ROLE OF OXIDATIVE STRESS IN TWO MODELS OF INSULIN RESISTANCE WITHIN PRIMARY RAT ADIPOCYTES

by

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Abstract

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Insulin's major action is to increase the flux of glucose into insulin sensitive tissues. A decreased biological response to insulin, termed insulin resistance, is induced in adipocytes by chronic incubation in high glucose and insulin (high G/I), or glucosamine, or hydrogen peroxide. The hypothesis that resistance to insulin stimulated glucose uptake caused by high G/I and glucosamine is due to enhanced production of reactive oxygen species or oxidative stress was tested. Isolated rat adipocytes incubated in high G/I or glucosamine showed insulin resistance while the antioxidant and glutathione (GSH) precursor N-acetylcysteine (NAC) and GSH monoethyl ester prevented resistance. However, the antioxidant α-tocopherol and ebselen had no significant effect. Similarly the MAPKK (MEK) inhibitor, PD98059, and the p38 MAPK inhibitor, SB203580, could not prevent insulin resistance. Intracellular GSH was decreased by glucosamine, and increased by high G/I. NAC prevented the decreased levels of GSH caused by glucosamine. The data support a role for GSH in the pathogenesis of insulin resistance but the role of oxidative stress is not clear.

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List of Abbreviations

AGE	advanced glycation end-products
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
BSO	L-buthionine-[S,R]-sulfoximine
DMEM	Dulbecco's modified Eagle's medium
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
ERK	extracellular signal regulated kinase
GDR	glucose disposal rate
GFAT	glutamine:fructose-6-phosphate amidotransferase
GlcNAc	N-acetyl-glucosamine
GLUT4	glucose transporter 4
GPx	glutathione peroxidase
GSH	glutathione
GSK3	glycogen synthase kinase 3
GSSG	glutathione disulfide (oxidized)
IGF-1	insulin-like growth factor 1
IRS-1	insulin receptor substrate-1
JNK	c-jun N-terminal kinase
KRBH	Kreb's ringer bicarbonate Hepes buffer
MAPK	mitogen activated protein kinase
MEK	mitogen activated/extracellular signal regulated kinase
NAC	N-acetylcysteine
NADPH	β-nicotinamide adenine dinucleotide phosphate (reduced form)
NIDDM	non-insulin dependent diabetes mellitus
OGTT	oral glucose tolerace test
PH	pleckstrin homology
PI3K	phosphoinositide-3 kinase
PIP3	phosphatidylinositol (3,4,5) phosphate
PKB	protein kinase B
PKC	protein kinase C
PTB	protein tyrosine binding
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SH2	src homology 2
SH3	src homology 3
Shc	src homology and collagen like protein
SOD	superoxide dismutase
SOS	son of sevenless
SSA	5-sulfosalicylic acid
TBARS	thiobarbituric-acid reactive substances

