UNIVERSITY OF KWAZULU-NATAL

Characterization of the insulin signalling pathway in skeletal muscle and skin of streptozotocin-induced diabetic male Sprague-Dawley rats: The effects of Oleanolic acid

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Characterization of the insulin signalling pathway in skeletal muscle and skin of streptozotocin-induced diabetic male Sprague-Dawley rats: The effects of Oleanolic acid.

BY

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Submitted in fulfillment of the requirements for the degree of Master of Science in the Discipline of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science

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DECLARATION

I, Andrew Mukundwa hereby declare that the dissertation entitled

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is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

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List of abbreviations

α	Alpha
γ	Gamma
δ	Sigma
Akt	Protein kinase B
ALT	Alanine transaminase
aPKC	Atypical protein kinase C
AS160	Akt substrate of 160 kDa
AST	Aspartate transaminase
β	Beta
BRU	Biomedical Resource Unit
BSA	Bovine serum albumin
bw	Body weight
$CuSO_4.H_2O$	Copper sulphate
DMSO	Dimethyl sulfoxide
DSP	Dual-specific phosphatases
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
GP	Glycogen phosphorylase
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3

HCl	Hydrochloric acid
HMIT	Highest myocardial ischemic threshold
i.p	Intraperitonial
IR	Insulin receptor
IRS	Insulin receptor substrate
IRTK	Insulin receptor tyrosine kinases
IU	Insulin units
KCl	Potassium chloride
K_2HPO_4	di-Potassium hydrogen orthophosphate
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
KOH	Potassium hydroxide
$KNaC_4H_4O_4.H_2O$	Potassium sodium tartate
LDH	Lactate dehydrogenase
MES	2-(N-morpholino) ethanesulfonic acid
MOPS	3-(N-morpholino) propanesulfonic acid
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NaF	Sodium fluoride
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
$Na_4P_2O_7$	Sodium pyrophosphate
$NaSO_4$	Sodium sulphate
Na_3VO_4	Sodium orthovanadate
OA	Oleanolic acid

OGTT	Oral glucose tolerance test
P70 S6K	p70 ribosomal s6 kinase
p- Akt	Phospho-Akt
PAS	Primer activation signal
PDK1	Phosphoinositide dependent protein kinase – 1
PGM	Phosphoglucomutase
p- GS	Phospho-GS
PH	Plekstrin homology
PI3-K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4-5-biphosphate
PIP_3	Phosphatidylinositol (3, 4, 5) triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
p.o	Oral
PP-1	Protein phosphatase 1
PTP	Protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase 1 B
s.c	Subcutaneous
SDS	Sodium dodecyl sulphate
SGK	Serum and glucocorticoid-induced kinase
SH2	Src homology 2

STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TEMED	N, N, N, N –Tetramethylethylenediamine
UDPG	Uridinediphosphoglucose
UDPGPP	UDP-glucose pyrophosphate
WHO	World Health Organization

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Abstract

Treatment of diabetes mellitus is mainly focused on glycaemic control regulated by insulin and takes place in insulin sensitive tissues like skeletal muscle which accounts for 75% of glucose metabolism. Plant derived compounds that have anti-diabetic potential are currently being investigated for diabetes treatment as they are cheap and non-toxic. Oleanolic acid (OA), a triterpene found in a wide variety of plants has been shown to have anti-diabetic effects but its mechanism of action, especially on the insulin signalling cascade has not been fully elucidated. The aim of the present study was to investigate the effects of OA on the PI3K/Akt insulin signalling cascade in skeletal muscle and skin of streptozotocin induced diabetic male Sprague-Dawley rats. Male Sprague-Dawley rats (non-diabetic and diabetic) were treated with insulin (4IU/ kg bw), OA (80 mg/kg bw) and a combination of OA + insulin in an acute and sub-chronic study. The study showed that OA does not reduce blood glucose levels in type 1 diabetic rats but enhances insulin stimulated hypoglycaemic effects. In the acute study OA was shown to activate Akt and dephosphorylate GS in skeletal muscle of streptozotocin induced diabetic rats. In the sub-chronic study OA and OA + insulin increased expression of GS in skeletal muscle of diabetic rats. GP expression was decreased by OA and OA + insulin treatments in skeletal muscle whilst in skin it was increased by both treatments. OA increased both GS and GP in skeletal muscle whilst in skin they were decreased. OA + insulin treatment increased GS and decreased GP activities in skeletal muscle and increased activity of both enzymes in skin of diabetic rats. OA increased the amount of glycogen in both muscle and skin whilst OA + insulin reduced the amount of glycogen. OA and OA + insulin treatment showed some protective effects against liver and muscle damage as there were reductions in serum LDH, ALT and AST levels. In conclusion, oleanolic acid in synergy with insulin can enhance activation of the insulin signalling pathway and there was evidence of OA activation of insulin signaling enzymes independent of insulin.

CHAPTER 1

LITERATURE REVIEW

1.0. PREAMBLE

Diabetes mellitus is one of the major diseases with a high mortality rate worldwide. It is caused by pancreatic β cell dysfunction and insulin resistance in peripheral tissues, and characterized by hyperglycaemia. People suffering from insulin dependent diabetes mellitus need insulin injections daily. The main role of insulin in the body is to lower blood glucose levels. Since the commercial production of insulin started, different insulin analogues have been produced namely; long acting insulin analogues, rapid acting insulin analogues and premixed analogues. These analogues mimic physiological insulin but with different pharmacodynamic effects. Insulin like many hormones in the body binds to specific receptors on cell surfaces triggering activation of a signalling cascade in that cell. In skeletal muscle (which accounts for approximately 75% of glucose metabolism) insulin leads to activation of the PI3-K/Akt (Phosphoinositide - 3 kinase/ protein kinase B) cascade that leads to increased glucose uptake from the blood and glycogen synthesis. The presence and activation of the upstream proteins of the PI3-K/Akt cascade has been observed in the skin but not much information is known about the downstream processes in relation to glycogen metabolism. Due to the side effects and inconvenience of taking synthetic drugs a lot of work is now being conducted into finding plant derived compounds that are less toxic but have anti-diabetic potential. Triterpenoids have been observed to have anti-diabetic activities. Oleanolic acid, a triterpenoid, has been observed to have hypoglycaemic activity and is already used clinically in the treatment of liver disorders. The aim of this study is to determine the effects of oleanolic acid on the PI3-K/Akt signalling cascade in muscle and skin of streptozotocin-induced diabetic rats.

1.1. Diabetes mellitus

Diabetes mellitus is a heterogeneous disease characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (Jacobsen *et al.*, 2009). According to recent statistics from the World Health Organization (WHO), about 3 million deaths per year are caused by diabetes and its complications. Characteristic symptoms of mild diabetes are thirst, polydipsia, and polyuria

and weight loss. In severe cases, ketoacidosis or hyperglycaemic-hyperosmolar states may occur and lead to disturbances of consciousness, coma and death unless treated appropriately (Kuzuya *et al*., 2002).

There are two types of diabetes, Type 1 and Type II diabetes. Type 1 diabetes (insulin-dependent Diabetes mellitus) usually begins in childhood and is characterized by atrophy of pancreatic β cells (Kuzuya *et al*., 2002; Varshosaz, 2007). Type 1 diabetes mellitus (T1DM) which accounts for 5-10% of diabetic patients is caused by extensive β-cell destruction, resulting in a decrease or cessation of insulin production, thus patients are absolutely dependent on exogenous insulin (Adams *et al*., 2011; Varshosaz, 2007). The condition can further be classified as immune-mediated diabetes (type 1A and idiopathic diabetes (type 1B) which affects the minority of patients (Adams *et al*., 2011). Type II diabetes mellitus (T2DM) (non-insulin-dependent diabetes mellitus) is far more common and generally occurs in patients older than 40 years. Patients normally have normal or high levels of insulin in their blood but exhibit low response to insulin. T2DM can be treated by controlling the diet of the patient although exogenous insulin can be used to supplement that produced by the pancreatic β cells (Varshosaz, 2007).Although T1DM and T2DM originate from different pathogenetic causes the effects are similar these being hyperglycaemia and diabetic microvascular complications (Jang *et al*., 2010).

Over time diabetes leads to complications involving small blood vessels that cause retinopathy, nephropathy and neuropathy, which lead to visual disturbance, renal failure and gangrene respectively (Kuzuya *et al*., 2002). T2DM has also been implicated in cardiovascular diseases that include atherosclerotic cardiovascular artery disease, stroke and diabetic cardiomyopathy (Raza and Movahed, 2003). The complications of diabetes mellitus are the main causes for its high mortality and morbidity rate (Kuzuya *et al*., 2002). Hyperglycemia is the main cause of the development and progression of these microvascular and macrovascular complications, suggesting that its control can prevent or reverse diabetic complications. Thus diabetes treatment mainly focuses on glycaemia control (DaSilva *et al*., 2010).

In healthy individuals, plasma glucose levels remain within the normal range despite large fluctuations in nutritional intake and physical activity. After healthy individuals eat, their plasma

glucose concentration increases rapidly, peaks in 30-60 minutes and returns to basal concentrations within 2-3 hours (Herbst and Hirsch, 2002). Initial insulin response to glucose intake during a meal is characterized by a rapid increase in insulin secretion that is completed within 10 minutes followed by a sustained secretion of insulin above basal rates that can last for hours. Patients with T1DM who no longer produce adequate endogenous insulin need replacement that mimics these phases of insulin production (Herbst and Hirsch, 2002).

1.1.1. Insulin

Various studies have revealed that many tissues in the body respond to insulin with effects on carbohydrate, lipids, protein and ion metabolism (Thirone *et al*., 2006).Thus in addition to the classical insulin responsive tissues (liver, muscle and adipose), insulin elicits responses in the kidney, brain and β cells (Thirone *et al*., 2006). The primary role of insulin is to remove excess glucose from the circulatory system. In mammals glucose is stored as glycogen and the conversion of glucose into glycogen in muscle and liver, and to fat in adipose tissue is a key event in the maintenance of glucose homeostasis (Newgard *et al*., 2000). In humans 80% of glycogen is stored in the skeletal muscle and its synthesis plays a key role in insulin action and glycaemic control (Jensen *et al*., 2011b; Jiao *et al*., 2001). Insulin is released from the pancreatic β cells in response to increased blood glucose and amino acids following ingestion of meals and exerts its actions through binding to specific receptors present on many cells of the body (Aronoff *et al*., 2004). There are three ways by which insulin controls postprandial glucose. Insulin signals the cells of insulin sensitive peripheral tissues, primarily skeletal muscle, to increase their uptake of glucose (Aronoff *et al*., 2004). Secondly insulin promotes glycogenesis synthesis in the liver. Thirdly insulin inhibits glucagon secretion from pancreatic α cells, thus signalling the liver to stop producing glucose via glycogenolysis and gluconeogenesis (Aronoff *et al*., 2004). Other actions of insulin include the stimulation of fat synthesis, promotion of triglyceride storage in fat cells, promotion of protein synthesis in the liver and muscle, and proliferation of cell growth (Aronoff *et al*., 2004).

Insulin is secreted as an inactive single-chain precursor, preproinsulin, with a signal sequence that directs its passage into secretory vesicles (Rains and Jain, 2011). Proteolytic removal of this signal sequence results in the formation of proinsulin. In response to an increase in blood glucose or amino acid concentration, proinsulin is secreted and converted into active insulin by special

proteases (Rains and Jain, 2011). The active form of insulin consists of two polypeptide chains (Figure 1.1). The A chain consists of 21 residues (A1-A21) and the B chain consists of 30 residues (B1-B30) (Derewenda *et al*., 1989).The two chains are held together by two disulfide bonds with another disulfide bond connecting A6 and A11 (Derewenda *et al*., 1989; Rains and Jain, 2011). There are also numerous non-covalent interactions between the amino acids of the two chains (Derewenda *et al*., 1989).

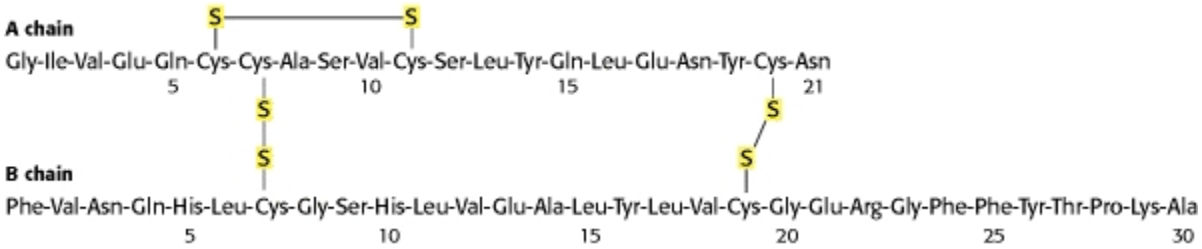


Figure 1.1: Amino acid sequence of insulin (adapted from Berg *et al*. (2002).

1.1.1.1. Insulin analogues

Insulin analogues are designed to mimic physiologic insulin profiles through improved pharmacokinetic characteristics which result in either rapid or prolonged pharmacodynamic effects (Hartman, 2008). Upon subcutaneous injection, insulin molecules form a depot from which absorption into the systemic circulation occurs. All insulin molecules have a tendency to self-aggregate into hexameric complexes and these clusters must dissociate into dimers and monomers to diffuse through interstitial fluid, penetrate the capillary wall, and enter the bloodstream (Hartman, 2008). The unique pharmacological features of individual insulin analog preparations largely involve the rate of hexamer dissociation and the subsequent movement of free insulin into the circulation (Hartman, 2008). The three types of insulin analogues are the long acting, short acting and premixed. Premixed analogues are a mixture of the long acting and rapid acting insulin analogues mixed in differing ratios. They are more convenient as they are a single product and have a further advantage of providing coverage from the beginning of a meal(Gómez-Pérez and Rull, 2005).

Rapid acting insulin analogues e.g. lyspro and aspart, are designed to offer a more rapid onset of action and shorter duration of activity than human insulin by making minor modifications to one or two amino acids on the insulin molecule (Gómez-Pérez and Rull, 2005; Hartman, 2008). The

analogues reach peaks higher than those obtained with regular insulin(Gómez-Pérez and Rull, 2005). The structural changes weaken the tendency to self-associate into hexamers, thereby facilitating more rapid absorption. The molecular changes do not alter the biological properties of the analogs in terms of binding to the insulin receptor, and the insulin analogues retain the same glucose lowering effects as human insulin (Hartman, 2008).

Examples of long acting insulin analogues are glargine and detemir. Glargine has got amino acid modifications in both chains. In the A chain Asn^{21} is substituted by glycine and the B chain is elongated at the C-terminus by addition of two arginine residues(Gómez-Pérez and Rull, 2005; Valla, 2010). Glargine is a molecule with a changed isoelectric point towards neutral, bearing decreased solubility at physiological pH (Valla, 2010). This causes precipitation after injection in subcutaneous tissue, stabilization of insulin hexamers, delay of their dissociation, and steady absorption into the circulation. This analogue bears a stable serum concentration without pronounced peaks and significantly elongated duration of action which covers the patient for 24 hours (Valla, 2010). Detemir is characterized by acylation of myristic acid to the Lys^{29} on the B chain and deletion Thr^{30} in the B chain(Gómez-Pérez and Rull, 2005; Valla, 2010). Its action is achieved through delayed resorption caused by increased self-association and reversible albumin binding at the injection site. Further albumin binding causes buffering of insulin concentration. A flat, prolonged pharmacodynamic profile is achieved which provides a metabolic profile for approximately 17 hours (Valla, 2010).

1.1.2. Synthetic hypoglycaemic agents

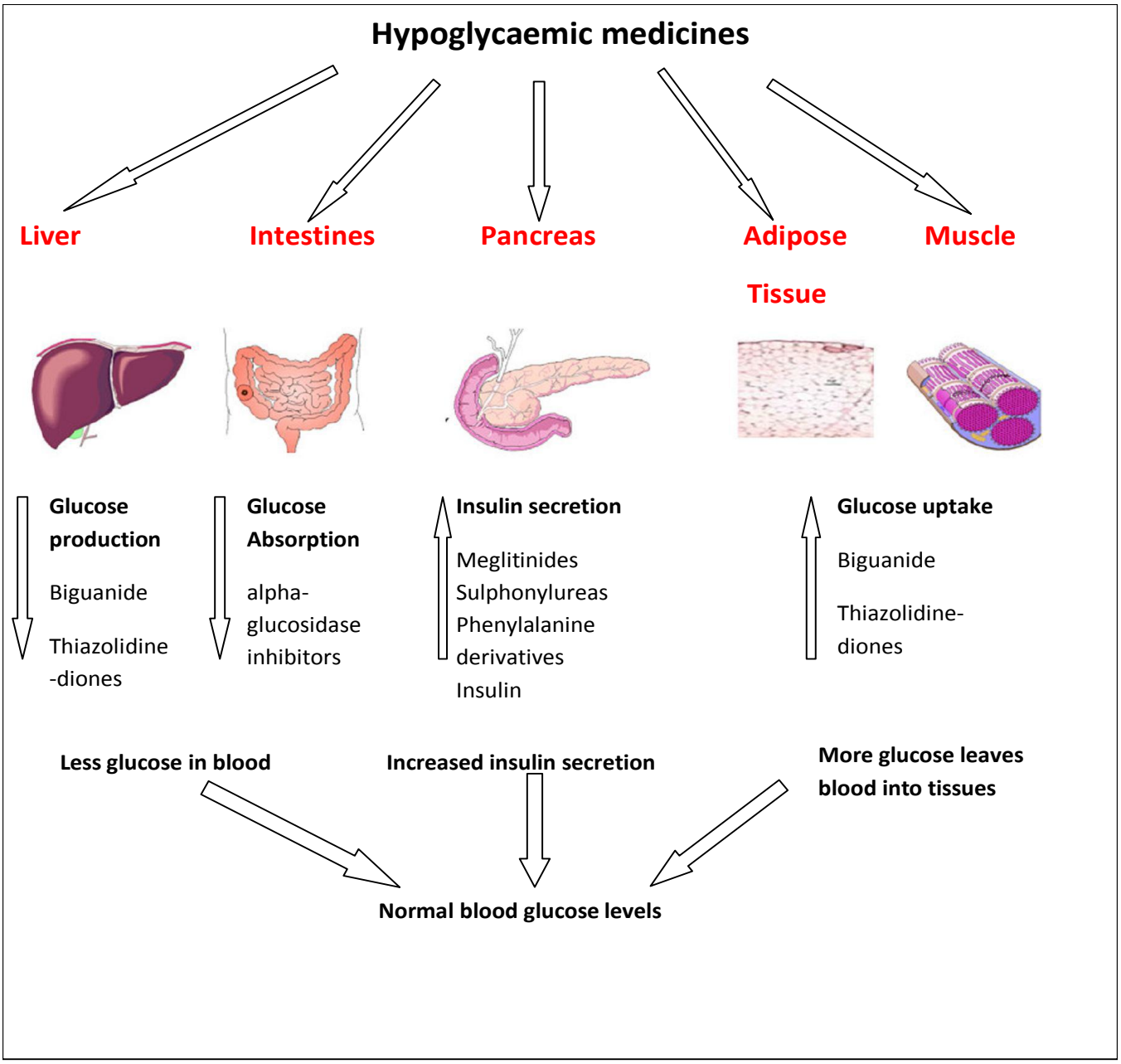


Figure 1.2: Hypoglycaemic action of different drugs and their target organs (Adapted from Hui *et al.* (2009).

Figure 1.2 shows the oral glucose-lowering agents in T2DM which include α-glucosidase inhibitors, metiglinides, sulphonylureas, biguanides and thiazolidinediones. α-Glucosidase inhibitors delay carbohydrate absorption, metiglinides and sulphonylureas increase insulin supply, and biguanides and thiazolidineodines enhance insulin action (Anselmino, 2009). The mechanism of action of α-glucosidase inhibitors relies on the reduction of glucose uptake by the

duodenum and jejunum through sites of competitive inhibition of the brush border α-glucosidase enzyme (Anselmino, 2009). Metiglinides and sulphonylureas are insulin secretagouges and depend on functioning pancreatic β-cells receptors to which they bind and enhance basal and postprandial glucose stimulated insulin secretion (Donner, 2006). Their effects might also be enhanced by peripheral effects that may include reduction of hepatic insulin clearance (Anselmino, 2009). Biguanides (e.g. metformin) depend on the availability of insulin. The drug decreases glucose production enhancing hepatic insulin response (via gluconeogenesis inhibition) (Anselmino, 2009)and to a lesser extent increase glucose utilization by skeletal muscle (Donner, 2006). Other effects include decreased appetite and food absorption and reduction in low-density lipoprotein cholesterol levels leading to weight loss (Anselmino, 2009; Donner, 2006). Thiazolidineodines action depends on the presence of insulin and resistance to its action (Anselmino, 2009). They act by enhancing insulin action in muscle and fat without increasing insulin secretion (Donner, 2006).

1.2. Glycogen metabolism in skeletal muscle and skin

In the fed-fasted transition, glycogen stores represent a major source of energy and serve to protect against hypoglycemia (Newgard *et al*., 2000). Glycogen concentrations depend on glycogen synthesis and degradation which are tightly regulated by hormones and metabolic signals via modulation of glycogen synthase and glycogen phosphorylase enzymatic activities (Jiao *et al*., 2001; Newgard *et al*., 2000; Toole and Cohen, 2007). Other rate-limiting steps in glycogen synthesis are increased glucose uptake via the insulin stimulated glucose transporter GLUT4 (Toole and Cohen, 2007) and the activity of hexokinase (James *et al*., 1998). In the rat model of diabetes, an increase in rate of glycogen synthesis in response to hyperglycaemia occurs via the dephosphorylation of glycogen synthase. However, there is no increase in glucose-6-phosphate levels (James *et al*., 1998). The insulin mediated PI3K/Akt signalling cascade regulates glucose homeostasis in the main insulin responsive tissues in the body.

