to uptake glucose triggered by insulin is higher than fast-twitch muscles (Henriksen et al. 1990).

Apart from GLUT4, Glucose transporter 1 (GLUT1), was also suggested to contribute to glucose uptake in skeletal muscle fibers, being the main transporter at the basal state (Guillet-Deniau et al. 1994; Marshall et al. 1999; Ciaraldi et al. 2005). In fact, this isoform seems to have an important role during the skeletal muscle growth and development process in order to sustain the high energetic demands, which characterize this stage of life. Moreover, the specific cellular expression of GLUT1 in mature skeletal muscle has been a topic of debate. For instance, several studies reported that GLUT1 immunoreactivity was absent in the sarcoplasma of muscle cross-sections, being located mainly outside the muscle fibers, specially in blood and endothelial cells (Santalucia et al. 1992; Martineau et al. 1999; Gaster et al. 2000; Gaster et al. 2000). In contrast to these observations, other studies showed the expression of GLUT1 in muscle fibers of adult rats and humans (Marette et al. 1992; Ciaraldi et al. 2005). Thus, exact GLUT1 localization and function in mature muscle fibers remains unclear.

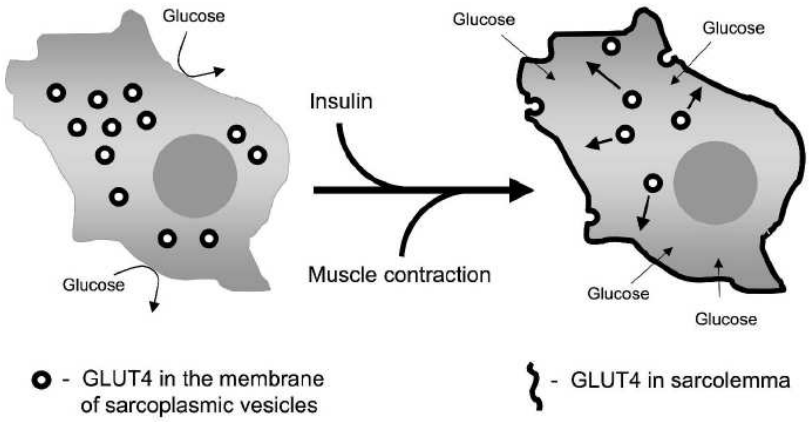

Figure 2.1- Insulin and muscle contraction stimulate the redistribution of isoform 4 of glucose transporter (GLUT4) from the membrane of cytoplasm vesicles through the sarcolemma, allowing the muscular uptake of glucose. Extracted from Santos et al. (2008).

2.2 The insulin dependent cascade

In skeletal muscle fibers insulin triggers the intracellular cascade by binding to insulin receptors (IR) at the cell membrane (Figure 2.2). The IR is a tetrameric protein with two α and two β subunits functioning as allosteric enzymes, with the α-subunit inhibiting the tyrosine kinase activity of the β-subunit. Insulin binding to the α-subunit activates the kinase activity in the β-subunit followed by the transphosphorylation of the receptor (Andreasson et al. 1991; Okada et al. 1994; Cheatham and Kahn 1995; Saltiel and Kahn 2001). The activated IR phosphorylates tyrosine residues of the insulin receptor substrate (IRS) family, initiating the so called "classical insulin cascade" (Goodyear et al. 1995; Saltiel and Kahn 2001; Chang et al. 2004) (Figure 2.2). Upon tyrosine phosphorylation, IRS proteins interact with the p85 regulatory subunit of phosphatidylinositol (PI) -3-kinase (PI3K) leading to the activation of the p110 catalytic

subunit of this enzyme, which has the membrane phospholipids as its main target (Cheatham et al. 1994; Okada et al. 1994). This key component of the classical insulin cascade generates the lipidic product phosphatidylinositol 3,4,5-trisphosphate (PIP3), which regulates the activity of downstream proteins, especially of the 3-phosphoinositide-dependent protein kinase (PDK). The PDK activates two protein kinases that have been postulated to be essential key factors for insulin-stimulated glucose transport: protein kinase B (PKB or AKT) and atypical protein kinase C-ζ and ι in humans and ζ/λ in rodents (aPKC) (Bandyopadhyay et al. 1997; Bandyopadhyay et al. 2001; Kanzaki et al. 2004; Kotani et al. 2004; Karlsson et al. 2005).

AKT is a signalling protein for several insulin actions, including the activation of glycogen synthesis, protein synthesis, and GLUT4 translocation (Bruss et al. 2005). In order to activate GLUT4 vesicles, AKT activates a AKT substrate with 160KD (AS160) (Kane et al. 2002; Eguez et al. 2005), which directly links the insulin signal with GLUT4 trafficking (Dugani and Klip 2005). The AS160 contains a domain for Rabs, which are small G-proteins required for vesicle trafficking process. Under basal conditions the unphosphorylated form of AS160 retains GLUT4 vesicles into the sarcoplasmma (Kramer et al. 2006). The activation of AS160 seems to remove the inhibitory link in GLUT4 vesicle allowing its translocation to the plasma membrane (Dugani and Klip 2005). Thus, AS160 is the identified most proximal step to GLUT4 vesicles in the pathway through which insulin induces increase in glucose uptake. It should be noted that this substrate is diminished in DMT2 patients (Karlsson et al. 2005).

The protein kinase C (PKC) families have been subdivided into three groups based on sequence homology and mechanisms of activation. While differentiated by their sensitivity to Ca^{2+}, both the conventional isoforms (cPKC) and novel (nPKC) seem to be dependent on diacylglycerol for full activation. The aPKC constitutes a third group

within PKC family which is activated by lipid binding (e.g. PIP3 or phosphatidic acid) (Newton 1995). Once activated the PKD phosphorylates aPKC and this protein acts on GLUT4 translocation in parallel with AKT and AS160 (Newton 1995; Hajduch et al. 2001; Litherland et al. 2001; Saltiel and Kahn 2001). Even being well established the importance of aPKC on GLUT4 translocation, the exact link of aPKC in this process is not well elucidated. Studies suggest that aPKC mediates the phopohorilation of the motor proteins KIF3 which coordinate the direction and the velocity of GLUT4 vesicle along the microtubule structure to the plasma membrane (Imamura et al. 2003). Defective activation of aPKC has been observed in muscle of DMT2 rats, monkeys and humans (Farese 2002; Farese et al. 2005; Farese et al. 2005; Farese et al. 2007).

The binding between IR and insulin also stimulates the mitogen activated protein kinases (MAPK), which include the extracellular signal-regulated kinases (ERK) and p38 MAPK (Furtado et al. 2002). This event results in the stimulation of different transcription factors, which initiate the program that leads to cell proliferation or differentiation. Such transcriptional-dependent adaptations also should have the potential to indirectly modify glucose uptake (Saltiel and Kahn 2001).

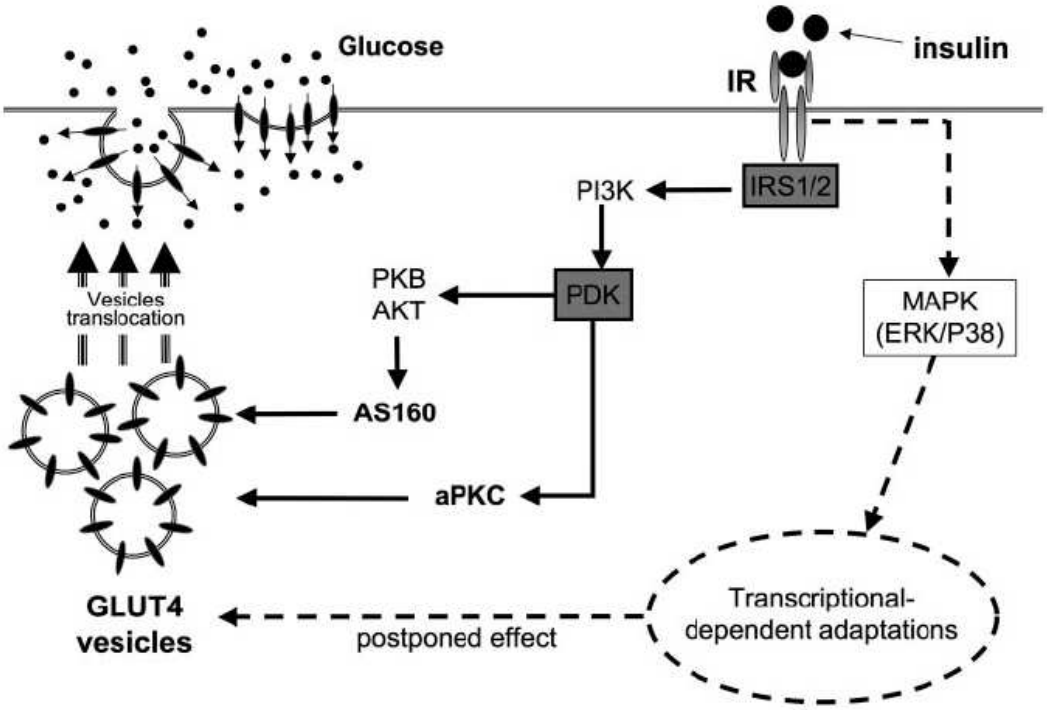

Figure 2.2- Signal transduction in insulin action: The autophosphorylation of the insulin receptor (IR) catalyses the phosphorylation of IRS 1/2 (insulin receptor substrates) that activates PI3K (Phosphatidylinositol 3-kinase), which interacts with AKT/AS160 (Protein kinase B/AKT substrate of 160 KD) and aPKC (atypical protein kinase C). The IR activation also triggers MAPK (mitogen-activated protein kinase) pathways. These pathways act in regulating GLUT4 (glucose transporter isoform 4) vesicle trafficking. Extracted from Santos et al (2008).

2.2.1 Insulin resistance

Insulin resistance is characterized by a diminished ability of skeletal muscle and fat cells to respond to physiological levels of circulating insulin, despite the normal GLUT4 cellular concentration. Disorders on glucose and lipid metabolism might compromise several steps of insulin cascade (Kim et al. 2008). Elevated free fatty acid (FFA) levels in blood lead to the accumulation of fat acid in nonadipose tissue as skeletal muscle. In fact, lipid infusion in rodents and high fat feeding diet in human

reduces insulin-stimulated glucose disposal suggesting the defect in lipid metabolism as the major contributor for insulin resistance (Boden and Jadali 1991; Kim et al. 1996).

Elevated FFA and glucose in the plasma impairs fatty acid oxidation due to an increased concentration of Malonyl-CoA into the sarcoplasmma. The intramyocellular lipid accumulation induces an accumulation of lipid intermediates such as diacylglycerols (DG), ceramides and long-chain fatty acid-Coenzyme A (LC-CoA). The concentration of lipid intermediates is directly linked to defects in the insulin signalling cascade. Ultimately, these changes in cellular signalling have been associated to a decrease in insulin-stimulated glucose uptake and metabolism (Kim et al. 2008).

Recent studies have shown that the accumulation of LC-CoA, DGs, ceramides, or any combination of these affect multiple sites of the insulin signalling pathway: (1) increase in serine phosphorylation of IRS proteins (2) decrease in PI-3K activity (Houmard et al. 2002; Straczkowski et al. 2007) and, (3) decreased activation of insulin receptor downstream molecules including aPKC and AKT (Saltiel and Kahn 2001; Farese 2002). In fact, reduction of phosphorylation of IRS family members in response to insulin stimulation has been well reported in insulin- resistant animal and humans (Bjornholm et al. 1997; Howlett et al. 2002). One possible mechanism to explain the decreased IRS response to insulin is the activation binding of this substrate by other molecules that do not integrate the insulin cascade which inhibits the IRS-PI3K interaction. In fact, it is known that FFAs stimulate a cellular receptor which mediates the proinflammatory signalling and activates IκB kinase β (IKKβ) and c-Jun N-terminal Kinase (JNK) which in turn phosphorylates IRS (Itani et al. 2002). This effect inhibits the interaction between IRS and PI3K with a resultant decrease in aPKC and AKT activity (Karlsson et al. 2005).

Nevertheless, the presence of lipid or triglyceride in muscle cells is not always related to insulin resistance. It was observed in high elite athletes of endurance sports, as marathon runners and long distance cycling, high concentration of intramyocellular lipids into skeletal muscle without any impairment on insulin sensitivity (van Loon et al. 2004). This accumulation of lipids was substantially greater compared to DMT2 and obese subjects. Researchers justify this phenomenon as being a physiological up-regulated compensation for the increased oxidative capacity of the muscle to chronic exercise (Rattarasarn 2006).

Mitochondrial dysfunction and the consequent increase in reactive oxygen species (ROS) is another potential mechanism to explain the insulin resistance. Furthermore, ROS also stimulates proinflamatory signalling by activation of IkB kinase β (IKKβ) which impaired insulin pathway (Nishikawa and Araki 2007). Mitochondrial dysfunction also results in accumulation of lipids intermediates as DG that activate some PKCs isoforms as PKC- β, θ, δ that also inhibit the interaction of IRS with PI3K (Schmitz-Peiffer and Biden 2008). The integral link between DG, PKC and insulin resistance has been confirmed in studies using PKC – θ deficient mice, where it was observed that these genetically modified animals presented a protection in fat-induced insulin resistance compared to wild rats (Serra et al. 2003; Gao et al. 2007).

In summary, lipid accumulation might contribute to insulin resistance by several mechanisms promoting a reduction on the interaction between IRS and PI3K with a consequent decrease of insulin-induced glucose uptake. Therefore, it is of clinical importance to identify physiologic and pharmacological mechanisms which activate GLUT4 or downstream insulin cascade proteins, as aPKC and AS160, independently of IRS and PI3K, which are impaired by lipid intermediates.

2.2.1 Age and Insulin Resistance

Advancing age constitutes an important risk factor for insulin resistance and for the development of DMT2 (2006; Nakhanakup et al. 2006). It is well documented that the ability to control the blood glucose level declines during the life span (Leighton et al. 1989; Houmard et al. 1995; Qiang et al. 2007). Phenotype modifications in skeletal muscle seem to play an important role in this decline (Young 1997; Guillet and Boirie 2005).

In rodent models, a progressive decline in the ability of skeletal muscle to uptake glucose, in the absence and in the presence of insulin, was observed during the phase of growth-related weight gains between 1 and 4 months of age (Goodman et al. 1983; Gulve et al. 1993; Cartee et al. 1997; Qiang et al. 2007). This premature and rapid decrease in glucose uptake by the skeletal muscle is followed by a long period of stabilization with further slower modifications taking place during the later ageing process (Goodman et al. 1983; Rowe et al. 1983; Qiang et al. 2007).

The exact underlying mechanism leading to the age-related decrease in the ability to uptake glucose is still lacking. Accumulation of lipid intermediates, sarcopenia and a decrease of GLUT1 and/or GLUT4 expression have been proposed as mechanisms to explain the aging-induced insulin resistance (Houmard et al. 1995; Nair 2005; Qiang et al. 2007).

With increasing age, fat mass tends to gradually increase, especially visceral fat, and the daily energy expenditure tends to decrease (Kim et al. 2008). The decreases of lipid oxidative capacity have been demonstrated in aged skeletal muscle of humans and animals (Conley et al. 2000; Peltoniemi et al. 2001). Mitochondria of old animals presented changes in morphology in addition to increased ROS production and decreased in ATP production (McCarroll et al. 2004). Thus the increase of ROS

production and modification of mitochondrial biogenesis contribute to mitochondrial dysfunction which increases the risk of insulin resistance development (Kim et al. 2008).

Many studies have reported a decrease in muscle mass, fiber and function with age which has been associated with a decrease in protein synthesis rate (Guillet and Boirie 2005; Nair 2005; Short et al. 2005). Therefore, this effect *per se* affects the clearance of plasma glucose since the skeletal muscle accounts for ~80% of glucose disposal under insulin-stimulated conditions in healthy young animals. Insulin also regulates the protein metabolism, inducing an increase in synthesis and a consequent muscle growth. This effect is impaired in DMT2 which contributes to an increase of sarcopenia triggered by age. Therefore, in a vicious cycle, sarcopenia plays a role on DMT2 and DMT2 contributes to sarcopenia (Guillet and Boirie 2005).

Modifications of glucose transporters in skeletal muscle have been reported during the life span. Concerning GLUT4, a positive correlation was found between its total amount and the capacity of muscle tissue from an adult healthy animal to uptake glucose under insulin stimulation (Henriksen et al. 1990). However, it is not clear whether the ontogenic decline in glucose uptake is directly related to the muscular GLUT4 content. On this subject, conflicting results were reported, either showing an enhanced GLUT4 expression in adult rats with advanced age compared to younger animals (Santalucia et al. 1992) or a decrease of this protein expression with increasing age (Gulve et al. 1993). In humans, several studies found a negative correlation between GLUT4 content and age (Houmard et al. 1995; Gaster et al. 2000). Regarding GLUT1, which is not regulated by insulin, its expression seems to decline rapidly after birth (Santalucia et al. 1992). This could partly explain the premature decline in glucose uptake previously described, but only few studies provided experimental support for this hypothesis (Gaster et al. 2000a; Postic et al. 1994). Similarly to the present knowledge

about the specific cellular GLUT1 localization, the effect of age in this transporter isoform also remains unclear.

2.2. Muscle contraction-enhanced glucose uptake

During exercise the turnover of ATP in skeletal muscle in the contracting muscle increases significantly being fuelled by the catabolism of carbohydrate (intramuscular glycogen and blood glucose) and fatty acids (intramuscular triglycerides, blood lipids). The contribution of blood glucose for ATP resynthesis at the beginning of exercise seems to be low. However, with the increased time of exercise the blood contribution of glucose becomes more substantial (Rose and Richter 2005).

