Investigation of the effects

Of thrombin and electrical pulse stimulation on metabolic function in cultured human skeletal muscle cells

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ABSTRACT

Hyper-coagulability (elevated thrombin) is a feature of T2D. There is an emerging evidence of a correlation between the genetic basis of hypercoagulation and T2D. We hypothesized that thrombin affects insulin activity and/or exercise responses in human skeletal muscle cells which could link the hypercoagulability and insulin resistance in T2D. Furthermore, we hypothesized that the metabolic benefits of exercise are decreased in cultured muscle cells from T2D patients.

Cultured human myotubes were used aiming to explore the effects of thrombin on insulin signalling and glucose uptake as well as the effect of thrombin on metabolic function in the presence and absence of EPS as an *in vitro* model of exercise. Furthermore, to explore the effects of EPS on metabolic function in muscle cell cultures derived from T2D and non-diabetic control subjects.

The findings of the first three chapters of this thesis demonstrated that thrombin was shown to have multiple metabolic effects represented by a decrease in insulin stimulated IRS1 and Akt activation which was mediated through PKCα, but thrombin had no effect on the parallel insulin-stimulated aPKC and AS160 pathway. Thrombin directly increased glucose uptake through an AMPK mediated mechanism. Furthermore, the increase in AMPK activity, elevation of glucose uptake and the rise in cytokine release in response to EPS (above basal values) that were noted with non-thrombin treated myotubes was lost upon thrombin treatment. The key findings of the last chapter there were, AMPK activation and glucose uptake increased in response to EPS in control myotubes, and EPS enhanced the effect of insulin on glucose uptake and distal insulin signalling pathway (AS160). In diabetic myotubes, EPS did not increase AMPK activation and glucose uptake, nor enhanced the action of insulin.

Thus, hypercoagulation associated with diabetes could be involved in multiple metabolic effects in skeletal muscle including insulin signalling, exercise signalling, proinflammatory pathways, and glucose uptake. There is an intrinsic defect in diabetic myotubes represented by defective AMPK, glucose uptake and distal insulin signalling in response to EPS that are consistent with the changes observed *in vivo*.

DECLARATION OF ORIGINALITY

I hereby declare that all the work presented in this thesis is my own unless stated otherwise within the text or acknowledged accordingly within the references. The data have not been submitted previously for any alternative degrees.

Ali Al-bayati

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PUBLISHED WORK

Published Abstracts:

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Conference Paper · October 2015

Conference: The North East Postgraduate Conference 2015

The responses to an *in vitro* exercise system are impaired in cultured human muscle cells from patients with Type 2 diabetes

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Effects of thrombin on insulin signalling and glucose uptake in cultured human myotubes

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LIST OF ABBREVIATIONS

ACC   Acetyl-CoA carboxylase

ADA   American Diabetes Association

ADP   Adenosine Diphosphate

AICAR   5-Aminoimidazole-4-Carboxamide1-B-D-Ribofuranoside

AMP   Adenosine Monophosphate

ANOVA   Analysis Of Variance

ATP   Adenosine Triphosphate

BMI   Body Mass Index

BSA   Bovine Serum Albumin

CAPS   N-cyclohexyl-3-aminopropanesulfonic acid

CCL2   C-C motif chemokine Ligand 2

cDNA   complementary DNA

CEE   Chick Embryo Extract

CO_2 Carbon dioxide

CoA   Coenzyme A

CT   Cycle Threshold

DMSO   Dimethyl sulfoxide

DNA   Deoxyribonucleic Acid

EDTA   Ethylenediamine Tetraacetic Acid

EPS   Electrical Pulse Stimulation

FBS   Foetal Bovine Serum

FFA   Free Fatty Acids

FPG   Fasting Plasma Glucose

G-6-P   Glucose 6-Phosphate

GBD   Glycogen Binding Domain

GIP	Gastric Inhibitory Polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GS	Glycogen Synthase
GSK	Glycogen Synthase Kinase
GWAS	Genome-Wide Association Studies
HbA1c	glycated haemoglobin (A1c)
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IRS	Insulin Receptor Substrates
kDa	kiloDalton
MODY	Maturity-Onset Diabetes of the Young
mTOR	mammalian Target Of Rapamycin
NGT	Normal Glucose Tolerance
PARs	Protease Activated Receptors
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PGC1-α	Peroxisome proliferator-activated receptor Gamma Coactivator 1- alpha
PH	Pleckstrin Homology domain
PI3K	Phosphatidylinositol-3 Kinase
PIP2	Phosphatidylinositol bisphosphate
PIP3	Phosphatidylinositol triphosphate
PKB	Protein Kinase B
RNA	Ribonucleic Acid
rpm	revolutions per minute

SLC2	Solute Carrier 2
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TBST	Tris Buffered Saline Tween
TNFα	Tumor Necrosis Factor alpha
WHO	World Health Organisation

CHAPTER 1 : GENERAL INTRODUCTION

1.1 Diabetes mellitus

Diabetes is defined as a chronic disease that is associated with abnormally high blood glucose levels. It represents a major health problem and a great challenge to healthcare provision worldwide. According to the World Health Organization (WHO), a diabetic person is characterised by abnormally chronically elevated blood sugar with disorders in carbohydrate, lipid and protein metabolism (American Diabetes Association, 2015). The chronic increase in blood glucose level is associated with a decrease in both life expectancy and quality.

The condition acquires great attention due to the rapid rise in prevalence supported by an increase in obesity and an inactive lifestyle. In an exceptional way for a non-infectious disease, in 2007 the Centres for Disease Control and Prevention in USA categorised the increase in diabetes incidence as an "epidemic" (Home *et al.*, 2008). According to the International Diabetes Federation (IDF), disease prevalence is expected to reach 642 million persons in 2040 compared to 415 million people who have diabetes worldwide in 2015 (Yensen and Naylor, 2016).

This rapidly growing problem of diabetes is causing an economic burden on healthcare providing services across the world. Most countries spend 5-20% of their total annual fund allocated for healthcare on diabetes related services (IDF Diabetes atlas, 2015). Therefore, every effort should be made in the prevention of the disease and improvement of its management.

Diabetes mellitus as a disease can be diagnosed by polyuria, polydipsia, and unexplained weight loss plus one of the following diagnostic criteria adopted by the WHO and American Diabetes Association (ADA) as the following; FPG ≥ 7.0 mmol/l (126 mg/dl) and/or 2-hours post glucose load ≥ 11.1 mmol/l (200 mg/dl) or a random venous plasma glucose concentration ≥ 11.1 mmol/l or HbA1c ≥ 6.5 %. If the person was asymptomatic, the diagnosis would be confirmed by repeating at least one of the above tests. Moreover, the patient is diagnosed as having impaired fasting glucose (IFG) if the FPG 6.1–6.9 mmol/l (110 to 125 mg/dl) (WHO) or FPG 5.6–6.9 mmol/l (100–125 mg/dl) (ADA). Furthermore, the patient is regarded as having impaired glucose tolerance (IGT) if FPG < 7.0 mmol/l (126

mg/dl) and 2-hours post glucose load ≥ 7.8 and < 11.1 mmol/l (140 and 200 mg/dl) (WHO) or only if the 2-hours post glucose load ≥ 7.8 and < 11.1 mmol/l (140 and 200 mg/dl) (ADA) (American Diabetes Association, 2015).

People with IFG and IGT are referred to as having "prediabetes". This is considered an intermediate state between the normal population and clinical diabetes and they at relatively high risk of developing future diabetes than normal (Gavin Iii *et al.*, 1997; Genuth *et al.*, 2003). Individuals with prediabetes can delay the onset or even prevent development of diabetes by interventions that includes changing lifestyle (decrease body weight and increase physical activity) and use of drugs like metformin or both (Buchanan *et al.*, 2002; Diabetes Prevention Program Research, 2002; Parker *et al.*, 2014).

1.2 Classification of diabetes mellitus

Diabetes mellitus can be classified into either two main types of diabetes or less common types. The two main types of diabetes are:

1.2.1 Type 1 diabetes (T1D)

Previously known as insulin dependent diabetes, juvenile onset diabetes or autoimmune diabetes the exact causes of type 1 diabetes are not fully elucidated, but pancreatic beta cell destruction is a final outcome that usually leads to absolute deficiency of insulin. Affected people are usually children and young adults but any age could be influenced. Due to total insulin deficiency, patients with T1D are managed with insulin injection for diabetes control and survival (American Diabetes Association, 2016).

1.2.2 Type 2 diabetes (T2D)

T2D was previously known as non–insulin-dependent diabetes or adult-onset diabetes. Insulin is produced by the pancreatic β-cells but the body becomes resistant to insulin and subsequently the insulin produced may become insufficient. Insulin resistance is the main prominent feature that leads to a decreased response to the secreted insulin in the target tissues, liver and skeletal muscle leading to elevated glucose levels. T2D is the most common type of the disease and accounts for 90–95% of all diabetes (American Diabetes Association,

2016). The affected individuals are usually adults but there are now increasing numbers of children and adolescents being diagnosed with T2D. The clinical course of T2D is usually gradual and cases undiagnosed for many years are not uncommon, as the symptoms are usually not severe enough to be noticed, therefore many patients present with complications due to damage to the cells by high glucose (IDF Diabetes atlas, 2015).

Gestational diabetes

Gestational diabetes is a clinical condition of elevated blood glucose levels above the normal values that is firstly recognised during pregnancy. It is established as a consequence of hormonal and inflammatory changes of pregnancy that lead to insulin resistance (Yensen and Naylor, 2016),

The normal consequences of gestational diabetes are as follow: 60% of cases of gestational diabetes return to normal non-diabetic state after termination of pregnancy, 36% develop T2D and only 4% convert to T1D. Therefore, gestational diabetes is regarded as an added risk factor for developing diabetes in the future (Yensen and Naylor, 2016).

The less common types of diabetes include:

- Monogenic diabetes: Examples are; Maturity-Onset Diabetes of the Young (MODY) and Neonatal Diabetes Mellitus which occur as a result of mutation to genes and accounts for 4% to 13% of diabetes in children (Kropff *et al.*, 2011; Fendler *et al.*, 2012).
- Secondary diabetes: which occur as a complication of treatment of other diseases or hormonal imbalances like Cushing's syndrome or acromegaly, or secondary to pancreatic disease (American Diabetes Association, 2014).

1.3 Aetiology of T2D

Insulin resistance and diminished insulin secretion are the main final features of patients with T2D. No single cause leads to these metabolic disorders, a complex interaction between genetic and environment factors are implicated in the development of the disease.

3

1.3.1 Genetics of T2D

The genetic component in T2D is now well established. Many observations point towards the importance of heritability factors as a cause of development of the disease. Firstly, T2D is more prevalent in certain ethnic groups. For example, people of South Asian origin and African and Africa-Caribbean origin are more likely to develop T2D (Oldroyd *et al.*, 2005). The rate of T2D is very low in Caucasians in Europe compared to the higher rate, approaching 50%, among Pima Indians in the USA (Diamond, 2003). Furthermore, studies show that the concordance rate of T2D in monozygotic twins is about 70 % in contrast to that in dizygotic twins which is 20-30 % (Medici *et al.*, 1999; Poulsen *et al.*, 1999). In addition, the risk of an individual to develop T2D approaches 70 % if both parents have diabetes while it is just 40 % if only one of them has the disease compared to a population prevalence of 7% (Meigs *et al.*, 2000).

The advances in genetic studies draw the attention to the new gene candidates associated with T2D. Prior to 2007, before the advances of genome-wide association studies (GWAS) only few candidate genes had been recognized to be linked with T2D based on functional, linkage and candidate gene approaches. The most obvious candidate genes discovered in this era include IRS1, PPARG, CAPN10 and TCF7L2 (Bonnefond *et al.*, 2010).

In 2007, multiple results of GWAS started to emerge. One of the earliest studies reported in 2007 confirmed the previous genetic associations and revealed two new alleles linked to early onset T2D (HHEX and SLC30A8) (Sladek *et al.*, 2007).

Genome-wide association studies (GWAS) have documented more than 80 loci linked to an increase in risk of T2D development (Mohlke and Boehnke, 2015). The genes associated with T2D are mostly related to insulin secretion and β-cell function rather than genes related to insulin resistance. This could be attributed to the insulin resistance phenotype being mostly related to lifestyle factors such as overeating and inactivity, and the genetic background is therefore mostly mixed with obesity related variants. The following tables list many of the loci and genes associated with impaired β-cell function (Table 1-1) and insulin resistance (Table 1-2) reported by different gene approaches (linkage analysis, candidate gene studies and GWAS).

Table 1-1: β-cell function related susceptibility genesas identified by candidate gene association studies, linkage analysis and GWAS (Bonnefond *et al.*, 2010; Qi and Hu, 2012).

SNP	gene	Chromosome	Official Full Name
rs5219	KCNJ11	11	potassium voltage-gated channel subfamily J member11
rs7903146	TCF7L2	10	Transcription factor 7-like 2
rs10010131	WFS1	4	wolframin ER transmembrane glycoprotein
rs4402960	IGF2BP2	3	Insulin-like growth factor 2 mRNA binding protein 2
rs10946398	CDKAL1	6	CDK5 regulatory subunit associated protein 1-like 1
rs13266634	SLC30A8	8	Solute carrier family 30 (zinc transporter), member 8
rs10811661	CDKN2A/B	9	Cyclin-dependent kinase inhibitor 2A/2B
rs1111875	HHEX/IDE	10	hematopoietically expressed homeobox / Insulin-degrading enzyme
rs7578597	THADA	2	Thyroid adenoma associated
rs864745	JAZF1	7	Juxtaposed with another zinc finger protein 1
rs1277979032	CDC123-CAMK1D	10	Cell division cycle 123 homolog (Saccharomyces cerevisiae)- Calcium/calmodulin-dependent protein kinase type 1D
rs7961581	TSPAN8-LGR5	12	Tetraspanin 8-Leucine-rich repeat-containing G protein coupled receptor 5
rs231362 K	KCNQ1	11	voltage-gated channel, KQT-like subfamily, member 1
rs10830963	MTNR1B	11	Melatonin receptor 1B
rs340874	PROX1	1	Prospero homeobox 1
rs4607517	GCK	7	Glucokinase (Hexokinase 4)
rs2191349 7	DGKB/ TMEM195	7	Diacylglycerol kinase, β 90 kDa/ Transmembrane protein 195 β-cell function
rs1552224	CENTD2 (ARAP1)	11	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1

Table 1-2: Insulin resistance related susceptibility genes as identified by candidate gene association studies, linkage analysis and GWAS (Brown and Walker, 2016).

SNP	gene	Chromosome	Official Full Name
rs13081389	PPARG	3	Peroxisome proliferator-activated receptor-γ
rs972283	KLF14	7	Kruppel-like factor 14
rs2943641	IRS1	2	Insulin receptor substrate 1
rs780094	GCKR	2	Glucokinase (hexokinase 4) regulator
rs8050136	FTO	16	Fat mass and obesity-associated protein
rs7903146	TCF7L2	10	Transcription factor 7-like 2
rs1208	NAT2	8	N-acetyltransferase 2
rs6723108, rs998451	TMEM163	2	transmembrane protein 163
rs35767	IGF1	12	insulin like growth factor 1
rs12970134	MC4R	18	melanocortin 4 receptor
rs17046216	SC4MOL	4	sterol-C4-methyl oxidase-like
rs7077836	TCERG1L	10	transcription elongation regulator 1 like
rs702634 , rs4311394	ARL15	12	ADP ribosylation factor like GTPase 15

1.3.2 Epigenetic Factors related to T2D

The term "Epigenetic" refers to the study of heritable changes in phenotype that does not involve changes in the underlying DNA sequences but can alter gene function. Environmental factors, such as nutrition and exercise, could precipitate epigenetic effects through different mechanisms such as DNA methylation, histone modification or microRNA activation. Knowledge about the epigenetic process and its ability to influence the development of T2D is not abundant but epigenetic mechanisms have been shown to have a role in the pathogenesis of the disease complication. For example, prior hyperglycemia leads to various methylation and demethylation events in vascular epithelial cells. These events were shown to have effects on gene activity that represented by sustained activation of pro-inflammatory pathways, which are likely playing a role in the progression of diabetic complications (Brasacchio *et al.*, 2009).

1.3.3 Environmental Factors increase risk of T2D

The most important environmental factors that increase risk of T2D are nutritional factors. Overeating and, as a consequence, positive energy balance leads to obesity and fat accumulation. Insulin resistance is a direct result of obesity (Samuel and Shulman, 2012). In addition, certain antioxidants and vitamins have a protective role against T2D. Studies show that vegetable intake, or dietary supplements containing carotenoids, vitamin C and tocopherol have an inverse relationship with the development of insulin resistance and T2D (Salonen *et al.*, 1995; Sargeant *et al.*, 2000; Coyne *et al.*, 2005).

Other environmental factors are physical factors, such as pollution and chemical exposure that could result in increased risk of T2D. Studies show that biological pollutants that are used as pesticides or herbicides can induce insulin resistance (Porta, 2006; Lee *et al.*, 2011).

1.4 Glucose homeostasis in normal versus T2D

Glucose homeostasis in a healthy individual is maintained in a narrow range by the effect of hormones. On the one hand is insulin and on the other hand are insulin antagonising hormones such as glucagon. The main organs involved in glucose regulation are the intestinal tract, pancreas, liver, skeletal muscle and adipose tissues. Normal glucose homeostasis is maintained by at least three

harmonious mechanisms and include; secretion of insulin from pancreatic β-cells, hepatic glucose production, and stimulation of glucose uptake by the liver and muscle.

After a meal, nutrients are absorbed from the gastrointestinal tract into the bloodstream via the hepatic portal circulation. Ingested food acts directly on the intestinal tract and stimulates its own endocrine cells to secrete groups of metabolic hormones known as Incretins. Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are well known examples of incretins. They act with the elevated circulatory glucose on pancreatic cells inhibiting alpha cells from releasing glucagon and stimulate β-cells to secrete insulin (Kreymann *et al.*, 1987; Nauck *et al.*, 2004).

The pancreatic β-cells secrete insulin in response to the increase in blood glucose concentrations. Insulin decreases blood glucose by increasing glucose uptake into skeletal muscle and adipose tissue and by lowering glucose production by the liver. Pancreatic β-cells produce insulin in two phases. In the first phase, insulin is directly secreted after a meal and this rise in insulin is thought to inhibit glucose production by the liver. The second phase is released within 1-2 hours (plateau) after a meal and is responsible for glucose uptake in insulin sensitive tissues (skeletal muscle and adipose tissue) (Moore *et al.*, 1991).

The liver is the first organ that the absorbed nutrients encounter. It plays an essential role in regulating glucose release into the circulation after a meal. Hepatic glucose production is inhibited by insulin and stimulated by glucagon. During fasting, liver produces glucose from glycogen stores via hepatic glycogenolysis until liver glycogen stores are depleted, then it is substituted by gluconeogenesis.

In the early stages of T2D, hepatic insulin resistance develops which means that the ability of insulin to suppress hepatic glucose production is reduced (Wei *et al.*, 2007). As the condition progresses, the balance between insulin and glucagon is disturbed leading to further increases in hepatic glucose production (Bagger *et al.*, 2011).

In resting muscle and adipose tissue upon insulin stimulation, insulin triggers a series of signalling cascades which lead to the translocation of the GLUT4 glucose

transporter to the cytoplasmic membrane. This process facilitates glucose uptake by facilitated diffusion (Holt *et al.*, 2011). Furthermore, insulin stimulates anabolic pathways that include glycogenesis, lipogenesis and protein synthesis. Simultaneously, insulin inhibits glycogenolysis, lipolysis. Moreover, insulin activates enzymes of glycolysis (glucose metabolism). In T2D, insulin-stimulated glucose uptake into the cells is impaired, with impairment in the action of enzymes involved in glucose utilisation. Muscle glycogen synthesis rate is also about 50% lower than the rate in normal subjects (Shulman *et al.*, 1990). Figure 1-1 shows the glucose homeostasis in normal and the possible abnormalities in different organs that could be existed in T2D.
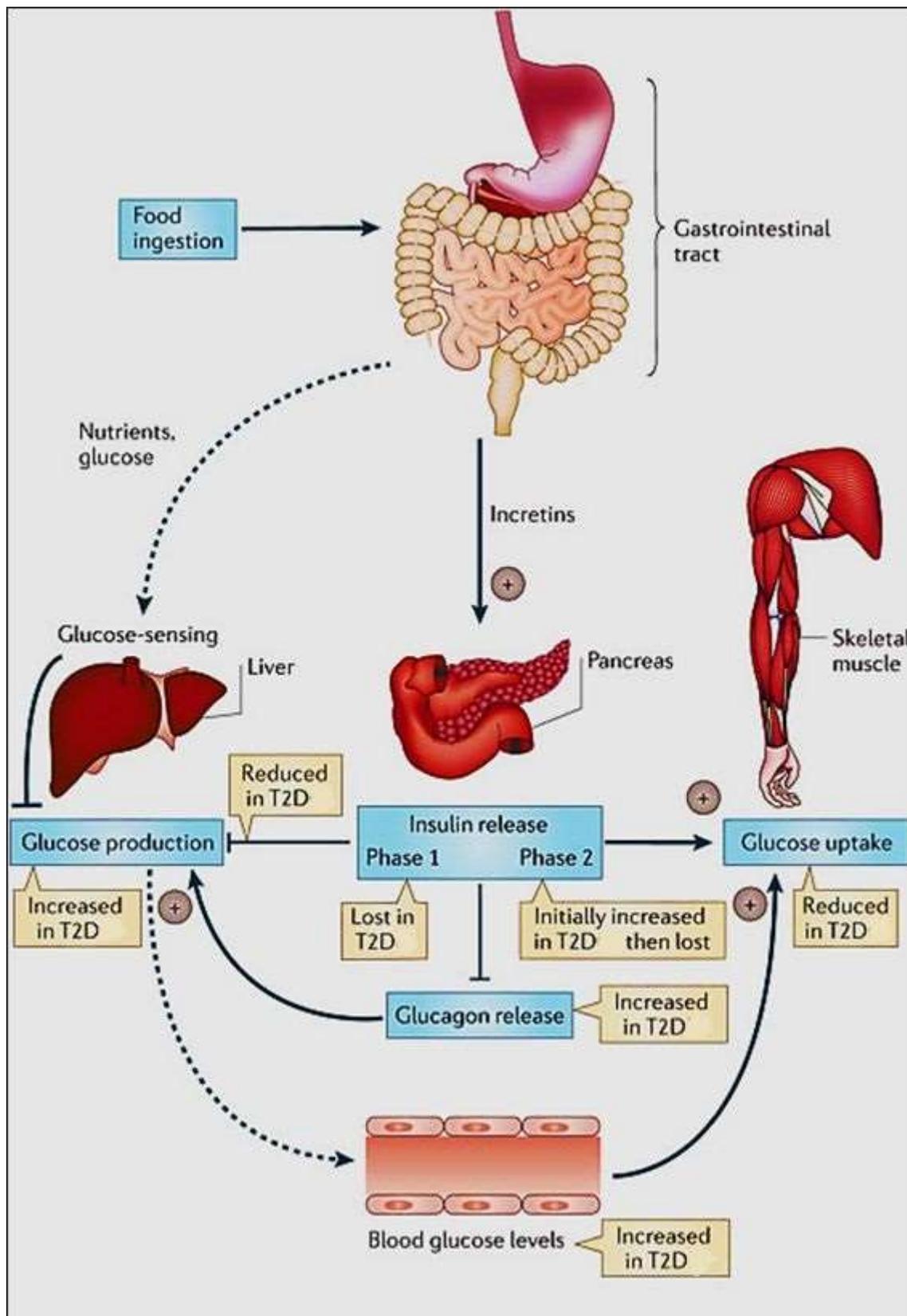
Figure 1-1: Glucose homeostasis in normal versus T2D adapted from (Grayson *et al.*, 2013).

1.5 Insulin secretion in normal versus in T2D

The cellular mechanism of insulin secretion from β-cell, as presented in Figure 1-2, begins with an influx of glucose into the cell via the glucose transporter, GLUT2. Subsequently, glucose is phosphorylated by the enzyme glucokinase to glucose 6-phosphate (G-6-P) for ATP generation and stimulates a transcription factor PDX1 for synthesis of new insulin. The ATP generated facilitates closure of ATP-sensitive potassium channels inhibiting the entry of potassium ions into the cell leading to depolarization of the resting membrane potential. As a result, the voltage-gated calcium channels in the membrane open and facilitate calcium ion entry. The increased intracellular calcium initiates insulin release through exocytosis of insulin-containing granules (Hall, 2015).
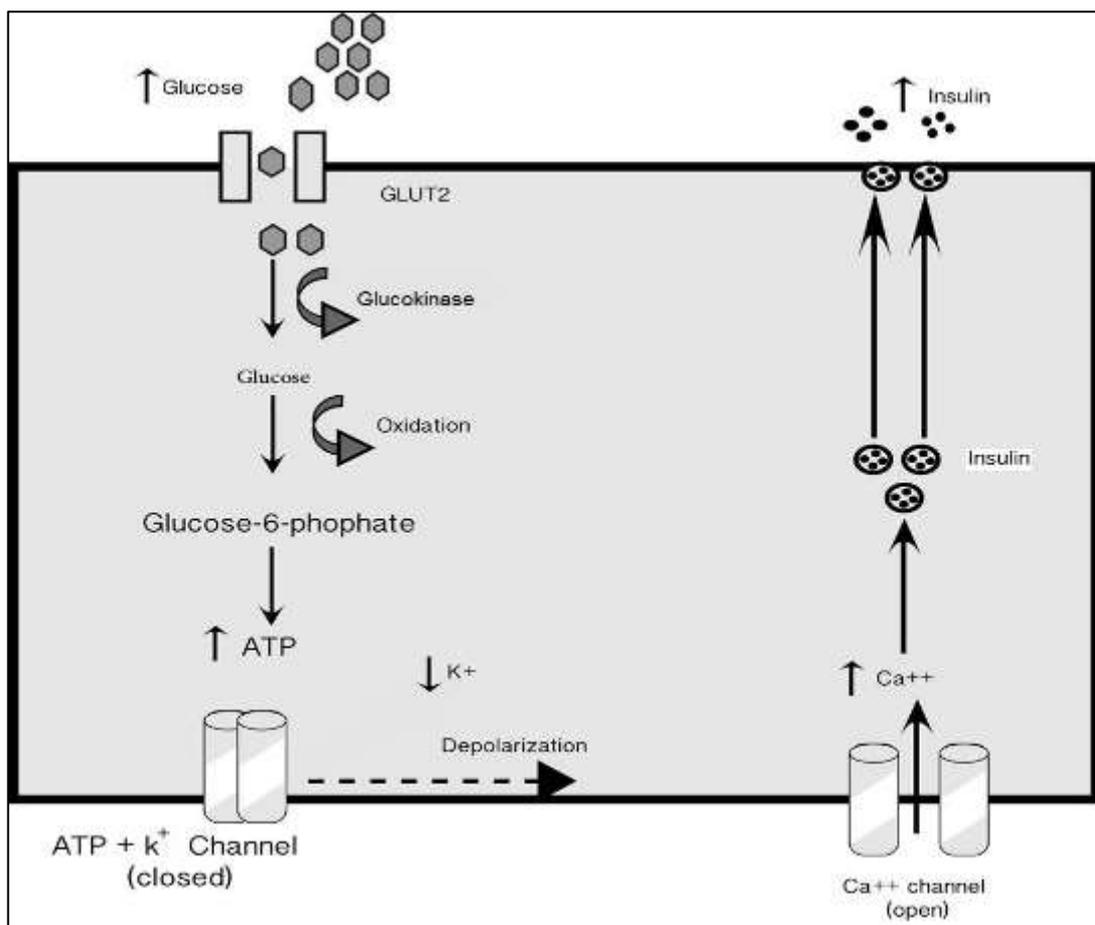
Figure 1-2: Mechanism of insulin secretion from β-cells.

It has been under debate whether insulin resistance or β-cell failure is the primary defect in the development of T2D. It was accepted for a long time that β-cell dysfunction follows insulin resistance in the progression to T2D. Numerous studies suggested that β-cell dysfunction is a result of exhaustion after a long period of a compensatory increase in insulin secretion (DeFronzo, 1988; Olefsky and Nolan, 1995; Reaven, 1995). However, more recently, it has been proposed that β-cell dysfunction is the primary defect, based on critical studies that measure β-cell function and insulin sensitivity (Polonsky *et al.*, 1996; Kahn, 2001; Pratley and Weyer, 2001). Furthermore, studies of first degree relatives of individuals with T2D who have no signs of insulin resistance (normal glucose tolerance) show reduced insulin secretion responses (Gerich, 1998; van Haeften *et al.*, 2000). Thus, β-cell dysfunction seems to be a key early defect that could lead to progression to IGT or even T2D if accompanied by insulin resistance.

When T2D is diagnosed, the degree of β-cell dysfunction becomes quite obvious. For example, using the homeostasis model assessment (HOMA), the UK Prospective Diabetes Study stated that at time of diagnosis of T2D β-cell activity was reduced by 50% and function continues to deteriorate irrespective of therapy (1995).

The secretory function of β-cells is also affected by aging. A decline in β-cell secretory function of 0.7 – 1% per year has been observed for both basal and glucose stimulated secretion in normal glucose tolerant adult individuals (Szoke *et al.*, 2008). In individuals with IGT, the secretory function decline rate is about double that of NGT individuals (Szoke *et al.*, 2008), and it is about 6% per year in T2D patients (Kahn *et al.*, 2006).

1.6 Whole body insulin resistance

Insulin resistance is described as the inability of target tissues to respond to a normal physiological level of insulin. Insulin resistance has detrimental effects on glucose homeostasis that can lead to the onset of metabolic abnormalities such as T2D. Regarding glucose haemostasis, two essential metabolic defects are recognised in insulin resistance. The first is an impairment of the suppression of hepatic glucose production in response to insulin and the second is the reduced peripheral uptake of glucose.

There is a substantial body of evidence indicating that insulin resistance is a fundamental feature of T2D. Many cross sectional studies have shown that almost all patients with T2D are insulin resistant, and many follow up studies have suggested that insulin resistance precedes the onset of clinical diabetes by many years as reviewed in detail by (Lillioja *et al.*, 1988; DeFronzo *et al.*, 1992).

Most individuals with insulin resistance are frequently hypertensive with abdominal obesity. The cluster of these abnormalities in an individual leads to increased risk of developing T2D and cardiovascular complications and is known as metabolic syndrome. The table in APPENDIX 1 shows the Definition criteria of diagnosis of metabolic syndrome as adopted by different associations. Individuals with metabolic syndrome have about a fivefold greater risk of developing T2D. Moreover, these individuals have double risk of death from cardiovascular disease compared to non-metabolic syndrome people (Alberti *et al.*, 2006).

1.7 Skeletal muscle

1.7.1 Skeletal muscle structure

Skeletal muscle is composed of numerous muscle fibres that in turn consist of multinucleated cells known as myotubes. The myotubes are developed by division and fusion of myoblasts. Skeletal muscle cells are covered by three layers. Sarcolemma is the term used to describe the plasma membrane of muscle fibres and represent the first layer. A number of fibres are organised into bundles (fasciculi) that are covered by connective tissue known as the perimysium. Lastly, there is a heavier connective tissue layer termed the fascia that sheaths the whole muscle (Standring, 2015).

1.7.2 Skeletal muscle metabolism

Both glucose and free fatty acids are used as energy sources by skeletal muscle. During fasting, muscle glucose uptake is low and the plasma FFA concentrations are raised. FFAs act as the principal source for energy production in skeletal muscle during fasting. After meals, the plasma glucose concentration is elevated leading to stimulation of insulin secretion from the β-cells in the pancreas. The resultant increased insulin level will facilitate inhibition of adipose tissue lipolysis leading to a decrease in the FFA plasma concentration and consequently, a decrease in the rate of lipid utilisation. At the same time, insulin stimulates increased glucose uptake into skeletal muscle and activation of key enzymes in glucose metabolism leading to a marked increase in muscle glucose oxidation (DeFronzo, 1997).

Glucose provides a source of cellular energy and substrate storage in skeletal muscle. Skeletal muscle glucose metabolism occurs through either aerobic or anaerobic pathways to produce ATP. The anaerobic pathway of glucose produces only a small proportion of ATP and occurs in the cell cytoplasm, and known as glycolysis which means splitting of the glucose molecule to form two molecules of pyruvic acid. Whereas the aerobic pathway yields the maximum amount of ATP production per glucose molecule by entering the tricarboxylic acid cycle within the mitochondria (Bouché *et al.*, 2004).

The first step upon entry of glucose into the cells is its phosphorylation by hexokinase to form glucose-6-phosphate. Phosphorylation of glucose is a vital

step as it ensures that the glucose is trapped inside the cell and does not diffuse back out. In muscle cells this step is irreversible due to the absence of glucose phosphatase enzyme that expressed predominantly in liver cells. Therefore, skeletal muscle unlike liver cells, cannot produced glucose into circulation during fasting or in absence of insulin (van Schaftingen and Gerin, 2002). Glucose-6-phosphate is converted into fructose 6-phosphate which is readily further phosphorylated into fructose 1, 6 diphosphate, a reaction catalysed by phosphofructokinase. The later molecule is cleaved into a three carbon compound glyceraldehyde-3-phosphate, and each of which is then converted over five further steps into pyruvic acid and only a small amount of the free energy. The pyruvic acid molecule has the ability to pass through mitochondrial membrane and further converting into a molecule of acetyl coenzyme A (acetyl-CoA) that enters citric acid cycle. The citric acid cycle is a sequence of chemical reactions in the mitochondrial matrix in which the acetyl-CoA is degraded to carbon dioxide, water and releasing large amounts of energy in the form of ATP (Hall, 2015).

1.8 Insulin signalling pathway in skeletal muscle cell

Insulin is the master regulatory hormone of glucose haemostasis in the mammalian body. It promotes transport and oxidation of glucose and other metabolites as well as regulating the synthesis of proteins, triglycerides and glycogen within the cells. Skeletal muscle was chosen by many studies as a target of insulin action because of its role as an organ that is responsible for about 80% of the whole body glucose disposal in response to insulin (DeFronzo *et al.*, 1981; Thiebaud *et al.*, 1982). For a better understanding of events at the molecular level that occur in insulin resistant states, it is crucially important to know the insulin signalling pathway. This helps to explore the possible defect in each step of the pathway to identify abnormalities that may occur in insulin resistance and T2D.

1.8.1 The Insulin Receptor

The insulin receptor is a tyrosine kinase receptor meaning its activation depends mainly on activation of tyrosine residues. Structurally, the insulin receptor is a cell membrane receptor consisting of four subunits, two α and two β subunits. α subunits lie totally outside the cell membrane and are linked together, and with the β subunits, by disulfide bonds. As shown in Figure 1-3, α subunits contain the ligand-binding domains whereas, the β subunits have both intracellular and extracellular with a transmembranous domains. The β subunits contain the tyrosine phosphorylation kinase domain that activates intracellular signalling pathways inside the cell (Lee and Pilch, 1994)
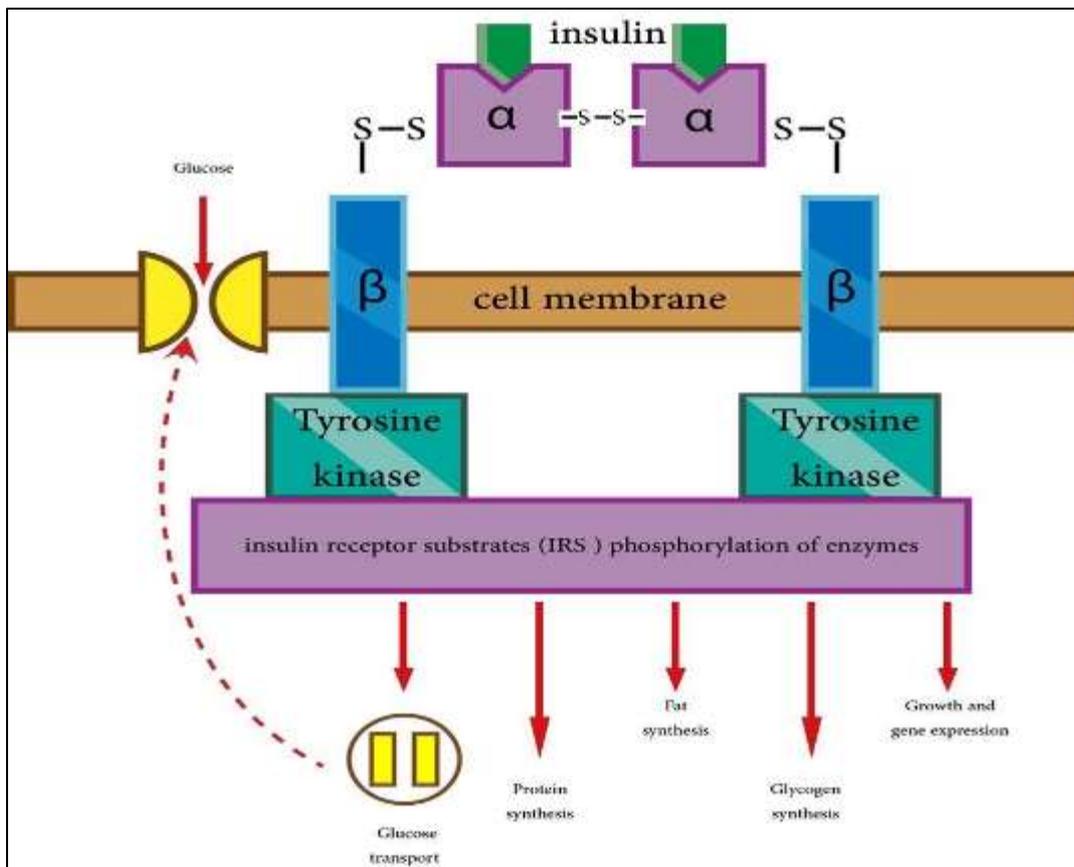
Figure 1-3: A graphical representation of insulin receptor structure and activation.

Binding of insulin to the binding domains of α subunits leads to a conformational changes in the insulin receptor resulting in an auto-phosphorylation of the tyrosine kinase domain of the β subunit. This tyrosine phosphorylation in turn phosphorylates and activates multiple intracellular enzymes, including a group called insulin-receptor substrates (IRS) (White *et al.*, 1984). These proteins are characterised by having Src Homology 2 (SH2) domains which are the phospho-tyrosine binding domains that have the ability to recognise the phosphorylated tyrosine. Examples of these proteins are; the IRS family (Sun and Liu, 2009), Shc proteins (Taniguchi *et al.*, 2005), Cbl CAP proteins (Baumann *et al.*, 2000), and phospholipase C gamma1 (PLCγ1) (Kwon *et al.*, 2003).

In addition to insulin receptor tyrosine phosphorylation, insulin receptors can be phosphorylated on serine and threonine residues leading to decreased insulin signalling. Phosphorylation on serine or threonine residues can act as a negative feedback signalling mechanism or even inhibitory signalling that could contribute to the pathogenesis of insulin resistance (Dunaif *et al.*, 1995; Barbour *et al.*, 2007).

1.8.2 The IRS molecules

These molecules are family of proteins perform important roles in insulin signalling. There are eight proteins belonging to this family and they display some degree of structural homology. Structurally, IRS proteins are made up of three regions; an amino terminal pleckstrin homology (PH) domain for receptor docking, a phosphotyrosine binding domain that recognizes the phosphotyrosine residues of the insulin receptor, and a COOH - terminal domain that contains multiple tyrosine and serine phosphorylation sites (Virkamäki *et al.*, 1999). Among the eight members, IRS1 and IRS2 are considered to be the most important proteins in glucose metabolism and insulin signalling, and their deficiency is associated with insulin resistance in humans (Zhande *et al.*, 2002). IRS1 is the major IRS in skeletal muscle while IRS2 is expressed predominantly in liver and β cells and is required for normal β cell function (Holt *et al.*, 2011). Following insulin stimulation, numerous tyrosine sites are phosphorylated but Tyrosine 612 (Y^{612}) and Tyrosine 632 (Y^{632}) are the critical residues for the downstream activation of PI-3 kinase for GLUT4 translocation (Esposito *et al.*, 2001). IRS can be negatively regulated by

serine threonine kinases through serine phosphorylation and protein phosphatases. In humans, variants within the gene responsible for the coding of IRS1 (IRS1 gene) are associated with T2D and insulin resistance (Laakso *et al.*, 1994; Rung *et al.*, 2009).

1.8.3 Phosphatidylinositol 3-Kinase (PI3K)

PI3- kinase is an enzyme consisting of two different macromolecules (hetero-dimer), the p110 catalytic subunit (110 kDa) that contains the activation sites and mediates the kinase activity, and the p85 regulatory (85 kDa) adaptor subunit which is responsible for binding with IRS1. PI3-kinase is involved in different signalling pathways, and this enzyme has an essential role in insulin signalling and cell metabolism (Engelman *et al.*, 2006).

The cascade of activation of this kinase is as shown in Figure 1-4 insulin auto phosphorylates the insulin receptor leading to phosphorylation and activation of IRS1 that directly binds to the p85 subunit of PI3K. This binding leads to phosphorylation and activation of the catalytic p110 subunit facilitating the recruitment of the complex to the cell membrane which is the site of action. Subsequently, activated PI3-kinase facilitates phosphorylation of membrane phospholipids, phosphatidylinositol 4, 5-bisphosphate (PIP2) on the 3' position forming phosphatidylinositol-3, 4, 5-triphosphate (PIP3). PIP3 then activates phosphoinositide-dependent protein kinase (PDK1 and PDK2). Activated PDK is responsible for phosphorylation and activation of protein kinase B (PKB/Akt) and atypical protein kinase C (aPKC) (Vollenweider, 2003; Engelman *et al.*, 2006).

Insulin has been shown to be a potent activator of PI-3 kinase (Shepherd *et al.*, 1998). Activation of PI3-kinase is a crucial step in the insulin signalling pathway for glucose uptake in skeletal muscle as proven by the use of specific chemical inhibitors of PI3- kinase, Wortmannin and LY294002 (Somwar *et al.*, 2001; Foster *et al.*, 2003).

1.8.4 Akt

Akt is a 56 kDa serine threonine kinase, also known as protein kinase B, and consists of an N-terminal PH domain followed by a catalytic kinase domain and C-terminal tail. Originally, this enzyme was associated with cancer development, by promoting cell proliferation and inhibition of apoptosis. However, Akt has also been

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shown to be activated acutely by insulin and other growth factors (Hemmings, 1997).

Akt has 3 isoforms; Akt1, Akt2 and Akt3, also known as Akt α, Akt β, and Akt γ, and these isoforms are expressed in different tissues (Björnholm and Zierath, 2005; Kim and Novak, 2007). Akt1 and Akt2 isoforms are predominantly expressed in insulin sensitive tissues (liver, adipose and skeletal muscle) whereas Akt3 has been shown to be expressed mainly in the brain (Brazil and Hemmings, 2001). In skeletal muscle, Akt2 is predominantly involved in glucose uptake and insulin responsiveness (Cho *et al.*, 2001), and studies showed that overexpression of the Akt2 isoform in skeletal muscle significantly enhances glucose metabolism (Cleasby *et al.*, 2007). Akt is activated by insulin via a PI3K-dependent pathway, and this activation facilitates its translocation and binding to the cell membrane. This translocation to the membrane is an essential step of enzyme activation (Gonzalez and McGraw, 2009). At the cell membrane, Akt is further activated by phosphoinositide-dependent protein kinase (PDK) and mTOR complex 2 (Mora *et al.*, 2004). The full activation of Akt requires phosphorylation at two activation sites. The first, is Thr^{308} at the kinase domain which is activated by the upstream kinase PDK and accounts for about 10 % of the kinase activity (Schultze *et al.*, 2011). The second and most critical activation site is the Ser^{473} at the carboxyl terminal domain. The mechanism of activation at this site is still unclear but mTOR complex 2 and others like PDK1, PDK2, and auto phosphorylation are implicated (Brazil and Hemmings, 2001; Hajduch *et al.*, 2001b).

Metabolically, Akt seems to regulate glucose metabolism in skeletal muscle and adipocytes (Hajduch *et al.*, 2001a; Vollenweider, 2003). Activation of Akt promotes translocation of the glucose transporter GLUT4 in response to insulin from the intracellular space to the plasma membrane. Studies have confirmed the role of the PI3K-Akt pathway in glucose transport in skeletal muscle (Wang *et al.*, 1999) and adipocytes (Kohn *et al.*, 1996; Bai *et al.*, 2007). Numerous studies have shown that Akt mediates its GLUT4 translocation activity through a downstream target Akt substrate 160 (AS160). Activation of this enzyme by Akt was shown to be through phosphorylation at multiple key residues (Ser^{588}, Thr^{642}, Ser^{318}, Ser^{570}, and Thr^{751}) (Sano *et al.*, 2003; Zeigerer *et al.*, 2004).

1.8.5 AS160 substrate

AS160 is a 160 kDa Rab GTPase-activating protein that is a downstream enzyme of Akt. It is also known as TBC1 domain family member 4 (TBC1D4) as it is encoded in humans by the TBC1D4 gene. AS160 emerges as a key protein in GLUT4 translocation and is implicated in the mechanisms of both insulin-dependent and independent glucose uptake (Cartee and Wojtaszewski, 2007; Sakamoto and Holman, 2008).

Rab proteins are highly involved in GLUT4 translocation processes (trafficking and exocytosis) (Moyers *et al.*, 1996). In unstimulated conditions, GLUT4 remains in intracellular sites by inhibition of translocation via the effects of Rab-GAP activity. Under basal conditions, Rab-GAP activity remains in an inactive form (GDP-loaded) and upon stimulation AS160 is phosphorylated leading to conversion of Rab-GAP activity into an active form (GTP-loaded). This alteration enables the translocation of GLUT4 vesicles from the cytoplasm to the plasma membrane (Sakamoto and Holman, 2008).

Numerous activation sites on AS160 have been identified. Thr^{642} and Ser^{588} are considered the major residues that are phosphorylated and activated in response to insulin (Sano *et al.*, 2003). However, Treebak *et al.* (2009) identified further sites involved in insulin-stimulated AS160 phosphorylation such as Ser^{318}, Ser^{570}, and Thr^{751}.

1.8.6 Atypical protein kinase C (aPKC)

PKC is a family of kinases that play essential roles in many cellular processes. There are several different isoforms and they are involved in events such as growth, differentiation, and glucose and lipid metabolism. PKC is classified into 3 subgroups according to structure and regulation; conventional (cPKC), novel (nPKC) and atypical (aPKC) (Bertram and Ley, 2011; Turban and Hajduch, 2011).

The (aPKC λ/ζ) are independent of both diacylglycerol (DAG) and Ca^{2+} for activation. In skeletal muscle, aPKC seems to be regulated by PDK1, the downstream target of PI3K (Etgen *et al.*, 1999). Studies show that, overexpression of aPKC leads to an increase in both basal and insulin-stimulated glucose transport. Furthermore, muscle-specific abolition of both isoforms of aPKC disturbs insulin-stimulated glucose transport and GLUT4 translocation (Farese *et al.*,

2007). These data suggest that aPKC is involved in the stimulation of glucose transport, independent of Akt.

1.8.7 CAP/Cbl/TC10 pathway

The CAP/Cbl/TC10 pathway was recognised to be activated by insulin in various insulin sensitive tissues (Ribon *et al.*, 1998; Baumann *et al.*, 2000; Chiang *et al.*, 2001). These proteins are involved in mediating insulin signalling independent of the PI3K-Akt pathway. It is reported that the CAP/TC10 pathway plays a role in glucose transport in adipocytes (Chiang *et al.*, 2001) but, in skeletal muscles the metabolic role of these proteins is not fully clarified.

1.8.8 MAPK pathways

The mitogen activated protein kinase (MAPK) pathway is another insulin controlled well-characterized signalling cascade. This pathway is activated in response to insulin, growth factors and cellular stress (Widegren *et al.*, 2001). The establishment of activation of the MAPK pathway occurs at the level of IRS, which activates a series of signals involving the Ras/ERK cascade (Weng *et al.*, 2001). Thus, activation of ERK-MAPK plays a pivotal role in regulation of gene transcription in response to insulin such as that involved in the cell cycle (Blenis, 1993).

1.9 Glucose transporter in skeletal muscles

Glucose transporters are a family of specialized membrane-embedded transporter proteins known as GLUTs which are members of the solute carrier 2 (SLC2) family (Watson and Pessin, 2006; Marín-Juez *et al.*, 2013). Different isoforms of GLUT have been identified in human skeletal muscle; GLUT1 and GLUT4 are the major contributors for glucose transport in basal and stimulated states respectively (Guillet-Deniau *et al.*, 1994; Hansen *et al.*, 1998; Ciaraldi *et al.*, 2005).