The skeletal muscle is the major site for the action of insulin on glucose uptake and utilization (Jensen *et al*., 2011b; Meynial-Denisa *et al*., 2005), with approximately 75% of insulin-dependent-glucose disposal taking place in this tissue (Saltiel and Kahn, 2001). The GLUT4 transporters are the main transporters responsible for glucose uptake in skeletal muscle (Jensen *et al*., 2011b). Hyperglycemia increases blood glucose levels leading to an increase in skeletal

muscle glucose disposal (Björnholm and Zierath, 2005). The major stimulators of glucose transporters in skeletal muscle are insulin, insulin-like growth factors, muscle contraction and cellular stress (Zierath and Kawano, 2003). In the muscle glucose is converted into glycogen. Glucose-6-phosphate (G-6-P) is converted to glucose-1-phosphate (G-1-P) by phosphoglucomutase (PGM) and finally into UDP-glucose by UDP-glucose pyrophosphate (UDPGPP) (Ferrer *et al*., 2003). UDP-glucose is the glycosyl donor in the reaction catalyzed by glycogen synthase (Ferrer *et al*., 2003).

The skin is not considered as a major insulin-responsive tissue, thus little information is known about the effects of the insulin family of proteins on regulation of metabolic processes in skin (Shen *et al*., 2000). Studies by Shen *et al*. (2000) indicated that insulin differentially regulated glucose uptake as well as expression and translocation of specific transporters in the skin. Functional insulin receptors (IR) have been expressed in cultured murine skin keratinocytes (Sadagurski *et al*., 2007). It has also been shown that insulin increases the glucose transport rate, induces proliferation, and enhances differentiation in epidermal cells (Sadagurski *et al*., 2007). Lack of IR expression abolishes these insulin-induced processes and there are several skin pathologies associated with impaired insulin signalling, e.g. wound healing (Sadagurski *et al*., 2007). Pelegrinelli and co-workers in 2001 demonstrated that proteins that are involved in early steps of insulin action are expressed in skin of intact rats and are quickly activated after insulin stimulation.

1.3. PI3-K/Akt signalling cascade

Figure 1.3 shows a schematic presentation of how insulin regulates the PI3K/Akt signalling pathway. Insulin binds to the extracellular α subunits of the insulin receptor, leading to intracellular autophosphorylation of the transmembrane β subunits and enhancement of their intrinsic tyrosine kinase activity (Watson and Pessin, 2001). The activated receptor, recruits and phosphorylates the substrate molecules, insulin receptor substrates 1 / 2 (IRS1/IRS2), adapter molecules that play a major role in coupling the insulin signal to the PI3K-Akt kinases (Fröjdö *et al*., 2009). Tyrosine phosphorylated IRS1/2 recruit the heterodimeric 85/p110 PI3K at the plasmamembrane, where it produces the lipid second messenger Phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3), which activates a serine/threonine phosphorylation cascade of PH (plekstrin

homology) domain containing proteins; phosphoinositide dependent protein kinase-1 (PDK1), Akt and atypical protein kinase C (aPKC) ζ/ λ.

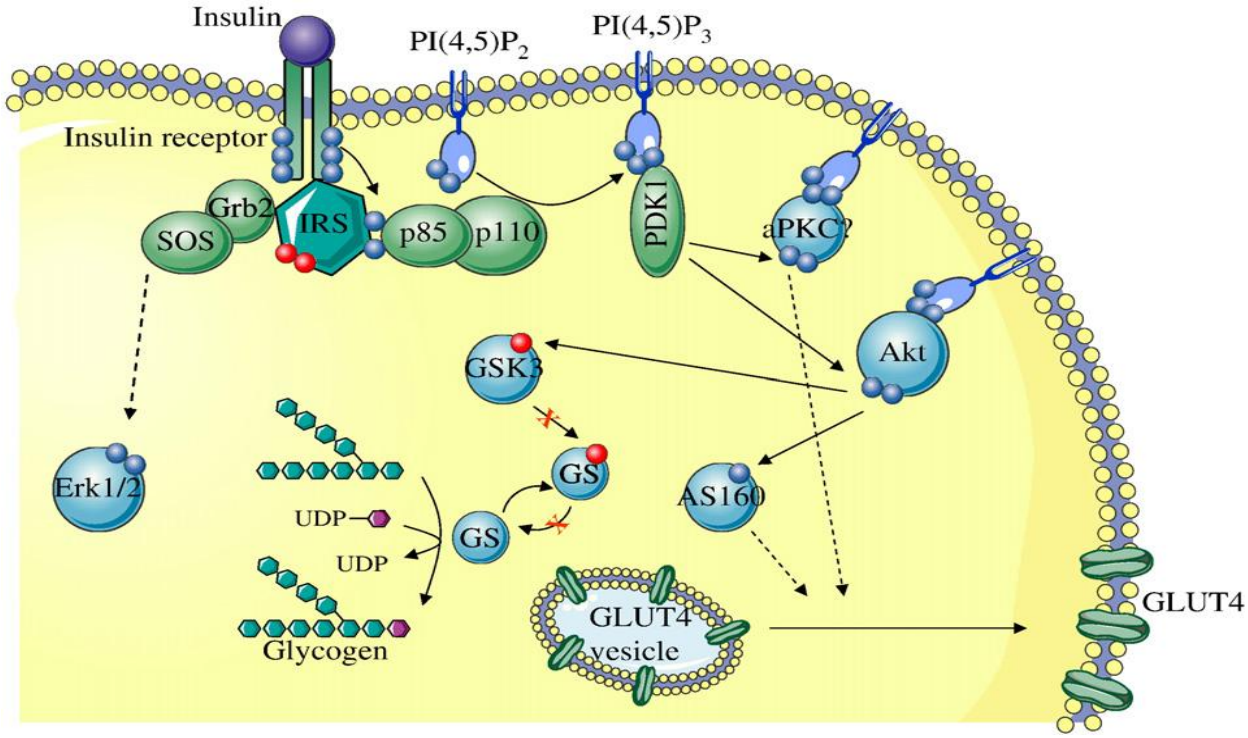


Figure 1.3: Schematic representation of the PI3K/Akt signalling pathway enzymes during glucose homeostasis (Fröjdö *et al*., 2009).

These proteins are recruited at the plasma membrane by binding to PIP_3 and thereafter, PDK1 phosphorylates Akt and aPKCs on a threonine residue located in the activation loop of the catalytic domain, causing their activation (Fröjdö *et al*., 2009). Activated Akt targets GSK-3 and AS160. Akt-mediated phosphorylation on Ser^9 inactivates glycogen synthase kinase 3 (GSK-3). This inactivation, in parallel to protein phosphatase-1 (PP1) activation, relieves the inhibitory phosphorylation of glycogen synthase (GS), which becomes activated and promotes glycogen synthesis. Akt also regulates the insulin-stimulated translocation of the glucose transporter GLUT4 at the plasma membrane, resulting in increased glucose uptake (Fröjdö *et al*., 2009). This pathway involves an inhibitory phosphorylation of the RabGTPase activating protein AS160. Inhibition of AS160 favours the GTP-loaded state of Rab and relieves an inhibitory effect towards GLUT4 translocation from intracellular compartments to the plasma membrane.

In addition to the role of Akt in controlling GLUT4 translocation, aPKCs act in parallel to /or can even be substitutive for Akt (Fröjdö *et al*., 2009).

1.3.1. Insulin receptor (IR)

Insulin receptors (IR) are found in virtually all vertebrate tissues and belong to a family of growth factor receptors, all with protein tyrosine kinase activity (Björnholm and Zierath, 2005; Pelegrinelli et al., 2001). The IR is a heterotetrameric membrane glycoprotein consisting of two α-subunits and two β-subunits that function as allosteric enzymes in which the α-subunit inhibits the tyrosine kinase activity of the β-subunit (Björnholm and Zierath, 2005; Saltiel and Kahn, 2001). Insulin binds to the extracellular α-subunit of the receptor and induces a conformational change that brings the α-subunits closer together leading to a rapid autophosphorylation of the receptor (Björnholm and Zierath, 2005; Pessin and Saltiel, 2000). The coming together of the α-subunits removes the β subunit inhibitory effect. Upon ligand binding and receptor dimerization, the kinase domain in one half of the dimer phosphorylates cytoplasmic tyrosine residues on the half of the receptor dimer. This results in a large increase in the catalytic activity of the receptor. Upon autophosphorylation of tyrosine residues at position 1158, 1162 and 1163 in the activation loop, this region swings out of the catalytic site and is stabilized in a conformation that gives unrestricted access to substrates (Nystrom and Quon, 1999). Tyrosine sites are phosphorylated on the IR creating a recognition motif for the phosphotyrosine-binding domain of the IRSs (insulin receptor substrates) (Björnholm and Zierath, 2005). In addition to tyrosine phosphorylation the IR proteins can undergo serine phosphorylation which may attenuate signalling by decreasing insulin-stimulated tyrosine phosphorylation (Saltiel and Kahn, 2001). Insulin action is also attenuated by protein tyrosine phosphatases (PTPs) which catalyze the rapid dephosphorylation of the receptor and its substances (Saltiel and Kahn, 2001).

1.3.2. Insulin receptor substrates (IRSs)

The insulin receptor substrates (IRSs) family of proteins are phosphorylated by the activated insulin receptors (Virkamäki *et al*., 1999). IRS1 is the best characterized of the IRSs, and lacks catalytic activity, but in order to effect the many biological activities of insulin, it serves as a docking protein to which different signalling proteins such as phosphatidylinositide-3-kinase

(PI3-K) bind through Src homology 2 (SH2) domains (Alonso *et al*., 2006). The IRS proteins contain a NH_2-terminal PH-domain and COOH-terminal PTB (phosphotyrosine binding) domain (Lee and White, 2004; Thirone *et al*., 2006; Virkamäki *et al*., 1999). The PH domain is involved in targeting of the IRS proteins to the membrane and the insulin receptor whilst the PTB domain is critical for recognition of the NPXpY (asparagine-proline-any amino acid-phosphotyrosine) sequence in the insulin receptor (Virkamäki *et al*., 1999). IRS1 also contains at least 21-22 tyrosine phosphorylation sites and also more than 30 serine/threonine phosphorylation sites (Björnholm and Zierath, 2005). A significant increase in the Ser/Thr phosphorylation of IRS proteins has been shown to act as a negative feedback-control mechanism that uncouples IRS proteins from their upstream and downstream effectors and therefore terminates signal transduction in response to insulin. IRS1 Ser/Thr phosphorylation is a potential molecular mechanism for insulin resistance (Alonso *et al*., 2006).

1.3.3. Phosphoinositide 3-kinase (PI3-K)

PI3-K plays an important role in many insulin-regulated mitogenic and metabolic processes including glucose uptake, general and growth-specific protein synthesis and glycogen synthesis (Pelegrinelli *et al*., 2001). The enzyme is activated by insulin, insulin-like growth factor and other growth factors. It is a heterodimeric lipid kinase capable of phosphorylating the D-3 position on the inositol ring in phosphoinositides (Shepherd, 2005). PI3-K consists of an 85kDa regulatory subunit (that binds to the IRSs) that associates with a 110 kDa catalytic subunit (phosphorylates phosphatidylinositol's) (Björnholm and Zierath, 2005; Lee and White, 2004; Shepherd, 2005). PI3-K phosphorylates phosphatidylinositol 4, 5-biphosphate (PIP_2) to phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3). Inhibition of PI3-K blocks the formation of PIP_3 within the cell and leads to the inhibition of several cellular events, most importantly GLUT 4 translocation, and thereby inhibiting insulin-stimulated glucose transport in the insulin sensitive tissues. Activation of PI3-K with insulin though is insufficient for insulin stimulated glucose transport (Björnholm and Zierath, 2005).

1.3.4. Phosphoinositide-dependent protein kinase-1 (PDK1)

PDK1 plays a central role in activating the AGC (cAMP-dependent, cGMP-dependent, protein kinase C) kinases subfamily members that include protein kinase B (PKB), p70 ribosomal S6 kinase (p70 S6K), serum and glucocorticoid-induced kinase (SGK) and protein kinase C (PKC) isoforms (Biondi, 2004; Biondi *et al*., 2000). It is a 556-amino acid containing enzyme possessing a kinase domain at its N terminus and a PH domain at its C-terminus which interacts with PIP_3 and PIP_2 (Mora *et al*., 2004). PDK1 appears to possess a hydrophobic binding site in the small lobe of its kinase catalytic domain that regulates its activity as well its interaction with substrates (Biondi *et al*., 2000). Akt also contains a PH domain and thus interacts with PIP_3/PIP_2. This allows for co-localization of Akt and PDK1 at the plasma membrane and enabling PDK1 to phosphorylate and activate Akt (Biondi, 2004; Mora *et al*., 2004). PDK1 phosphorylates Akt at Thr^{308} and the equivalent residues on PKC isoforms (Biondi *et al*., 2000).

1.3.5. Protein kinase B (Akt)

Akt is a serine/ threonine kinase that belongs to the AGC subfamily of kinases. Three isoforms have been identified, Akt 1, Akt 2 and Akt 3, all of which are ubiquitously expressed. The protein contains three functional domains: an N-terminal PH domain, a central kinase domain and a C-terminal regulatory domain containing the hydrophobic motif (HM) phosphorylation site. Full activation of Akt 1 requires phosphorylation of two specific sites, Thr^{308} in the activation loop of the kinase domain and Ser^{473} in the HM domain (Fayard *et al*., 2005). The unphosphorylated form of Akt is inactive and PDK1 phosphorylation stimulates its activity by at least 100-fold. Phosphorylation of Ser^{473} augments the activity of PDK1 phosphorylated Akt by 7- to 10 fold (Yang *et al*., 2002).The mechanism for phosphorylation of Ser^{473} is unclear, but PDK2 is believed to be involved(Björnholm and Zierath, 2005). PKB is activated as a consequence of the stimulation of PI3-K and the generation of PIP_3. PIP_3 interacts with the PH domain of Akt, recruiting the kinase to the plasma membrane and exposing a pair of serine and threonine residues for phosphorylation by PDK1 (Yang *et al*., 2002).

1.3.6. Glycogen synthase kinase 3 (GSK-3)

GSK-3 is a serine/threonine protein kinase that is highly conserved and is ubiquitously expressed. It exists in 2 isoforms, GSK-3α (51kDa) and GSK-3β (47 kDa) (Patel *et al*., 2008). GSK-3 plays a role in a wide variety of cellular functions, including several components of fuel metabolism and transcriptional regulation (Sakamoto and Goodyear, 2002). A major regulatory mechanism for GSK-3α and GSK-3β activity occurs via phosphorylation of serine residues near the amino terminus (Ser^{21} on GSK-3α, Ser^{9} on GSK-3β), which results in deactivation of the enzyme. Insulin stimulation of PI3-kinase leads to Akt activation, leading to phosphorylation of GSK-3 on $Ser^{9/21}$, and deactivation of GSK3. Deactivation of GSK-3 is then thought to promote activation of glycogen synthase (Sakamoto and Goodyear, 2002).

1.3.7. Glycogen synthase (GS)

Glycogen synthase catalyzes the incorporation of UDP-glucose into glycogen as illustrated in Figure 1.4. Phosphorylation of multiple sites on GS results in progressive inactivation whereas insulin stimulates the dephosphorylation of glycogen synthase resulting in maximal stimulation of glycogen synthesis (Brady *et al*., 1999). Increase in intracellular levels of glucose, UDP-glucose, and glucose-6-phosphate which is an allosteric effector for the activation of GS (Mandarino *et al*., 1987), also contribute to the regulation of GS (Brady *et al*., 1999). Activation of glycogen-targeted protein phosphatase 1 (PP1) by insulin produces the dephosphorylation of both glycogen synthase and phosphorylase resulting in enzymatic activation of GS and inhibition of GP (Brady *et al*., 1999). Insulin also decreases GSK-3 activity, which phosphorylates and inactivates GS. The relative contribution of PP1 activation versus GSK-3 inactivation in the regulation of glycogen synthase by insulin remains uncertain, and might vary among cell types (Brady *et al*., 1999). Glycogen synthase is the key-regulating enzyme for glycogen synthesis.

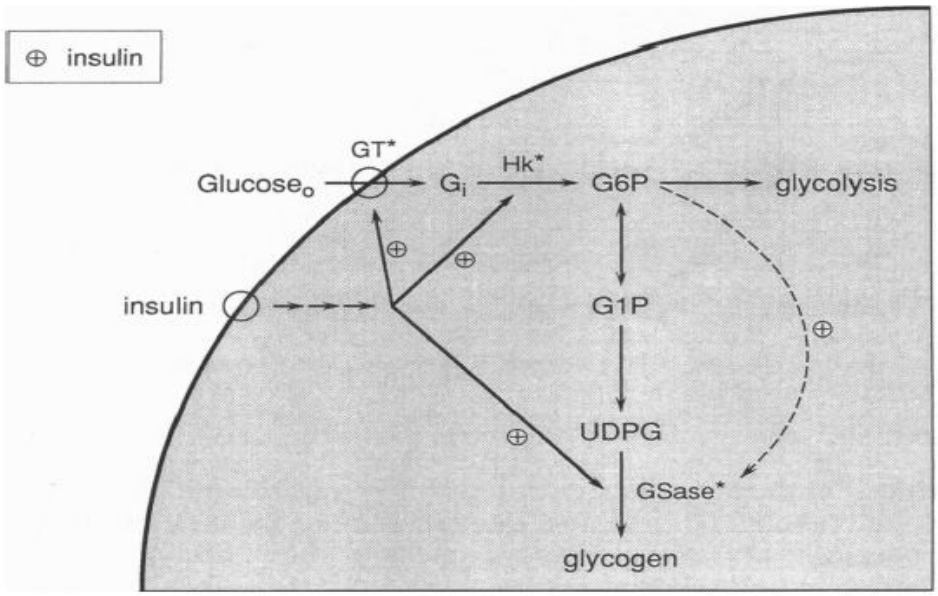


Figure 1.4: Schematic of the muscle glycogen synthesis pathway indicating the allosteric control of GS by glucose 6-phosphate (G6P). GT (glucose transporter); HK (hexokinase); Gi (intracellular glucose); GSase (glycogen synthase) Adapted from Shulman *et al.* (1995)

A correlation of glucose-6-phosphate with glycogen synthesis in rat muscle shows that, when insulin is increased from 1 to 100 mU/L, the rate of glycogen synthesis is increased but the content of glucose-6-phosphate remains unaltered, suggesting a direct effect of insulin on glycogen synthase (Dimitriadis *et al*., 2011). However this effect of insulin would not result unless glucose transport and hexokinase activity are both increased and the activity of glycogen phosphorylase is strongly inhibited by insulin (Dimitriadis *et al*., 2011).

1.3.8. Atypical PKCs (aPKCs)

Studies in animals have implicated aPKCs (ubiquitously expressed and evolutionarily conserved in many different species) in insulin-stimulated GLUT4 translocation and glucose transport in muscle and adipose tissue. In human isolated adipocytes, expression of PKC-ζ or PKC-λ increased glucose transport (Fröjdö *et al*., 2009). In lean, non-diabetic human subjects, insulin stimulation leads to a two fold increase in aPKC activity in muscle and adipose tissue. A positive relationship was also seen between insulin-stimulated PKC- ζ / λ activity and insulin-stimulated glucose disposal rate (Fröjdö *et al*., 2009). The activation of aPKCs is unclear although there are

two mechanisms that are thought to be involved (Liu *et al*., 2006). APKCs are activated in two steps: unfolding the folded state, then autophosphorylation of specific threonine residues of the aPKCs. Inactivated aPKCs exist in a folded state in which auto-inhibitory pseudo-substrate sequences at aPKCs NH_2-terminal covers the substrate binding site of catalytic domain at the COOH-terminal, not only hampering autophosphorylation of specific substrates, but also of specific threonine residue of aPKCs (Liu *et al*., 2006). The second mechanism is through the disassembly of freed kinase domain by the ubiquitin-proteasome system (Liu *et al*., 2006). After unfolding and activation, limited hydrolysis of aPKCs subunits, produce a short lived constituted active M-type kinases (PKM) that are not inhibited by pseudosubstrate-mediated auto-inhibition (Liu *et al*., 2006).

1.3.9. Akt substrate of 160 kDa (AS160)

AS160 is a 160-kDa substrate of Akt which was first identified in 3T3L1 adipocytes. It is a protein containing a RabGTPase-activating protein (GAP) domain. Phosphorylation of AS160 is required for the insulin induced translocation of GLUT4 to the plasma membrane in 3T3L1 adipocytes (Karlsson *et al*., 2005). The substrate has been shown to regulate GLUT 4 translocation in insulin sensitive 3T3-L1 adipocytes and L6 myoblasts. AS160 also contains two phosphotyrosine-binding domains and multiple putative phosphorylation sites, including six phospho-Akt substrate (PAS) targeted by Akt, AMPK, and potentially other upstream kinases (Kramer *et al*., 2006). GLUT4 vesicles are dynamic complexes that are directed by GTP-bound Rab proteins and other molecular chaperones. Thus, AS160 GAP activity could inactivate a critical, still unidentified Rab protein as part of the mechanism for controlling basal GLUT4 trafficking (Kramer *et al*., 2006). When cells are treated with insulin, however, AS160 is rapidly phosphorylated at PAS (primer activation signal) motifs and dissociates from GLUT4 vesicles. This is associated with accelerated rates of GLUT4 vesicular exocytosis, such that GLUT4 manifests predominantly at the cell surface and enhances glucose transport (Kramer *et al*., 2006).