It was well reported that a single bout of exercise promotes a decrease in blood glucose even when insulin action is impaired and moreover, it is also well supported that regular exercise in a chronic way can diminish in insulin blood concentration with a consequent improvement of the glycemic control (Chibalin et al. 2000; Christ et al. 2002; Holloszy 2005; Engler 2007). These effects are resultant from the acute increase of glucose uptake on contracting skeletal muscle and from the enhanced insulin sensitivity consequent in addition to intramuscular adaptation triggered by chronic exercise. Nevertheless, the underlying mechanism that contributes to the acute and chronic modification those results in an enhancement in glucose uptake in skeletal muscle triggered by contraction is not completely identified.

In the absence of insulin, muscle contraction, *in vitro*, increases glucose transport to a degree similar to that described during exercise *in vivo* (Wallberg-Henriksson et al. 1988; Richter et al. 1989; Cartee and Holloszy 1990; Douen et al. 1990; Helmrich et al. 1991; Dolan et al. 1993). Additionally, a great number of studies reported a synergistic effect of muscle contraction- and of insulin-induced glucose uptake hypothetically

triggered by multiple signalling cascades (Wallberg-Henriksson et al. 1988; Richter et al. 1989; Cartee and Holloszy 1990; Douen et al. 1990; Helmrich et al. 1991; Dolan et al. 1993). Therefore, different research groups initially suggested the existence of a pool of molecules completely different, triggered by these two stimuli. For instance, it has been shown during a marathon run, by using a continuous glucose monitoring system, that the intracellular mechanism triggered by exercise was sufficiently effective to maintain glucose transport in diabetes type 1 subjects (Cauza et al. 2005) suggesting a low requirement of insulin to maintain transport of glucose during long periods of exercise.

On the other hand, several results have demonstrated that this potential of exercise to increase glucose transport is reduced in type 1 diabetic subjects compared to normal individuals (Tuominen et al. 1997; Riddell et al. 2000; Peltoniemi et al. 2001; Peltoniemi et al. 2001). This data suggests the role of an insulin dependent component on the mechanism whose contraction enhance glucose uptake. Keeping this idea in mind, recent findings have shown that insulin downstream regulators, such as AS160 and aPKC, become more active on contracted skeletal muscle (Beeson et al. 2003; Deshmukh et al. 2006). It is therefore also reasonable to suggest that exercise triggers an additional effect on the insulin cascade to enhance glucose uptake probably through one or more components of the insulin cascade.

2.2.1 Mechanisms of glucose transport induced by muscle contraction

Despite the endocrine (Wicklmayr et al. 1979; Kishi et al. 1998; Tatar et al. 2003; Bobbert et al. 2007) and paracrine (Higaki et al. 2001; Kingwell et al. 2002) influence on skeletal muscle glucose uptake during exercise, the intracellular mechanism triggered by muscle contraction appears to be the major responsible candidate for acting

straight on the enhancement of surface GLUT4 levels. Two main intracellular mechanisms have been suggested to explain the contraction-dependent glucose transport. The first one is based on the assumption that glucose transport might be related to the metabolic strain imposed on skeletal muscle during exercise (Kurth-Kraczek et al. 1999; Fujii et al. 2004) and the second one explains the enhanced glucose uptake by the depolarization of the sarcolemma and T-tubule membranes through calcium-mediated second messengers (Wright et al. 2004; Wright et al. 2005).

2.2.1.1 Metabolic stain dependent mechanisms

A key progress to understand the molecular events of how the muscle contraction affects glucose transport came from the identification of a protein kinase activated by adenosine-5´-monophosphate (AMP), which resulted in the denomination of AMPK (Hardie and Carling 1997; Hardie and Sakamoto 2006). In the past decade, many studies have been conducted associating the role of this enzyme on contraction-stimulated glucose uptake *in vivo* (Rasmussen and Winder 1997; Musi et al. 2001), *in situ* (Hutber et al. 1997) and *in vitro* (Hayashi et al. 1998). AMPK is a heterotrimeric protein consisting on one α subunit and two non-catalytic subunits β and γ. Two isoforms of the α-subunit have been identified, α1 and α2, with different distribution among tissues, with the highest expression of α2 in skeletal muscle (Stapleton et al. 1996; Kemp et al. 2003). Besides, a pharmacologic activator of AMPK, namely 5-aminoimidazole-4-carboxamide-riboside (AICAR), activates the glucose transport in resting rat muscle (Kurth-Kraczek et al. 1999; Fisher et al. 2002; Musi and Goodyear 2003). This drug mimics the effect of AMP on AMPK without alterations of ATP stores or of earlier steps of the insulin cascade (Musi et al. 2003; Long and Zierath 2005). Additionally to

AICAR, hypoxia and metformin (an oral antidiabetic) also seem to act on glucose uptake through AMPK (Nesher et al. 1985; Zhou et al. 2001) (Figure 2.3).

In ob/ob rats (obese and insulin resistant), a subcutaneous administration of AICAR was associated with a decrease in glucose intolerance (Halseth et al. 2002). The same phenomenon has been reported with the use of AICAR in different models of insulin resistance as KKAy-CETP mice and Zucker rats (Fiedler et al. 2001; Buhl et al. 2002). In agreement with these animal studies, Musi et al. (2002) observed a correlation between the increase in AMPK activity and glucose transport in DMT2 patients after exercise. An augmentation of AMPK expression has also been reported after metformin treatment (Musi et al. 2001). These studies suggested that insulin resistance did not inhibit the effect of AMPK on glucose uptake.

Recent findings convincingly propose the tumor suppressor kinase LKB1 (serine/threonine kinase 11) as the major upstream regulator of AMPK in skeletal muscle. The development of a muscle-specific LKB1 knockout mouse by Sakamoto et al. (2004) evidenced the role played by this enzyme in AMPK activation. It was demonstrated in this elegant study that the glucose uptake and AMPK α2 activation induced by AICAR, by phenformin (a metformin analogue) and by muscle contraction were diminished in LKB1 deficient *extensor digitorum longus* (EDL) muscle (Sakamoto et al. 2004). In addition, different methodologies have also emphasized the role of LKB1 as a dependent molecule for AMPK-induced glucose uptake (Taylor et al. 2005; Sriwijitkamol et al. 2007). Therefore, LKB1 seems to catalyze the phosphorylation of the AMPKα subunit turning into active AMPK complexes (Hardie 2005). Thus, convincing evidences are supporting the role of LKB1 on AMPK activation, but it is still unknown, whether LKB1 is the only kinase responsible for AMPK activation.

In spite of the well-established evidences about the role of AMPK as an underlying substance for contraction-induced glucose uptake, the relationship between glucose uptake and this protein does not appear to represent a simple dose-dependent response. It could be suggested that a component of the insulin cascade may be a downstream regulator of the AMPK pathway for increasing glucose uptake.

The majority of investigations have failed to demonstrate an association between earlier components in the insulin cascade such as IR, IRS, and PI3K and the mechanisms of contraction-stimulated glucose uptake. *In vivo* studies demonstrated that PI3K expression decreased some minutes after exercise (Wojtaszewski et al. 1997; O'Gorman et al. 2000). It was observed in isolated muscle that Wortmannin (a PI3K inhibitor) did not influence contraction- and AICAR-stimulated glucose transport but, on the other hand, this inhibitor impaired the insulin action (Goodyear et al. 1995; Lee et al. 1995; Whitehead et al. 2000; Wright et al. 2006). These reports suggest that molecules responsible for that muscle contraction-induced glucose uptake act in a downstream step regulated by PI3K.

Consistent findings have established a convincing connection between AS160 and AMPK. It was demonstrated in isolated *epitrochlearis* muscle that contraction and AICAR, as well as insulin, stimulated AS160 phosphorylation (Bruss et al. 2005). Additionally, Kramer et al. (2006) showed a synergic effect among contractile activity and AICAR on AS160 phosphorylation, without Wortmannin impairment. In addition, it was demonstrated that the effect of AICAR on AS160 was abolished in AMPK α2-inactive transgenic mice; in contrast, contraction-stimulated AS160 phosphorylation was only partially affected in these animals (Treebak et al. 2006). This result highlights the role of AMPK on AS160 phosphorylation but also suggests an alternative mechanism, independently of AMPK, for AS160 activation triggered by muscle contraction.

Supporting studies using an *in vitro* methodology reported, an enhancement of AS160 phosphorylation in *vastus lateralis* of humans`muscle was observed after endurance training (Deshmukh et al. 2006). Such approaches strongly support the idea that AS160 is an important AMPK downstream regulator and also as a potential convergence point integrating the insulin- and contraction-stimulated glucose uptake pathway(s).

Regarding aPKC, several studies have consistently shown that the aPKC activity is higher in contracting muscle, as demonstrated in table 2.1, suggesting the role of this protein on contraction induced-glucose uptake (Beeson et al. 2003; Nielsen et al. 2003; Perrini et al. 2004; Rose et al. 2004). An increase in aPKC activity was observed in the exercised muscles in comparison to resting muscles in human DMT2 subject (Beeson et al. 2003). In addition, just like in AS160, it was suggested that this increase was independent of PI3K activity (Beeson et al. 2003). Using a similar methodology, Perini et al (2004) reported an increase of phospho-aPKC expression in the sarcolemma of healthy subjects after muscle contraction. Additionally, the authors suggested a translocation of aPKC to the membrane after being phosphorylated. Moreover, althougt Ritcher et al (2004) reported an increase on aPKC activity after bike exercise, however this effect was not related to the energy demand (Richter et al. 2004). These studies pointed out the effect of muscle contraction on aPKC activity, however none of these reports have directly associated aPKC activity with glucose uptake. Thus more research is needed to verify if aPKC is really required to contraction-enhanced glucose uptake in skeletal muscle.

Even being described that aPKC can be activated by muscle contraction, the second messager required for contraction-induced aPKC activation is unknown. In contrast to AS160, little research has been conducted with the aim of analysing the association between AMPK and aPKC. Chen et al. (2002) suggested that AICAR and

muscle contraction activate aPKC without the requirement of PI3K. Instead, aPKC activity induced by AMPK appears to be dependent on MAPK-ERK, which activates phospholipase D (PLD) to generate phosphatidic acid (PA) that directly activates aPKC (Chen et al. 2002). The correlation between the activation of aPKC and ERK was also observed by two studies after endurance exercise (Nielsen et al. 2003; Richter et al. 2004). These results demonstrate a convincing connection between aPKC and ERK and also propose the role of AMPK on aPKC activation. In agreement with this idea, data demonstrated in skeletal muscle of DMT2 subjects that metformin therapy also increases aPKC activity, suggesting a common effect of aPKC and of AMPK for the enhancement on glucose uptake in skeletal muscle (Luna et al. 2006). Furthermore, like AS160, aPKC may also represent a point of convergence in insulin and contraction pathways, although additional research is required to address this hypothesis sufficiently.

A valuable approach to elucidate the molecular mechanisms of contraction-induced glucose uptake *in vivo* was originated from transgenic mice with an inactive (dominant-negative) AMPK protein expressing an inhibitory effect on the AMPK α2 subunit. Mu et al. (2002), using these transgenic mice, observed a reduction of only 30% in the glucose transport in response to hind limb contraction evoked by electrical stimulation (Mu et al. 2001). Additionally, in α1 or α2 AMPK isoforms knockout mice it was demonstrated that contraction-induced glucose transport in isolated muscle was not altered despite the inability of AICAR to stimulate glucose transport in α2 knockout mice (Jorgensen et al. 2004). These important findings suggest that a small activation of AMPK may be enough to induce normal glucose uptake and, moreover, it may only be a part of the mechanisms leading to contraction-stimulated glucose transport. It should therefore be feasible to assume the existence of parallel or compensatory pathways

acting independently of AMPK, which may be triggered by a calcium dependent mechanism (Figure 2.5) (Derave et al. 2000; Musi and Goodyear 2003; Fujii et al. 2005).

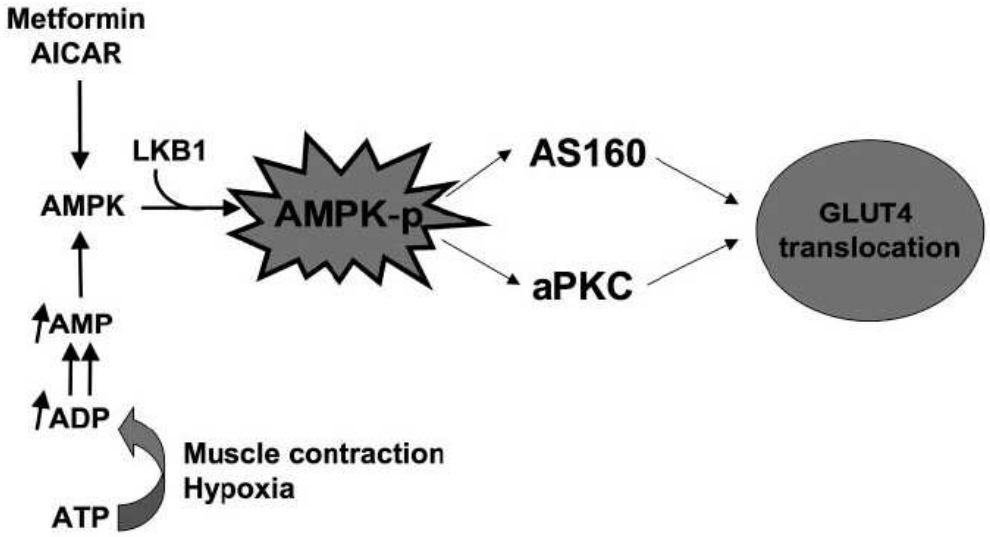

Figure 2.3 Phosphorylation of adenosine monophosphate-activated protein kinase (AMPK-p) dependent of LKB1 (serine/threonine kinase 11), with AMPK upstream and downstream regulators (ATP - Adenosine 5'-triphosphate; ADP - Adenosine diphosphate; AMP - Adenosine monophosphate). Possible interaction between aPKC (atypical protein kinase C) and AS160 (160 KD substrate of protein kinase B) on GLUT4 (glucose transporter isoform 4) translocation. Extracted from Santos et al (2008).

Studies that suggested convergence points between insulin- and contraction-induced glucose uptake				
Stimuli	**Muscle type and population**	**Convergence point Insulin and exercise**	**Characteristic of this response**	**References**
Bicycle exercise	not mentioned/ DMT2 humans	aPKC activity	Independent of PI3K	Benson et al. (2003)
	not mentioned/ healthy humans	p-aPKC expression	Activated isoform located at membrane fractions	Perini et al. (2004)
	vastus laterlis/ healthy humans	aPKC activity	Enhance on aPKC activity not p-aPKC expression	Ritcher et al. (2004)
	vastus laterlis/ healthy humans	aPKC activity	Independent of cPKC	Rose et al. (2004)
	not mentioned/ healthy humans	aPKC activity	In parallel with ERK	Niels et al. (2003)
	vastus lateralis/ athletes	AS160 activity	Endurance rather than strength exercise	Deshmukh. et al. (2006)
Treadmill run	mice/ ELD	aPKC activity	Dependent on AMPK and ERK	Chen et al. (2002)
AICAR	L6 muscle cells	aPKC activity	Dependent on AMPK and ERK	Chen et al. (2002)
	L6 muscle cells	p-AS160 expression AS160 activity	Directed related on GLUT4 translocation	Farah et al. (2007)
	mice/ EDL	p-AS160 expression AS160 activity	Additive of insulin and AMPK independent of PI3K	Kramer et al. (2006)
	mice AMPK (-/-)/ ELD	p-AS160 expression AS160 activity	Partial dependent on AMPK and independent of PI3K	Treebak et al. (2006)
Metformin	*vastus lateralis*/ DMT2 humans	aPKC activity	Dependent on AMPK	Luna et al. (2006)
Electrical-stimulation	Wistar rats/ epitrochlearis	p-AS160 expression AS160 activity	Additive of insulin and independent of PI3K	Bruss et al. (2005)
	Wistar rats/	p-AS160	Parallel of	Kramer et

	EDL and in situ hind limb	expression AS160 activity	insulin and AMPK independent of PI3K	al. (2006)
	mice AMPK (-/-)/ ELD	p-AS160 expression AS160 activity	Partial dependent on AMPK and independent of PI3K	Treebak et al. (2006)
K+ depolarization	L6 muscle cells	p-AS160 expression AS160 activity	Direct related on GLUT4 translocation. Partial dependent on c/n PCK and AMPK.	Tong et al. (2007)

Table 2.1 – Studies demonstrating that proteins activated by insulin can also be activated by exercise (*in vivo*), muscle contraction (*in vitro*), AMPK (adenosine monophosphate-activated protein kinase) specific pharmacological activators and K+ depolarization in skeletal muscle. AS160 (160 KD substrate of protein kinase B) and aPKC (atypical protein kinase C) activities are referred to studies that have used an immunopreciptation methodology; p-AS160 (phosphorylated AS160 isoform) or p-aPKC (phosphorylated aPKC isoform) are referred to studies that have quantified these proteins by western blotting.

2.2.2.2 Calcium dependent mechanisms

The increase in cytoplasmic calcium concentration due to membrane depolarization also seems to activate several substances involved in the translocation of GLUT4 vesicles. Several studies have shown that glucose transport is increased in mammalian muscle when cytoplasmic calcium concentrations are raised independently of insulin or of the energy status (Cleland et al. 1989; Henriksen et al. 1989; Youn et al. 1991; Jessen and Goodyear 2005). Two proteins have been identified in the context of this phenomenon: the Ca/calmodulin-dependent protein kinase (CaMK) and the PKC (new and classical) (Ojuka et al. 2002; Wright et al. 2004; Wright et al. 2005; Thong et al. 2007). In an attempt to associate the mechanisms only with calcium in the absence of

muscle contraction, studies have used pharmacological calcium activators and inhibitors such as caffeine and KN-62, respectively (Brozinick et al. 1999; Wright et al. 2004).