1.9.1 GLUT1

Glucose transporter isoform 1 (GLUT1) is located at the plasma membrane and is responsible for basal glucose uptake into cells. GLUT1 plays a role in skeletal muscle development and it is expressed in abundance in myoblasts and expression tends to decrease dramatically after muscle differentiation (Gaster *et al.*, 2000; Al-Khalili *et al.*, 2003). In human skeletal muscle fibres, GLUT4/ GLUT1

expression is about 400/1 (Stuart *et al.*, 2006). A study in adipocytes shows that prolonged exposure to insulin causes a steady increase in expression at both the mRNA and protein level of GLUT1 (Sargeant and Pâquet, 1993).

1.9.2 GLUT4

Glucose transporter isoform 4 (GLUT4) is the major glucose transporter in adult insulin responsive tissue such as skeletal muscle and adipose tissue. GLUT4 is activated in response to distinct stimuli including insulin and exercise (Marette *et al.*, 1992; Lund *et al.*, 1995). This transporter is well expressed in differentiated myotubes and its presence in myoblasts is scanty (Gaster *et al.*, 2000; Al-Khalili *et al.*, 2003). Under basal unstimulated conditions, GLUT4 remains within intracellular GLUT storage vesicles in the intracellular space (cytoplasm) and less than 5% of total GLUT4 cell content is located at the cell surface (Gould and Holman, 1993). Upon insulin stimulation these GLUT4 containing vesicles translocate to the cell membrane (increased exocytosis). For example, in brown adipose tissue insulin stimulation leads to a 40-fold increase in cell surface GLUT4 compared to unstimulated cells (Slot *et al.*, 1991). Simultaneously, myotubes previously stimulated with insulin have a 2-3 fold increase in plasma membrane levels of GLUT4 compared to non-stimulated cells (Fazakerley *et al.*, 2010).

1.10 Cascades of glucose transport into skeletal muscle cells

The major contributors of glucose transport activity in skeletal muscle are insulin and contraction. Under basal conditions, GLUT1, and to a lesser extent GLUT4, on the cell surface facilitate glucose transport. Upon insulin stimulation, insulin initiates a series of signalling events that involve insulin receptors, insulin receptor substrates, PI3K, PIP3 and/or PDK, AKT, aPKC and AS160. These events lead to translocation of GLUT4 storage vesicles from the intracellular space to the cell surface facilitating glucose transport.

During exercise, glucose uptake into skeletal muscle increases independent of the insulin pathway. The increased glucose uptake into skeletal muscle is achieved by muscle contraction and not by increased blood flow associated with exercise. This was confirmed by *in vitro* contraction studies carried out in the absence of insulin, showing the same GLUT4 recruitment to the cell membrane (Goodyear *et al.*, 1990; Brozinick *et al.*, 1994; Gao *et al.*, 1994). It seems that exercise mediates its activity at least partially through the AMPK-dependent pathway. Studies show that muscle contraction or exercise mimetics such as AICAR induce GLUT4 translocation from intracellular vesicles (cytoplasmic location) to the cell surface (Koistinen *et al.*, 2003; Friedrichsen *et al.*, 2012). Figure 1-4 summarises the glucose uptake mechanisms into skeletal muscle.
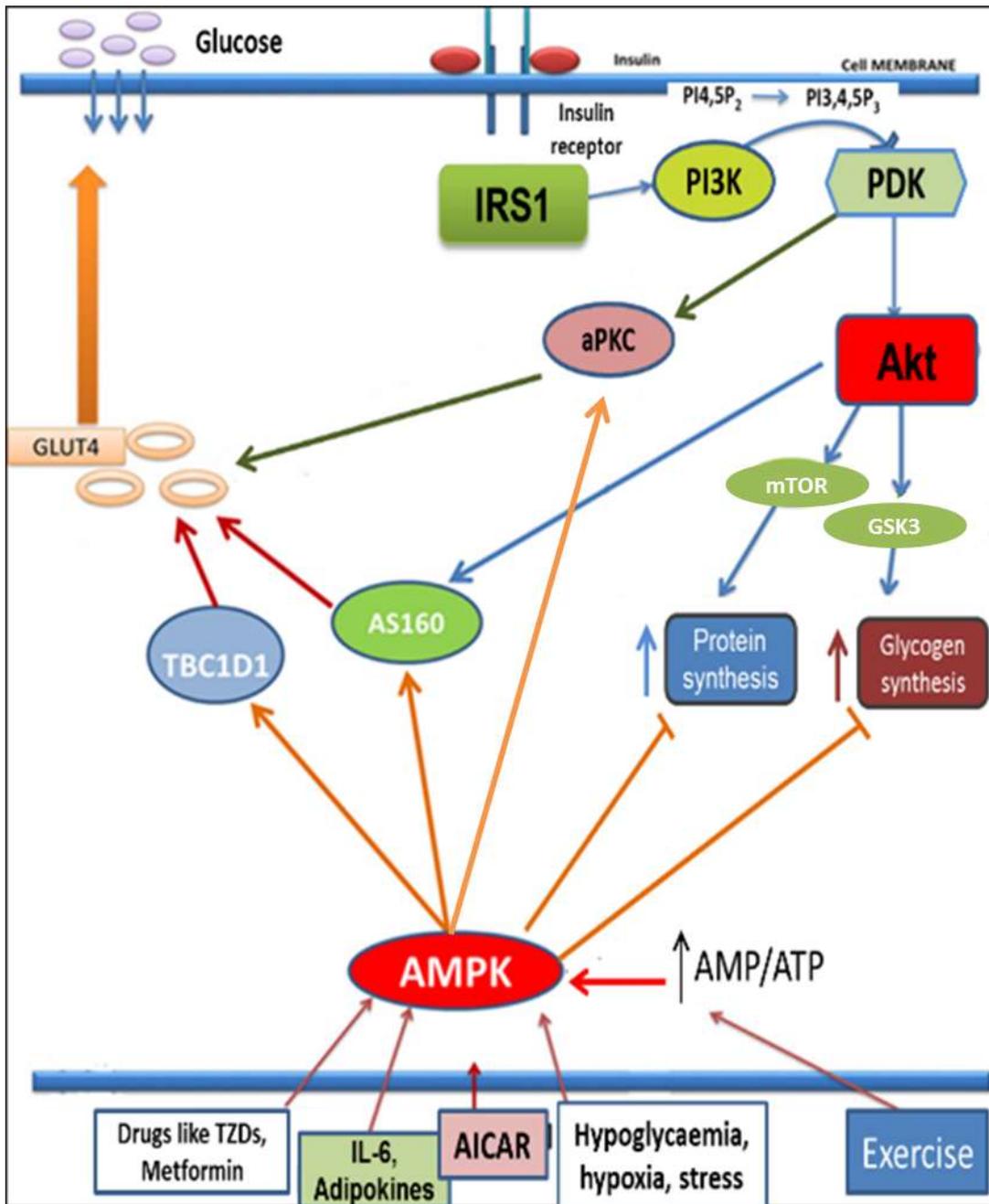
Figure 1-4: Presumed mechanisms of insulin/AMPK signalling and glucose uptake into skeletal muscle cells.

1.11 Insulin resistance in skeletal muscle

As previously mentioned, the term insulin resistant (IR) means an impairment of insulin action in insulin sensitive tissues such as skeletal muscle, liver and adipocytes. Regarding skeletal muscle, the primary action of insulin is enhancement of glucose uptake and metabolism. If a comparison takes place between healthy lean and insulin resistant individuals, the result will be a marked reduction in insulin stimulated glucose uptake as well as a delay in the insulin response in the insulin resistant individual as shown in Figure 1-5. Many studies show that the abnormality in insulin resistant skeletal muscle is not only a decrease in the magnitude of insulin action, but also a delayed onset of insulin to stimulate glucose uptake (Sargeant and Pâquet, 1993; Stuart *et al.*, 2006; Abdul-Ghani and DeFronzo, 2010).
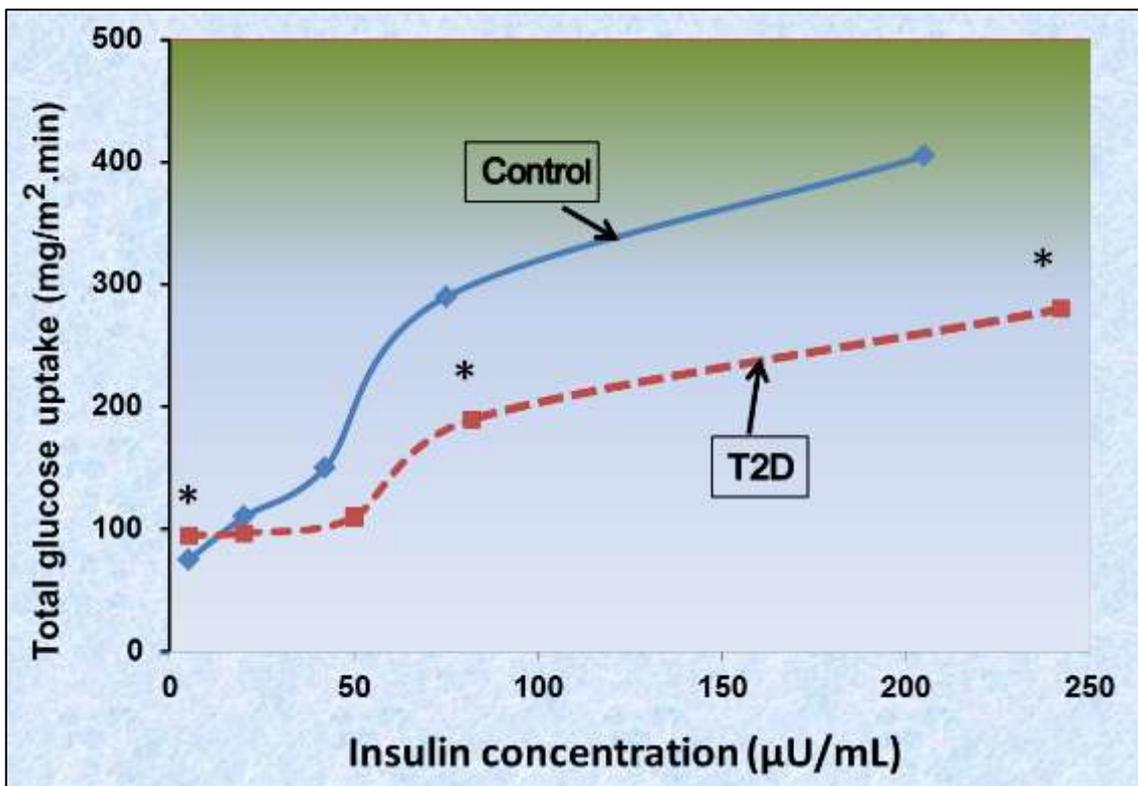
Figure 1-5: Dose-response curve of insulin-glucose uptake in response to an increasing dose of insulin ∗P < 0.01 adopted from (Groop et al., 1989; Abdul-Ghani and DeFronzo, 2010).

1.12 Defects in insulin signalling in insulin resistant skeletal muscle

Generally, it is well accepted that the ability of insulin to facilitate glucose disposal in skeletal muscle is impaired in T2D patients (DeFronzo *et al.*, 1985; Hepburn *et al.*, 1994).

In skeletal muscle of insulin resistant individuals, insulin receptor tyrosine kinase activity has been shown to be impaired but this is regarded as an acquired secondary defect to abnormal glucose metabolism (Pratipanawatr et al., 2001). Reversing this metabolic defect by weight loss can restore normal insulin receptor tyrosine kinase activity (Freidenberg et al., 1988).

Reduced IRS1 activity in skeletal muscle is associated with either decreased tyrosine phosphorylation or increased serine phosphorylation. In both *in vitro* (muscle culture or muscle strips) or *in vivo* (during hyperinsulinemic clamp) studies, skeletal muscle from T2D subjects was shown to have a reduced insulin-stimulated IRS1 tyrosine phosphorylation activity (Krook *et al.*, 1998; Cusi *et al.*, 2000; Kim *et al.*, 2003). In addition, animal and human studies have demonstrated increased serine phosphorylation of IRS1 in the skeletal muscle of insulin resistant subjects (Yu *et al.*, 2002; Morino *et al.*, 2005).

Aberrant activity of PI3K in human skeletal muscle from T2D subjects has been reported (Björnholm *et al.*, 1997; Krook *et al.*, 2000; Bandyopadhyay *et al.*, 2005). Furthermore, binding of the p85 regulatory subunit of PI3K to IRS1 is also diminished in obese and T2D compared to normal lean subjects (Pratipanawatr *et al.*, 2001).

Data about the defects in Akt activity in insulin resistant subjects is debatable. On the one hand, a number of studies reported an impaired phosphorylation of Akt at Ser^{473} or Thr^{308} in skeletal muscle obtained from T2D and insulin resistant individuals (Krook *et al.*, 1998; Meyer *et al.*, 2002; Karlsson *et al.*, 2005; Hojlund *et al.*, 2008). On the other hand, no such impairments have been found in skeletal muscle of T2D (Kim *et al.*, 1999; Krook *et al.*, 2000; McIntyre *et al.*, 2004).

PI3K/Akt signalling to GLUT4 translocation is linked via AS160. In skeletal muscle from T2D subjects, the insulin-stimulated phosphorylation of AS160 is significantly decreased (Karlsson *et al.*, 2005). Moreover, the decreased AS160

phosphorylation in T2D skeletal muscle is correlated with impairment in glucose transport (Karlsson *et al.*, 2006).

There is significant impairment in GLUT4 translocation from the intracellular sites to the membrane in skeletal muscle from T2D compared to control (Ryder *et al.*, 2000; Koistinen *et al.*, 2003). No differences in GLUT4 content have been observed between skeletal muscle from T2D subjects and normal controls (Pedersen *et al.*, 1990; Eriksson *et al.*, 1992). Thus, the defects in insulin stimulated glucose uptake in skeletal muscles from T2D have been shown to be in insulin signalling affecting GLUT4 translocation and not in GLUT4 expression.

1.13 Role of inflammation in insulin resistance development

Insulin resistance has been identified to be associated with a state of low grade inflammation and therefore it is suggested that inflammation plays an essential role in the development of insulin resistance (Glass and Olefsky, 2012). Studies showed that patients with sepsis and septic shock were in an insulin resistant state (Clowes *et al.*, 1978; Raymond *et al.*, 1980). In addition, insulin resistance is observed in patients with chronic inflammatory diseases such as active rheumatoid arthritis (Svenson *et al.*, 1987). Furthermore, induction of acute inflammation induces systemic insulin resistance in humans (Mehta *et al.*, 2010).

 One of the first and most important inflammatory cytokines to be associated with insulin resistance is TNFα. An early observation by Hotamisligil et al. (1993) primarily linked the increased expression of this cytokine in adipose tissue of different animal models, with insulin resistance. The same study showed that neutralisation of high TNFα enhances insulin sensitivity. In humans, a similar pattern of increased expression of TNFα in adipose tissue of individuals with insulin resistance has been observed (Kern et al., 1995), and after weight loss cytokine expression returned to normal levels (Dandona et al., 1998; Moschen et al., 2010). At the cellular level, TNFα has been shown to deactivate insulin signalling by inhibiting IRS1 activation (Aguirre et al., 2000). The classical isoform of PKC is involved in the TNFα inhibitory action (Sampson and Cooper, 2006).

IL-6 is another important cytokine associated with insulin metabolism. The role of IL-6 in peripheral insulin resistance is debatable. On the one hand, like TNFα, raised serum concentrations of IL-6 is linked to obesity and T2D (Hotamisligil,

2006). Adipose tissue is the major source of IL-6 in the body so an increased secretion and expression of the cytokine in obese subjects is expected, and is correlated with impaired whole-body insulin sensitivity (Bastard *et al.*, 2002). On the other hand, acute exposure of insulin sensitive tissues (cultured skeletal muscles) to IL-6 is associated with improvement in insulin action (Al-Khalili *et al.*, 2006; Carey *et al.*, 2006). Furthermore, circulatory levels of IL-6 seem to be elevated with muscle contraction and this is associated with an improvement in insulin action (Steensberg *et al.*, 2000; Febbraio *et al.*, 2004). Moreover, neutralisation of IL-6 in humans has been shown to improve insulin action and glucose levels (Schultz *et al.*, 2010).

In conclusion, acute and chronic inflammation is associated with whole body insulin resistance. Chronic elevation of these cytokines is associated with insulin resistance while the acute increase in response to exercise or direct treatment has positive insulin sensitisation effects.

1.14 AMP-activated protein kinase (AMPK)

Since 1987 the name AMP-activated protein kinase has been adopted for the serine-threonine kinase, but it was discovered long before that as an enzyme regulating FFA and cholesterol synthesis (Steinberg and Beck Jorgensen, 2007). Recently, AMPK emerged as an enzyme regulating several metabolic processes associated with glucose and lipid metabolism in skeletal muscle (Hardie *et al.*, 2012).

Activation of AMPK by exercise leads to increased glucose uptake into skeletal muscle and enhances insulin stimulated glucose uptake. These effects of AMPK draw attention to its ability to improve glucose metabolism and control metabolic abnormalities associated with T2D (Hardie, 2013). In addition to metabolism-related functions, AMPK is associated with cell growth, gene expression, protein synthesis, and other important cell functions (Richter and Ruderman, 2009). The fully functional enzyme kinase consists of three subunits, α (catalytic), and β and γ regulatory units. Activation of AMPK occurs in response to an increase in the AMP/ATP ratio either due to decreased ATP production such as in hypoxia and ischemia, or increased energy consumption as observed in muscle contraction (Witczak *et al.*, 2008).

1.15 Structure of AMPK

Structurally, AMPK consist of three subunits and each one of these subunits is present in different isoforms, there are two α subunits (α1 and α2), two β subunits (β1 and β2) while γ has three isoforms (γ1, γ2 and γ3). The ratio of these subunits is 1 α: 1β: 1γ, Thus, human cells could contain 12 different isoform combinations of AMPK (Hardie and Sakamoto, 2006),and this isoform diversity may have an influence on the activation of AMPK in response to different stimuli and also determine the subcellular localization of the kinase (Hardie, 2007; Oakhill *et al.*, 2009). In human skeletal muscle the predominant isoform expression is the α2β2γ3 heterotrimer (Chen *et al.*, 1999).

1.15.1 α subunit

In skeletal muscle, this subunit has two isoforms, α1 and α2. Both have similar catalytic activities. The α subunit is highly conserved consisting of an N-terminal catalytic domain that contains the Thr^{172} residue that represents the activation or deactivation point by phosphorylation and dephosphorylation respectively, followed by an auto inhibitory domain. The C-terminus in this subunit is responsible for binding to the β and γ subunits (Oakhill *et al.*, 2009). The phosphorylation and dephosphorylation of the Thr^{172} residue are achieved by its by upstream kinases and phosphatases, respectively (Gowans *et al.*, 2013). The presence of different isoforms of α subunit suggests diverse actions and subcellular localization within mammalian cells. For example, the α1 subunit is presents in non-nuclear location of AMPK, while the **α**2 subunit has nucleus, cytoplasmic and membrane activities (Salt *et al.*, 1998).

1.15.2 β subunit

The β subunit is one of the two regulatory subunits of the kinase complex, has two isoforms (β1and β2), and each comprises three biochemical parts. The N-terminal is responsible for membrane docking of AMPK (Oakhill *et al.*, 2009). The C-terminal is required for the binding with α and γ subunits. The β subunit contains the central region which is termed the glycogen binding domain (GBD) (Hardie and Sakamoto, 2006) or it is sometimes called the carbohydrate binding module (CBM) (Oakhill *et al.*, 2009). The binding of glycogen to the β subunit of AMPK seems to

directly regulate the kinase activity, the more abundant the glycogen store the less the AMPK activation (Wojtaszewski *et al.*, 2002).

1.15.3 γ subunit

The AMPK allosteric binding sites of AMP or ATP are located within the γ subunit, which has an N-terminal region followed by four highly conserved cystathionine-beta-synthase (CBS) sequence motifs. Each one of the CBS acts as a potential site for binding with adenine nucleotides and each two of these motifs together form a Bateman domain. So the γ subunit contains two Bateman domains which represent the functional site of this subunit (Towler and Hardie, 2007; Witczak *et al.*, 2008).

Mutations in the γ2 subunit in cardiac muscle results in a type of arrhythmic disorder known as Hereditary Wolff-Parkinson–White syndrome (WPW) which might be caused by high glycogen content of the cardiac muscle due to inactivation of AMPK (Scott *et al.*, 2004). The γ3 subunit is expressed exclusively in skeletal muscle and mutation in the Bateman domain of this isoform is also associated with high glycogen content within muscles cells but the clinical outcome of this accumulation is not fatal like that of the cardiac muscle. Instead, the affected muscle seems to be more tolerant to high glycogen levels and becomes more resistant to fatigue (Barnes *et al.*, 2005).

1.16 AMPK allosteric activation

This is achieved by the binding of adenine nucleotide in AMP/ADP or ATP to the Bateman domains in γ subunit of the kinase. The binding of the different adenine nucleotides to the AMPK supports the kinase characteristic energy sensor of the cell (Hardie and Sakamoto, 2006). AMP should compete with ADP and ATP to the binding sites on the γ subunit of the kinase complex because the γ binding sites have the same affinity for binding adenine nucleotides. The binding of AMP or ADP, but not ATP, causes conformational changes in the γ domains that facilitate activation of the catalytic α subunit by upstream kinases through phosphorylation of Thr[172] and simultaneously inhibiting dephosphorylation by upstream phosphatases (Xiao *et al.*, 2011).

1.17 AMPK upstream kinases activation

AMPK activation is achieved by phosphorylation and activation of the Thr[172] residue of α catalytic subunit of the kinase molecule. The activity of the kinase can be increased up to 100 times above its basal level through activation of α subunit (Thr[172]) by upstream kinase, in addition to the allosteric activation that result from the binding of AMP to γ subunit that may magnify the effect of the kinase up to 10 times. The amplification of the kinase activity in response to exercise could reach up to 1000 time the enzyme basal activity (Hardie *et al.*, 2012). Figure 1-6 is a representative diagram for AMPK activation mechanisms.
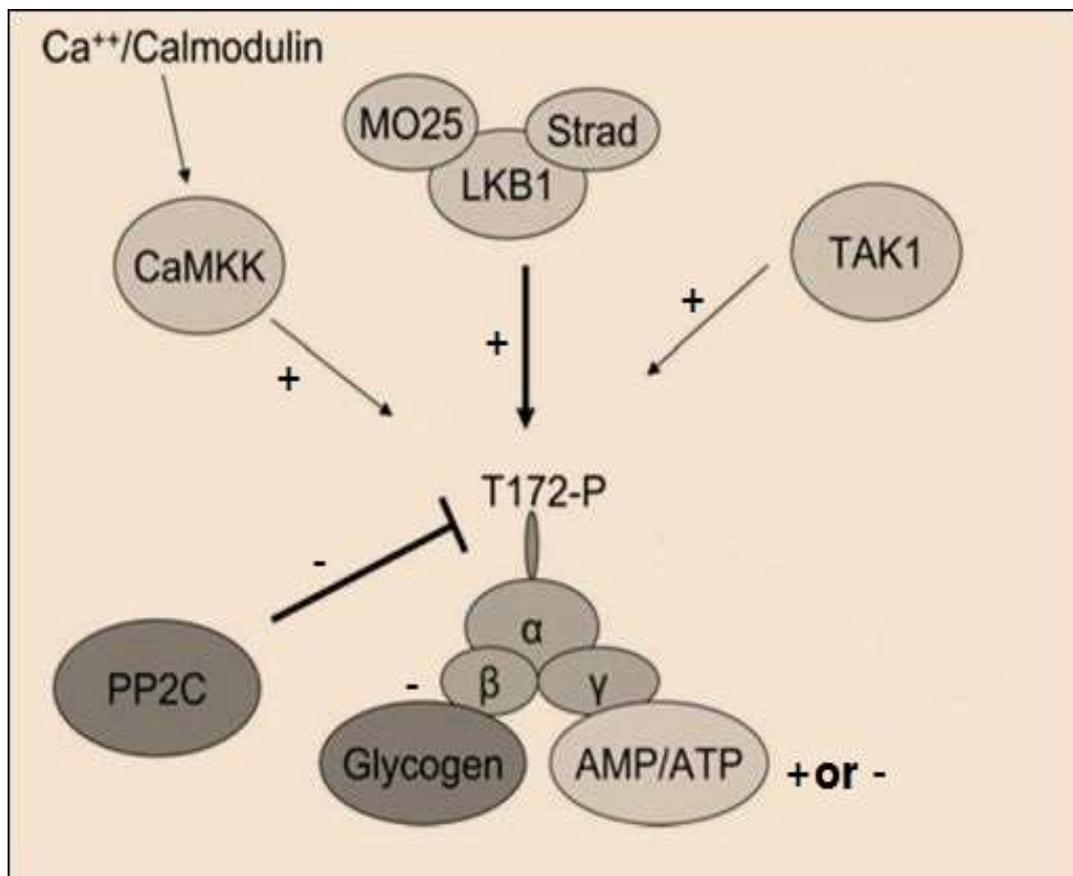
Figure 1-6: A schematic representation of AMPK activation showing both allosteric activation by AMP binding to γ subunit and activation of the α subunit (Thr172) by upstream kinases. The figure was adapted from (Richter and Ruderman, 2009).

1.17.1 LKB1 (liver kinase beta 1)

LKB1 is the major upstream kinase that phosphorylates Thr^{172} residue on the α subunit of the AMPK complex. It was first recognized as a complex with tumour suppressor activity, and suppressed LKB1 activity is recognised in a rare autosomal dominant disorder, Peutz-Jeghers syndrome, which is characterized by multiple benign tumours in the gastrointestinal tract (Hemminki *et al.*, 1998; Jenne *et al.*, 1998). In addition to this rare syndrome, gene defects of LKB1are related to sporadic cases of cancer (Sanchez-Cespedes *et al.*, 2002).

LKB1 has a complex structure and requires binding with two accessory protein subunits to be fully activated; these proteins are STRAD and MO25 (Hawley *et al.*, 2005). Experiments in human skeletal muscle cultured cells show that both of these subunits are essential for AMPK activation by AICAR (Woods *et al.*, 2003). LKB1 represents the most important upstream activator of AMPK but it requires the presence of allosteric activation of the γ subunit by AMP. In other words LKB1 acts as an activator only in the presence of AMP (Suter *et al.*, 2006; Witczak *et al.*, 2008).

1.17.2 CaMKKs (calcium/Calmodulin-dependent protein kinase kinases)

In some experiments where LKB1 activity is inhibited or blocked, AMPK could be still activated (phosphorylated) in response to Ca^{2+} ions or contraction and this led to the discovery of CaMKKs (Woods *et al.*, 2005; Fogarty *et al.*, 2010). Activation of CaMKKs depends on an increase in intracellular Ca^{2+} levels. Elevated Ca^{2+} leads to the binding of Ca^{2+} with calmodulin, the resultant complex binds to CaMKK leading to an increase in its activity (Hawley *et al.*, 1995). Studies show that CaMKK activates AMPK in an AMP-independent way as reviewed in Witczak *et al.* (2008).

1.17.3 TAK1 (Transforming factor –beta- activated kinase 1)

TAK1 is a serine/threonine protein kinase. TAK1 is expressed in different tissues in the mammalian body including skeletal muscle (McGee *et al.*, 2008). In mammalian cells, TAK1 is activated by a number of cytokines such as transforming growth factor beta and TNFα, and is also activated by chemical agents that either alter or mimic AMP/ATP levels like AICAR and metformin (Xie *et al.*, 2006). In cell-free studies, TAK1 directly phosphorylates the Thr^{172} residue of

the α subunit of AMPK in the same way that LKB1 and CaMKKs do (Kondo *et al.,* 1998).

1.17.4 Upstream phosphatases (Protein phosphatases 2A and 2C)

Protein phosphatases 2A and 2C mediate dephosphorylation of Thr^{172} of the AMPK complex (Suter et al., 2006). The effects of these phosphatases depend on the intracellular levels of AMP and lipid content within the cell (Wu et al., 2007). The adenine nucleotides indirectly regulate dephosphorylation of Thr^{172} via upstream phosphatases. By binding of adenine nucleotides (AMP or ADP) to the γ subunit a conformational change results that permits access of upstream kinases to activate Thr^{172} residue and prevent upstream phosphatases from causing dephosphorylation.

1.17.5 Inhibition of AMPK by glycogen

Glycogen plays a critical role in AMPK activity regulation in skeletal muscle. This regulatory role might be mediated by direct binding of glycogen to glycogen binding domain (GBD) on β subunit of the kinase (Hardie and Sakamoto, 2006). In experimental work on rodent muscle, AMPK activity was suppressed in response to AICAR or contraction in a high glycogen containing environment (Derave *et al.*, 2000; Wojtaszewski *et al.*, 2002). In human studies, activation of AMPK was greater in glycogen-depleted compared to a glycogen-loaded muscles (Wojtaszewski *et al.*, 2003). AMPK regulation is not only determined by energy availability (ATP) but also by fuel reserves (glycogen).

1.18 Physiological activators of AMPK

Leptin is a protein secreted from adipocytes. It plays an important role in whole body energy haemostasis, immunity, and inflammation (Antuna-Puente *et al.*, 2008). In skeletal muscle, leptin increases FFA oxidation and also reduces the lipid content inside the cell in an AMPK-mediated way (Minokoshi *et al.*, 2002). Leptin also induces activation of AMPK in skeletal muscle by direct interaction of leptin with its receptor at the cell surface (Minokoshi *et al.*, 2002).

Adiponectin is an adipokine secrete from adipocytes. In skeletal muscle, adiponectin induces activation of AMPK and its downstream target ACC causing an enhancement of FFA oxidation (Yamauchi *et al.*, 2002). In addition, adiponectin

causes an increase in glucose uptake in a mechanism independent of insulin (AMPK mediated) (Ceddia *et al.*, 2005).

During exercise, IL-6 is released from skeletal muscle into the circulation and its level increases up to 100 fold (Al-Khalili *et al.*, 2006). Elevated IL-6 enhances glucose uptake via an insulin-independent and AMPK-dependent mechanism (Carey *et al.*, 2006). Furthermore, IL-6 has an additive effect to insulin in enhancing glucose uptake (Geiger *et al.*, 2007). Nieto-Vazquez *et al.* (2008) clarified the complexity of IL-6 behaviour with glucose uptake and insulin sensitivity. They found that, chronic treatment induces insulin resistance, while acute exposure enhances insulin stimulated glucose uptake via an AMPK dependent mechanism as discussed in1.13.

1.19 Pharmacological activators of AMPK

Studies on AMPK became more productive after the use of AICAR (5-aminoimidazole-4-carboxamide 1-b-D-ribofuranoside) due to its ability to produce an exercise-like effect in skeletal muscle (Witczak *et al.*, 2008). AICAR is internalized to the cell by the adenosine transporter, and metabolized by the enzyme adenosine kinase into 5-aminoimidazole-4-carboxamide 1-b-D-ribofuranosyl monophosphate (ZMP), an AMP analogue. ZMP acts like endogenous AMP by binding to the Bateman domains of the γ subunit of AMPK and promoting allosteric activation of the kinase (Gadalla *et al.,* 2004). In cultured muscle cells from rodents or humans with T2D, treatment with AICAR causes an enhancement of glucose uptake and GLUT4 translocation via an insulin-independent mechanism (Hayashi *et al.*, 1998; Kurth-Kraczek *et al.,* 1999).

Thiazolidinediones (TZDs) are insulin sensitising drugs such as rosiglitazone and epioglitazone. Studies demonstrate that addition of TZDs to muscle cells in culture activates AMPK as well as its downstream effects including glucose uptake and FFA oxidation (LeBrasseur *et al.*, 2006). Moreover, chronic treatment of skeletal muscle cells from obese mice that display a reduced AMPK activity leads to restoration of the normal kinase activity (Lessard *et al.*, 2006).

Metformin is the most widely used antidiabetic agent all around the world. It is derived from a plant extract (galegin) that was used in medieval Europe for treatment of diabetic-like conditions (Hardie *et al.*, 2012). The therapeutic effects

of the drug are mediated through inhibition of gluconeogenesis in liver cells as well as activation of AMPK in skeletal muscle. The exact molecular mechanism of activation of AMPK by metformin is not fully clarified. Studies showed that metformin treatment in rat skeletal muscle leads to AMPK activation and increased skeletal muscle glucose transport (Zhou *et al.*, 2001). In human skeletal muscle of T2D subjects, metformin has been shown to increase phosphorylation of AMPK (Musi *et al.*, 2002).

1.20 Downstream events and targets of AMPK

Activation of AMPK is achieved by contraction or any cellular events associated with increased energy demands. The downstream events of AMPK activation can be divided into two phases; an acute phase that consists of phosphorylation of metabolic enzymes which subsequently lead to either activation or deactivation of these enzymes (such as inhibition of fatty acid synthesis), and a chronic phase which comprises changes in gene expression (either increased or decreased), followed by specific protein production or cellular responses such as mitochondrial biogenesis and increased GLUT4 expression (Hardie and Sakamoto, 2006).

1.21 Regulation of lipid metabolism by AMPK

AMPK activation increases FA uptake by facilitating the translocation of FA transporters (FAT/CD36) to the cell membrane (Coort *et al.*, 2004). Furthermore, activated AMPK mediates an inhibition of acetyl-CoA carboxylase2 (ACC2). This inhibition leads to a drop in malonyl-CoA levels which represents the key mediator for both FA synthesis and FA oxidation (Winder *et al.*, 1997). Low malonyl-CoA has been associated with activation of Carnitine palmitoyltransferase-1 (CPT-1), an enzyme responsible for transferring cytosolic long chain fatty acyl group (LCFA-CoA) into the mitochondria (a rate limiting step in FA oxidation) (McGarry and Brown, 1997). Thus, activated AMPK causes a reduction in malonyl-CoA levels, enhancement of internalization, and oxidation of FA in mitochondria.

AMPK directly inhibits phosphorylation of ACC1 (acetyl-CoA carboxylase 1), which is the key enzyme in FA synthesis. Furthermore, hydroxymethylglutaryl-coenzyme A reductase (HMGR-CoA) that regulates cholesterol synthesis is inhibited by AMPK as well and glycerol phosphate transferase, the important enzyme in TGL

synthesis and hormone-sensitive lipase (HSL) (Carling *et al.*, 1987; Garton *et al.*, 1989).

AMPK blocks the activation of transcription factors involved in lipogenic pathways. For example, SREBP1c (sterol regulatory element-binding protein 1c) which is a key transcription factor in lipogenic and FA synthesis (Foretz *et al.*, 2005).

1.22 Exercise

1.22.1 Effects of exercise on glucose homeostasis

A sedentary lifestyle represents a modifiable cause of various diseases including insulin resistance and T2D. Regular physical activity (exercise) combined with dietary intervention are recommended by health providers as a first line for prevention and treatment of T2D and could be more successful than drug treatment (Knowler *et al.*, 2002). Both acute (single bout of exercise) and regular exercise training are known to correct the metabolic abnormalities associated with insulin resistance and T2D, and regular exercise training is recognised as a cornerstone in the prevention and management of T2D (Knowler *et al.*, 2002; Haskell *et al.*, 2007).

Independently of each other, insulin and exercise can increase glucose uptake via an improvement in GLUT4 translocation from the cytoplasm to the cell membrane (van Dijk *et al.*, 2012). During rest, skeletal muscle glucose uptake after meal is totally dependent on insulin action, whereas during exercise, the glucose flux into skeletal muscle is an insulin independent process. Muscle contraction stimulates GLUT4 translocation and glucose uptake in the complete absence of insulin and the effects of contraction and insulin are additive. These observations reveal that contraction and insulin stimulate glucose uptake by separate pathways (Holloszy and Hansen, 1996; Sakamoto and Goodyear, 2002; Holloszy, 2003).

Muscle contraction stimulates glucose uptake by increase GLUT4 translocation into the cell surface. The particular mechanisms that involve the increase in GLUT4 expression at the cell membrane in response to contraction are not fully clarified but it is generally believed that AMPK is involved. However, some studies

on AMPK knockdown mice show a normal increase in GLUT4 expression level at cells membrane in response to contraction (Maarbjerg *et al.*, 2009).

When AMPK kinase is activated, it serves as an energy controlling enzyme by switching off cellular processes that cause energy consumption like protein, lipid, and glycogen synthesis as well as promoting and enhancing ATP production through increasing glucose uptake and metabolism and FFA uptake and oxidation (Hardie *et al.*, 2006).

1.22.1 Role of AMPK in exercise mediated glucose metabolism

During exercise, intracellular glucose and glycogen are the main energy substrates in skeletal muscle and their consumption is related to the degree of exercise intensity (Holloszy *et al.*, 1998). The sources of muscle glucose are either from plasma glucose or from degradation of muscle glycogen.

Activation of AMPK by contraction or AICAR regulates glucose uptake by increasing GLUT4 translocation from intracellular vesicles (cytoplasmic location) to the cell surface (Merrill *et al.*, 1997; Koistinen *et al.*, 2003).

Studies showed that the distal insulin signalling pathway could be involved in AMPK-stimulated glucose uptake. The identification of AS160 as a key substrate for the insulin signalling pathway was a critical challenge and represents the link between insulin signalling and GLUT4 translocation (Kane *et al.*, 2002; Sano *et al.*, 2003). Moreover, skeletal muscle expressing mutant TBC1D4/ TBC1D1 at the key activating sites exhibit impaired insulin-stimulated glucose uptake (Kramer *et al.*, 2006b; Cartee and Wojtaszewski, 2007) and impaired contraction and AICAR stimulated glucose transport (Sakamoto and Holman, 2008; Pehmøller *et al.*, 2009)

In addition, AMPK has an essential role in long term glucose uptake by increasing the expression of both GLUT4 and hexokinase (Holmes *et al.*, 1999). The pathway by which AMPK activates GLUT4 expression involves the transcription coactivator PGC1-α (Jäger *et al.*, 2007). This increase in GLUT4 expression and thereby production might contribute to the enhancement in glucose uptake in response to insulin after exercise and explain the additive effects of AMPK on insulin-activated glucose uptake.

AMPK activation leads to the inhibition of glycogen synthesis and promotes glycogen breakdown (glycogenolysis). By inhibiting glycogen synthesis, AMPK achieves two effects; it decreases energy consumption and provides a source of glucose supplement by degradation of the stored glycogen. *In vitro* studies show that AMPK phosphorylates glycogen synthase (GS) at site 2 (Ser[7]) and this leads to deactivation of the enzyme (Wojtaszewski *et al.*, 2002). Moreover, β-subunit of AMPK has the ability to bind to glycogen at specific binding sites and the high glycogen contents inside the cell can inhibit AMPK activity (McBride *et al.*, 2009).

1.22.2 Exercise stimulates inflammatory cytokines release

Skeletal muscle is considered an endocrine organ that releases a variety of cytokines which are called (myokines). Muscle contraction during exercise is supposed a key stimulus for both expression and release of myokines such as IL-6 (Steensberg *et al.,* 2000) and IL-8 (Steensberg *et al.,* 2007).

Cytokines are a family of polypeptides or proteins that play an essential role in cell to cell signalling. Once secreted, they can act on the same cells or on distant tissues. Cytokines produce from different tissues of the body including adipose tissue, immune cells and skeletal muscle. It is well accepted now that skeletal muscle contraction during exercise increases the production and release of particular cytokines into the circulation leading to elevated their blood levels (Pedersen *et al.*, 2003; Febbraio and Pedersen, 2005). However, the increase in inflammatory cytokines such as IL-6 has been reported with metabolically disturbed events such as obesity and/or insulin resistance (Hotamisligil, 2006) and sepsis (Pedersen and Febbraio, 2008). The difference noted is that, unlike obesity that leads to insulin resistance, the increase of IL-6 with exercise is not continuous. In other words, it is variable with a rapid increase to reach the peak at the end of exercise or shortly after followed by a rapid decrease to return to the pre-exercise level (Steensberg *et al.*, 2000). Another difference between cytokine response to exercise and sepsis is that the increase in inflammatory cytokines in response to exercise is not preceded by an elevation in plasma TNFα such as that observed in sepsis which could be responsible for the negative metabolic effects (Steensberg *et al.*, 2000).

1.22.3 Electrical pulse stimulation (EPS) as an in vitro system to exercise cultured myotubes

Exercise is a complex process at the cellular level and to fully understand this complexity, an *in vitro* system for studying exercise would be of value. The creation of an *in vitro* model of exercise helped to explore the molecular mechanisms and the cellular responses as well as the therapeutic implications of the exercise. For the last few decades, *in vitro* models of exercise comprised of skeletal muscle fibres activated by nerve impulse. Recently, a new technique involving electrical pulse stimulation (EPS) of cultured myotubes has been developed. Since the 1970s, early attempts can be tracked of the use animal primary skeletal cells in an *in vitro* model of exercise (Nikolić *et al.*, 2016). The first published study that used EPS in human myotubes was in 2002. The results of that study showed an increase in glucose uptake in human cultured myotubes in response to EPS which was similar to that observed during *in vivo* muscle contraction (Aas *et al.*, 2002).

Two main protocols described for electrical stimulation. The first is comparable to an acute bout of exercise, with high-frequency stimulation for a short-duration (Egan and Zierath, 2013). The second, chronic low-frequency EPS is used as an analogue for chronic exercise training (Nikolić *et al.*, 2012), and the data obtained from the latter model of EPS makes it more convenient for studying skeletal muscle contraction. Observations of studies like that performed by Nikolic *et al.* (2012) showed that chronic low frequency EPS causes increased glucose uptake as well as improving myotubes oxidative capacity for both glucose and FFA. These observations are similar to the adaptive changes observed during exercise training *in vivo* (Hayashi *et al.*, 1997; Santos *et al.*, 2008). However, variable metabolic effects of exercise were observed using chronic low-frequency EPS and certain adaptations observed with *in vivo* exercise cannot be reproduced by EPS, as shown in detail in Figure 1-6.
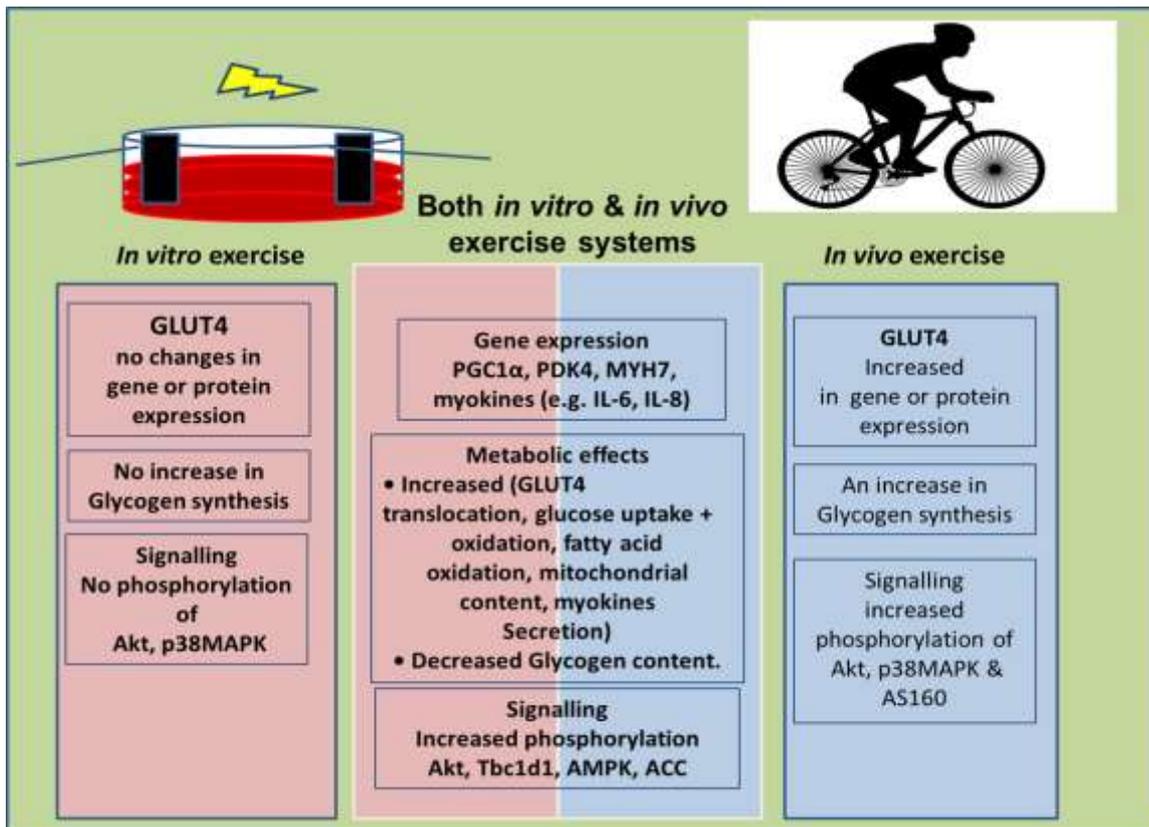
Figure 1-6: A comparison of the effects of different exercise models(*In vitro* and *in vivo*). The left column shows some gene, metabolic and signalling changes observed in response to *In vitro* exercise model (EPS) in cultured cells. The right column shows gene, metabolic and signalling changes observed in response to *in vivo* exercise studies. The middle column shows the changes in gene, metabolic and signalling responses that observed in common both *in vitro* and *in vivo* systems (Nikolić *et al.*, 2016).

1.23 Thrombin

Thrombin (factor IIa) is a serine protease that converts fibrinogen into fibrin in blood coagulation. It is involved in several biological processes in addition to its role in the coagulation cascade (Bailey *et al.*, 1951). Thrombin is produced from the pro-enzyme prothrombin which is a vitamin K-dependent protein synthesized by the liver. It is proteolytically produced from prothrombin by the action of the coagulation factor Xa. Thrombin then converts the soluble fibrinogen into insoluble fibrin that acts on platelet aggregation in blood clot formation, the vital step in the body's protective mechanism (Licari and Kovacic, 2009). In addition to clot formation and coagulation, thrombin has been observed to affect several cellular processes such as cell division (Chen and Buchanan, 1975), chemotaxis (Bar-Shavit *et al.*, 1983), cancer growth (Tsopanoglou and Maragoudakis, 2004), and inflammation and tissue repair (Johnson *et al.*, 1998).

Thrombin acts via its proteolytic activity, on the cell-surface receptor family of seven transmembrane G-protein-coupled receptors known as protease activated receptor family (PARs). PAR1, PAR3 and PAR4 are activated by thrombin whereas PAR2 is shown to be targeted by other proteases (trypsin) (Wang *et al.*, 2002). Thrombin acts as a protease enzyme on the cell surface receptors. Normally, the binding of thrombin to the PARs receptors leads to proteolytic degradation of the N-terminal of the receptor domain leading to formation of a new N-terminus that activates the receptor itself and initiates the intracellular signalling processes (Vu *et al.*, 1991). This binding and activation facilitates different cellular downstream responses that vary according to cell types and need. Thus, stimulation of cells by thrombin leads to enzyme activation or deactivation, induction of gene expression, cell migration, and division in different cell types.