1.3.10. GLUT 4 transporters

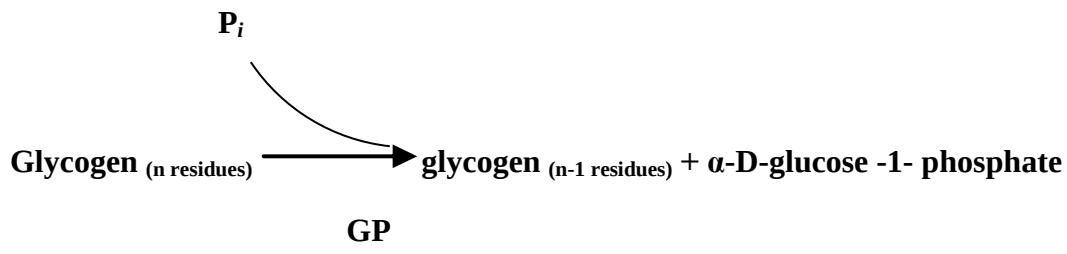
GLUT 4 is one of 13 sugar transporter proteins (GLUT1-GLUT12, and HMIT (Highest Myocardial Ischemic Threshold) encoded in the human genome. These transporters facilitate

hexose transport across cell membranes through an ATP-independent, facilitative diffusion mechanism (Huang and Czech, 2007). GLUT 4 is expressed primarily in muscle and adipose tissue and is the major insulin-responsive glucose transporter isoform (Watson and Pessin, 2001). The transporter displays mostly intracellular disposition in the unstimulated state that is acutely redistributed to the plasma membrane in response to insulin and other stimuli (Huang and Czech, 2007). GLUT4 contains unique sequences in its N- and COOH- terminal cytoplasmic domains that direct its characteristic membrane trafficking capability. These include a distinctive N-terminal sequence with a potentially critical phenylalanine residue as well as dileucine and acidic motifs in the COOH terminus. These motifs likely govern kinetic aspects of both endocytosis and exocytosis in a continuously recycling system (Huang and Czech, 2007). In the basal state GLUT 4 cycles slowly between the plasma membrane and multiple multicellular compartments, with the vast majority of the transporter residing within the cell interior. Activation of the insulin receptor stimulates the rate of GLUT 4 vesicle exocytosis, concomitant with a smaller decrease in the rate of GLUT 4 endocytosis, resulting in increased glucose uptake (Brady *et al*., 1999). The exocytic steps regulated by insulin potentially include stimulation of the formation of GLUT 4 transport vesicles, mobilization of a pool of GLUT 4 vesicles, increased docking at the plasma membrane of GLUT 4 vesicles, and/or increased fusion of GLUT 4 vesicles with the plasma membrane (Gonzalez and McGraw, 2006)

1.4. Regulation of the PI3-K/Akt insulin signalling cascade

1.4.1. Glycogen phosphorylase (GP)

GP is a dimer of identical 842-residue (97 kDa) subunits that catalyzes the rate-controlling step in glycogen degradation (Voet *et al*., 2006). It catalyzes the conversion of α (1-4) linked glucose in glycogen to glucose-1-phosphate (Toole and Cohen, 2007; Wallis *et al*., 1998).



Reaction 1: Breakdown of glycogen by glycogen phosphorylase

The enzyme is regulated both by allosteric interactions and by covalent modifications (phosphorylation and dephosphorylation). Phosphorylase *a* is the activated form and has a phosphoryl group esterified to Ser^{14} and the dephosphorylated form is called phosphorylase *b (Voet et al., 2006)*. Phosphorylase *b* is activated by phosphorylase kinase through phosphorylation at Ser^{14}. Phosphorylase kinase is in-turn activated by protein kinase A (PKA) (Voet *et al*., 2006). Phosphorylase kinase is 1300 kDa protein with four non-identical subunits (α, β, γ, δ), with the γ subunit containing the catalytic site and the other subunits having regulatory functions. The enzyme is activated by Ca^{2+} and by the phosphorylation of its α and β subunits by PKA (Voet *et al*., 2006). Type 1 diabetic humans and STZ-induced diabetic rats exhibit increased expression of (GP) gene expression in muscle. This decreases in rats after insulin treatment suggesting that the GP gene is negatively regulated by glucose uptake (Wallis *et al*., 1998). For optimal glycogen deposition to occur it is suggested that it is not only necessary to activate glycogen synthase but also the proportion of glycogen phosphorylase in the active phosphorylated form must decrease (Brau *et al*., 1997).

1.4.2. Protein Tyrosine Phosphatase (PTP)

PTPs are involved in the negative regulation of receptor tyrosine kinases (RTKs) of which the insulin receptor belongs to (Östman and Böhmer, 2001). PTPs are characterized by the presence of a signature motif, H-C-X-X-G-X-X-R. They are further categorized as dual-specificity phosphatases (DSPs) and classical PTPs (Tonks and Neel, 2001). Classical PTPs contain one or two 'PTP domains', conserved stretches of 240-250 amino acids that surround the signature motif (Tonks and Neel, 2001). The structure of classical PTPs, especially the depth of the active site cleft renders the specificity of PTPs for pTyr residues, compared with pSer or pThr (Tonks and Neel, 2001). PTP1B is a 435 amino acids form of 50 kDa, with an N-terminal catalytic domain (PTP domain), followed by two proline rich domains. It localizes in the endoplasmic reticulum through a small hydrophobic stretch found in its C terminus (Dubé and Tremblay, 2005). DSPs belong to a heterogeneous subgroup of the type-1 cysteine-based PTP superfamily. These phosphatases can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within one substrate (Patterson *et al*., 2009). PTP1B has been implicated in the attenuation of several protein tyrosine kinase (PTK) signalling pathways including the insulin receptor (Dubé and Tremblay, 2005; Elchebly et al., 1999). It has

been discovered that mice in which PTP1B gene was deleted had reduced plasma insulin and glucose levels. This was ascribed to enhanced insulin signalling through the insulin receptor and insulin receptor substrate-1 in the absence PTP1B (Hooft-van-Huijsduijnen *et al*., 2002).

1.4.3. Protein phosphatase 1 (PP1)

PP1 catalyzes the insulin-dependent dephosphorylation of glycogen synthase and also inactivation of glycogen phosphorylase, further contributing to stimulation of glycogen deposition (Newgard *et al*., 2000). The enzyme removes the phosphoryl groups from glycogen phosphorylase *a* and the α and β subunits of phosphorylase kinase (Voet *et al*., 2006). In muscle the catalytic subunit of the protein phosphatase-1 is called $PP1_c$. In its active state it is bound to a 124 kDa glycogen-binding protein, G_M, forming the PP1c-G_M phosphatase haloenzyme (PP1-G) (Aggen *et al*., 2000). The activity PP1c and its affinity for the G_M subunit are regulated by phosphorylation of the G_M at two separate sites. PP1c is activated by insulin-stimulated protein kinase (a homolog of PKA and the γ subunit of phosphorylase kinase) at site 1. PKA (which can also phosphorylate site 1) then activates PPIc at a second site, site 2. This phosphorylation causes $PP1_c$ to be released into the cytoplasm where it cannot dephosphorylate the glycogen-bound enzymes of glycogen metabolism (Voet *et al*., 2006).

1.5. Oleanolic acid

Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) shown in Figure 1.5, is a triterpenoid compound which exists widely in natural plants in the form of free acid or aglycones from triterpenoid spooning (Liu, 1995) and is marketed in China as an oral drug for human liver disorders (Song *et al*., 2006). The compound has been isolated from more than 1620 plant species and is mainly prevalent in plants belonging to the *Oleaceae* family. The main source of commercial oleanolic acid is the olive (*Olea europaea*) from which the compound derives its name (Pollier and Goossens, 2012). Insulin and synthetic agents used in the treatment of diabetes are good in controlling hyperglycaemia, but these drugs have prominent side effects such as hypoglycaemia, dropsy, drug resistance and weight gain (Gao *et al*., 2009). Compounds derived from natural resources have been determined to function in the regulation of diabetic pathophysiological signalling pathways (Liu *et al*., 2010) and being less toxic they have received more attention as antihyperglycaemic agents (Gao *et al*., 2009).

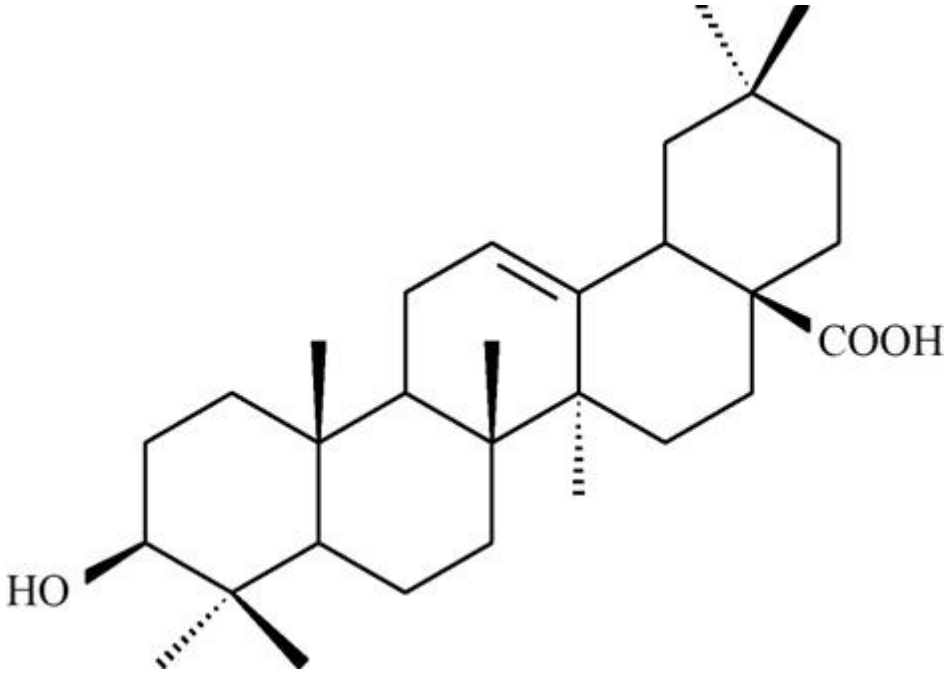


Figure 1.5: Structure of oleanolic acid (Melo *et al*., 2010)

1.5.1. Pharmacology of oleanolic acid

OA has been used as an oral remedy for the treatment of acute and chronic liver diseases in China (Feng *et al*., 2011). In a study conducted by Feng *et al*. (2011), it was shown that OA might have a beneficial effect on the vascular system by up-regulating heme oxygenase-1 (HO-1) via the PI3K/Akt and ERK (extracellular signal-regulated kinase) pathways. Due to its low solubility, OA has low bioavailability in the system. *In vitro* Caco-2 cell permeation studies showed that OA was a low permeability compound with no directional effects suggesting a low *in vivo* absorption mediated by a passive diffusion (Jeong *et al*., 2007). *In vivo* experiments on rats confirmed that OA was poorly absorbed with an absolute oral bioavailability of 0.7% (Jeong *et al*., 2007). An experiment conducted by Jeong *et al*. (2009) showed that OA was not stable in rat liver microsomes in the presence of NADPH as 60% of the OA disappeared at the end of the 1 hour incubation period. This suggested that the compound is extensively metabolized in rat liver (Jeong *et al*., 2007).

1.5.2. Hypoglycaemic effects of oleanolic acid

Gao and co-workers in 2009 showed a significant hypoglycaemic activity of OA, derived from *Ligustrum lucidum Ait*, on alloxan induced type II diabetic rats over a period of 40 days at doses

of 60 and 100 mg/kg of body weight. Hypoglycaemic activity has also been shown in adrenaline-induced type II diabetic mice which had hyperglycaemia due to increased hepatic glycogenolysis (Chen *et al*., 2006). OA was administered orally at 100 mg/kg/day for 7 days and on the last day blood glucose levels were measured after administering adrenaline intravenously. The *in vivo* test results showed that OA effectively inhibited the increase of fasted plasma glucose of diabetic mice induced by adrenaline (Chen *et al*., 2006). In experiments conducted by Wang and co-workers in 2011, preventive and therapeutic treatment of streptozotocin induced type I diabetic rats with 100 mg/kg of body weight resulted in glycaemic control. Animals that were treated with OA as a preventive measure had normal fasting blood glucose levels throughout the experiment whilst those treated after diabetic induction had fasting blood glucose levels dropping to normal after a week (Wang *et al*., 2011).

1.6. Oleanolic acid regulation of the PI3-K/Akt insulin signalling pathway

Oleanolic acid has been shown to be a natural inhibitor of glycogen phosphorylase (GP) *in vitro*, the enzyme responsible for glycogen breakdown to produce glucose and other metabolites for energy supply (Chen *et al*., 2006; Cheng *et al*., 2009; Cheng *et al*., 2010). Chen and coworkers in 2006 outlined the importance of OA being a therapeutic drug. Firstly OA may lower blood glucose levels in part by inhibiting GP, thereby reducing hepatic glucose production. Secondly OA is already a safe non-prescription drug used for the treatment of hepatitis and may find its new clinical uses in treating diseases caused by defects in glycogen metabolism (Chen *et al*., 2006; Cheng *et al*., 2010). Finally OA is widely distributed in the plant kingdom and easily available in bulk. Chemical modifications of OA could further mechanistic studies and drug development (Chen *et al*., 2006; Cheng *et al*., 2010).

Oleanolic acid has also been found to be a competitive inhibitor of protein tyrosine phosphatase1B (PTP1B) (Liu *et al*., 2010). PTP1B is a negative regulator of insulin signalling, and inhibits insulin signalling by dephosphorylating and deactivating IRTK and IRS (Liu *et al*., 2010). Studies carried out with knock-out mice suggest that lack of PTP-1B enzyme would result in activated insulin receptors, improved sensitivity to insulin, and stimulated glucose uptake (Ramírez-Espinosa *et al*., 2011). Oleanolic acid has been reported to be an inhibitor of PTP-1B with an IC_{50} value of 3.02 µM. The inhibition mechanism was determined by studying the effects of increasing inhibitors concentration on main kinetics parameters, K_m and V_{max}. The results

suggested that the inhibition mechanism of oleanolic acid could be a linear mixed-type inhibition model, suggesting a possible binding in a different cavity from the catalytic site (Ramírez-Espinosa *et al*., 2011). A derivative of oleanolic acid 3-ß-(2-carboxybenzoly)-oleanolic acid (NPLC441) was also found to be a competitive inhibitor of PTP1B, potently stimulated glucose uptake through multi-target pathways as it could increase IR and Akt phosphorylation and upregulate GLUT4 expression (Liu *et al*., 2010).

1.7. Rationale of study

Diabetes mellitus leads to disturbances in insulin mediated glucose metabolism and is characterized by hyperglycaemia. Treatment of the disease focuses on glycaemic control. Natural compounds have been investigated and OA has be shown to be anti-diabetic (Gao *et al*., 2009). Skeletal muscle account for most of the body's insulin stimulated glucose uptake (Saltiel and Kahn, 2001). The largest organ in the body is the skin which can make up to 15% of the body weight and thus a reduction in the glucose uptake would contribute to increased blood glucose levels (Spravchikov *et al*., 2001). Glucose metabolism in the skin can therefore play an important role in glycaemic control. Glucose taken up by cells is mainly converted into glycogen with GS and GP being the two regulatory enzymes that catalyze the rate-limiting steps of glycogen synthesis and degradation respectively (Rao *et al*., 1995). An understanding of how OA affects the insulin signalling cascade *in vivo* is not well elucidated. Therefore the study investigated the effects of OA on the insulin signalling cascade and the activities of GS and GP in skeletal muscle and skin.

1.8. Aims and Objectives

Aim: To determine the effects of OA on the PI3-K/Akt insulin signalling cascade in muscle and skin of streptozotocin induced diabetic male Sprague-Dawley rats.

Objectives

1. To determine the acute effect of OA administration on the expression of PI3-K/Akt insulin signalling cascade enzymes using SDS-PAGE Western Blot analysis.
2. To determine, in sub-chronic studies, the effect of OA on the expression of the PI3-K/Akt insulin signalling cascade enzymes using SDS-PAGE Western Blot analysis.
3. To measure the activities of glycogen phosphorylase and glycogen synthase in skeletal muscle and skin after treatment with OA in sub-chronic studies.
4. To determine the effect of OA on muscle and skin glycogen.
5. To measure serum lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) levels after treatment with OA.

CHAPTER 2

METHODS AND MATERIALS

2.1. MATERIALS

2.1.1. Chemicals

The oleanolic acid (OA) was purchased from Shaanxi King Stone Enterprise Company Limited, China and SIGMA-ALDRICH, South Africa. The insulin (Humalog Mix50 Kwikpen) was purchased from Eli Lilly, South Africa The following chemicals were purchased from SIGMA-ALDRICH, South Africa: Ethylene diaminetetraacetic acid (EDTA), Sodium pyrophosphate ($Na_4P_2O_7$), Sodium fluoride (NaF), Sodium orthovanadate (Na_3VO_4), Nonidet P-40, Leupeptin hemisulphate, Benzamidine hydrochloride hydrate, Phenylmethanesulfonylfluoride (PMSF), Dithiothreitol (DTT), Glycogen, 3-(N-morpholino) propanesulfonic acid (MOPS), 2-(N-morpholino) ethanesulfonic acid (MES), α-D-Glucose 1-phosphate disodium salt hydrate, Glucose-6-phosphate disodium salt, Adenosine monophosphate, Uridinediphosphoglucose (UDPG), Glycerol, Bromophenol blue, Glycine, Acrylamide, N'N'- bis- methylene-acrylamide, Dimethyl sulfoxide (DMSO), Potassium chloride (KCl), Phenol solution, di-Potassium hydrogen orthophosphate (K_2HPO_4), Methanol, β-Mercaptoethanol, Potassium dihydrogen phosphate (KH_2PO_4) and Tween-20, Streptozotocin (STZ), Sodium chloride (NaCl), Triton-X 100 and Bovine serum albumin (BSA). Copper sulphate ($CuSO_4.H_2O$), Potassium iodide (KI), Sodium hydroxide (NaOH), Citric acid, Sodium citrate, Disodium hydrogen phosphate (Na_2HPO_4), Sulphuric acid, Potassium sodium tartate ($KNaC_4H_4O_4.H_2O$), Sodium sulphate ($NaSO_4$), Hydrochloric acid (HCl), Potassium hydroxide (KOH), were purchased from MERCK, South Africa. Sodium dodecyl sulphate (SDS), Tris, Ammonium persulphate, N, N, N, N - Tetramethylethylenediamine (TEMED) and 10X Tris/Glycine/SDS buffer were purchased from either BIORAD, South Africa or SIGMA-ALDRICH, South Africa. The antibodies for Glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH), anti-phospho-Akt, anti-phospho-GS and anti-GS were obtained from Cell Signalling Technology, South Africa. The anti-GP and anti-phospho-IRS1antibodies were purchased from ABCAM, South Africa whereas the secondary antibodies, Goat anti-Rabbit IgG Dylight 650 conjugate and Goat anti-Rabbit IgG Alkaline phosphatase conjugate were purchased from Thermo Scientific, South Africa and from

BIORAD, South Africa, respectively. The radionuclides U-C^{14}-uridine diphosphoglucose (C^{14}-UDPG) and α-D-[U-^{14}Cglucose-1-phosphate were purchased from BIOTREND, Germany.

2.1.2. Animals (Ethical clearance number 081/11/Animal and 018/12/Animal)

Male Sprague-Dawley rats (250-300 g body weight) were procured from the Biomedical Resource Unit (BRU) at Westville Campus of the University of KwaZulu-Natal. The animals were fed a commercially available rat chow diet chow (Meadows, Pietermaritzburg, South Africa), *ad libitum* for the entire experimental period. Drinking water was provided for all the animals *ad libitum* for the duration of the experiment. The animals were maintained according to the rules and regulations of the University of KwaZulu-Natal (UKZN) Animal Ethics Committee.

2.2 METHODS

2.2.1. Preparation of OA

An oleanolic acid (OA) suspension was prepared in a vehicle containing DMSO: physiological saline solution (1:2). The OA was first suspended in DMSO and then diluted by an equal volume of saline. Different lower concentrations of DMSO (10% and 25%) were tried until the one that provided the most uniform slurry was found.

2.2.2. Induction of diabetes

Diabetes was induced with a single intraperitoneal (I.P) injection of streptozotocin (STZ, 60 mg/kg. body weight) dissolved in freshly prepared cold 0.1 M citrate buffer (pH 4.5). Animals with a fasting blood glucose concentration of ≥18 mM after one week were considered as stable diabetic animals before experimental procedures began. The fasting blood glucose in tail vein blood was measured using an ACCU CHEK Aviva Nano glucometer, (USA).