CaMK seems to integrate the pathway by which calcium stimulates the translocation of GLUT4 vesicles (Fukunaga et al. 1988; Brozinick et al. 1999; Olson and Williams 2000; Wright et al. 2004). Wringht et al. (2005) analyzed *in vitro* the interaction between muscle contraction, AICAR, and caffeine, and described a synergistic effect of these factors on glucose transport. Additionally, the combination of these two drugs increased glucose transport rates to the same degree as muscle contraction. In concordance, the role of CaMK, using different tissues, was suggested to parallel AMPK independent mechanisms (Corcoran and Means 2001; Wright et al. 2005). During steady state exercise, resulting in a modest activation of AMPK, Ojuka (2002) proposed that the primary adaptive stimulus for GLUT4 vesicles translocation might be linked to the rise of cytoplasmic calcium. On the other hand, intensive exercise results in a more powerful stimulus for GLUT4 expression and translocation by activating both, CaMK and AMPK (Figure 2.4) (Yu et al. 2001; Ojuka et al. 2002).

In contrast with the idea of an AMPK independent pathway, recent studies have shown that calcium may activate AMPK by CaMK kinase (CaMKK) (Hawley et al. 2005; Woods et al. 2005; Jensen et al. 2007). It was demonstrated that the inhibition of Ca/calmodulin-regulated protein kinases potently inhibits AMPK phosphorylation independently of the influence of LKB1 in muscle at rest and during contraction (Jensen et al. 2007). The same group has demonstrated in isolated *soleus* muscle that the mechanism underlying caffeine-induced glucose uptake (through a Ca^{+2} increase) is also dependent on the AMPK α1 activation (Jensen et al. 2007). These data, support CaMKK as an upstream regulator of AMPK, and also suggest that calcium and AMPK signals are at least partially connected rather than exclusively parallel as proposed before. This

assumption, however, was partially contradicted by Witczak et al. (2007) using an innovating methodology with electroporated vectors containing constitutively active CaMKK. The consequent overexpression of CaMKK increased the *in vivo* glucose transport in wild strain and in AMPK deficient animals, demonstrating that CaMKK-stimulated glucose uptake may act independently of AMPK through one or more unknown components (Witczak et al. 2007). The apparent contradiction between the studies of Witczak et al. (2007) and of Jessen et al. (2007a and 2007b) may be due to the different methodologies used. Nevertheless, it is important to keep in mind that although the *ex vivo* muscle contraction model remains a valuable tool to study the components of contraction signalling, it is unknown whether the *ex vivo* findings entirely mimic the conditions of *in vivo* studies.

To our best knowledge, the association between the earlier insulin cascade components and calcium-activated proteins has received little attention in the literature. It was observed *in vitro* that KN-62, a specific Ca/calmodulin-dependent protein kinase inhibitor, induced a decrease in glucose transport triggered by hypoxia and insulin, without parallel alterations in the expression or activity of PI3K and AKT (Brozinick and Birnbaum 1998; Brozinick et al. 1999). These data suggest that the association between CaMK and GLUT4 may not be explained by an interaction with AKT or PI3K, however, it is important to highlight that CaMK might interact within a cascade step below these substances start to act (Brozinick and Birnbaum 1998; Brozinick et al. 1999). Since its identification, AS160 was assumed to be a candidate for the convergence point between CaMK and insulin cascade components as shown in figure 2.5, however, few findings have rejected this hypothesis; in one of the first studies testing this hypothesis, it was demonstrated in the adipocyte that Ca/calmodolin is not a downstream regulator for AS160 (Kane and Lienhard 2005). In agreement, an increase

of AS160 phosphorylation in skeletal muscle was also not observed with CaMKK overexpression, suggesting that CaMKK trigger metabolic reactions, such as glucose uptake, independent of AS160 (Witczak et al. 2007). Additional research is certainly required to identify the link between Ca/calmodulin-regulated protein kinases and insulin cascade components.

Although PCKθ negatively affects GLUT4 translocation (Griffin et al. 1999; Yu et al. 2002; Haasch et al. 2006), the other new PCK isoforms have been associated with the promotion of glucose uptake. Using a PKC inhibitor (Calphostin C), a decrease of glucose transport was observed after muscle contraction suggesting a major role of conventional and novel (c/n) PKCs in glucose uptake (Ihlemann et al. 1999). However, this result should be interpreted with caution, because Calphostin C inhibits all PKC isoforms without specificity. With the aim to find a possible candidate for PKC downstream regulation, Thong et al. (2007) demonstrated that (n/c) PKC activation due to K^+ depolarization and a consequent increase of intracellular calcium increased AS160 phosphorylation, which contributed for the regulation of the GLUT4 traffic in cultured L6 cells (Thong et al. 2007). Thus, there is a possibility that PKC stimulated by muscle contraction acts on glucose transport through AS160. Controversially, a recent study using muscles from PKC α (a conventional isoform) (-/-) mice demonstrated a significant decrease in contraction-stimulated glucose uptake when compared to muscles from wild type animals. The authors concluded that PKC α, which represents ~97% of conventional PKC in skeletal muscle, is not required for contraction-stimulated glucose uptake (Jensen et al. 2009). Therefore it will be important to identify in further studies what calcium- activation proteins are underlying on calcium-induced glucose uptake and if this protein is also related with the action of any components which mediate insulin cascade.

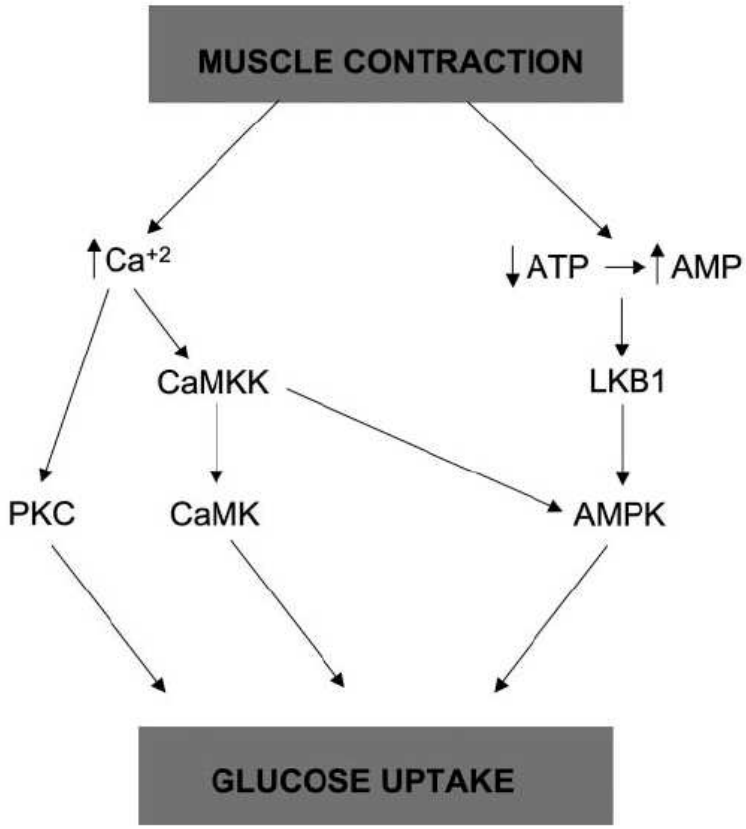

Fig. 2.4. Calcium activated proteins (PKC - protein kinase C; CaMK - Ca/calmodulin-dependent protein kinase; CaMKK - Ca/calmudulin-dependent protein kinase kinase) act on glucose transport parallel and/or connected to the AMP-activated protein kinase (AMPK) pathway (ATP - Adenosine 5'-triphosphate; AMP - Adenosine monophosphate; LKB1 - serine/threonine kinase 11). Extracted from Santos et al. (2008).

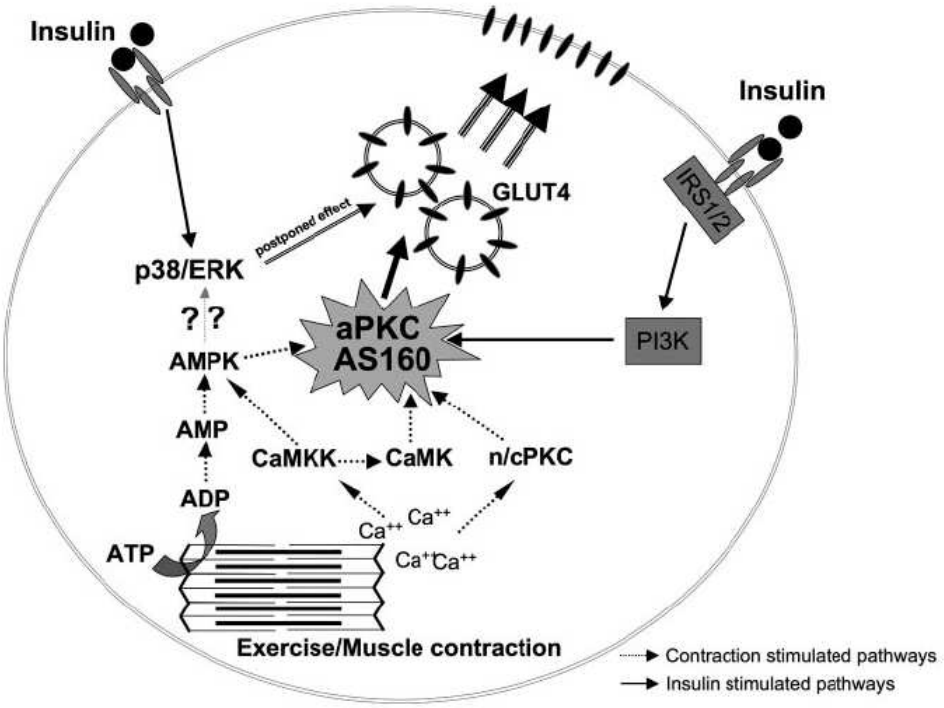

Figure 2.5 Proposed pathways to explain the increase of glucose transport triggered by muscle contraction (PI3K - Phosphatidylinositol 3-kinase; GLUT4 - glucose transporter isoform 4; CaMK - Ca/calmodulin-dependent protein kinase; CaMKK- Ca/calmodulin-dependent protein kinase kinase; AMPK – Adenosine monophosphate-activated protein kinase; ERK - Extracellular signal-regulated kinase; AS160 - 160 KD substrate of protein kinase B; n/cPKC - novel and conventional protein kinase C; IRS 1/2 - insulin receptor substrate; ATP - Adenosine 5'-triphosphate; ADP - Adenosine diphosphate; AMP - Adenosine monophosphate). Extracted from Santos et al. (2008)

2.3. Post-exercise adaptations of skeletal muscle – Effect on glucose uptake

In 1966, Bergström and Hultman defined "improve in insulin sensitivity induced by exercise" as being the decrease on the amount of insulin required to skeletal muscle at post-exercise state (rest) to uptake the same rate of glucose observed before exercise. (Bergstrom and Hultman 1966). This definition was later confirmed by Hollozy (Holloszy 2005).

An early study, carried out by Richter et al. (1989) used an isolated perfused rat hindquarter preparation to demonstrate that after 45 minutes of treadmill run the physiological concentration of insulin had enhanced during several hours post exercise

the glucose uptake. This effect was reproduced the when hindquarter muscle was electrically stimulated through the sciatic nerve (Richter et al. 1989). This increase in insulin sensitivity after contraction and exercise was later confirmed in human studies (Richter et al. 1982; Cartee and Holloszy 1990). It was observed a 2-fold increase in glucose uptake in response to low physiological insulin concentrations 4 hours after one-leg exercise compared with the rested leg (Mikines et al. 1988). Schineider et al. (1984) showed that glucose tolerance was substantially better 12 and 17h after a single bout of exercise (Schneider et al. 1984). Furthermore, it was also shown that an improvement in insulin sensitivity could be detected during few days after a single session of exercise in people with obesity and DMT2 (Devlin et al. 1987; Cartee et al. 1989). Supporting these conclusions, two studies done with athletes demonstrated that the high insulin sensitivity observed after exercise diminishes during the following days after ceasing exercise (Heath et al. 1983; Burstein et al. 1985). In summary, these reports established that a single bout of exercise enhances the sensitivity and the responsiveness of skeletal muscle to insulin in both humans and experimental animals.

This persistent effect of exercise on glucose uptake for promoting a glycogen resynthesis has been characterized in two phases (Zierath 2002). While the first phase seems to be independent of insulin or its pathway in the early components (e.g. IRS and PI3K), in the second phase it is dependent of insulin, IRS and PI3K (Richter et al. 1982; Devlin et al. 1987; Chibalin et al. 2000; Zierath 2002). Zouh and Dohn (1997) noticed that exercise increases the ability of insulin to activate PI3K (Zhou and Dohm 1997). In agreement, Howlett et al. (2002) observed an increase in IRS and PI3K activity after exercise (Howlett et al. 2002). In addition, it was demonstrated that the enhancement on insulin sensibility is only partially dependent of IRS and PI3K, suggesting an additional mechanism to explain this effect (Howlett et al. 2002). Therefore, on the basis of these

findings, the early components of insulin cascade seem to be necessary to enhance the insulin sensibility post-exercise, however, other mechanisms also appear to contribute to this process.

2.3.1 Increase GLUT4 expression

An adaptation that may contribute to increase insulin action and, therefore, increase glucose uptake and glycogen synthesis post exercise is an increase in skeletal muscle GLUT4 protein expression (Ren et al. 1994; Kuo et al. 1999). Early data demonstrated a rapid enhancement of GLUT4 expression which persists 2 days after one prolonged swimming session (Ren et al. 1994). In accordance, recent data demonstrates an increase of GLUT4 content and mRNA of GLUT4 after 60 minutes, independent of the exercise intensity (Kraniou et al. 2006). Hou et al. (2003) observed the effect of two bouts of three hours swimming and carbohydrate supplementation in diabetic and control rats. The effect of exercise on GLUT4 expression was higher in control rats with lower plasmatic insulin, which demonstrates a partial dependence of insulin on this phenomenon (Hou et al. 2003). On the other hand, it was reported that GLUT4 expression did not accompany the increase of insulin sensibility observed after 1 and 5 bouts of swimming exercise, which suggests that GLUT4 expression is not a rate limit to increase insulin sensitivity after exercise (Chibalin et al. 2000). In addition, the prevention of protein synthesis, with cycloheximide (and thus the prevention of an increase in GLUT4 content) did not avoid the increased action of insulin after exercise. Therefore, the increase of GLUT4 content might play a role to the late adaptive response to exercise and not to the immediate increase in insulin sensitivity observed few hours after exercise.

2.3.2 Lipid metabolism on increased insulin sensitivity- Role of AMPK

The increased action of insulin immediately after exercise may simply be a result of an increased FA oxidation (Turcotte and Fisher 2008). This notion is consistent with recent findings in cultured myotubes and animal muscle, which showed an increased action of insulin under conditions of increased fat oxidation (Koves et al. 2008). Thus, the more important question regarding the effects of exercise on intramuscular lipid intermediates is whether intramuscular levels of ceramides, DGs, or both, are reduced by regular exercise. Few studies have examined the effects of short-term or regular exercise on the content of intracellular lipid intermediates, and the results have been conflicting. In line with the beneficial role of exercise in muscle insulin sensitivity, exercise training was shown to decrease intramuscular levels of DGs and ceramides in obese people (Bruce et al. 2006). Conversely, short-term exercise was found to increase rather than decrease ceramide levels in lean people (Helge et al. 2004) which was also observed in endurance athletes as previously mentioned (van Loon et al. 2004). Thus, more research is required to clarify the changes induced by exercise (acute, chronic and both) in intracellular lipid accumulation and to verify if these changes in exercise and lipid intermediates in fact play a role in skeletal muscle insulin sensitivity. However, it seems likely to suggest that there is a different response of intracellular accumulation of lipid intermediates in DMT2 people comparing to the response on high performance athletes on lean people.

In order to elucidate the mechanisms underlying lipid metabolism interaction with insulin sensitivity, the activity of AMPK was verified after muscle contraction and AICAR treatment. AMPK seems also to be a key protein to increase fatty acid oxidation by inducing a depletion on malonyl-CoA concentration (Rattarasarn 2006). Thus, the activation of AMPK for muscle contraction and AICAR subsequently leads to an

enhanced insulin action (Buhl et al. 2001; Saltiel and Kahn 2001; Fisher et al. 2002). In obese rats the AMPK activity triggered by muscle contraction was correlated with the enhancement of mitochondrial oxidation which results on activating lipolysis and lipid catabolism, thereby lowering the levels of lipid toxic intermediates, and therefore ameliorated insulin resistance (Thyfault et al. 2007). Indeed, AMPK activation leads to a decreased muscular LC-CoA (Hardie and Sakamoto 2006). Furthermore, insulin resistance has been associated to a decrease of AMPK expression (Liu et al. 2006). In fact, in insulin resistant Zucker fa/fa rats, a single subcutaneous injection of AICAR improves insulin sensitivity (Iglesias et al. 2002). Having gathered all the presented results, it is acceptable to suggest that AMPK might contribute to an increase in glucose uptake during muscle contraction by activation of AS160 and aPKC, and post exercise this protein plays a role on the activation of lipid catabolism which removes the inhibitory action of lipids intermediate on IRS, increasing the sensibility of skeletal muscle to insulin.

CHAPTER 3

METHODOLOGY

3. METHODOLOGY

In order to achieve the aims of the present dissertation three studies were carried out involving male Wistar rats. Animals from the first and the third study were purchased from Charles Rivers laboratory (Barcelona, Spain) while rats from the second study were purchased from CRIFA (Barcelona, Spain). Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR 1996). The local Ethics Committee approved the study and the experiments were complied with the current national laws.