1.23.1 Role of thrombin in coagulation and fibrinolysis

Disruption of the endothelial surface leads to activation of the coagulation cascades, which is a balance between coagulation and fibrinolysis. Thrombin plays a central role in maintaining this balance. The coagulation process is reviewed by Licari and Kovacic (2009) and summarized in Figure 1-7. In brief, the process starts when circulating factor VII comes into contact with tissue factor (TF)

in the presence of Ca^{2+} and phospholipids. This contact is responsible for activation of factor X to produce Xa which leads to production of only trivial amounts of thrombin from prothrombin. The small amount of the produced thrombin activates factor VIII that results in more Xa production and activation of more Va. The combination of Xa, Va, Ca^{2+} and phospholipids represents the prothrombinase activity which ultimately catalyses more thrombin production. Most of the produced thrombin is on the surface of the platelets in order to localize the activation of thrombin rather than systemic activation which is prevented by other mechanisms. The main process mediated by thrombin is the conversion of fibrinogen into insoluble fibrin that is the cornerstone in thrombus formation. However, the positive feedback effect of thrombin on the production of the Va, VIIa, VIIIa and Xa results in amplification of thrombin production and recruitment of the coagulation cascades, but simultaneously thrombin exerts negative feedback on its production and by this means it takes part in the process of fibrinolysis or anti-coagulation. Thereby thrombin mediates a central role in preventing spread of clot to nearby or even remote tissues.
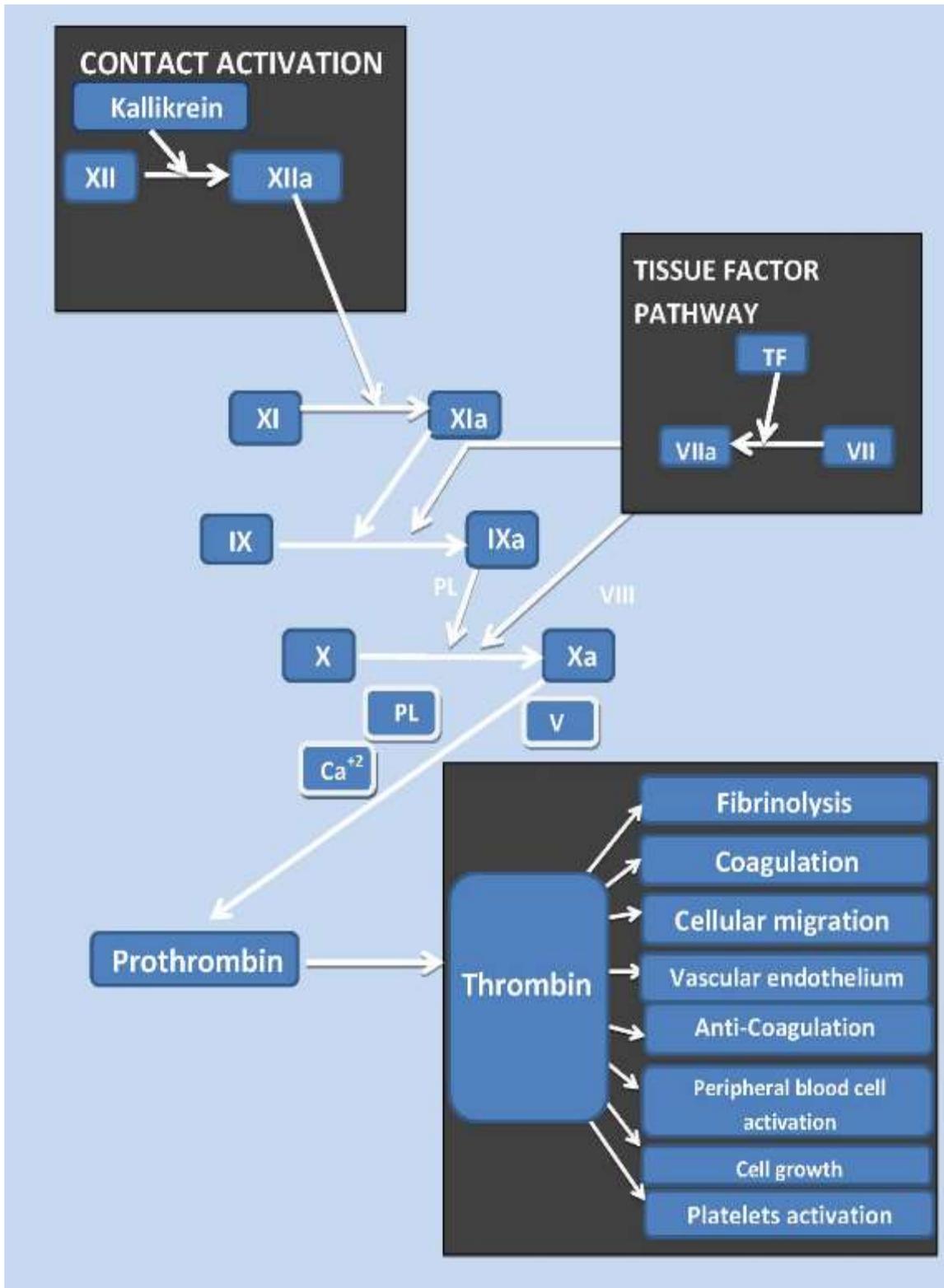
Figure 1-7: Thrombin production and functions, thrombin is produced via coagulation system activation and variable reported functions, adapted from (Carr, 2001; Mann, 2003).

1.23.2 Action of thrombin at the cellular level in skeletal muscle cells

Thrombin acts as a protease enzyme on the cell surface receptors. Distinct PARs are expressed in different tissue types, including skeletal muscle cell. PAR-1 activation has diverse cellular responses including mobilisation of intracellular Ca^{2+}, changes in the metabolism of membrane phospholipids, inhibition of adenylate cyclase, activation of c-Jun N-terminal kinase, protein kinase C, and mitogen-activated protein (MAP) kinases (Mackie *et al.*, 2002; Ossovskaya and Bunnett, 2004; Arora *et al.*, 2007).

Expression of PAR1 in skeletal muscle cells depends on the developmental stage. First, receptor expression is mainly observed in myoblasts with minimal expression after cell differentiation (myotubes stage). In skeletal muscle myoblast cells, stimulation of the cells with thrombin results in activation of PAR-1 and mobilisation of intracellular Ca^{2+} from its store inside muscle cells while its treatment in myotubes does not cause Ca^{2+} mobilization (Suidan *et al.*, 1996). However, other researchers reported that treatment of the cultured myotubes with thrombin causes Ca^{2+} mobilization from the intracellular stores and this activation depends on the age of the subject, the developmental stage and culture environment (Chevessier *et al.*, 2001; Mbebi *et al.*, 2001). Thrombin, through its binding to PAR-1, facilitates important biological processes in myoblasts which include inhibition of cell fusion and differentiation, inhibition of apoptosis, and increase in the rate of cell growth and division (De Niese *et al.*, 2002).

As mentioned earlier, clot formation is not the only function of thrombin. To specify, thrombin has been shown to stimulate increased expression of different cytokines, chemokines and other substances in different cell types. For example, in human adipocytes, thrombin enhances the production of IL-1β, IL-6, TNF-α and other cytokines (Strande and Phillips, 2009). Different cellular pathways are suggested to be involved in this process, including Ca^{2+} ion mobilization from intracellular stores as seen in the myoblast model (Suidan *et al.*, 1996). PI-3 kinase is also involved in PAR activation pathways as observed in mouse mast cells (Gordon *et al.*, 2000). Tanaka *et al.* (2004) suggested that the p38 MAPK and tyrosine kinase pathways are involved in IL-6 secretion in response to PAR-1 activation in human gingival fibroblast. A recent study on bone cells suggested an

AMPK-mediated pathway as a mediator for increasing cytokine production in response to receptor activation by thrombin (Tokuda *et al.*, 2012). Therefore, it seems that activation of thrombin receptors (PARs) stimulates multiple sets of pathways sometime in the same cell line to produce different biological actions.

1.23.3 Thrombin and diabetes

Hypercoagulation is a feature of patients with T2D who are at high risk of thrombotic and vascular events (Carr, 2001). Firstly, T2D subjects are typically associated with increased markers of coagulation system activation (Tripodi *et al.*, 2011). Furthermore, T2D is accompanied by increased clotting factor levels. For example, fibrinogen is higher in T2D subjects compared to normal control (Acang and Jalil, 1992). Tissue factor (TF), Factor VII, Factor VIII, Factor XI and Von Willebrand factor are shown to be elevated in T2D individuals (Carr, 2001). The exact pathogenesis of hypercoagulability in patients with T2D or insulin resistance has been shown to be multifactorial and not fully clarified but two underlying major abnormalities are clear, chronic hyperglycemia and hyperinsulinemia.

T2D is currently regarded as a low grade inflammatory state (Lopez *et al.*, 2006; Daar *et al.*, 2007) and simultaneously, inflammation has been shown to induce insulin resistance. To specify, T2D is associated with an increase in production of inflammatory cytokines such as TNFα, IL-6 and others (Hotamisligil, 2006). Thrombin, a final product of coagulation cascades, has an influence in inflammation. Different cytokines and chemokines expression were shown to be upregulated in response to thrombin in various tissue types. For example, in human adipocytes thrombin increases secretion of interleukin IL-1β, IL-6, TNFα and growth factors (Strande and Phillips, 2009). In addition, thrombin has been shown to induce an increase in inflammatory factors that mediate bladder inflammation (Vera *et al.*, 2010). Therefore, high thrombin levels are associated with T2D and could play a role in inflammation and insulin resistance.

In T2D, there is a strong association between metabolic abnormalities such as hyperglycaemia and increased coagulation (elevated thrombin level) (Rao *et al.*, 1999). In addition, a decreased insulin sensitivity is associated with enhanced thrombin production (Romano *et al.*, 2003). Thus, a combination of factors such as

hyperglycaemia, hyperinsulinemia, increased inflammatory cytokines and hyper-coagulation would predispose T2D individuals to vascular complications.

The impairment of insulin sensitivity, elevated inflammatory markers, and elevated thrombin production are fundamental characteristics of T2D. In an animal model of insulin resistance, inhibition of thrombin action by 4 weeks administration of Argatroban, a selective thrombin inhibitor, results in an enhancement of insulin action in those animals (Mihara *et al.*, 2010). Thus, the enhanced thrombin action in diabetic muscle might play a significant role in insulin resistance.

Furthermore, there is emerging evidence of a correlation between the genetic basis of hypercoagulation and T2D. Genetic correlations were reported in a cohort of Mexican Americans for a number of coagulation factors traits, including prothrombin which was strongly associated with T2D risk (Warren *et al.*, 2005). These observations are consistent with the proposal by Jarrett and Shipley (1988) and Stern (1995) about the common genetic origin of both T2D and cardiovascular disease, the ''common soil'' hypothesis. In addition, GWAS and other gene studies show multiple gene variants in common for T2D or insulin resistance, and coagulopathy. Zhang *et al.*(2016) showed that the IRS1 gene is associated with a high platelet reactivity in T2D individuals, and the platelets hyper-reactivity is strongly linked to high thrombin levels (Nylander and Mattsson, 2003). Additionally, carriers of the minor allele of TCF7L2 gene variants are associated with elevated procoagulant, impaired insulin secretion, and insulin resistance (Delgado-Lista *et al.*, 2011).

1.23.4 Effects of thrombin on glucose uptake

The knowledge of the metabolic effect of hypercoagulation (increased thrombin) on glucose uptake and metabolism in human skeletal muscle is not well known. In other tissue types, thrombin was shown to have multiple metabolic effects on glucose uptake and metabolisms.

In animal study by Mihara *et al.* (2010) showed that, inhibition of thrombin by selective thrombin inhibitor Argatroban in mice model of insulin resistance enhances whole body glucose clearance and improves insulin signalling in fat cells

of these mice. Furthermore in vascular smooth muscle cells, thrombin causes an increase in glucose uptake through p38 MAPK activation and this increase is shown to be insulin independent (Kanda and Watanabe, 2005).

Moreover, thrombin has been shown to stimulate glucose uptake and metabolism in human platelets which could be a key metabolic step for the generation of energy required for thrombus formation (Heijnen *et al.*, 1997).

In mesangial cells of kidney, thrombin is considered as a regulator of glucose uptake and metabolism through its effects on Hexokinase enzyme activity which is the rate limiting enzyme of cellular glucose uptake and metabolism (Robey *et al.*, 2000)

1.24 Skeletal muscle cell culture

Human skeletal muscle satellite cells be isolated from muscle biopsies, nourished, grown *in vitro* and differentiated in to multinucleated myotubes. The cells in culture replicate individual human properties that open a way to examine important human physiology, diseases and treatment in the form of treatment response manner (Aas et al., 2013). Moreover, the cell culture system provides a great opportunity to study muscle metabolism and response to different stimuli due to two important causes; first, the cell culture system is entirely under strictly controlled conditions so that the serum and other environmental factors can be excluded. The second important feature is that the human muscle cells in culture retain phenotypic characteristics such as abnormal glucose metabolism in primary myotubes cultured from insulin resistant individuals (Thompson *et al.*, 1996; McIntyre *et al.*, 2004). Therefore, the human skeletal muscle cell culture is regarded as an invaluable model for studying metabolism in response to different stimuli and contributes impressively to the current understanding of cellular metabolism.

1.24.1 Cultured muscle cells metabolism

Regarding substrate metabolism, by comparing both cultured myotubes and adult skeletal muscle fibres the following findings have been observed:

1. Basal level of glucose uptake tends to be higher in cultured cells than in adult human muscle fibres due to the presence of more GLUT1 transporters (Sarabia *et al.*, 1992).

2. There is a decrease in glucose uptake in response to insulin in cultured cells compared to muscle fibres. This seems to relate to the higher GLUT1/ GLUT4 ratio in cultured muscle cells, and other reasons such as loss effects of *in vivo* nerve stimulation and growth factors. Usually, the increase in glucose uptake in response to insulin is about 40%-50% in cultured myotubes while it is much higher in muscle fibres. This decrease in insulin response in cultured cells leads to the use of higher concentrations of insulin that is more than the physiological level to get the same glucose uptake response in culture (Aas *et al.*, 2002; Al-Khalili *et al.*, 2003).

3. Glycogen synthesis in response to insulin shown to be the same as that noted in adult muscle cells homogenates which is about double the basal level (Al-Khalili *et al.*, 2003; Aas *et al.*, 2004; Al-Khalili *et al.*, 2005).

4. Regarding lipid metabolism, cultured myotubes represent an appropriate model for studying oxidative metabolism in muscle mitochondria based on the observations that both long chain FFA oxidation and enzymes involved in lipid metabolism are similar in both cultured cells and adult muscle fibres (Zuurveld *et al.*, 1985; Jacobs *et al.*, 1987).

1.24.2 Preservation of metabolic features in cultured skeletal muscle

Several studies have shown that some metabolic abnormalities observed in certain disease states are retained in cultured skeletal muscle cells grown from muscle biopsies from those subjects. For example, muscle cultures from diabetic patients display several metabolic features that characterise the disease state (Henry *et al.*, 1996; Thompson *et al.*, 1996; Gaster *et al.*, 2002; McIntyre *et al.*, 2004). The exact mechanism by which cultured cells retain the metabolic characteristics of native muscle *in vivo* is not known till now but both genetic and epigenetic factors could be involved (Aas *et al.*, 2013).

Limitation of cell culture system

In cell culture, the interaction between muscle cells and other cell types that are present in human muscle fibres is lost because cultured cells are isolated from other accompanied cells. This results in loss of cell-cell communication that naturally occurs (Trayhurn *et al.*, 2011). Furthermore, as mentioned some of metabolic features are not exactly the same as that of the adult skeletal muscle such as glucose uptake in both basal and insulin stimulated. In addition, the effects of muscle fibre type are lost in cultured myotubes compared with that of the adult muscle fibres. As glucose uptake and insulin sensitivity is increased in oxidative slow twitch muscle fibres if compared with glycolytic fast twitch fibres (Zierath and Hawley, 2004). Moreover, some gene differences are observed in both groups (myotubes in culture versus adult muscle fibres) (Lund *et al.*, 1997; Nehlin *et al.*, 2011).

Several genes that studied in muscle homogenates are over expressed compared to that in cultured skeletal muscles cells. This could be attributed to less numbers of cells are matured and act as active myotubes and also cells might be in dormant state in culture rather than active (Lund *et al.*, 1997).

Finally, there is a decrease in metabolic and phenotypic features as the number of passages of the cultured cells increases (Nehlin *et al.*, 2011).

1.26 General Hypothesis

Thrombin impairs insulin action in human skeletal muscle cells and this could illuminate the link between the hypercoagulability and insulin resistance in T2D.

Furthermore, I explored the hypothesis that thrombin affects the metabolic response to exercise in skeletal muscle cells from control non-diabetic human subjects using a recently developed *in vitro* system to exercise the cultured muscle cells by electrical pulse stimulation (EPS).

Moreover, skeletal muscle cell cultures have been established from insulin resistant diabetic patients, and shown that defects of insulin action are retained in cultured skeletal muscle cells (McIntyre *et al.*, 2004). I investigated the hypothesis that in these cell cultures contraction mediated metabolic responses was preserved in response to EPS as a model of exercise.

1.27 Aims of the PhD study

1. To explore the effects of thrombin on insulin signalling pathway in cultured human skeletal muscle cells.

2. To study the effects of thrombin on insulin stimulated glucose uptake as a metabolic parameter in cultured human skeletal muscle cells.

3. To examine the effects of thrombin on exercising human skeletal muscle myotubes using an *in vitro* exercise model (EPS).

4. To study the effect of an *in vitro* exercise (EPS) alone and in combination with insulin on skeletal muscle cell cultures from insulin resistant T2D patients, measuring AMPK activation, cytokines release, glucose uptake, and distal insulin signalling.

CHAPTER 2 : GENERAL MATERIALS AND METHODS

2.1 Chemicals and reagents

Thrombin was purchased from Sigma-Aldrich, human actrapid insulin was from Novo Nordisk (Crawley, UK), both the PKC general inhibitor GF109203X (Cat. No. 0741) and compound C (Cat. No. 3093) were from Tocris Bioscience and the classical PKC specific inhibitor GÖ 6976 (Cat. No. 365253) was from Calbiochem. Laboratory chemicals were either bought from Sigma-Aldrich (Dorset, UK) or British Drug House (BDH, Poole, UK). Protease inhibitor cocktail and standard BSA were from Thermo Scientific. 40% acrylamide was from Melford Laboratory Ltd (Suffolk, UK), imaging film was from Thermo Scientific, catalogue number #34089. Coomassie, Coomassie plus Protein Assay reagents, Restore TM western blot stripping buffer, SuperSignal West Pico Luminol Enhancer solution, SuperSignal West Pico Stable Peroxide solution and Western Blot Stripping Buffer all were purchased from Thermo Fisher Scientific. Ponceau's solution (Sigma), nitrocellulose blotting membranes was from GE Healthcare Life science (Germany).

Material of Cell culture and media will be mentioned separately in the subsequent sections. Phospho- IRS1^{Y612} (ab66153), Phospho-AS160^{Thr642} [EPR2733 -2] and Phospho-AS160^{S588} sheep antibody (ab65754) were purchased from Abcam. Phospho-Akt^{Ser473} (D9E), total Akt rabbit antibodies, Phospho-PKCζ/λ$^{Thr410/403}$ antibody (9378),total-PKCζ antibody (9372S), phospho-AMPK^{Thr172} (40H9) rabbit antibodies and AMPKα (F6) mouse antibodies, total AS160 rabbit (C96A7)and total IRS1 (L3D12) mouse were supplied by New England Biolabs (Herts, UK). Anti-Rabbit IgG (whole molecule) antibody was raised in Goat and peroxidase-conjugated with Catalogue number (A0545), Anti-Mouse IgG (whole molecule) peroxidase antibody was raised in rabbit Catalogue number (A9044), Anti-sheep IgG (whole molecule) peroxidase antibody produced in donkey and has Catalogue number (A3415) all were supplied by Sigma- Aldrich. Anti-human thrombin receptor IgG (Abcam, UK), Cy3-conjugated anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (711-165-152). 2-Deoxy-D-[2, 6-3H] glucose was

purchased from Hartmann Analytic (Germany). Scintillation liquid was from National Diagnostic (Atlanta, USA). LDH Cytotoxicity Kit II (PK-CA577-K313) was from PromoKine, IL-6 and IL-8 ELISA kits were obtained from Qiagen (Sussex, UK).

2.2 Cell culture material and reagents

Tissue culture plastic wares were obtained from Greiner Bio-One Ltd (Gloucestershire, UK), Pipettes were from Sarstedt (Leicester, UK). Coverslips and microscope slides were obtained from BDH (Leicester, UK). Ham's F-10 media, Minimum Essential Media (MEM), penicillin/streptomycin (100units/ml and 100µg/ml respectively), trypsin-EDTA (0.25%-0.02%) and foetal bovine serum (FBS) were purchased from Thermo Fisher Scientific. Chick embryo extract (CEE) was purchased from Sera Labs International (Salisbury, UK). PBS (Dulbecco without calcium and magnesium, PAA Laboratories). DMSO was obtained from Sigma (Poole, UK). For cell sorting system, magnet with stand, Minimac® beads and separation columns were purchased from Miltenyi Biotec (Bisley, UK).

2.3 Cell culture media preparations

All cell culture procedures were carried out in a sterile environment in a Class II microbiological cabinet and all media were warmed to 37˚C in a water bath before nourishing cells. Table 2-1shows different media used and the recipe of each one.

Table 2-1: Media recipes used in cell culture.

Growth media – myoblasts	**Ham's F-10 medium with L-glutamine** **20% (v/v) foetal bovine serum (FBS)** **2% (v/v) chick embryo extract (CEE)** **1% (v/ v) antibiotic penicillin (100units/ml) /streptomycin (100µg/ml)**
Differentiation media – myotubes	**Minimum Essential Medium Eagle's Medium with L-Glutamine (MEME)** **2% (v/v) FBS** **1% antibiotic penicillin/streptomycin.**
Reduced serum media – myotubes	**Minimum Essential Medium Eagle's Medium with (MEME)** **0.5% FBS.**
Freezing media	**50% (v/v) growth media** **40% (v/v) FBS** **10%(v/v) DSMO**

2.4 Muscle biopsy and isolating satellite cells

All the cells used in the study were kept in liquid nitrogen and were previously grown from samples taken from the vastus lateralis muscle biopsy either under general anaesthesia at time of hip surgery or needle biopsy under local anaesthesia.

For control human muscle cells (thrombin work), samples were taken from the vastus lateralis muscle of healthy subjects with no family history of T2D and with normal glucose tolerance. Local Ethic Committee approval was obtained before beginning the sample collection and all participants gave informed written consent.

For the diabetes work, muscle biopsies were taken from T2D subjects with strong clinical evidence of insulin resistance. To specify, all patients taking >100 U insulin/day and had at least one first-degree relative with T2D. After diagnosis, patients had been treated with diet and oral hypoglycaemic drug for more than 3 yr before starting insulin. All enrolled patients had a body mass index (BMI) < 32 kg/m2. Skeletal muscle biopsies were taken from six age- and BMI-matched nondiabetic control subjects with no family history of T2D. The study was approved by the Newcastle and North Tyneside Joint Ethics Committee and all the participants gave written informed consent.

Satellite cells were isolated as described previously (Blau and Webster, 1981; Jackson *et al.*, 2000). In brief, biopsies were collected in myoblast growth media. Fibrous or fat tissues were carefully dissected from the muscular tissue, and muscle tissue was cut into approximately 1mm diameter pieces. The tissue was then digested with trypsin-EDTA (0.25%-0.02%) at 37°C for 15 minutes after washing with phosphate buffered saline (PBS) 4 times to remove adherent blood cells. This digestion process was repeated for a maximum of 4 times depending on the biopsy sample size. After each trypsinisation, the resultant supernatant was removed and neutralised with FBS, centrifuged at 1700 rpm for 5 minutes then 1 ml of myoblast growth media added to the cell pellet. Finally, the resuspended cell pellets were seeded in a T25 culture flask and incubated at 5% (v/v) CO_2, 37°C in a humidified atmosphere. The next day, growth media was replaced with fresh media.

2.5 Purifying myoblasts from Mixed Cell Culture

The established primary cell culture from the aforementioned digestion steps of the mixed tissue can be enriched for the myoblast fraction. Cultures were enriched as described previously (McIntyre *et al.*, 2004) by the use of a magnetic bead system (Miltenyi Biotec). Briefly, cells were harvested with trypsin, re-suspended in 50-80 µl of separation buffer (5% (v/v) FBS in PBS, 2 mM EDTA. PBS without magnesium and calcium) and incubated in the hood for 5 minutes. The appropriate amount of primary antibody (20 µl of NCAM CD56 antibodies, a cell surface protein marker present on muscle cells) was added and incubated at 4 $^{\circ}$C for 10 minutes and then incubated in the hood for a further 5 minutes. The cell suspension was then washed twice with 1.5 ml of separation buffer and centrifuged at 1700 rpm for 3 minutes. After being resuspended in 80 µl of buffer, 20 µl of appropriate secondary antibodies attached to magnetic beads was added. The mixed cell suspension was left in the hood for 10 minutes then washed again with 2 ml of buffer and centrifuged at 1700 rpm for 5 minutes. The cell pellet was re-suspended in 500 µl of separation buffer and added to the magnetic column. The suspension was permitted to run through, labelled muscle cells were retained in the column by the magnetic beads within the magnetic field. Following washing of the cells on the column three times with 500 µl of separation buffer, the column was removed from the magnetic field and the labelled cells were eluted from the column using another 550 µl of separation buffer. The resultant cells were resuspended in an appropriate volume of growth media and seeded in a T25 flask.

2.6 Human muscle cell culture

Cells (myoblasts) resuspended in 1ml Freezing media were stored in Cryovials in liquid nitrogen. Cryovials were thawed at 37°C (water bath), and the contents transferred to a 75 cm^2 flask (T75) containing an appropriate volume of growth media. Flasks were placed in a humidified incubator and the cells were maintained at 37°C under 5% CO_2 atmosphere.

2.6.1 Cell proliferation

Growth medium was changed every 3-4 days to ensure cells were maintained in a proliferation phase. Cell concentration and wellbeing were checked every day

under a light microscope until cells reached 70-80 % confluence, at which point they were ready to be passaged.

2.6.2 Cell passaging

Cultured cells in T75 flasks were ready to be passaged when they were 70%- 80% confluent. Growth medium was aspirated, and cells rapidly washed with 5mL PBS. Cells were incubated with 3mL 1x trypsin-EDTA / flask for 4-6 minutes at 37°C, until the cells had rounded up and detached from the bottom of the flask. At this point trypsin was neutralised by addition of growth media. Cells were centrifuged (1750 rpm for 3 minutes), and the supernatant was aspirated. The cell pellet was re-suspended in an appropriate volume of growth media, plated and returned to the incubator. Confluent cell cultures of passage 5-8 were used in this study.

2.6.3 Cell differentiation

When myoblasts fuse together in culture, they form multinucleated myotubes that display key metabolic characteristics of mature human skeletal muscle cells. Myoblast cultures needed to be 80-90% confluent to facilitate fusion into myotubes. When this level of density was reached, the growth media was replaced by differentiation media. Media was changed frequently (every two days) and cells differentiated for seven days. All experiments were performed on day 7 myotubes.

2.7 Immunofluorescence staining

Myoblasts were plated in cover slips in 6-well plates and fed with growth media until cells became confluent, and then allowed to differentiate by substitution of the media with differentiation media (MEME) for 7 days. All steps were then carried out at room temperature, on a shaker set to low speed. Fully differentiated myotubes (on cover slips) were washed with PBS three times for 5 minutes each and fixed with 10% formalin for 20 minutes. Cells were washed with PBS then permeabilized with 0.2% t-octylphenoxy polyethoxyethanol (Triton X-100, Sigma-Aldrich) for 45 minutes. After washing with PBS, cells were incubated in blocking buffer (20% FBS in PBS) for 1 hour to block the non-specific binding sites. Cells were then incubated with rabbit anti-human thrombin receptor IgG (Abcam, UK), diluted 1:500 in 0.05% FBS in PBS for 1 hour (primary antibody). After washing with PBS, cells were incubated in Cy3-conjugated anti-rabbit IgG (secondary antibody), diluted 1:500 in 0.05% FBS in PBS for 1 hour in the dark (wrapped in aluminium foil). PBS washes were repeated in the dark then, cover slips were attached onto slides using a drop of Vectashield with DAPI to visualise nuclei and the edges glued to slides with varnish. Fluorescent microscopy was performed on an Olympus CKX41 to visualise staining and images taken using QCapturePro60 software.

2.8 Western Blot analysis

Western blotting is a technique often used in research to identify proteins that are separated on the basis of their molecular weight differences through gel electrophoresis. The detection of protein is achieved by specific antibodies targeting the protein of interest and then the bound antibody is detected by developing the film. The antibodies detect specific proteins with the intensity of the bands reflecting the amount of protein present (Mahmood and Yang, 2012). Different recipes for buffers and solutions used for western blotting experiments are listed in Table 2-2 below.

Table 2-2: Different buffers used in SDS-gel electrophoresis.

Protein extraction buffer	100 mM Tris-HCl, pH 7.4 (at 4°C) 100 mM KCl 1 mM EDTA 25 mM KF 0.1mM Na$_2$VO$_4$ 0.1% Triton Protease inhibitor cocktail supplied by Thermo Scientific
Running buffer	144.2g/l glycine 30.3 g/l Tris base 0.1% (w/v) SDS
Transfer buffer	10mM CAPS (3 cyclohexylamino-1-propanesulphonic acid), pH 11.1 10% (v/v) Methanol/l dH$_2$O
Sample loading buffer	0.25M Tris-HCl, pH 6.8 8% (w/v) SDS 40% (v/v) glycerol 0.008 % (w/v) bromophenol blue 200 µl 2-mercaptoethanol/ml

2.8.1 Protein extraction

Cells were rapidly washed with ice cold PBS 5 times. the remnants of PBS was removed and 150 µl/ well of protein extraction buffer added, cells were scraped to ensure all cells detached from the wells (by using a plastic cell scraper) and lysates were collected in screw-cap Eppendorf tubes on ice. Cell lysates were then briefly sonicated for 10-15 seconds at 5µm amplitude to ensure cell lysis by using a sonicator (Sonipath, Dawe), and immediately snap frozen in liquid nitrogen, prior to storage at -80° C. Samples were thawed when further analysis was required and protein concentration in each sample determined by dye binding with Coomassie reagent.

2.8.2 Protein concentration determination

Before protein concentration determination, samples were defrosted on ice and centrifuged at maximum speed for 5 min at 4°C to exclude cell debris that could interfere with the determination and forthcoming processes. Spectrophotometric analysis of cell lysates was performed according to the modified Bradford dye binding colorimetric method for protein quantification (Bradford, 1976). A 2 mg/ml BSA stock (Thermo Scientific) was used to construct a standard curve in duplicate with the final volume made up to 10µl with protein extraction buffer. Duplicates of 10µl of cell lysate samples were plated in a 96 well plate, 200µl of Coomassie blue stain (Thermo Scientific) was added to each well before being read on a plate reader at 595nm, and the protein concentration determined by plotting a standard curve as shown in the following Figure 2-1.
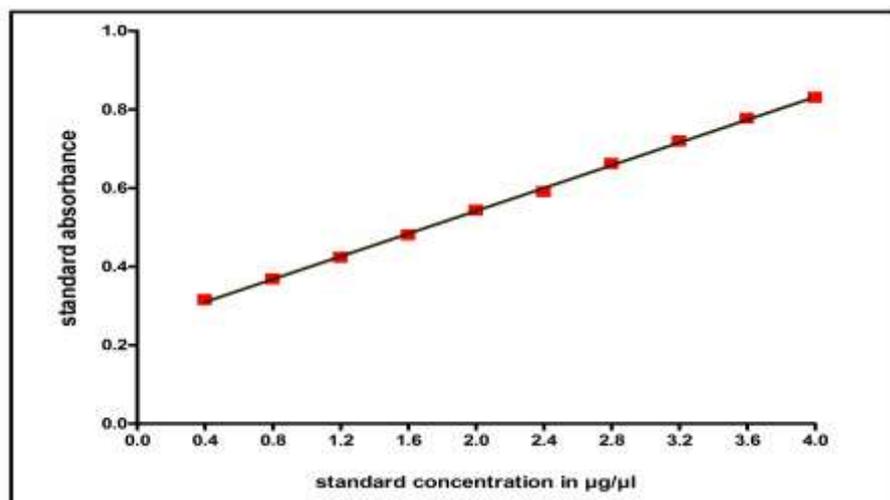
Figure 2-1: Example of standard curve for protein concentration determination.

2.8.3 Protein sample preparation for SDS-PAGE

The calculation of the protein concentration in each sample was performed and each sample was then normalised to contain a total of 10µg protein per well. The same volume (1/4 of the total volume) of loading buffer (was added to each sample, and the final volume was made up with PE buffer. Samples were heated to 95°C to denature the proteins for 3 minutes, and then the protein samples were loaded on the SDS–PAGE gel.

2.8.4 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a method of separation of proteins according to their size and molecular weight by applying an electrical current. A 10% running gel, and 4% stacking gel was used in these experiments.

Table 2-3 shows the recipes that were used to make the 2 gels. The running gel solution was left to polymerise in a gel cassette (30-minutes). Then 4% stacking gel solution was added, a plastic comb was inserted to create wells, and left to set for about 1 hour. The gel cassette was then positioned onto the appropriate apparatus, with 1x electrode buffer. A molecular weight marker, (Pre-stained Broad Range 7-175 kDa, New England Biolabs, catalogue number P7708S), and all protein samples (10µg/well) were loaded into individual wells. The gel was run at 40 mA, the gel was electrophoresed until the tracking dye had migrated to the bottom of the gel and good separation of the molecular weight markers had been obtained.

Table 2-3: SDS-acrylamide gel recipes used to prepare 2 gels.

	10%	6%
dH2O	11.8ml	9.4ml
acrylamide (40% solution)	6.4ml	1.5ml
Buffer B (1.5M Tris, pH 8.8)	6.3ml	-
Buffer D (0.5M Tris, pH 6.8)	-	3.8ml
10% SDS	250µl	150µl
10% APS	250µl	150µl
TEMED	12.5 µl	7.5µl

2.8.5 Gel transfer

After SDS-PAGE, the separated proteins in the gel were transferred onto a nitrocellulose membrane (0.45 µm pore size), pre-wet with transfer buffer and then placed between 2 layers of 3 mM filter paper also pre-wet with transfer buffer. The 'sandwich' was then inserted between the plates of the gel holder cassette and the transfer was performed using a mini-Hoeffer gel transfer tank (2-3 h, 250V) containing transfer buffer (10mM CAPS, 10% methanol). Once transfer was complete the membrane was briefly stained with Ponceau's solution (Sigma) for 5 minutes to check whether transfer was successful and equal loading of protein had been achieved.

2.8.6 Blocking of membranes and treating with antibodies

Non-specific sites on the nitrocellulose membranes were blocked by incubation with shaking in Tris buffered saline tween (TBST) /5% (w/v) milk or 3% (w/v) BSA as a blocking buffer for 1 hour at room temperature. Afterwards, the membrane was incubated with monoclonal primary antibody which was diluted in TBST containing 1% milk or BSA and the membranes were incubated overnight on a shaker at 4°C. Different antibodies targeting specific proteins were used in this

work and will be mentioned accordingly in their related chapters. Following primary antibody incubation, membranes were washed with TBST (3 x 5 min) on a shaker. They were then incubated with secondary antibody conjugated to horseradish peroxidase (HRP) diluted in TBS-Tween containing 1% milk or BSA for 2 hours on a shaker at room temperature. The membrane was washed with TBST (3 x 5 min) before addition of enhanced chemiluminescent (ECL) solutions.

2.8.7 Chemiluminescence detection

Membranes were soaked in chemiluminescence reagents, 2.5mL SuperSignal West Pico Peroxide Solution and 2.5mL Luminol/Enhancer Solution, for 5-minutes with shaking. HRP is used to catalyse the oxidation of luminol in the presence of hydrogen peroxide. Immediately after the oxidation, the luminol is in an excited state which emits light and the intensity of this light is directly proportional to the concentration of the bound antibody. By exposing a photographic film to the membrane, an image of the protein bands can be obtained. Once developed, dark bands show the protein of interest, to which the HRP was bound. The entire process must be achieved in a dark room using red lights.

X-Ray film (CL-XPosure™ Film, Thermo Scientific, catalogue number #34089) was placed over the nitrocellulose membrane and the cassette lid closed. The film was exposed to the membrane for a certain time before being put into developing solution (Carestream® Kodak® autoradiography GBX developer/replenisher, Sigma-Aldrich, catalogue number P7042) for 1 minute. The film was washed briefly in water before being placed in fixer solution (Carestream® Kodak® autoradiography GBX fixer/replenisher, Sigma-Aldrich, catalogue number P7167) for 1 minute. The film was washed again with water and left to dry. The developer and fixer solutions were diluted 1:10 in water.

2.8.8 Membrane stripping

The membrane was treated with the stripping buffer Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) (5 ml for 15minutes) when more than one primary antibody was to be used. Following detection of the first protein of interest the membrane was washed with TBST, stripping buffer was added to remove both primary and secondary antibodies from the membrane. This allows another re-probing of the membrane. Following incubation with the stripping buffer,

membrane was then re-blocked with blocking buffer before the next primary antibody incubation.

2.8.9 Densitometric analysis

Images were analysed by densitometry, performed using a Bio-Rad Molecular Imager GS-800 calibrated densitometer, and Quantity One software was used to quantify target protein bands. All proteins analysed were normalised to native proteins and expressed as a mean of phospho/native proteins.

2.9 Glucose uptake assay

Glucose uptake was measured by the incorporation of tritium (^{3}H) labelled 2-deoxyglucose (2-DG) into myotubes in culture in the presence or absence of 100 nM insulin. In each experiment, 10 µM Cytochalasin B was added to one of the wells to measure non-specific deoxyglucose uptake as it causes inhibition of glucose transport through glucose transporters. 2-DG uptake was measured by liquid scintillation counting to quantify radioactivity. The radiolabelled 2-deoxyglucose is not metabolised nor broken down upon entry into the cell. The assay was performed on 7 day differentiated myotubes that were cultured in either 6well plates or 35mm dishes.

 The differentiation media was changed to reduced serum media before the beginning of the assay. Cells were incubated with or without stimulus that could be treatment or EPS for a given time at 37°C. After treatment, cells were washed twice with warm Kreb's buffer, and then cells were incubated in Krebs' buffer (see Table 2-4) with or without 100nM insulin or cytochalasin B (10µM) for 20 minutes in the incubator at 37°C and 5% CO_2. Glucose transport was initiated by the addition of hot glucose 50µl of 2-[3H]-deoxyglucose (Final concentration 50 µmol/l and 1 µCi/ml) to each well for 10 minutes. The reaction was stopped by inverting the plates rapidly to remove the incubation buffer, and then washing the plates 5 times with ice-cold PBS. Subsequently, 250 µl of 0.05% SDS was added to the cell monolayers as a lysis solution and incubated at 37°C and 5% CO_2 for 15-30 minutes. Wells were scraped with a pipette to ensure all cell lysate was transferred to 1.5 ml Eppendorf' tubes. 200µl of the cell lysate was used for scintillation counting, added to scintillation fluid (2.5ml) with vigorous vortexing before counting

via liquid scintillation counting. The other 50µl of cell lysate was saved for protein determination as described previously.

Table 2-4: Solutions used in glucose uptake assay.

Kreb's buffer (KRH) pH 7.4 500ml	NaCl 136mM = 3.97g in 500ml KCl 4.7mM = 0.175g $MgSO_4 7H_2O$ 1.25mM = 0.154g $CaCl_2 H_2O$ 1.2mM = 0.088g Hepes 20mM = 2.383g BSA 1g
Hot glucose solution	2-deoxyglucose (2-DG) 2mM 10 µCi 2-[3H]-deoxyglucose/ml Kreb's buffer

2.10 Gene expression analysis

2.10.1 RNA extraction

Total RNA was extracted by using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) following the manufacturer's instructions. For each 250 µl of lysis solution needed, 2.5 µl of β-mercaptoethanol was added to fully inactivate RNase enzymes. Media was removed from dishes and a single wash with PBS performed before addition of lysis solution (250µl per well). The cells were scraped using the end of a pipette tip, and the lysis solution in the wells was gently pipetted over the surface of the well numerous times to ensure most of the cells were lysed. The cell lysates either directly underwent RNA extraction, or were collected in tubes, snap frozen in liquid nitrogen and stored at -80°C until required.

The first step of RNA extraction is to filter the lysate using filtration columns to eliminate cellular fragments and shear DNA. Lysates were centrifuged at maximum speed for 2 minutes and the filtration column discarded. Secondly, an

equal volume of 70% ethanol was added to the filtered lysate, this was vortexed thoroughly to prepare the mixture for the binding step. Thirdly, the lysate was added to the binding column and centrifuged at maximum speed for 15 seconds. The column was washed with wash solution 1 before running a DNase digest. To ensure that the resultant RNA samples were free from DNA contamination, DNase enzyme was added to the binding column using RNase-Free DNase Set (Qiagen). DNase 1 stock solution was prepared by dissolving the provided solid DNase I in the RNase-free water. To each binding column, an appropriate volume of DNase incubation mix (10µl DNase 1 stock solution added to 30µl of the supplied DNase Digest Buffer per RNA binding column) was added and incubated at room temperature for 15 minutes. After DNase treatment, a series of washes was carried out including a last wash with 100% sterile filtered ethanol. Finally, RNA was eluted using the provided elution solution.

2.10.2 RNA Quantification

The concentration of RNA (ng/µL) in each sample was measured using a Thermo Scientific NanoDrop 2000 Spectrophotometer, together with the Nanodrop 2000 software for analysis. 1µl of each sample was loaded onto the receiving fibre (end of the fibre optic cable) and the equipment acquired absorbance readings at 260 nm for RNA, and assessed purity of samples. The volume of each RNA sample needed for cDNA synthesis was adjusted according to the obtained RNA concentrations.

2.10.3 cDNA Synthesis

Complementary DNA (cDNA) is double-stranded DNA synthesized from a single stranded RNA via a reaction catalysed by the reverse transcriptase enzyme. The synthesis of cDNA was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Firstly, RNA samples were standardised to the same concentration (ng/µL) in a 10µL total volume; where necessary, RNase-free water was added. An appropriate volume of master mix was made up of the components that are presented in Table 2-5. For every set of samples a negative control was prepared, where the RT enzyme was replaced by RNase-free dH$_2$O, hence cDNA could not be synthesised.

Table 2-5: Reverse Transcription (RT) master mix components for cDNA synthesis.

Components	Volume per sample
10x RT Buffer	2.0µl
25x dNTP Mix	0.8µl
10x RT Random primers	2.0µl
MultiScribe™ Reverse Transcriptase	1.0µl
Nuclease-free H_2O	4.2µl
total	10 µl

10µl of the RT master mix was then added to 10µl of the previously standardised RNA and pipetted gently to mix. The tubes were briefly centrifuged and loaded to a Thermocycler Gene AMP PCR System 9700 (Applied Biosystems) and the instrument set to the conditions defined in Table 2-6 that allowed reverse transcription. The produced cDNA was stored at -20 ^{o}C until further use.

Table 2-6: Thermal cycler temperature settingsfor reverse transcriptase reaction.

Steps	Stage	Temperature (°C)	Duration (min)
1	Annealing	25	10
2	Extension	37	120
3	Final Extension	85	5
4	Cooling	4	∞

2.10.4 Real time PCR

Quantitative real-time PCR was performed on a Lightcycler 480 (Roche) using TaqMan primers and probes. β2-microglobulin (β2M) was used as a reference gene. For each assay two master mixes were prepared, one for the target gene

and the other for the reference gene. Five serial dilutions were performed to generate a standard curve. A 96-well plate was used and each well held a 20µL reaction mix: 2µL cDNA and 18µL master mix, with each sample run in triplicate. The details of primers concentration and the probes used will be mentioned when appropriate. After loading of all samples, the plate was sealed with a transparent LightCycler®480 Sealing Foil, briefly centrifuged and loaded into the qPCR machine. The LightCycler®480 was programmed for thermal condition as shown in Table 2-7.

Table 2-7: LightCycler®480 program setting for qPCR.

Detection	Stage	No. of Cycles	Temperature (°C)	Duration (mim:sec)	Genes
TaqMan (Qiagen)	**Pre-incubation**	1	95	02:00	GLUT1 GLUT4 *B2M*
	Amplification	45	95	00:10	
			60	00:30	
	Cooling	1	40	Continuous	

TaqMan probes were used in qPCR for quantification of the genes of interest. The principle action of this type of detection is based on 5´–3´ exonuclease activity of Taq polymerase. The probe contains a reporter dye attached to the 5'-end of the probe and a quencher at the 3'-end. The quencher molecule diminishes the fluorescence of the reporter when the reporter and the quencher are in close proximity. During the extension phase of the PCR cycle, the Taq enzyme synthesises and extends the targeted piece of DNA strand and when the extension reaches the 5´–3´ region of the probe the enzyme degrades the probe and separates the reporter from the quencher permitting the emission of the fluorescence, and the amount of the fluorescent light emitted in the exponential phase is proportional to the quantity of the targeted gene (Bustin, 2000).

2.10.5 Quantification of Gene Expression

The results of qPCR were calculated by measuring the cycle threshold, CT value, which is the cycle number at which the fluorescence increases above the baseline. This value is used for quantitative analyses either indirectly (standard curve

method) or directly (ΔΔCT method). For the standard curve method, standard curves were generated from a 5-fold serially diluted cDNA sample. The standard curve was plated in duplicate with every qPCR run. Two separate standard curves were generated, one for the target and the other for the reference gene. Figure 2-2 is an example of the standard curve generated for calculation of GLUT1 gene expression in response to thrombin.
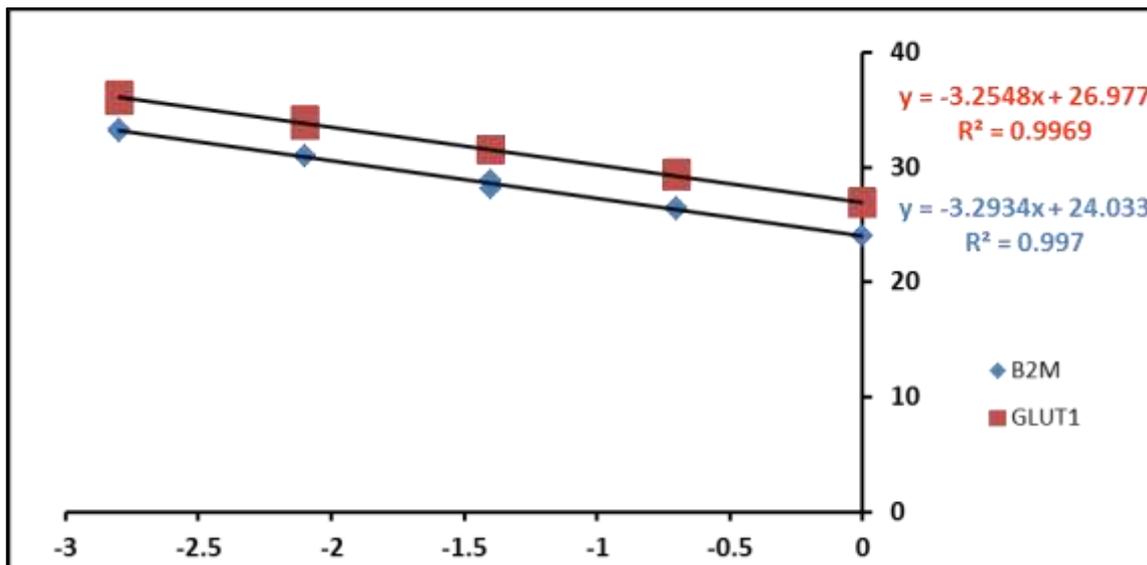
Figure 2-2: Standard curve for gene quantification used in GLUT1 gene expression in response to thrombin.

The *ΔΔCT* method

The comparative CT method was used for calculation of gene expression for GLUT4 in response to thrombin due to difficulties in constructing standard curves as this gene is poorly expressed in our cell system. In case that the reaction efficiencies between the target gene and the reference gene are relatively equivalent, and as TaqMan® Gene Expression Assays was used relative quantification can be achieved by using this method (Biosystems, 2004).

A series of arithmetic formulas was applied to obtain the relative expression of the gene. In brief, after obtaining average CT and standard deviation for both target sample and reference, ΔCT was calculated.

ΔCT = CT target – CT reference.

After obtaining ΔCT for each sample, the ΔΔCT was calculated by applying the following formula;

ΔΔCT = ΔCT treated - ΔCT untreated.

Subsequently, fold changes were determined by using;

Fold change = $2^{-\Delta\Delta Ct}$

When all the experiments were completed, standard deviation and standard error of the mean (SEM) were determined and expressed in the results.

2.11 Enzyme Linked Immunosorbent Assay (ELISA)

Conventional Enzyme Linked Immunosorbent Assay (ELISA) was used to measure cytokine release into media in response to a stimulus. The analysis is based on the interaction of target protein (antigen) with protein-specific capture antibodies. Samples were prepared from an experiment by removing the media and centrifuging at 1000g for 10min. Media was assayed for secretion of IL-6 and IL-8 according to the manufacturer's protocol. Briefly, following the preparation of all reagents needed, a standard curve was constructed using the provided standard antigen (1µg/ml) and a suitable dilution of the sample with sample dilution buffer was performed in order to ensure the samples would fall within the range of the standard curve. All samples and standard were analysed in duplicate. Equivalent volumes of sample and standard were added to the pre-coated plate and incubated for 2 hours followed by 3 washes, and then detection antibodies were added and incubated for 1 hour and followed by 3 washes. Subsequently, Avidin-HRP was added and incubated for 30 minutes and followed by 4 washes. Development solution was added and incubated for 15 minutes in the dark and finally stop solution was added and the absorbance was read at 450nm. Background absorbance was subtracted from the values and the protein concentrations of the samples calculated from the standard curve.