Chapter 1: Background

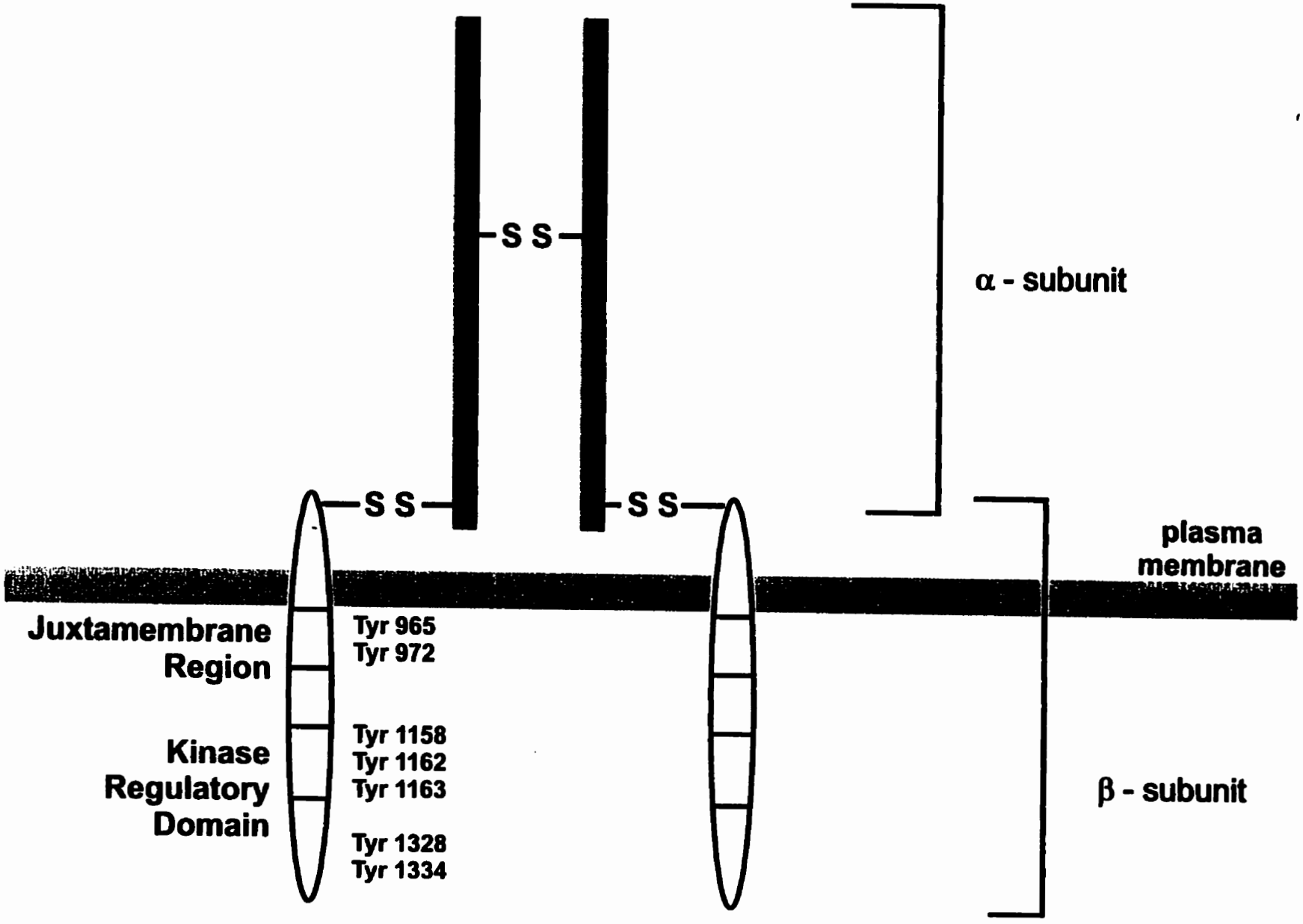
A.1. Insulin Action

To a large extent, glucose homeostasis and fuel metabolism within the body falls under the regulation of the hormone insulin. Insulin is synthesized and released from the β-cells of the pancreas in response to a rise in plasma glucose concentrations (for review see ref. 19). Within the plasma, insulin normally circulates at a concentration of 10^{-10} to 10^{-9} M. Of its several actions, insulin's major function is to act upon peripheral insulin sensitive tissues such as muscle, adipose, and liver to cause a general uptake of glucose from the plasma into these tissues. This is achieved at the cellular level in muscle and fat by insulin's ability to cause a translocation of glucose transporters (primarily GLUT4) from an intracellular storage compartment to the plasma membrane (76,149,132). Once fused into the plasma membrane, these glucose transporters facilitate the uptake of glucose from the circulation into the cell, at which point, the glucose is free to undergo glycolysis, glycogen synthesis, and other anabolic reactions. This translocation of glucose transporters is achieved through a complex cellular signaling cascade which begins with insulin binding to its specific plasma membrane receptor and then proceeds via several different downstream enzymes and proteins.

A.1.1. Insulin Receptor

The insulin receptor is expressed in almost all mammalian tissues although the number of receptors per cell varies from tissue to tissue. They are most abundant on the insulin sensitive tissues adipose, muscle, and liver (for review see ref. 19,87). The insulin

Figure 1: Insulin Receptor



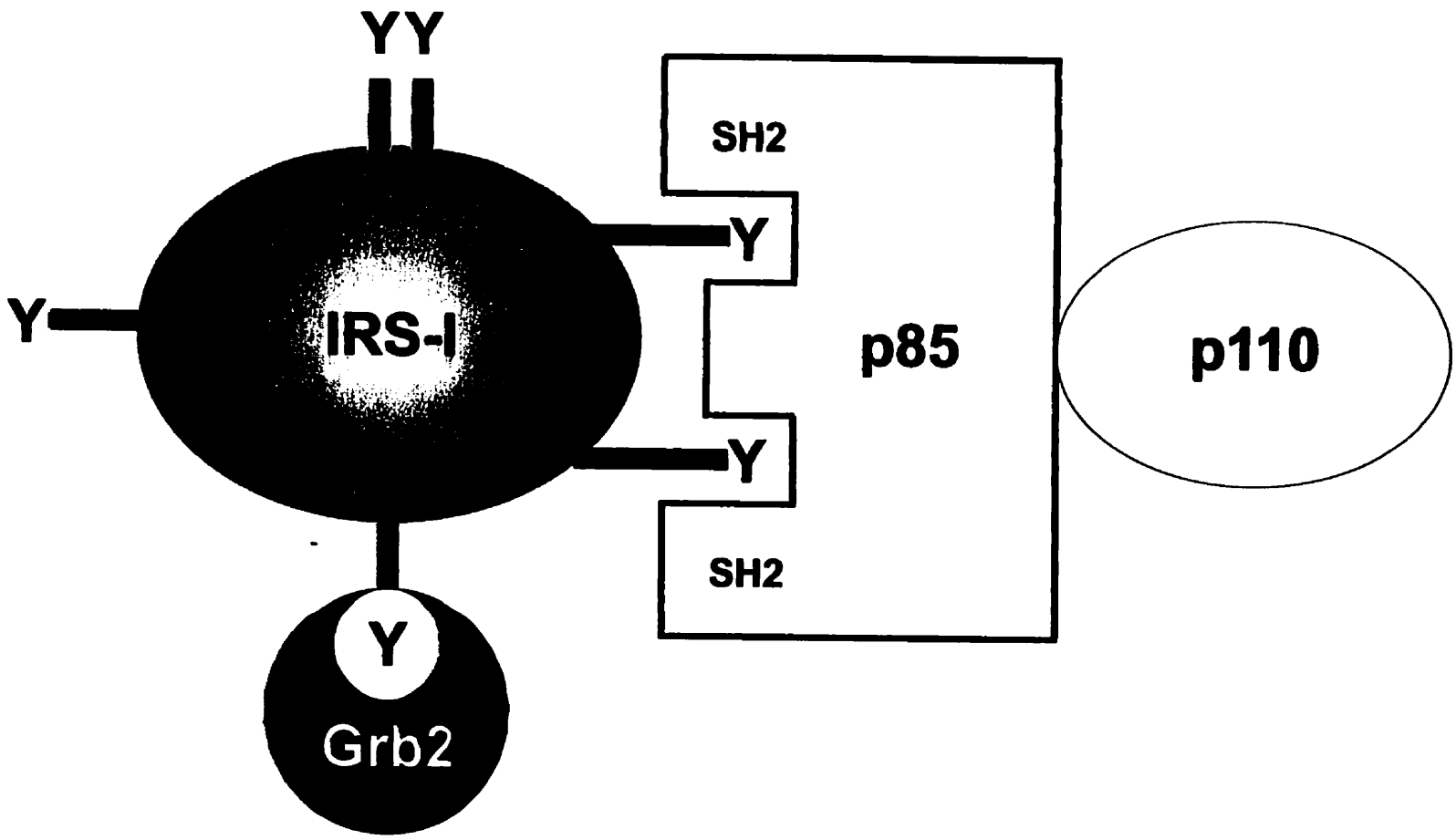
receptor consists of two α-subunits and two β-subunits linked through disulfide bonds to form a heterotetramer (19) (see fig. 1). In this arrangement, the α-subunits, which are located entirely extracellularly, contain the ligand or insulin binding domain while the β-subunits contain a tyrosine kinase domain. The β-subunit is composed of a short extracellular domain, a 23 amino acid transmembrane domain, and an intracellular domain which contains an ATP binding site and tyrosine kinase activity (19). Although it is unclear exactly how the insulin signal is transduced through the receptor to result in increased kinase activity, it is known that insulin binding causes the autophosphorylation of the insulin receptor (19). The autophosphorylation occurs on seven possible tyrosine residues. Three of these residues, Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}, comprise the kinase regulatory domain and, upon their phosphorylation, are responsible for further activating the tyrosine kinase activity of the insulin receptor (19). There are at least four other possible tyrosines (two in the juxtamembrane region and two at the C-terminal) which may play important roles upon their phosphorylation (19). The importance of the C-terminal phosphotyrosines lies in their ability to act as binding or docking sites for several src homology 2 (SH2) domain containing proteins. Tyr^{972} within the juxtamembrane region is the phosphotyrosine which has been implicated to play an important role in insulin stimulated glucose transport (see below) (165). The insulin receptor is also known to contain many sites of serine and threonine phosphorylation, the importance of which is currently being pursued although there is evidence that phosphorylation on these residues may decrease or inhibit the insulin receptor tyrosine kinase activity (19,157).