Animals were housed (two per cage) in a room with constant temperature (21-23ºC) and exposed to a light cycle of 12h light/12h dark. Commercial chow and tap water were provided *ad libitum*. In all three studies, 12h before the experimental protocol food were removed.

3.1 First Study

3.1.1 Animal handling

Eighteen Wistar rats (weighting 334.89±7.36 g, aged 11-14 weeks) were randomly divided into 2 different groups according to the frequency of electrical stimulation to be applied: the 10Hz group (n=9) and the 100Hz group (n=9). Rats were sacrificed by decapitation and intact *soleus* and *gastrocnemius* muscles (n=36) were excised. *Gastrocnemius* muscles were only used to evaluate the water content in skeletal muscle tissue. One s*oleus* muscle from each animal was assigned to the electrical stimulation protocol (10Hz or 100Hz) while the contralateral muscle served as incubated rested control. Four extra animals with similar characteristics (age and gender) like those

used in the *in vitro* protocol were further used to control potential *soleus* muscle alterations induced by the incubation process itself.

3.1.2 Muscle preparation, stabilizing period, and experimental groups

The muscles were placed vertically suspended in an organ bath between 2 electrodes attached to an isometric transducer under a passive tension of 10g. The muscles were incubated in a Krebs Buffer with the following composition (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO4, 1.2 $MgSO_4$, 24.6 $NaHCO_3$, 5.5 glucose, 2 pyruvic acid and 5 HEPES, gassed with a mixture of 95% CO_2 and 5% CO_2 and kept at 30ºC (Aslesen et al. 2001). After a stabilization period of 60 min, one muscle from each animal was submitted to electrical stimulation protocol ("stimulated") whereas the contralateral muscle, was not stimulated and remained incubated during the time required for the stimulation protocol ("incubated controls"). Immediately before the application of electrical stimulation the buffer of both muscles was refreshed with Krebs Buffer containing 0.25μCi/ml of 2-deoxy-D -[1-^{3}H]-glucose (8.50 Ci/mmol; Amersham, UK) and 0.061 μCi/ml of [1-^{14}C]-D mannitol (61 mCi/mmol; Amersham, UK). *Soleus* muscles obtained from the 4 animals which were not submitted to the *in vitro* protocol, constituted the "non-incubated controls".

3.1.3 *In vitro* muscle electrical stimulation

The protocols of electrical stimulation consisted of trains of pulses (10V, 0.2ms pulse width) delivered at 100Hz for periods of 10 seconds with 50 seconds interval during 10 minutes, or at 10Hz for periods of 10 seconds with 50 seconds interval during 30 minutes. After each protocol, muscles were assigned to biochemical (n=6 animals per group) or morphological assays (n=3 animals per group).

3.1.4 Muscles biochemical assays

Stimulated and rested incubated muscles were blotted on filter paper and homogenized on ice in 1 ml of 10 mM Tris- HCl buffer containing 1 mM EDTA and protease inhibitors (500 μM of phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, and 1 μg/ml pepstatin). A part of the homogenate (500 μl) was frozen at -80ºC for further assay of cleaved caspase-3 while the remaining was digested in 2 ml of 1 M NaOH during 10 minutes at 80ºC for ^{3}H and ^{14}C quantification. Digested samples were neutralized with 2 ml of 1 M HCl mixed with 10 ml of scintillation cocktail (Optiphase HiSafe 3, PerkinElmer) and ^{3}H and ^{14}C in the samples were evaluated by scintillation spectrometry (Beckman LS 6500, Beckman Instruments).

The intracellular water content was calculated for each muscle taking into account the measured interstitial water volume and the percentage of total muscle water content assessed in a 5-day lyophilized *gastrocnemius* (EZ Dry Benchtop Freeze Dryer-FTS Systems). Total muscle water content was calculated by the difference between the initial and the final muscle weight. Glucose uptake was normalized by the calculated intracellular water volume and expressed as absolute values for each group of muscles, being the effect of electrical stimulation on glucose uptake presented as a percentage of control incubated muscles.

To assess the muscular expression of cleaved caspase-3, samples were centrifuged at 14.000g at 4ºC for 10 minutes and the supernatant protein concentrations were determined according to the Lowry protocol (Lowry et al. 1951). Supernatant volumes containing 30 μg of protein were separated on 12.5% SDS-PAGE gel and transferred to a PVDF membrane. Membrane was blocked in Tris Buffered Saline – Tween (TBS-T), pH 7.6 containing 5% dry milk and incubated with rabbit anti-cleaved caspase-3 antibody (1:1000, Cell Signaling Technology) followed by incubation with

anti-rabbit IgG HRP conjugated (Amersham Biosciences). Bands were detected using a chemiluminescence ECL-kit (Amersham Biosciences), quantified in Image J software (version 1.41) and expressed as percentage of enhancement from the incubated control muscle.

3.1.5 Morphological assays

Muscles from 3 animals of each group (and from the 4 animals not submitted to electrical stimulation – the non-incubated controls) were fixed overnight in a 4% formaldehyde/phosphate-buffered saline (PBS pH 7.4) buffer. Muscles were then dehydrated through a graded series of ethanol to xylene and imbedded in paraffin following the conventional protocol. Transversal and longitudinal sections of 4 μm were prepared for immunohistochemistry of cleaved caspase-3 and GLUT4 or were stained with hematoxylin/eosin for light microscopy. Briefly, after being deparaffinised and rehydrated, slices were placed in 10 mM citrate buffer in a microwave oven (700W) during 20 min for antigen retrieval. Tissues were then submitted to a block solution of 3% bovine serum albumin (BSA) in TBS-T at 37º C during 60 minutes. After several washes, sections were incubated over night at 4ºC with cleaved caspase-3 primary antibody (rabbit polyclonal, 1:100 Cell Signaling, Technology) or with GLUT4 primary antibody (rabbit polyclonal 1:200 Chemicon, International) at 37ºC for 1h. After washing, all sections were incubated at 37ºC for 1 hour with alkaline phosphatase conjugated anti-rabbit antibody (1:100, Santa Cruz Biotechnology). Fast Red reagent (Sigma-Aldrich) was used to detect the alkaline-phosphatase from secondary antibodies and slides were counterstained with hematoxylin. Negative controls were prepared by replacing primary or secondary antibodies by TBS-T. For cleaved caspase-3, the results were expressed as the percentage of fibers stained red in at least 300 muscle

fibers/skeletal muscle. The analysis of GLUT4 slides was merely qualitative taking into consideration the distribution of red stain within muscle fibers. In slides stained with hematoxylin/eosin, the percentage of swelled and eosinophilic fibers was quantitatively assessed after observing at least 300 muscle fibers/skeletal muscle.

The non-incubated control muscles were specifically used for morphological analysis in order to control immunohistochemistry data and morphological alterations of the *soleus* muscle, which might have been induced by the incubation process itself.

3.1.6 Statistical Analysis

Data were calculated as means ± standard deviation (SD)**.** Statistical analyses were carried out using SPSS 15.0 for Windows software. Comparisons between groups were performed using the Mann-Whithney U test since the normal distribuition test (Kolmogorov-Smirnov test) detected an abnormal distribution in our data. Significance level was set at $P<0.05$.

3.2 Second Study

3.2.1 Animal handling

Twenty eight animals aged of 4 (n=7), 10 (n=7), 22 (n=7) and 42 (n=7) weeks (wks) were classified, respectively, as childhood group, juvenile group, adult young group and adult old group. They were sacrificed by concussion followed by decapitation with exsanguinations and both intact *soleus* and *gastrocnemius* muscles were excised; the latter were only used to evaluate the water content in skeletal muscle tissue.

3.2.2 Electrical stimulation

S*oleus* muscles were split longitudinally in pieces with similar weight and placed in organ baths and incubated as previously described in section 3.1.2. After the stabilization period of 60 min one muscle from each animal was electrically stimulated with trains of pulses (20 V, 0.2 ms width) delivered at 10 Hz for periods of 10 seconds with 50 seconds interval during 30 min, referred as contracted muscles; the contralateral muscle was submitted to the same conditions but the electrical stimulation was omitted, being referred as unstimulated muscles. At the end of stimulation period muscles were collected and processed to quantify the expression of GLUT1, GLUT4 and glucose uptake.

3.2.3 Glucose uptake assays

Before application of electrical stimulation, the incubation medium was replaced by a buffer containing 0.25 μCi/ml of 2-[^{3}H] deoxy-D-glucose (8.50 Ci/mmol; Amersham Biosciences), which was used as an indicator of glucose uptake, and 0.061 μCi/ml of [1-^{14}C]-D mannitol (61 mCi/mmol; Amersham Biosciences), used to calculate the interstitial muscle volume. At the end of the stimulation period muscles were blotted on filter paper and homogenized on ice in 1 ml of buffer containing 1% Triton X-100, 0.1% SDS, in PBS (pH 7.4) and protease inhibitors (500 μM of phenylmethylsulfonyl fluoride, 5 μg/ml of leupeptin, 1 μg/ml pepstatin and 2 μg/ml of aprotinin; (Ben-Abraham et al. 2003). A fraction of the homogenate (500 μl) was frozen at -80 ºC for further evaluation of GLUT expression (see below). The remaining 500 μl were processed as previously described in 3.1.4.

Glucose uptake was expressed in mol/L/30min, corresponding to the amount of glucose uptake by the muscle during the 30 min incubation normalized by the calculated intracellular mean of water volume from each group as described in section 3.1.4.

3.2.4 Western blotting assays

To assess the muscular expression of GLUT4 and GLUT1 samples were centrifuged at 10,000g at 4ºC for 10 min and the protein concentration in the supernatant determined according to Lowry et al. (1951). Supernatant volumes containing 30μg of protein were separated on 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked with Tris buffer with 0.01 % of tween 20 (TBS-T, pH 7.4) containing 5% dry milk and incubated with rabbit anti-GLUT1 antibody (1:500, ABCam) and anti-GLUT4 antibody (1:1000, Chemicon International) followed by incubation with conjugated anti-rabbit IgG HRP (Amersham Biosciences). Immunoreactive bands were detected using a chemiluminescence ECL-kit (Amersham Biosciences), quantified in Image J software (version 1.41) and expressed in percentage of the expression observed in 4 wks old rats.

3.2.5 Immunohistochemistry assays

Transversal sections of unstimulated muscles were processed for GLUT4 and GLUT1 immunohistochemistry assays as decribed in section 3.1.5. Sections were incubated overnight at 4ºC with GLUT1 primary antibody (rabbit polyclonal 1:25, Abcam) or with GLUT4 primary antibody (rabbit polyclonal 1:200; Chemicon International) at 37ºC for 1h. After washing, all sections were incubated at 37ºC for 1 hour with alkaline phosphatase conjugated anti-rabbit antibody (1:100, Santa Cruz Biotechnology). Fast Red reagent (Sigma-Aldrich) was used to detect the alkaline-

phosphatase from secondary antibodies and slides were counterstained with hematoxylin. Analyses of the intracellular and extracellular distribution of GLUT4 and GLUT1 in the tissue were merely qualitative.

3.2.6 Statistical analysis

Results were presented as means ± standard deviation (SD)**.** Statistical analyses were carried out using Sigma Stat software (version 3.5). All data were tested for normal distribution (Kolmogorov-Smirnov test). The Wilicoxon test was used to compare contracted and unstimulated muscles glucose uptake within group. Comparisons between groups were performed using the multifactorial ANOVA test for variables with normal distribution such as muscle water content, muscle weight and body weight, or with the Kruskal-Wallis test for variables with abnormal distribution such as glucose uptake, GLUT1 and GLUT4 content. The Spearman's test was used for correlations between muscle glucose uptake vs. GLUT1 or GLUT4 expression and age vs. glucose uptake or GLUT1 and GLUT4 expression. Significance level was set at $P < 0.05$.

3.3 Third Study

3.3.1 Animal handling

Sixteen-week-old rats (n=28) were randomly divided into 4 different groups according to the *in vitro* incubation treatment: not treated (n=7); incubated with Insulin (n=7); incubated with Wortmannin (n=7); and incubated with Insulin plus Wortmannin (n=7) being referred as Control, Wortmannin, Insulin and Insulin+Wortmannin respectively. After being designated they were sacrificed by concussion followed by decapitation with exsanguination and both intact *soleus* muscles and *gastrocnemius* were excised. The latter were only used to evaluate the water content in skeletal muscle tissue.

3.3.2 Muscle Incubation

S*oleus* muscles were placed in organ baths, between 2 electrodes, attached to an isometric transducer under a passive tension of 10 g and incubated in a Krebs buffer with the following composition (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO4, 1.2 $MgSO_4$, 24.6 $NaHCO_3$, 5.5 glucose, 2 pyruvic acid and 5 HEPES, gassed with a mixture of 95% O_2 and 5% CO_2 and kept at 30 ºC during a stabilization period of 60 min. During the incubation period muscles from each animal were treated on the following conditions as shown in figure 3.1: a) not treated (Control); with b) 500nM of Wortmannin (Sigma-Aldrich) dissolved in 0.05% DMSO for 30 min (Wortmannin); c) 10mU/ml of insulin (Insulin) for 10 min; and d) both drugs, Insulin+Wortmannin.

3.3.3 Electrical Stimulation

After stabilization period, 0.2 mCi/ml of 2-[^{3}H]-deoxy-D-glucose and 0.06 μCi/ml of [^{14}C]–D mannitol were added to the baths and one muscle from each animal was electrically stimulated with trains of pulses (20 V, 0.2 ms width) delivered at 10 Hz for periods of 10 seconds with 50 seconds interval during 30 min, referred as contracted muscle; the contralateral muscle was submitted to the same conditions but the electrical stimulation was omitted, being referred as uncontrated muscle. Therefore, 8 conditions were analysed, 4 in the study uncontracted muscles (Control, Insulin, Wortmannin, Insulin+Wortmannin); and 4 in contracted muscles (Control, Insulin, Wortmannin, Insulin+Wortmannin).

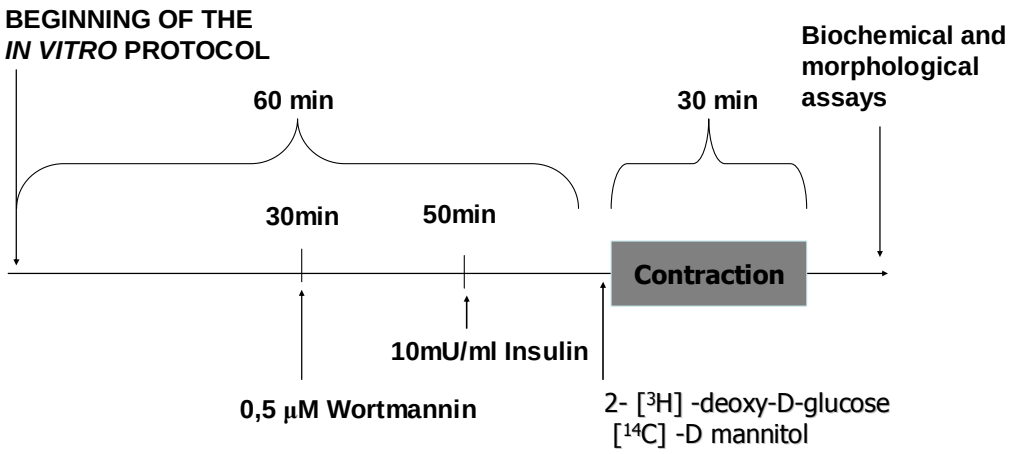

Fig 3.1- Representative Illustration of the *In vitro* protocol. 30 minutes (min) before electrical stimulation 0.5 μM of Wortmannin were added into the buffer in the Wortmannin and Insulin+Wortmannin condition. On the 50th min of incubation 10mU/L Insulin was added to the buffer in the Insulin and Insulin+Wortmannin groups. At the end of the incubation period, 60 min, the buffer was replaced with a fresh Krebs buffer containing 0.2 mCi/ml of 2-[^{3}H]-deoxy-D-glucose and 0.06 μCi/ml of [^{14}C]–D mannitol with or without (Control condition) Insulin and/or Wortmannin accordingly to the different groups.

3.3.4 Glucose uptake assays

Afterwards, muscles were homogenised in a buffer containing 50mM tris-HCl, 250mM sucrose, 1mM EDTA, 1mM EGTA, 1mM PMSF, 1mM 2-mercaptoetanol, 5 mM sodium fluoride, 5mM sodium vanadate, 10% glycerol, 2 μg/ml aprotin, 5 μg/ml leupetin, 2 μg/ml of pepstatin and 0.5 mM PMSF. A fraction of the homogenate (500 μl) was frozen at -80 ºC for further evaluation of aPKC, and phospho-aPKC expression (see below) and processed as explained in section 3.1.4.

3.3.5 Immunohistochemistry assays

Muscles of all groups (n=2) were prepared for immunohistochemistry of GLUT4 as described previously in section 3.1.5. As in the previous study, the analysis of the GLUT4 intracellular distribution was merely qualitative.