2.12 Measurement of lactate dehydrogenase (LDH) release

Lactate dehydrogenase (LDH) enzyme is found inside nearly all cell types and any injury to the cells that lead to cell membrane damage can result in the release of the enzyme into the culture medium making it a good marker of cytotoxicity. Samples were prepared by collecting the media from the appropriate experiments and LDH release into the media was determined colorimetrically at 450 nm according to the manufacturer's instructions, LDH Cytotoxicity Kit II (PK-CA577-K313) was from PromoKine. In brief, the lactate dehydrogenase assay working solution was prepared by mixing WST substrate with a suitable volume of LDH assay buffer. An equal volume of LDH assay working solution and the media sample were added and incubated in the dark for 30 min at room temperature. The colour intensity produced from the reaction was measured spectrophotometrically at 450nm.

2.13 Statistical analysis

All data values presented in the results sections are expressed as mean ± standard error of the mean (SEM). Where (n) value is given, n represents the number of experimental repeats from different cell lines (individuals) of primary cell cultures. No power calculation has been performed due to the limited number of samples available but in all experiments more than three primary cell lines in each group were investigated which is the lowest number required for statistical repeats.

All data were tested for normal distribution using the Kolmogorov-Smirnov test. The variables presented were shown to be normally distributed, data were analysed using one-way ANOVA, for multiple comparisons especially for the glucose uptake and distal insulin signalling pathway data analysis when the multiple comparison between means were required. The unpaired t-test was used in all data to compare between means of two groups. A p-value of <0.05 was considered significant. Statistical analyses were performed using GraphPad Prism (California) software.

CHAPTER 3 : EFFECTS OF THROMBIN ON INSULIN SIGNALLING AND GLUCOSE UPTAKE IN CULTURED HUMAN MYOTUBES.

3.1 Introduction

Thrombin is a multifunctional serine protease enzyme. It is involved in several biological functions such as cell division (Chen and Buchanan, 1975), chemotaxis (Bar-Shavit *et al.*, 1983), cancer growth and inflammation (Licari and Kovacic, 2009), with its primary role in the coagulation cascade. It is generated from the precursor molecule prothrombin by proteolytic cleavage and thrombin acts on the cell surface receptors known as protease activated receptors (PARs) (Bailey *et al.*, 1951). The binding of thrombin to the PARs leads to proteolytic degradation of the N-terminal of the receptor domain leading to formation of new N-terminus that activates the receptor itself and initiates the intracellular signalling processes (Vu *et al.*, 1991).

PAR1, PAR3 and PAR4 are considered to be activated by thrombin, whereas PAR2 has been shown to be targeted by other proteases such as trypsin (Wang *et al.*, 2002). The binding of thrombin to its receptors on the cell surface leads to receptor activation which facilitates different cellular downstream responses that vary according to cell type and need. The mobilisation of intracellular Ca^{2+}, activation of membrane phospholipids, metabolic enzyme alterations and PKC activation are important second messengers that facilitate transmission of thrombin action (Mackie *et al.*, 2002; Ossovskaya and Bunnett, 2004).

Distinct PARs are expressed in different tissue types, including skeletal muscle cells. In skeletal muscle cells PAR expression depends on developmental stage and cellular needs. For example, PAR1 is expressed more abundantly in myoblasts than in myotubes and receptor activation with thrombin or PARs activators at this stage plays an important role in muscle development (Suidan *et al.*, 1996) and regeneration after injury (Bedair *et al.*, 2007).

Skeletal muscle is the primary peripheral tissue site of insulin stimulated glucose uptake (DeFronzo *et al.*, 1981) and the key peripheral site of insulin resistance in T2D (Cline *et al.*, 1999). In skeletal muscle cells, insulin stimulates glucose uptake by initiation of insulin signalling cascades that start with activation of insulin

receptors and end with translocation of insulin sensitive glucose transporters GLUT4 from the cytoplasm to the plasma membrane as described in details in section 1.8.

Insulin resistance is defined as a reduction in the ability of the target cell or tissue to respond to a normal physiological level of insulin. Insulin resistance has detrimental effects on glucose homeostasis and is considered a characteristic early feature of T2D (DeFronzo *et al.*, 1992). The mechanism of development of insulin resistance is multifactorial; genetics, obesity, and activation of proinflammatory pathways are all involved. Two important aspects of insulin activity altered during insulin resistance are, insulin signalling and insulin stimulated glucose uptake.

Hypercoagulation (increased thrombin production and action) is a feature of T2D patients who are shown to be at high risk of thrombotic and vascular events (Carr, 2001). In T2D, a strong association between metabolic abnormalities such as hyperglycaemia and increased coagulation (elevated thrombin level) has been recognised (Rao et al., 1999). Additionally, a decline in insulin sensitivity is linked with elevated thrombin production (Romano et al., 2003). Furthermore, T2D is currently regarded as a low grade inflammatory state (Lopez *et al.*, 2006; Daar *et al.*, 2007). Strong links have been identified between increased inflammatory mediators with both increased hypercoagulability and reduced insulin sensitivity in insulin resistant individuals (Romano *et al.*, 2003). In addition, thrombin receptor activation has been shown to stimulate increased expression of different proinflammatory cytokines. For example, in human adipocytes thrombin enhances the expression and secretion of IL-1β, IL-6, TNF-α and other cytokines(Strande and Phillips, 2009). Finally, inhibition of thrombin in insulin resistant animals has been shown to enhance insulin sensitivity (Mihara *et al.*, 2010).

In addition, there is a strong genetic association between haemostasis factors of hypercoagulation and T2D development. Warren et al. (2005) showed that genetic traits leading to increased thrombin activity is a risk factor for T2D. These observations are consistent with the proposal by Jarrett and Shipley (1988) and Stern (1995) about the common genetic origin of both T2D and cardiovascular disease, the "common soil" hypothesis. GWAS also show that multiple gene variants are common to both T2D or insulin resistance and coagulopathy. The

IRS-1 gene and some variants of TCF7L2 have been linked to both increased coagulopathy and insulin resistance (Delgado-Lista *et al.*, 2011; Zhang *et al.*, 2016).

Very little is known about the metabolic effects of hypercoagulation (increased thrombin) on insulin signalling and glucose metabolism in human skeletal muscle. Therefore this study set out to explore the effects of thrombin on insulin signaling and glucose uptake in cultured human skeletal muscle cells

We hypothesize that thrombin directly contributes to the insulin resistant state.

The aims of the current chapter are:

1- To explore the effects of thrombin on proximal insulin signalling pathway in cultured human skeletal muscle cells.

2- To explore the effects of thrombin on basal glucose uptake or in response to insulin in cultured human skeletal muscle cells.

3.2 Methods

3.2.1 Cell culture

Muscle biopsies were taken from Vastus lateralis muscles of healthy human subjects with no family history of T2D. Satellite cells were prepared as described previously in general methods sections 2.42.5. Cells were treated with thrombin on day 7 of differentiation. All experiments were performed on day 7 after initiation of differentiation of myotubes; passages were between 5 and 8.

3.2.2 Immunofluorescent Staining

This procedure is explained in detail in general Materials and Methods section 2.7. Briefly, fully differentiated myotubes were grown on cover slips and fixed with 10% formalin for 20 minutes, cells were permeabilized with 0.2% t-octylphenoxy-polyethoxyethanol (Triton X-100, Sigma-Aldrich) for 45 minutes and incubated in blocking buffer (20% FBS in PBS) for 1 hour. Cells were then incubated with rabbit anti-human thrombin receptor IgG diluted 1:500 in 0.05% FBS in PBS for 1 hour. After washing with PBS, cells were incubated in Cy3-conjugated anti-rabbit IgG, diluted 1:500 in 0.05% FBS in PBS for 1h in the dark. After washing in PBS again cover slips were mounted onto slides using a drop of Vectashield with DAPI.

A negative control is performed by incubating cells without primary antibody, followed by incubation with secondary antibodies (Cy3-conjugated anti-rabbit IgG). This to ensure that staining is produced from detection of the antigen by the primary antibody and not by the experimental artefacts.

No positive control has been performed as the expression of thrombin receptors at both gene and protein level (Immunofluorescent Staining) in primary muscle cells (myoblast) has been examined by a previous member of our lab, Dhanisha Lukka, as part of her MRes project.

Fluorescent microscopy was performed on an Olympus CKX41 to visualise staining and images taken using QCapturePro60 software.

3.2.3 Thrombin preparation

Thrombin from human plasma (Sigma) was obtained in the form of lyophilised powder, and reconstituted with 100μL sterile PCR grade water to give a final

concentration of 1unit/µL thrombin, which was then aliquoted into small Eppendorf tubes and stored at -20°C.

3.2.4 Adjusting thrombin dose and duration in cultured muscle cells

The work of choosing the appropriate dose and duration of thrombin treatment was performed by a previous member of our lab, Dhanisha Lukka, as part of her MRes project and the results of the work are described below:

Myotubes were grown and differentiated in 6-well plates and at day 7 of differentiation were incubated with reduced serum media, before being treated with thrombin. Three different concentrations of thrombin were used; 1unit, 5 units or 10units. The controls were 1µL, 5µL or 10µL/mL of PCR grade water. Treated cells were incubated at 37°C, 5% CO_2 for either 6 hours or 24 hours. After the given time, cell morphology after thrombin treatment was examined, media was taken to run LDH cytotoxicity tests and RNA was extracted to measure cytokine expression levels (dose effects measurement).

The obtained data helped to decide both the optimal concentration and incubation period of thrombin that could be tolerated by the myotubes without adverse cytotoxic effects. Under the light microscope cultured myotubes were examined for their tolerance to treatment, changes in morphology, and cell death. Cell images were taken at the end of each incubation period, as illustrated in Figure 3-1.

Light microscope images of cells incubated for 6 hours show no visual differences in cell morphology between control upper panel image and thrombin treated lower panel image (Figure 3-1 A), both show healthy muscle cells which remained attached to the bottom of the plates with an elongated spindle shape. On the other hand, 24 hours of thrombin treatment was shown to be detrimental to cultured myotubes. Cells adopted rounded shapes and eventually become suspended in the culture media as cell debris particularly with 10 units/ml thrombin (Figure 3-1 B). Overall, cells were more confluent and healthier in appearance after 6 hours thrombin treatment compared with 24 hours treatment.
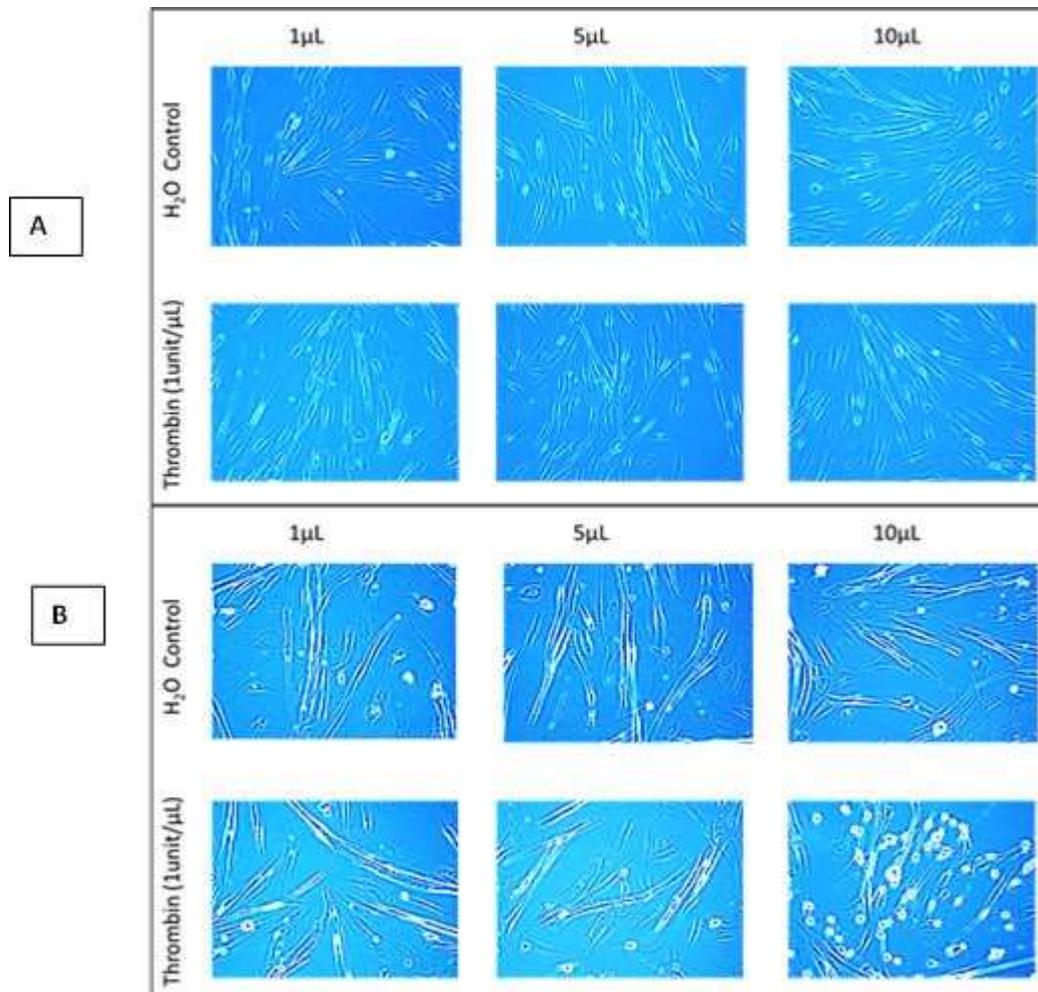
Figure 3-1: Light microscope images of cultured human myotubes after thrombin treatment with different concentrations of thrombin for variable durations. A: Myotubes with 6 hours thrombin treatment. B: Myotubes with 24 hours thrombin treatment.

3.2.5 LDH cytotoxicity results in response to thrombin treatment

LDH cytotoxicity measurements were performed on media collected from 4 different thrombin-treated and 4 control cultures. Results showed that 24 hours treatment released significantly more LDH into the media than 6 hours treatment at all thrombin concentrations. Moreover, 24 hours LDH release from cells into the media was significantly higher even in non-thrombin treated myotubes. Furthermore, at 6 hours application of thrombin, 10 U/ml thrombin treatment caused a significant increase in LDH release compared to 1 and 5 U/ml (# $p<0.05$). The LDH assay was performed in triplicate and the results showed in Figure 3-2.
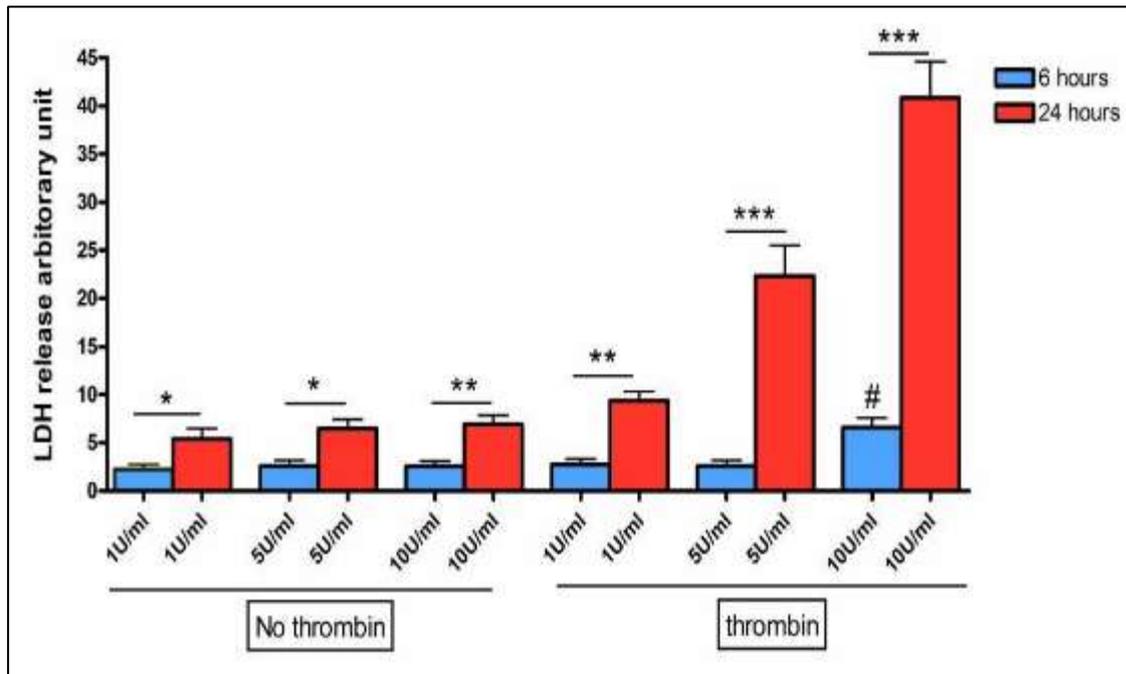
Figure 3-2: LDH cytotoxicity datafrom 4 different cultures of myotubes, treated with varying concentrations of thrombin for 6 hours and 24 hours, performed in triplicate. *p<0.05, **p<0.01, ***p<0.01 (comparing 24 hours versus the corresponding 6 hours treatment) and # p<0.05 (comparing 10U/ml treatment with 1U/ml and 5U/ml for 6 hours thrombin treatment).

3.2.6 Effect of thrombin on pro-inflammatory cytokine gene expression

IL-8, IL-6 and CCL2 gene expression were evaluated in response to increasing concentrations of thrombin for 6 and 24 hours to assess the effective concentration of thrombin with regard to its action. From 3 different cultures of myotubes performed in triplicate, the results were as shown in the following Figure 3-3.

1 U, 5 U, and 10 U/ml of thrombin for 6 hours showed a significant increase in gene expression of IL-8, IL-6 and CCL2 in myotubes compared with non-thrombin treated matched controls ($p < 0.01$). In addition, the use of 5 U/ml thrombin treatment for 6 hours showed more stimulation of gene expression than 24hour treatment for the three examined cytokines. Furthermore, the highest expression for IL-8 and CCL2 was associated with the use of 5 U/ml thrombin treatment for 6 hours, unlike IL-6 whose expression continued to increase with the use of 10 U/ml. Based on these results (examination under microscope, LDH cytotoxicity assay and cytokine gene expression) the decision was made to treat muscle cells with 5 units/mL thrombin for 6 hours.
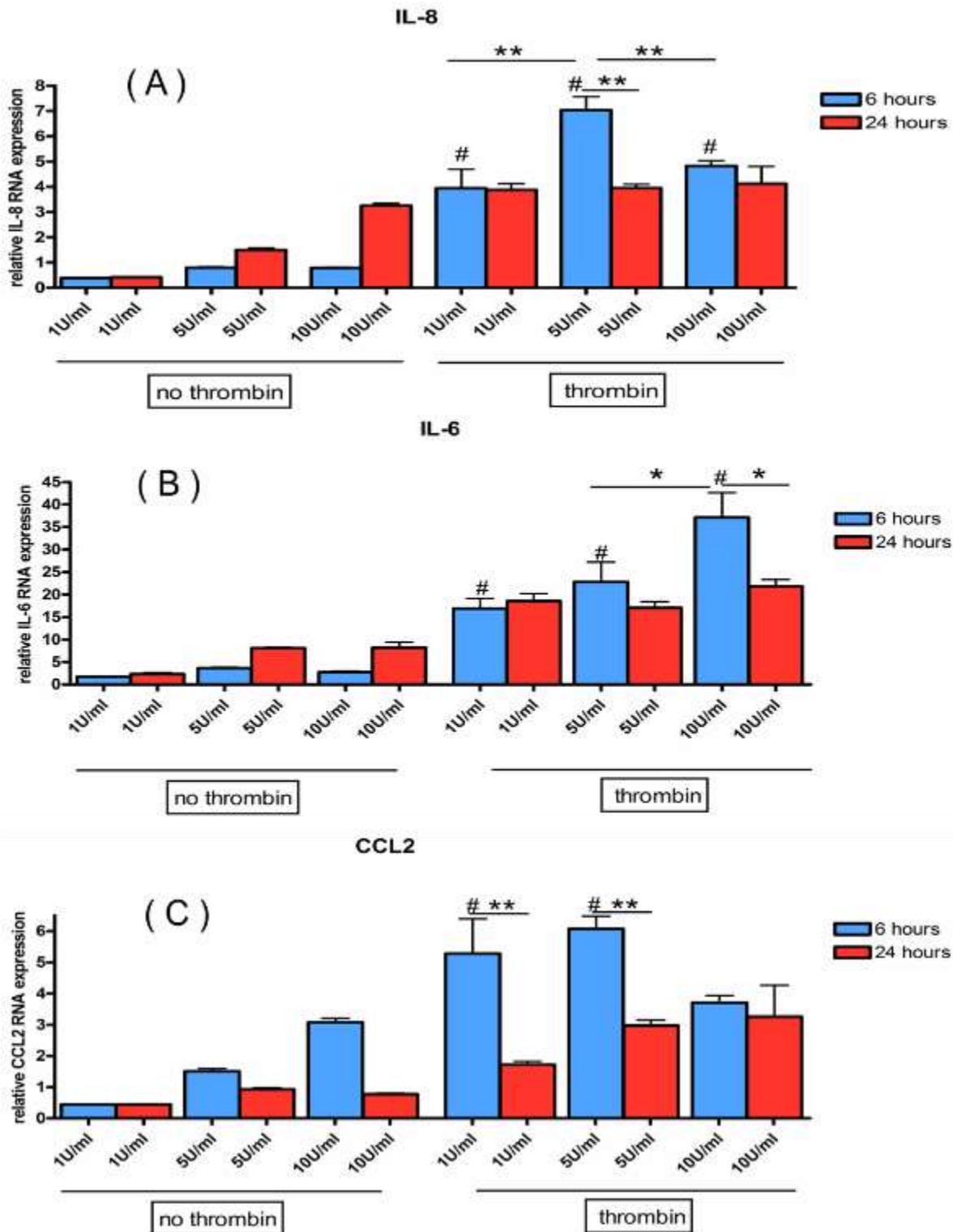
Figure 3-3: qPCR data for determining optimal thrombin dose and duration. (A) IL-8, (B) IL-6, and (C) CCL2 relative gene expression in cultured human myotubes treated with varying concentrations of thrombin for 6 and 24 hours. *$p<0.05$, **$p<0.01$, and # $p<0.01$ comparing thrombin treated cultures to the non-thrombin treated corresponding controls.

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3.2.7 Western Blot

The detail of the protocol was discussed in general material and methods section 2.8. Membranes were incubated with monoclonal primary antibody (1°Ab) diluted in 5% (w/v) skimmed milk powder in TBST, overnight at 4°C. After washing, membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody IgG, diluted in 5% blocking buffer. Detection was done using enhanced chemiluminescence and densitometry measurements performed. Phospho-Akt^{Ser473} antibody was used at a 1:10000 dilution while native Akt was used at a 1:2000 dilution and Phospho-IRS1^{Y612} and native-IRS1 were used at 1:1000 dilutions.

3.2.8 Glucose uptake assay

Cells were cultured on 6 well plates and incubated with or without thrombin, at 37°C in serum free media for 6 hours prior to use. After treatment, cells were washed twice with Kreb's buffer, then treated with or without 100nM insulin, or 10μM Cytochalasin B for 30min. Hot glucose solution (0.1mM 2-deoxy-glucose and 0.5μCi (2, 6-^{3}H) 2-deoxyglucose) was added for the last 10 minutes before the reaction was stopped by washing with ice-cold phosphate buffered saline (PBS). The detail of the protocol was discussed in general material and methods chapter section 2.9.

3.3 Results

3.3.1 Expression of thrombin receptor proteins

Generally, the protease-activated receptors, PAR1, PAR3 and PAR4, are all activated by thrombin, a serine protease, and referred to as thrombin receptors. Immunofluorescent staining of human myotubes was performed using a general anti-thrombin receptor antibody (rabbit anti-human thrombin receptor IgG), to visualise the distribution of thrombin receptor on the surface of human skeletal muscle cells as shown in (A). From the images taken using fluorescent microscopy, it is clear that thrombin receptors (in red) are abundant and widely spread across the cell membrane. Nuclei were visualised using DAPI (blue). (B) Represents the negative control of the staining, cells incubated with no primary antibody and no thrombin receptors can be visualised.

(A)
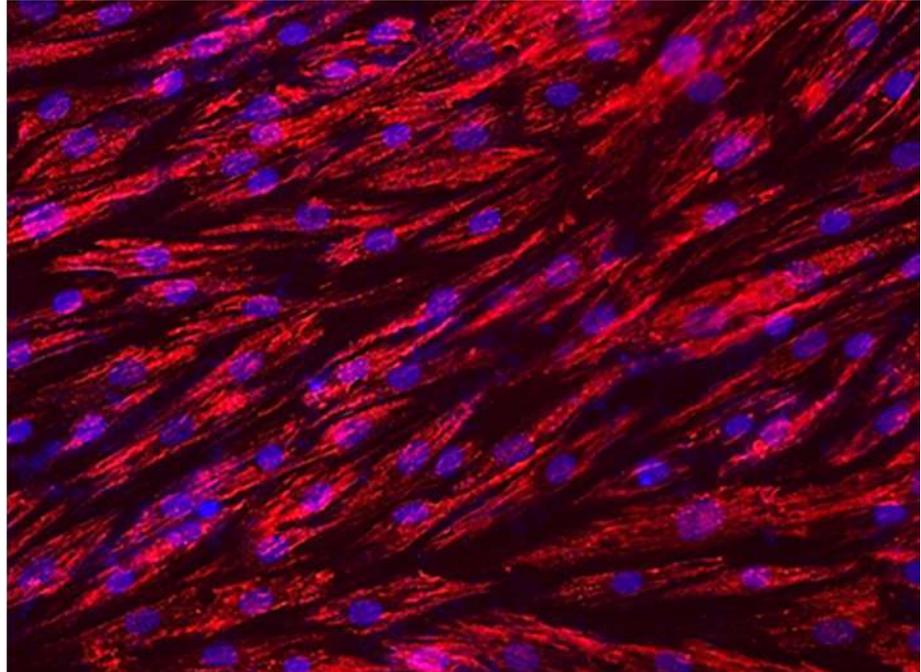
(B)
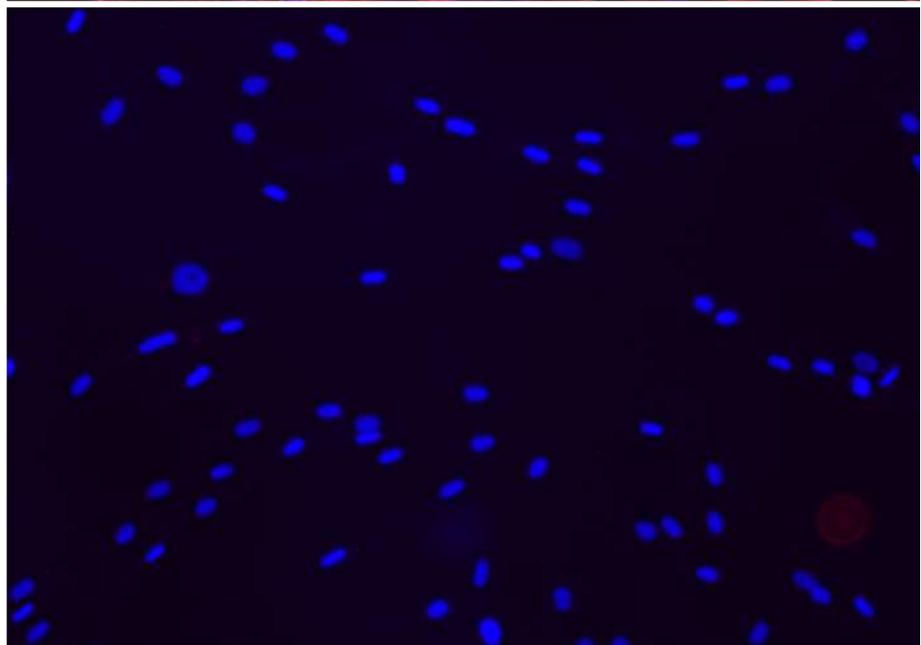
Figure 3-4: Immunofluorescent staining of thrombin receptorson the cell membrane of human skeletal muscle cells. (A) Showing: anti-thrombin receptor antibody (Cy3-red) and nuclei (DAPI-blue); staining in myotubes at x20 magnification. (B) A negative control of the image when no primary antibody was added to cultured myotubes and no thrombin receptors (in red) can be detected.

3.3.2 Effects of thrombin on insulin signalling

The effect of thrombin on the insulin signalling pathway was investigated by treating human muscle cells in culture with thrombin then stimulating these cells with insulin and examining the degree of activation of the key insulin signalling molecules, IRS1 and PKB (Akt) at the well-known activation residues.

IRS1 is the major IRS in skeletal muscle. Upon insulin stimulation, multiple tyrosine sites are phosphorylated with tyrosine 612 (Y^{612}) found to be the critical residue for downstream activation of the PI-3 kinase for GLUT4 translocation (Esposito *et al.*, 2001).

As shown in Figure 3-5 A and B, treatment of myotubes with 5 U/ml of thrombin for 6 hours resulted in a significant reduction ($p < 0.01$) in the phosphorylation of IRS1^{Y612} following insulin treatment of 100 nM for the last 10 minutes of thrombin exposure.

Akt phosphorylation and activation is a crucial step for insulin signalling and glucose uptake in skeletal muscle. The most critical activation site is Ser^{473} at the carboxyl terminal domain of the Akt enzyme. Following insulin stimulation, Akt^{Ser473} phosphorylation was examined in both thrombin and non-thrombin treated cells. Figure 3-6 A and B shows that treatment of the cells with 5 U/ml thrombin for 6 hours resulted in a significant reduction ($p < 0.01$) in the phosphorylation of Akt^{Ser473} following insulin treatment of 100 nM for the last 10 minutes of thrombin exposure.

(A)

Thrombin	+	+	−	−
Insulin	−	+	−	+

(B)
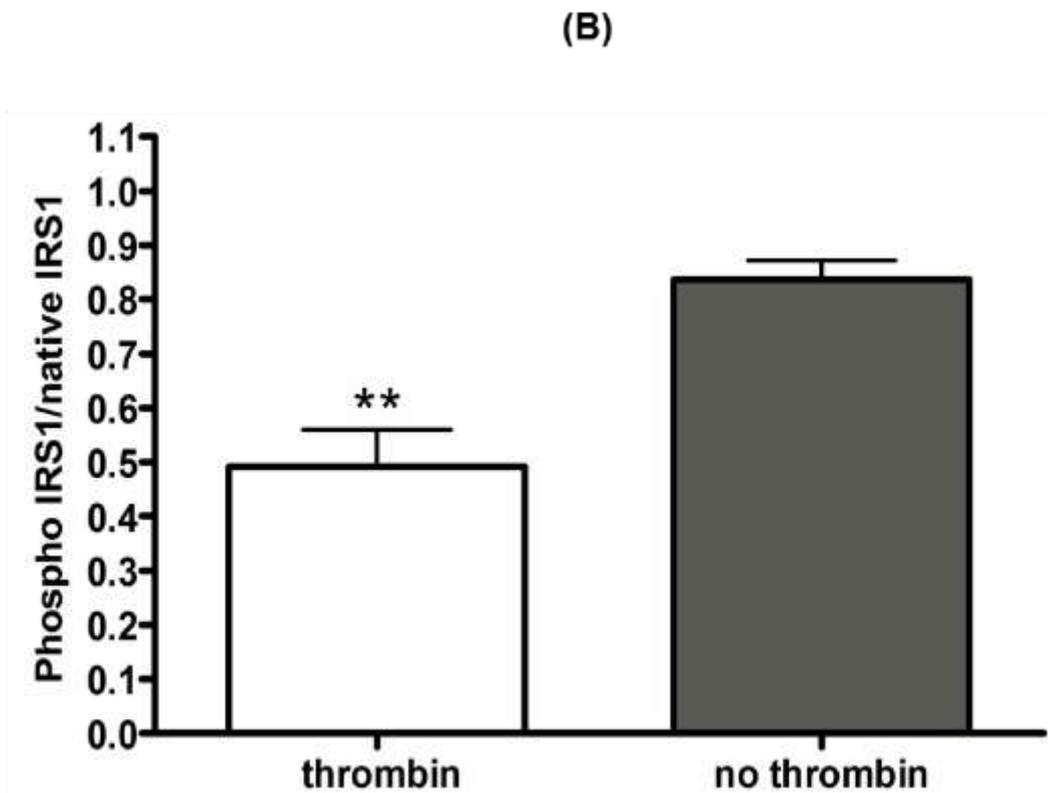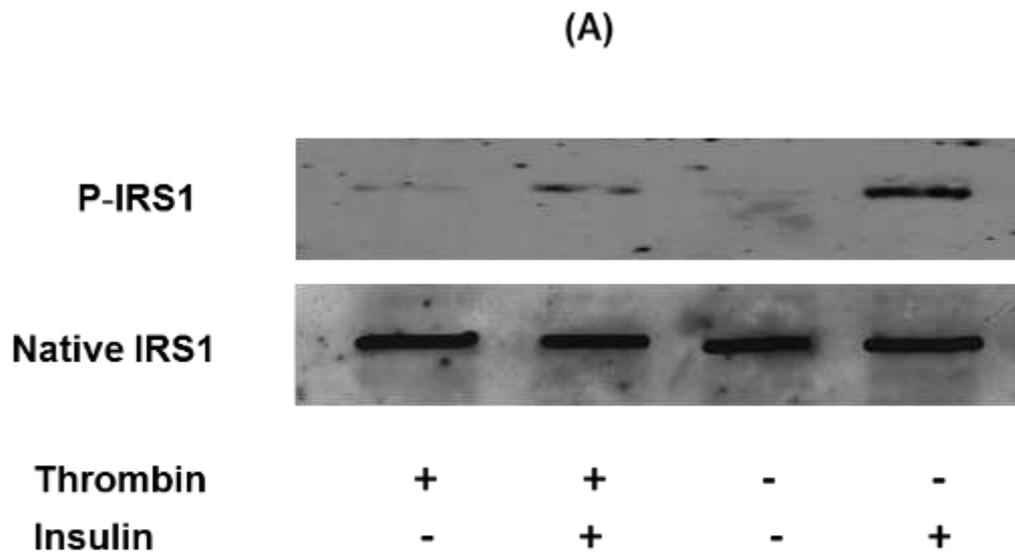
Figure 3-5: Thrombin inhibits insulin stimulated IRS1 activation in cultured myotubes. IRS1 phosphorylation at Y^{612} was measured by Western blot analysis. Cells were treated with thrombin (5 U/ml) for 6 hours then stimulated with insulin (100 nM) for the last 10 minutes of thrombin treatment. (A) Shows representative blots of phosphorylated IRS1 protein and blots of native IRS1. (B) A graph shows the results of densitometry analysis of P-IRS1 (phosphorylated IRS1)/N-IRS1 (Native IRS1) in insulin stimulated cells from both thrombin treated and non-treated cells. Values are expressed as the mean ± SEM, n=4, **$p<0.01$.

(A)

Thrombin	+	+	-	-
Insulin	-	+	-	+

(B)
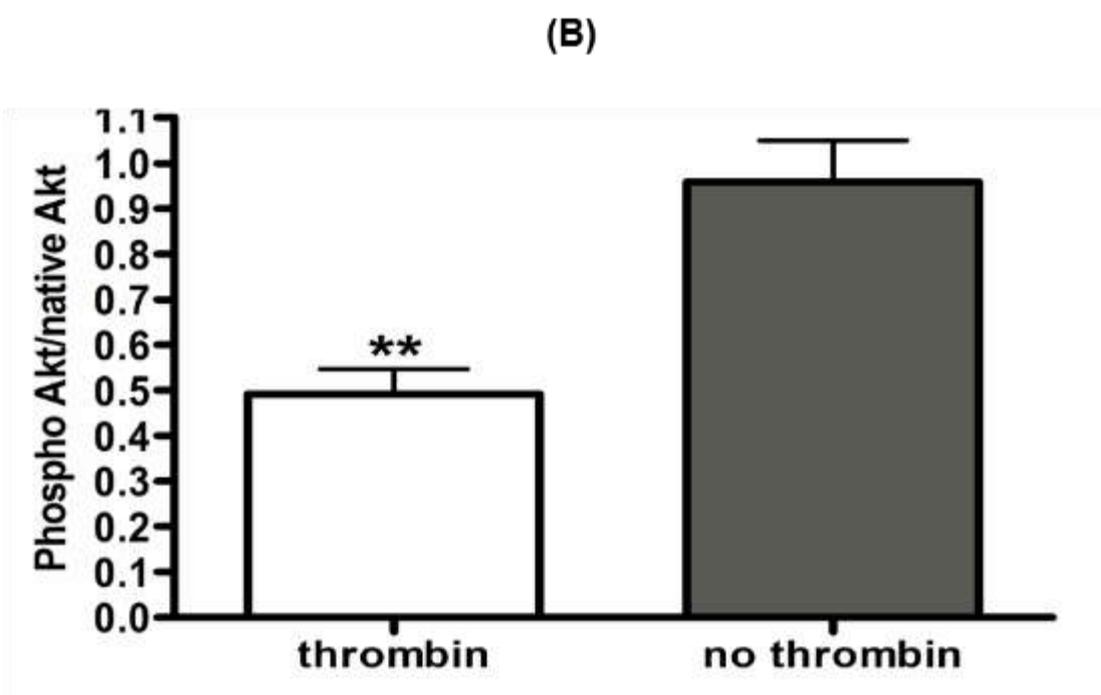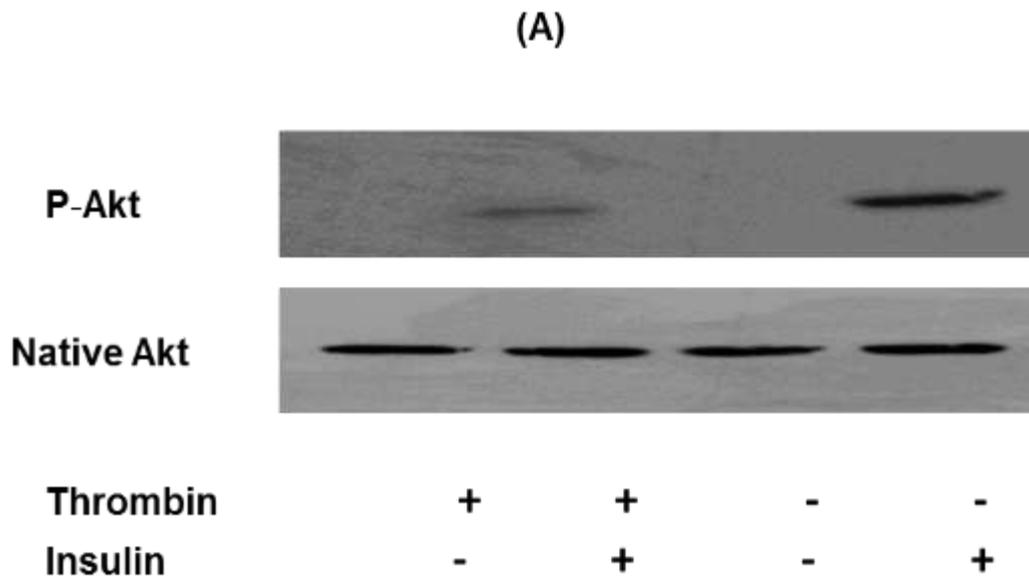
Figure 3-6: Thrombin inhibits insulin stimulated Akt activation in cultured myotubes. Akt phosphorylation at Ser^{473} was measured by Western blot analysis. Cells were treated with thrombin (5 U/ml) for 6 hours then stimulated with insulin (100 nM) for the last 10 minutes of thrombin treatment. (A) Shows representative blots of phosphorylated Akt and blots of native Akt. (B) A graph shows the results of densitometry analysis of P-Akt (phosphorylated Akt)/N-Akt (Native Akt) in insulin stimulated cells from both thrombin treated and non-treated cells. Values are expressed as the mean ± SEM, n=8, **p<0.01.

3.3.3 Role of PKC in mediating thrombin inhibitory effect on insulin signalling

3.3.4 PKC general inhibitor GF109203X

PKCs are involved in the insulin signalling pathway in skeletal muscle cells. To explore whether the inhibitory effect of thrombin on insulin-mediated Akt phosphorylation involves PKCs, human skeletal muscle cells in culture were treated with thrombin and increasing concentrations of the PKC general inhibitor GF109203X that is reported to inhibit all PKC isoforms. Final concentrations of 1, 10 and 100 nM GF109203X were used in both thrombin and non-thrombin treated cells for the last 30 minutes of thrombin treatment before stimulating cells with 100 nM insulin for the final 10 minutes. Subsequently, Akt^{Ser473} phosphorylation was measured to explore whether PKCs mediate the inhibitory effects of thrombin on insulin signalling.

Where cells were not treated with thrombin and subjected to the same doses of the PKC general inhibitor, no inhibition of Akt^{Ser473} phosphorylation in response to insulin was observed as shown in Figure 3-7. 10 and 100nM of GF109203X reversed the inhibitory effect of thrombin on insulin-mediated Akt^{Ser473} phosphorylation whereas 1nM displays no enhancement in Akt activation as shown in Figure 3-8.
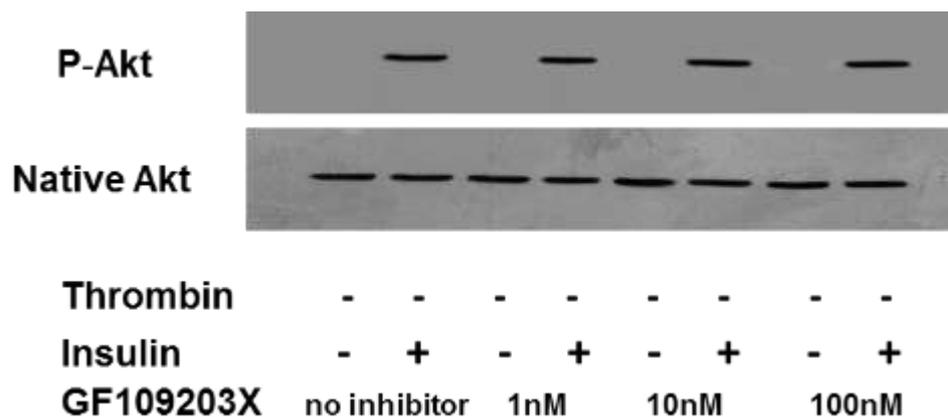
Figure 3-7: PKC general inhibitor GF109203X in non-thrombin treated cells has no effect on Akt^{Ser473} phosphorylation. A representative blot of phosphorylated Akt^{Ser473} and native Akt in non-thrombin treated cells with or without 100 nM insulin stimulation with increasing doses of inhibitor, n =1.

(A)

	P-Akt							
Native Akt								

Thrombin	+	+	+	+	+	+	+	+
Insulin	-	+	-	+	-	+	-	+
GF109203X	no inhibitor		1nM		10nM		100nM	

(B)
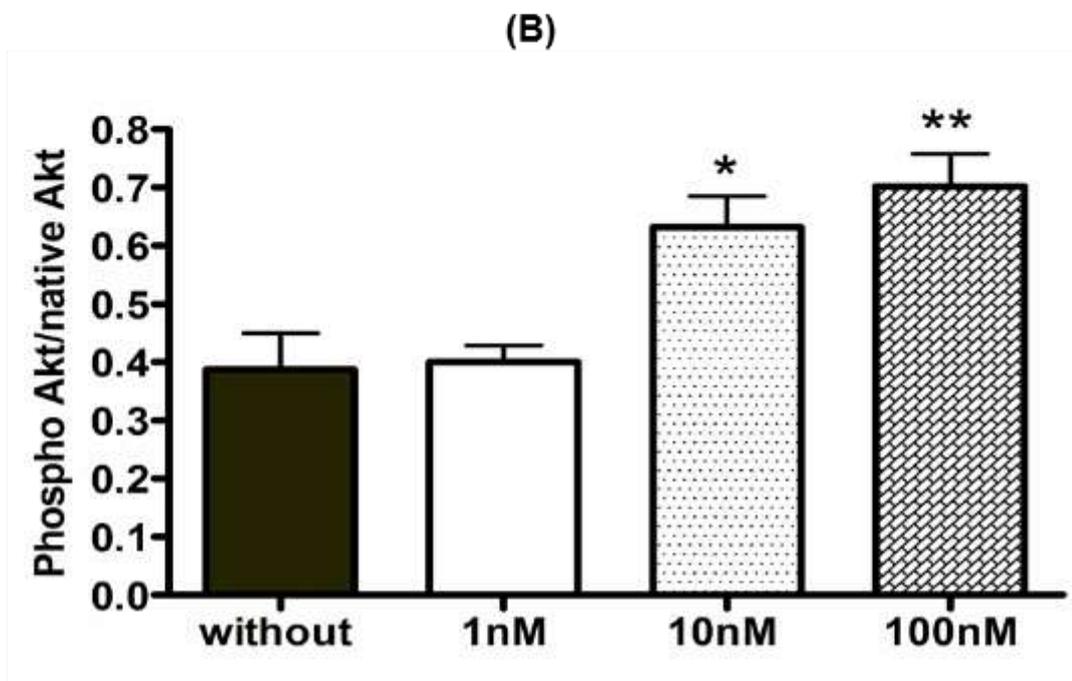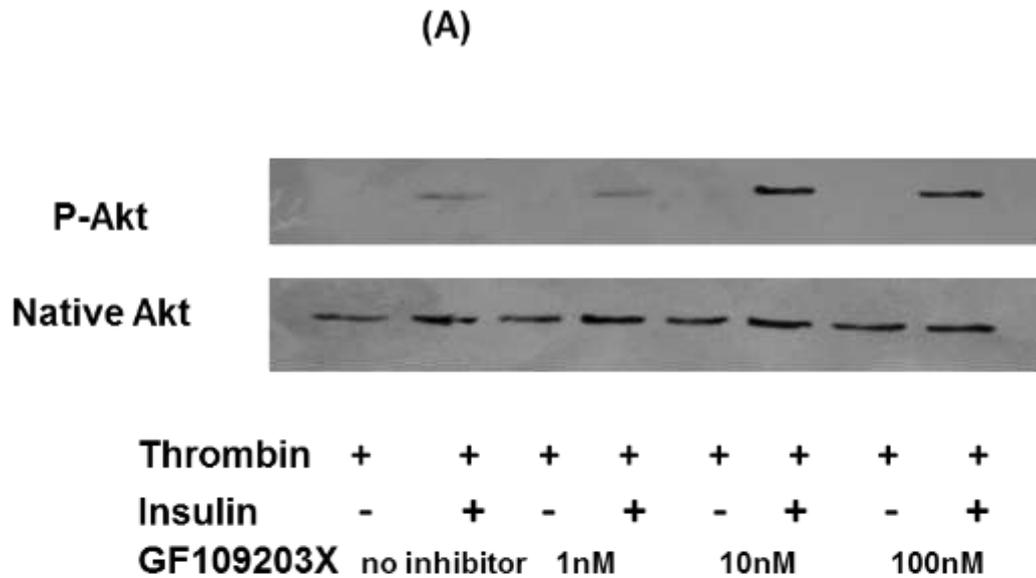
Figure 3-8: PKC general inhibitor GF109203X in thrombin treated myotubes.Cells were treated with thrombin (5 U/ml) for 6 hours; different doses of GF109203X were added for the final 30 min before cells were stimulated with 100 nM insulin for 10 mins. (A) Representative blots of phosphorylated Akt [Ser473] and native Akt in thrombin treated cells with or without 100 nM insulin stimulation with increasing doses of inhibitor. (B) Graph shows the results of densitometry analysis of P-Akt (phosphorylated Akt)/N-Akt (Native Akt) in insulin stimulated cells with different doses of PKC inhibitor. Values are expressed as the mean ± SEM, n = 6, *p< 0.05, **p<0.01.

3.3.5 PKC Conventional isoform selective inhibitor (GÖ 6976)

PKC has many isoforms and several of them have been linked to the insulin signalling pathway. The conventional PKC selective inhibitor Indolocarbazole (GÖ 6976), which is specific for the Ca^{2+} dependent isoforms α and β, was used in this study to investigate the inhibitory effects of thrombin on insulin signalling.