2.2.3. Preliminary studies to investigate the blood glucose lowering effect of OA

Male Sprague-Dawley rats were divided into 4 groups (n = 8): diabetic control (DC), diabetic OA (DO), diabetic insulin (DI) and diabetic OA + insulin (DOI). Each group was further divided into 2 sets (n = 4) and an oral glucose tolerance test (OGTT) was conducted. In the first set the animals

were fasted for 18 hours and then given glucose orally (0.86 g/kg body weight). The DC group did not receive any treatment, whilst the DO group was given 80 mg/kg body weight of OA orally. The DI group was given a subcutaneous injection of insulin (4 IU/kg body weight) and the DOI group was given 80 mg/kg body weight of OA orally + subcutaneous injection of insulin (4 IU/ kg body weight). Blood glucose levels were measured at time 0, 15, 30, 60 and 120 minutes using an ACCU CHEK Aviva Nano glucometer, (USA). The second set of animals were not fasted or given a glucose load before OGTT was performed but received the same treatment.

2.2.4. Acute effect of OA on the insulin signalling cascade.

Male Sprague-Dawley rats were divided into 8 groups as illustrated in Table 2.1 below: non-diabetic control (NC), non-diabetic insulin (NI), non-diabetic OA (NO), non-diabetic OA + insulin (NOI), diabetic control (DC), diabetic insulin (DI), diabetic OA (DO) and diabetic OA + insulin (DOI). The NC and the DC groups were orally dosed with the vehicle composed of DMSO: physiological saline (3 ml/kg body weight). The NI and DI groups were treated with insulin subcutaneously 4 IU/kg body weight. The NO and DO groups were treated with OA (80 mg/kg body weight). The NOI and DOI groups were treated with both OA and insulin. All the groups were euthanized at the following time intervals after giving them their treatment: (n = 2) at 5, 15, and 30 minutes and (n = 6) for 60 minutes. The skin and skeletal muscle were harvested, snap frozen and stored at -20 ºC.

Table 2.1: Representation of the different treatments for the acute studies

Male Sprague-Dawley rats	**Non- diabetic**				**Diabetic**			
Groups	NC	NI	NO	NOI	DC	DI	DO	DOI
Treatments	DMSO: Saline (p.o)	4 IU/kg bw (s.c)	80 mg/kg bw OA (p.o)	80 mg/kg bw OA (p.o) +4IU/kg bw (s.c)	DMSO: Saline (p.o)	4 IU/kg bw (s.c)	80 mg/kg bw OA (s.c)	80 mg/kg bw OA (p.o) +4IU/kg bw (s.c)
Treatment periods	15 minute (n = 2)							
	30 minutes (n = 2)							
	60 minutes (n = 6)							
Rats euthanized after treatment periods. Skeletal muscle and skin collected snap frozen and stored at -20 ºC for furt analysis.								

p.o; oral, s.c; subcutaneous, bw; body weight, IU; insulin units

2.2.5. Sub-chronic studies

For acute studies there were 7 day and 14 day treatments periods. In each treatment period there were 8 groups (n = 6) of male Sprague-Dawley rats designated as follows: non-diabetic control (NC), non-diabetic insulin (NI), non-diabetic OA (NO), non-diabetic OA + insulin (NOI), diabetic control (DC), diabetic insulin (DI), diabetic OA (DO) and diabetic OA + insulin (DOI). The NC and the DC groups were orally dosed with the vehicle composed of DMSO: physiological saline (3 ml/kg body weight). The NI the DI groups were treated with insulin subcutaneously (4 IU/kg body weight). The NO and DO groups were treated with OA (80 mg/kg body weight). The NOI and DOI groups were treated with both OA and insulin. All the groups were treated daily and were euthanized after 7 or 14 days. The skin and skeletal muscle was harvested, snap frozen and stored at -20 ^{0}C for further analysis. Blood was also collected in Vacutainer blood collection tubes containing thrombin and kept on ice. Serum was obtained after blood that was kept on ice for 2 hours was centrifuged at 3000 rpm

for 10 minutes using a Heraeus Labofuge 200 centrifuge, Thermoscientific (USA). The clear serum was collected in Eppendorf tubes and stored at 20 ^{0}C for further analysis

2.2.6. Tissue processing for western blots

Skeletal muscle was processed according to a method adapted from Sakamoto *et al.* (2003). Muscles samples were homogenized using an OMNI TH2 tissue homogenizer (USA) at 35 000 rpm in 4 volumes of lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Nonidet P-40, 7 µM leupeptin, 3 mM benzamidine, and 1 mM PMSF. Homogenates were then centrifuged at 14000 *g* for 10 min at 4 °C using a Beckman Coulter J26-XPI centrifuge (USA) and the supernatant collected. The protein concentrations of the supernatants were determined by the Biuret method with BSA as a standard.

The skin tissue was processed according to a method adapted from Pelegrinelli *et al*. (2001). Skin was homogenized in 4 volumes of buffer containing 1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM , $Na_4P_2O_7$, 100 mM NaF, 10 mM EDTA, 10 mM Na_3VO_4, 2 mM PMSF and 3 mM benzamidine, using an OMNI tissue homogenizer (USA) at 35 000 rpm at 4 ^{0}C. The homogenates were centrifuged at 27216 *g* and 4 ^{0}C for 45 minutes using a Beckman Coulter J26-XPI centrifuge (USA). The supernatant was collected and protein concentration was determined using the Biuret method with BSA as a standard.

2.2.7. SDS-PAGE and Western Blot analysis

SDS-PAGE was conducted according to the method adapted from Laemmli (1970). Equal amounts of proteins were resolved by SDS-PAGE using a Bio-Rad Mini - PROTEAN 3 Electrophoresis cell, BIORAD (South Africa) and BG-VerMINI Marine Electrophoresis cell from Vacutec, South Africa. The protein was electrophoresed in Tris/Glycine/SDS buffer, pH 8.3 at 110 V. The proteins on the gels were transferred to nitrocellulose membranes in Tris/Glycine/Methanol buffer, pH 8.3 using a Bio-Rad Trans-Blot Electrophoretic Transfer Cell, BIORAD (South Africa) run at 100V for 2 hours. The membranes were blocked with Phosphate buffered saline (10 mM Phosphate, 137 mM NaCl, 2,7 mM KCl, pH 7.4) containing 0.1%

Tween 20 and 1% BSA, at 4 ^{0}C overnight. The membranes were incubated in Phosphate buffered saline containing 0.1% Tween 20 (PBST) with the indicated antibodies (anti-GAPDH which acts as a loading control, anti-phospho-Akt, anti-phospho-GS, anti-GS and anti-GP) for 1 hour at room temperature. Membranes were probed with Goat anti-Rabbit IgG Dylight 650 conjugate (1:5000) and Goat anti-Rabbit IgG Alkaline phosphatase conjugate (1:1500 in PBST for 1 hour at room temperature, and antibody-bound proteins were visualized using a Syngene G-BOX Chem XR5, Vacutec (South Africa). Images were captured using the GeneSys software and the bands were quantified using GeneTools analysis software.

2.2.8. Determination of enzyme activity

2.2.8.1. Glycogen synthase activity assay

The glycogen synthase assay was adapted from Mandarino *et al*. (1987). Samples were weighed still frozen and immediately homogenized (50 mg tissue/ml) in an ice-cold buffer consisting of 2 mM DTT, 20 mM NaF, 20 mM EDTA, and 50 mM potassium phosphate buffer, pH 7.4 using an OMNI tissue homogenizer (USA) at 35 000 rpm. The homogenates were centrifuged at 20000 *g* for 20 minutes using a Beckman Coulter J26-XPI centrifuge (USA). Protein concentration was determined using the Folin-Lowry method and BSA used as a standard. The supernatant was diluted 1:5 in a buffer consisting of 20 mM EDTA, 25 mM NaF and 50 mM Tris-HCl, pH 7.8. Reactions were started by the addition of 50 µl of the sample to 50 µl of a reaction mixture composed of 20 mM EDTA, 25 mM NaF, 50 mM Tris-HCl (pH 7.8), 1% glycogen, 1 µCi/ml [U-^{14}C]uridinediphosphate glucose (UDPG), 5 mM UDPG, and 10 mM glucose-6-phosphate (G6P). The reaction mixture was incubated for 20 minutes at 30 °C. The reactions were terminated by adding 50 µL of the reaction mixture onto a 2 x 2 cm square filter paper which is immediately immersed in 70% (v/v) ethanol to precipitate glycogen. Filter papers were washed twice in 70% ethanol for 30 minutes, dried and placed into scintillation vials containing 3 ml FLUKA scintillation cocktail and the amount of ^{14}C determined. For each set of assays three blanks were prepared using buffer instead of tissue extract to determine background counts, which were subtracted from the tissue extract counts. The activity was expressed per mg of protein

2.2.8.2. Glycogen phosphorylase assay

The glycogen phosphorylase assay was adapted from Taylor *et al*. (2006). Samples were weighed still frozen and homogenized (100 mg/ml) in an ice cold solution of 10 mM MOPS, 50 mM NaF, 5mM EDTA, and 1 mM DTT, pH 7.0. The mixture was homogenized using an OMNI TH2 tissue homogenizer (USA) using a speed of 35 000 rpm at 4 °C. The homogenates were centrifuged at 4°C for 10 minutes at 9000 *g* using a Beckman Coulter J26-XPI centrifuge (USA). Protein concentration was determined using the Folin-Lowry method and BSA used as a standard. Total GP activity was measured in the reverse direction by the incorporation of radiolabelled D-glucose-1-phosphate into glycogen at 30 °C. The reaction was started by adding 50 µL of sample to 50 µL of assay buffer containing 33 mM MES, 0.34% glycogen, 22.3 mM G-1-P and 1 µCi/ml D-[U^{14}C]-glucose-1-phosphate and incubating the mixture for 20 min. The reactions were terminated by adding 50 µL of the reaction mixture onto a 2 x 2 cm square filter paper which is immediately immersed in 70% (v/v) ethanol to precipitate glycogen. The filter papers were washed twice for 30 minutes in 70% ethanol, dried and placed into scintillation vials containing 3 ml FLUKA scintillation cocktail and the amount of ^{14}C determined. For each set of assays three blanks were prepared using buffer instead of tissue extract to determine background counts, which were subtracted from the tissue extract counts. The activity was expressed per mg of protein.

2.2.8.3. Serum enzymes analysis

Serum alanine transaminase (ALT) aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured using a Labmax Plenno biochemical analyzer, Brazil. AST catalyzes the transfer of the amino group from L - aspartate to α - ketoglutarate to yield oxaloacetate and L - glutamate. Malate dehydrogenase then catalyzes the reduction of oxaloacetate with simultaneous oxidation of NADH to NAD^+. The resulting decrease in absorbance at 340 nm is directly proportional to AST activity. The ALT catalyzes the conversion of L-alanine and α - ketoglutarate to pyruvate and L-glutamate. LDH then catalyzes the oxidation of pyruvate with simultaneous oxidation of NADH to NAD^+. The rate of decrease in absorbance of the reaction mixture at 340 nm, due to the oxidation of NADH is directly proportional to the ALT activity. LDH catalyzes the reversible oxidation of L - lactate to pyruvate with concurrent reduction of

NAD^+ to NADH. The rate in change of absorbance at 340 nm is directly proportional to LDH activity.

2.2.9. Biuret and Folin-Lowry assays for protein determination

Protein concentration was determined using a modified biuret assay (Gornall *et al*., 1949). Protein standards were prepared using bovine serum albumin (BSA). The biuret reagent was prepared by dissolving 3 g of $CuSO_4.H_2O$ and 9 g of $KNaC_4H_4O_4.H_2O$ in 500 ml of 0.2 M NaOH. 5 g of KI was added and volume made up to 1 liter with 0.2 M NaOH. 3 ml of the biuret reagent was added to 2 ml of protein sample. The resulting solution was mixed and incubated for 10 minutes at 37 ^{0}C in a water bath. The tubes were cooled and the absorbance was read at 540 nm using a Varian *Cary 50 UV/Vis* Spectrophotometer, USA. The protein standards were plotted against their known protein concentrations in the range of 0-10 mg/ml. A standard curve was drawn and the protein concentrations calculated.

Protein concentration was also determined by using the Lowry method (Lowry *et al*., 1951). Solutions were prepared as follows: Alkaline sodium carbonate reagent (20 g/litre Na_2CO_3 in 0.1 M NaOH) and copper sulphate-sodium potassium tartrate solution (5 g/litre $CuSO_4.5H_2O$ in 10 g/litre Na, K tatrate). On the day of the experiment alkaline reagent was prepared by mixing alkaline sodium carbonate solution and copper sulphate–sodium potassium tartrate solution 50:1 whereas the Folin Ciocalteu reagent was prepared by diluting stock 1:2 with deionized water. To 0.5ml of sample, 5 ml of alkaline reagent was added and incubated at 40 °C for 15 min. Samples were cooled followed by the addition of 0.5 ml Folin's reagent, vortexing and incubation at room temperature for 30 minutes. Absorbance was read at 600 nm. BSA was used as a standard range (20 - 100 µg). The concentration of each sample was extrapolated from the standard curve graph.

2.2.10. Determination of glycogen content

The skeletal muscle and skin glycogen content was determined by a method adapted from Nader and Esser (2001). The tissues (0.5 g) were weighed and boiled for approximately 30 minutes in 1

ml of 30% KOH saturated with $NaSO_4$. After the tissues were completely digested, glycogen was precipitated with 4 ml of 95% ice-cold ethanol and incubated on ice for 30 minutes. The tubes were centrifuged for 30 minutes at 20000 *g*. The pellets were re-suspended in 1 ml H_2O followed by the addition of 5% phenol. A known concentration of glycogen was used in place of the sample for the standards. A colorimetric reaction was obtained by the addition of 5 ml sulphuric acid followed by incubation on ice for 30 minutes. The absorbance was measured at 490 nm using an UVmini-1240, UV-VIS spectrophotometer (Shimadzu, Corporation, Kyoto, Japan). Water was used as a blank. The standard curve was constructed using glycogen in the range of 0-100 µg/ml.

2.2.11. Data analysis

All data was expressed as mean ± standard error of mean (SEM). Statistical analysis was done using Graph Pad InStat Software (version5, GraphPad Software, San Diego, California, USA). Statistical comparisons of data between groups were done using one way one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test. Values of $p< 0.05$ were taken as statistically significant.

CHAPTER 3

RESULTS

3.1. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopic studies of OA

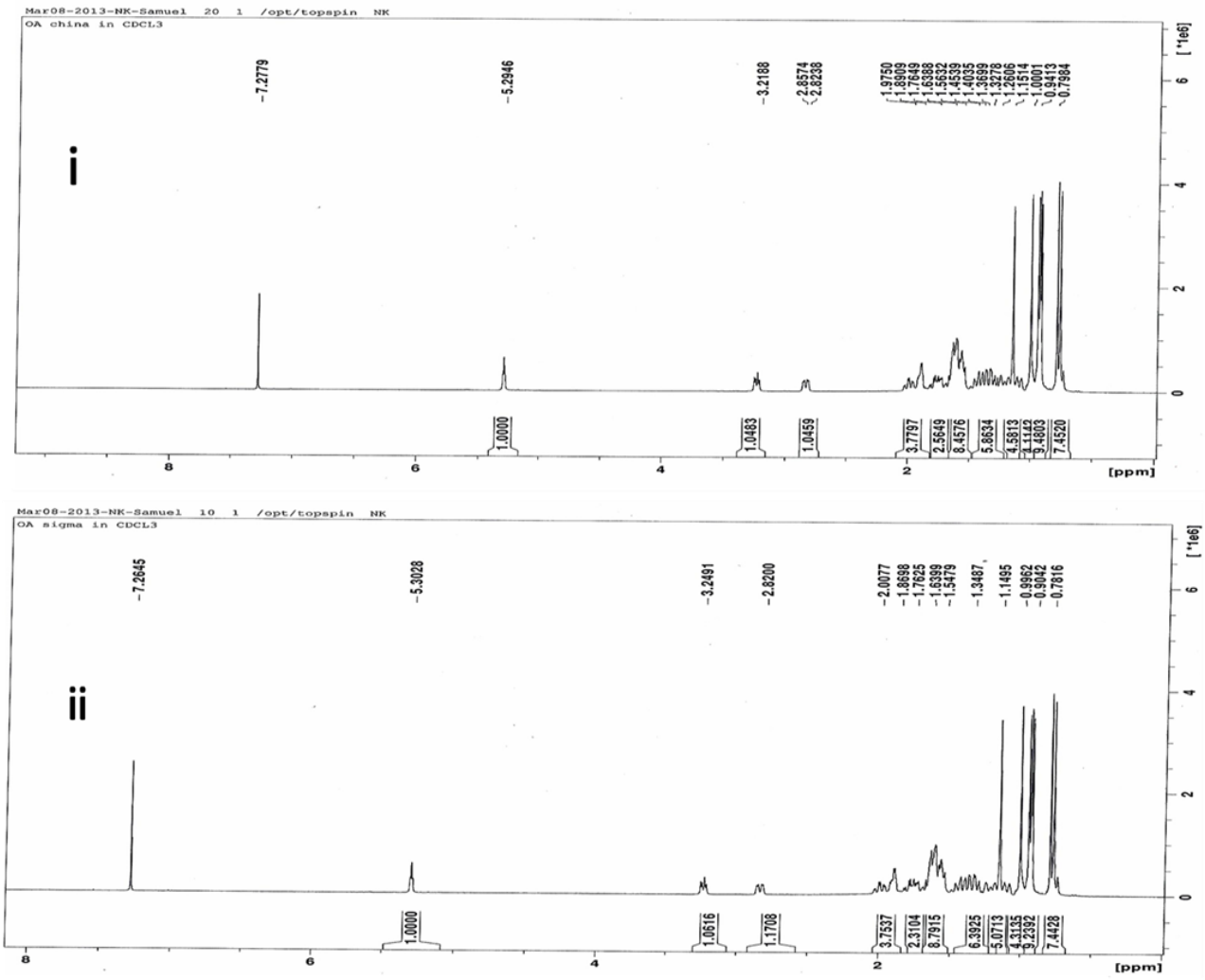


Figure 3.1.1: ^{1}H NMR spectra of OA dissolved in deuterated chloroform (i) from Sigma Aldrich and (ii) Shaanxi Kingston Enterprise Company Limited.

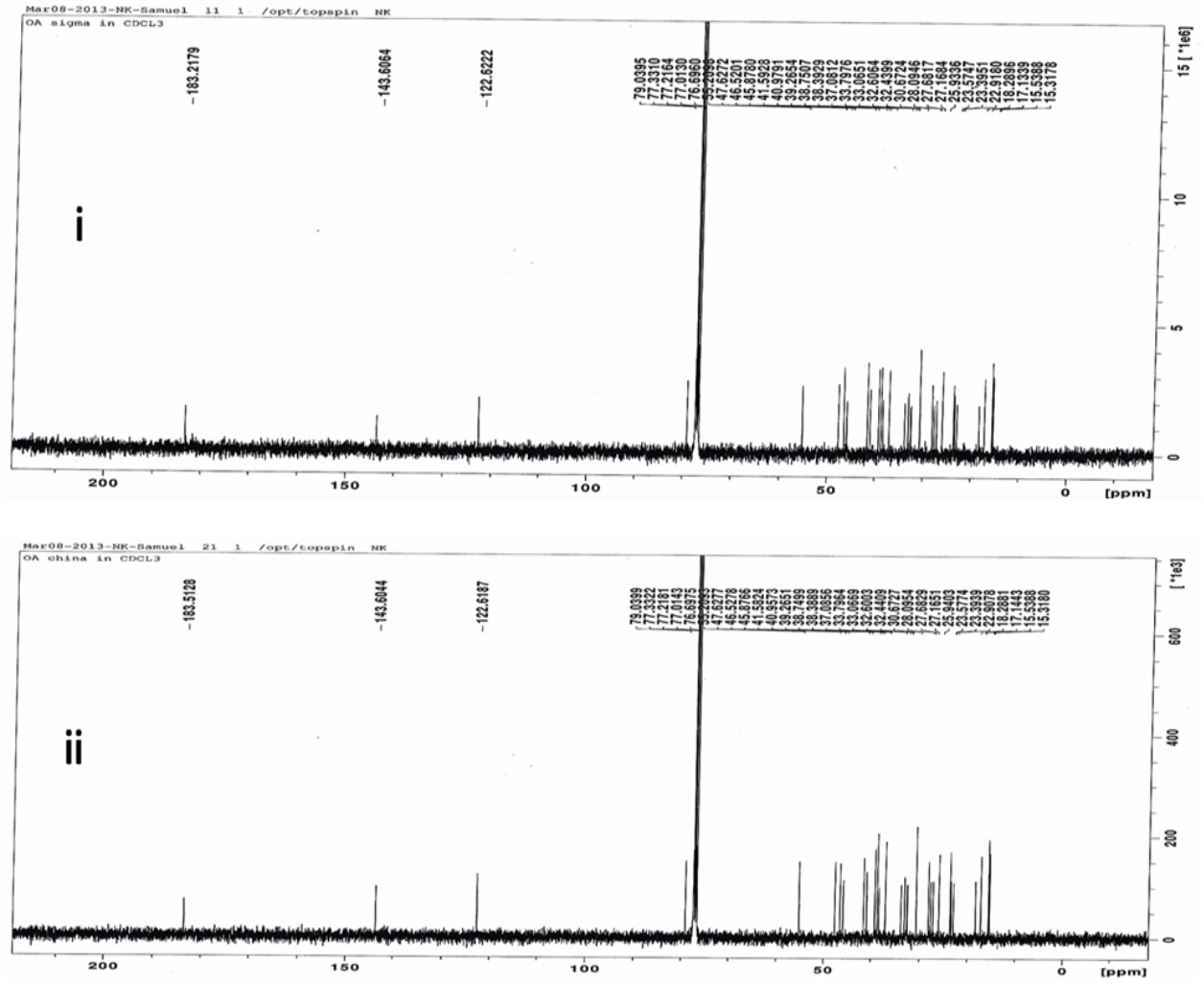


Figure 3.1.2: ^{13}C NMR spectra of OA in deuterated chloroform (i) from Sigma Aldrich and (ii) Shaanxi Kingston Enterprise Company Limited.