A.1.2. Insulin Receptor Substrates

Immediately downstream of the insulin receptor lies a protein termed the insulin receptor substrate (IRS) (for review see ref. 19,166). IRS-1, a protein of approximately 185kDa on SDS-PAGE when fully phosphorylated, associates with the insulin receptor through its protein tyrosine binding (PTB) domain which functions by binding to the phosphorylated tyrosines on the insulin receptor (specifically tyr^{972} in the juxtamembrane region) (165) (see fig. 2). Tyr^{972} appears to play a specific role in binding downstream substrates of the insulin receptor as mutation of this particular tyrosine residue did not affect insulin binding, autophosphorylation, or kinase activation, but it did prevent phosphorylation of IRS-1 indicating that there was no association between the two molecules (165). Another protein domain which has been postulated to play an important role in the association between IRS-1 and the insulin receptor is the pleckstrin homology (PH) domain which is linked to the amino-terminus of the PTB domain (12,171). PH domains function by binding to phosphoinositides and are speculated to play a role in targeting molecules to different cell membranes (101). IRS-1 also has another domain near the N-terminus which contains a consensus sequence for a nucleotide binding site, but since this molecule does not contain any catalytic activity, the exact function of this region remains unknown (19).

In addition to these various protein modules, IRS-1 contains greater than 20 potential tyrosine phosphorylation sites which are conserved between IRS-1 homologs (19). When phosphorylated, these tyrosine residues would serve as a potential binding site for many proteins which contain a src homology 2 (SH2) domain. SH2 domains are protein modules

Figure 2: Insulin Receptor Substrate and PI3 Kinase



of approximately 100 amino acids which recognize and bind specific phosphotyrosine residues. Two sequences known to be recognized by SH2 domains of PI3-Kinase are pYMXM and pYXXM, both of which can be found in the IRS-1 protein (there are six pYMXM and three pYXXM motifs) (166). Thus, IRS-1 serves as a docking protein which is capable of recruiting several other proteins into its signaling cascade via its phosphotyrosines.

IRS-1 is also known to contain approximately 40 potential sites of serine and threonine phosphorylation (19,153). In the basal state, IRS-1 is known to be predominantly phosphorylated on serine residues and to a lesser extent on threonine residues (153). Upon insulin stimulation, IRS-1 undergoes an increase in both tyrosine and serine phosphorylation (152). The exact function of such serine phosphorylation is unknown thus far, but it has been proposed that serine phosphorylation of IRS-1 may decrease the ability of the insulin receptor to tyrosine phosphorylate IRS-1 or that it may interfere with the binding between the SH2 domains of downstream substrates and the phosphotyrosines of IRS-1 (166).

IRS-2 is similar to IRS-1 both structurally and functionally although there are slight, and possibly, significant differences (166). Like IRS-1, IRS-2 is also phosphorylated by the activated insulin receptor and is therefore capable of causing the translocation of glucose transporters to the cell surface, resulting in a net influx of glucose into the cell (177). In IRS-1 deficient mice, it was found that IRS-2 could act as a compensatory molecule capable of transducing the insulin signal and ameliorating the negative effects of IRS-1 loss (128). Although IRS-2 may be similar in structure to IRS-1 and is capable of playing a similar role in insulin signaling, there is increasing evidence that perhaps IRS-2 has a functional significance and specificity which is quite distinct from that of IRS-1. Evidence for this

conclusion arises from a comparison of the IRS-1 and IRS-2 knockout mice. IRS-2 knockout mice developed overt diabetes with a defect in β-cell function, however, a similar defect was not seen in IRS-1 knockout mice.

A.1.3. Phosphoinositide 3-kinase

Phosphoinositide 3-kinase (PI3-kinase, PI3K) is made of two different subunits, the p85 adaptor or regulatory subunit and the p110 catalytic subunit (for review see ref. 150,144). The p85 subunit is known to contain two SH2 domains which allow it to associate with phosphotyrosines on either the IRS-1 (tyr^{460}, tyr^{608}, tyr^{939}, tyr^{987} on IRS-1), IRS-2, or growth factor receptors (76). In addition to its primary ability to phosphorylate phosphoinositides, the p110 catalytic subunit is known to have limited serine kinase activity towards the p85 subunit and IRS-1 which may serve as an autoregulatory feedback mechanism to control the association between these two proteins or perhaps to regulate the catalytic activity of the p85/p110 complex (41).