Non-thrombin treated cells were subjected to the same doses of the inhibitor GÖ 6976 and this shows no inhibition of Akt^{Ser473} phosphorylation in response to insulin as shown in Figure 3-9. The inhibitory effect of thrombin on insulin-mediated Akt phosphorylation was reversed in a dose dependent manner by GÖ 6976 treatment. There was a significant ($p< 0.05$) increase in Akt^{Ser473} phosphorylation even with the lowest concentration of inhibitor as shown in Figure 3-10.
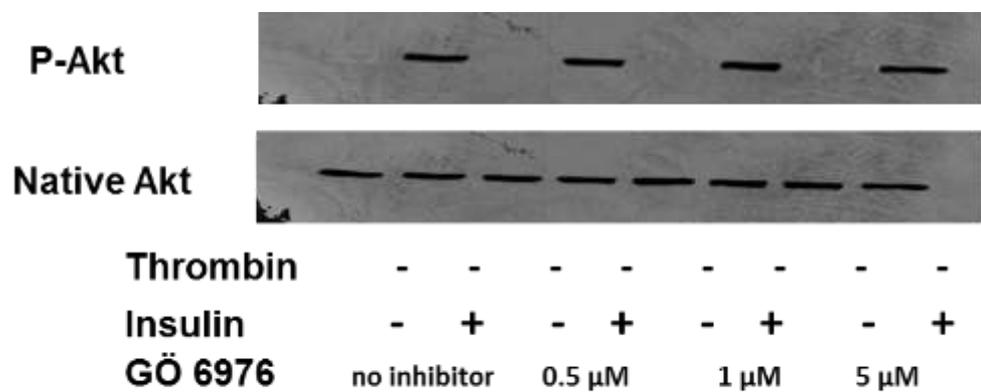
Figure 3-9: PKC selective inhibitor (GÖ 6976) in non-thrombin treated cells has no effects on phosphorylation of Akt^{Ser473}. Representative blots of phosphorylated forms of Akt, native Akt in non-thrombin treated cells with or without 100 nM insulin stimulation with increasing doses of inhibitor, n =1.

(A)

Thrombin	+	+	+	+	+	+	+	+
Insulin	-	+	-	+	-	+	-	+
GÖ 6976	no inhibitor		0.5 μM		1 μM		5 μM	

(B)
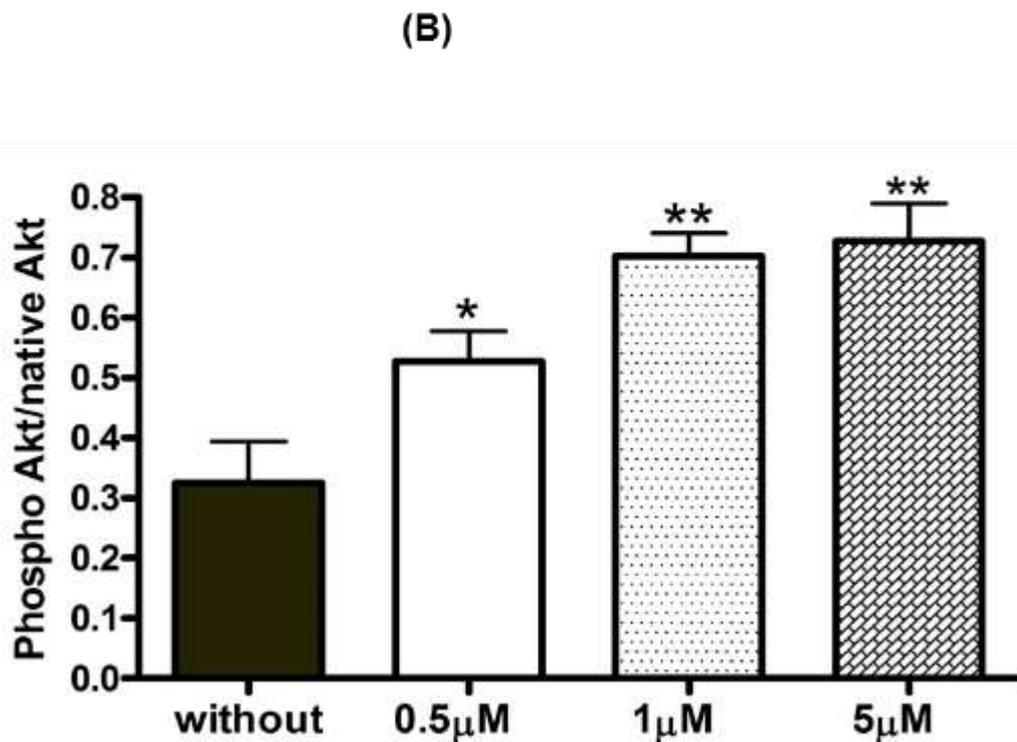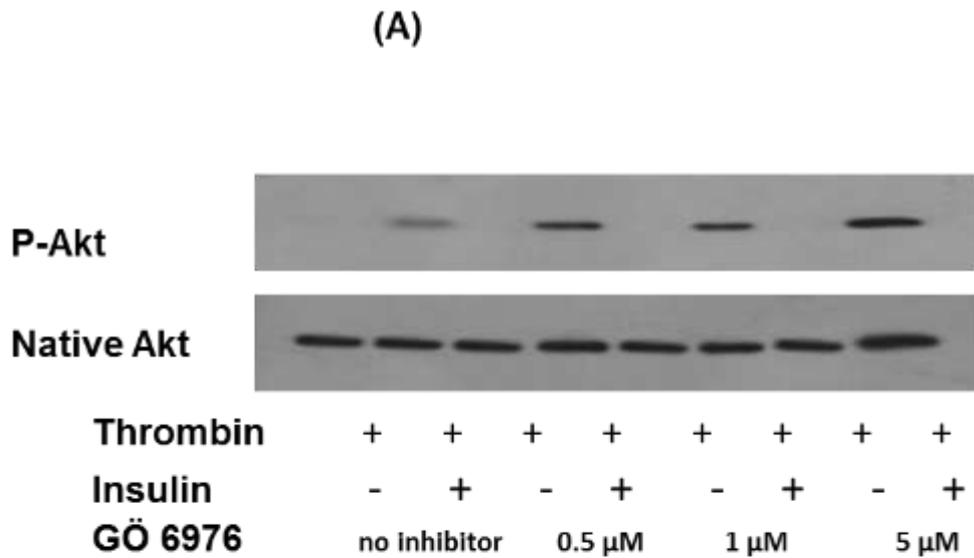
Figure 3-10: PKC selective inhibitor (GÖ 6976) in thrombin treated myotubes. Cells were treated with thrombin then different doses of the inhibitor were applied, finally cells were stimulated with 100 nM insulin. (A) Shows a representative blot of phosphorylated forms of Akt^{Ser473} and blot of native Akt (B) Graph shows the results of densitometry analysis of P-Akt (phosphorylated Akt)/N-Akt (Native Akt) in insulin stimulated cells with different doses of the inhibitor. Values are expressed as the mean ± SEM, n = 4, *p< 0.05, **p< 0.01.

3.3.6 Effects of thrombin on glucose uptake in cultured muscle cells

To examine the effect of thrombin on glucose uptake in cultured human muscle cells, radiolabelled glucose uptake was measured in the presence and absence of 5 U/ml of thrombin and 100nM of insulin. As shown in Figure 3-11 A, interestingly, thrombin increases glucose uptake to 659.8 ± 102.4 pmol/min/mg which is significantly above basal glucose uptake 501.6 ± 61.66 pmol/min/mg ($p<0.05$). Furthermore, in non-thrombin treated myotubes insulin increased glucose uptake to 780.7 ± 132.2 pmol/min/mg which was significantly higher than basal uptake in the absence of thrombin ($p<0.05$).

Insulin stimulated glucose uptake in thrombin treated myotubes continues to increase to 888.3 ± 166.3 pmol/min/mg; however, this was statistically not significant.

Fold change represents an alternative way of expressing glucose uptake. It is used to overcome the increased basal glucose uptake with thrombin treatment. The fold changes computed as the ratio of insulin over basal glucose uptake in both thrombin and non-thrombin treated cells showed that there was no significant difference in fold change between thrombin (1.3 fold) and non-treated myotubes (1.5 fold) as shown in Figure 3-11B.
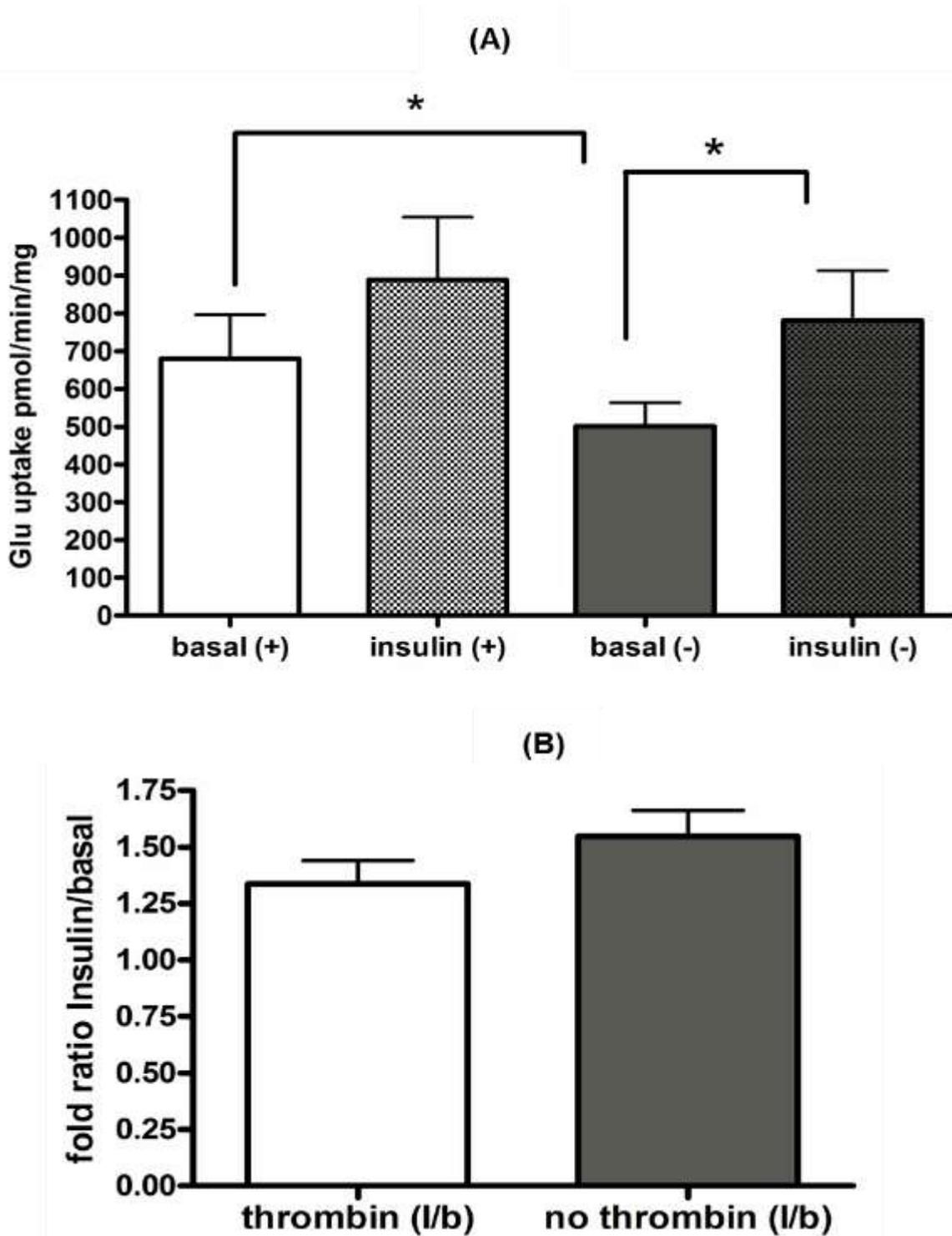
Figure 3-11: Glucose uptake in skeletal muscle myotubes. (A) Represents the absolute values of glucose uptake, basal and insulin stimulated for both the thrombin (+) and non-thrombin (-) treated myotubes. (B) Fold change in glucose uptake, I/b (glucose uptake in response to insulin/basal uptake) in the presence or absence of thrombin. The values are presented as mean ± SEM, n=5, *p<0.05.

3.4 Discussion

To our knowledge, this is the first work to examine the metabolic effects of thrombin treatment in skeletal muscle. In the present study, elevated thrombin causes a reduction in insulin signalling characterised by a decrease in IRS1 and Akt phosphorylation and this reduction in human myotubes was shown to be mediated, at least in part, through classical PKC. In addition, thrombin was found to directly increase glucose uptake with evidence that this is facilitated through an insulin independent pathway.

The activation of IRS1 tyrosine phosphorylation and PI3K-Akt pathways by insulin are fundamental steps of insulin signalling, insulin stimulated glucose uptake and metabolism (Pessin and Saltiel, 2000). In this study, thrombin decreased insulin-stimulated $IRS1^{Y612}$ and Akt^{Ser473} phosphorylation. These results are consistent with the results obtained from other studies measuring Akt activity in response to insulin and thrombin in adipocytes (Mihara *et al.*, 2010) and growth factor and thrombin in endothelial cells (Thors *et al.*, 2003).

However, the results differ from some published studies on platelets which show that thrombin directly causes an increase in phosphorylation of Akt and activation of platelets (Kroner *et al.*, 2000; Barry and Gibbins, 2002). This appears to be different to the situation in skeletal muscle, as we observed no activation of Akt by thrombin in myotubes in the absence of insulin. This inconsistency in the results of thrombin effects on insulin signalling between platelets and other cell lines, could be attributed generally to the fact that platelets are directly stimulated by thrombin and have no need of insulin for activation. In addition, in these studies there was a difference in duration of thrombin treatment. Moreover, Akt activation in platelets has been shown to be transient and only for short duration and it seems to return to the deactivated state after 1 hour of thrombin treatment. This differs from the method used in this study in which thrombin treatment was applied for 6 hours before measuring the response.

The inhibition of Akt activation by thrombin seems to be via a PKC-related mechanism. Protein kinase C is a serine-threonine kinase that is expressed abundantly in skeletal muscle. Different PKC isoforms are expressed and classified according to their structure and regulatory cofactors of activation into

three families as conventional, novel, and atypical (Parker and Murray-Rust, 2004). PKC isoforms play different roles in modulating biological activities such as growth, differentiation and signal transduction in different cell lines including human skeletal muscle (Buchner, 1995).

With regard to the insulin signalling pathway, different PKC isoforms exert different effects, that range from an activation of signal transduction in response to insulin such as for the PKC delta and zeta isoforms, to an inhibition of signal transduction such as for the PKC theta isoform (Sampson and Cooper, 2006). The diversity of functions of PKC isoforms in response to insulin could be summarized as below (Figure 3-12).
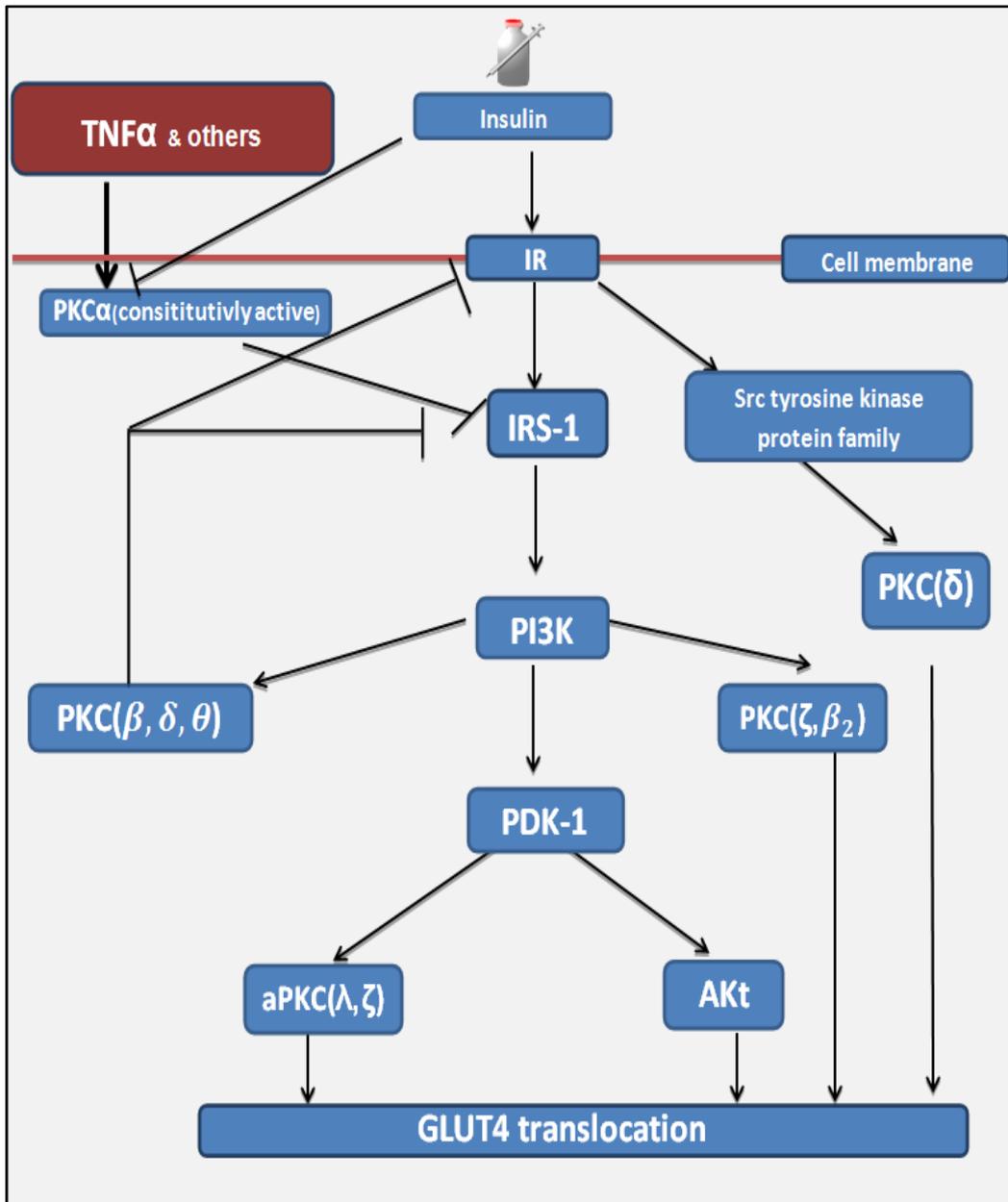
Figure 3-12: A summary of PKC isoforms role in the insulin signalling pathway in skeletal muscle.

GF109203X is a PKC general inhibitor that has been shown to inhibit the activation of PKC in skeletal muscle (Kim *et al.*, 2002; Wright *et al.*, 2003). The addition of the PKC general inhibitor improves insulin sensitivity in human skeletal muscle through an improvement of IRS1 tyrosine phosphorylation (Cortright *et al.*, 2000). The results of the present study show that GF109203X treatment in thrombin-treated cells reversed the inhibitory effects of thrombin on insulin signalling through an enhancement in Akt phosphorylation. These findings are in line with those observed in endothelial cells in which GF109203X reversed the inhibitory effect of thrombin on growth factor stimulation of Akt (Thors *et al.*, 2003).

In order to clarify the specific PKC isoform involved in the inhibitory effects of thrombin on insulin action, a specific PKCα isoform inhibitor was used. Studies found that activation of PKCα causes inhibition of insulin action (causes insulin resistance), and PKCα knockout mice show an improvement in insulin signalling and sensitivity compared to wild type (Letiges *et al.*, 2002). In addition, PKCα has been shown to be constitutively associated with IRS1 and this linkage causes a reduction in insulin activation, but upon insulin stimulation there is a dissociation of PKCα from IRS1 and an increase in downstream activity towards increased insulin signalling (Chin *et al.*, 1994; Liu and Roth, 1994; Andreozzi *et al.*, 2005). Inflammatory cytokines, like TNFα, have been shown to increase PKCα-IRS1 association, leading to insulin resistance. Sampson and Cooper (2006) in their review, suggested that any stimuli that strengthens the PKCα -IRS1 association would inhibit insulin signalling and could contribute to insulin resistance. Experiments in this study were designed to explore whether thrombin might act through the same mechanism, and the results show that selective inhibition of the cPKC isoform using the GÖ 6976 (cPKC specific inhibitor) reversed the inhibitory effect of thrombin on insulin-mediated Akt phosphorylation. This would suggest that PKCα is involved in the inhibitory effects of thrombin on the insulin signalling pathway.

While clear evidence was found that thrombin decreased insulin signalling in human skeletal muscle cells, it was also found that thrombin directly increases glucose uptake in both the basal and to some extent the insulin stimulated state. This is consistent with the findings of Kanda and Watanabe (2005) who showed that thrombin causes an increase in glucose uptake in vascular smooth muscle

cells. Furthermore, thrombin has been shown to have some similarity with other proinflammatory cytokines such as TNFα, which, despite interference with proximal insulin signalling pathways (decreased IRS1 and Akt activation), does not downregulate insulin-induced glucose transport, and even has a positive effect by inducing an increased basal glucose transport (Storz *et al.*, 1999).

In conclusion, this is the first study to look at thrombin and insulin action/signalling in human skeletal muscle. It was found that thrombin decreased insulin-stimulated IRS1 and Akt activation which was mediated through PKCα and simultaneously, thrombin directly increased glucose uptake and did not downregulate insulin-induced glucose transport.

CHAPTER 4 : THE EFFECTS OF THROMBIN ON DISTAL INSULIN SIGNALLING AND STIMULATION OF AMPK IN CULTURED HUMAN MYOTUBES.

4.1 Introduction

Thrombin is a multifunctional proteolytic enzyme and stimulation of cells with thrombin leads to protein phosphorylation, gene expression, contractility, and proliferation in a variety of cells, including skeletal muscle cells (Mackie *et al.*, 2008). In the previous chapter, it was shown that thrombin receptors were expressed in cultured human skeletal muscle cells and that thrombin has multiple metabolic and signalling effects represented by a decrease in proximal insulin signalling as assessed by measuring phosphorylation of IRS1 and Akt, without a concomitant decrease in insulin stimulated glucose uptake. In addition, thrombin by itself exerts a positive effect by enhancing basal glucose transport.

Glucose uptake into skeletal muscle cells is carried out by facilitated transport via glucose transporters. GLUT1 and GLUT4 are the most important members of the glucose transporter family in skeletal muscle cells.

GLUT1 in skeletal muscle cells is postulated to be responsible for the majority of basal glucose uptake, and this assumption is supported by studies in transgenic mice, where GLUT1 is overexpressed in skeletal muscle (Marshall *et al.*, 1993). An abnormal cell surface content of GLUT1 has been reported in human cases of insulin resistance and T2D (Miele *et al.*, 1997). However, Vogt *et al.* (1992) and Pedersen *et al.* (1990) found no differences in GLUT1 expression between T2D patients and control subjects. Furthermore, factors such as TNFα which have been shown to increase basal glucose uptake in skeletal muscle is reported to cause an increase in expression of GLUT1 at both gene and protein levels (Storz *et al.*, 1999).

GLUT4 was discovered in the late 1980s by James *et al.* (1988), and since then it has received the greatest attention of all transporter proteins due to its importance in whole body glucose homeostasis. The results of transgenic overexpression and knock-out of muscle-specific GLUT4 in mice increases basal and insulin-stimulated whole body glucose disposal. This observation confirms the crucial role

of this transporter in regulating muscle-specific and whole body glucose metabolism (Ren *et al.*, 1995; Zisman *et al.*, 2000). The critical step in glucose transport in response to stimuli is the translocation of GLUT4 from the cytoplasm to the plasma membrane. AS160 is a Rab GTPase activating protein that acts as an upstream mediator for GLUT4 translocation in response to insulin (Gonzalez and McGraw, 2006) and contraction or exercise mimetics, such as AICAR (Kramer *et al.*, 2006a). Atypical PKC has also been implicated in insulin stimulated GLUT4 translocation but its precise pathway is not fully understood (Farese *et al.*, 2007). Other PI3K-independent kinases have also been proposed to contribute to GLUT4 activation in response to insulin such as c-Cbl, CAP, and TC10 (Saltiel and Pessin, 2003). Skeletal muscle contraction also induces translocation of GLUT4 from intracellular compartments to the plasma membrane that presumably occurs via the activation of AMPK kinase, which is considered a sensor of intracellular energy (Jessen and Goodyear, 2005). Furthermore, studies show that factors that activate AMPK such as muscle contraction, AICAR, and others result in a rapid increase in GLUT4 gene and protein expression (Sherman *et al.*, 1993; Zheng *et al.*, 2001; Ojuka *et al.*, 2002).

It is reported that in many cell lines, thrombin exerts its effects via AMPK activation. For example, in human umbilical vein endothelial cells, inhibition of AMPK using compound C, an AMPK inhibitor, ameliorates thrombin-induced action (Gunduz *et al.*, 2015). Moreover, in human platelets, studies found that AMPK-α1 was directly activated by thrombin. Thrombin activates AMPK via the Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ)-dependent pathway and the use of a chemical inhibitor of CaMKKβ blocks thrombin-induced platelet response (Onselaer *et al.*, 2014; Randriamboavonjy and Fleming, 2014). In addition, in bone cells (osteoblasts) thrombin-stimulated IL-6 synthesis was proposed to be an AMPK dependent, and the use of compound C produces a dose dependent suppression in thrombin-stimulated IL-6 release (Tokuda *et al.*, 2012).

Having observed an inhibitory effect of thrombin on insulin signalling, it was surprising to observe that insulin stimulated glucose uptake tended to increase in the presence of thrombin although this was not statistically significant.

Therefore the experiments described in this chapter were designed to explore the possible pathway by which thrombin exerts its effects on glucose uptake.

Our hypothesis was that, thrombin increased glucose uptake in an insulin independent pathway that would bypass its inhibitory effects on proximal insulin signalling.

Aims of this chapter:

1- Exploring the effects of thrombin on distal insulin signalling pathway.

2- Examining other pathway by which thrombin exerts its effects on glucose uptake.

4.2 Methods

4.2.1 Experimental design

Skeletal muscle cells were seeded in 6 well plates, grown to confluence and allowed to differentiate for 7 days. Media was changed to reduced serum media (minimal essential media supplemented with 0.25 % (v/v) FBS), the cells treated with 5 U/ml thrombin or the same volume of water as a control, according to the previous optimisation of the dose and duration. For all experiments insulin was added at a concentration of 100nM for the last 10 minutes. For AMPK inhibitor compound C, all cells were treated with thrombin for six hours then for the last 30 minutes 40 µM of compound C was applied.

4.2.2 Western Blot

The detail of the protocol was discussed in general material and methods chapter. section 2.8, with the following exception; 10µg samples were loaded on 10% SDS-PAGE gels in loading buffer for the AMPK and aPKC experiments and 8% SDS-PAGE gels for AS160 were used for better separation of high molecular weight protein "about 160 kDa". After separation, proteins were transferred onto a nitrocellulose membrane (0.45 µm pore size), with a longer period for transfer (3 hours) for the high molecular weight proteins. Non-specific antibody binding sites on the nitrocellulose membranes were blocked by incubation in Tris buffered saline tween (TBST) containing 5% (w/v) milk for AMPK and aPKC and 3% (w/v) BSA for AS160 for 1 hour at room temperature. Membranes were incubated with monoclonal primary antibody (1°Ab) diluted in 5% (w/v) milk in TBST or 1 % BSA accordingly, overnight at 4°C. After washing, membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody IgG, diluted in blocking buffer. Detection was done using enhanced chemiluminescence and densitometry measurements performed. Phospho and native aPKC antibodies were used at a 1:1000 dilution, phospho and native AMPK antibodies were used at a 1:1000 and phospho and native AS160 were 1:500 dilutions.

4.2.3 Glucose uptake assay

Cells were cultured and grown on 6 well plates and allowed to differentiate and, when confluent media was changed to differentiation media. On day seven, the media was changed to reduced serum media and cells were incubated with or

without 5 U/ml of thrombin, at 37°C for 6 hours prior to use. Glucose uptake was then performed as described in general Materials and Methods chapter, section 2.9.

4.2.4 RNA isolation and cDNA synthesis

Total RNA was extracted from human skeletal muscle cells using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma) following the manufacturer's instructions that are described in detail in general material and method chapter, section 2.10. Total RNA was treated with DNase and 200ng was reverse-transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems) in a final volume of 20µl.

4.2.5 Quantitative real-time PCR

Quantitative real-time PCR was performed on a Lightcycler 480 (Roche) using TaqMan primers and probes. GLUT4 (Hs.380691) and GLUT1 (Hs.473721) were obtained from Applied Biosystems as predesigned TaqMan primer-probe mixes and were used at the recommended 1:20 dilution. β2-microglobulin (β2M) was used as a reference gene with the following sequences:
Forward primer; GCCTGCCGTGTGAACCAT,
Reverse primer; TTACATGTCTCGATCCCACTTACCTATC,
Probe; FAM-TGACTTTGTCACAGCCCA-TAMRA.
The concentration of both primers was 300 nM per reaction and 250 nM for the probe. 10µl of Gene expression mastermix (Applied Biosystems) was added to each reaction with 20ng of template. Results were analysed either by the standard curve method for GLUT1 or by the ΔΔCT method for GLUT4 as it is sparsely expressed in myotubes and failed to achieved a readable standard curve. Relative quantification was performed with data normalised to β2-microglobulin.

4.2.6 ELISA

Secretion of IL-6 from day 7 differentiated myotubes was determined by enzyme-linked immunosorbent assay (ELISA) using the Single-Analyte ELISArray (Qiagen). At day seven differentiated myotubes, media was changed to reduced serum media before treatment. Cells were treated with or without 5 U/ml of thrombin for 6 hours. The media of both thrombin treated and non-thrombin treated cells was collected, centrifuged at 1000g for 10 minutes and analysed for

secretion of IL-6 according to the manufacturer's protocol. A standard curve was generated by serial dilution of the antigen provided. Standards and test absorbance were read at 450nm. Background absorbance was subtracted from the values.

4.3 Results

4.3.1 Effect of thrombin treatment on glucose transporter GLUT1 expression

In order to assess the stimulatory effects of thrombin on glucose transport, glucose transporter gene expression was examined using qPCR methods. GLUT1 is one of the major glucose transporters predominantly expressed in human muscle cells and represents the main way by which basal glucose uptake takes place (Ciaraldi *et al.*, 2005). The addition of thrombin to fully differentiated myotubes causes a significant increase in GLUT1 gene expression ($p < 0.05$) compared with non-thrombin treated cells as displayed in Figure 4-1.

4.3.2 Effect of thrombin treatment on glucose transporter GLUT4 expression:

GLUT4 is the principal transporter involved in insulin- and muscle contraction-responsive glucose uptake in skeletal muscle. Upon stimulation, GLUT4 translocates from the cytoplasm to the cell membrane facilitating increased glucose uptake. GLUT4 is highly expressed in myotubes compared to myoblasts as a reflection of functional demands (Santalucia *et al.*, 1992). From the data in Figure 4-2, it is apparent that GLUT4 gene expression is significantly higher ($p < 0.05$) in response to thrombin treatment compared to myotubes without added thrombin.
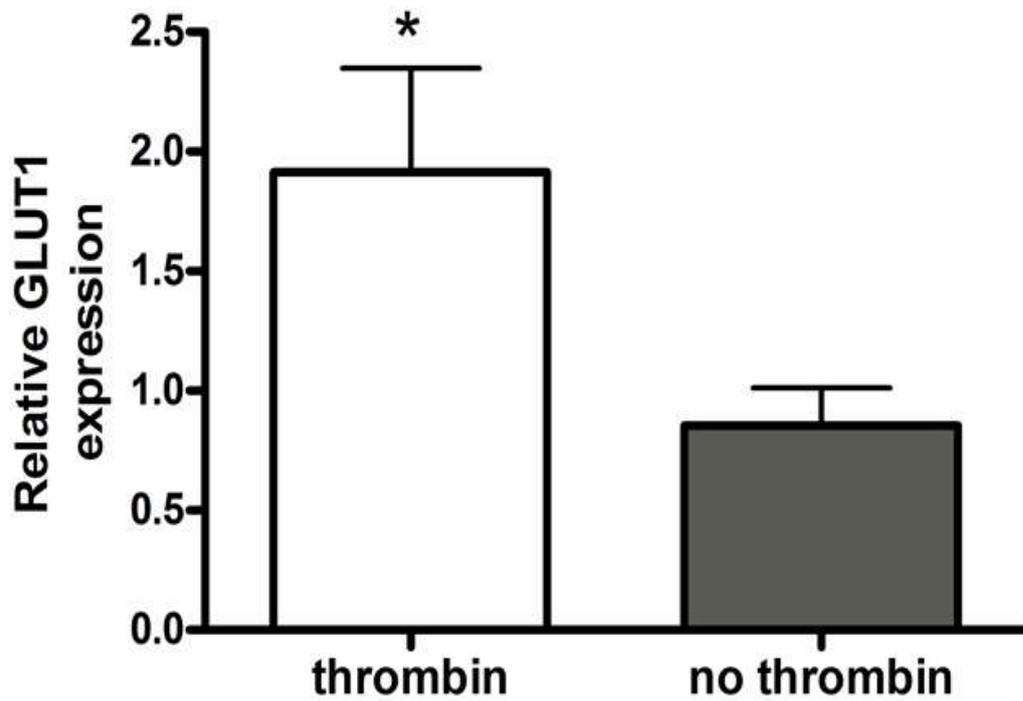
Figure 4-1: Effect of thrombin on GLUT1 gene expression. An increase in GLUT1 (glucose transporter) gene expression is observed in myotubes treated with thrombin compared to non-thrombin cells. Values are normalized to the level of β2-microglobulin mRNA as a reference gene and expressed as the mean ± SEM, n =4, *p<0.05.
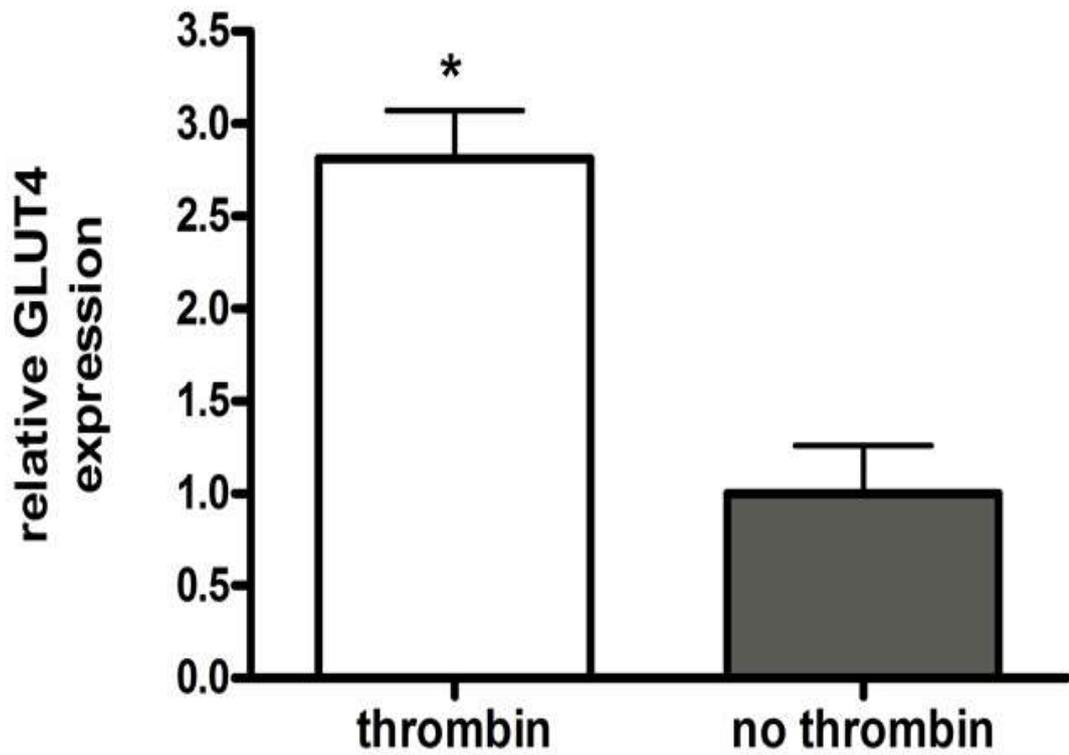
Figure 4-2: Effect of thrombin on GLUT4 gene expression. GLUT4 expression in myotubes is increased in response to thrombin. Values are normalized to the level of β2-microglobulin mRNA as a reference gene. Data expressed as mean ± SEM, n =5, *p<0.05.

4.3.3 Effects of thrombin on the distal insulin signalling pathway

The results of the work in the previous chapter showed that thrombin causes a decrease in the proximal insulin signalling pathway, represented by a reduction in the phosphorylation of both IRS1^{Y612} and Akt^{Ser473} following insulin treatment, without a concomitant reduction in insulin stimulated glucose uptake. Furthermore, thrombin per se causes an increase in basal glucose uptake. Therefore the work in this chapter was designed to explore alternative pathways by which thrombin exerts its effects.

4.3.4 Effects of thrombin on aPKC activation

Insulin continued to increase glucose uptake in the presence of thrombin, which was unexpected in view of the inhibitory effect of thrombin on insulin-mediated Akt activation. We therefore explored the impact of thrombin on insulin activated aPKC by measuring the phosphorylation of PKC ζ/λ$^{Thr410/403}$. Thrombin did not affect aPKC stimulation with insulin compared to non-thrombin treated cells as shown in Figure 4-3.
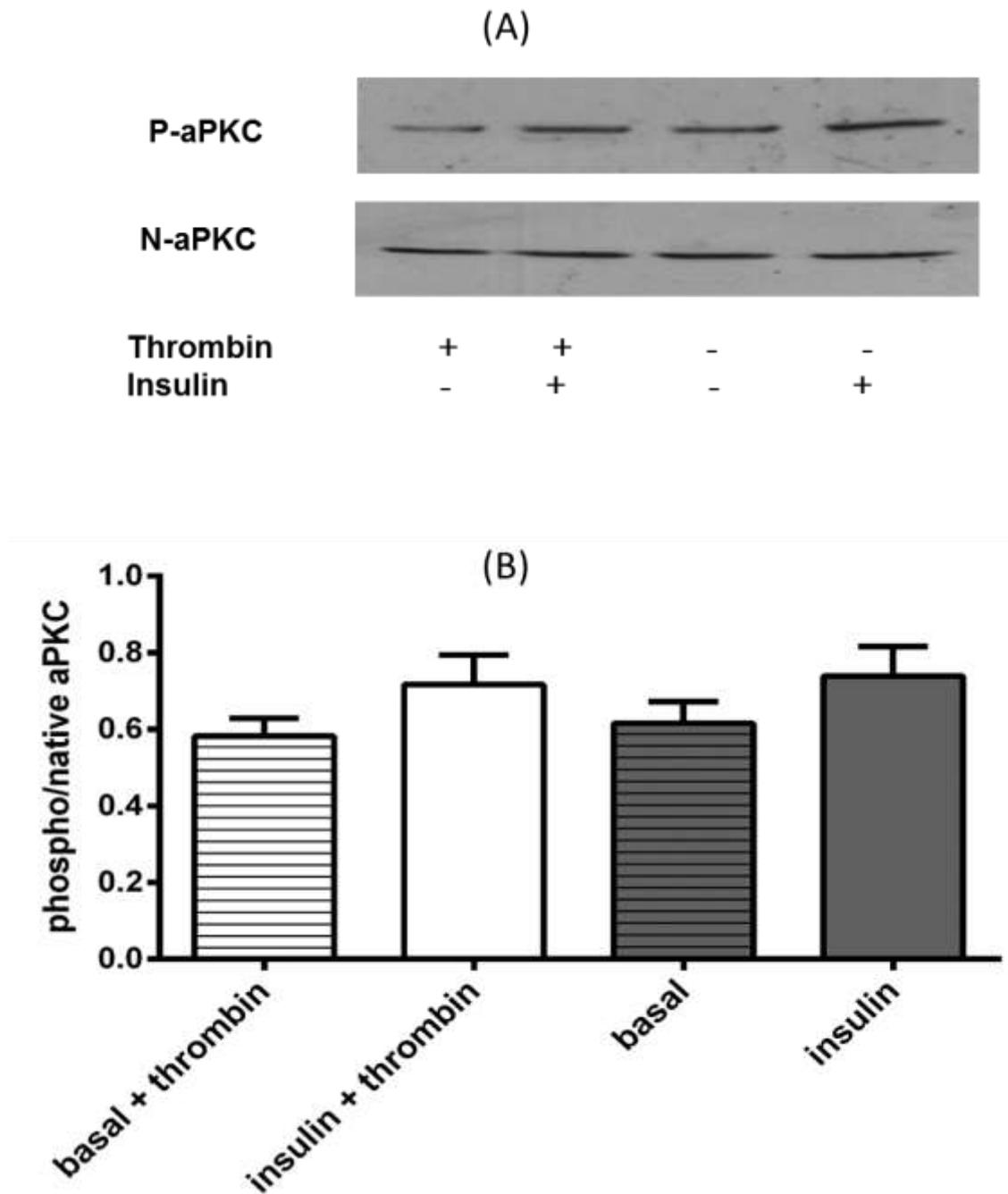
Figure 4-3: Effect of thrombin on insulin stimulated aPKC activation in cultured myotubes. The phosphorylation of PKC ζ/λ$^{\text{Thr410/403}}$ was measured by Western blot analysis. Cells were treated with thrombin (5 U/ml) for 6 hours then stimulated with insulin (100 nM) for the last 10 minutes of thrombin treatment. (A) Representative blots of phospho-PKC ζ/λ (Thr$^{410/403}$) and blots of native aPKC. (B) Graph showing the densitometric analysis of P-aPKC (phospho-PKC ζ/λ Thr$^{410/403}$)/N-aPKC (native aPKC) in basal and insulin stimulated cells in thrombin treated or non- treated cells. Values are expressed as the mean ± SEM, n=5.

4.3.5 Effect of thrombin on AS160 activation

AS160 is the direct downstream signalling molecule activated by both insulin and contraction, which results in an increase in glucose uptake. The effect of thrombin on AS160 activation was investigated by measuring Thr^{642} phosphorylation. Thrombin showed no inhibitory effect on insulin-mediated AS160 activation compared with untreated cells and simultaneously thrombin caused an increase in basal AS160 activation compared to non-thrombin treated cells ($p < 0.05$) (Figure 4-4).

(A)

P-AS160

N-AS160

| Thrombin | + | + | − | − |
| Insulin | − | + | − | + |

(B)
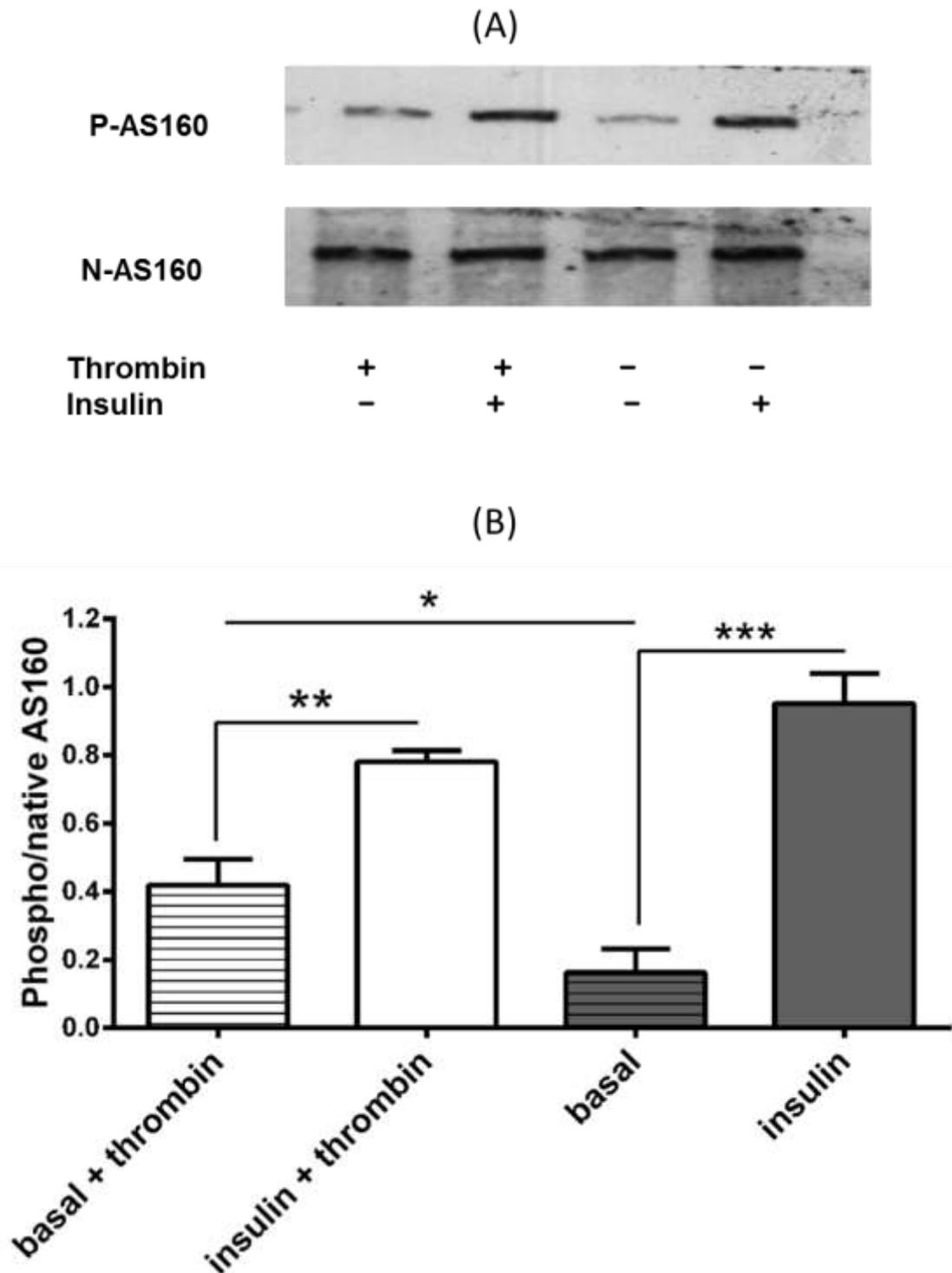
Figure 4-4: Effect of thrombin on insulin stimulated AS160 activationin cultured myotubes. Phosphorylation of AS160^{Thr642} was measured by Western blot analysis. Cells were treated with thrombin (5 U/ml) for 6 hours then stimulated with insulin (100 nM) for the last 10 minutes of thrombin treatment. (A) Representative blots of phospho- AS160^{Thr642} and native AS160. (B) Graph showing the densitometric analysis of P-AS160 (phospho- AS160^{Thr642})/N-AS160 (Native AS160). Values are expressed as the mean ± SEM, n=4, *p<0.05, **p<0.01, ***p<0.001.

4.3.6 Thrombin increases AMPK activation

To explore the possible mechanisms resulting in increased basal glucose uptake, the effect of thrombin on AMPK activation was assessed by measuring AMPK^{Thr172} phosphorylation.

As shown in

Figure 4-5, thrombin significantly increased AMPK activation ($p < 0.001$) compared to untreated cells.

Furthermore, to prove that thrombin mediates its action via AMPK activation, compound C, an inhibitor of AMPK, was used. Incubation with 40µM compound C for the last 30mins of thrombin treatment resulted in a partial reversal of the stimulatory effect of thrombin on AMPK phosphorylation ($p < 0.01$) (Figure 4-6).

In addition, the decrease in AMPK phosphorylation after compound C treatment was also associated with a partial reversal of the stimulatory effect of thrombin on glucose uptake (Figure 4-7). Glucose uptake decreased from 813±113.38 pmol/min/mg after thrombin treatment to 484.6±80.24 pmol/min/mg ($p < 0.05$) after treatment with both thrombin and compound C. Taking these data together, thrombin increases glucose uptake that is in part mediated by the activation of AMPK.