Figures 3.1.1 and 3.1.2 show, respectively, the ^{1}H and ^{13}C (NMR) spectrums to compare OA from Sigma Aldrich that was used for the oral glucose tolerance test (OGTT) and that from Shaanxi Kingston Enterprise Limited Company which was used in the rest of the study due to the prohibitive cost of the former. In Table 3 are listed the ^{13}C (NMR) peaks obtained for OA from the two suppliers. The OAs' were similar as they had peaks at the same ppm values.

Table 3.1: ^{13}C (NMR) data comparison of OA from (i) Sigma Aldrich and from (ii) Shaanxi Kingston Enterprise Limited Company.

(i) δ (ppm)	**(ii) δ (ppm)**
183.2179	183.5128
143.6064	143.6044
122.6228	122.6187
79.0395	79.0399
77.3310	77.3322
77.2164	77.2181
77.0130	77.0143
76.6960	76.6975
55.2090	55.2093
47.6272	47.6277
46.5278	46.5278
45.8780	45.8766
41.5928	41.5824
40.9791	40.9573
39.2654	39.2651
38.7507	38.7499
38.3929	38.3889
37.0812	37.0856
33.7976	33.7964
33.0651	33.0669
32.6064	32.6003
32.4399	32.4409
30.6724	30.6727
28.0946	28.0954
27.6817	27.6829
27.1684	27.1651
25.9336	25.9403
23.5747	23.5774
23.3951	23.3939
22.9180	22.9078
18.1896	18.2881
17.1339	17.1443
15.5388	15.5388
15.3176	15.3180

3.2. Oral glucose tolerance test (OGTT)

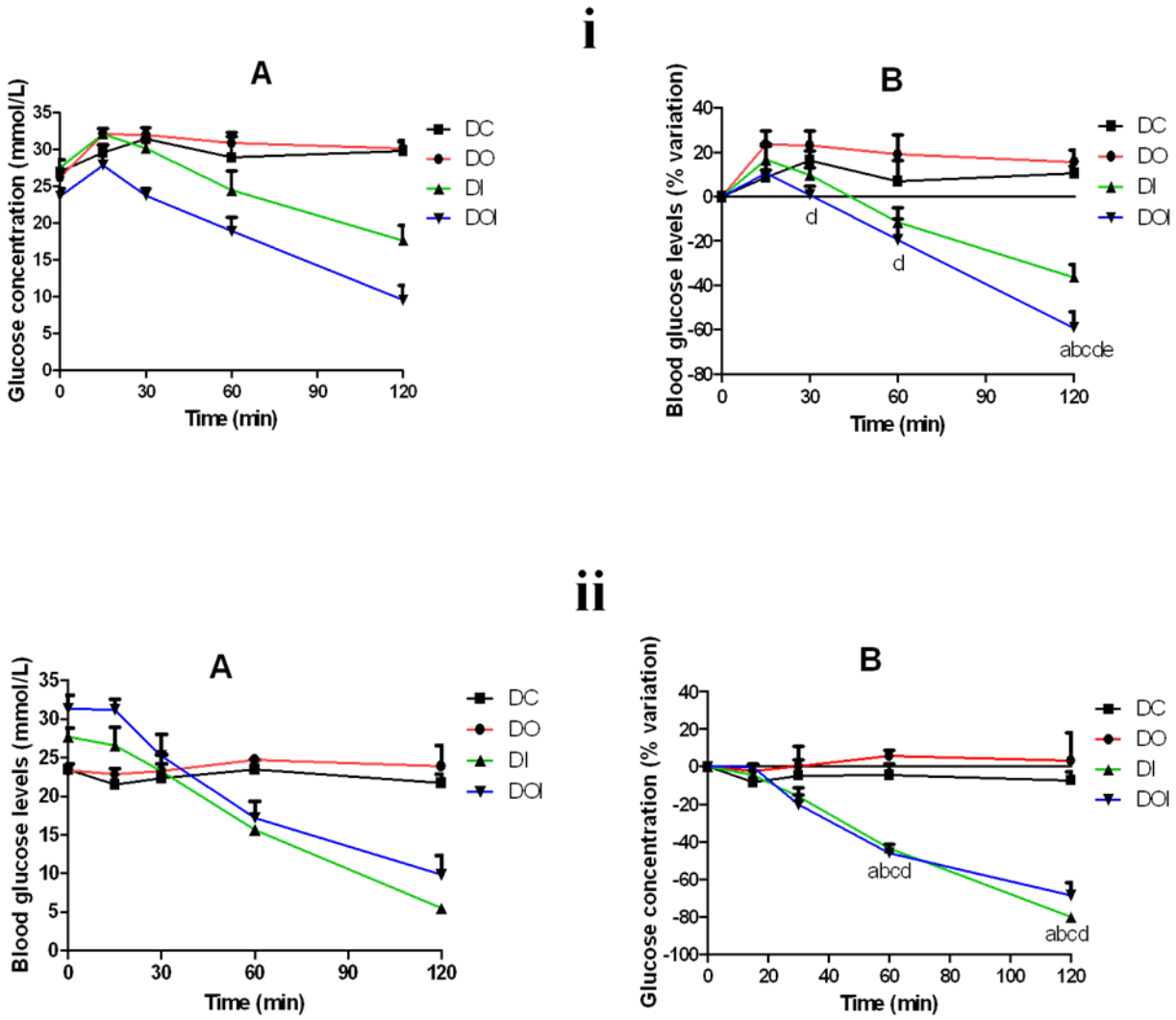


Figure 3.2: Fasting (i) and non-fasting (ii) blood glucose levels of diabetic male Sprague –Dawley rats (n = 4) over a 2 hour period. (A) Shows the blood glucose concentrations expressed in mmol/L. (B) shows the blood glucose levels expressed as percentage variations from the blood glucose levels at time 0. The data is represented as mean ± SEM. a $p<0.05$, DI compared to DC, b $p<0.05$, DOI compared to DO, c $p<0.05$, DI compared to DO , d $p< 0.05$, DOI compared to DO and e $p<0.05$ DOI

The effects of OA on fasting and non-fasting blood glucose levels of STZ induced diabetic rats were investigated. Figure 3.2 above shows the fasting and non-fasting blood glucose levels of diabetic male Sprague-Dawley rats over a 2 hour period. The blood glucose levels are expressed as mmol/L (**A**) and also as % variation in blood glucose levels from time 0 (**B**). OA did not reduce the blood glucose levels over the duration of the experiment in both the fasted and non-fasted animals. The treatment of animals with insulin alone or with insulin in combination with OA caused a reduction in blood glucose levels. The fasted rats that were treated with OA + insulin had significantly lowered blood glucose levels ($p<0.05$) after 30 and 60 minutes of treatment when compared to the OA treated group. After 120 minutes, there was a significant lowering of blood glucose levels ($p<0.05$) by insulin ± OA when compared to the OA treatment and control in both the fasted and non-fasted animals. In the fasted rats OA enhanced the blood glucose lowering effect of insulin as there is a significant decrease in blood glucose levels ($p<0.05$) after 120 minutes in insulin + OA treated animals compared to those treated with insulin alone.

3.3. SDS-PAGE gels for muscle and skin homogenates

Figure 3.3 below shows a representative SDS-PAGE gel of skeletal muscle and skin homogenates. The protein distribution in a 10% polyacrylamide reducing gel is shown for all the treatment groups. Of note are the high molecular weight proteins in skin homogenates which are largely absent in muscle homogenates. This type of gel was subsequently used for Western blot experiments.

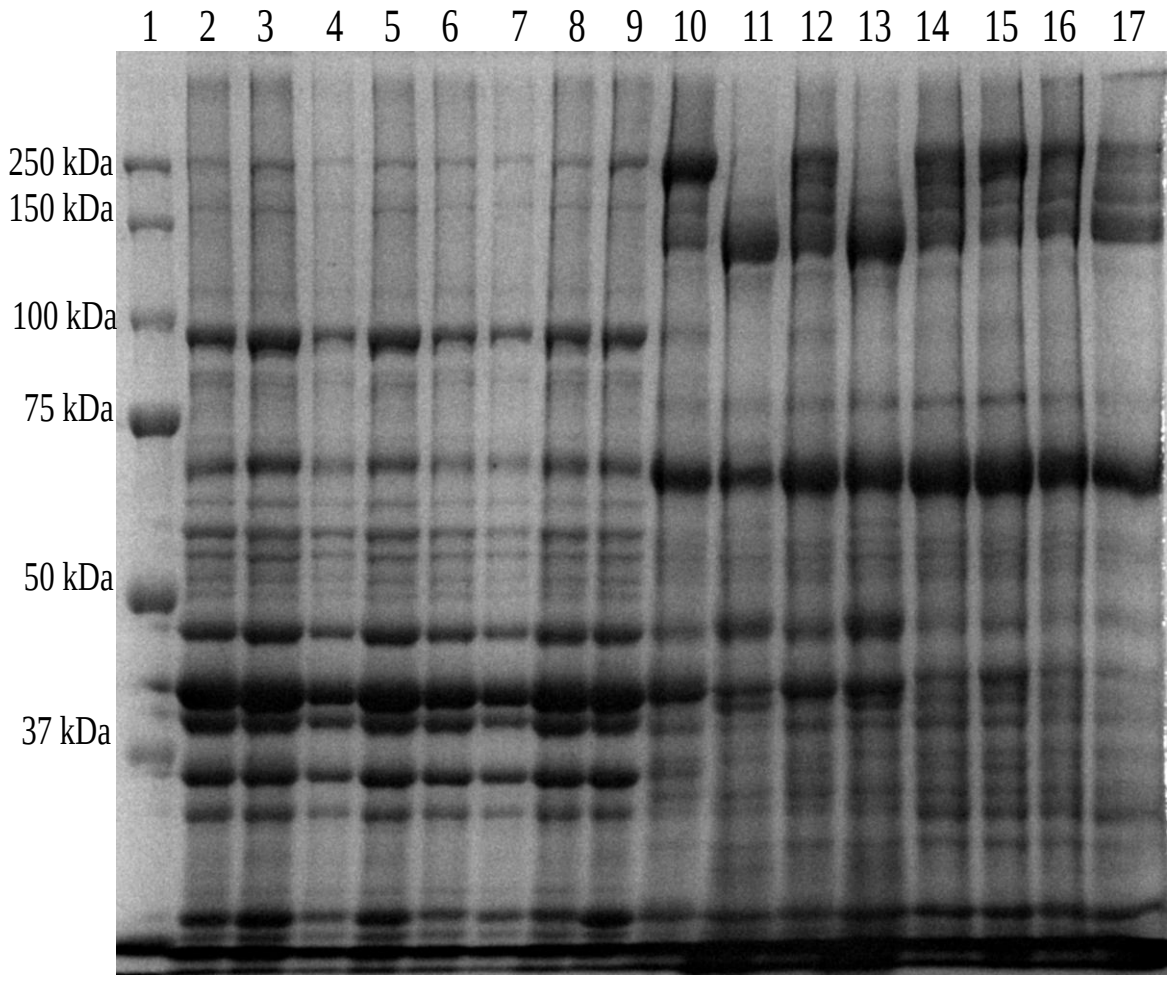


Figure 3.3: Coomassie stained 10% polyacrylamide SDS-PAGE reducing gel of skeletal muscle (lanes 2-9) and skin homogenates (lanes 10-17). Lanes: 1 (molecular weight marker) 2 and 10 (NC), 3 and 11(DC), 4 and 12 (NI), 5 and 13 (DI), 5 and 16 (NO), 7 and 14 (DO), 8 and 16 (NOI), 9 and 17 (DOI). 40 µg o proteins was load or muscle and 110 µg o protein or skin loaded.

3.4. Acute studies on effects of OA on expression of enzymes of the insulin signalling pathway

Effects of OA on the activation of selected enzymes involved in the PI3-K/Akt insulin signalling cascade in muscle and skin were studied. The enzymes were phospho-Akt (Ser 473), phospho-

GS (Ser 641) and GS with GAPDH used as an loading control. Phospho-GS and GS were not detected in skin homogenates but phospho-Akt was detected at one time interval (30 minutes).

3.4.1. Effects of OA on expression of phospho-Akt (p-Akt) in muscle

After 15 minutes following treatment the expression of p-Akt was higher in diabetic animals compared to their respective treatment groups in non-diabetic animals (Figure 3.4.1 below). The highest expression of p-Akt was to be found in diabetic animals treated with insulin followed by the diabetic group treated with insulin plus OA and then the normal group treated with OA plus insulin and finally the diabetic group treated with OA. The level of p-Akt expression generally declined with time in all groups to reach lowest levels after 60 minutes. It is noted that expression levels were low in both control groups throughout the experimental period as did those in the normal groups treated within insulin or OA. Also of note is the independent activation of Akt phosphorylation by OA that declined with time to reach the lowest levels at 60 minutes following treatment.

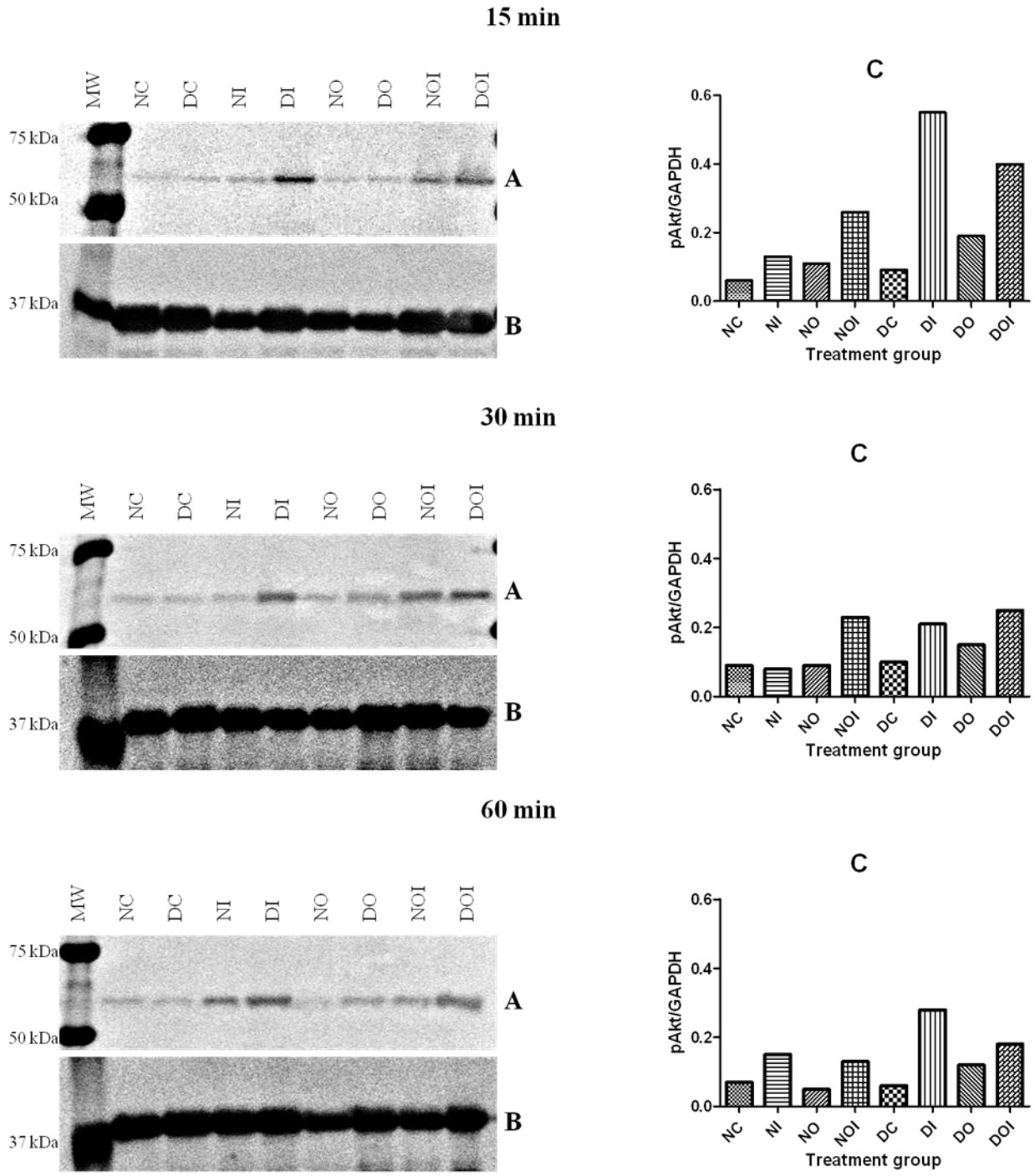


Figure 3.4.1: Western blots of (A) phospho-Akt (Ser 473) and (B) GAPDH in muscle homogenates 15, 30 and 60 minutes following treatment. 40 µg of protein was loaded for A and 20 µg for B. (C) is the normalized data showing the ratio of phospho-Akt/GAPDH. The normalized data is grouped as non-diabetic and diabetic animals for clarity.

3.4.2. Effect of OA on expression of phospho-GS (p-GS)

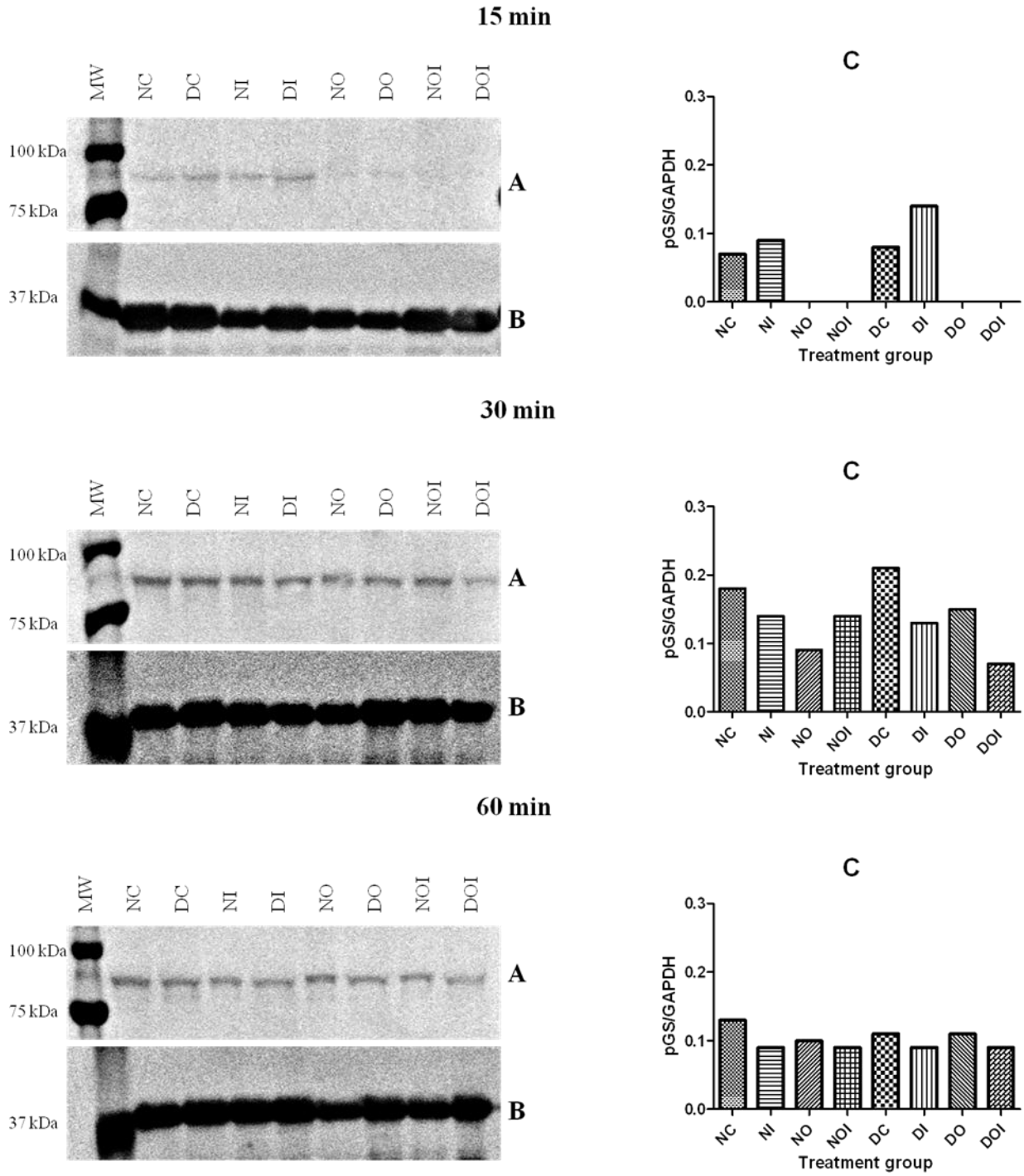


Figure 3.4.2: Western blot of (A) phospho-GS (Ser 641) and (B) GAPDH in muscle homogenates 15, 30 and 60 minutes following treatment. 40 µg of protein was loaded for A and 20 µg for B. (C) is the normalized data showing the ratio of phospho-GS/GAPDH. The normalized data is grouped as non-diabetic and diabetic animals for clarity.