3.3.6 Western Blotting assays

In order to assess aPKC and phospho-aPKC expression in membrane and cytosolic fractions, samples were centrifuged at 100,000g at 4ºC for 60 min. The cytosolic fractions, the supernatant, were removed for further analysis, whereas the remaining pellet contains, membrane fractions, was resuspended in a similar homogeneizated buffer containing 1% of Triton-X. The resuspendend samples were centrifuged at 15,000g at 4ºC for 15 min and the supernatant with membrane fractions was removed. Protein concentration was determined according to Bradford et al. (1976). Supernatant volumes containing 30μg of protein were separated on 12.5% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked with Tris buffer with 0.01 % of tween 20 (TBS-T, pH 7.4) containing 5% dry milk and incubated with rabbit polyclonal anti-phospho aPKC antibody (1:500 Cell Signaling) and mouse

monoclonal anti-aPKC (1:10,000 Santa Cruz). Followed by incubation with conjugated anti-rabbit IgG HRP (1:1,000, Santa Cruz) and anti-mouse IgG HRP (1:1,000 Amersham Biosciences) in phospho-aPKC and aPKC membranes respectively. Immunoreactive bands were detected using a chemiluminescence ECL-kit (Amersham Biosciences), quantified in Image J software (version 1.41) and expressed in percentage of the expression observed in uncontracted Control muscles.

3.3.7 Statistical Analysis

Data were calculated as means ± standard deviation**.** Statistical analyses were carried out using SPSS 15.0 for Windows software. All data were tested for normal distribution (Kolmogorov-Smirnov). As the variables presented abnormal distribution the Wilcoxon test was used to compare uncontracted and contracted muscles glucose uptake within the same group. Comparisons between groups were performed using the Kruskal-Wallis test. Significance level was set at $P<0.05$.

CHAPTER 4

RESULTS

4. RESULTS

Results of each study will be presented independently as further described:

4.1 First Study

4.1.1 Glucose Uptake

After the adjustment of muscular glucose content to the respective incubation period of each protocol (10 or 30 minutes), the incubated controls for the muscles stimulated at 10Hz and 100Hz presented a basal glucose uptake rate of 42.5±8.6 (n=6) and 40.6±7.3 (n=6) nmol/ml/min, respectively. In muscles submitted to electrical stimulation these rates increased significantly to 61.3±15.5 (n=6) and 71.7±17.1 nmol/ml/min (n=6), which correspond to a variance of 143±16 and 180±24% for muscles stimulated at 10Hz and 100Hz, respectively (Figure 4.1.1).

4.1.2 GLUT4 localization

As depicted in figure 4.1.2, a notorious difference between the incubated control and stimulated muscles regarding the location of GLUT4 within the muscle fibers was observed. This transporter protein was spread all over the fiber in incubated control muscles while it was mainly positioned near the sarcolemma in stimulated muscles. In both protocols of stimulation (10Hz and 100Hz), there was no qualitative difference concerning the GLUT4 distribution at the periphery of the fibers, suggesting that both frequencies of stimulation have a similar effect on the translocation of this protein towards the muscle fiber membrane. Compared to non-incubated control muscles, the incubated control muscles also showed some discrete increase in concentration of GLUT4 near the periphery of fibers, suggesting that the incubation process itself might have constituted a weak stimulus for GLUT4 translocation (Figure 4.1.2).

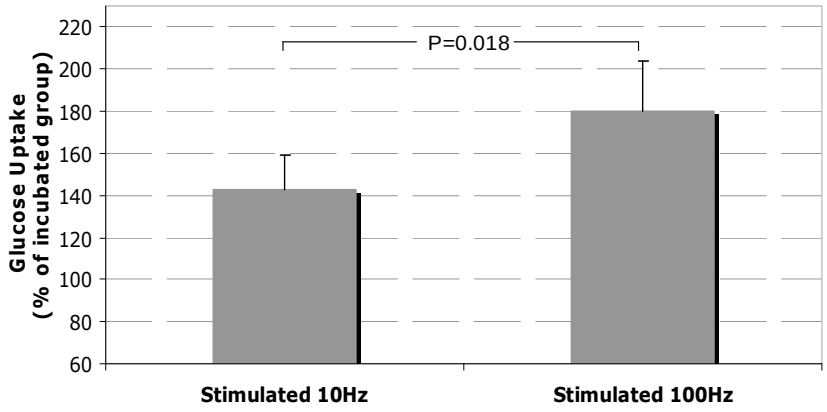

Figure 4.1.1 Glucose uptake induced by muscle contraction triggered by two different frequency of electrical stimulation: 10Hz and 100Hz. Contraction-stimulated glucose uptake was calculated as the percentage of variance between rested and stimulated muscle. Values are means ± SD for 6 muscles per group, $p<0.05$.

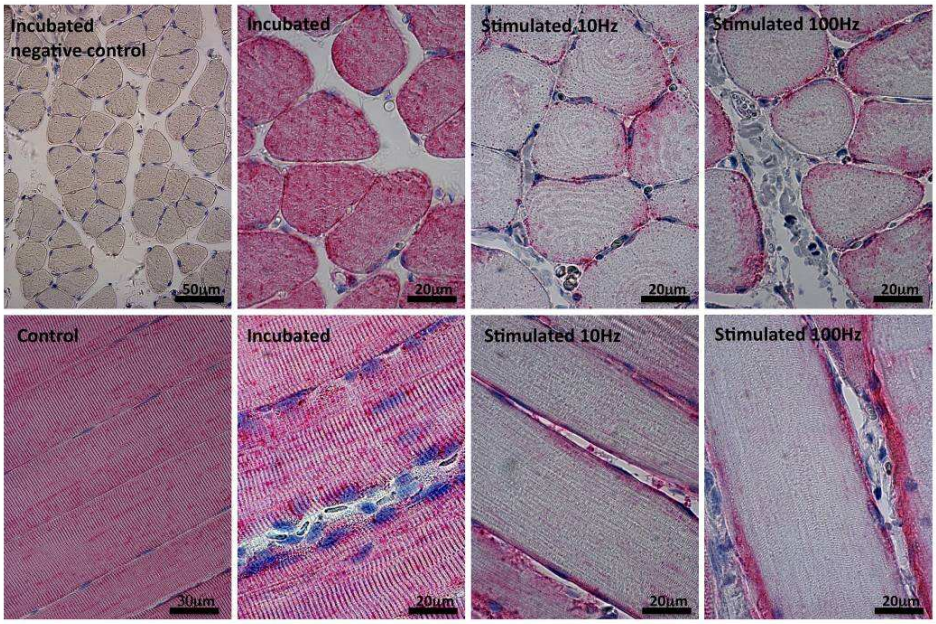

Fig 4.1.2 Representative light micrographs with GLUT4 immunoreactivity of all studied groups stained with fast red and counterstained with hematoxylin. Cross-section of negative control form muscle stimulated with 100Hz; at longitudinal section of control muscle (non-incubated control) it was observed high amount of red staining at the sarcoplasma; Incubated are cross- and longitudinal- sections, respectively, obtained from a muscle which was not stimulated to electrical stimulation, presence of few red stained at the muscle membrane. It was not observed presence of red staining into the muscle in cross- and longitudinal-sections of muscle stimulated with 10Hz frequency; and from cross- and longitudinal-sections of 100Hz stimulated muscle.

4.1.3 Morphological alterations

The *in vitro* protocol induced several morphological alterations in *soleus* muscle fibers. The incubated control muscles demonstrated the presence of a few swelled fibers (3.2±1.8%, n>300 fibers/muscle) located mainly in the centre of the muscle (Figure 4.1.3A), whereas such alteration was not found in non-incubated control muscles. Both electrical stimulation protocols, at 10Hz and 100Hz, induced a significant increase of swelled fibers compared to the incubated muscle. Quantitative analysis revealed highest

numbers of swelled fibers in muscles stimulated at 100Hz (44.6±11.5%, n>300 fibers/muscle), while only 18.2±6.6% (from n>300 fibers/muscle) of the fibers showed this feature in muscles stimulated at 10Hz (Figure 4.1.3B). Concerning the severed damage of fibers, as evaluated by eosinophilic staining, such was only observed in the stimulated muscles (Figure 3A), and the percentage of affected fibers was significantly higher in muscles stimulated at 100Hz (5.2±2.2%, n>300 fibers/muscle) compared to 10Hz (0.8±0.8%, n>300 fibers/muscle, Figure 4.1.3B).

4.1.4 Cleaved caspase-3

The absence of cleaved caspase-3 inside the fibers of non-incubated and of incubated control muscles demonstrated that the incubated process *per se* did not induce any proteolysis signal. However, the discrete presence of this protein in its active form in the interstitial space of the incubated muscles suggests the occurrence of some proteolysis in endothelial cells (Figure 4.1.4A). In contrast, the fibers of muscles stimulated either at 10Hz or 100Hz showed the presence of cleaved caspase-3 (Figure 4.1.4A). This was more frequent in the muscles stimulated at 100Hz (38.3%±7.6%, n>300 fibers/muscle) than in the 10Hz-stimulated muscles (5.1±0.8%, n>300 fibers/muscle; Figure 4.1.4B). In order to confirm the immunohistochemistry results, the western blot technique was used to detect the cleaved caspase-3 content in *soleus* muscles. In agreement with immunohistochemical data, muscles submitted to electrical stimulation presented a higher expression of cleaved caspase-3 than incubated control muscles. Furthermore, the expression of cleaved caspase-3 was significantly higher in muscles stimulated at 100Hz than in muscles stimulated at 10Hz (Figure 4.1.5).

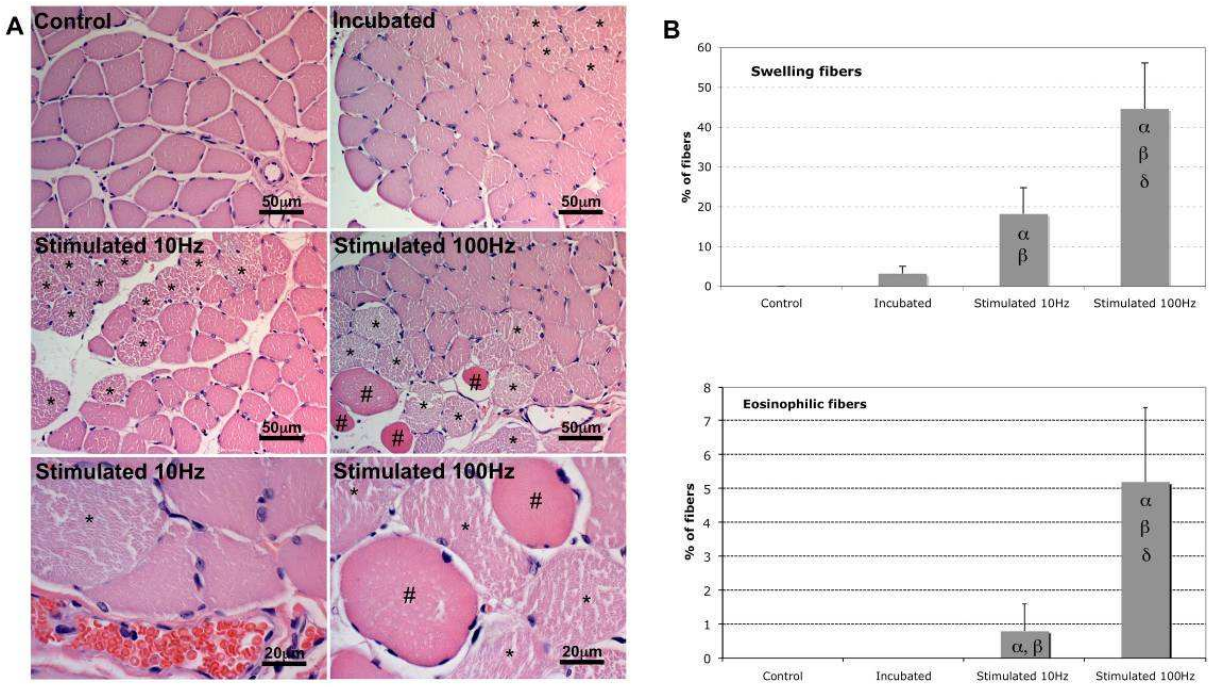

Fig 4.1.3 Representative light micrographs from sections of *soleus* muscles stained with Hematoxylin/Eosin (A) and percentage of muscle fibers evidencing swelling and degenerative signs of damage (B) observed in all the studied groups. In (A) the incubated muscles evidence a slight swelling particularly at the center of the muscle, which is depicted in the photograph at the upper position fibers; swelled fibers (*) are evident in muscles stimulated with 10Hz and 100Hz; several round huge eosinophilic fibers (#) are also depicted in 100Hz stimulated muscles. In (B) the values are given as mean ± SD (n=6 slides/group). α $p<0.05$ vs. control group; β $p<0.05$ vs. incubated group; δ $p<0.05$ vs. stimulated 10Hz group.

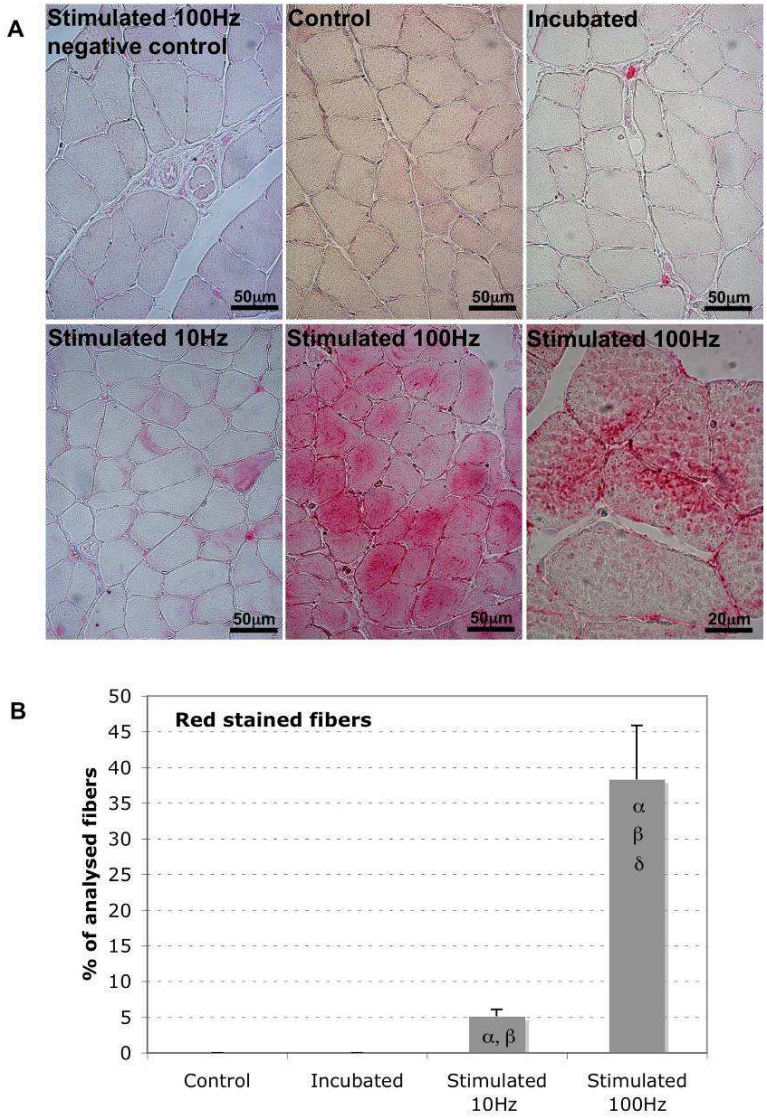

Figure 4.1.4 Representative light micrographs (A) of all studied groups stained with fast red and counterstained with hematoxylin and percentage of red stained muscle fibers (B); represents a negative control from a muscle stimulated with 100Hz; the control muscles did not evidence any red staining; in the incubated muscles red stained were observed in several interstitial cells (apparently endothelial cells). The red staining affecting muscle fibers from stimulated groups showing a granular pattern distribution in all over the fiber being predominantly in 100Hz than in 10Hz groups. In (B) the values are given as mean ± SD (n=6 slides/group). α $p<0.05$ vs. control group; β $p<0.05$ vs. incubated group; δ $p<0.05$ vs. stimulated 10Hz group.

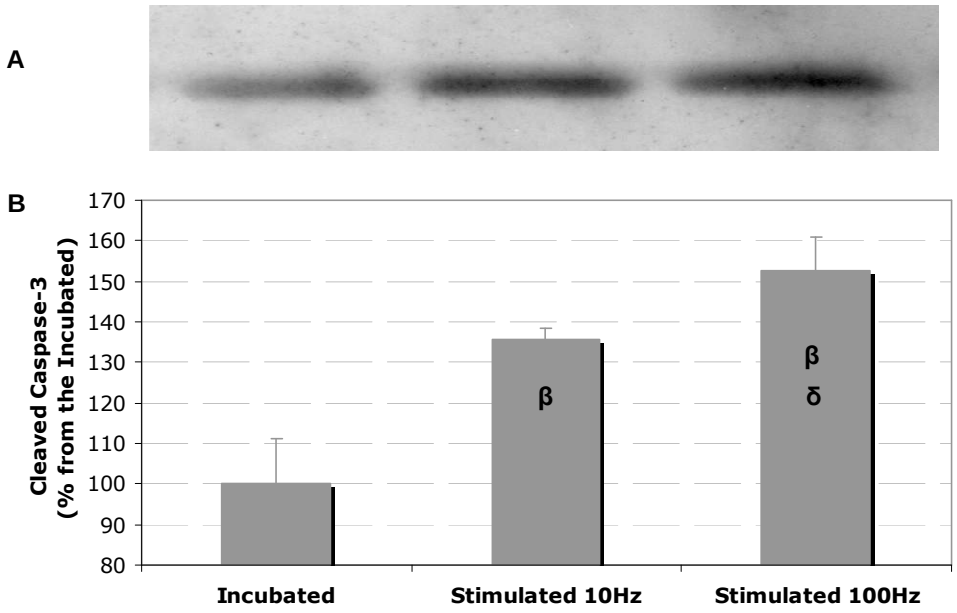

Fig. 4.1.5 Representative effect of electrical stimulation triggered by 10Hz and 100Hz protocols on cleaved caspase-3 expression in *soleus* muscle. A represents an immunoblot against cleaved caspase-3 on incubated, 10Hz and 100Hz muscles. B represents cleaved caspase-3 quantified by scanning densitometry, data are means ± SD (n=4) in incubated, 10Hz and 100Hz muscles; β $p<0.05$ vs. incubated group and δ $p<0.05$ vs. stimulated 10Hz group.