4.3.7 Thrombin increases IL-6 secretion

From the same experiments that show that thrombin causes an increase in AMPK activation, media was kept to measure the effects of thrombin on IL-6 secretion. As shown in Figure 4-8, thrombin causes a significant increase in IL-6 liberation into the media ($p < 0.05$) compared to non-thrombin treated cells.
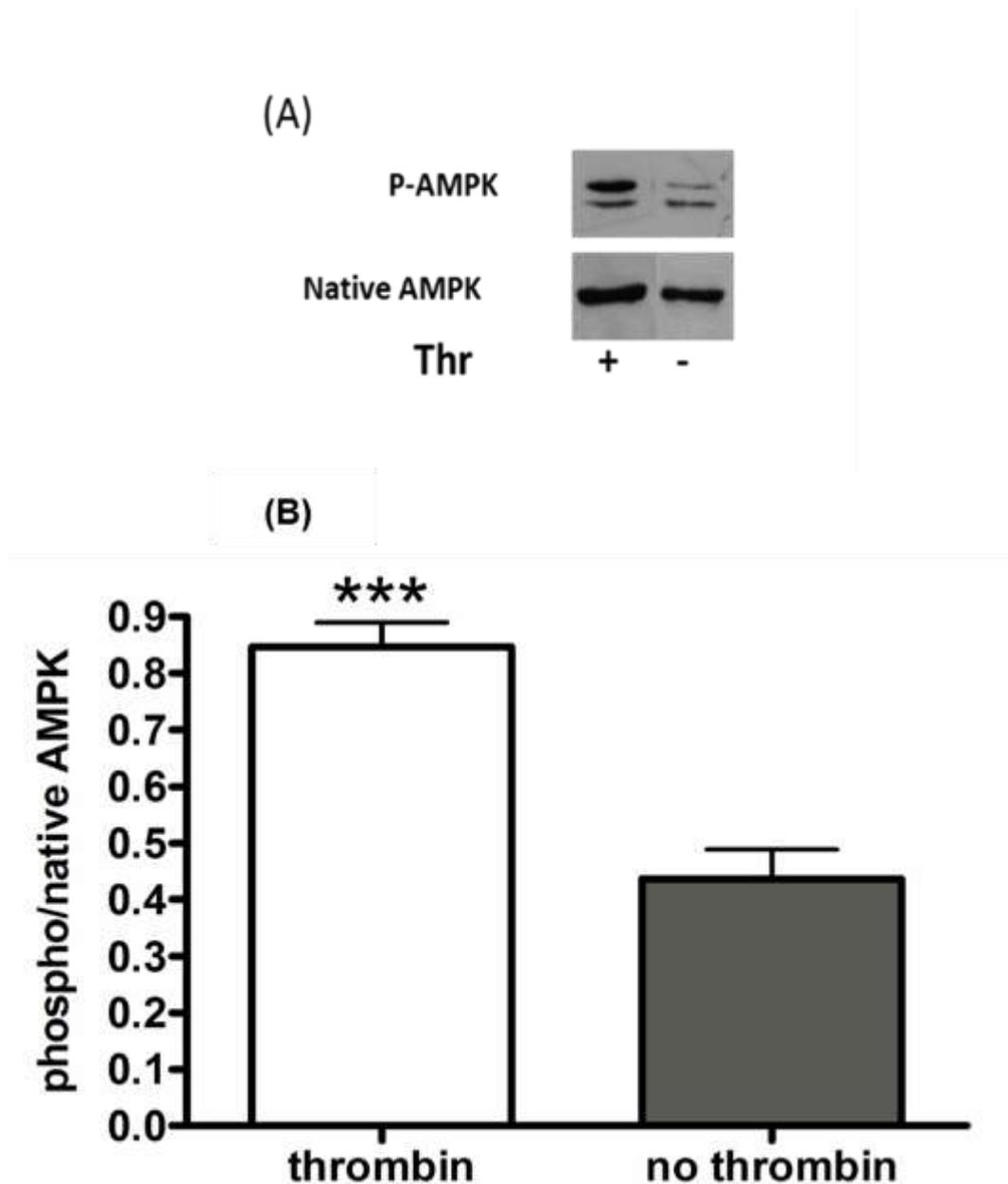
Figure 4-5: Effect of thrombin on AMPK activation in cultured myotubes. AMPK activation assessed in myotubes treated with thrombin (5 U/ml) for 6h and measurement of AMPK^{Thr172} phosphorylation by Western blot. (A) Shows the representative blots of phospho- AMPK^{Thr172} and total AMPK in both thrombin and non-thrombin treated cells. (B) Graph of densitometric analysis results of AMPK activation at Thr^{172}. Values are expressed as the mean ± SEM, n=5, ***p<0.001.

(A)

P-AMPK

T-AMPK

| • Thrombin | - | + | - | + |
| • Cc | + | + | - | - |

(B)
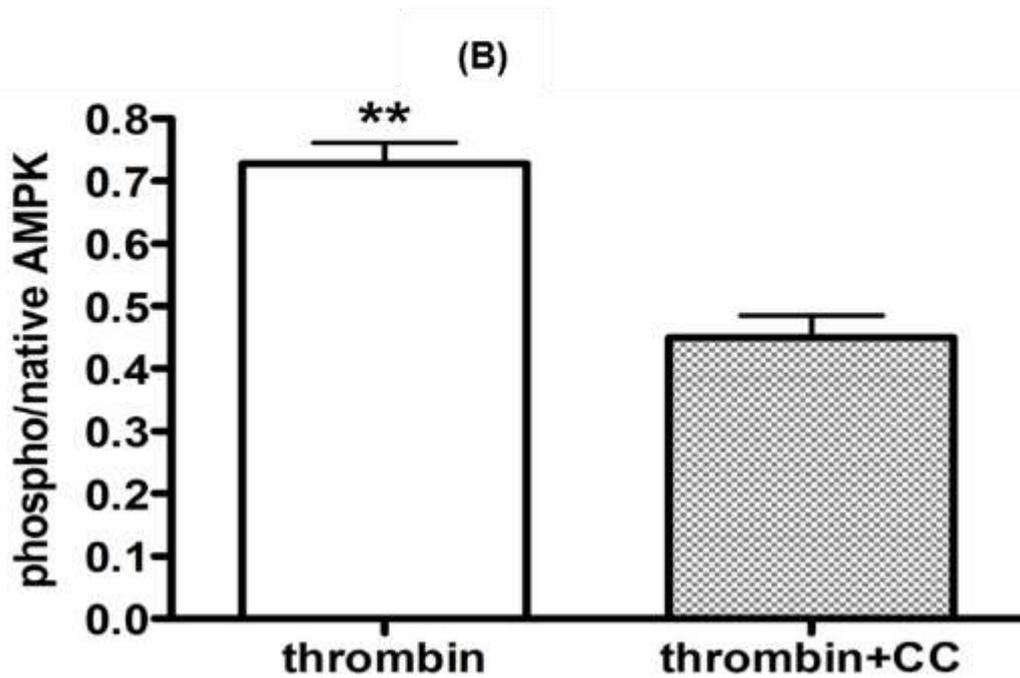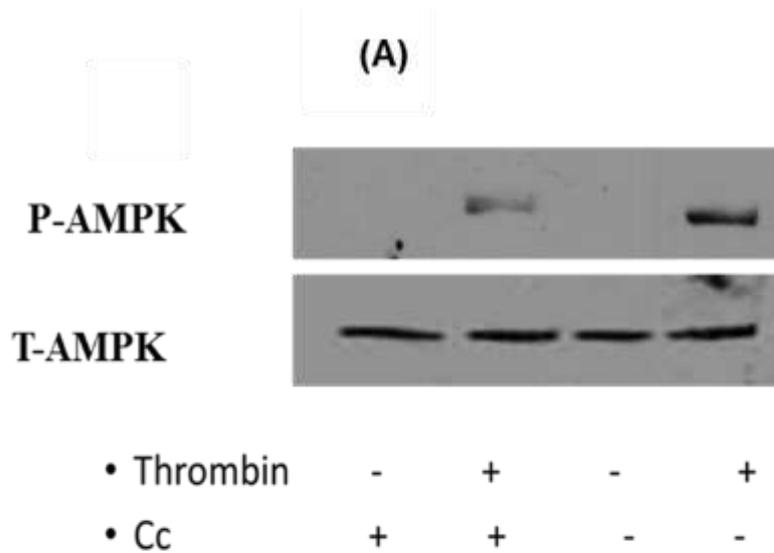
Figure 4-6: Use of the AMPK chemical inhibitor compound C on thrombin-treated cells. Cells were treated with thrombin (5 U/ml) for 6 hours with or without 40µM compound C that was added for the last 30 minutes before measuring AMPK^{Thr172} phosphorylation by Western blot. (A) Shows the representative blots of phospho-AMPK^{Thr172} and total AMPK in both thrombin and compound C treated and non-treated cells. (B) Graph of densitometric analysis of AMPK activation at Thr^{172} for the above treatment. Values are expressed as the mean ± SEM, n=4, **p<0.01.
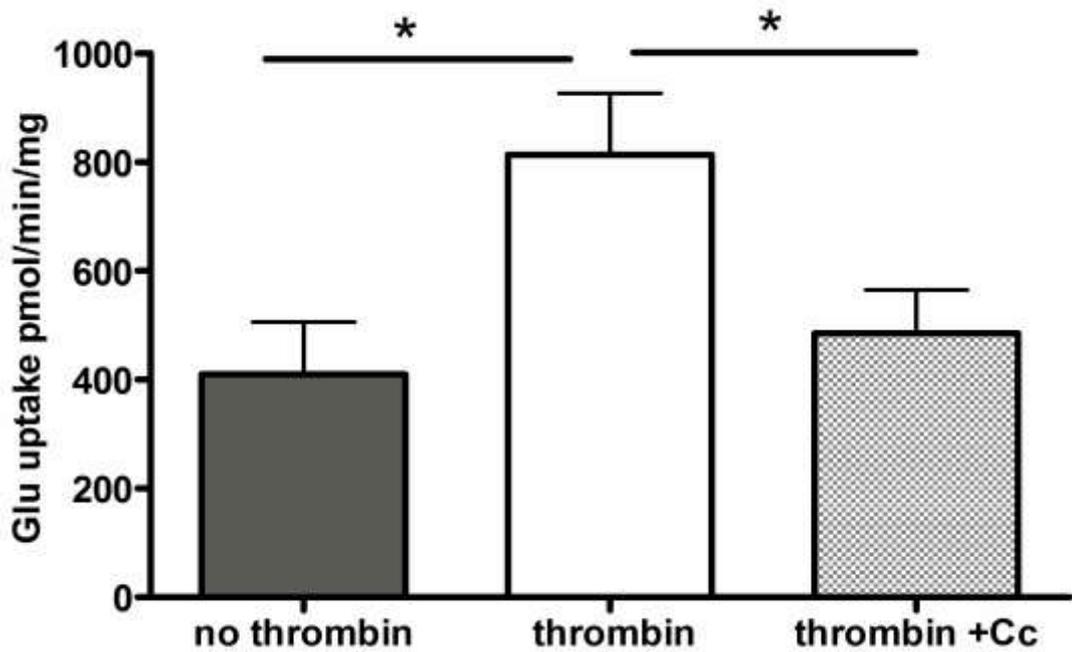
Figure 4-7: Effect of compound C on thrombin induced glucose uptake. Cultured human myotubes treated with thrombin (5 U/ml) for 6 hours, and then 40µM compound C was added for the last 30 minutes followed by measurement of radiolabelled glucose uptake into cells. The graph is representative of basal glucose uptake in skeletal muscle myotubes for control (no thrombin), thrombin and thrombin with compound C. Values are expressed as mean ± SEM, n=5, *p<0.05.
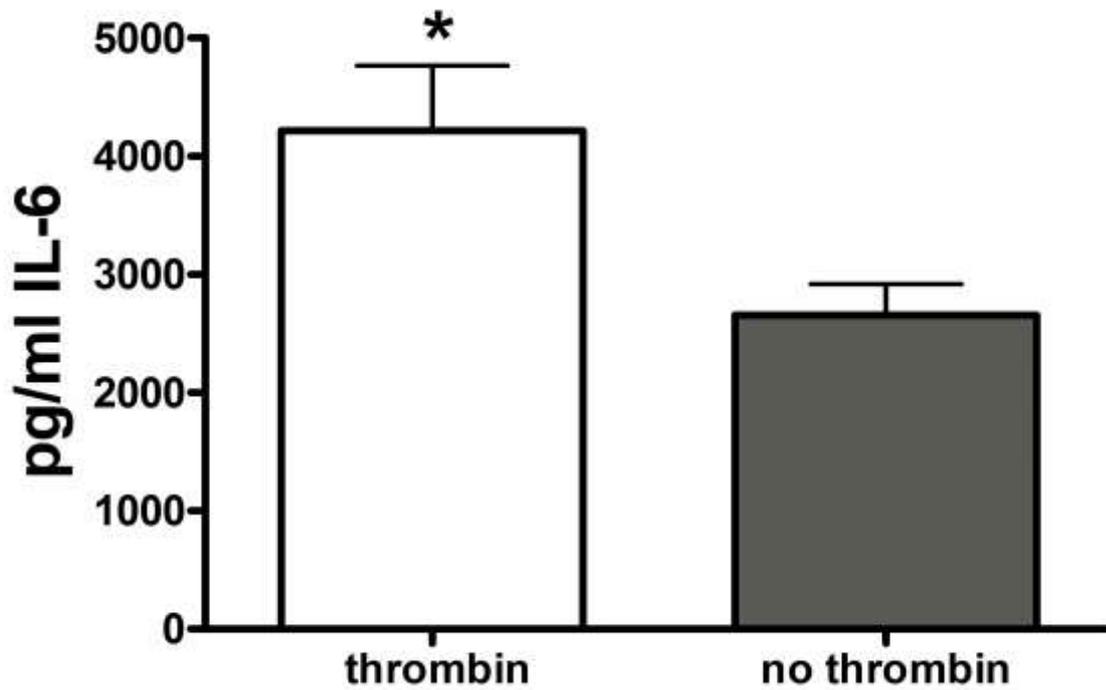
Figure 4-8: IL-6 secretion in response to thrombin treatment in human myotubes. Graph of the ELISA results of IL-6 secretion from muscle cells in response to thrombin treatment, day 7 differentiated myotubes were treated with or without thrombin (5 U/ml) for 6 hours then the media collected and IL-6 measured by ELISA. Values are expressed as mean ± SEM. n=4, *p<0.05.

4.4 Discussion

The present study provides new insights into the mechanisms of the effects of thrombin on insulin signalling and glucose uptake in human skeletal muscle cells. These data showed that thrombin, despite interference with the proximal insulin signalling pathway, does not downregulate insulin-induced glucose transport, and even exerts a positive effect by enhancing basal glucose transport. In addition, it was shown that thrombin directly increased glucose uptake with evidence that this is facilitated through AMPK activation.

Glucose is transported into the cell via a family of specialized transporter proteins (GLUTs). Different isoforms of GLUT have been identified in human skeletal muscle; GLUT1 and GLUT4 are the major contributors to glucose transport in basal and stimulated states, respectively. In this study, thrombin was recognised to enhance gene expression of both GLUT1 and GLUT4, which would be predicted to contribute to the observed increase in both basal and insulin mediated glucose uptake. GLUT4 translocation to the cell membrane is the critical step of stimulated glucose uptake, therefore measurement of the GLUT4 translocation or protein expression of the membrane fraction is considered more important than gene expression. However, GLUT4 protein is particularly difficult to detect in cultured primary human skeletal muscle cell cultures and so investigating such measures would be highly unfeasible in such a cell system. These results suggest that thrombin stimulates basal glucose transport by upregulation of GLUT1 gene expression in human skeletal muscle cells. Many metabolic effects of thrombin are in common with other proinflammatory cytokines used in other studies such as that observed by Storz *et al.* (1999) who used TNF and found that the increase in basal glucose uptake is associated with increased protein expression of GLUT1 in skeletal muscle cells.

It is interesting to note that thrombin had inhibitory effects on insulin signalling as represented by decreased activation of both IRS1 and Akt phosphorylation. What is surprising is that insulin stimulated glucose uptake tended to increase in the presence of thrombin however, this was not statistically significant.

Akt exists at a central hub in the regulation of insulin stimulated glucose uptake but a substantial evidence also indicates aPKC acts downstream of PDK1 and

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displays a role in insulin signalling to GLUT4 translocation (Farese *et al.*, 2005; Farese *et al.*, 2007). Additionally, other studies state that aPKC can be activated in response to muscle contraction (Chen *et al.*, 2002; Santos *et al.*, 2014). Further downward dissection of the signalling pathway towards increased insulin stimulated glucose uptake is also required and represented by the direct target of Akt, AS160. Therefore experiments were designed to examine the effect of thrombin on insulin stimulated phosphorylation of both aPKC and AS160. In the current study, no inhibitory effect of thrombin treatment was found with either signalling molecule. These findings would suggest that insulin action is not inhibited in the presence of thrombin through the activation of both AS160 and aPKC that effectively bypasses the inhibitory effect of thrombin on IRS1 and Akt.

Previous studies in many cell lines report that AMPK activation is involved in mediating the function of thrombin. In this study, it was demonstrated that thrombin markedly induced the phosphorylation of AMPK α-subunit (Thr^{172}). AMPK is a serine threonine heterotrimeric complex that consists of three subunits, α, β and γ, and is a kinase that acts as a metabolic sensor and a key regulator of cellular energy homeostasis in skeletal muscle and other cells (Hardie *et al.*, 2006). AMPK is activated upon any condition that causes the cell to be in a state of decreased ATP either from decreased energy production or from an increased consumption. Activated AMPK causes switching off of the metabolic processes that cause increased energy consumption (protein synthesis, lipid synthesis and glycogen synthesis) and simultaneously, switching on the processes that increase energy production including glucose uptake (Hardie *et al.*, 2012).

The increased AMPK activation upon thrombin treatment in human skeletal muscle cells is consistent with results obtained from studying different cell lines. In vascular endothelium, thrombin directly increases $AMPK^{Thr172}$ phosphorylation and activation with an increase in AMPK downstream effects (Thors *et al.*, 2004). In platelets, thrombin is a potent platelet activator and plays a central role in platelet aggregation and clot retraction (Lundblad and White, 2005), and this activation is recognized to be AMPK-mediated (Randriamboavonjy *et al.*, 2010; Onselaer *et al.*, 2014). In bone forming cells (osteoblasts), chemical inhibition and siRNA AMPK

knockdown cause a remarkable reduction in AMPK mediated action (Tokuda *et al.*, 2012).

The increase in AMPK activity in response to thrombin treatment was attenuated with the use of an inhibitor of AMPK (compound C). Although compound C is likely to inhibit other kinases, in this study it was used at a concentration to ensure maximal effects on AMPK action (Bain *et al.*, 2007). The usage of compound C as an inhibitor of AMPK activation in human skeletal myotubes in this study, is consistent with data obtained from other studies (Thors *et al.*, 2004; Nieto-Vazquez *et al.*, 2008; Benziane *et al.*, 2012). The observed decrease in AMPK activity by using compound C was associated with a decrease in thrombin-mediated basal glucose uptake.

Previous studies (Kramer *et al.*, 2006a; Kramer *et al.*, 2006b) have suggested that AS160 activation is not restricted to the insulin-Akt pathway but can also be activated by the AMPK pathway. It is interesting to note that thrombin causes an increase in AS160 phosphorylation at Thr^{642} in the basal state which is in line with the increase in basal glucose uptake after thrombin treatment.

The role of thrombin in inducing inflammation has been investigated. Thrombin can activate cellular signalling in many cell types and can stimulate the production of different pro-inflammatory mediators such as cytokines, chemokines and growth factors (Ma and Dorling, 2012). Thrombin has been shown to increase IL-6 production in many tissues including fibroblasts (Chiu *et al.*, 2008), adipocytes (Mihara *et al.*, 2010), and bone cells (Tokuda *et al.*, 2012). In reviewing the literature, no data was found showing the effects of thrombin treatment on skeletal muscle IL-6 release. The current study found that thrombin increased IL-6 secretion compared to non-thrombin treated cells. A further study with more focus on whether AMPK is involved in IL-6 secretion is therefore suggested.

In conclusion, very little is known about the metabolic effects of hypercoagulation (increased thrombin) on insulin signalling and glucose metabolism in human skeletal muscle. This is the first study to look at thrombin and insulin action/signalling in human skeletal muscle. Thrombin was shown to have multiple metabolic effects represented by a decrease in insulin stimulated IRS1 and Akt activation which was mediated through PKCα, but thrombin had no effect on the

parallel insulin-stimulated aPKC and AS160 pathway. Thrombin directly increased glucose uptake through an AMPK mediated mechanism.

CHAPTER 5 : EFFECTS OF THROMBIN AND EXERCISE ON HUMAN CULTURED SKELETAL MUSCLE MYOTUBES.

5.1 Introduction

Exercise and regular physical activity have favourable health benefits, and it is reported that regular exercise has an essential role in the prevention and treatment of metabolic disease, cardiovascular disease and mental health issues (Pedersen and Saltin, 2015). Exercise has both local and systemic benefits, the local benefits represented by skeletal muscle growth and positive metabolic adaptations whereas the systemic outcomes appear as improvements in cardiovascular function and decreased fat mass (Neufer *et al.*, 2015). In an insulin resistant state such as T2D, exercise improves whole body glucose tolerance and insulin sensitivity (Rogers *et al.*, 1988; Dela *et al.*, 1995). In skeletal muscle cells, a single bout of exercise increases glucose uptake (Richter *et al.*, 1989), and improves insulin sensitivity in the post-exercise period (Henriksen, 2002).

For the last few decades, different *in vitro* models of exercise have been used to study the exact molecular mechanisms and the cellular responses as well as the therapeutic implications of the exercise. Details about the use of EPS models and implications were discussed previously in general introduction in section 1.22.3.

As mentioned earlier in general introduction section 1.22.1, muscle contraction stimulates glucose uptake by increase GLUT4 translocation into the cell surface. The particular mechanisms that involve the increase in GLUT4 expression at the cell membrane in response to contraction are not fully clarified but it is generally believed that AMPK is involved. However, some studies on AMPK knockdown mice show a normal increase in GLUT4 expression level at cells membrane in response to contraction (Maarbjerg *et al.*, 2009).

Nedachi *et al.* (2008) subjected C2C12 myotubes to EPS and showed an increase in activation of AMPK and increased glucose transport. Human myotubes in culture have also been shown to respond to EPS in a metabolically similar way to that of skeletal muscle exercise *in vivo*. Examples of these metabolic adaptations include; activation of AMPK and improvement in glucose uptake and oxidation (Lambernd *et al.*, 2012; Nikolić *et al.*, 2012; Christensen et al., 2015; Evers-van

Gogh et al., 2015). Even for short-duration, the low frequency model of EPS increased AMPK activation compared to resting muscles (Goto-Inoue *et al.,* 2016; Miyatake *et al.*, 2016).

Skeletal muscle myotubes become metabolically active during exercise. Variable metabolic activities have been reported in human myotubes in response to EPS Contraction-stimulated glucose uptake was markedly elevated in response to EPS compared to resting cells in cultured human myotubes (Nikolić *et al.*, 2012; Brown *et al.*, 2015a). Furthermore, glycogen metabolism in cultured myotubes is affected by EPS. Glycogen content in response to EPS decreased in myotubes (Manabe *et al.*, 2012; Farmawati *et al.*, 2013) and this observation is also described in *in vivo* exercise (Jensen and Richter, 2012). Feng *et al.* (2015) reported that insulin-stimulated glycogen synthesis in human myotubes was increased after EPS. Glucose oxidation is another metabolic characteristic that is shown to increase in response to EPS in human myotubes (Nikolić *et al.*, 2012; Feng *et al.*, 2015).

Currently, it is accepted that skeletal muscle is an endocrine organ that releases a variety of myokines. Muscle contraction during exercise is considered a key stimulus for both expression and release of myokines such as IL-6 (Steensberg *et al.*, 2000) and IL-8 (Steensberg *et al.*, 2007). The use of EPS models as an *in vitro* exercise protocol provides invaluable evidence that skeletal muscle cells per se are the definite source of specific myokines. Lambernd *et al.* (2012) showed that IL-6 secretion is significantly increased in response to EPS in cultured human muscle cells. Furthermore, Scheler *et al.* (2013) highlight the significance of skeletal muscle cells as an endocrine organ and showed that both IL-8 and IL-6 are significantly increased in response to EPS both at gene and protein levels in cultured human muscle cells.

Insulin and exercise are the most important metabolic stimuli in skeletal muscle cells. Treatment with thrombin was shown to cause multiple metabolic effects on insulin signalling and insulin induced glucose uptake in cultured human myotubes. Therefore, this study set out to investigate the effects of elevated thrombin and EPS on human cultured myotubes, exploring the effects of thrombin on the release of inflammatory cytokines (IL-6 and IL-8) in response to EPS.

Our hypothesis was that, hypercoagulation that is associated with T2D affects in the metabolic response to exercise in skeletal muscle cells.

Aims of this chapter:

1- Exploring the effects of thrombin on exercise signalling represented by AMPK^{Thr172} in response to EPS.

2- Examining the effects of thrombin and/or EPS on insulin stimulated glucose uptake.

3- Examining the effects of thrombin and/or EPS on cytokines release from cultured skeletal muscle cells

5.2 Methods

5.2.1 Experimental design

All experiments were performed on passage 6 myotubes on day seven of differentiation. Media was changed to serum reduced media (minimal essential media supplemented with 0.25 % (v/v) FBS), and the cells either treated with thrombin (5U/ml for 6 hours) according to previous optimisation of the dose and duration as discussed in sections 3.2.4, 3.2.5 and 3.2.6. After the 6 hour thrombin treatment, media was changed to fresh starvation media and 16 hours of EPS applied. For glucose uptake experiments, insulin was added at a concentration of 100 nM for the last 10 minutes of the exercise protocol`.

5.2.2 In vitro model of exercise (EPS)

Electrical pulse stimulation (EPS) was performed using a C-Pace EP cell culture pacer (IonOptix, Dublin) using a two-step protocol (alternation between a period of high frequency and low frequency electrical pulses). Cells were plated in 35mm dishes, grown to confluence and differentiated for 7 days. At day 7 differentiation, cells were treated as described above before subjecting to EPS. Electrical pulse stimulation (EPS) was applied via carbon electrodes at 5 volts, 24 milliseconds, 2 Hz for 1 hour followed immediately by 5 volts, 24 milliseconds, 0.2 Hz for 1 hour. This alternation in frequency was continued for 16 hours (period of exercise), the 16 hours EPS period has chosen based on previous work in our lab on control cultured myotubes showing a maximal AMPK activation after 16 hours EPS (Brown *et al.*, 2015a). The following Figure 5-1 shows the photographic picture of the C-Pace EP cell culture pacer that used in this experiment.

Figure 5-1: IonOptix C-PACE electrical stimulator (An *in vitro* model of exercise). (A): is an image of a culture dish electrode used for EPS which is suitable to hold 6 individual 35 mm dishes. (B): the C-Pace EP a multi-channel stimulator used for EPS of cells in culture. Images captured from (IonOptix, 2011).

5.2.3 Glucose uptake

Seven days differentiated myotubes in 35 mm plates were set into Control and thrombin treated cells both were divided into two groups; with or without 16 hours EPS at 37 °C in serum reduced media. Subsequently, 2-deoxy-D-[2, 6-3H] glucose uptake was measured as described previously in general Materials and Methods chapter, section 2.9.

5.2.4 Western blot

After 16 hours incubation with or without EPS, western blot was performed as described previously in general Materials and Methods chapter, section 2.8. The non-specific sites on the nitrocellulose membranes were blocked by incubation in Tris buffered saline tween (TBST) containing 5% (w/v) milk as a blocking buffer for 1 hour. Membranes were incubated with monoclonal primary antibody (1°Ab) diluted in 0.5% blocking buffer, overnight at 4 °C. Phospho AMPK antibodies were used at a 1:1000 dilution while native AMPK was 1:2000.

5.2.5 Measurement of lactate dehydrogenase (LDH) release

Lactate dehydrogenase levels were measured in the media from cells with or without thrombin and subjected to -/+ 16 hours exercise (EPS) and -/+ compound C using the colorimetric Lactate Dehydrogenase kit (PromoKine). Cells were incubated in serum reduced media then treated with or without thrombin for 6 hours and with or without 40 µM compound C that was added for the last 30 minutes of thrombin treatment. Media was then changed to fresh media to start the exercise protocol. After 16 hours of EPS, media was collected and LDH release into the media was determined colorimetrically at 450 nm according to the manufacturer's instructions. LDH release was measured as described in general Materials and Methods chapter, section 2.12.

5.2.6 ELISA

Secretion of IL-6 and IL-8 was determined by enzyme-linked immunosorbent assay (ELISA) using the Single-Analyte ELISArray (Qiagen). After 7 days of differentiation, media was changed to serum-reduced MEM, and cells were treated or untreated with 5U/ml thrombin for 6 hours after which fresh media was put on cells. Cells were then either subjected to EPS or kept in the incubator for 16 hours.

After EPS, media was removed, centrifuged at 1000g for 10 minutes and assayed for secretion of IL-6 and IL-8 according to the manufacturer's protocol that described in general Materials and Methods chapter, section 2.11.

5.3 Results

5.3.1 AMPK activation in response to 16 hours EPS

AMP-activated protein kinase (AMPK) is key sensor of energy status in skeletal muscle and is activated during exercise. AMPK activation was measured by Western blot using phospho-specific antibodies against Thr^{172}, phosphorylation of which is essential for the full activity of the kinase (Scott *et al.*, 2002). Figure 5-2 shows that, in non-thrombin treated cells, phosphorylation was increased significantly after 16 hours EPS ($p < 0.01$). In contrast, thrombin treated myotubes subjected to the same EPS protocol showed no activation of AMPK over its basal activity. Thrombin was shown to significantly increase basal AMPK activation compared to control non-thrombin treated myotubes ($p < 0.001$). However, there was no further activation of the thrombin treated cells in response to EPS, however, AMPK activation in these cells was still significantly higher than resting control muscle ($p < 0.001$).

(A)

| 16 hours EPS | - | + | - | + |
| Thrombin | - | - | + | + |

(B)
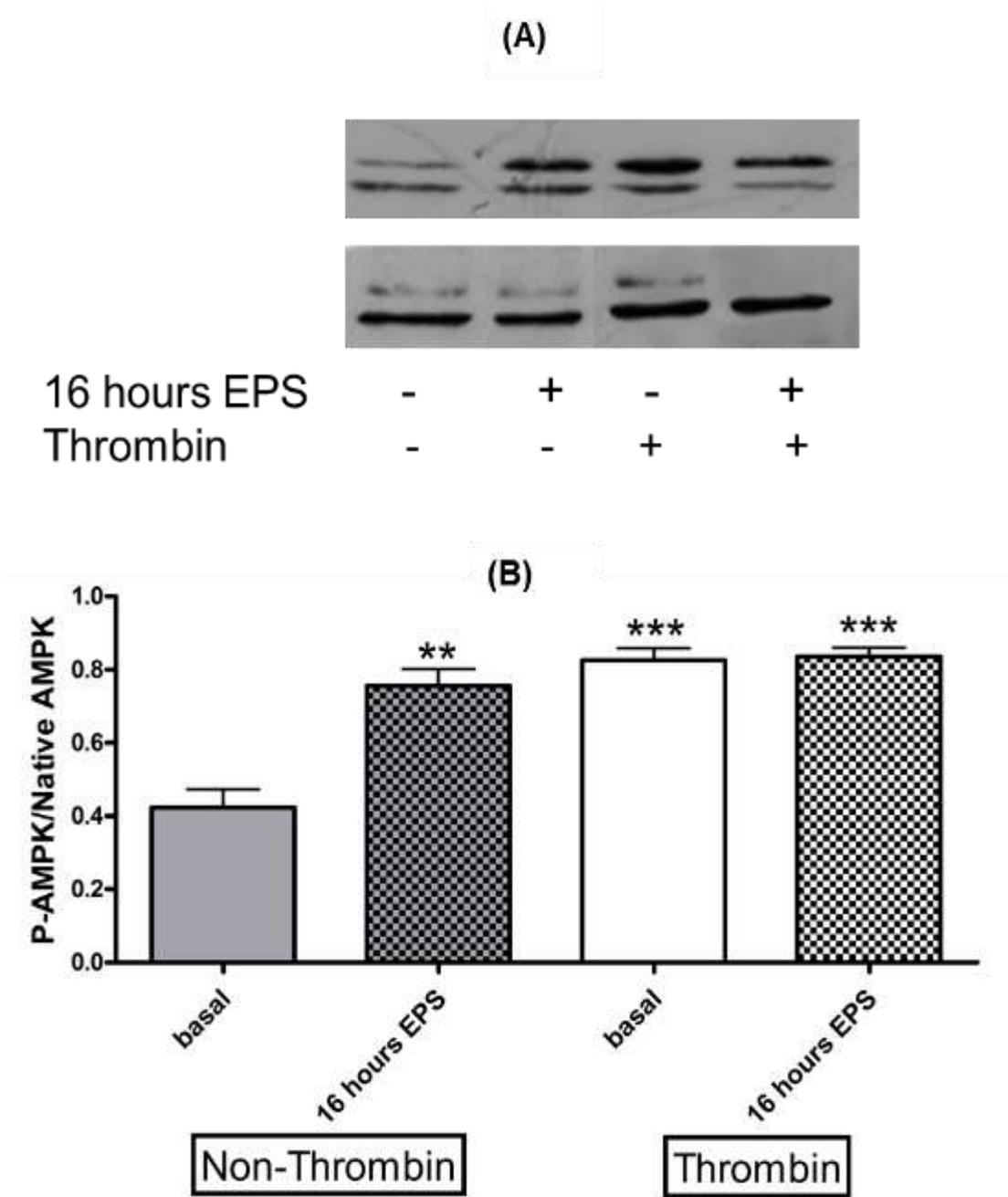
Figure 5-2: Effects of EPS and thrombin on AMPK activation. (A) Shows the blots of AMPK activation at Thr172 for basal and 16 hours EPS with and without thrombin, cells were treated with thrombin (5 U/ml) for 6 hours then subjected to EPS or kept in incubator. (B) The results of densitometry analysis of P-AMPK/total AMPK for both thrombin and non-thrombin treated cells with or without exercise (16 hours EPS). Values are expressed as mean ± SEM. n = 4, **p< 0.01, ***p< 0.001 comparing to the basal untreated myotubes.

5.3.2 Glucose uptake

To examine the metabolic effects of the *in vitro* exercise protocol (16 hours EPS), radiolabelled glucose uptake was measured in both thrombin and non-thrombin treated myotubes.

As shown in Figure 4-5 (A), in non-thrombin treated cells, insulin increased glucose uptake significantly ($p < 0.05$) over basal. Furthermore, 16 hours EPS also caused a significant increase in glucose uptake over basal ($p < 0.05$). Additionally, an additive effect was observed when cells were stimulated with both insulin and 16 hours EPS with a significant increase observed over both basal ($p < 0.001$) and insulin alone ($p < 0.05$).

In thrombin treated myotubes, there was an increase in basal glucose uptake compared to non-thrombin treated myotubes ($p < 0.01$). In addition, as we showed previously in chapter 3, insulin-stimulated glucose uptake did not increase over basal in thrombin-treated cells. In addition, 16 hours EPS failed to stimulate an increase in glucose uptake over basal to a significant level as observed in non-thrombin treated cells. Thrombin treatment caused myotubes to lose the additive effects of insulin and 16 hours EPS observed in non-thrombin treated cells. There were no statistical differences comparing absolute levels of glucose uptake in response to insulin, 16 hours EPS and insulin plus 16 hours EPS between thrombin and non-thrombin treated myotubes.

By calculating fold change (glucose uptake in response to stimulus over basal) Figure 5-3 (B) shows that there was a significant difference in fold change with insulin and EPS together in thrombin treated versus non-treated cells ($p < 0.05$). No such differences were observed in fold change for insulin or EPS alone.

(A)

(B)
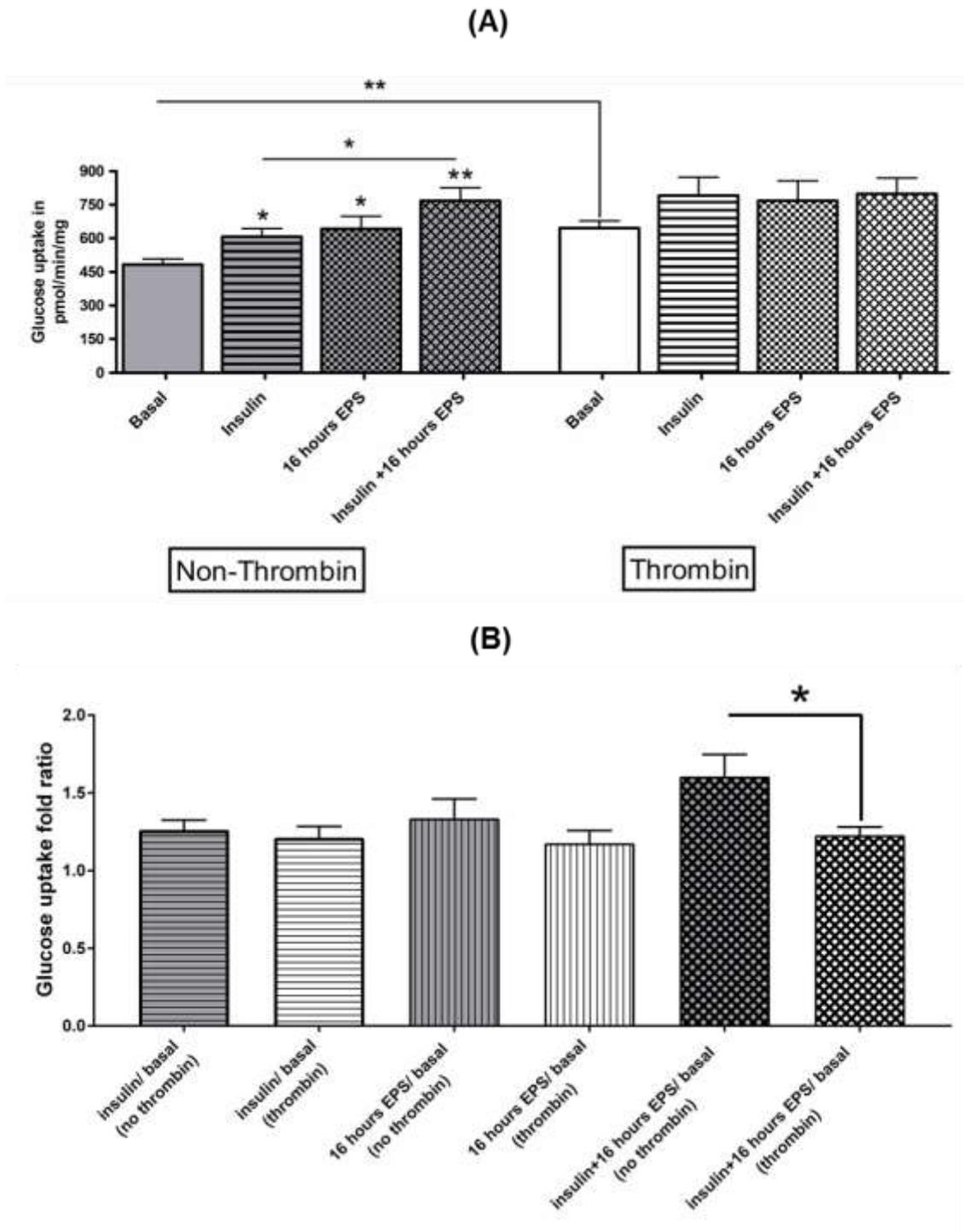
Figure 5-3: Effects of EPS and thrombin on glucose uptake in myotubes. Both thrombin treated and control (non-thrombin treated) with or without 16 h EPS and with or without 100 nM insulin for 10 min. (A) Graph shows the absolute glucose uptake values in non-thrombin and thrombin treated myotubes in basal, insulin, 16 hours EPS and insulin plus EPS. (B) Graph representative of the fold ratio in glucose uptake with stimulus over the basal in both control and thrombin treated myotubes. Values are expressed as the mean ± SEM, n = 5, *p< 0.05, **p< 0.01.

5.3.3 Lactate dehydrogenase (LDH) release

As described in Chapter 4, section 4.3.6, thrombin was shown to increase both AMPK and basal glucose uptake, while compound C partially reversed these effects. For the current experiment, the design was similar to that described previously 4.2.1. Myotubes were treated with thrombin (5 U/ml for 6 hours) and for the last 30 minutes, 40µM compound C was added. Fresh starvation media was added to cells before 16 hours EPS. The results of 3 successive experiments showed cell death in dishes treated with combination of thrombin and compound C as noted morphologically (under microscopic examination) and by examining LDH release as an indicator of cytotoxicity. LDH assay results showed that the combination of thrombin and compound C for the given time followed by EPS caused cell death as shown in Figure 5-4 (A). There was a pronounced difference between cells treated with a combination of thrombin and compound C together compared to that with or without thrombin whether they were subjected to EPS or not ($p < 0.05$). Morphological changes were noted in cells treated with a combination of thrombin and compound C, cells looked unhealthy and adopted rounded shapes and eventually become suspended in the culture media, whereas, myotubes without compound C treatment remained healthy looking and attached to the bottom of the plates with an elongated spindle shape (Figure 5-4 B).

(A)

No thrombin | Thrombin | Thrombin + Cc

(B)

No thrombin + EPS | Thrombin + EPS | Thrombin + Cc + EPS
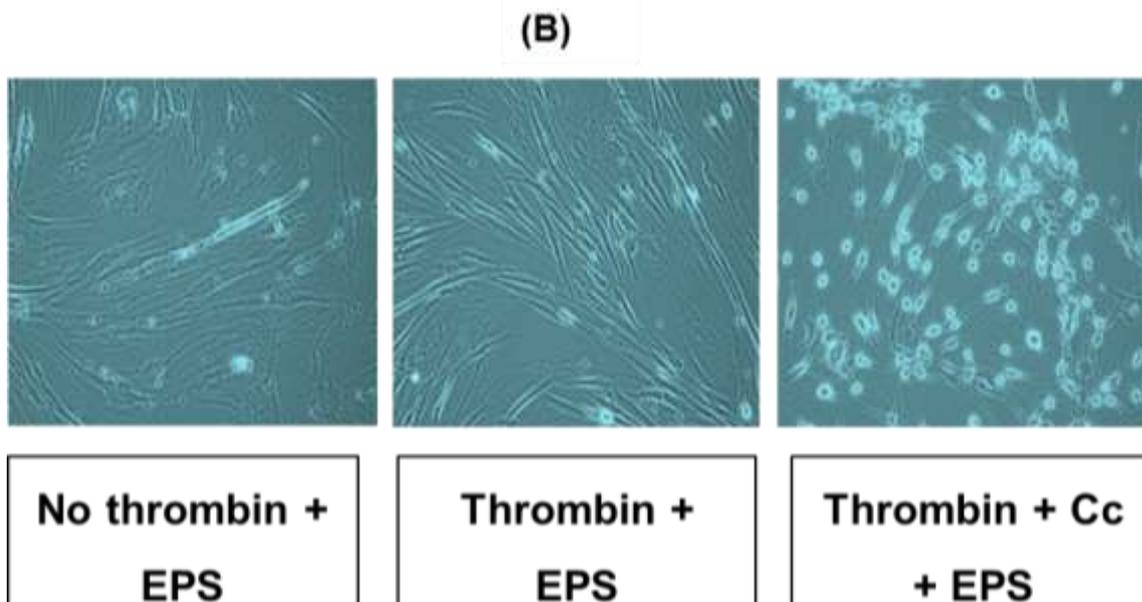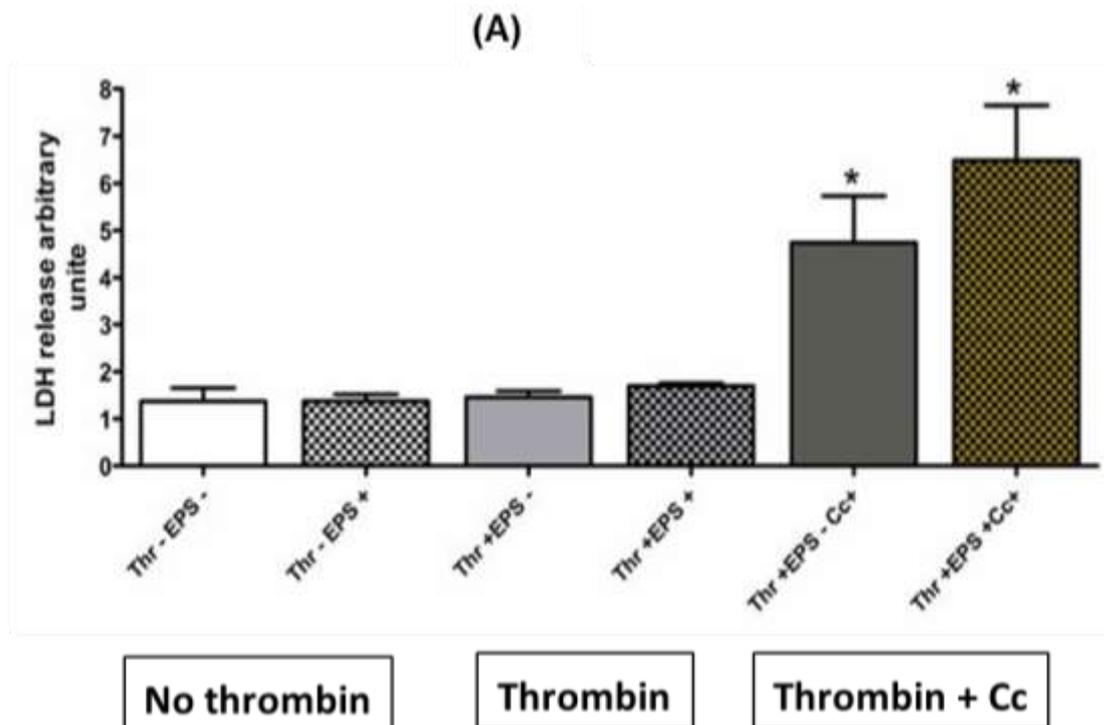
Figure 5-4: LDH cytotoxicity release in response to EPS and compound C treatment in cultured myotubes. LDH release from the cells into the media was used as a sign of cytotoxicity, cells were either control (non-thrombin treated) or treated with thrombin for 6 hrs and for the last 30 minutes, 40µM compound c was added then media changed to fresh starvation media before 16 hours EPS. (A) Graph of LDH release in arbitrary units with different treatments. Values are expressed as the mean ± SEM, n = 3, *p< 0.05. (B) Representative light microscope images of myotubes cultures after 16 hours EPS without or with thrombin ± compound C.

5.3.4 Cytokine release

Skeletal muscle produces cytokines in response to contraction. The effect of EPS on IL-6 and IL-8 secretion is shown in Figure 5-5 and Figure 5-6. Basal IL-6 release was significantly higher in thrombin-treated compared with non-thrombin treated cells ($p < 0.05$). EPS increased IL-6 secretion in non-thrombin treated cells. Thrombin was shown to inhibit further increase of IL-6 secretion in response to EPS over basal levels. IL-8 showed a similar pattern to that noted for IL-6, when addition of thrombin caused a significant increase in basal IL-8 secretion into the media compared to non-thrombin treated cells ($p < 0.05$). 16 hours EPS caused a significant increase in IL-8 secretion into the media only in control non-thrombin treated cells ($p < 0.05$), while no further increase in cytokine release in thrombin-treated cells subjected to EPS was observed.