Activation of PI3-kinase begins with the tyrosine phosphorylation of the receptor or IRS-1. Phosphorylation of either of these molecules on a tyrosine within the specific YXXM motif facilitates the association between these phosphotyrosines and the two SH2 domains of the p85 subunit (144). This association or recruitment of the p85 subunit to the phosphotyrosines then leads to activation of cellular PI3-kinase activity through an increase in the catalytic activity of the p110 subunit and by moving the PI3-kinase complex in close proximity to membranes, and therefore to its substrate (144,150). Stimulation of cellular PI3-kinase activity results in a transient increase in the intracellular levels of PIP3 (144).

The mechanisms by which increased levels of PIP3 can propagate the insulin signal are unknown however, and are presently being actively pursued.

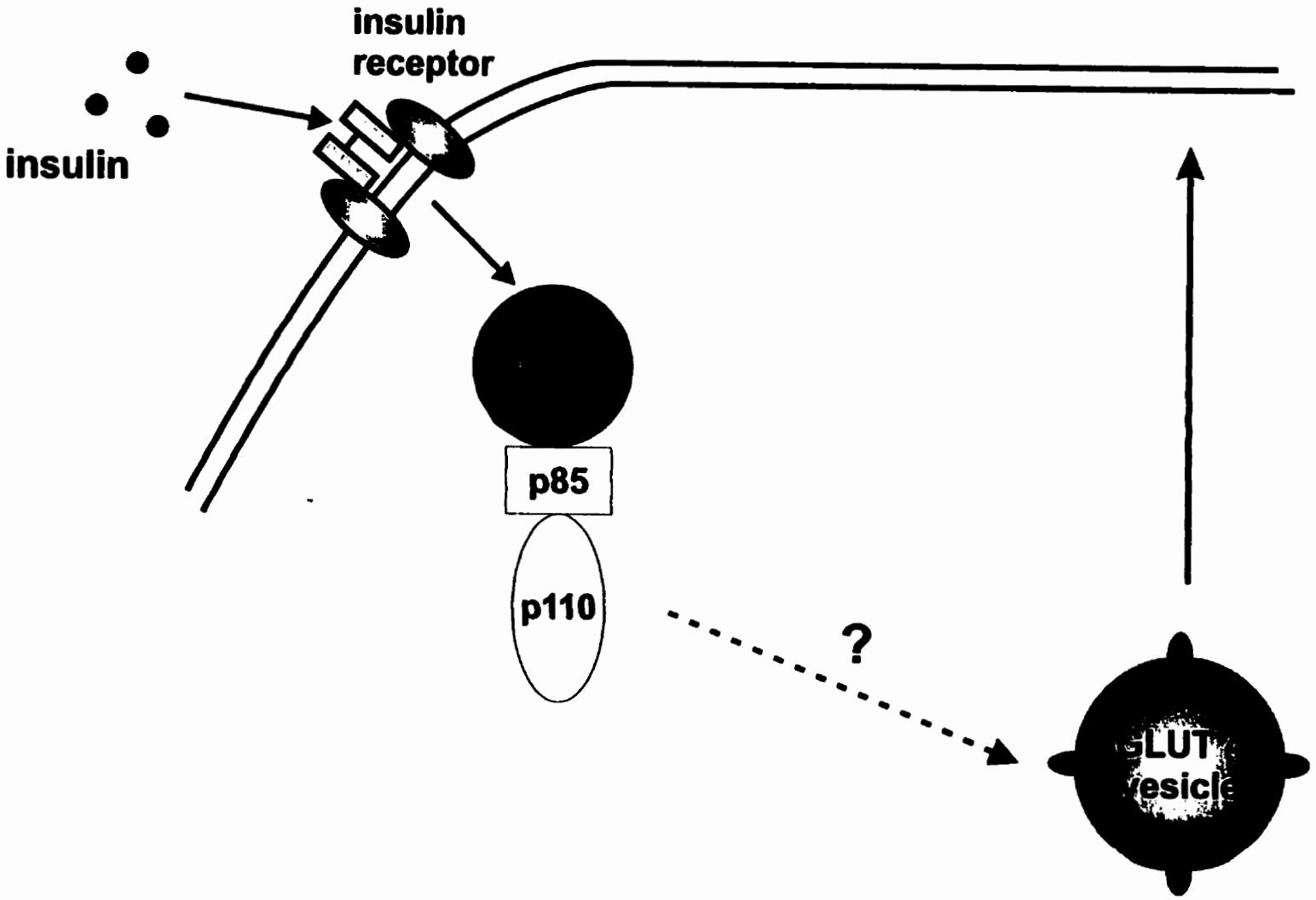
Most evidence to date would seem to indicate that PI3-kinase plays an essential role in insulin stimulated glucose metabolism. The strongest evidence implicating this enzyme in insulin signaling results from the observation that two structurally independent PI3-kinase inhibitors, wortmannin and LY294002, were capable of blocking insulin stimulated glucose transport and GLUT4 translocation (20,123,169).

While most evidence would indicate the importance and necessity of PI3-kinase in insulin signaling, some observations provide evidence which confound or complicate the data observed. For example, while both activation of the insulin receptor and the platelet-derived growth factor receptor can stimulate PI3-kinase activity, only the insulin receptor can stimulate glucose transport (119). Therefore, the exact role or mechanism by which PI3-kinase activation leads to a translocation of glucose transporters to the cell surface remains elusive, but the answers to this question should become more tangible as the downstream substrates of the lipid products of PI3-kinase are identified. Understanding the mechanism by which different growth factors achieve specificity through PI3-kinase in their signaling would also help to elucidate the role of this enzyme in insulin action.

A.1.4. Translocation of Glucose Transporters

One of the end metabolic effects of insulin is an increased flux of glucose into the cell which is achieved through a translocation of glucose transporters to the cell surface. To date there are seven different facilitative glucose transporters that have been cloned, but GLUT4 appears to be the major insulin sesnsitive transporter. GLUT4 is expressed mainly

Figure 3: Glucose Transporter Translocation



within the insulin sensitive tissues of fat, cardiac, and skeletal muscle (for review see ref. 149). Another glucose transporter which is ubiquitously expressed in most tissues including the insulin sensitive ones are the GLUT1 transporters (149). Functionally, both molecules are proteins of high hydrophobicity which lack any catalytic activity except when present within a membrane bilayer (149).