4.2 Second Study

4.2.1 General observations

Animal characteristics of body weight, muscle weight and percentage of muscular water content in the studied groups are presented in Table 4.1. The body weight and muscular weight increased between animals of 4 wks and those of 10 and 22 wks, but no change was observed in these parameters between animals of 22 and 42 wks old. The muscular water content was similar in animals of 4 and 10 wks, declined in animals of 22 and 42 wks and remained unchanged between animals of 22 and 42 wks.

4.2.2 Glucose uptake

Regarding glucose uptake on unstimulated state no differences were observed between *soleus* muscles from rats of 10, 22 or 42 wks, but it was higher in the younger group, rats with 4 wks, compared to all other groups (Fig. 4.2.1). Muscle contraction increased glucose transport in all studied groups. A higher rate of glucose uptake was induced by muscle contraction in the childhood group (4 wks) when compared to juveniles (10 wks), young adults (22 wks) and the adult old group (42 wks). A decline in glucose uptake was also observed in contracted muscles of 42 wks compared to 10 wk rats.

4.2.3 Expression of GLUT1 and GLUT4

The presence of GLUT1 and GLUT4 transporters was detected in *soleus* muscle homogenates from all studied groups. Representative blot images of GLUT1 and GLUT4 protein expression are displayed in Fig. 4.2.2. Analysis of GLUT1 expression revealed no differences between groups (Fig. 4.2.2A). Regarding the expression of GLUT4, an inverse correlation was observed between the animal's age and the level of expression (Fig. 4.2.2B). Muscles from 4 wks old rats displayed higher levels of GLUT4 in comparison to those observed in the 10, 22 and 42 wks old. A decline in GLUT4 expression was also found between muscles of 10 and 42 wks old rats, whereas no difference was found between 10 and 22 wks old rats.

Table 4.1. Animal characteristics of body weight, muscle weight and percentage of muscular water content

Groups	Body weight (g) (N=7)	Muscular weight (mg) (N=7)	Muscular Water (%) (N=14)
4 wks	83,3 ± 22,0	43,02 ± 2,39	76,6 ± 2,22
10 wks	304,0 ± 27,3 *	152,04 ± 32,8 *	76,6 ± 2,01
22wks	494,3 ± 19,99*#	203,5 ± 27,8 *	73,1 ± 1,39 *#
42 wks	558,0 ± 43,9 *#	213,34 ± 25,4 *	73,3 ± 0,52 *#

Data are mean ± Standard deviation; N represents the number of observations per group; * P < 0.05 vs. 4 wks group; # P < 0.05 vs. 10 wks group.

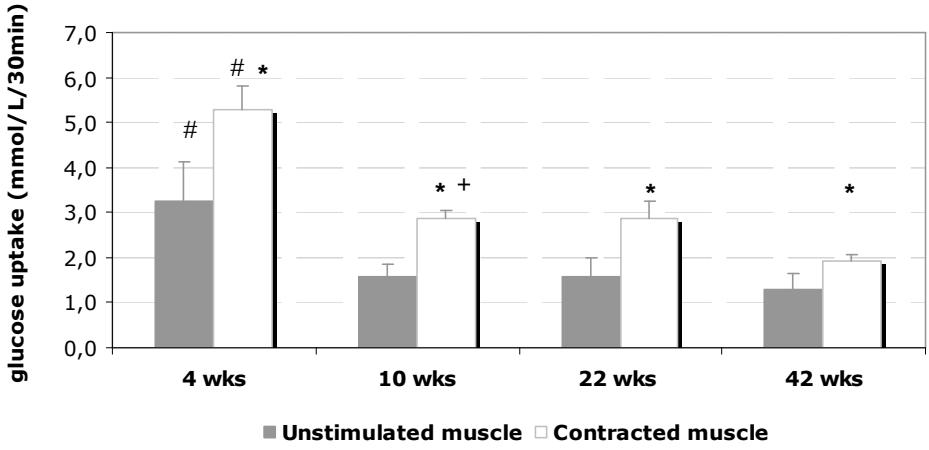

Fig. 4.2.1 Glucose uptake from *soleus* muscle in the absence (unstimulated muscle) and during muscle contraction (contracted muscle) of 4, 10, 22, 42 wks old rats. Results were obtained from 5 animals of each group: * P<0.05 vs. unstimulated muscle; # P < 0.05 vs. 10, 22, 42 wks unstimulated and contracted muscle; + P < 0.05 compared with the 42 wks contracted muscle group.

4.2.4 Localization of GLUT1 and GLUT4

GLUT1 and GLUT4 immunoreactivity was detected in sections from *soleus* muscle from all studied groups (Fig. 4.2.3). Transversal sections demonstrated that in all studied groups the GLUT1 signal (red stained) was higher in erythrocytes, interstitial cells and in the perineural layer (arrows) than within the muscle fibers. Unlike western blotting results, an age-related decrease was observed regarding GLUT1 presence within muscle fibers. A dispersed granular distribution, with a predominant location at the peripheral perinuclear areas, was observed within muscle fibers of 4 wks old rats for GLUT1 (Fig. 4.2.3). However, in older animals (10, 22 and 42 wks) the peripheral GLUT1 expression was weak. A qualitative difference of GLUT1 immunoreactivity was observed between animals of 10, 22 and 42 wks, but in all groups the difference appeared to be localized within the skeletal muscle fibers. Regarding GLUT4 localization, a scattered granular intrafiber distribution without stromal staining was detected in all studied groups. The qualitative analysis was in line with the western blot results, suggesting an apparent progressive decline of GLUT4 expression related to increasing age (Fig. 4.2.3).

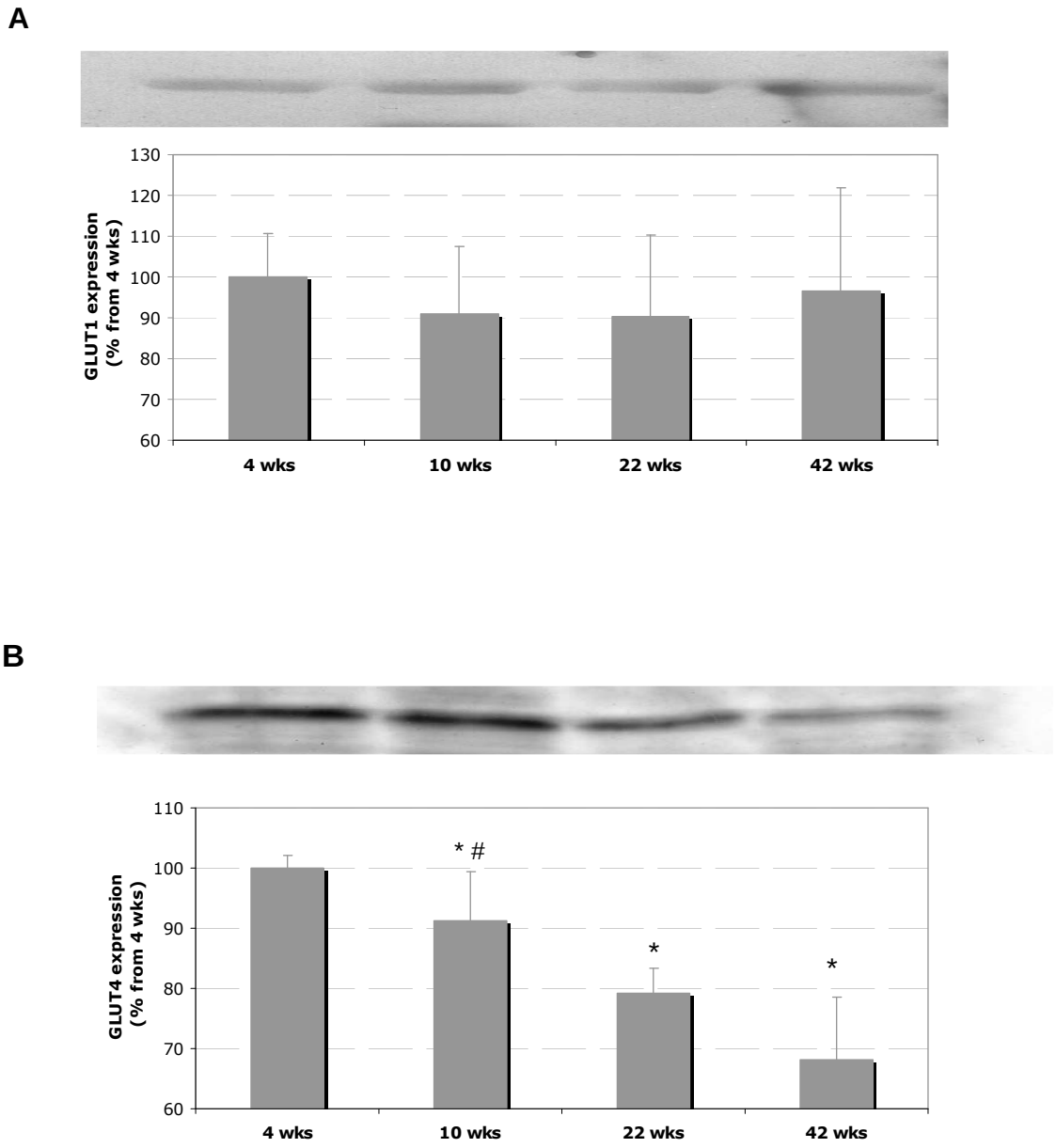

Fig. 4.2.2 GLUT1 (A) and GLUT4 (B) immunoreactive protein quantified by western blotting in *soleus* muscle from rats with 4, 10, 22 and 42 wks old. Results were obtained from 5 animal of each group and are expressed in percentage of the amount of GLUT1 and GLUT4 present in the *soleus* of 4wks old rats; * $P < 0.05$ vs. 4 wks groups; # $P < 0.05$ vs. 42 wks group.

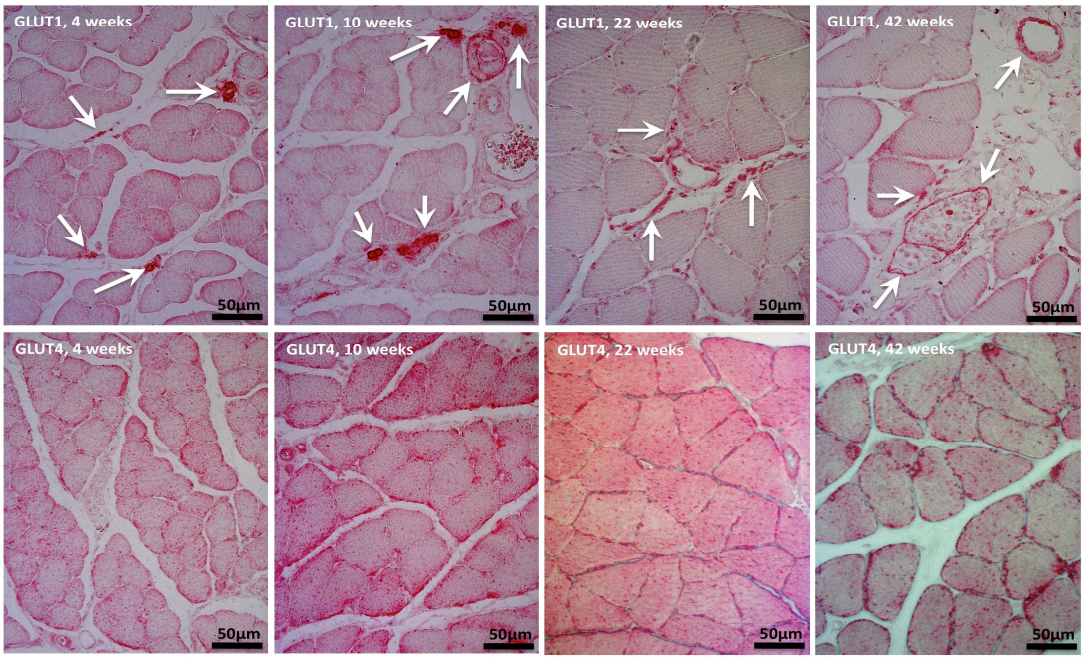

Fig. 4.2.3 Representative light micrographs of GLUT1 and GLUT4 immunoreactivity in slices of *soleus* muscles from rats of 4, 10, 22 and 42 wks old. GLUT1 and GLUT4 are stained with fast red. GLUT1 immunoreactivity is more evident in perineural sheaths and capillaries (arrows) than at the sarcolemma, with a noticeable qualitative age-related decline between groups. Considering GLUT4 immunoreactivity the red stain was spread within muscle fibers in all groups; additionally an apparent age-related decline was observed, in particular between rats of 4 wks and the other age groups.

4.2.5 Correlation between GLUTs expression and glucose uptake

No correlation was found between GLUT1 expression and glucose uptake either at the unstimulated or contracted muscle and age (Spearman test: $p<0.05$). However, a positive correlation was found between the GLUT4 expression and the rate of glucose uptake in muscles at the unstimulated muscle ($r=0.61$, $p<0.05$) or contracted muscle ($r=0.63$, $p<0.05$). In addition, a negative correlation was observed between glucose uptake in unstimulated and contracted muscle vs. age ($r=-0.76$ and $r=-0.62$ respectively, $p<0.05$) and between GLUT4 expression vs. age ($r=-0.83$, $p<0.05$).

4.3 Third Study

4.3.1 Glucose uptake

Muscle contraction induced an increase in glucose uptake compared with the uncontracted muscle in groups Control, Wortmannin and Insunin+Wortimannin conditions ($p<0.05$). But no differences in the Insulin conditions were observed as demonstrated in figure 4.3.1. However, highest rates of glucose uptake were detected in Insulin condition on the uncontracted muscle compared to the other uncontracted groups, (Control, Wortmannin and Wortmannin+Insulin) ($p<0.05$); in addition, glucose uptake after muscle contraction was also higher in Insulin groups comparing to Wortmannin and Wortmannin+Insulin groups ($p<0.05$).

4.3.2 GLUT4 localization

GLUT4 immunoreactivity was detected in sections from *soleus* muscle from all studied groups. Due to technical problems, we were not able to demonstrate immunohistochemistry data from Wortmannin and Insulin+Wortmannin (uncontracted and contracted) which might be a result of the hypothetical interference of Wortmannin with one or more substances used during the immunohistochemistry protocol. In uncontracted Control, uncontracted Wortmannin and uncontracted Insulin+Wortmannin groups, transversal and longitudinal sections, demonstrated a granular distribution of GLUT4 into the muscle fiber. Regarding uncontracted Insulin group and contracted Control, contracted Wortmannin and contracted Insulin+Wortmannin groups GLUT4 expression was located at the periphery of the fiber which clearly confirms the migration of GLUT4 to the muscle membrane. No qualitative difference was observed between these groups. These results suggest that Insulin and muscle contraction evoked by

electrical stimulation have a similar effect on the translocation of GLUT4 towards the muscle fiber membrane.

4.3.3 aPKC, phospho- aPKC expression and distribution

In the present study aPKC was expressed in all studied groups in cytosolic and in membrane fractions as demonstrated in figure 4.3.3A and 4.3.4A respectively. We were unable to detect imunoreactivity of phospho-aPKC in cytosol fraction as depicted in Fig. 4.3.3B even being detected the presence of other proteins as aPKC (Fig 4.3.3B) and β-actin in the same portion (Fig 4.3.3C). In contrast, phospho-aPKC was detected and quantified in membrane fractions of all studied groups. Representative blot images of membrane fraction are displayed in Fig. 4.3.4B. Analysis of phospho-aPKC expression in the membrane fraction demonstrated a significative difference between treatment (uncontracted vs. contracted muscles) in Control and Wortmannin groups ($p<0.05$). In uncontracted groups differences were found in Insulin treatment comparing with the Control, Wortmannin and Wortmannin+Insulin groups ($p<0.05$).

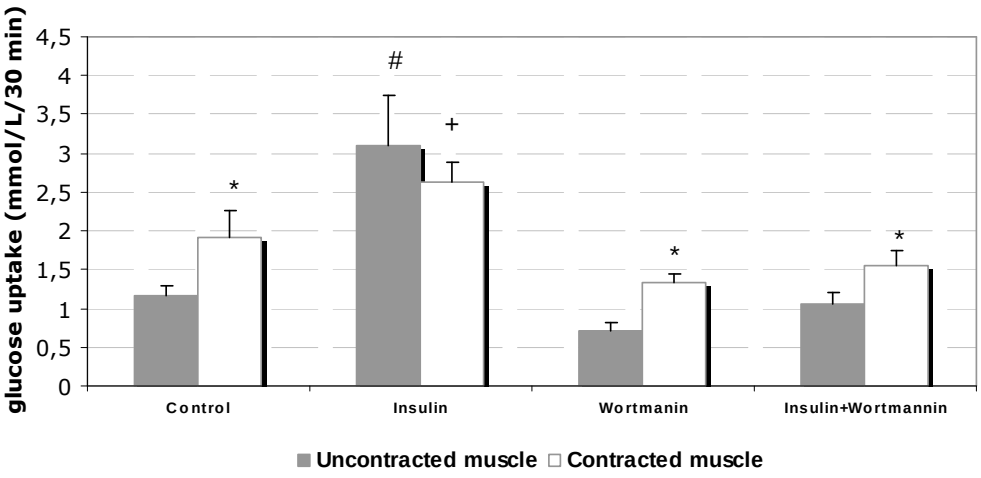

Fig 4.3.1 Glucose uptake from *soleus* muscle in uncontracted muscle (Control, Insulin, Wortmannin, Insulin+Wortmannin) and contracted muscle (Control, Insulin, Wortmannin, Insulin+Wortmannin) groups. Results were obtained from 5 animals of each group: * $p<0.05$ vs. uncontracted muscles; # $p< 0.05$ vs. uncontracted Control muscles, Wortmannin and Insulin+Wortmannin groups; + $p< 0.05$ vs. contracted Wortmannin and Insulin+Wortmannin groups.