Figure 3.4.2 above shows the expression p-GS 15, 30 and 60 minutes following treatment. After 15 minutes the expression of p-GS was higher in diabetic control animals and diabetic animals treated with insulin compared to non-diabetic animals that received the same treatment whilst p-GS expressed in diabetic and non -diabetic animals that were treated with OA or OA plus insulin could not be quantified by densitometry. The p-GS was expressed in all the treatment groups after 30 minutes following treatment and all treatments had lower enzyme expression than the respective group controls showing net suppression of GS phosphorylation. The lowest p-GS expression was in the diabetic group treated with OA plus insulin, followed by the non-diabetic animals treated with insulin and then the diabetic animals treated with insulin. Non-diabetic animals that were treated with insulin or OA as well as diabetic animals treated with OA had the same p-GS expression. The p-GS expression after 60 minutes was generally low in all treatment groups.

3.4.3 Effect of OA on expression of GS in muscle

Figure 3.4.3 below shows the total GS expression 15, 30 and 60 minutes following treatment. After 15 minutes, the insulin treated diabetic and non-diabetic animals had high GS expression, with the highest expression being in the diabetic animals. Of note is that the enzyme expressed in diabetic and non-diabetic animals treated with OA plus or minus insulin could not be quantified by densitometry after 15 minutes following treatment. All the treatment groups had GS expressed after 30 minutes following treatment with expression being lower than that in the group controls. It was noted that GS expression in the normal OA plus insulin treated group, diabetic insulin and OA treated groups remained almost the same after 30 and 60 minutes following treatment. The changes in GS expression from 30 to 60 minutes following treatment showed that insulin treated normal animals had a slight decrease whilst there was a slight increase in normal OA treated animals. In diabetic OA plus insulin treated animals the change in expression was almost a 2 fold increase. The GS expressed in control groups after 30 and 60 minutes following treatment was almost the same.

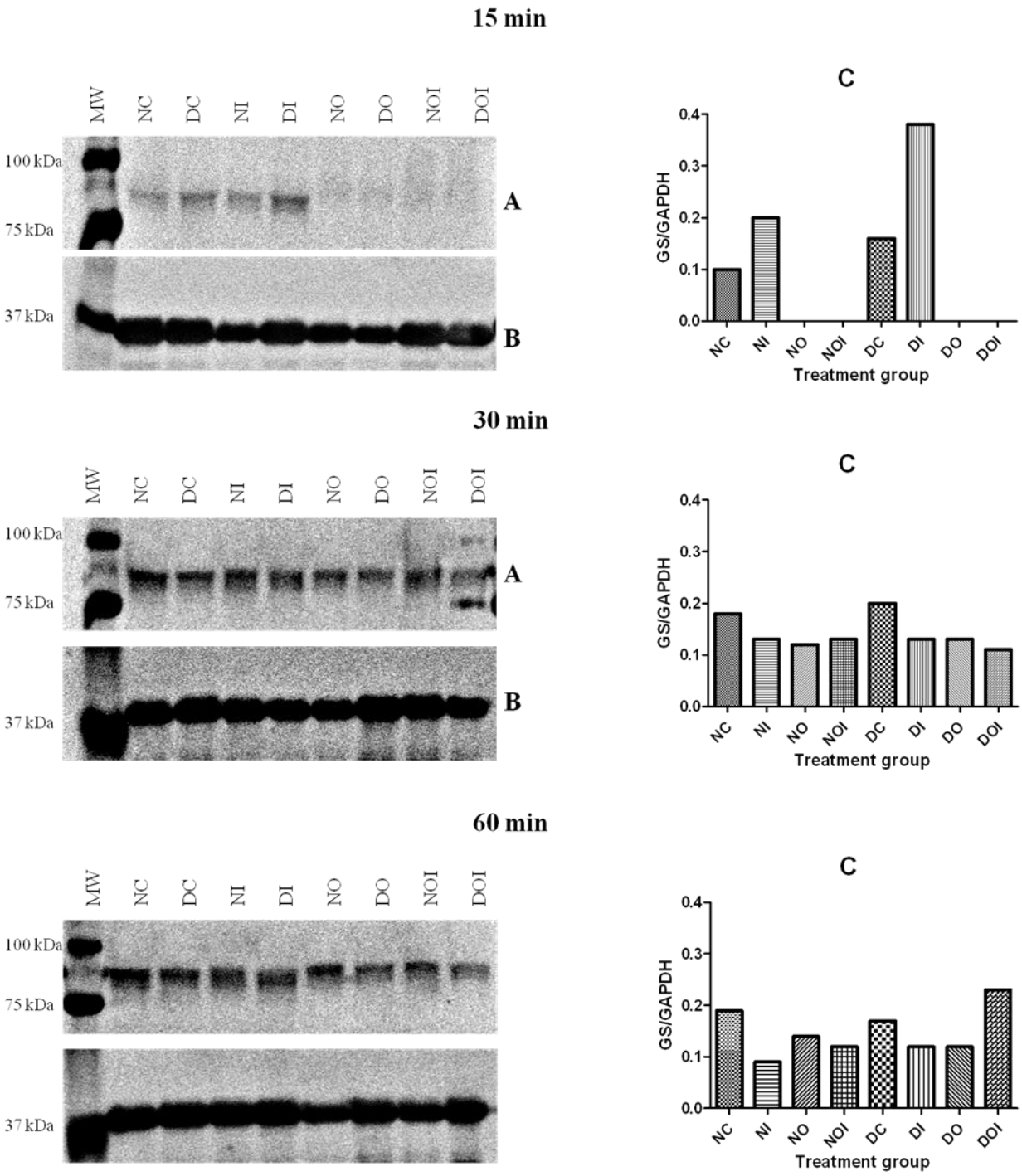


Figure 3.4.3: Western blot of (A) GS and (B) GAPDH in muscle homogenates 15, 30 and 60 minutes following treatment. 40 µg of protein was loaded for A and 20 µg for B. (C) is the normalized data showing the ratio of GS/GAPDH. The normalized data is grouped as non-diabetic and diabetic animals for clarity.

3.4.4. Effect of OA on expression of p-Akt in skin

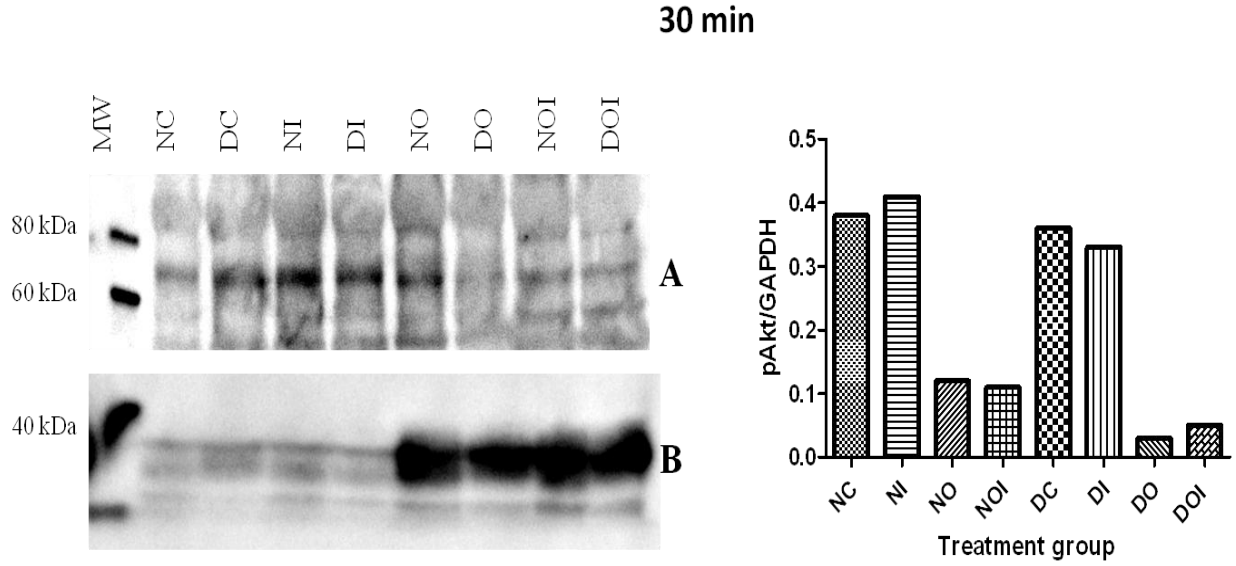


Figure 3.4.4: Western blots of (A) phospho-Akt (Ser 473) and (B) GAPDH in skin homogenates following 30 minutes treatment. 160 µg of protein was loaded for A and 80 µg for B. (C) is the normalized data showing the ratio of phospho-Akt/GAPDH. The normalized data is grouped as non-diabetic and diabetic animals for clarity.

The effect of OA on the expression of p-Akt in skin was investigated after 30 minutes following treatment (Fig 3.4.4). The p-Akt expression was higher in the normal treatment groups in compared to the respective treatments in diabetic animals. The highest p-Akt expression was that in insulin treated normal animals, followed by insulin treated diabetic animals, then OA treated normal animals, OA plus insulin treated normal animals, OA plus insulin treated diabetic animals and lastly OA treated diabetic animals. Of note is that, the loading control, GAPDH was not uniformly expressed and this might invalidate the normalization.

3.5. Sub-chronic studies on expression of glycogenic enzymes

Effects of OA on the expression of glycogen synthase and glycogen phosphorylase were investigated in skeletal muscle after 7 and 14 days of treatment. In skin the enzyme expression was investigated for animals treated for 14 days. GAPDH was used as a loading control. Glycogen synthase was not detected in skin homogenates.

3.5.1 Effect of OA on expression of GS in muscle

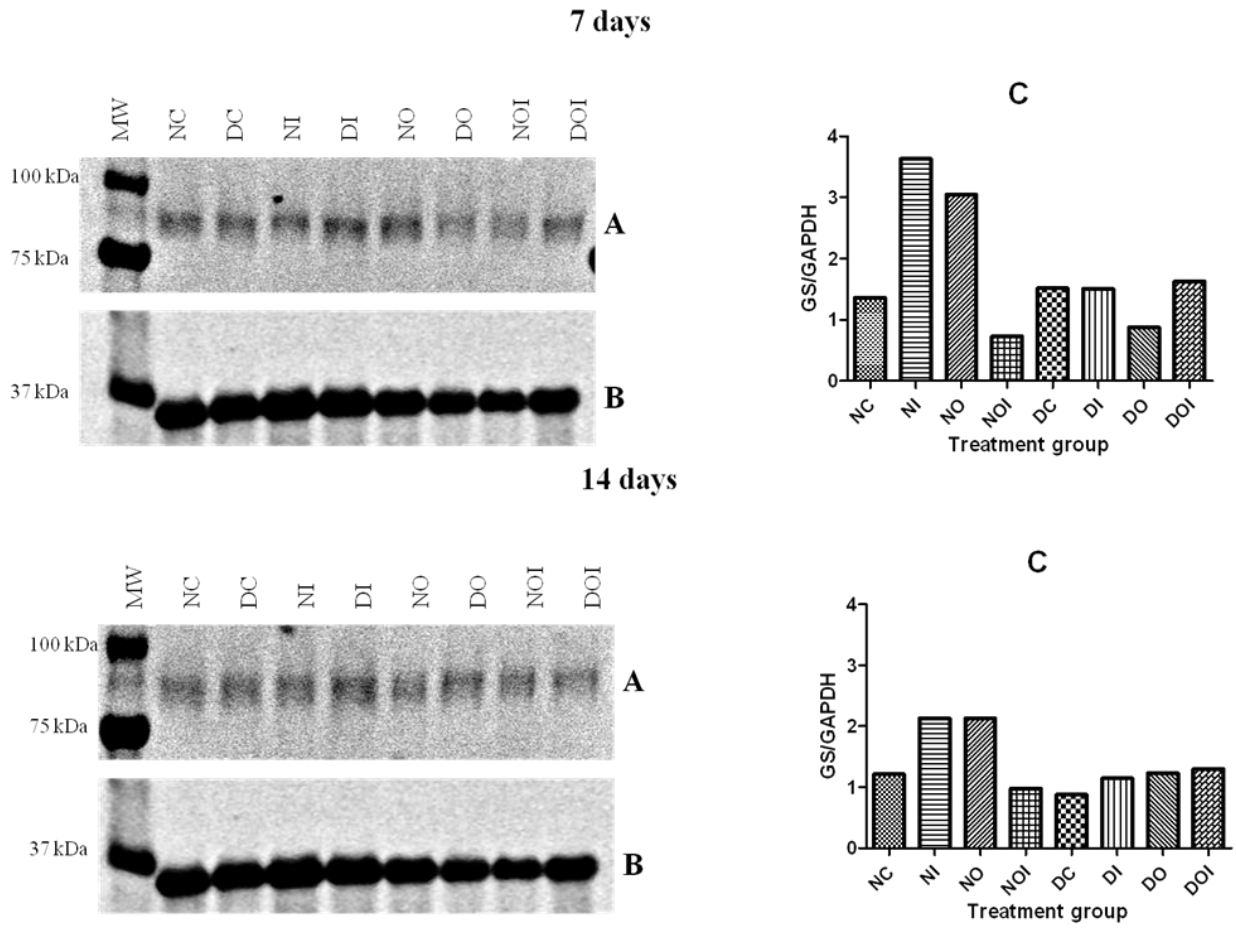


Figure 3.5.1: Western blots of (A) GS and (B) GAPDH in muscle homogenates after 7 and 14 days of treatment. 40 µg of protein was loaded for A and 20 µg for B. (C) is the normalized data showing the ratio of GS/GAPDH. The normalized data is grouped as non-diabetic and diabetic animals for clarity.

Figure 3.5.1 above shows the GS expression following the sub-chronic treatment of the rats for 7 and 14 days. Expression of GS was highest after 7 days of treatment in the normal animals treated with insulin and OA and this was the same trend after 14 days of treatment although GS expression had declined for these two groups. Normal animals treated with OA plus insulin had the lowest expression of GS amongst all groups after 7 days of treatment. The expression of GS in normal controls did not change over the 14-day period but that in diabetic control animals declined. There was an increase of GS expression in OA treated diabetic animals after 14 days of treatment.

3.5.2 Effect of OA on expression of GP in muscle

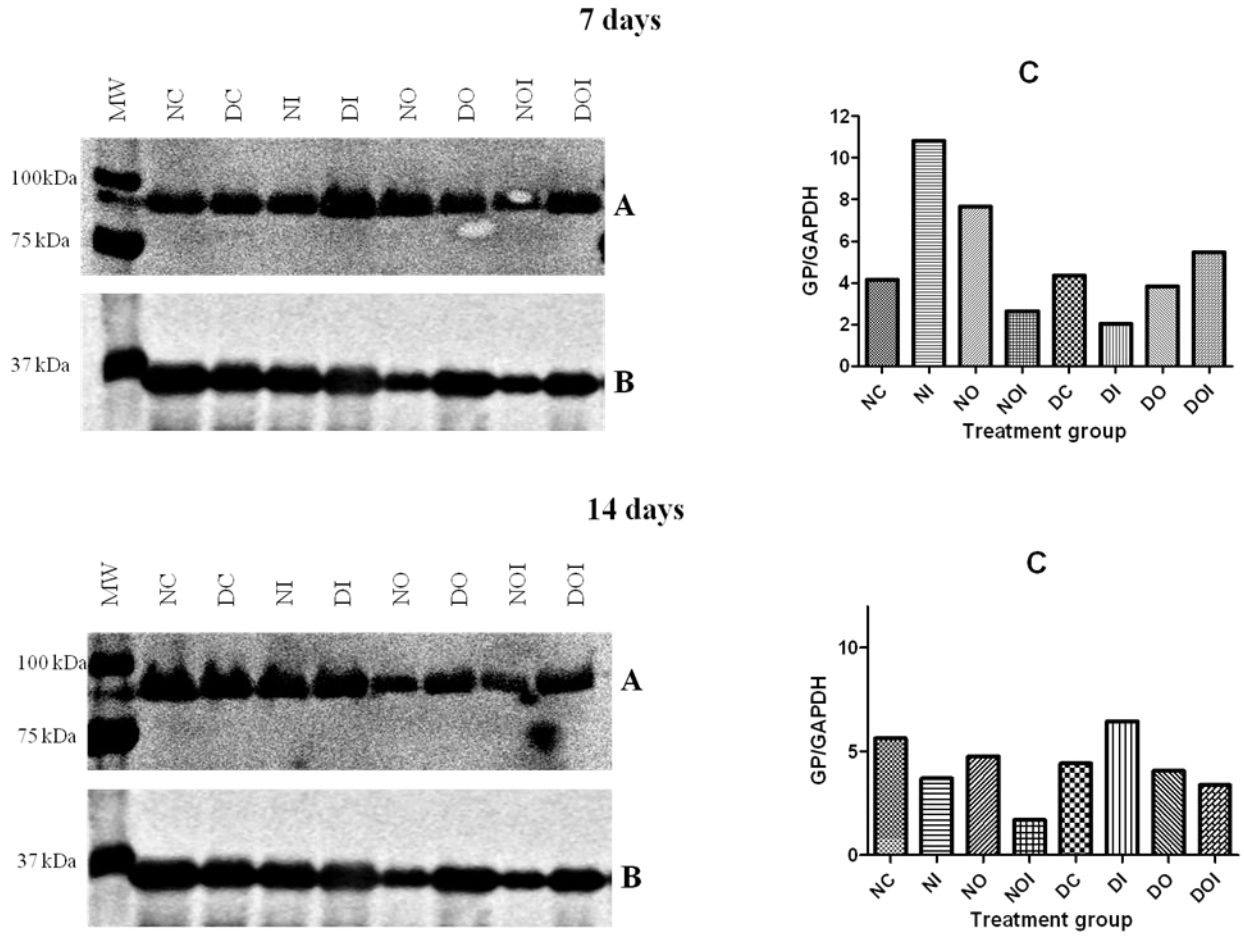


Figure 3.5.2: Western blots of (A) GP and (B) GAPDH in muscle homogenates after 7 days of treatment. 40 µg of protein was loaded for A and 20 µg for B. (C) is the normalized data showing the ratio of GP/GAPDH. The normalized data is grouped as non-diabetic and diabetic animals for clarity.

The results in figure 3.5.2 show GP expression levels following sub-chronic studies for 7 and 14 days in skeletal muscle. After 7 days of treatment GP expression was highest in normal animals treated with insulin, followed by normal animals treated with OA and diabetic animals treated with OA plus insulin. The GP expression levels in these groups declined drastically after 14 days. The GP expressed in all the treatment groups was lowered after 14 days except in the

insulin treated diabetic animals in which it increased substantially. The GP expressed in the control animals was more or less the same after 7 days and after 14 days.

3.5.3 Effect of OA on expression of GP in skin

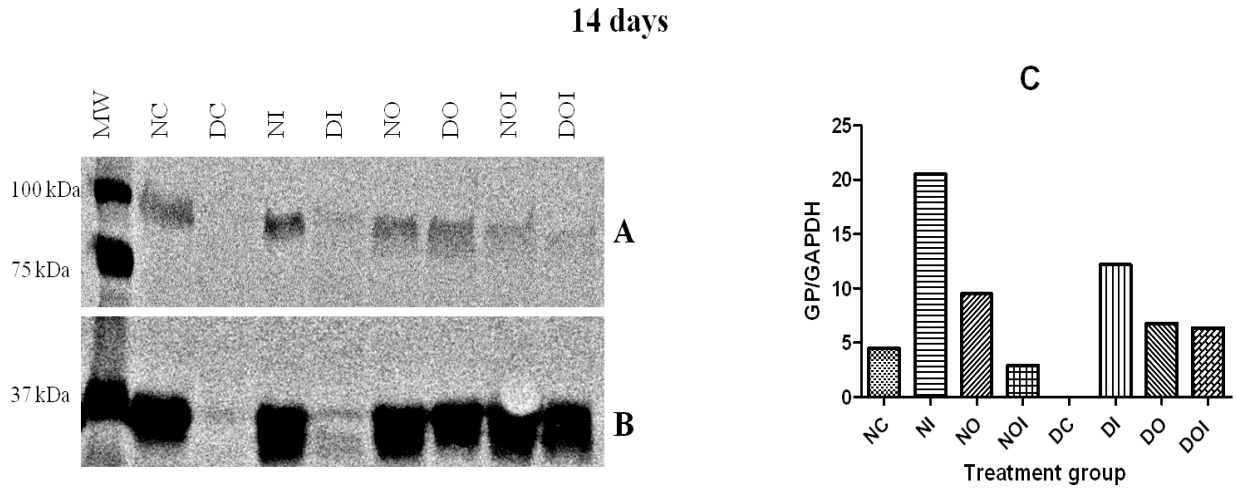


Figure 3.5.3: Western blots of (A) GP and (B) GAPDH in skin homogenates after 14 days of treatment. 220 µg of protein was loaded for A and 20 µg for B. (C) is the normalized data showing the ratio of GP/GAPDH. The normalized data is grouped as non-diabetic and diabetic animals for clarity.

Figure 3.5.3 above shows the GP expression in skin following 14 days of treatment. GP expression was highest in insulin treated non-diabetic animals, followed by insulin treated diabetic animals and OA treated non-diabetic animals. Treatment of non-diabetic and diabetic animals with OA plus insulin caused a decline in GP expression when compared to animals treated with insulin. GP expressed in the diabetic control group could not be quantified by densitometry. The low expression of GAPDH in the diabetic control and insulin treated diabetic groups is noted and might invalidate these quantifications.