(A)

standard curve

(B)
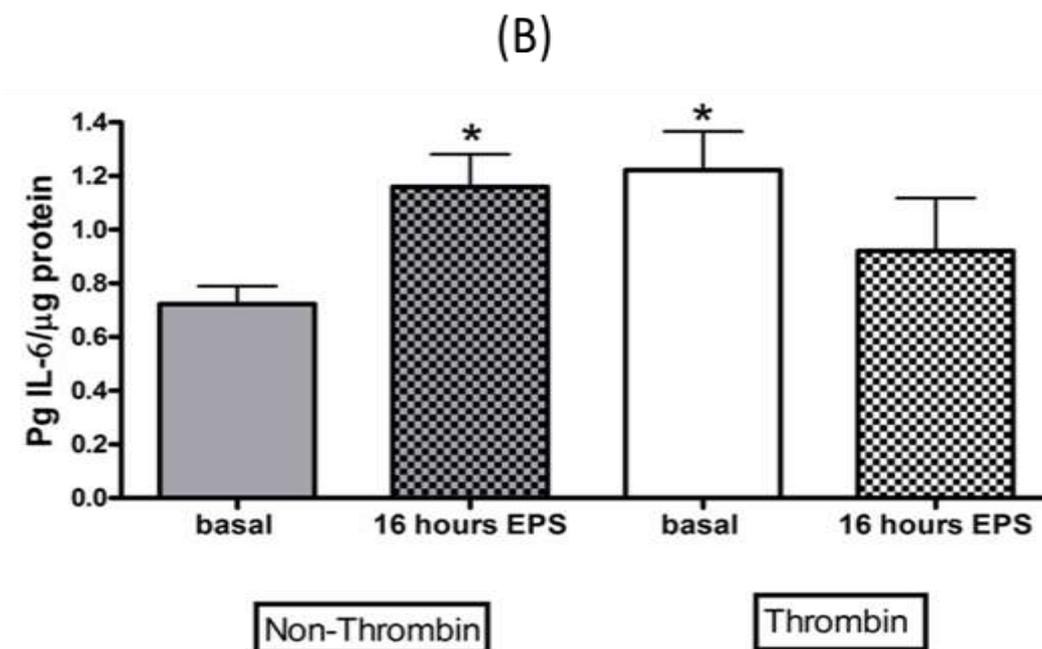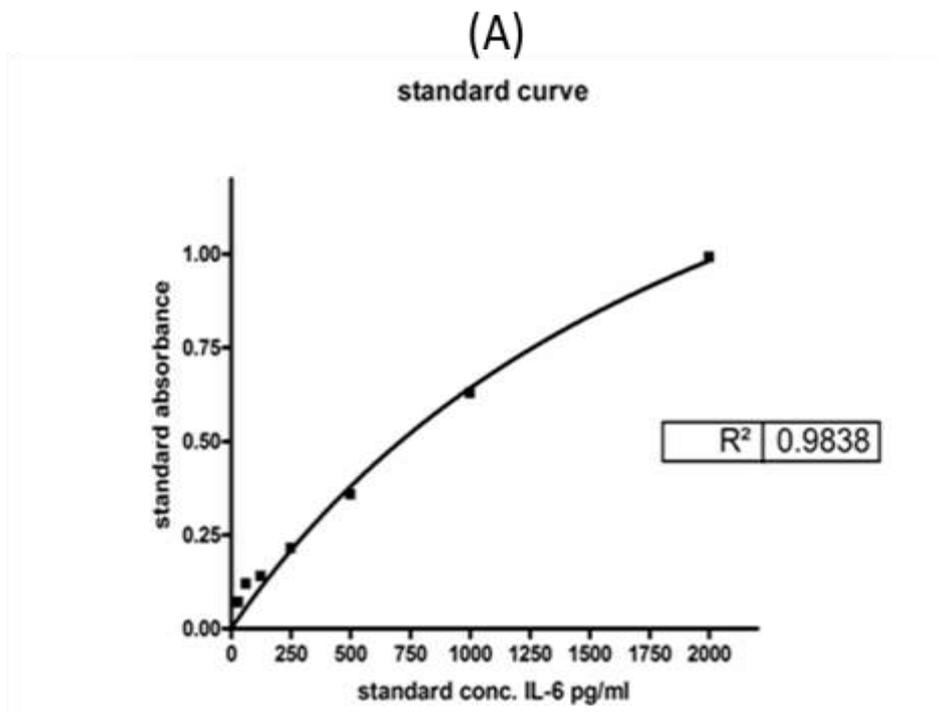
Figure 5-5: IL-6 secretion in response to EPS and thrombin cultured myotubes.Fresh media was added to 4 controls and 4 thrombin treated cultures and subjected to EPS for 16 hours. Media was collected and assayed for IL-6 secretion by ELISA. (A) Represents a standard curve of IL-6 prepared from provided standard IL-6 which was diluted by serial dilution. (B) Normalised IL-6 secretion to total protein content of the cells. Results are expressed as mean ± SEM. n=4, * p<0.05.
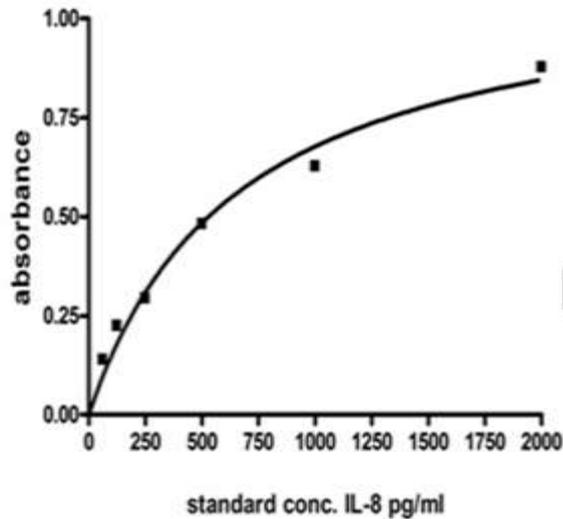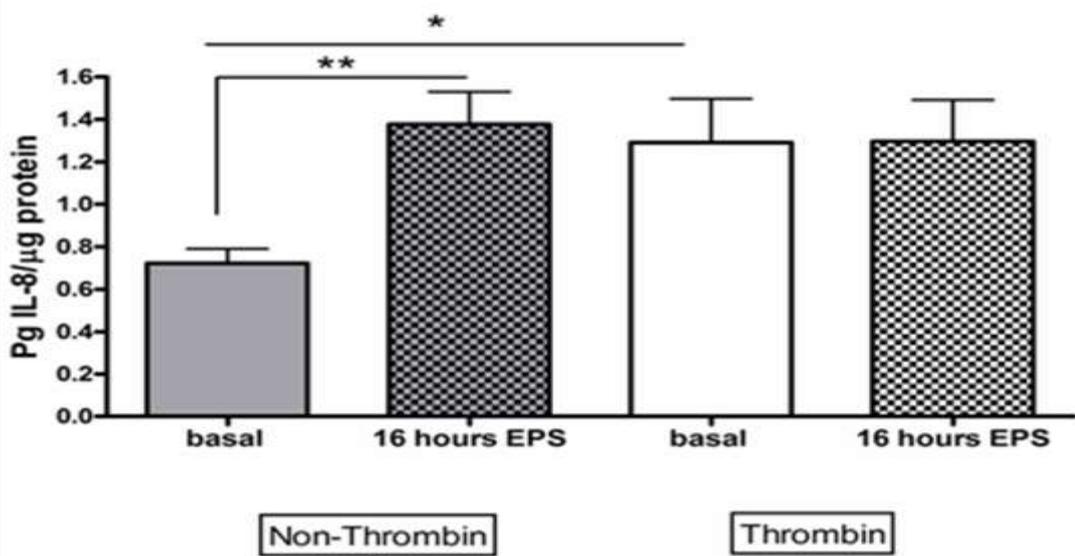
Figure 5-6: IL-8 secretion in response to EPS and thrombin cultured myotubes. Fresh media was added to 4 controls and 4 thrombin treated cultures and subjected to EPS for 16 hours. Media was collected and assayed for IL-8 secretion by ELISA. (A) Represents a standard curve of IL-8 prepared from provided standard IL-8 which was diluted by serial dilution. (B) Normalised IL-8 secretion to total protein content of the cells. Results are expressed as mean ± SEM, n=4, *p<0.05, **p<0.01.

5.4 Discussion

Thrombin is a serine protease enzyme that carries out a variety of biological processes, as well as its role in coagulation, such as cell division, cancer growth, and inflammation. T2D is regarded as a hypercoagulable state. An elevated thrombin level has been recognised in association with metabolic abnormalities of T2D like hyperglycaemia and hyperinsulinemia (Carr, 2001). In chapters 3 and 4, thrombin was shown to have effects on the insulin signalling pathway and glucose uptake in cultured human myotubes. The aims of this chapter were to assess the effects of thrombin on exercise related metabolic and signalling events. The most obvious findings to emerge from the study are that AMPK activation and glucose uptake increase in response to EPS in non-thrombin treated myotubes, and EPS enhanced the effect of insulin on glucose uptake. Treatment with thrombin increased basal AMPK activation, glucose uptake and cytokine release, and these effects were not significantly increased by EPS.

EPS is an *in vitro* model system for exercising cultured muscle cells. The use of EPS gave the great opportunity to explore the intrinsic mechanisms of exercise in skeletal muscle cells, while excluding the systemic and serum factors that might have an impact on metabolism in response to physical activity. The low frequency-long duration EPS used in this study is similar to that used in many other studies (Lambernd *et al.,* 2012; Nikolić *et al.,* 2012; Brown *et al.,* 2015a) as *in vitro* model that can mimic some of the effects of exercise seen in whole body studies.

AMPK is activated in response to contraction in skeletal muscle and mediates insulin independent glucose uptake (Mu *et al.*, 2001). In the current study, stimulation of cultured human control myotubes by EPS leads to an increase in AMPK^{Thr172} phosphorylation. These results are consistent with data obtained from other studies using the EPS system in both mouse C2C12 myotubes (Nedachi *et al.*, 2008; Manabe *et al.*, 2012) and human cells (Lambernd *et al.*, 2012; Brown *et al.*, 2015b; Christensen *et al.*, 2015). In thrombin treated cells, thrombin was shown to significantly increase basal AMPK activation, with no further activation after EPS. The possible explanations for this might be that AMPK activation with thrombin reached maximal activation and cannot rise beyond this point. Supporting this opinion is that AMPK phosphorylation with thrombin under basal

conditions was comparable to that seen in EPS subjected cells in the absence of thrombin (Figure 5-2). The other possible explanation is that, thrombin inhibits further activation of AMPK above basal activity.

The early metabolic response of skeletal muscle cells to exercise is to increase glucose uptake. Previous studies on cultured muscle cells have shown that EPS boosts glucose uptake (Nikolić *et al.*, 2012; Brown *et al.*, 2015a). The results of this study in control non-thrombin treated cells are consistent with these previous reports, and the increase in glucose uptake in response to EPS is similar to that observed in response to *in vivo* exercise. EPS failed to stimulate glucose uptake to a significant level above basal in thrombin treated cells. It is interesting to note that basal glucose uptake in response to thrombin treatment was significantly higher than non-thrombin treated cells. It could be argued that the positive effect of EPS on glucose uptake is lost upon thrombin treatment, although no significant statistical differences in the response to EPS between thrombin treated and non-treated cells was observed. These results are consistent with the AMPK data that also fails to increase with EPS in thrombin-treated cells.

Exercise and insulin increase glucose uptake in skeletal muscle using two independent pathways. Both a single bout of exercise and exercise training are reported to enhance insulin action in human skeletal muscle (Howlett *et al.*, 2002; Hawley and Lessard, 2008). In line with these observations, glucose uptake in non-thrombin treated myotubes increased in response to insulin or EPS and there was an additive effect of insulin plus EPS over insulin alone. These results are in agreement with those obtained by Brown *et al.* (2015a).

In cells treated with thrombin, the ability of insulin or EPS to increase glucose uptake was lost. The insulin sensitising effect of EPS on glucose uptake was also absent. Thrombin appears to impair the beneficial effect of EPS (increasing glucose uptake) in skeletal muscle cells.

Results in chapter 4 showed that compound C AMPK inhibitor can reverse the stimulatory effect of thrombin on AMPK activation and could also partially reduce the thrombin-mediated increase in basal glucose uptake. Studies in this chapter suggested that thrombin inhibited the effects of EPS in cultured skeletal muscle cells. Therefore, myotubes were treated with compound C to assess if AMPK

inhibition could reverse the thrombin inhibitory effects in response to EPS. The combination of thrombin and compound C treatment together with EPS was detrimental to cell health and caused cell death as confirmed by LDH enzyme release and microscopically.

It is not surprising to know that compound C is cytotoxic and has been used in cancer research to inhibit cancer growth and induce cell death in different tumor cells (Jin *et al.*, 2009; Yang *et al.*, 2012). The inhibitory effects of compound C in chapter 4 without any signs of cell death could be due to the short duration of compound C application (30 minutes) followed by immediate assay. However, the use of compound C in this study was involved keeping cells for another 16 hours for EPS.

Exercise is a positive regulator for many metabolic parameters, and currently, the role of skeletal muscle contraction in elevated serum cytokines during exercise is accepted. Skeletal muscle cells express and secrete different proteins (cytokines) during and shortly after muscle contraction which are known as myokines (Febbraio and Pedersen, 2005). Some of these cytokines could be involved as essential factors for mediating the health promoting effects of exercise (Pedersen and Febbraio, 2012).

IL-6 and IL-8 secretion were chosen to be investigated in response to *in vitro* exercise for many reasons such as; both cytokines are shown to increase in response to exercise (Peake *et al.*, 2015). The Gene sets of these proinflammatory cytokines are shown to be upregulated in diabetic myotubes compared to control (Brown *et al.*, 2015b). Finally, both cytokines are involved in thrombin effects on different cell lines.

The current study found that both IL-6 and IL-8 release from non-thrombin cultured cells are increased in response to EPS. These results are in accord with studies indicating that *in vitro* exercise causes an increase in IL-6 and IL-8 secretion from skeletal muscle (Lambernd *et al.*, 2012; Scheler *et al.*, 2013).

Cytokine release in response to thrombin treatment is disturbed. Thrombin increases the release of IL-6 and IL-8 significantly, and the increase in cytokine release after EPS that is noted with non-thrombin treated cells is lost.

The increase of cytokine release with thrombin has been reported before with other cell types but, this could be the first study highlighting the increase in cytokine release after thrombin treatment in skeletal muscle cells. Thrombin has been shown to stimulate IL-6 production in different cell types including fibroblasts (Chiu *et al.*, 2008) and adipocytes (Mihara *et al.*, 2010). Additionally, IL-8 production has been shown to rise in response to thrombin in human lung epithelial cells (Lin *et al.*, 2015) and cultured human fetal astrocytes (Simmons *et al.*, 2013).

In conclusion, this is the first study looking at the effects of thrombin in cultured human skeletal muscle subjected to an *in vitro* exercise protocol. The metabolic changes with EPS were no longer apparent in the presence of thrombin. The increase in AMPK activity, elevation of glucose uptake and the rise in cytokine release (above basal values) in response to EPS that were noted with non-thrombin treated muscle cells are lost upon thrombin treatment. Additionally, myotubes respond to thrombin by increasing basal activity of multiple metabolic, signalling and inflammatory pathways. Thus, hypercoagulation associated with diabetes could be involved in multiple metabolic effects in skeletal muscle including insulin signalling, exercise signalling, proinflammatory pathways, and glucose uptake.

CHAPTER 6 : EFFECTS OF *IN VITRO* ELECTRICAL PULSE STIMULATION (EPS) ON CULTURED DIABETIC HUMAN MUSCLE CELLS.

6.1 Introduction

T2D is a worldwide health problem that is rapidly growing (da Rocha Fernandes *et al.*, 2016). Resistance to insulin action in addition to reduced insulin secretion are the cardinal features of T2D subjects. Skeletal muscle is the main organ that accounts for insulin resistance, as it is responsible for more than 80% of glucose disposal after meals in response to insulin secretion (DeFronzo *et al.*, 1981; Thiebaud *et al.*, 1982). The principal function of insulin in skeletal muscle of a normal glucose tolerant individual is the enhancement of glucose uptake and metabolism. In general, it is accepted that in T2D individuals, insulin loses its ability to facilitate glucose uptake into skeletal muscle *in vivo* (DeFronzo *et al.*, 1985; Hepburn *et al.*, 1994). This defect in insulin action has also been reported in cultured diabetic skeletal muscle cells (Henry *et al.*, 1995; McIntyre *et al.*, 2004; Brown *et al.*, 2015b).

The phenotypic characteristics of the native skeletal muscle fibres are retained in multinucleated myotubes in culture (van der Ven *et al.*, 1992), which attracted researchers to use muscle cell cultures from different pathological conditions as well as from healthy control subjects. These cultures are subjected to the same standardised conditions and excluding the possible interfering systemic and serum factors, the defects that emerged reflects the intrinsic phenotypic abnormalities. There is a growing body of literature comparing human myotubes from diabetic versus non-diabetic control subjects in terms of metabolic and signalling pathways (McIntyre *et al.*, 2004; Chen *et al.*, 2005; Brown *et al.*, 2015b).

High levels of regular physical activity are associated with a lower incidence of diseases such as T2D, cancer, obesity, hypertension, and depression. Physical exercise is demonstrated to reverse the metabolic abnormalities associated with insulin resistance and T2D, especially regular exercise training (Knowler *et al.*, 2002; Haskell *et al.*, 2007). In skeletal muscle, exercise corrects the metabolic defects associated with diabetes via different pathways that include AMPK

activation and increased/decreased gene expression and/or protein synthesis leading to an enhancement in insulin sensitivity (Stallknecht *et al.*, 2000; Hawley, 2002; Park *et al.*, 2002). Acute exercise and regular exercise training are regarded as a first line treatment in the prevention of obesity and/or T2D (Henriksen, 2002; Zanuso *et al.*, 2010).

For the last few decades, *in vitro* models of exercise have been performed using skeletal muscle fibres activated by nerve impulse and more recently by electrical pulse stimulation (EPS) of cultured myotubes. EPS provides researchers the opportunity to study the molecular mechanism of exercise under completely controlled conditions in both animal and human muscle cells (Fujita *et al.*, 2007; Burch *et al.*, 2010).

The use of EPS in human myotubes has been reported to increase in glucose uptake and improve metabolic activity (Nikolić *et al.*, 2012). AMPK has been reported to be activated in response to EPS in both mouse C2C12 myotubes and human myotubes (Nedachi *et al.*, 2008; Lambernd *et al.*, 2012). Feng et al. (2015) used the EPS system with diabetic myotubes to examine some metabolic process associated with insulin sensitivity like glycogen synthesis. Glycogen synthesis was shown to be unaffected by EPS in both control and T2D myotubes, whereas insulin-stimulated glycogen synthesis after EPS was increased in myotubes of T2D compared to control. Feng et al. (2015) didn't explore whether these effects involved AMPK activation. No previous studies have examined the effects of EPS on AMPK activation in cultured human diabetic myotubes.

The insulin sensitisation effect is a crucial health benefit of exercise. Exercise-mediated enhancement of glucose uptake is preserved for 2–3 hours post-exercise. Furthermore, after a single bout of exercise, enhanced insulin sensitivity at the level of both skeletal muscle and whole body is retained for up to 24-48 hours (Cartee *et al.*, 1989; Cartee and Holloszy, 1990; Arias *et al.*, 2007). In skeletal muscle cells the enhancement of insulin sensitivity can be demonstrated in two forms; the first is the preservation of the increased insulin mediated glucose uptake, and the second is the increased phosphorylation and activation of kinases that mediate insulin action after exercise. Studies on isolated muscles show that the enhancement in insulin sensitivity is independent of increased blood flow or

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other systemic influences (Gulve *et al.*, 1990; Hamada *et al.*, 2006), and the same was observed in cultured muscles subjected to EPS which show increased insulin stimulated glucose uptake (Brown *et al.*, 2015a) and increased insulin stimulated glycogen synthesis (Feng *et al.*, 2015).

The exact molecular mechanisms by which the beneficial effects of exercise are mediated in skeletal muscle are still not clearly defined. The enzyme AMPK plays an essential role in the cellular response to exercise, where AMPK is activated in the cellular state in which the cell is in need of energy as observed in exercise (decreased ATP and increased AMP/ADP) (Hardie and Sakamoto, 2006). The exact status of AMPK activity and expression in skeletal muscle of diabetic and insulin resistant subjects is still controversial. Therefore, an *in vitro* exercise protocol using human skeletal myotubes from T2D and control subjects was established in order to examine clearly the activation of AMPK, and the increase in cellular glucose uptake and cytokine levels, and most importantly the insulin sensitising effects in response to exercise. Under these highly regulated conditions the effects of other factors such as serum factors that could change in response to exercise in an *in vivo* exercise protocol could be excluded.

Our hypothesis was that, contraction mediated metabolic responses is preserved in cultured human skeletal muscle cells obtained from T2D individuals using EPS as an *in vitro* model to exercise cultured cells.

Aims of this chapter:

1- Exploring the activity of AMPK in diabetic versus control at basal and in response to EPS.

2- Examining the effects of EPS and/or insulin on glucose uptake in diabetic versus control cultured skeletal muscle cells.

3-Examining the effects of EPS and/or insulin on distal insulin signalling pathway (AS160 activation) in diabetic versus control cultured skeletal muscle cells.

4- Examining the effects of EPS on cytokines release diabetic versus control cultured skeletal muscle cells.

6.2 Materials and methods

6.2.1 Study subjects

Muscle biopsies were taken from T2D subjects with strong clinical evidence of insulin resistance. To specify, all patients were taking >100 U insulin/day and had at least one first-degree relative with T2D. After diagnosis, patients had been treated with diet and oral hypoglycaemic drug for more than 3 years before starting insulin. All enrolled patients had a body mass index (BMI) < 32 kg/m^2 to exclude the effects of marked obesity. Skeletal muscle biopsies were also taken from six, age and BMI-matched nondiabetic control subjects with no family history of T2D. Metabolic and anthropometric features of recruited subjects have been documented before (McIntyre *et al.*, 2004; Brown *et al.*, 2015b). The following Table 6-1 shows the clinical characteristics of subjects from which muscle cells were taken.

Table 6-1: Metabolic and anthropometric characteristics of subjects of study.

	T2D	Control
Age (years)	59 ± 7	59 ± 11
Sex (males/females)	5/1	3/3
Time to insulin treatment (years)	10 ± 5	
Units of insulin/day	131.2 ± 9.6	
Hb A1c, %	9.0 ± 0.5	5.2 ± 0.1 **
BMI, kg/m^2	30 ± 0.7	28.5 ± 1.0
Waist/hip ratio	1.0 ± 0.03	0.9 ± 0.02 **
Total cholesterol, mmol/l	4.8 ± 0.2	5.9 ± 0.3 **
Triglycerides (mmol/liter)	3.3 ± 0.5	1.6 ±0.4
Fasting serum insulin (mU/liter)		7.1 ± 0.6
Fasting plasma glucose (mmol/liter)		5.4 ± 0.2

Data are presented as means ± SE; T2D vs. control. T2D subjects had significantly higher Hb A1c, waist/hip ratio, and triglycerides. Controls had significantly higher total cholesterol. **P< 0.01; *P< 0.05.

6.2.2 Cell culture

Muscle biopsies were obtained from the vastus lateralis muscle of diabetic subjects by needle biopsy under local anesthesia while control muscle was obtained from the vastus lateralis muscle at the time of hip surgery under general anesthesia and the cells isolated and purified as mentioned in sections 2.5 and 2.5. All experiments were performed on cells at passages 6 and 7 on day 7 differentiated myotubes.

6.2.3 In vitro model of exercise (EPS)

Electrical pulse stimulation (EPS) was performed using a C-Pace EP cell culture pacer (IonOptix, Dublin) using a two-step protocol (alternation between a period of high frequency and low frequency electrical pulses). Cells were plated on 35mm dishes, grown to confluence and differentiated by changing the media to differentiation media. At day 7 of differentiation, media was changed to starvation media and electrical pulse stimulation (EPS) applied via carbon electrodes at 5volts, 24ms, 2 Hz for 1 hour followed immediately by 5V, 24ms, 0.2Hz for 1h. This alternation in frequency was continued for 16 hours (period of exercise).

6.2.4 Glucose uptake

Measurement of 2-deoxy-D-[2, 6-3H] glucose uptake was carried out as described in Materials and Methods chapter section 2.9, and was performed on day 7 differentiated cells in 35 mm plates. Cells were divided into two groups for treatment; with or without 16 hours EPS at 37 °C.

6.2.5 Western blot

After 16 hours incubation with or without EPS, protein was extracted and western blotting was carried out as mentioned in Materials and Methods, section 2.8, with the following differences. Non-specific sites on the nitrocellulose membranes were blocked by incubation in Tris buffered saline tween (TBST) containing 5% (w/v) milk as a blocking buffer for 1 hour for AMPK and 3% (w/v) BSA for AS160 at room temperature. Membranes were incubated with monoclonal primary antibody (1°Ab) diluted in 0.5% blocking buffer TBST/0.5% (w/v) milk or BSA, overnight at 4 °C. After washing, membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody IgG, diluted in 0.5% blocking buffer.

Detection took place using enhanced chemiluminescence and densitometry measurements performed using the Bio-RAD Molecular Imager GS-800 calibrated densitometer and Quantity One software. Phospho-AMPK, native AMPK and native AS160 antibodies were used at a 1:1000 dilution and phospho-AS160 was used at a 1:500 dilution.

6.2.6 ELISA

Secretion of IL-6 and IL-8 were determined by enzyme-linked immunosorbent assay (ELISA) using the Single-Analyte ELISArray (Qiagen). Media was changed to starvation media and the day 7 differentiated cells were either subjected to EPS or kept in the incubator for 16 hours. After 16 hours EPS, media was removed, centrifuged at 1000g for 10 minutes and assayed for secretion of IL-6 and IL-8 according to the manufacturer's protocol and described in detail in Materials and Methods section 2.11.

6.3 Results

6.3.1 Effects of EPS on glucose uptake in control and T2D myotubes

To examine the metabolic effects of the *in vitro* exercise protocol (16 hours EPS), radiolabelled glucose uptake was measured in both diabetic and non-diabetic (control) myotubes.

As shown Figure 6-1 (A), in non-diabetic myotubes, there was a statistically significant increase in glucose uptake in response to insulin from 539.8 ± 42.1 to 744.3 ± 59.3 pmol/min/mg ($p < 0.05$). Furthermore, 16 hours EPS had the same effect in these cells when it caused an increase in glucose uptake to significant level 782.9 ± 68.1 pmol/min/mg over basal uptake ($p < 0.05$). There is an additive effect of combining insulin and 16 hours EPS on glucose uptake to a highly significant level 931 ± 58.7 pmol/min/mg over basal ($p < 0.001$) and to a significant level as well over insulin stimulated glucose uptake ($p < 0.05$).

In diabetic myotubes Figure 6-1(A), the level of glucose uptake in response to 16 hours EPS was 736.5 ± 59.3 pmol/min/mg which is comparable to that of the control cells. However, unlike the control cells, the increase in glucose uptake in response to 16 hours EPS against basal uptake, which was 644.7 ± 51.6 pmol/min/mg, did not reach to significance level due to the higher basal glucose uptake in diabetic myotubes. In addition, insulin failed to stimulate an increase in glucose uptake (670.5 ± 45.7 pmol/min/mg) over basal glucose uptake. EPS in combination with insulin did not increased glucose uptake in comparison to insulin alone. Due to an increase in basal glucose uptake in diabetic cells, the expression of fold changes of stimulus (insulin, 16 hours EPS and insulin plus 16 hours EPS) over basal glucose uptake were examined.

In Figure 6-1(B) the fold changes of insulin over basal glucose uptake in control cells was 1.4 fold which is significantly higher than that 1.1 fold observed in diabetic ($p < 0.01$). The same was observed for insulin plus 16 hours EPS; the 1.5 fold increase was significantly higher ($p < 0.01$) in control compared to1.1 fold increase observed in the diabetic myotubes.
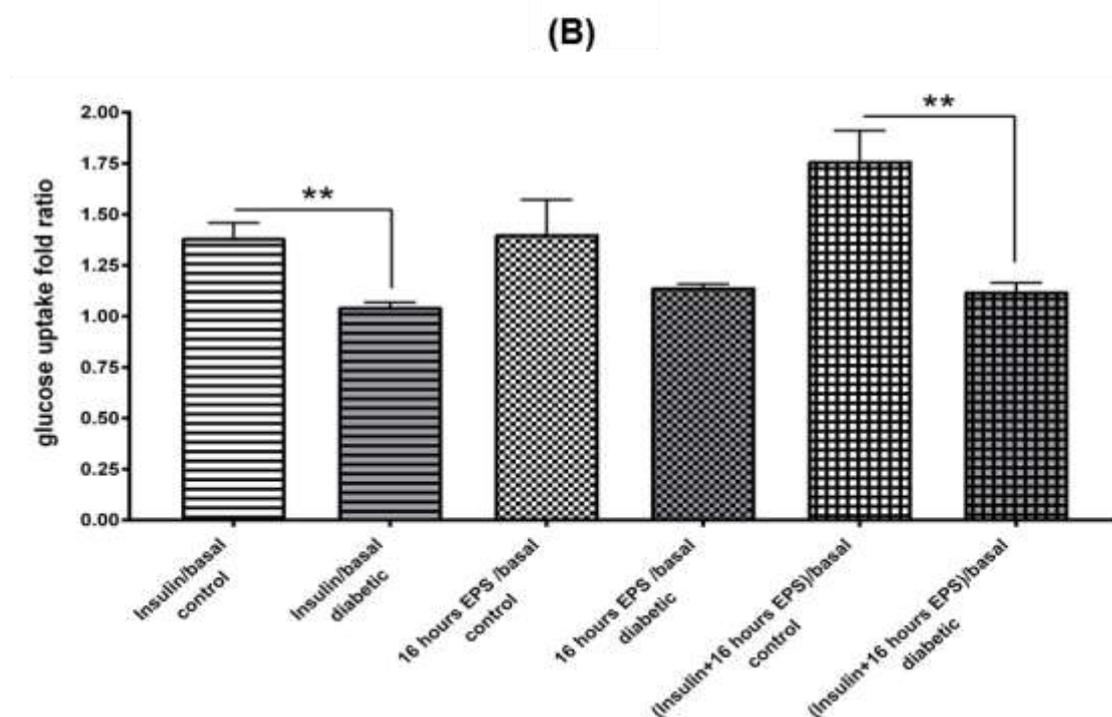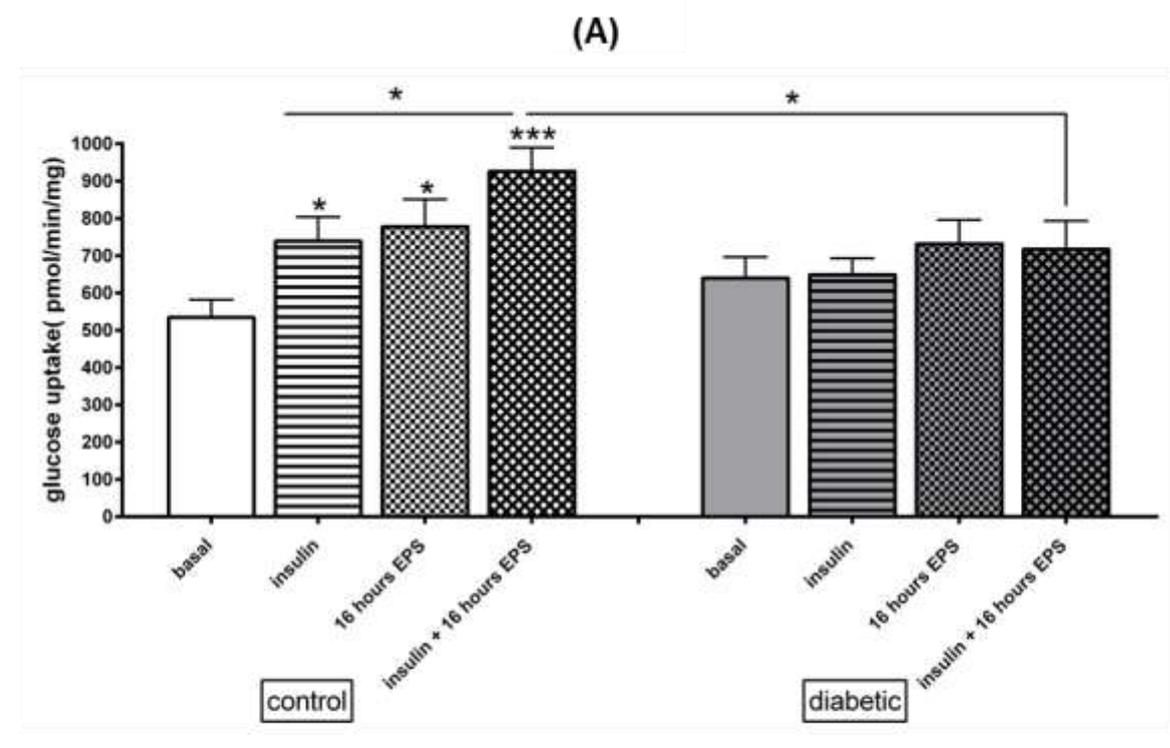
Figure 6-1: Glucose uptake in both control and diabetic myotubes with or without 16 hours EPS and with or without 100 nM insulin for 10 minutes. (A) Graph shows the glucose uptake in control and diabetic myotubes at basal levels, after insulin stimulation, 16 hours EPS and insulin plus 16 hours EPS. (B) Graph representative of the fold changes in glucose uptake with stimulus over basal in both control and diabetic. Values are expressed as the mean ± SEM, n=6 for each group, *p< 0.05, **p< 0.01, ***p< 0.001.

6.3.2 AMPK activation in control and diabetic myotubes after EPS

AMP-activated protein kinase (AMPK) is a key sensor of energy status in skeletal muscle. AMPK activation was measured by western blot using phospho-specific antibodies against threonine residue (Thr^{172}), and activation is essential for the full activation of the kinase activity (Scott *et al.*, 2002).

As shown in Figure 6-2, in control myotubes cultures, phosphorylation of AMPK^{Thr172} was shown to increase significantly with 16 hours EPS (p<0.01). In contrast, diabetic myotubes subjected to the same EPS protocol showed no activation of AMPK over basal. There was a significant increase in AMPK activation in the basal state in the diabetic versus control cultures (p<0.01). However, there was no further activation of AMPK in the diabetic cells in response to exercise. However, AMPK activation in diabetic cells was still significantly higher than in resting control muscle (p<0.05).

(A)

P-AMPK			
T-AMPK			

16 hours EPS   -   +   -   +

(B)
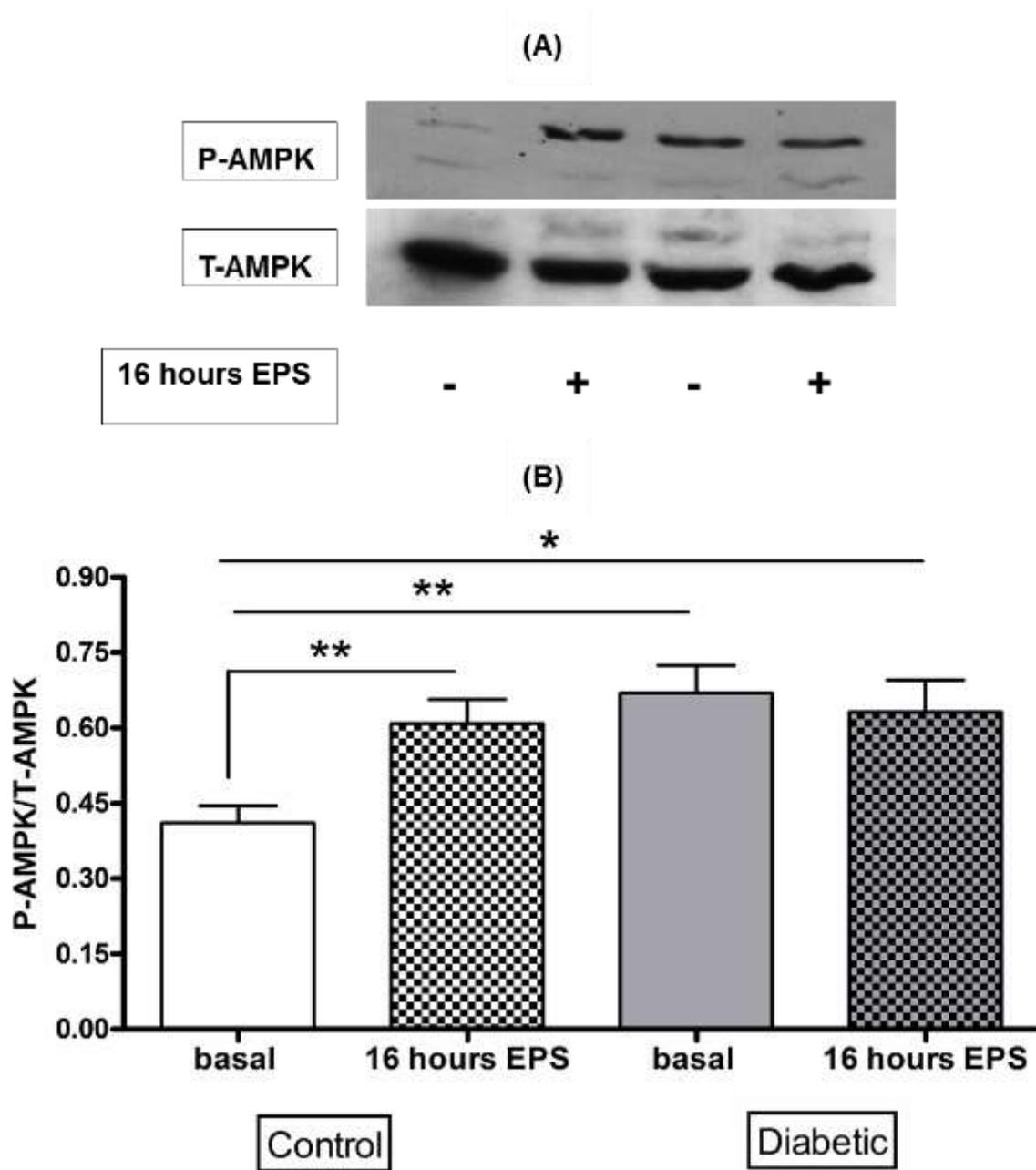
Figure 6-2: AMPK activation in response to 16 hours EPS in cultured myotubes. (A) Shows the blots of AMPK activation at Thr^{172} for basal state after 16 hours EPS for both control and diabetic. (B) Graph represents the densitometric analysis results of phospho/total AMPK for both control and diabetes with and without 16 hours EPS. Values are expressed as the mean ± SEM, n=6 for each group, *p< 0.05, **p< 0.01.

6.3.3 Effects of EPS on AS160^{Thr642} phosphorylation in control and T2D myotubes

AS160 is the direct downstream signalling molecule of Akt, activated by insulin and responsible for an increase in glucose uptake. The effect of 100 nM insulin, 16 hours EPS and a combination of both insulin and EPS on AS160 activation were investigated by Western blot measuring Thr^{642} phosphorylation. Thr^{642} is one of the most important phosphorylation sites activated by Akt.

Figure 6-3 shows that in control myotubes, insulin significantly increased AS160 activation by increasing Thr^{642} phosphorylation compared to the basal state ($p<0.01$). In addition, there was an additive effect of insulin and EPS together over insulin alone ($p<0.05$). In diabetic cells, insulin increased AS160 phosphorylation to a significant level ($p< 0.01$) compared to basal, but there was no additive effect of the combination of insulin and exercise on Thr^{642} phosphorylation. Furthermore, there was a significant difference between control and diabetic myotubes after stimulation with a combination of both insulin and EPS with increased activation of AS160 in control compared to diabetic myotubes ($p< 0.01$).
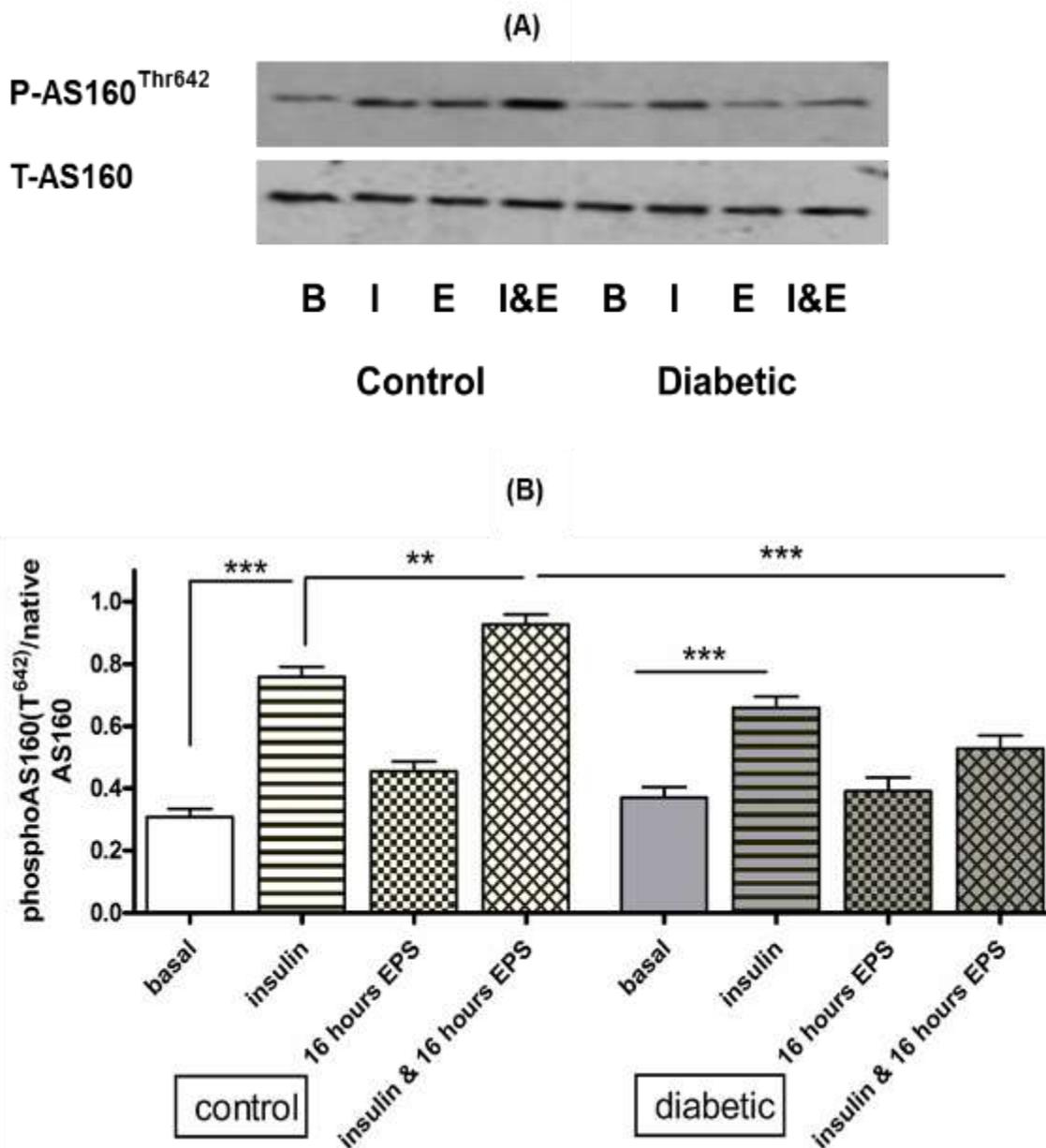
Figure 6-3: AS160^{Thr642} activation in cultured myotubes. (A) Shows the blots of AS160 activation (phosphorylation) on AS160^{Thr642} for cells subjected to the following conditions; rest (basal), 100 nM insulin for 10 minutes, 16 hours EPS, and a combination of 16 hours EPS and 10 minutes insulin at the end of EPS for both control and diabetic myotubes. (B) Graph represents the densitometric analysis results of phospho Thr^{642}/native AS160 for both control and diabetes for the same conditions. Values are expressed as the mean ± SEM, n=6 for each group, **p< 0.01, ***p< 0.001.

6.3.5 Effects of EPS on AS160^{Ser588} phosphorylation in control and T2D myotubes:

AS160^{Ser588} is the other direct downstream target site of Akt, activated by insulin and responsible for an increase in glucose uptake. The effect of 100 nM insulin, 16 hours EPS and the combination of EPS and insulin on AS160^{Ser588} activation was investigated by Western blot measuring Ser^{588} phosphorylation in both control and diabetic myotubes.

As can be observed in Figure 6-4, insulin significantly increased AS160^{Ser588} phosphorylation above basal in control cells but not in diabetic myotubes, and there was a significant difference ($p<0.05$) between control and diabetic cells in response to insulin stimulation. Furthermore, in control myotubes 16 hours EPS significantly increased AS160 activation by increasing Ser^{588} phosphorylation compared to basal ($p< 0.05$), but there was no additive effect observed by combining EPS and insulin. In diabetic cells, EPS alone did not increase AS160^{Ser588} phosphorylation, and did not enhance insulin stimulated S^{588} phosphorylation.
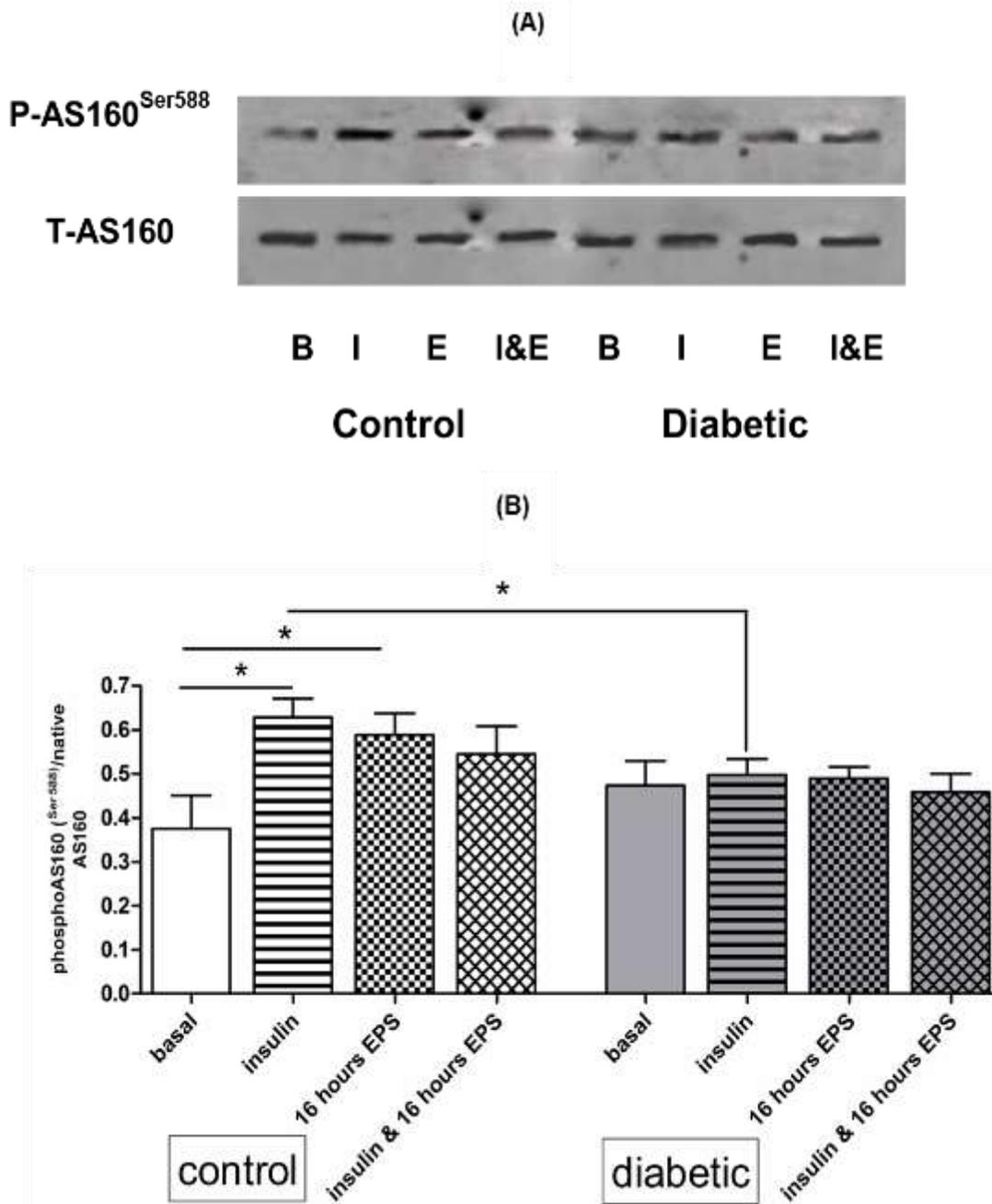
Figure 6-4: AS160^{Ser588} activation in cultured myotubes. (A) Shows the blots of AS160 activation (phosphorylation) of AS160 ^{Ser588} for cells subjected to the following conditions; rest (basal), 100 nM insulin for 10 minutes, 16 hours EPS, and a combination of 16 hours EPS followed by 10min insulin treatment for both control and diabetic myotubes. **(B)** Graph represents the densitometric analysis results of phospho S 588 /native AS160 for both control and diabetes for the same conditions. Values are expressed as the mean ± SEM, n=6 for each group, *p< 0.05.