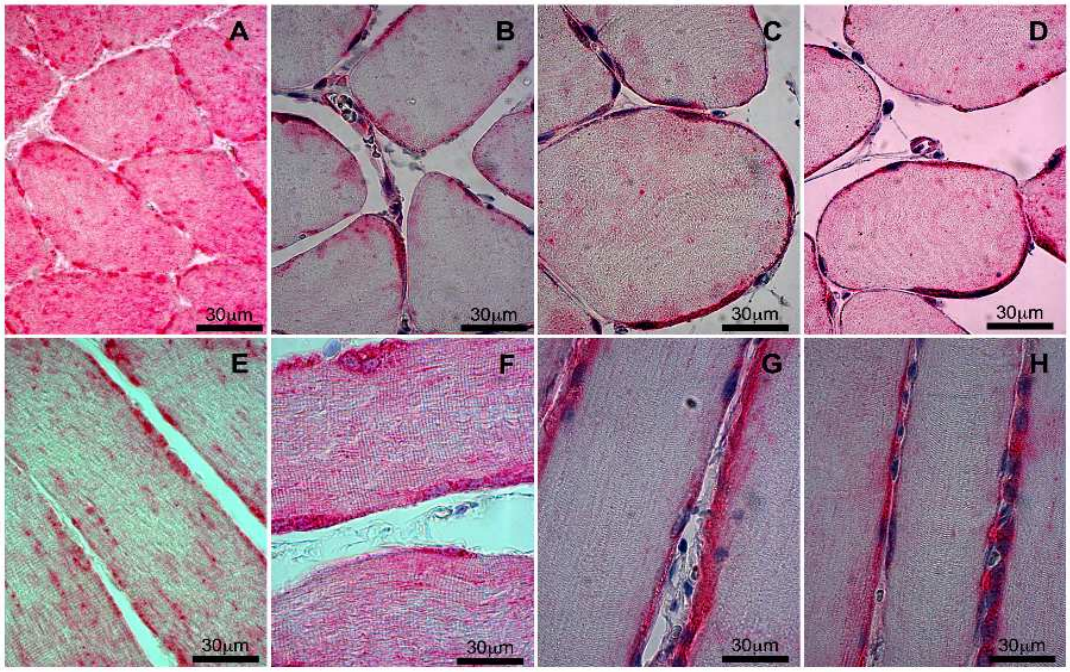

Fig 4.3.2 Representative light micrographs of GLUT4 immunoreactivity in transversal and longitudinal section of *soleus* muscle stained with fast red and counterstained with hematoxylin. Letter A and E represents transversal and longitudinal sections of uncontracted Control group; letter B and F represents transversal and longitudinal sections of contracted Control group; letter C and G represents transversal and longitudinal sections of uncontracted Insulin group; letter D and H represents transversal and longitudinal sections of contracted Insulin groups. Comparing to B and F it is notorious the GLUT4 migration to the peripheral fiber areas induced by muscle contraction (B and F), insulin (C and G) and both stimuli (D and H)**.**

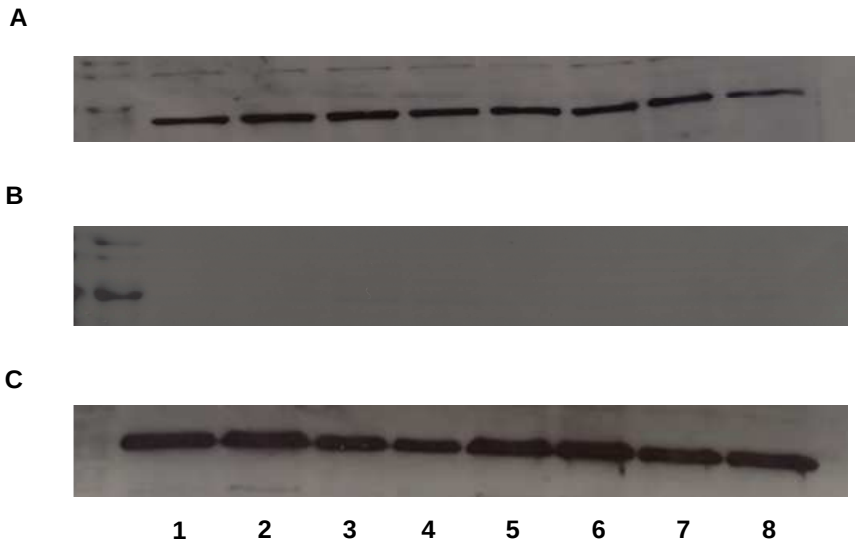

Fig. 4.3.3 Immunoreactive of aPKC (A), phopho-aPKC (B) and β-actin (C) protein from cytosolic fraction of all studied groups: uncontracted Control (1), contracted Control (2), uncontracted Insulin (3), contracted Insulin (4), uncontracted Wortmannin (5), contracted Wortmannin (6), uncontracted Insulin+Wortmannin (7) and contracted Insulin+Wortmannin (8). Even detecting the presence of aPKC and β-actin, we were unable to detect immunoreactivity of phospho-aPKC in cytosolic fraction of *soleus* muscle in three samples of each group.

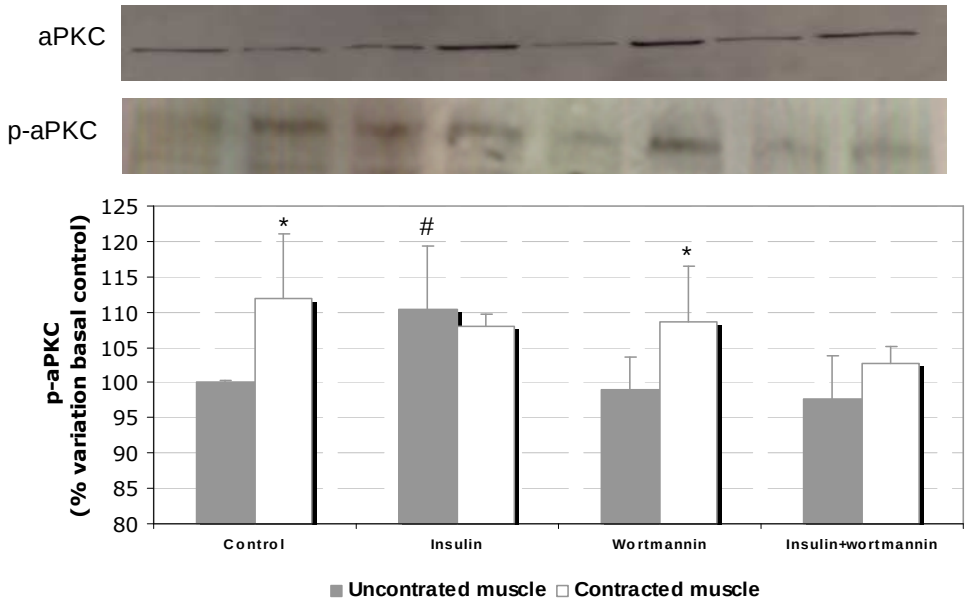

Fig 4.3.4 aPKC (A) and Phospho- aPKC (B) immunoreactivity protein quantified by western blotting in membrane fraction from all studied groups. Data from Phospho-aPKC (p-aPKC) were obtained from 3 sample from each group and are expressed in percentage of the amount of p-aPKC present from the uncontracted Control group; * $p < 0.05$ vs. respective uncontracted into the treatment; # $p < 0.05$ vs. uncontracted Control, Wortmannin, Insulin+Wortmannin.

CHAPTER 5

DISCUSSION

5. DISCUSSION

The present work demonstrates that muscle contraction triggered by electrical stimulation at frequency of 100Hz induces higher rates of glucose uptake in the isolated *soleus* muscles than when the muscle is stimulated at 10Hz. The higher rates of glucose uptake could be merely explained through physiologic mechanisms associated both with the higher GLUT4 vesicle translocation to the sarcolemma or with the higher number of activated fibers triggered by stimuli at 100Hz. However, the percentage of damaged muscles fibers, the expression of caspase-3 and the similar distribution of GLUT4 in both stimulation groups, suggest that other mechanisms related to increased sarcolemma permeability may contribute to explain differences of glucose uptake between muscles stimulated at 100Hz and 10Hz, respectively.

In addition, this work also shows that age has a negative impact on muscle glucose uptake either at basal conditions and when muscle contraction was induced by electrical stimulation. This decline is justified, at least in part, by an age-related decline of the GLUT4 expression. In addition, our results also demonstrate that GLUT1 expression does not seem to contribute to the negative correlation between increasing age and in muscle glucose uptake.

Finally, it was demonstrated that both muscle contraction and insulin promote an enhancement on glucose uptake. However, an additional effect of these stimuli on the enhancement on glucose uptake in *soleus* muscle was not observed, which supports the hypothesis for the existence of a common point between these two stimuli. Moreover, it was also observed that both insulin and muscle contraction promote an increase in aPKC phosphorylation, the active form of aPKC, without a synergetic effect of these two stimuli on aPKC activity. Accordingly, these findings point out aPKC as a convergence point between muscle contraction and insulin. Thus, these results do not support the

hypothesis of the existence of two independent pathways for increase glucose uptake in skeletal muscle, one triggered by insulin and another triggered by contraction, as previously suggested by other authors (Douen et al. 1990; Goodyear and Kahn 1998).

Considering the protocols and objectives of the three studies, we will firstly discuss the option for a low frequency protocol used in the second and the third studies; following we will discuss the age influence on skeletal muscle GLUTs (GLUT1 and GLUT4) expression and the relationship of these proteins with glucose uptake; finally the role of aPKC on insulin- and contraction- induced glucose uptake and the possible role of this protein as a convergence point between these stimuli will be discussed.

5.1 Glucose uptake and muscular damage

Early studies demonstrated that the rate of glucose uptake in a muscle exposed to insulin stimulus is dependent on the fiber-type composition, with muscles dominantly composed by type I (slow-twitch), as *soleus*, displaying the highest comparing with those composed by type II fibers (Henriksen et al. 1990). This relationship is directly dependent upon the relative abundance of GLUT4 being higher in slow-twitch muscles (Henriksen et al. 1990). Considering these findings, which clearly demonstrated the high capacity of *soleus* muscle to uptake glucose, this muscle was chosen for the present work.

It has been demonstrated in *soleus* muscle that muscle contraction triggered by different protocols promotes the translocation of GLUT4 with a consequent enhancement of glucose transport (Lund et al. 1995; Aslesen et al. 2001; Sakamoto et al. 2004; Jensen et al. 2008). However, it is important to highlight that none of these studies concomitantly analysed the effect of the *in vitro* contraction protocol on muscular integrity. The data presented in this work noticeably demonstrated that the increase in

glucose uptake rates was coincident with several morphological and biochemical modifications in skeletal muscle fibres.

For instance, the presence of eosinophilic fibers, which are a good marker of fiber damage (Guo et al. 2006), was observed only in muscles submitted to electrical stimulation, with a higher percentage of affected fibers in muscles stimulated at 100Hz. Additionally, the presence of swelled fibers, suggesting an increased sarcolemma permeability (Shennan 2008), was also one of the changes observed, in particular in muscles stimulated at 100Hz. These results clearly suggest that the *in vitro* stimulation is harmful to muscles, and the contraction evoked by electrical stimulation at 100Hz is more deleterious to cell structure and membrane permeability than the contraction evoked by 10Hz stimulation. It appears noteworthy to mention that even the *in vitro* incubation itself has slightly harmful effects on muscle fibers.

It is widely accepted that the loss of cellular membrane integrity promotes efflux of intracellular substances such as creatine kinase and acid lactic dehydrogenase (Fredsted et al. 2005; Fredsted et al. 2007). Fredsted et al. (2007) demonstrated that inappropriate *in vitro* electrical stimulation protocol promotes loss of skeletal muscle cell integrity inducing an efflux of acid lactic dehydrogenase (LDH). Accordingly, if LDH leaks out of the fiber by modification of membrane permeability, it might be expected that substances with lower molecular weight located in the incubation medium, such as glucose, might also passively cross the membrane according to its gradient and enter into the fibers without the interference of the GLUT4 carrier. The similarity of GLUT4 distribution at the periphery of fibers in both 100Hz and 10Hz stimulated muscles and the percentage of damage fibers, support the assumption that other mechanisms apart from GLUT4 (a non-physiological) might have contributed to the enhanced rate of glucose uptake observed in muscles stimulated at 100Hz.

The arguments supporting this non-physiological mechanism are strengthened by the results of cleaved caspase-3 assays. Electrical stimulation acutely induces an increased expression of cleaved (activated) caspase-3, as it was previously demonstrated (Biral et al. 2000). Our study also showed that when a frequency of 100Hz is applied, the presence of cleaved caspase-3 is observed in almost half of muscle fibers, suggesting a reasonable catabolic state in muscles stimulated at 100Hz. Although the activation of caspase-3 might be closely associated with the phenomenon of apoptosis (Fuentes-Prior and Salvesen 2004; Chung and Ng 2006) it is important to refer that it may also be the consequence of an abnormal concentration of Ca^{2+} within muscle fibers. Compared to fast twitch fibers (which contract at high frequencies), slow twitch muscle fibers, like those present in *soleus*, are characterized by lower quantity of sarcoplasmic reticulum (SR)-Ca^{2+} pumps and lower levels of calcium buffering proteins, such as parvalbumin, which reduce the ability of these muscle fibers to re-establish Ca^{2+} homeostasis during high-frequency induced contractions (Berchtold et al. 2000; Bassel-Duby and Olson 2006). Therefore, it seems possible that Ca^{2+}-overload in the fiber also explains the loss of muscle integrity and the release of intracellular proteins, in accordance with previous reports (Fredsted et al. 2005; Fredsted et al. 2007). Calcium overload also activates phospholipase A_2 (PLA_2) which metabolizes membrane phospholipids to arachidonic acid and lysophospholipid, thereby promoting the loss of membrane integrity and function (Cummings et al. 2000).

As to our experimental protocol we should recall the fact that stimulation at 10Hz lasted 30 minutes while at 100Hz lasted only 10 minutes. This protocol was chosen in accordance with other research groups working on this topic (Dolan et al. 1995; Hayashi et al. 1999; Wright et al. 2004; Wright et al. 2005). The difference in the duration of the stimulation period reinforces our considerations about the harmful effects

of high frequency stimulation of slow-twitch muscles. Tripling the duration of physiological stimulation (stimulation at 10Hz, see above) did not bring about a reasonable "structural failure" like that observed in muscles stimulated at 100Hz. Moreover, in this first study the option to use the complete and therefore intact *soleus* instead of small splits of this muscle, as used in other glucose transport studies (Henriksen et al. 1990; Aslesen et al. 2001) was chosen because cutting the muscle for this procedure will certainly damage many muscle fibers. Additionally, the split muscle procedure is recommended for easy comparisons of muscles with different sizes and fiber-types (Henriksen and Holloszy 1991), which was not the case in our experimental design. In fact, in all groups of the first study the used, *soleus* muscle, did not show significant difference of weight between groups. We cannot exclude the possibility of limited substrate diffusion into different zones of the muscle, especially for the centre of the muscles as suggested by the morphological analysis of incubated muscles. However, if such occurred in our muscles, the existence of a control group (unstimulated muscle) in the experimental design allowed neglecting this condition.

Considering the results of the first study which clearly demonstrate that the *in vitro* muscle contraction at 10Hz induced increase in glucose uptake with only a slight injury to *soleus* fibers, while stimulation at higher frequencies (100Hz) is harmful to these muscle fibers the 10Hz frequency was the preferred protocol to induce muscle contraction for the next studies.

5.2 The influence of age on glucose uptake

Regarding the effect of age on glucose uptake, a negative correlation between insulin stimulus and age have been well documented by studies developed during the last decades (Fink et al. 1983; Gulve et al. 1993; Ryan 2000; Nair 2005; Watters 2005;

Qiang et al. 2007), showing the occurrence of a decrease in muscular glucose transport during growth and development. In hindlimb perfusion studies, muscle glucose transport, either in the presence of insulin or in its absence, rapidly decreases over the first weeks of life (Goodman et al. 1983; Ivy et al. 1989; Gulve et al. 1993). Our results at basal conditions (unstimulated muscles) glucose uptake are consistent with these findings since in contrast to the group aged 4 wks, a significant reduction of glucose uptake was observed in older animals. In addition, it was also demonstrated that, like the effect mediated by insulin, the contraction-stimulated glucose uptake is affected by growth as evidenced by its reduction in juvenile (10 wks) and adult rats (22 wks and 42 wks) compared to rats in early development (4 wks old). Moreover, age seems to have an influence on muscle contraction-induced glucose uptake since a difference between juvenile (10 wks) and an old adult (42 wks) rat was observed. In our opinion, this difference might be better explained by the growing process itself rather than the aging process *per se* since no difference was observed between adult young (22 wks) and adult old (42 wks) rats.

The exact mechanism leading to the age-related decrease in the ability to uptake glucose is still lacking. Regarding the age associated insulin resistance, several mechanisms have been proposed such as fatty acid toxicity, increase in muscle atrophy and a decrease of GLUT1 and/or GLUT4 expression (Houmard et al. 1995; Nair 2005; Qiang et al. 2007). Lipid toxicity does not seem to influence muscle glucose uptake induced by contraction (Wheatley et al. 2004) and skeletal muscle of the juvenile and adult animals did not show any atrophy process since no difference of intact muscle weight was observed in rats 22 and 42 wks old. Therefore, the most likely explanation for the age related decline on glucose transport stands on a reduction of GLUT4 and/or GLUT1 expression.