3.6. Glycogen synthase and phosphorylase activity

Effects of OA on the activities of glycogen synthase and phosphorylase in skeletal muscle and skin after 14 days of treatment were determined.

3.6.1 Glycogen synthase activity

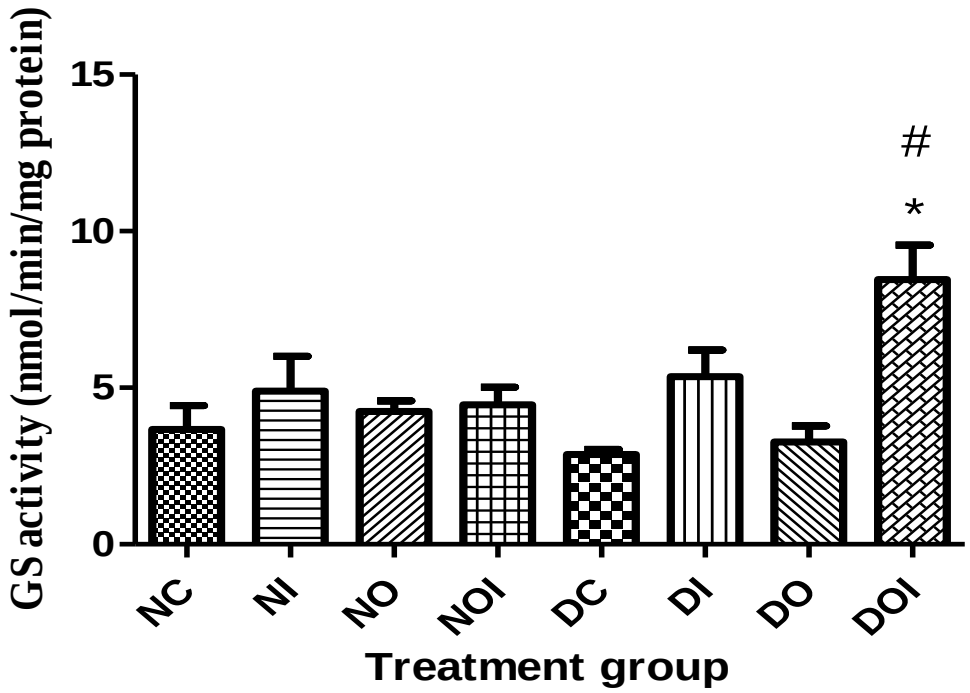


Figure 3.6.1a: Muscle glycogen synthase activities after 14 days of treatment in non-diabetic and diabetic male Sprague-Dawley rats (n = 6). Data is represented as mean ± SEM. * p<0.05 compared to normal control (NC) and # p<0.05 compared to diabetic control (DC)

The results in figure 3.6.1a show skeletal muscle GS activity following 14 days of treatment. GS activity was slightly lower in diabetic control animals compared to normal control animals. The diabetic animals treated with OA plus animals had significantly elevated GS activity in comparison to the controls. Treatment of normal and diabetic animals with insulin also lead to higher GS activity while treatment with OA caused a slight increase in GS activity compared to their respective controls.

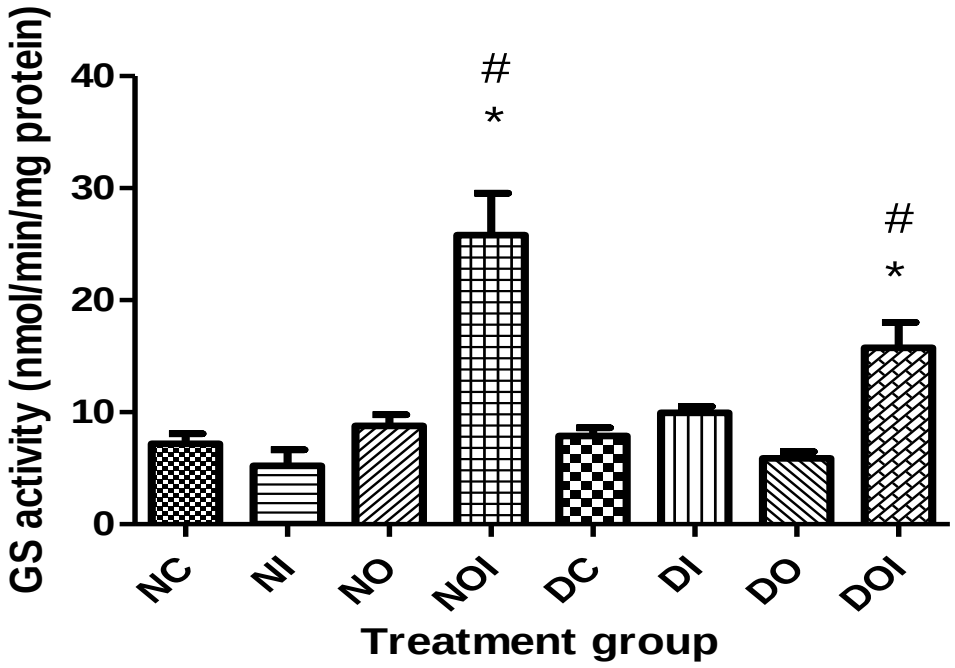


Figure 3.6.1b: Skin glycogen synthase activity after 14 days of treatment in non-diabetic and diabetic male Sprague-Dawley rats (n = 6). Data is represented as mean ± SEM. * p<0.05 compared to normal control (NC) and # p<0.05 compared to diabetic control (DC)

Figure 3.6.1b above shows skin GS activity following the 14 day sub-chronic study. There was slightly higher GS activity in diabetic control animals compared to normal control animals. In both non-diabetic and diabetic animals the OA plus insulin treatment groups had significantly elevated GS activity when compared to their respective control groups. The highest GS activity was in the non-diabetic insulin treated group which was followed by diabetic OA plus insulin treated animals. The insulin treated diabetic animals and OA treated non-diabetic rats had slightly higher GS activities compared to their controls. The non-diabetic insulin treated and diabetic OA treated animals had lower GS activities compared to their controls.

3.6.2 Glycogen phosphorylase activity

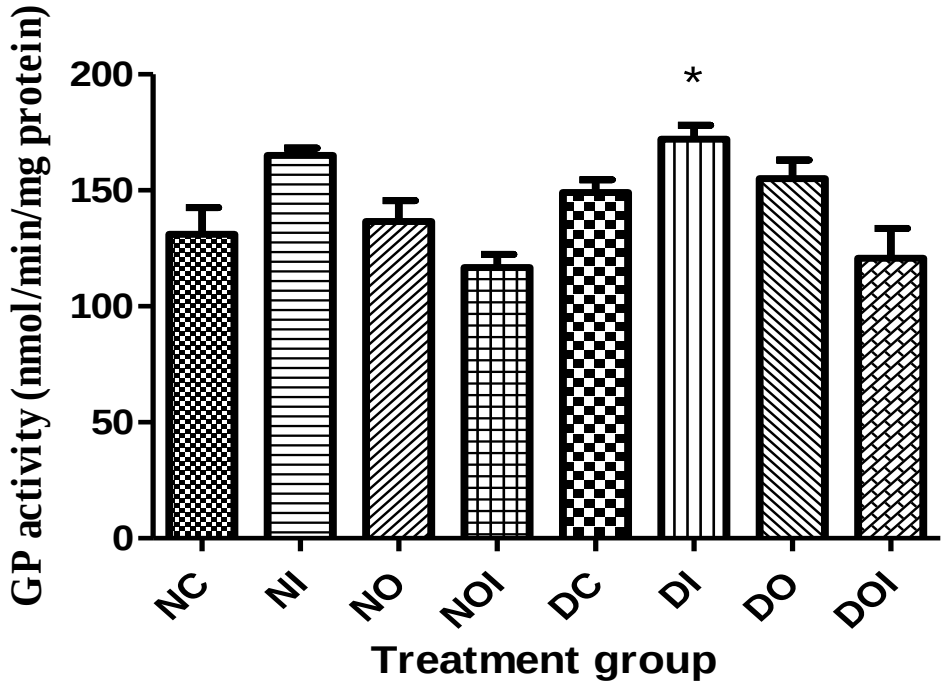


Figure 3.6.2a: Muscle glycogen phosphorylase activity after 14 days of treatment in non-diabetic and diabetic male Sprague-Dawley rats (n = 6). Data is represented as mean ± SEM. * p<0.05 compared to normal control (NC).

The results in figure 3.6.2a show the skeletal muscle GP activity after the 14 day sub-chronic study. GP activity was slightly higher in normal control animals compared to diabetic control animals. Treatment with OA did not cause a change in GP activities for both diabetic and non-diabetic animals but treatment with OA plus insulin reduced GP activity to levels below their controls. The GP activity of insulin treated diabetic animals was significantly higher when compared to the GP activity of normal control animals.

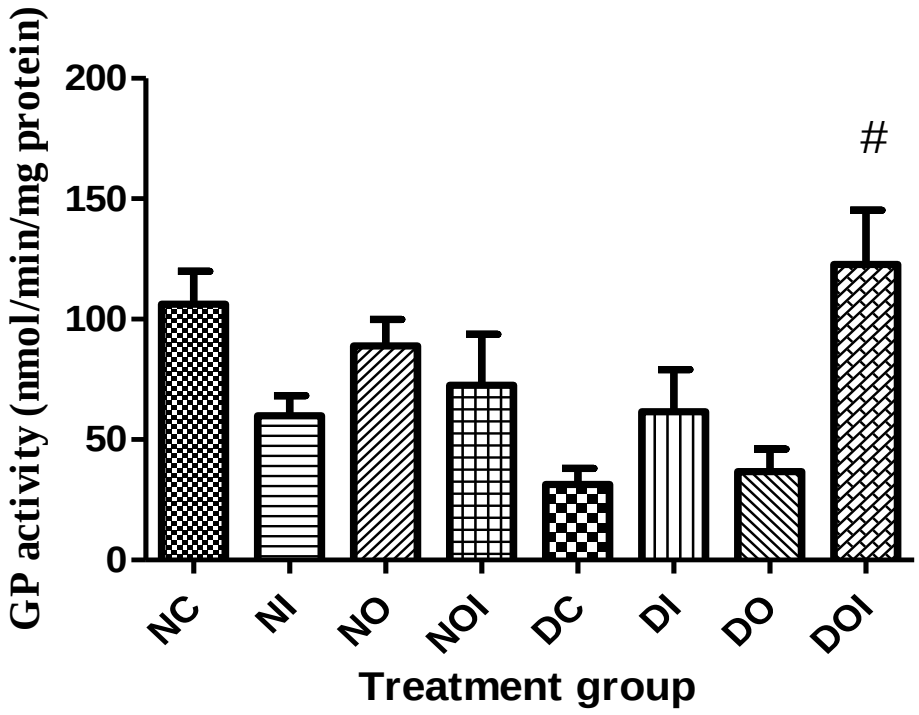


Figure 3.6.2b: Skin glycogen phosphorylase activity after 14 days of treatment in non-diabetic and diabetic male Sprague-Dawley rats (n = 6). Data is represented as mean ± SEM. # p<0.05 compared to diabetic control (DC).

The activity of GP in the skin is shown in Figure 3.6.2b. The diabetic control animals had lower GP activity compared to normal control animals. GP activity was significantly elevated in the OA plus insulin treated diabetic animals when compared to diabetic control animals. Treatments in the non-diabetic animals reduced GP activity whilst in diabetic animals treatments increased the activities compared to their respective controls

3.7. Glycogen content

Table 3.2 below shows the amount of glycogen in skeletal muscle and skin following the 14 days sub-chronic study. Diabetic control animals had a slightly higher amount of glycogen compared to the non-diabetic control animals. The amount of muscle glycogen was slightly higher in the treated diabetic animals except the OA plus insulin treated diabetic animals when compared to the diabetic control animals. The highest amount of glycogen was in the insulin treated diabetic animals and followed by the OA treated diabetic animals. All the other treatment groups had lower amounts of glycogen than the non-diabetic control animals. The highest amount of glycogen was in non-diabetic insulin treated animal and diabetic OA plus insulin treated animals which had the same amount of glycogen. This was followed by the normal animals treated with insulin and lastly normal animals treated with OA plus insulin. The amount of glycogen in skin was slightly elevated in diabetic animals compared to their respective treatments in non-diabetic animals. The amount of glycogen was higher in the diabetic OA treated animals compared to the diabetic control group whilst OA plus insulin treated diabetic animals and insulin treated diabetic animals had lower amounts of glycogen. The treated non-diabetic animals had slightly lower amounts of glycogen compared to the non-diabetic control animals. The highest amount was in the OA plus insulin treated group, followed by insulin treated animals and lastly OA treated animals.

Table 3.2: The effect of different treatments on skeletal muscle and skin glycogen levels after 14 days of treatment in non-diabetic and diabetic male Sprague-Dawley rats. Values are represented as mean ± SEM (n=5)

	NC	NI	NO	NOI	DC	DI	DO	DOI
Muscle (mg/g tissue)	0.93 ± 0.07	0.80 ± 0.06	0.74 ± 0.08	0.68 ± 0.03#	1.12 ± 0.04	1.28 ± 0.12	1.21 ± 0.16	0.80 ± 0.09
Skin (mg/g tissue)	1.08 ± 0.07	1.02 ± 0.06	1.05 ± 0.04	1.07 ± 0.04	1.32 ± 0.12	1.15 ± 0.09	1.37 ± 0.12	1.21 ± 0.04

# $p < 0.05$ compared to DC

3.8. Serum enzyme analysis

The results in Table 3.3 below show the levels of LDH, ALT and AST after the sub-chronic study. The diabetic animals had higher LDH levels than their respective treatment groups in the normal animals. Insulin and OA treated animals showed slightly higher LDH levels in both non-diabetic and diabetic animals compared to their respective controls. However LDH levels were decreased in OA plus insulin treated normal and diabetic animals compared to their respective controls. The level of serum ALT was higher in all the diabetic animals, compared to their respective treatment groups in the normal animals. The ALT levels were decreased in all the treatment groups for non-diabetic and diabetic animals compared to their respective controls except for the insulin treated diabetic animals. Insulin treated diabetic animals had increased ALT levels compared to the diabetic control group. Non-diabetic treated animals had lower ALT levels compared to the non-diabetic control animals. In all the groups, there was a higher level of AST in the diabetic treatment groups compared to the respective treatments in non-diabetic animals. The level of AST was higher in all the normal treatment groups compared to the non-diabetic control group. The highest AST level was in the insulin treated animals, followed by OA treated animals and then OA plus insulin treated animals. All the treatment groups in the diabetic animals had reduced AST levels compared to the diabetic control group. Of note is that OA reduced LDH, ALT and AST in both diabetic and non-diabetic animals compared to their respective control and this reduction was enhanced when both OA plus insulin are used for treatment.

Table 3.3: The effect of different treatments on serum LDH, ALT and AST levels after 14 days of treatment in non-diabetic and diabetic male Sprague-Dawley rats Values are represented as mean ± SEM (n=5)

	NC	NI	NO	NOI	DC	DI	DO	DOI
LDH (U/L)	1035 ± 130	1258 ± 54.4	1063 ± 86.1	788.4 ± 50.6	1167 ± 52.5	1270 ± 139	1045 ± 92.4	786.5 ± 97
ALT (U/L)	75.8 ± 5.9#	67.17 ± 1.4#	71 ± 2.8#	69.4 ± 3.2#	170.6 ± 12.3*	188.3 ± 10.1*	156.7 ± 8.6*	150.2 ± 6.0*
AST (U/L)	133.3 ± 12#	185.5 ± 7.6	158.2 ± 8.5#	138.8 ± 7.6#	237.8 ± 21.4*	217 ±12.5*	163.8 ± 14.8#	145.5 ± 8.7#

* $p < 0.05$ compared to NC

# $p < 0.05$ compared to DC

CHAPTER 4

DISCUSSION

Diabetes mellitus is one of the diseases with a high mortality rate of about 3 million deaths per year according to World Health Organization (WHO). Type 1 diabetes which is caused by pancreatic β cell destruction accounts for 5-10% of all diabetes cases and patients suffering from the disease need exogenous insulin (Adams *et al*., 2011). Diabetes (both type 1 and type 2) is characterized by chronic hyperglycaemia, thus treatment of diabetes focuses on glycaemic control. Chronic hyperglycaemia can directly contribute to glucose-induced insulin resistance (glucose toxicity) in both forms of diabetes. The high levels of glucose can result in this resistance due to activation of protein kinase (PKC) which leads to increased serine/threonine phosphorylation of the IR. Serine/threonine phosphorylation of the IR reduces its tyrosine phosphorylation which in turn reduces downstream insulin-stimulated IRS1 phosphorylation and PI3K activation (Zierath and Kawano, 2003). Hyperglycaemia can also directly impair insulin action on Akt. High concentrations of glucose are associated with modifications of PI3K, Akt and GS suggesting a mechanism by which glucose can directly alter the signalling properties of signalling intermediates and enzymes (Steiler *et al*., 2003). Hyperglycaemia also induces oxidative stress, which leads to tissue damage (Giacco and Brownlee, 2010), and has been hypothesized to contribute to oxidative stress by the direct generation of reactive oxygen species (ROS) or by altering the redox balance (Rains and Jain, 2011).

The study investigated the effects of OA *in vivo* on the insulin signalling cascade in different diabetic pathologies in muscle and skin. Normal animals were subjected to hyperinsulinaemic conditions by being given an insulin dose. Hyperinsulinaemia is implicated in causing insulin resistance in skeletal muscle (Björnholm and Zierath, 2005). Treatment of normal animals with insulin plus OA investigates whether OA can prevent insulin resistance due to hyperinsulinaemia. OA treatment of normal animals investigated whether OA might have detrimental effects in non-diabetic as it is used to treat other ailments such as hepatitis (Song *et al*., 2006). Treatment of diabetic animals with OA investigated whether the compound has insulin mimetic capabilities. It has been suggested that OA enhances the effects of insulin *in*

vitro (Wang *et al*., 2011) and thus treatment of diabetic rats with OA + insulin investigated the hypothesis *in vivo*. The skeletal muscle accounts for 75% of glucose disposal (Jensen *et al*., 2011a) and most of the glucose is converted to glycogen storage. The activities of enzymes (GS and GP) that regulate the levels of glycogen were investigated as well as the deposition of glycogen after treatments. The skin is the largest tissue in the body and its contribution to glucose utilization has not been elucidated. The skin was studied as it might contribute to glucose homeostasis in normal and diabetic animals particularly those undergoing treatment.

The results in Figure 3.2 suggest that OA does not reduce fasting and non-fasting blood glucose levels in STZ induced diabetic rats. An experiment by Zeng *et al*. (2012) also showed that OA did not reduce blood glucose levels after treatment for 2 weeks in a type 1 diabetes model in mice, though it did reduce blood glucose levels which was sustained for six weeks in a type 2 model of diabetes. Studies conducted by Gao *et al*. (2009) and Ramírez-Espinosa *et al.* (2011), showed a decrease in blood glucose levels *in vivo* in a type 2 model of diabetes starting an hour after treatment. One of the investigated hypoglycaemic actions of OA is its inhibition of PTP1B (Elchebly *et al*., 2000; Li *et al*., 2012; Liu *et al*., 2010; Ramírez-Espinosa *et al*., 2011). PTP1B inhibition improves insulin sensitivity by blocking the PTP1B-mediated regulation of the insulin signalling pathway (Thareja *et al*., 2012). Our results suggest that when OA is used in combination with insulin in type 1 diabetic rats there is a synergistic enhancement of the insulin mediated reduction of fasting blood glucose levels.

Results in Figure 3.4.1 showed that *in vivo* insulin mediated Akt phosphorylation, was achieved in both non-diabetic and diabetic rats. Insulin stimulated Akt phosphorylation is higher in diabetic animals compared to non-diabetic animals. This might be due to an increase in insulin receptor expression or sensitivity in diabetes. It has been reported that there is an increase in insulin receptor expression and tyrosine kinase activity in the heart of STZ induced type 1 diabetic rats (Laviola *et al*., 2001), and an increase in insulin stimulated IR and IRS-1 phosphorylation has been demonstrated in skeletal muscle and liver of diabetic rats (Wang *et al*., 1999). An experiment by Oku and co-workers in 2001 showed that insulin stimulated tyrosine phosphorylation of the insulin receptor was increased by 39 % STZ induced diabetic rats compared to normal animals (Oku *et al*., 2001). Insulin stimulated phosphorylation of IRS1 and

IRS2 was also increased by 76 an 89 % respectively in STZ induced diabetic rats compared to normal rats (Oku *et al*., 2001). This may lead to the higher Akt phosphorylation levels due to insulin treatment in the diabetic animals. Insulin treatment of non-diabetic rats can also lead to hyperinsulinaemia. Hyperinsulinaemia has been implicated in causing insulin resistance (Thirone *et al*., 2006) which can lead to a decrease in insulin stimulated phosphorylation of Akt (Wang *et al*., 2011) which might account for the low pAkt expression in normal animals treated with insulin. Studies by Feng *et al*. (2011) showed that OA can activate Akt in vascular smooth muscle cells. OA has also been shown to enhance insulin stimulated activation of Akt in hepatocytes at certain concentrations (Wang *et al*., 2011). Our results show that OA can activate Akt phosphorylation which suggests that it can influence certain proteins of the insulin signalling pathway independently of insulin. In the current study we also show that a combinational treatment of the diabetic rats with OA and insulin leads to the enhancement of the insulin mediated Akt activation in 15 minutes following treatment and this might be when the OA concentration is optimum in the animal. This is quite remarkable considering that OA was administered orally.