6.3.7 Effects of EPS on Cytokines release in control and T2D myotubes

Skeletal muscle produces cytokines in response to contraction. The effect of EPS on IL-6 and IL-8 secretion is shown in Figure 6-5 and Figure 6-6. IL-6 secretion was significantly increased from both control and diabetic myotubes after 16 hours EPS ($p<0.05$). There was no difference in IL-6 secretion levels between control and diabetic myotubes. The same pattern was observed with IL-8 secretion, the increase in IL-8 secretion observed from the control and diabetic myotubes after 16 hours EPS compared to basal resting corresponding cultures was statistically significant ($p<0.05$). The values of both IL-6 and IL-8 secretion were normalised to total protein concentration.
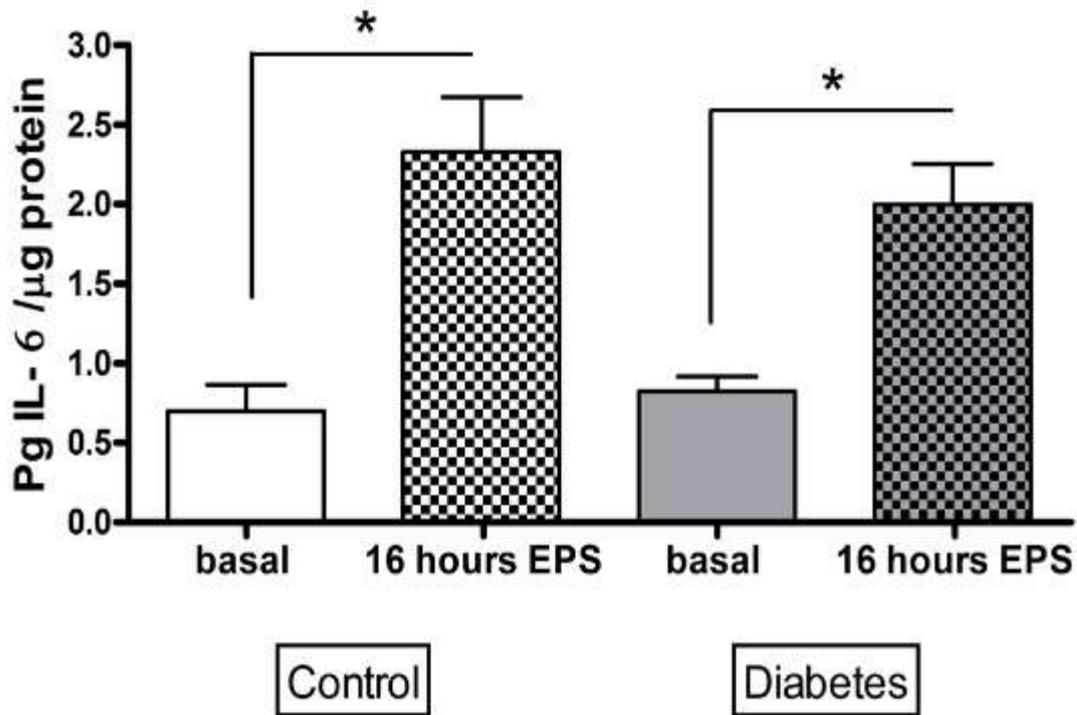
Figure 6-5: Effects of EPS on IL-6 secretion in control and diabetic cell cultures. Fresh reduced serum media was added to 6 controls and 6 diabetic cultures and subjected to EPS for 16 hours. Media was collected and assayed for IL-6 secretion by ELISA. The graph represents the IL-6 secretion into the media in response to contraction normalised to the total protein concentration of the cells. Results are expressed as mean ± SEM. * $p < 0.05$.
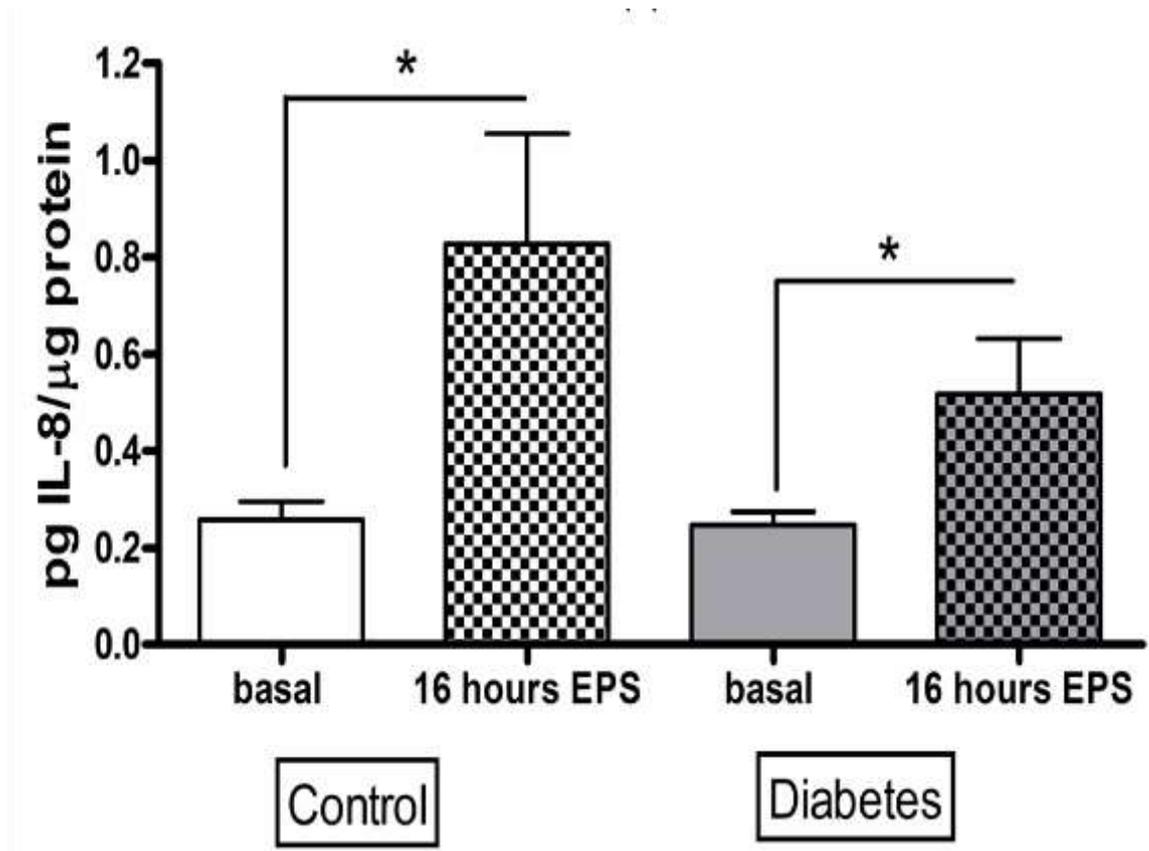
Figure 6-6: Effects of EPS on IL-8 secretion in control and diabetic cell cultures. Fresh reduced serum media was added to 6 controls and 6 diabetic cultures and subjected to EPS for 16 hours. Media was collected and assayed for IL-8 secretion by ELISA. The graph represents the IL-8 secretion into the media in response to contraction normalised to the total protein concentration of the cells. Results are expressed as mean ± SEM. * p<0.05.

6.4 Discussion

Glucose uptake in skeletal muscle is achieved by distinct ways; insulin signalling and contraction, or exercise-related pathways are involved. In T2D, skeletal muscle is characterized by decreased insulin sensitivity which is associated with a decrease in glucose uptake in response to insulin (Zierath *et al.*, 1996; Bennett *et al.*, 2000; Brown *et al.*, 2015b). The contraction mediated or exercise mimetic-mediated (AICAR) glucose uptake in these cells has been reported to be preserved (Lee *et al.*, 1995; Lund *et al.*, 1995; McIntyre *et al.*, 2004).

To date, there are few studies that have investigated the effects of EPS in human cultured diabetic myotubes. To our knowledge, this is the first study that measured AMPK activation in cultured diabetic myotubes using an *in vitro* system of exercise (EPS). The most interesting findings of this study are; there is an intrinsic molecular defect in diabetic myotubes represented by defective AMPK and glucose uptake in response to EPS. AMPK activation and glucose uptake increased in response to EPS in control myotubes, and EPS enhanced the effect of insulin. Conversely, EPS did not increase AMPK activation and glucose uptake, nor enhanced the action of insulin in cultured diabetic muscle cells.

Activation of AMPK by exercise draws the attention to its ability to improve glucose control and the possibility of therapeutic use in T2D patients through its effects on glucose and lipid metabolism (Hardie, 2013). A thorough examination of related literatures reveals that whole body insulin sensitivity and/or glucose uptake are improved in insulin resistant subjects in response to exercise or exercise mimetics. Table 6-2 shows details of the studies related to AMPK and the metabolic effects of exercise in insulin resistant skeletal muscle compared to insulin sensitive controls. It is recognised that the AMPK level and activity vary among different studies. Studies can be divided into three categories, the first with normal basal AMPK activity and a normal response (increase) in response to stimulation (studies 1-6 in Table 6-2). The second group are those with abnormally elevated resting AMPK levels in insulin resistant muscle and failure to increase further with stimulation (studies 7 and 8). Finally, the third group represents studies that show an abnormal low level of AMPK in insulin resistant muscle compared to insulin

sensitive muscle that returns to normal with exercise or other stimulation (studies 9-11).

Table 6-2: A review of literatures about AMPK level and activation in response to exercise or other stimulus in insulin resistant versus insulin sensitive skeletal muscle cells.

studies	AMPK activity In insulin resistant		Measurement method	Exercise type	Subject and Muscle tissue	obesity	Insulin sensitivity in type 2 DM
	Basal	stimulation					
1- (**Musi** *et al.*, **2001**)	↔	↑	Activity by immunoprecipitation & immunoblotting	45 minutes cycling	Diabetes versus control before and after exercise	Non-obese	Improved (decrease plasma glucose and insulin
2- (**Højlund** *et al.*, **2004**)	↔	No stimulation Only basal measured	immunoprecipitation & α Thr^{172} phosphorylation immunoblotting	No exercise	Diabetes versus control	obese	Not measured
3- (**Wojtaszewski** *et al.*, **2005**)	↔	↑	immunoblotting α Thr^{172} phosphorylation	6-week training	Diabetes versus control Muscle trained and resting side (after 6 weeks)	Diabetic obese Vs lean control	Improve insulin action (glucose removal rate)
4- (**Chen** *et al.*, **2005**)	↔	↑	immunoblotting α Thr^{172} phosphorylation	2 mM AICAR	muscle biopsy taken during surgery (muscle culture)	Lean Vs Obese Vs Obese diabetic	Not measured
5- (**Kjobsted** *et al.*, **2016**)	↔	↑	immunoprecipitation	60 minutes cycling	3 muscle biopsies, before , immediately after and 3hours after exercise	Obese control Vs Obese diabetic	decreasing insulin level compared with pre-exercise
6- (**Koistinen** *et al.*, **2003**)	↔	↑	α Thr^{172} phosphorylation by immunoblotting	1 mM AICAR	Skeletal muscle strips Diabetes versus control	Non-obese	Improved insulin action by increased glucose uptake (additive effect)

7- (Sriwijitkamol *et al.*, **2007)**	↑	**NO** ↑	Immunoprecipitation And immunoblotting	40 minutes cycling	4 muscle biopsies 0, 10, 40, and 90 minutes after exercise	Lean Vs Obese Vs Obese diabetic	Improved insulin action by decreasing plasma glucose concentrations
8- (Christopher *et al.*, **2003) Animal study**	↑	**NO** ↑	Immunoprecipitation.	30 minutes	Muscle biopsy before and after exercise on 2 occasions before and after induction of DM	Weight matched animal	Not measured
9- (Bandyopadhyay *et al.*, **2006)**	↓	↑	Immunoprecipitation and immunoblotting	Rosiglitazone treatment 3 month	biopsy before and after 3 month rosiglitazone therapy	Lean Vs Obese Vs Obese diabetic	Improved insulin action
10- (Cao *et al.*, **2012) Animal study**	↓	↑	Western Blotting	2 groups Acute 90 minutes & chronic Swimming	Biopsy after exercise protocols compared to controls	Control lean Vs Obese HFD Vs HFD diabetic	Improve insulin action
11- (Lessard *et al.*, **2006) Animal study**	↓	↑	Immunoprecipitation and immunoblotting	rosiglitazone treatment 6 weeks	obese Zucker and age-matched lean rats	Lean Vs obese Vs obese with treat	Improved metabolic parameters associated with increased AMPK
12-(Barnes *et al.*, **2002) Animal study**	↔	**NO** ↑ **AMPKα1 but increase AMPKα2**	Immunoprecipitation	*In vitro* contraction	Epitrochlearis Muscle from Female obese male Zucker and age-matched lean rats	Lean Vs obese	Improved insulin action

Vs.: Versus , HFD: high fat diet

6.4.1 Control non-diabetic myotubes

In the present study, in control non-diabetic myotubes the main findings were as follow; AMPK activation increased with EPS. Glucose uptake was increased in response to insulin and EPS each one separately. Furthermore, combined stimulation (EPS+insulin) caused additive effects to the action of insulin on glucose uptake and distal insulin signalling pathway.

Previous studies reported that an *in vivo* single bout of exercise increases AMPK activation in healthy non-diabetic muscle cells (Musi *et al.*, 2001; Wojtaszewski *et al.*, 2005; Kjobsted *et al.*, 2016). Furthermore, *in vitro* EPS was shown to increase AMPK activity in control myotubes (Nikolić *et al.*, 2012; Brown *et al.*, 2015a) which are consistent with the results obtained in the control myotubes used in In the present study.

Glucose uptake into skeletal muscle is achieved by two principal independent mechanisms. These are either insulin dependent or insulin independent pathways. Exercise (muscle contraction) represents the main insulin independent mechanism. A single bout of *in vivo* exercise raises glucose uptake into skeletal muscle (Richter *et al.*, 1989), and improves insulin sensitivity in the post-exercise period (Henriksen, 2002). Previous studies on cultured human muscle cells have shown that EPS stimulates glucose uptake (Lambernd *et al.*, 2012; Nikolić *et al.*, 2012; Brown *et al.*, 2015a). In line with these observations, in this study glucose uptake in non-diabetic skeletal muscle myotubes increased in response to insulin, EPS and there was an additive effect of combining insulin plus EPS.

Exercise or physical activity increases peripheral insulin sensitivity. The ability of insulin to stimulate glucose uptake in skeletal muscle in response to a prior bout of exercise is increased several hours thereafter (Richter *et al.*, 1982; Frøsig *et al.*, 2009). During exercise and shortly after, the increased insulin-mediated glucose uptake depends on systemic and local cellular factors. The cellular processes that are involved in insulin sensitisation are mediated by an increase in GLUT4 translocation and availability at the cell membrane (Hansen *et al.*, 1998). The enhanced insulin sensitivity in both cultured muscle cells and isolated muscle fibres in response to EPS or AICAR is an important observation. This confirms that the sensitisation effect is not entirely dependent upon systemic influences such as

enhanced blood flow, and that it is an intrinsic characteristic of the muscle cells (Hamada *et al.*, 2006; Brown *et al.*, 2015a).

Upon insulin stimulation, a series of signalling events occur ending with GLUT4 translocation. The increased GLUT4 translocation is mediated by activation of both insulin dependent and independent signalling pathways. The increased insulin-stimulated glucose uptake is not associated with enhancement of the proximal insulin signalling pathway. Studies show that prior exercise does not increases IR tyrosine kinase activity, IRS1 tyrosine phosphorylation, PI3K, Akt Ser[473] and Thr [308] phosphorylation (Wojtaszewski *et al.*, 1997; Wojtaszewski *et al.*, 2000; Frøsig *et al.*, 2007). On the other hand, several studies have reported that insulin stimulated AS160 phosphorylation and glucose uptake are both enhanced after exercise (Arias *et al.*, 2007; Treebak *et al.*, 2009; Schweitzer *et al.*, 2012; Cartee, 2015).

AS160 is a family of kinases that are directly activated by Akt upon insulin or exercise stimulation. TBC1 domain family member 1 (TBC1D1) and 4 (TBC1D4) are members of this family and act as GTPase activating proteins that serve to control GLUT4 translocation. It is suggested that these proteins act as a convergence points for both insulin dependent and independent signalling pathways (Cartee and Wojtaszewski, 2007). Insulin and exercise significantly increase AS160 phosphorylation (otherwise known as TBC1D4) in human skeletal muscle (Mîinea *et al.*, 2005; Howlett *et al.*, 2008). Phosphorylation of AS160 was shown to increase in response to exercise in both rat (Funai *et al.*, 2009) and human skeletal muscle cells (Treebak *et al.*, 2009) and was associated with enhanced insulin stimulated glucose uptake several hours after exercise.

Multiple phosphorylation sites of AS160 have been reported to be activated by distinct upstream kinases including Akt and AMPK (Geraghty *et al.*, 2007; Howlett *et al.*, 2008). Activation of these sites on AS160 leads to inhibition of the GTPase-activating protein, eliminating their inhibition on GLUT4 translocation process and increasing glucose uptake. Thr[642] and Ser[588] are key residues regulating insulin mediated GLUT4 translocation (Sano *et al.*, 2003). Studies showed that mutations of AS160 on Thr[642] and Ser[588] reduce insulin stimulated GLUT4 translocation (Sano *et al.*, 2003; Mîinea *et al.*, 2005). Thus, reasonably these sites were

investigated to study the effects of exercise-induced insulin sensitisation and the responses of cultured myotubes to insulin, EPS and both.

In the current study, AS160 phosphorylation at Thr^{642} was increased in response to insulin in control myotubes, and EPS causes an enhancement in insulin stimulated AS160 phosphorylation in these cells, and was associated with enhanced glucose uptake (additive effect). These results are in agreement with that obtained by various studies in both human and animal models that showed a sustained post exercise activation in distal insulin signalling molecules in skeletal muscles (Arias *et al.*, 2007; Treebak *et al.*, 2014; Kjøbsted *et al.*, 2015). The following Table 6-3 summarises the effects of different models of exercise on different AS160 phosphorylation sites in skeletal muscle cells.

Furthermore, in this study $AS160^{Ser588}$ phosphorylation was increased in control myotubes in response to both insulin and exercise but no additive effect has been observed for the co-activation of insulin and EPS.

Schweitzer *et al* (2012) and Castorena *et al.* (2014), both using control rat skeletal muscles, similarly showed that insulin and exercise stimulate $AS160^{Ser588}$ phosphorylation. Both fore mentioned studies showed an additive effect for the co-activation of insulin and exercise as measured with insulin stimulation after 3 hours post exercise. The differences from the results of the current study could be attributed to the differences in exercise protocol (*in vivo* vs. *in vitro*). In a human *In vivo* study, Treebak *et al.* (2009) showed that $AS160^{Ser588}$ activation was increased in response to exercise and physiological insulin (after food) which is similar to the results of the control myotubes in the current study.

In summary, insulin and EPS alone increased glucose uptake, and EPS enhanced the effect of insulin on glucose uptake so mimicking the key changes observed *in vivo*. EPS caused an increase AMPK phosphorylation at Thr^{172}. Interestingly; I found that insulin increased $AS160^{Thr642}$ phosphorylation, and that this was enhanced by EPS. This raises the intriguing possibility that the enhanced effect of exercise on insulin action during the post-exercise period involves $AS160^{Thr642}$ phosphorylation. Both insulin and EPS individually increased phosphorylation at $AS160^{Ser588}$, but there was no additive effect of EPS and insulin.

Table 6-3: A review of literatures about AS160 activation in response to exercise and/or insulin in different models of exercise.

Study	Animal/ human	Tissue/cell culture	Exercise type/ duration	Insulin	AS160 phospho sites activated by insulin		AMPK activation after exercise	Additive effects of AS160 activation in response to combined (Ex+Ins)
1- (Castorena *et al.*, 2014)	Animal (rats)	Isolated muscle fibers	*In vivo* swimming 4 bouts (30-min)	100 µU/mL	Thr642 and Ser588	Controls	increased	Additive effects of both Thr642 and Ser588
						insulin-resistant	increased	No Additive effects of both Thr642 and Ser588
2-(Funai *et al.*, 2009)	Animal (rats)	Isolated muscle fibers	*In vivo* swimming 4 bouts (30-min)	50 µU/mL	Thr642	Controls	Not measured	Additive effects in Thr642
						No diabetes		
3-(Schweitzer *et al.*, 2012)	Animal (rats)	Isolated muscle fibers	*In vivo* swimming 4 bouts (30-min)	50 µU/mL	Thr642 and Ser588	Controls	Not measured	Additive effects of both Thr642 and Ser588
						No diabetes		
4-(Kjøbsted *et al.*, 2016)	Animal mice	Isolated muscle fibers	*In vitro* In Situ Contraction 10 min	100 µU/mL	Thr649 Ser711 Ser324 Ser595	Controls WT	Highly increased	Additive effects in Thr642and Ser711
						AMPK KO/KD	Not increased	No Additive effects in any residues
5-(Kjøbsted *et al.*, 2015)	Animal mice	Isolated muscle fibers	AICAR 4-6 hours	100 µU/mL	Thr649 Ser711 Ser324 Ser595	Controls WT	Highly increased	Additive effects in Thr642 and Ser711
						AMPK KO/KD	Not increased	No Additive effects in any residues

						Controls	increased	Additive effects in PAS-AS160
6-(Arias *et al.*, 2007)	Animal (rats)	Isolated muscle fibers	*In vivo* swimming 4 bouts (30-min)	50 µU/ml	PAS-AS160	No diabetes		
7-(Treebak *et al.*, 2009)	human	Isolated muscle fibers	*In vivo* one-legged knee extensor for 60 min	100min euglycaemic–hyperinsulinemic clamp	Ser318 Ser341 Ser751 Thr642 Ser588	Controls	Not measured	Additive effects of Ser341, Ser751 and Ser588
						No diabetes		
8-(Kjobsted *et al.*, 2016)	human	Isolated muscle fibers	*In vivo* cycle ergometer for 60 min	4h euglycaemic-hyperinsulinemic clamp	Ser318 Ser341 Thr642 Ser588	Controls	increased	No Additive effects in any residues
						diabetes	increased	No Additive effects in any residues
9-(Treebak *et al.*, 2014)	human	Isolated muscle fibers	*In vivo* one-legged knee extensor for 60 min	Physiological insulin after food	Ser318 Ser341 Ser704 Thr642	Controls fasted	increased	Ser588 and Ser751 only activated by contraction and not activated by Physiological insulin(fed state)
						Controls fed	increased	Additive effects of Ser341, Ser704 and Thr642

❖ In animal studies; Thr[649] corresponds to Thr[642], Ser[595] corresponds to Ser[588], Ser[711] corresponds to Ser[704], and Ser[324] corresponds to Ser[318].

6.4.2 Diabetic myotubes

In the present study, one of the most important findings was that the basal activity of AMPK in diabetic cultured skeletal muscle was higher than that of control. Furthermore, the elevated AMPK activity at rest failed to further increase with exercise. This result is consistent with other studies in human (Sriwijitkamol *et al.*, 2007; Green *et al.*, 2011) and in diabetic dog skeletal muscle (Christopher *et al.*, 2003). In these studies, the elevated basal AMPK activity in diabetes was attributed to the metabolic decompensation that associates with diabetes (Christopher *et al.*, 2003). High basal AMPK activity was attributed as well to serum factors that are well known to be elevated in the diabetic state like IL-6 and leptin (Sriwijitkamol et al., 2007). Previous work confirmed that AMPK in skeletal muscle is activated by IL-6 (MacDonald *et al.*, 2003; Kelly *et al.*, 2004) or in response to leptin (Minokoshi *et al.*, 2002). The elevated level of these proteins in T2D individuals could have a role in an elevated basal AMPK.

In the current study, it could not be confirmed or refuted that leptin or IL-6 levels (in subjects from whom the muscle cells were taken) were responsible. However, it is possible that insulin resistance and/or the diabetic state cause epigenetic changes in skeletal muscle cells that may be responsible for the elevated basal AMPK level. These changes could be a compensation for the metabolic events associated with diabetes. Furthermore, this elevated basal AMPK activity in diabetic myotubes is associated with a slight increase in basal glucose uptake; however, the increased basal glucose uptake in T2D myotubes was statistically not significant. This is in agreement with our previous work on control myotubes in which a stimulus (thrombin) causes increased basal AMPK activity associated with an increase in basal glucose uptake (Al-Bayati *et al.*, 2016).

In diabetic myotubes, EPS did not increase AMPK activation above its resting activity which was already high. This failure to activate AMPK in response to EPS in diabetic myotubes may partly be explained by the possibility of reaching highest activation limits. In other words, AMPK activation in diabetic muscle cell cultures at rest reached the maximum activation capacity and could not rise beyond this point. Another possible explanation might be that, the long standing insulin resistance in the diabetic subject from whom the samples were taken could be accompanied by

metabolic abnormalities that inhibit AMPK further activation in response to EPS. In previous work on these cells, McIntyre *et al.(2004)* found that glucose uptake was increased in response to AICAR in both control and diabetic cells. They supposed that the increased in glucose uptake is related to AICAR-activated phosphorylation of AMPK. However, they did not measure AMPK activation in their work. In this study, EPS was shown to increase AMPK activation in control but not in diabetic myotubes and the increased AMPK activation in control myotubes was associated with a concomitant increase in glucose uptake.

In addition, hyperglycaemia and abnormal glycogen content in diabetic skeletal muscle are metabolic disturbances that accompany diabetes and have been shown to have an effect on AMPK levels and activation in response to exercise. The results of increased basal AMPK activity and failure to stimulate by EPS match those observed in earlier *in vivo* exercise studies. For example, Christopher *et al.* (2003) who examined AMPK activation in response to *in vivo* exercise diabetic dogs and Sriwijitkamol *et al.* (2007) in diabetic human skeletal muscle cells.

In the present study, it is interesting to observe that glucose uptake in diabetic myotubes failed to increase significantly in response to insulin which confirms that the diabetic phenotype has been preserved in cultured myotubes. These results are in agreement with those obtained in previous studies using diabetic cell cultures (Thompson *et al.*, 1996; McIntyre *et al.*, 2004). In addition, EPS failed to stimulate glucose uptake in diabetic myotubes and no additive effect of glucose uptake was observed by stimulation with both EPS and insulin. In agreement with these results, Stephens and Sparks (2014) claimed in their review that some subjects, including some T2D and severely insulin resistant individuals, tend to be more "exercise resistant" compared to insulin sensitive persons. Those individuals seem to have a diminished metabolic response to exercise due to different genetic and epigenetic causes.

In the current study, AS160 phosphorylation at Thr^{642} was increased in response to insulin in both control and diabetic myotubes. In other words, the diabetic phenotype has no effect on insulin stimulated $AS160^{Thr642}$ phosphorylation. These results match those observed in earlier studies that compared enzyme activity in

control with that of T2D individuals and their insulin resistant relatives (Karlsson *et al.*, 2006; Vind *et al.*, 2011).

No enhancement in the AS160^{Thr642} phosphorylation was observed in response to combined insulin and EPS in diabetic myotubes. This is consistent with that obtained from an animal *in vivo* exercise protocol performed by Castorena *et al.* (2014) who showed that impaired AS160^{Thr642} phosphorylation in high-fat diet rats (insulin-resistant) compared to low-fat diet rats (normal insulin sensitivity) 3 hours post exercise. Furthermore, EPS alone did not cause activation of AS160^{Thr642} in both diabetic and control which is consistent with the results of Chen et al. (2011). In the later study, they suggest that AS160^{Thr642} is more important for insulin than contraction-stimulated glucose uptake.

Another important finding was that, there is reduced insulin stimulated AS160^{Ser588} phosphorylation in skeletal muscle of patients with T2D compared to control. This could help to explain the failure of diabetic myotubes to increase glucose uptake above basal after insulin or EPS stimulation. These results are in line with those of a previous study carried out comparing the effects of insulin stimulation in human muscle cells in obese T2D, compared with matched controls. The insulin stimulated AS160^{Ser588} was lower in T2D than in control individuals (Vind *et al.*, 2011).

Kjobsted *et al.*(2016) showed that, there are no differences between obese T2D and BMI matched control skeletal muscle AMPK activation and insulin sensitisation in the both examined phosphorylation sites, AS160^{Thr642} and AS160^{Ser588}, in response to *in vivo* exercise bout. Three main differences have been observed in Kjobsted *et al.*(2016) from the current study that could be attributed; first is the duration of T2D which was (3.5 ± 1.2) years which is shorter than that of the current study. Furthermore, type of exercise (*in vivo*) versus EPS (*in vitro*) in ours. Finally, the type of treatment used for T2D which was diet and/or metformin. Whereas high doses of insulin 131.2 ± 9.6 units/ day were used for T2D subjects of the current study which reflect the severity of insulin resistance. The differences in activation in response to combined EPS and insulin between diabetic and control are in agreement with the glucose uptake data that showed

increased insulin mediated glucose uptake after EPS in control but not in diabetic myotubes.

In summary, basal AMPK activation was significantly increased in diabetic versus control muscle cell cultures, and both basal glucose uptake and AS160^{Ser588} phosphorylation tended to be higher in the diabetic muscle cells. EPS failed to increase AMPK activation above baseline, and neither insulin nor EPS alone increased glucose uptake and AS160^{Ser588} phosphorylation in the diabetic muscle cells. Insulin alone did increase phosphorylation at AS160^{Thr642}, suggesting that this is not implicated in the decreased effect of insulin on glucose uptake in the diabetic muscle cells. However, the effect of EPS to enhance the action of insulin on glucose uptake and AS160^{Thr642} phosphorylation observed in the control muscle cells was not evident in the diabetic cultures.

The long standing insulin resistant state in T2D subjects of the current study could be the cause of the inability of the diabetic cells to respond to EPS and/or insulin and can be attributed to the abnormal signalling.

Regular exercise is a key regulator for many metabolic processes, and plays a crucial role in prevention and treatment of many diseases. Many cellular and molecular regulators have been suggested to mediate exercise function at the level of skeletal muscle. Skeletal muscle cells express and secrete a range of biologically active peptides and proteins during and shortly after muscle contraction, known as myokines which have both local and systemic functions (Febbraio and Pedersen, 2005). The health promoting effects of exercise are largely attributed to some of these peptides (Pedersen and Febbraio, 2012).

IL-6 and IL-8 are important cytokines implicated in the exercise process in skeletal muscle cells. Contracting skeletal muscle has been shown to increase expression and secretion of these cytokines in response to exercise (Peake *et al.*, 2015). In addition, IL-6 and IL-8 gene expression has been shown to be upregulated in insulin resistant cells (Brown *et al.*, 2015b). In the current study, skeletal muscle cells from diabetes conserved their ability to increase cytokine release in response to exercise, and no differences were observed between diabetic and control skeletal muscle cells after 16 hours EPS. This is in keeping with the observation that IL-6 secretion from skeletal muscle in response to contraction was mediated

in part through an AMPK independent mechanism (Lauritzen *et al.*, 2013). These results are consistent with data obtained from previous *in vitro* exercise studies performed on control human muscle cells that showed an increased IL-6 and IL-8 secretion with EPS (Lambernd *et al.*, 2012; Scheler *et al.*, 2013). For the diabetic cells this is the first study that has examined cytokine secretion after *in vitro* exercise and currently, there are no available data with which to compare our results.

Earlier studies reported a difference in gene expression of IL-6 and IL-8 between diabetes and control in response to exercise. Tantiwong *et al.*(2010) and Feng *et al.* (2015) showed that both IL-6 and IL-8 gene expression are increase in response to an *in vivo* or EPS respectively in control but not in T2D skeletal muscle. Furthermore, gene expression of both of these cytokines is shown to increase in diabetes skeletal muscle in the basal state (Brown *et al.*, 2015b). The differences between cytokine gene expression and protein release in response to EPS highlights to an important remark. There are defects in diabetic muscle cells that make them resistant to exercise, but some metabolic processes such as cytokine release could be preserved. Future further efforts are needed focusing on the secretory function of skeletal muscle cells in response to exercise and the effect of these cytokines on metabolic processes.

In conclusion, control and diabetic myotubes respond in a different way to insulin, EPS and insulin plus EPS. In controls glucose uptake was significantly increased after insulin, 16 hours EPS and a combination of insulin and EPS. In contrast, diabetic muscle cell cultures failed to increased glucose uptake above basal in response to these stimuli. There are intrinsic defects that prevent diabetic myotubes from responding normally to EPS and/or insulin. The main differences are the basal increase in AMPK that failed to further increase with EPS. Furthermore, in control myotubes, AS160 activation at Thr^{642} increased in response to insulin and the combined stimulation (EPS+insulin) further increased Thr^{642} phosphorylation. However, some responses to exercise were preserved such as cytokine release after EPS stimulation that showed no differences between diabetic and control myotubes.

CHAPTER 7 : GENERAL DISCUSSION

T2D is a major global health problem, characterised by chronic hyperglycemia and peripheral insulin resistance. Resistance to the effects of insulin in insulin sensitive tissues is an early manifestation of T2D. Skeletal muscle represents the major insulin sensitive tissue in the human body, and insulin resistance in skeletal muscle is a core defect in T2D (Wu and Ballantyne, 2017).

Diabetic individuals have shown to be hypercoagulative. An association has been suggested between hyperglycaemia and high thrombin production (Rao *et al.*, 1999), and decreased insulin sensitivity correlates with elevated thrombin levels (Romano *et al.,* 2003). Key inflammatory cytokines such as IL-6, TNF-α and others that are linked to insulin resistance are reported to be increased in response to thrombin (Strande and Phillips, 2009). Inhibition of thrombin action in insulin resistant animals results in increased whole body glucose clearance, reversal of the suppression in the insulin signalling molecules IRS1 and Akt, and normalisation of elevated inflammatory markers (Mihara *et al.*, 2010).

Genetically, high heritability indices were reported in a cohort of Mexican Americans for a number of coagulation cascade's factors, including prothrombin which was strongly associated with T2D (Almasy *et al.*, 2005). A common genetic origin of both T2D and vascular disease, the "common soil" hypothesis has been proposed as well. Finally, the IRS1 gene and some variants of the TCF7L2 gene have been linked to both increased coagulopathy and insulin resistance as discovered by new genetic studies (Delgado-Lista *et al.*, 2011; Zhang *et al.*, 2016).

The studies in chapters 3, 4, set out with the aim of answering our first research question that was;

Does thrombin affect insulin action in human skeletal muscle cells and contribute directly to the insulin resistant state?

The main findings were; thrombin causes a reduction in insulin signalling characterised by decreased insulin-stimulated IRS1^{Y612} and Akt^{Ser473} phosphorylation. In addition, thrombin directly increased glucose uptake with

evidence that this is facilitated through an insulin independent pathway (AMPK mediated). The following, Figure 7-1 summarises the action of thrombin on glucose uptake in cultured human myotubes.
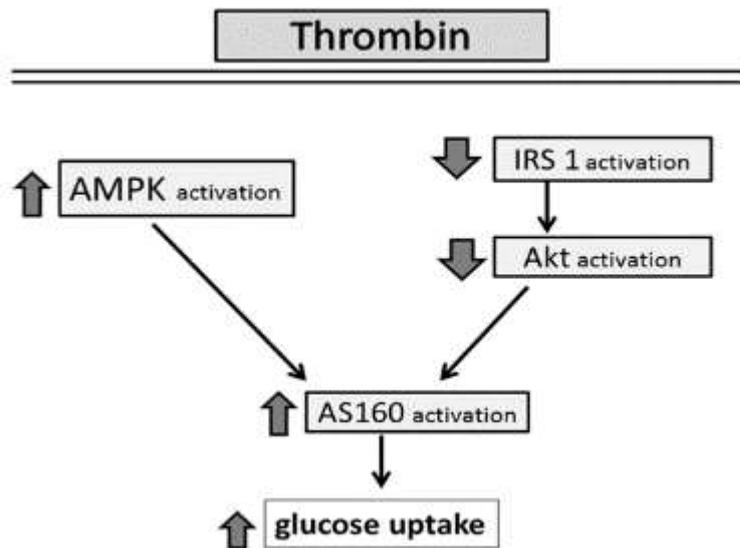
Figure 7-1: Summary of thrombin action on cultured human skeletal muscle, shaded arrows shows the effects of thrombin on activation of AMPK and insulin signalling molecules and glucose uptake.

The effect of thrombin on insulin signalling and glucose metabolism led as to ask our second research question which was:

Does thrombin affect the metabolic response to exercise in human skeletal muscle cells?

By using (EPS) as an *in vitro* system to exercise skeletal muscle cells in culture, main results were as follows; in control non-thrombin treated myotubes, 16 hours EPS causes a significant increase in both AMPK^{Thr172} phosphorylation and glucose uptake and the EPS facilitates an additive effect on insulin stimulated glucose uptake. IL-6 and IL-8 release into media were shown to increase in response to EPS. Whereas, thrombin treated cultures lose the metabolic and signalling responses to EPS that are seen in non-thrombin treated myotubes.

The nice response to EPS that were observed in control myotubes in thrombin work and the fact that primary skeletal muscle cell cultures from a diabetic person retain the key metabolic and genetic features of T2D, encourage as to ask third research question which was:

Is the contraction stimulated glucose uptake preserved in cultured muscle cells from T2D patients assessed using EPS?

The main findings were that;

In control myotubes, AMPK increased in response to EPS, glucose uptake increased after insulin and EPS. Furthermore, exercise enhanced the effect of insulin. In contrast, no significant increase in glucose uptake in diabetic myotubes was observed after insulin or EPS and EPS did not enhance the effect of insulin. In diabetic myotubes, AMPK activity was shown to be increased at rest with no further increase following EPS. The insulin enhancement after exercise was shown to be due to the distal insulin signalling pathway, AS160^{Thr642} phosphorylation was similar in both diabetes and control in response to insulin, but differs in that in control myotubes there was an additive effect of EPS on insulin stimulated AS160^{Thr642} phosphorylation that was not evident in the diabetic myotubes.

7.1 Clinical implications

Thrombin has multiple metabolic effects on insulin signalling and glucose metabolism. Pulling all these effects together, thrombin inhibited the classical insulin signalling pathway. In terms of glucose metabolism, this inhibition is by-passed through an AMPK mediated pathway. However, it is evident that insulin exerts many actions beyond glucose uptake and metabolism, and it may well be that the inhibitory effect of thrombin on the classical insulin signalling pathway decreases the action of insulin on these other non-glucose functions.

Based on our results of thrombin effects on exercise signalling, the possible clinical consequences would be a normalisation of the hypercoagulable state in T2D patients could improve the metabolic responses to exercise.

A new study by Kopec *et al.* (2017) showed that, a mutant mouse model of elevated thrombin procoagulant activity was significantly more prone to develop insulin resistance in response to high fat diet compared with wild type mice. Furthermore, treatment of these mice with a direct thrombin inhibitor, Dabigatran, reduced the high fat diet induced obesity and suppression of metabolic consequences of insulin resistance.

Inhibition of thrombin action using a chemically selective thrombin inhibitor in an insulin resistance animal model like that used by Mihara et al. (2010) and Kopec *et al.* (2017) could be considered as well. In the aforementioned studies, they treated an insulin resistant mouse model with a selective thrombin inhibitor (Argatroban and Dabigatran) and found that treated animals showed improved whole body glucose clearance and an enhancement in insulin signalling in fat cells compared with untreated animals. A similar approach might be conducted to examine both insulin signalling in skeletal muscle of such model and even the response to *in vivo* exercise protocol.

The safety of these chemically selective thrombin inhibitors should be considered and examined whether they can be used in human subjects with different hypercoagulable states including T2D.

To our knowledge, no previous studies on human have been performed to examine the effects of the different antithrombin and anticoagulants therapies that are used for treatment of variable hypercoagulable states on insulin sensitivity and/or exercise response and metabolism. Thus, it seems worthy to conduct such studies comparing whether targeting thrombin may be a beneficial therapeutic approach and could improve exercise performance and insulin sensitivity.

Primary skeletal muscle cell cultures from diabetic individuals retain the key metabolic and genetic features of T2D. Under highly controlled conditions of the cell culture system and excluding of all serum and systemic factors that can encounter. The use of EPS for comparing the metabolic response to contraction between diabetic versus control myotubes would be of great value. Our observations not only help to delineate the nature and location of the retained

molecular defects in diabetic muscle, but also help to identify normal metabolic responses that might be used therapeutically to by-pass these defects. Although our study revealed a presence of metabolic and signalling abnormalities in diabetic cells, some responses to exercise like cytokines release was preserved. Thus, even individual with severe diabetes and insulin resistance can gain some benefits from exercise.

7.2 Studies advantages

1. This is the first work to investigate the metabolic effects of thrombin on skeletal muscle cells using 2D cultures of myotubes. The work could emphasise the link between hypercoagulability and insulin resistance in T2D as we showed that increased coagulability in T2D could result in multiple metabolic effects beyond increased thrombogencity, and includes direct effects on insulin signalling and glucose metabolism.

2. The use of electrical pulse stimulation as an *in vitro* system of exercise enables pre-clinical examination and allows understanding and dissecting the molecular mechanisms of the exercise in insulin resistance and other pathology. This is the first study that use EPS in thrombin treated cultured myotubes as a model of hypercoagulation. The results of this study revealed that hypercoagulability associated with T2D might interfere with the metabolic response to contraction and exercise and could be translated clinically.

3. This work is one of the earliest studies using *in vitro* exercise system (EPS) in human cultured diabetic muscle cells. For the first time, the activity of AMPK and glucose uptake in response to EPS has been examined, excluding the effects of systemic and serum factors. EPS system showed that there are metabolic abnormalities that prevent skeletal muscle of severe insulin resistant subjects to respond to exercise in the same manner as the control. The work done so far can be extended further to examine cultures from diabetic subjects with different durations and type of therapy and has set a solid basis to examine different therapeutic approaches.

7.3 Studies limitations

Although the research reaches its aims, there were some unavoidable limitations:

First.   Cells were taken from control individuals who were not diabetic with no family history of T2D (thrombin work), and there are always inter-individual variations that might increase variability of the results of signalling and metabolic parameters. We tried to overcome this by dividing each cell lines (cultures taken from one subject) into control and treated so that only the effects of the treatment were encountered. For better results and to reduce this variability muscle biopsy to be taken from age, sex, level of activity and BMI matched individuals.

Second. The experiments were conducted on cultured cells and the limitations of cell culture systems are well known and discussed thoroughly in chapter 1 section 1.25. The lack of cell to cell contact and the interaction with other cell types via direct contact and bioactive molecules represents one of the major differences between cell culture and *in vivo* study, but the use of co-cultures with adipocytes might solve this limitation.

Third.   Third, there is a lack of knowledge of the exact physiological level of thrombin in serum *in vivo*. Most studies have determined thrombin activity rather than thrombin level. Thus, it is difficult to compare the dose used in cell culture with physiological levels. In our work, we depended on dose response and viability to determine the suitable dose that exerts effects without causing cell death. Further efforts and studies needed to determine what the physiological level is and try to use this level.

Fourth. Thrombin was used only for a short period (6 hours) while the elevated thrombin during the insulin resistant state is a chronic phenomenon. Therefore, chronic treatment of thrombin is suggested to be examined in order to explore the effects of chronic hypercoagulation on insulin sensitivity.

Fifth. The fact that contraction of skeletal myotubes in culture is non-neural mediated and so lacks the input of neurotransmitters. Moreover, EPS-induced contraction of control skeletal muscle cell cultures has been imaged as video as shown in the following link, but not in diabetic cultures yet. https://doi.org/10.1371/journal.pone.0122982.s002

However, the results related to cytokines release in response to EPS showed similar results in both controls and diabetes.

Sixth. In cultured diabetic versus control skeletal muscle cells, an issue that was not addressed in this study was whether the long duration of T2D and type of therapy could be the causative factors of failure to respond to EPS as control cultures, the solution will be discussed in future research recommendations.

7.4 Future research recommendations

Thrombin treatment was carried out for only 6 hours. It would be interesting to explore thrombin impact after a longer treatment time, and then investigating the effects of chronic thrombin treatment on insulin sensitivity. This can be achieved by different ways including either animal studies or in cell culture. The insulin resistance animal model like that used by Mihara *et al.* (2010) could be considered. However, longer duration of thrombin treatment with dose used in our work is shown to cause cell death. For example, cultures can be treated with low doses of thrombin from the day one differentiation for every day until day seven where the cell become fully differentiated to perform the experiments.

Effect of thrombin on glucose uptake was shown to be mediated via AMPK and we used a chemical inhibitor of AMPK (compound C) to explore the pathway. Compound C can affect other untargeted proteins; therefore, it would be interesting to specifically knock down AMPK expression using techniques such as siRNA as a clean way of inhibiting AMPK activity. This could help to investigate whether the observed high AMPK activity in response to thrombin treatment could be the cause of decreased metabolic response to EPS.

Furthermore, it would be worth exploring the effects of thrombin on muscle cells obtained from insulin resistant individuals. This will help to show the effects of hypercoagulation on individuals who were already T2D and if thrombin causes further resistance to the effects of insulin or further impairment of the response to EPS.

The response to EPS in cultured diabetic versus control skeletal muscle cells could be extended in different ways. To explore the steps proximal to AMPK such as upstream kinases like LKB1, CaMKKs and Calcium ions availability or the other causes of the basal elevation of AMPK.

Cytokine release in response to EPS was shown to be preserved even with the presence of the observed signalling defects. This could be utilised for further research that the pathway by which the diabetic cells preserved its ability to secrete cytokines and the metabolic benefits after these cytokines secretion.

Moreover, more research is required to determine the effects of duration of T2D and type of therapy. This can be achieved by examining cultures from diabetic and insulin resistant subjects of variable duration and different therapy and then analysing the metabolic and signalling processes that were examined in this study.

Conclusions

This is the first study that looked at thrombin and insulin action/signalling in human skeletal muscle. We found that thrombin activation results in multiple metabolic effects beyond increased thrombogenicity but does not include a decrease in insulin sensitivity (glucose uptake) in cultured human skeletal muscle cells. Furthermore, thrombin treated cultures lose the metabolic and signalling responses to EPS compared to untreated cultures. Thus, hypercoagulation associated with T2D could be involved in metabolic responses of skeletal muscle to exercise affecting both signalling, proinflammatory pathways, and glucose uptake. Additionally, metabolic abnormalities were observed in skeletal muscle of severe insulin resistant subjects. These defects prevent the diabetic muscle cells to respond to exercise in the same manner as that of the control muscle cells. However, some of the effects of exercise are preserved in diabetic myotubes represented by the inflammatory cytokines secretion in response to EPS.

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APPENDIX 1: Definition criteria of diagnosis of metabolic syndrome as considered by different associations. Adapted from (O'Neill and O'Driscoll, 2015).

WHO; World Health Organization; EGIR, European Group for the Study of Insulin Resistance; NCEP: ATPIII, National Cholesterol Education Program –

WHO	EGIR	NCEP:ATPIII	AACE	IDF
High insulin level + Two of the following: 1. Abdominal obesity WC > 37″, BMI > 30 kg/ m^2 2. Triglycerides >150 mg/ dL, HDL Cholesterol (male <35 mg/ dL) (female <39 mg/ dL) 3. BP ≥ 140/90 mm Hg 4. Microalbuminuria >30 mg/ g	High fasting insulin concentrations (insulin resistance) + Two of the following: 1. WC ≥ 94 cm (male), ≥80 cm (female) 2. Triglycerides >2mol/L, HDL Cholesterol <1.0 mmol/L or treated for dyslipidaemia 3. BP ≥ 140/90 mmHg hypertensive medication 4. Fasting glucose ≥6.1 mmol/ L	Any three of the following: 1. WC > 40″ (male) >35″ (female) 2. Triglycerides ≥150 mg/ dL, HDL Cholesterol , <40 mg/ dL (male), <50 mg/ dL (female) 3. BP ≥ 130/85 mm Hg 4. Fasting plasma glucose ≥110 mg/ dL	high risk of being insulin resistant * + Two of the following: 1. Triglycerides ≥150 mg/ dL 2. HDL Cholesterol <40 mg/ dL (male), <50 mg/ dL (female) 3. BP ≥ 130/85 mm Hg 4. Fasting glucose ≥6.1 mmol/ L or IGT	Central obesity (ethnicity and gender specific) + Two of the following: 1. Triglycerides ≥150 mg/ dL HDL Cholesterol <40 mg/ dL (male) <50 mg/dL(female) 2. BP ≥ 130/85 mm Hg 3. Fasting plasma glucose ≥5.6 mmol/ L or treatment of T2D

Third Adult Treatment Panel; AACE: American Association of Clinical Endocrinology; IDF: International Diabetes Federation. WC: west circumference. BMI: body mass index *: the AACE definition means being of high risk of insulin resistant existence of at least one of the followings: being diagnosed as hypertensive or have cardiovascular diseases; family history of T2D or history of gestational diabetes or glucose intolerance; BMI > 25 kg/m2 or WC > 94 cm (men), >80 cm (women); age >40 years.