GLUT4 is expressed exclusively in skeletal muscle and in fat cells, being the main targets for glucose uptake in these tissues (Guillet-Deniau et al. 1994). Moreover, it is also known that insulin and exercise are the main stimuli inducing an increase in the rate of glucose uptake in skeletal muscle (Saltiel and Kahn 2001; Santos et al. 2008). In healthy adult animals, a positive correlation was found, for both stimuli, between the total amount of intracellular GLUT4 and muscular ability to uptake glucose (Henriksen et al. 1990; Lund et al. 1995). Therefore, changes in GLUT4 expression were explored in several studies analysing the age-related changes in insulin resistance. These studies showed that the decrease of muscular GLUT4 content accounts for the decrease in glucose uptake observed during the growth phase (Gulve et al. 1993), which is in accordance with our results on muscular glucose uptake and GLUT4 expression in the childhood group and adult old group. A decline in GLUT4 expression with increasing age is not a consensual issue (Santalucia et al. 1992; Gulve et al. 1993; Houmard et al. 1995), being the different methodologies used a potential reason to explain these divergent results. When membrane fractions were used as models, no correlation was found between GLUT4 expression and increasing age (Santalucia et al. 1992), contrasting with studies in which the GLUT4 expression was quantified in whole homogenized tissues (Gulve et al. 1993; Houmard et al. 1995). In agreement with previous results (Gaster et al. 2000c) our imunohistochemistry analysis of *soleus* muscle suggests a decrease of muscular GLUT4 expression with age, showed by the granular pattern in the central areas of fibers, resembling GLUT4 vesicles, which in adult animals are mainly located in the subsarcolemmal zone near to the nuclei. This observation is also supported by western blotting analyses of the GLUT4 expression in animals of different ages. In addition, a significant correlation could be drawn between the age-

related changes of GLUT4 expression and glucose uptake in the contracted muscles and the unstimulated muscles, supporting the involvement of this protein on glucose uptake.

GLUT1 has an ubiquitous distribution and it was suggested that it may give an important contribution to glucose uptake in skeletal muscle, being the main transporter in the basal state (unstimulated) (Guillet-Deniau et al. 1994; Marshall et al. 1999; Ciaraldi et al. 2005). Considering this information, the GLUT1 expression and localization was also investigated in the current study in an attempt to explain variations on glucose uptake under unstimulated conditions. A weak expression of GLUT1 in the total muscle was found in all groups under investigation, without significant differences between them. However, our morphological results suggest that variations in GLUT1 content in the muscle fibers may occur with ageing. A careful analysis of the immunohistochemical results demonstrated that the main GLUT1 immunoreactivity is located outside the muscle fibers, suggesting that contamination with interstitial cells, which contain high amounts of GLUT1, masked the possibility to detect small variations of GLUT1 expression within muscle fibers in western blot.

In order to discriminate the localization of GLUT1 in the muscle fibers, highly sensitive immunohistochemistry protocols were used, excluding the presence of this protein inside the sarcoplamma of adult animals (Gaster et al. 2000a; Gaster et al. 2000b). According to these studies, the presence of GLUT1 can only be observed within muscle fibers in animals during growth and development phases, suggesting that its presence is confined to this stage in order to ensure an insulin independent glucose transport to sustain the high energetic demands characterizing this stage of life (Gaster et al. 2000a; Gaster et al. 2000b). Our morphological results confirm these observations showing the presence of GLUT1 in the muscle fibers in the childhood group, which supports the results of unstimulated muscles glucose uptake, but the signal almost

disappeared with increasing age. Unlike GLUT4, no correlation was found between GLUT1 expression and age or glucose uptake under unstimulated conditions or after muscle contraction. Therefore, the results obtained with the present study support the hypothesis that the main responsible for unstimulated glucose uptake in mature skeletal muscle fibers is GLUT4 (Handberg et al. 1992; Santalucia et al. 1992; Martineau et al. 1999; Gaster et al. 2000a; Gaster et al. 2000b) rather than GLUT1, as previously suggested (Marette et al. 1992; Marshall et al. 1999; Ciaraldi et al. 2005).

It has been suggested that the increased rate of muscle glucose uptake observed *in vitro* in childhood rats at unstimulated basal state and after muscle contraction might be explained by the small muscle size at this age (Henriksen and Holloszy 1991). According to this proposal, in smaller muscles, like those of 4 wks old rats, the central areas of the muscles might have a better contact with interstitial glucose than the central areas of larger muscles. Therefore, to reduce any possible overestimated result of glucose uptake promoted by the difference of diffusion distances, muscles of older animal (10, 22 and 42 wks) were split in order to analyse samples with similar volume (same wet weight).

In summary, the *soleus* muscles evidenced an age-related reduction of skeletal muscle GLUT4 expression that paralleled its ability to uptake glucose at the unstimulated state and after muscle contraction. In addition, it was demonstrated with the second study that the GLUT1 expression was mainly confined to muscle fibers in muscles in growth and development, which might contribute in part to the increased rates of glucose transport observed during this phase of life. Although other mechanisms may contribute, our findings clearly indicate that in *soleus* muscle of rats the age-related decline in glucose uptake is directly dependent on the level of GLUT4 expressed by muscle fibers. Since any modification on glucose uptake and GLUT4 expression

between 10 and 22 wks was observed, in the third study we chose *soleus* muscle of rats between these ages.

5.3 The role of aPKC on glucose uptake

The regulation of glucose transport is complex and requires multiple signalling molecules which result on GLUT4 vesicle translocation through the plasma membrane of the skeletal muscle. As in the first and in the second studies of this work, the third study also demonstrated an increase in glucose uptake triggered by muscle contraction in absence of insulin. This result is consistent to previous findings (Lund et al. 1995; Aslesen et al. 2001; Sakamoto et al. 2004; Jensen et al. 2008). It was also observed a similar increase in glucose uptake rates on muscles that have been activated by insulin as depicted in figure 4.3.1. As expected, these enhancements were directly related to redistribution on GLUT4 from the cytoplasm to the muscle membrane, observed in the present work at immunohistochemistry analysis. No qualitative difference was observed between insulin and contraction regarding GLUT4 translocation.

Moreover, a great number of studies reported a synergistic effect of muscle contraction- and of insulin-induced glucose uptake triggered by multiple signalling cascades (Wallberg-Henriksson et al. 1988; Richter et al. 1989; Cartee and Holloszy 1990; Douen et al. 1990; Helmrich et al. 1991; Dolan et al. 1993). These results initially suggested the existence of a pool completely different from the molecules triggered by these two stimuli: insulin and muscle contraction. However, it is important to highlight that none of these studies analysed glucose uptake when both stimuli (*in vitro* muscle contraction and insulin stimuli) were applied in simultaneously. Therefore, since several authors suggest the existence of a completely different pathway, it could be expected an additive effect of muscle contraction when muscle was already being stimulated by

insulin. Contrary to this idea, the data of the present work did not observe any cumulative effect of these stimuli on the rates of glucose uptake in *soleus* muscle. In agreement to the glucose uptake result, the immunohistochemistry data did not detect any difference on GLUT4 localization between muscles which has been activated by insulin and by muscle contraction. Furthermore, no synergetic effect of these two stimuli was observed on GLUT4 translocation. This result clearly contradicts the earliest concept of different routes to induce glucose uptake in skeletal muscle. The absence of an additive effect glucose uptake might be better justified by the existence of convergence points between these two stimuli. Therefore one or more substances activated either by insulin and muscle contraction would limit the maximal glucose uptake and consequently the ability to further enhancement of glucose when these stimuli act simultaneously.

It is important to emphasize that the hypothesis of an additive effect between these stimuli came from studies which stimulated *soleus* muscle with a high frequency of stimulation (Douen et al. 1990; Hansen et al. 1994). Thus, we can not discard the possibility of an over estimated result of glucose uptake on these studies triggered by an artificial entrance of glucose to the fiber from a non-physiological mechanism (loss of membrane integrity) as it was demonstrated in our first study of this. Therefore, the difference in the applied protocol could be the potential reason to explain the disagreement on the results of the third study and the previously reports (Douen et al. 1990; Hansen et al. 1998) regarding the additive effect of insulin and muscle contraction.

Since PI3K has been well depicted as an essential protein on insulin cascade and the inhibition of this protein represents a model to mimic insulin resistance on *in vitro* studies (Saltiel and Kahn 2001; Sigal et al. 2004), different laboratories observed the

effect of a selective PI3K inhibitor, Wortmannin, on muscle contraction-induced glucose uptake (Goodyear et al. 1995; Lee et al. 1995; Wojtaszewski et al. 1996; Whitehead et al. 2000; Wright et al. 2006). As a result, the majority of them did not observe any impairment on muscle contraction-induced glucose uptake in presence of Wortmannin (Goodyear et al. 1995; Lee et al. 1995; Whitehead et al. 2000; Wright et al. 2006). However, Wojtaszewski et al. (1996) demonstrated that high concentration of Wortmmanin blocks the effect of muscle contraction to induce an increase in the rates of glucose uptake by the muscle. Nevertheless, it was also observed a diminution on the capability of muscle to contract in response to electrical stimulation which might explain the difference between these studies. In agreement with Goodyear et al. (1995), Lee et al. (1995) Whitehead et al. (2000) and Wright et al. (2006), the present study also did not report an impairment on contraction induced glucose uptake in presence of Wortmannin. In addition, we observed that muscle contraction attenuate the inhibitory effect of Wortmannin on insulin-induced glucose uptake as reported previously (Lee et al. 1995). Alltogether, the results suggest that PI3K is not a convergence point between muscle contraction and insulin cascades. However, this finding did not point out the existence of a common swift between these stimuli, instead suggests that it is one of downstream regulators of PI3K on insulin cascade as aPKC and/or AS160.

Several recent studies well establish that AS160 can be activated by other stimuli apart from insulin, such as: muscle contraction *in vivo* (Deshmukh et al. 2006), *in vitro* (Bruss et al. 2005) and *in sito* (Kramer et al. 2006)*;* by AMPK pharmacological activators as AICAR (Treebak et al. 2006); and by the increase in sarcoplasmatic calcium, triggered by K^+ depolarization (Thong et al. 2007). These results clearly point out the role of AS160 as a potential connection between the mechanisms which contraction-induced enhance on glucose uptake (activates AMPK and increase in

sarcoplasmatic calcium) and as being a convergence point in skeletal muscle between the cascade induced by muscle contraction and insulin for induce GLUT4 translocation to the plasma membrane.

Regarding PKC, early data demonstrated that PKC become more active during contracted skeletal muscle of rat suggesting that such effect occurs with the translocation of PKC to a membrane-enriched fraction from a cytosolic fraction (Cleland et al. 1989). However, the last study did not designate which PKC isoform was more active after contraction. Moreover, using a more refined methodology, it was also suggested that the presence of the specific aPKC isoform in muscle membrane fractions was confined to skeletal muscle which received a glucose uptake stimulus, insulin or muscle contraction (Perrini et al. 2004). In contrast to these results, the present study observed the presence of aPKC in membrane fraction in all groups, even in the muscles that were not submitted to any type of stimulus (insulin or muscle contraction). This result is in agreement to data reported by Beeson et al. (2003). Moreover, in our study the presence of aPKC was also detected in the cythosol of all the studied groups, which is in accordance with previous results (Perrini et al. 2004; Rose et al. 2004).

In this decade several studies reported *in vivo* that aPKC become more active after exercise (Beeson et al. 2003; Nielsen et al. 2003; Perrini et al. 2004; Richter et al. 2004; Rose et al. 2004). Using a similar exercise methodology, bicycle exercise, four studies reported an increase on aPKC activity when comparing the contracted with the resting leg (Beeson et al. 2003; Nielsen et al. 2003; Richter et al. 2004; Rose et al. 2004). In agreement to these studies, Perrini et al. (2004) detected an increase in aPKC-phosphorylation (p-aPKC) expression in membrane fractions of skeletal muscle from subjects after muscle contraction. Using a western blotting technique these authors also suggested a translocation of p-aPKC activated by muscle contraction. Additionally, a

weak signal of p-aPKC was detected in cytosolic fraction the in rested leg muscle (Perrini et al. 2004). This result suggested that the increase in the concentration of p-aPKC in membrane fraction is a valid toll to analyse a modification on aPKC activity induced by muscle contraction in skeletal muscle. In contrast, Rose et al (2004) did not observe modification on p-aPKC expression triggered by exercise either in membrane or in cytosolic fraction despite modification in aPKC activity being found between controls and exercised muscle. Our results are in agreement with Perrini et al`s (2005) findings since no expression of p-aPKC was detected in cytosolic fraction and the increase on p-aPKC expression was found in the sarcolemma of contracted muscles. Furthermore, the present data also demonstrated an increase in p-aPKC in membrane fraction of *soleus* muscle after insulin stimulus and no cumulative effect of muscle contraction and insulin stimuli on p-aPKC translocation. Moreover, it was demonstrated that this increase of p-aPKC expression induced by muscle contraction was in similar magnitude to insulin stimulus. Therefore, these results clearly point out aPKC as a potential convergence point between pathways triggered by muscle contraction and by insulin and also suggested aPKC as a rate limited protein on the mechanism through these stimuli which induce an increase in glucose uptake in skeletal muscle.

The exact mechanism through which muscle contraction activates aPKC is not completely identified. When activated by insulin, aPKC activation is a product of the generation of phospholipids as phosphatidylinositol trisphosphate, PIP3, which is dependent on PI3K activity (Beeson et al. 2003). Diminished IRS-1/PI3K induced a decrease on aPKC activity in skeletal muscle during insulin resistance and DMT2 in monkeys, humans and rodents (Farese 2002; Farese et al. 2005A; Farese et al. 2005B; Farese et al. 2007). Besides which, Beeson et al. (2003) demonstrated that aPKC activity is defective in humans DMT2 and the inhibitory effect on this protein can be attenuated

by exercise without modification on PI3K activity. In addition, it was demonstrated that phosphatidic acid increases aPKC activity *in vitro* via phospholipase-D production in a mechanism independent of PI3K (Chen et al. 2002). Moreover, the increase on phosphatidic acid concentration was also previously demonstrated in hindlimb muscles of rodents after nerve electrical stimulation (Cleland et al. 1989). The referred results suggest that the activation of aPKC induced by muscle contraction does not seem to be mediated by PI3K instead dependent on phosphatic acid. In the present study no change was observed on the ability of muscle contraction to activate aPKC in presence of Wortmannin. In addition, the present work demonstrated that Wortmannin inhibited insulin-induced aPKC activity confirming the role of PI3K on this process. In fact, despite not being statistically significant a tendency on p-aPKC expression was verified between uncontracted and contracted muscles on the insulin plus Wortmannin group, suggesting that the inhibitory effect of Wortmannin on insulin-activated aPKC was attenuated by muscle contraction. Thus, in agreement with previous studies (Chen et al. 2002; Beeson et al. 2003) our results suggested that PI3K activation is unnecessary to the mechanism whose contraction induces aPKC activity as well as in contraction-induced glucose uptake since the effect of contraction was not impaired with PI3K inhibition by Wortmannin.

In summary, the results of this work clearly demonstrated that the rates of glucose uptake in the skeletal muscle in response of muscle contraction and insulin stimuli is directly related to the increase in aPKC phosphorylation in membrane fraction of *soleus* muscle from Wistars rats. On balance, as AS160, aPKC seems to be a common terminal switches that leads to GLUT4 translocation and enhance of glucose uptake triggered by insulin and muscle contraction. In addition, these results suggest the role of aPKC as a rate limiting protein on glucose uptake process. Keeping this idea in mind the

initial concept of two independent pathways for these two stimuli should be modified, the enhancement on glucose uptake induced by muscle contraction might be better explained by the effect of contraction stimulus on molecules which mediate insulin cascade. Therefore, this dissertation does not support the concept of the existence of two independent pathways to increase glucose uptake in skeletal muscle induced by insulin and by contraction.

CHAPTER 6

FINAL CONCLUSIONS

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Based in the general conclusions of each of the studies presented in this dissertation, it seems possible to emphasize the following major conclusions:

a) The rates of glucose uptake by *soleus* muscle increase with enhance of the frequency of stimulation. However, in slow-twitch muscles the increased intramuscular glucose concentration triggered by electrical stimulation at 100Hz might not only be explained by glucose uptake promoted through GLUT4 but also by the artificial enhancement of muscle membrane permeability;
b) The expression of GLUT4 in *soleus* muscle decreases during the life span which contributes to a decrease in the ability of this muscle to uptake glucose at the unstimulated state and after muscle contraction;
c) GLUT1 expression into muscle fibers is mainly confined to muscles in growth and development, which might contribute in part to explain the increased rates of glucose transport observed during this phase;
d) Muscle contraction enhances glucose uptake in a similar rate of insulin independently of PI3K. There is no additive effect of insulin and muscle contraction on inducing an enhancement on glucose uptake;
e) Phospho-aPKC translocates to the plasma membrane when activated by insulin and by muscle contraction and seems to be associated with the ability of these stimuli to increase glucose uptake in *soleus* muscle.

In summary, this dissertation presents evidences supporting the concept that aPKC is a convergence point between the pathways triggered by muscle contraction and

by insulin to activate GLUT4 translocation and to increase glucose uptake in *soleus* muscle of adult Wistars rats. These findings suggest the non existence of two independent cascades triggered by insulin and by contraction to increase glucose uptake in skeletal muscle.

CHAPTER 7

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