Mechanistically, insulin is able to stimulate an increase in glucose transport by causing a translocation of GLUT4 containing vesicles from an intracellular storage pool to the plasma membrane (35,154) (see fig. 3). Most evidence to date would suggest that GLUT4 is stored in a unique intracellular compartment (132). The translocation of GLUT4 essentially brings about an increase in the Vmax of glucose transport, but there is speculation that an increase in the intrinsic activity of the transporter may also be involved (149,180). Stimulation by insulin seems to cause an increase in transporter exocytosis while not having a major effect on transporter endocytosis (although it may cause a slight inhibition of endocytosis), the end result being a large flux of glucose transporters being incorporated into the plasma membrane (36,170). The biochemical processes which mediate the translocation event are currently under investigation and are slowly being elucidated.

A.1.5. Alternative Pathways of Insulin Signaling

In addition to increasing glucose uptake, insulin is able to stimulate other intracellular signaling pathways. Perhaps the best characterized alternative signaling pathway of insulin is the activation of the mitogen activated protein (MAP) kinase pathway. Activation of this pathway can begin at either the insulin receptor or at IRS-1. Src-homology-collagen like

protein (Shc), an adaptor molecule without any enzymatic activity, is capable of binding to the insulin receptor through its PTB domain and can form a complex with two other proteins, Grb2 and son-of-sevenless (SOS) (83,96,99). Alternatively, Grb2, also an adaptor protein, can bind directly to IRS-1 (through its SH2 domain) and associate with the guanine nucleotide exchange factor SOS (through its SH3 domain) (5). SOS, in turn, associates with a 21kDa protein referred to as Ras (112). Ras has the ability to bind and activate a cytoplasmic serine kinase termed Raf (97). However, Ras can only activate Raf when it is bound to GTP to form the active Ras-GTP complex (88). The binding of GTP to Ras is regulated by guanine nucleotide exchange factors such as SOS and the hydrolysis of GTP is regulated by GAPs (although Ras itself can hydrolyze GTP) (88). Once activated, Raf can serine phosphorylate the mitogen-activated protein kinase/extracellular signal regulated kinase kinase (MEK1 and MEK2) (22). These two kinases are known as dual specificity kinases and can phosphorylate both tyrosine and threonine residues as they do to their downstream substrates, the MAPKs also known as extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) (22). Once activated, the ERKs can phosphorylate p90 ribosomal S6 kinase as well as transcription factors, most of which are involved in cell growth and proliferation (22).

A.2. Insulin Resistance

Insulin resistance can be defined as a reduced biological response to a given concentration of insulin. The causes of insulin resistance have been classified by disease processes, e.g.) obesity and Type 2 Diabetes Mellitus (NIDDM) (see below) (106), by the initiating factor e.g.) elevated free fatty acids, TNF-α, or by site of the defect in the insulin signaling pathway, e.g.) insulin receptor, glucose transporter. In many situations *in vivo*, the etiology of insulin resistance is complex and both genetic and acquired causes may coexist. For example, in Type 2 Diabetes, insulin resistance of skeletal muscle exists prior to the onset of overt diabetes (90). However, hyperinsulinemia and hyperglycemia can also cause insulin resistance. There is evidence *in vivo* that hyperinsulinemia and hyperglycemia may be responsible for the induction of defects in insulin action in target tissues (52,106,162). The precise cellular and molecular mechanism leading to insulin resistance in these conditions remain incompletely understood. Conversely, insulin resistance can be ameliorated upon attaining tight control of diabetes (ie. by reducing the hyperglycemic state) (137). Glucose toxicity is often the term used for these adverse metabolic consequences of hyperglycemia. When primary adipocytes were cultured under conditions of hyperglycemia and hyperinsulinemia, they underwent a pronounced loss of both maximal insulin responsiveness and insulin sensitivity (52,160,161). This abnormality was the result of an impairment in the ability of glucose transporters to translocate to the cell surface in response to insulin (52). The efficacy of the insulin-responsive protein synthesis system was studied under these same conditions of hyperglycemia and hyperinsulinemia, but this system was found to be unaffected (108). This discovery suggested that any desensitization

which was caused by glucose and insulin was localized to the glucose transport system without an effect on insulin's stimulatory effect on protein synthesis. Conversely, particular amino acids (serine and isoleucine) were found to modulate insulin action through an enhancement of insulin responsiveness and insulin sensitivity of the protein synthesis system (107). Thus, such discoveries provided some explanation as to how insulin responsive cells regulate the ultimate responsiveness of each insulin effector system when the action of insulin is mediated by a common initial event.