In the skin (Figure 3.4.4) Akt phosphorylation was slightly increased by insulin treatment in non-diabetic animals whilst it was slightly decreased in diabetic animals compared to their respective controls. Insulin stimulated Akt activation has been shown in intact rat skin fragments from normal rats (Pelegrinelli *et al*., 2001). Our results show Akt phosphorylation was after 30 minutes following treatment thus insulin might not have reached the target organ and thus delayed insulin-stimulated Akt phosphorylation in diabetic rats or diabetes might also cause desensitization of insulin receptors in the skin. However the loading control, GAPDH was not fully expressed in the control and insulin treated animals of both the non-diabetic and diabetic animals. This might affect the normalization of our data. OA treatment also led to a suppression of insulin stimulated-Akt phosphorylation in both non-diabetic and diabetic rats.

Insulin is attributed with the net suppression of GS phosphorylation thus activating the enzyme (Mandarino *et al*., 1987; Villar-Palas and Guinovart, 1997). Our results in Figure 3.4.2 show that there was little non quantifiable expression of p-GS in OA or OA plus insulin treated animals after 15 minutes following treatment. In animals where OA was not included in the treatment,

expression of p-GS was low for this time period. After 30 minutes following treatment, p-GS was expressed in all animal groups at generally elevated levels with diabetic control animals having the highest expression followed normal control animals. At 60 minutes, expression levels of phosphorylated of GS were almost the same across all animal groups. Thus there appears to be biphasic expression in which activation is followed by suppression. What is clear is that at 30 minutes treatment of normal and diabetic animals with insulin, OA and OA plus insulin suppresses phosphorylation of GS which would be expected to activate GS and hence promote glycogen synthesis. Our results have shown that OA activates Akt on its own which might lead to the downstream suppression of GS phosphorylation and this appears to be the case at 30 minutes following treatment of animals with OA.

Sub-chronic studies on GS expression studies showed a decrease in GS expression in the diabetic control rats compared to non-diabetic control rats after 14 days of treatment. After treatment with insulin or OA, GS expression in skeletal muscle of normal animals was increased. Skeletal muscle glycogen synthase mRNA expression has been shown to increase due to insulin treatment (Huang *et al*., 2000). Treatment of non-diabetic rats with a combination of both insulin and OA led to an inexplicable decrease in GS expression. In the diabetic animals GS expression, after 14 days, was increased by all the treatments. The shorter time period of treatment and diabetes development might account for the differing GS expression levels after 7 days.

Our results in figure 3.5.2 show that GP expression levels in muscle are lower in diabetic control animals after 14 days compared to the non-diabetic animals. GP mRNA expression is destabilized by diabetes in rat liver leading to lower protein levels (Rao *et al*., 1995). After 14 days the treatments in normal animals reduce the GP expression levels. In rat liver GP mRNA expression in diabetic animals is normalized with chronic insulin treatment (Rao *et al*., 1995). Our results show that treatment of diabetic animals with insulin increases GP expression whilst OA + insulin reduces the expression. To our knowledge expression of GP and the effects of diabetes or insulin on GP expression in the skin have not been documented. The results (Figure 3.5.3) show that the GP encoded by the PYGM gene (present in skeletal muscle) was also present in the skin. GP which was expressed in the diabetic control animals could not be quantified. In the diabetic control and diabetic animals treated with insulin, there was also a

matching low expression of the loading control, GAPDH. Insulin and OA ± insulin treatment increased GP expression in both normal and diabetic animals. We cannot be certain on the effect of treatments the diabetic animals as we could not quantify the GP expressed in the control group could not be quantified as well as the low expression of the loading control..

Skeletal muscle GS activity has been shown to be decreased in diabetic rats (Farrace and Rossetti, 1992; Vestergaard *et al*., 1994) and this is confirmed by our results in Figure 3.6.1a which show a 22% decrease in GS activity in the diabetic animals when compared to the non-diabetic rats. Treatment of the normal animals with insulin, OA and OA + insulin leads to a 33%, 16% and 21% increase in GS activity respectively when compared to the normal control animals. Treatment of the diabetic animals with insulin, OA and OA + insulin leads to an 87%, 14% and 195% increase in GS activity respectively with the increase in the OA plus insulin treatment group being significant. In diabetes insulin-stimulated GS activation is impaired, though under hyperglycaemic conditions the insulin-stimulated GS activity is enhanced opposite has been shown (Farrace and Rossetti, 1992). Hyperglycaemia has been shown to activate GS (Wright *et al*., 1988) due to its increase of G-6-P concentrations (Farrace and Rossetti, 1992; Wallis *et al*., 1998). G-6-P is an allosteric activator of GS and an increase in its content has been shown to increase GS activity (Yki-Jirvinen *et al*., 1987). In studies by Farrace and Rossetti in 1992 there was a marked stimulation of GS activity in pancreatomized type 1 diabetic rats compared to normal rats which is due to the hyperglycaemic condition that can stimulate GS activity. In our study there was stimulation of GS activity in diabetic animals by insulin and OA treatments while OA plus insulin treatment resulted in a synergistic effect that increased the high GS activity.

GS activity has been measured in dog and mouse skin samples and found to be a fifth of the activity in the liver (Adachi, 1961). To our knowledge the effect of diabetes or insulin on the skin GS activity has not been documented. Figure 3.6.1b shows that in skin there is a 9% increase in GS activity for the diabetic control group when compared to the normal control group. Treatment in the normal animals with insulin leads to a 28% decrease in GS activity when compared to the normal control animals. OA and OA plus insulin treatment of normal animals lead to a 22% and 259% increase in the glycogen synthase activity respectively when compared

to the normal control animals. Our results show that in diabetic animals, insulin and OA +`insulin treatment increases GS activity (26% and 100% respectively) compared to the diabetic control group. OA treatment on the other hand leads to a 25% decrease GS activity when compared to the diabetic control group. The OA plus insulin treated diabetic animals had high GS activity which corresponds with the high GS activity observed in our muscle samples after the same treatment. The results also suggest that there was insulin stimulated GS activity in the skin of diabetic animals as there was an elevation of GS activity.

Glycogen phosphorylase activity is increased by 14% in the diabetic control group when compared to the normal control group as shown in Figure 3.6.2a. Non-diabetic and diabetic animals showed increased GP activity but very little change occurred with OA treated animals. However, a combination of OA plus insulin caused a 9% and 11% decrease in GP in normal animals and diabetic animals, respectively when compared to their respective controls. Insulin treatment of the non-diabetic and diabetic animals, lead to a 30% and 15% increase in GP activity respectively when compared to the respective control groups. GP activity has been shown to be increased in STZ-induced diabetes (Sadek, 2011). In liver insulin increases the stability of phosphorylase mRNA thereby increasing protein levels and total activity (Rao *et al*., 1995). The results from the study suggested that there was an increase in GP activity due to diabetes and also an insulin stimulated increase in GP activity. Our results also show that OA has no inhibitory effects of GP activity *in* vivo. This is contrary to studies that have reported the inhibitory activity of OA on GP in *in vitro* studies (Liu *et al*., 2010; Wen *et al*., 2008).

The activities of both the *a* and *b* form of GP been demonstrated in skin of dogs and mouse (Adachi, 1961). The effect of diabetes or insulin on the GP activity in skin has not been documented. In the skin GP activity was lowered in diabetic control animals by 70% when compared with the normal control animals as shown in Figure 3.6.2b. Treatment with insulin, OA and OA $\pm$ insulin causes a 44%, 16% and 32% decrease in GP activity, respectively in the normal animals. Insulin and OA treatment of diabetic animals increases GP activity by 97% and 17% respectively when compared to the diabetic control animals. OA + insulin treatment in diabetic animals caused a significant 291% increase in GP activity when compared to the diabetic control group. Our results suggested that insulin was able to increase the activity of GP

and this effect was enhanced by OA. On its own OA was also able to slightly increase GP activity. The results suggest that in skin GP is regulated differently muscle GP.

The results in Table 3.1 show that muscle glycogen is increased in diabetic control animals by 20% when compared to the normal control animals. In human diabetes glucose stimulates its own uptake in skeletal muscle with normal or improved efficiency and hyperglycaemia may exert a compensatory homeostatic mechanism that normalizes glucose disposal (Farrace and Rossetti, 1992). It has been shown that hyperglycaemia stimulates glucose storage in diabetic individual (Farrace and Rossetti, 1992) which can account for the higher glycogen content in our results. Treatment in normal animals with insulin, OA and OA + insulin decreased the glycogen content by 15%, 20% and 26%, respectively. When the glycogen store in muscle is replete, the glucose taken up is converted to lactate, in order to maintain enhanced glucose utilization. The lactate is taken up by the liver and converted to glycogen (Dimitriadis *et al*., 2011). This might account for the decrease in glycogen content in the non-diabetic animals treated with insulin and there was also an increase in the GP activity which would contribute to the decrease in the amount of glycogen. A combination of OA plus insulin treatment in the diabetic animals leads to a significant decrease (28%) in the glycogen content compared to the diabetic control group. Though there is an increase in GS activity and decrease in GP activity there was a decrease in glycogen content with OA + insulin treatment. This might be due to the maximum catalytic capacity of glycogen phosphorylase being approximately 50-fold greater than that of glycogen synthase in human skeletal muscle. Consequently, phosphorylase must be inhibited by approximately 99% of its maximum activity for net glycogen synthesis to occur (Dimitriadis *et al*., 2011). Work conducted by Ngubane *et al*. (2011) also showed that treatment with OA and insulin leads to a decrease in hexokinase activity in skeletal muscle of diabetic rats which would lead to a decrease in G-6-P. G-6-P is an allosteric activator of GS (Bouskila *et al*., 2008), therefore reduction in its concentration would lower glycogen synthesis. Individual insulin and OA treatment lead to an increase in glycogen content (14% and 8% respectively) in the diabetic animals with when compared to the diabetic control group. In the diabetic state there is a lower plasma insulin concentration and this might lead to a slight increase in glycogen content. Dimitriadis and co-workers in 2011 suggested that at low physiologic plasma insulin levels,

insulin increases the net rate of glycogen synthesis by inhibiting phosphorylase activity rather than activating glycogen synthase.

Our results show that there is GS and GP activity as well as expression of GP in skin which suggests the presence of glycogen in this tissue. The presence of glycogen in skin dogs was shown by (Adachi, 1961) and this glycogen is used in hair growth and production of sweat. The effect of diabetes and insulin on skin glycogen deposition has not been documented though. Results in Table 3.2 show that skin has glycogen content which is 22% higher in the diabetic control animals compared to the normal control animals. In our study we observed that GP expression levels in the skin of the diabetic control group are also very low which might lead to limited breakdown of the glycogen present. Our results also show that there was higher GS activity and lower GP activity for diabetic control animals. The amount of glycogen in normal and diabetic animals is not significantly altered by the different treatments when compared respective control groups. Our study also showed that the glycogen levels in skin are comparable to muscle glycogen in terms of amount. These are 5 times higher than the glycogen amounts found in dog skin by Adachi in 1961 probably due to improved extraction methods over the years.

Muscle and liver dysfunction is frequently associated with diabetes mellitus. Many clinical reports have indicated that activities of serum enzyme derived from the muscle such as creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) are elevated. A study conducted on 20 hyperglycaemic alloxan induced diabetic male white New Zealand rabbits showed that LDH levels were significantly increased (Celik *et al*., 2002). In our study untreated diabetic animals had a 13% increase in LDH compared to the normal control animals. Normal animals treated with insulin or OA had 22% and 3% increase in LDH respectively compared to the normal control whilst OA plus insulin treatment of the normal animals lead to a 24% decrease. Compared to the diabetic control group insulin treatment in diabetic animals increased the LDH levels by 9% whilst OA or OA plus insulin decreased the LDH levels by 10% and 37% respectively. The results suggest that OA and insulin might have synergistic protective effects against muscle damage caused by diabetes.

In STZ induced type 1 diabetes mellitus ALT levels are elevated showing liver damage (Ragbetli and Ceylan, 2010; West *et al*., 2006). Table 3.3 shows that there is a significant increase of ALT levels (125%) in the diabetic control group when compared to normal control group. Insulin, OA and OA + insulin reduces the ALT levels by 11%, 6% and 8% respectively when compared to the normal control group. In the diabetic animals insulin increases the ALT levels by 10% when compared to the diabetic control group. OA and OA + insulin treatments reduce the ALT levels by 11% and 12% respectively when compared to the diabetic control group. Work conducted by Takaike *et al*. (2004) showed that liver transaminases are slightly elevated at the start of insulin treatment in diabetic ketosis or diabetic ketoacidosis. Echo-histogram analysis on patients with transient elevation of liver transaminases after insulin treatment was conducted and the results suggested fat deposition in the liver. They suggested that fat deposition might be one of the causes of the mild increase in AST/ALT after initial insulin treatment (Takaike *et al*., 2004). The deposition of fat in the liver leads to increase in gluconeogenesis and a decrease in storage of glucose as glycogen in the liver (Ford *et al*., 2008). Increases in serum transaminases in diabetic animals has been attributed to cetogenesis and/or hepatic lesions (Ragbetli and Ceylan, 2010). Our results suggest that OA can ameliorate liver damage and prevent leaking of the enzymes from hepatocytes as evident from its lowering of serum ALT levels. In a study by Gao and co-workers in 2009, ALT was significantly increased the alloxan induced diabetic rats compared to the normal rats. After 40 days of treatment with OA, the ALT levels were significantly reduced compared to those in the diabetic control group.

AST is an enzyme primarily localized not only in the liver but also in skeletal and myocardial muscle. Our results show an elevation of the enzyme in the diabetic groups compared to the non-diabetic groups. There is a significant 78% increase in the serum AST levels of the diabetic control group when compared to the normal control group as shown in Table 3.3. Treatment of normal animals with insulin lead to a 39%, 19% and 4 % increase in AST levels respectively when compared to the normal control group whereas treatment of the diabetic animals with insulin, OA and OA + insulin leads to a 9%, 31% and 39% decrease in serum AST levels respectively when compared to the diabetic control group. Rhabdomyolysis (the breakdown of skeletal muscle tissue rapidly) can result from direct and indirect damage to muscle membrane and may lead to leakage of intracellular muscle components into the extracellular fluid

(Brancaccio *et al*., 2010). Diabetic ketoacidosis is one of the causes of rhabdomyolysis. Disorders in carbohydrate metabolism or an augmented metabolic demand resulting in a hyper metabolic state lead to a reduction in the availability of ATP and the impairment of Na-K ATPase. This results in decreased membrane resistance from increased internal free calcium ions which promotes the activation of intracellular proteases (Brancaccio *et al*., 2010). Our results suggest that OA and OA + insulin might reduce muscle damage as shown by the reduction in AST levels.

CONCLUSION

From the study OA was shown to have no hypoglycaemic effect in a type 1 diabetic model over a 2 hour period but might enhance insulin stimulated glucose uptake. It was observed that OA can activate Akt and suppresses phosphorylation of GS in skeletal muscle of diabetic rats in an insulin independent manner. OA in synergy with insulin was able to enhance insulin-stimulated suppression of GS phosphorylation skeletal muscle of diabetic rats. In the skeletal muscle of diabetic animals OA increases GS expression and lowers GP expression and GP encoded by the PYGM gene was expressed the skin. The treatment of diabetic animals with OA leads to an increase in both GS and GP activities in skeletal muscle, whilst in skin the activities of both GS and GP were lowered. In both skeletal muscle and skin of diabetic rats, OA increased the glycogen content. OA might also have tissue protective effects as it lowers LDH, AST and ALT levels in diabetic rats. In conclusion, OA in synergy with insulin can enhance activation of the insulin signaling pathway and thus has potential in treatment of type 2 diabetes, and secondly OA can also potentially regulate enzymes involved in the insulin signaling pathway in an insulin independent manner *in vivo*.

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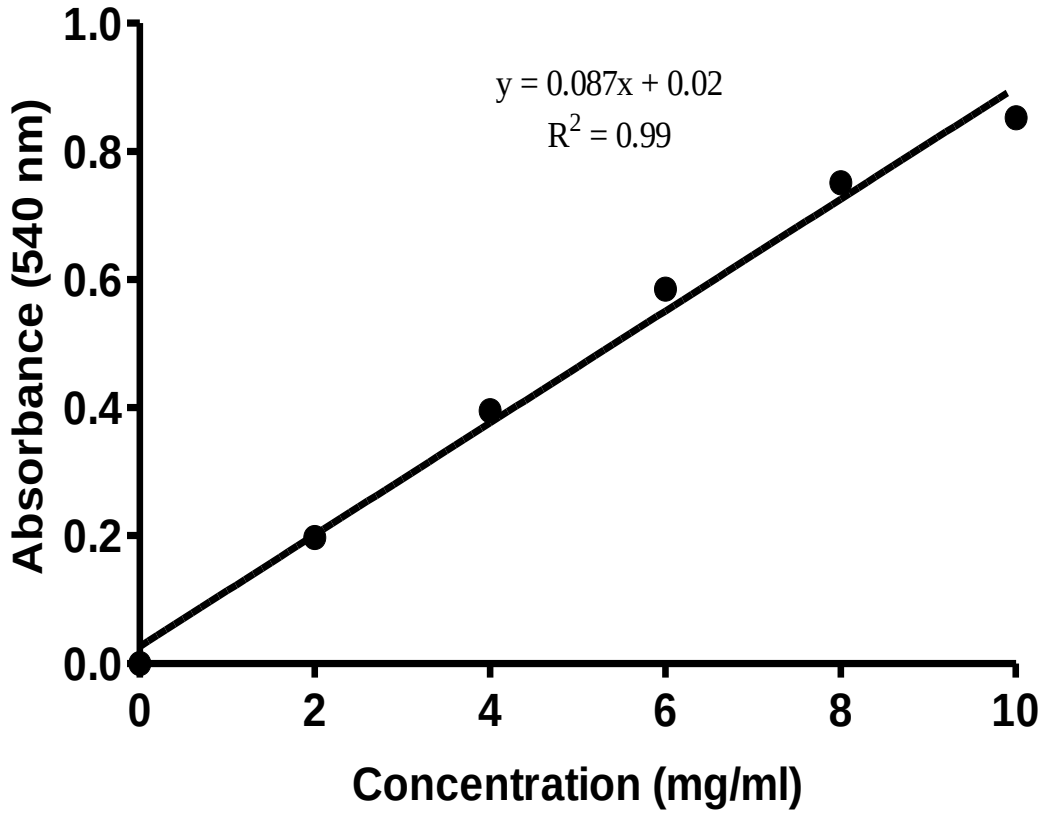
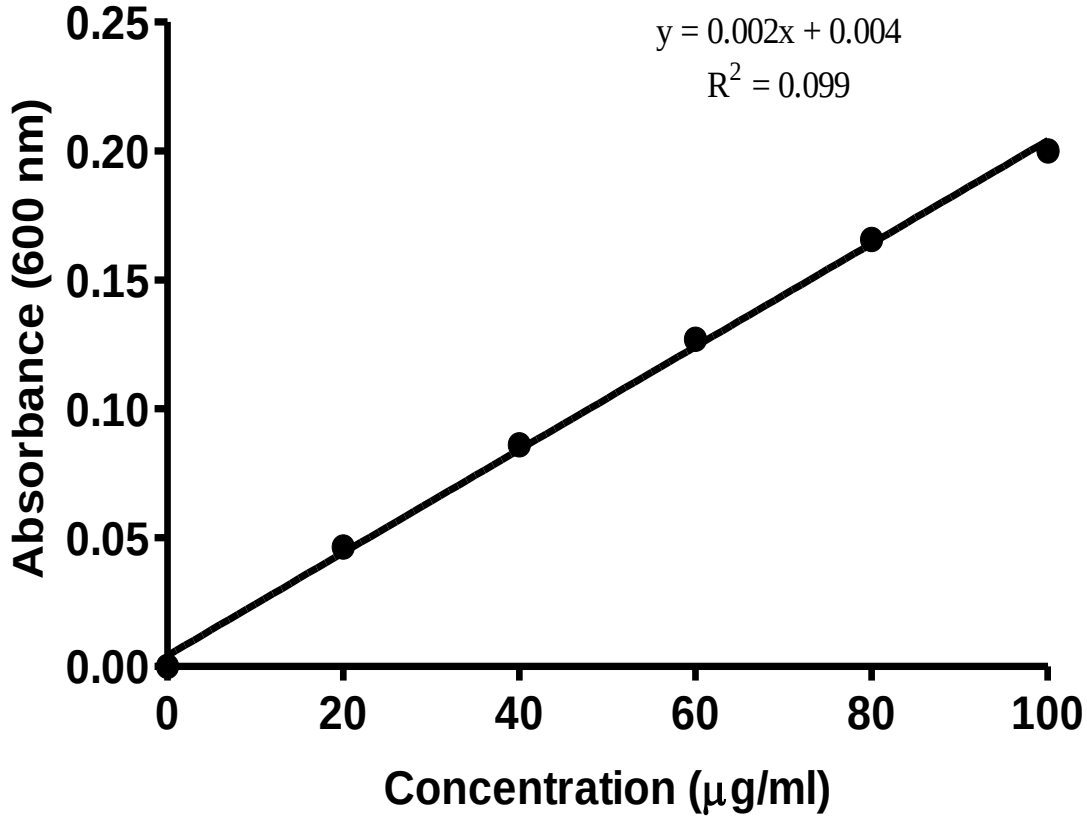
Appendix 1: Biuret assay standard curve



Appendix 2: Folin-Lowry assay standard curve



Appendix 3: Glycogen determination standard curve