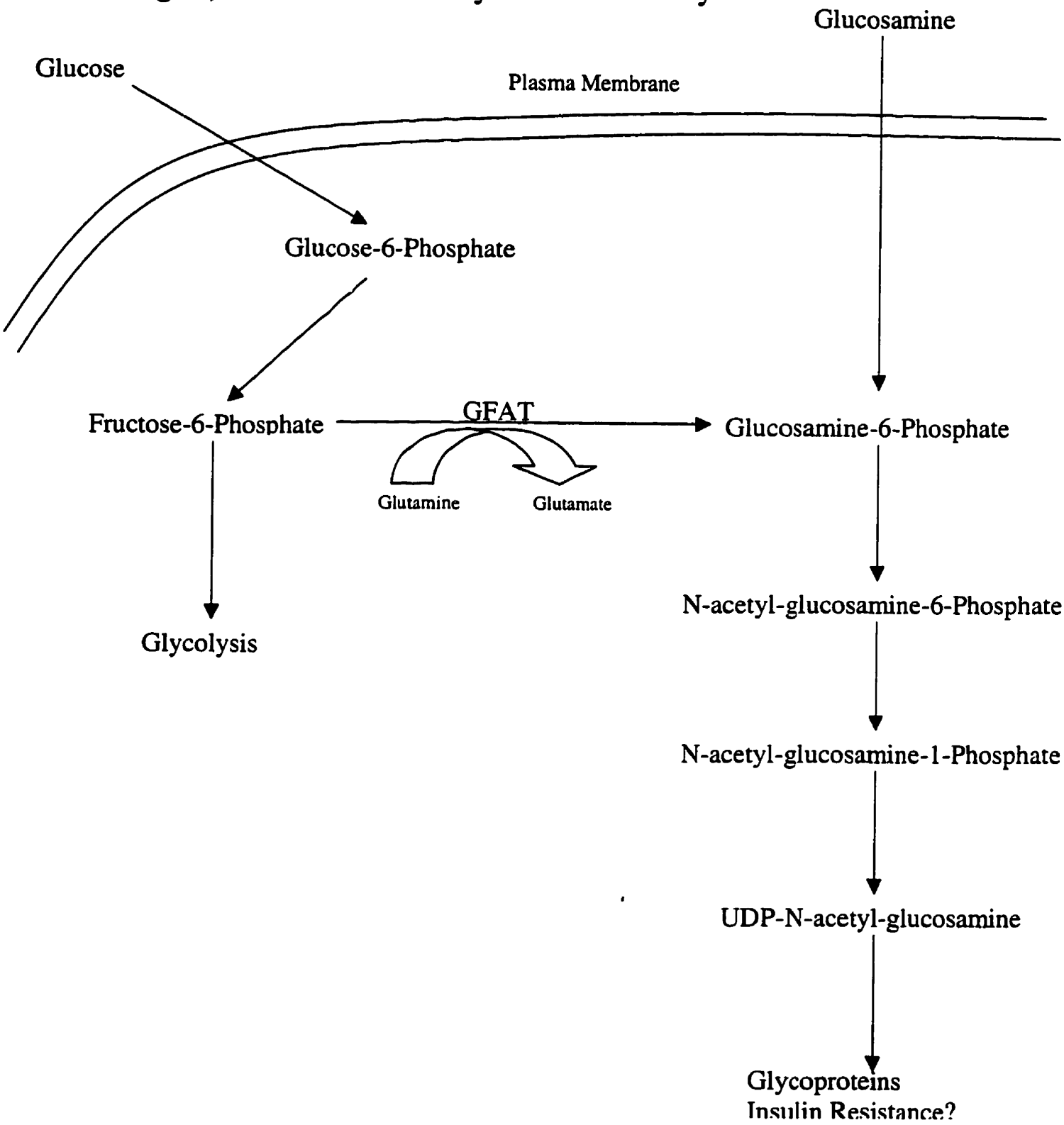
Not only could hyperglycemia and hyperinsulinemia induce insulin resistance within adipocytes, but this desensitization of the glucose transport system was found to be reversible (160). The recovery from insulin resistance was also found to be linear over time with a $t_{1/2}$ of 3.3 h (160). When the recovery rates were plotted against a function of the extent of desensitization, a high correlation emerged ($r=0.98$) which suggested that the recovery of insulin responsiveness is related to the extent of desensitization rather than the treatment time (160). Within the same study, cycloheximide inhibition of protein synthesis was unable to prevent desensitization of the glucose transport system which indicated that the insulin resistance was not mediated by the de novo synthesis of proteins (160). Subsequently, the integral role of amino acids in modulating glucose-induced insulin resistance became apparent when a Hepes-buffered salt solution was substituted for the more physiological Dulbecco's Modified Eagle's Medium (DMEM) (161).

When adipocytes were incubated under conditions of hyperglycemia and hyperinsulinemia in DMEM containing 15 different amino acids, there was a considerable decrease in the insulin responsiveness and sensitivity of these cells (161). However, when these amino acids were removed, little or no desensitization of the glucose transport system

was seen, although the absence of amino acids appeared to have no influence over dexamethasone-induced insulin resistance (160). Upon further analysis, it was discovered that L-glutamine was the amino acid responsible for the induction of glucose-induced insulin resistance (161). From this discovery, it was concluded that glucose, insulin, and glutamine were necessary components for the glucose-induced desensitization of the glucose transport system although other glutamine-free mechanisms of inducing insulin resistance were known to exist.

The necessity of glutamine to induce resistance led to the hypothesis that the hexosamine biosynthesis pathway was in some way implicated in the mechanism of glucose-induced resistance (105). The first and rate-limiting enzyme within this pathway is glutamine:fructose-6-phosphate amidotransferase (GFAT) which catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate by using the amide group of glutamine (105). This enzyme has high levels of expression of protein and mRNA in insulin sensitive tissues such as fat and muscle (120). The end products of this pathway include uridine diphosphate (UDP)-N-acetyl-glucosamine (GlcNAc) and other nucleotide hexosamines which are thought to invoke feedback inhibition upon the GFAT enzyme (110,94). In order to test this hypothesis, glutamine analogs (O-diazoacetyl-L-serine or azaserine, and 6-diazo-5-oxonorleucine) which had the capability of inhibiting glutamine-requiring enzymes such as GFAT were used. Both inhibitors of GFAT were able to prevent glucose-induced insulin resistance in 18hr treated cells while having no effect over maximal insulin responsiveness in control cells (105). A second method used to test the hypothesis was the use of glucosamine, which had the ability to enter the hexosamine biosynthesis pathway at a point distal to the enzymatic amidation by GFAT. Glucosamine is known to cross the cell

Fig. 4) Hexosamine Biosynthesis Pathway



membrane by being transported through the glucose transporters at which point it is subsequently phosphorylated by hexokinase (110). However, the Km or affinity of the glucose transporters for glucosamine is approximately three times less than what it is for glucose (110). When used in conjunction with the GFAT inhibitors, glucosamine could bypass the enzyme to induce a desensitization of the glucose transport system in a dose dependent manner (105). Furthermore, glucosamine was found to be approximately 40-fold more potent than glucose. This is indicated by the lower concentrations of glucosamine needed to induce insulin resistance in spite of the lower affinity between the glucose transporters and glucosamine. As well, it did not require the presence of glutamine as a co-factor (105). Lastly, two inhibitors of mRNA synthesis (actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)) were used to estimate GFAT turnover (104). Both mRNA inhibitors caused a rapid loss in GFAT activity which indicated that this enzyme had a short half-life (104). These inhibitors could also prevent or block glucose-induced insulin resistance, but glucosamine was able to overcome this blockade (104). From these observations, it was concluded that glucose-induced insulin resistance was mediated through the GFAT enzyme and that the mechanism of resistance did not require the synthesis or transcription of mRNA (104). Furthermore, while the discrepancy in induction of insulin resistance with cyclohexamide and the inhibitors of mRNA synthesis were noted by Marshall et al., a resolution could not be reached which could account for this difference (104). Thus, it appeared from this evidence that GFAT and the hexosamine biosynthesis pathway were closely implicated in glucose-mediated peripheral insulin resistance (see fig. 4). However, recent evidence has raised the possibility that glucose and glucosamine induce insulin resistance through two different pathways or mechanisms. This idea stems from one

study which demonstrated that troglitazone, an orally active hypoglycemic agent, could prevent hyperglycemia-induced insulin resistance, but not that induced by glucosamine when both agents were infused into rats and a hyperinsulinemic-euglycemic clamp was performed (113).

Since these initial studies into the nature of glucose-induced insulin resistance, several alternative approaches have been used in order to better elucidate the mechanism by which the hexosamine biosynthesis pathway affects the insulin signaling pathway. In other in vitro studies, the hexosamine biosynthesis pathway appeared to have no effect in L6 myotubes (37). However, when skeletal muscle (rat hemidiaphragm) was preexposed to glucosamine, there was a marked induction of insulin resistance which was attributed to a defect in translocation of glucose transporters, since the total GLUT4 pool was unaffected (135). Furthermore, insulin-stimulated glycogen synthesis was decreased within these cells (135). An alternative approach used to study this system on a cellular level was a genetic approach of transfecting cDNAs into various cellular systems. In rat-1 fibroblasts transfected with the yeast cDNA for GFAT, there was a loss in insulin-stimulated glycogen synthase activity, but no change was seen in insulin binding and receptor number (30). As well, the overexpression of GFAT had no effect on GLUT1 mediated glucose uptake (30). Similar studies were performed with the human cDNA for GFAT being transfected into rat-1 fibroblasts (32,178). Again, these cells were found to be insulin resistant for the stimulation of glycogen synthase activity in response to a given concentration of insulin (31,32,). The mechanism of this inhibition is thought to occur by glucosamine downregulating the activity of the glycogen-bound form of type-1 protein phosphatase (PP1G) (31). PP1G can activate glycogen synthase by dephosphorylating this enzyme, but glucosamine significantly reduced

insulin's ability to activate PP1G and in turn, glycogen synthase (31). Thus, it appeared that glucosamine could affect the phosphorylation state of particular enzymes within the insulin signaling cascade although no alteration of MAP kinase or S6 kinase was brought about by glucosamine within rat muscle cells (135). There is evidence that the glucose and glucosamine-induced insulin resistance is mediated through PKC (47). Both inducers of insulin resistance were able to cause a three-fold increase in PKC activity and the effects of hyperglycemia and glucosamine were reversed by the PKC inhibitor Ro-31-8220 as glucose transport was restored to control levels (47). Thus, the inhibition of glucose transport by glucose and glucosamine may be mediated by PKC, but as there are several different isoforms of PKC, further investigation needs to be done within this area.

When the effect of glucosamine on glucose metabolism in vivo was examined, the results appeared to support in general what was discovered in vitro although there were some inconsistencies. For example, muscle glycogen synthase was found to be unaffected by glucosamine infusion, but there was a marked decrease in the glucose disposal rate (GDR) of these animals after 7 hr of glucosamine infusion (138). Furthermore, hyperglycemia and glucosamine were found to be nonadditive within this system as glucosamine could not lead to a further reduction in the GDR of partially pancreatectomized diabetic rats (138). This observation indicated that the suppression of glucose disposal by hyperglycemia or glucosamine may operate through the same mechanism or pathway in vivo as they appear to do in vitro. Additionally, glucosamine infusion was shown to impair the translocation of GLUT4 to the cell surface in rat skeletal muscle (6). An important qualification of studies performing the infusion of glucosamine is that measurements of GDR need to be done under euglycemic and hyperinsulinemic conditions (4). A maximal

concentration of exogenous insulin is needed during the glucose clamp because glucosamine has been shown to be an inhibitor of glucokinase which has the effect of impairing β-cell glucose sensing and endogenous insulin secretion (4). Glucosamine also had the ability to impair the effect of insulin-like growth factor 1 (IGF-1) on glucose uptake, glycolysis, and glycogen synthesis (70). Thus, stimulation with IGF-1 could not overcome the insulin resistance brought about by glucosamine infusion which would indicate that such peripheral insulin resistance is mediated by the same mechanism or incurred at a site common to both IGF-1 and insulin signaling pathways (70).

The in vivo insulin resistance brought about by the infusion of glucosamine is proposed to be mediated by an increase in muscle UDP-N-acetyl-hexosamines (69). Since glucosamine is normally found in negligible concentrations within the blood (in normal and diabetic individuals), nonphysiological or pharmacological concentrations need to be infused in order to facilitate the movement of glucosamine down the hexosamine biosynthesis pathway (110). Infusing such high concentrations of glucosamine can deplete the cellular levels of UTP due to an increased production of nucleotide-hexosamines (105). Subsequently, depleting cellular UTP would prevent the formation and availability of UDP-glucose (therefore preventing glycogen synthesis) and markedly alter the utilization of intracellular glucose (135). Such an alteration would tend to confound any direct effect of glucosamine on glucose homeostasis. However, co-infusion of uridine and glucosamine was able to prevent a decrease in skeletal muscle UDP-glucose levels while further increasing the levels of UDP-N-acetyl-glucosamine (69). These effects of uridine co-infusion augmented the insulin resistance normally seen when glucosamine is infused alone (69). In fact, infusing uridine alone could significantly increase the UDP-N-acetyl-

glucosamine concentrations and induce insulin resistance which provided validity to the argument that glucosamine-induced resistance was a result of increased nucleotide-hexosamines rather than a decrease in UDP-glucose (69). Recently however, in vitro evidence has been published which suggested that glucosamine-induced resistance could be due to a depletion in intracellular ATP (79). In 3T3-L1 adipocytes, incubation with glucosamine was able to cause an inhibition in insulin receptor autophosphorylation, IRS-1 phosphorylation, IRS-1 associated PI-3kinase activity, PKB activation, and ultimately, translocation of GLUT1 and GLUT4 (79). Intracellular ATP levels were also reduced upon incubation with glucosamine and mimicking this ATP depletion with the use of azide could reproduce the insulin resistance induced by glucosamine (79). When inosine was added to the glucosamine incubation as an alternative energy source and ATP levels maintained, the insulin resistance was prevented indicating a dissociation between UDP-N-acetyl-glucosamine levels and insulin resistance (79). Therefore, the cellular mechanism by which the hexosamine biosynthesis pathway causes insulin resistance is not readily understood and further studies are warranted.

One approach which has been used to resolve the aforementioned debate is the use of transgenic mice overexpressing the GFAT enzyme (they are less likely to cause significant shifts in nucleotides or UDP-glucose levels). GFAT overexpression in cultured cells could double the levels of UDP-hexosamines while having no effect over nucleotide triphosphate levels (110). Transgenic animals overexpressing GFAT in muscle and fat tissue became hyperinsulinemic at approximately 6 months of age and had a marked decrease in their GDR (72,120). In a related study, transgenic mice overexpressing GLUT1 developed insulin resistance while mice overexpressing GLUT4 did not (14). The GLUT1

overexpressing mice had increased levels of GFAT activity (GFAT mRNA was unchanged), and increased levels of nucleotide-linked sugars (14). From this evidence, the authors concluded that chronic increased glucose flux increases GFAT activity and hence hexosamine biosynthesis leading to insulin resistance (14). Conversely, when rats experienced chronic hyperglycemia due to streptozotocin-induced diabetes, GFAT activity appeared to increase (136), while the opposite was true when muscle biopsies were taken from control and NIDDM patients (172). Thus, it becomes apparent that further investigation needs to be done in order to elucidate the relevance of the hexosamine biosynthesis pathway in insulin resistance and NIDDM as well as the mechanism by which the pathway works.

A.3. Oxidative Stress

A.3.1. Definition

Damaging and harmful free radicals or reactive oxygen species (ROS) are produced by all living aerobic organisms. Consequently, these same organisms have evolved strategies to remove ROS through the intake, production, and use of antioxidants. The overproduction of these ROS or the inability to clear them away is known as oxidative stress. A free radical is defined as any species which can exist independently which contains one or more unpaired electrons (59). Free radicals are most commonly generated by homolytic bond cleavage or by the transfer of an electron from one molecule to another molecule (158). Furthermore, because the unpaired electron of a ROS is not spin paired with a second electron as part of a chemical bond, it causes the ROS to be extremely

reactive and short-lived unless the unpaired electron is delocalized over several atoms (158). Possessing such a high reactivity is biologically hazardous as this facilitates oxidation and subsequent molecular damage of various biological molecules.

ROS can arise from many different endogenous and exogenous sources. Ongoing cellular reactions such as mitochondrial electron transport are known to be continuous sources of free radicals (109). In addition, enzymes such as the many oxidase enzymes are also known to produce ROS. Oxidative stress through the overproduction of free radicals can also arise as a result of inflammation, drug metabolism, or radiation (109). Therefore, it becomes apparent that free radicals can arise from several sources and that the production of these ROS must be balanced by the action of antioxidants in order to prevent the onset of oxidative stress.

A biologically relevant molecule, dioxygen (O_2), is a potential free radical as there is a single unpaired electron associated with each of the two oxygen atoms (158). However, the reactivity of O_2 is masked as a result of the two unpaired electrons possessing the same spin quantum number (59). Overcoming this spin restriction would greatly enhance the reactivity of dioxygen (59). Nevertheless, O_2 serves as the terminal electron acceptor in cellular respiration and is consequently involved in many biologically important free-radical processes (59). In addition, dioxygen also serves as the precursor for many other biologically relevant and more potent free radicals, some of which have been characterized. To date, the most thoroughly characterized organic free radicals have been the superoxide anion, hydrogen peroxide, and the hydroxyl radical. However, there are an increasing number of free radicals being discovered at a rapid rate, some of which have been shown to

play important biological roles such as the free radical peroxynitrite. Nevertheless, the focus of the following discussion will be upon the three aforementioned free radicals.

A.3.2. Superoxide Anion

A free radical or ROS which is naturally produced in almost all aerobic cells is the superoxide anion, $O_2^{\cdot}$. The major source of superoxide is due to the leakage of electrons onto O_2 (an electron must enter one of the π^*2p orbitals of oxygen) from different components of the cellular electron transport chain involved in respiration (50). These electron transport chains can often be found on such cellular organelles as the mitochondria, and chloroplasts (65). As well, the rate of $O_2^{\cdot}$ production increases in reaction to an increase in O_2 concentration due to a greater amount of electron leakage (48).

The chemistry and properties of $O_2^{\cdot}$ can vary greatly depending on its environmental solution. For example, $O_2^{\cdot}$ will behave as a strong base and nucleophile in organic solvents (134). However, in aqueous solution, superoxide possesses limited reactivity and is extensively hydrated (65). In this environment, $O_2^{\cdot}$ behaves mainly as a reducing agent and also as a weak oxidizing agent to such molecules as ascorbic acid (65). Despite its weak ability to oxidize molecules however, $O_2^{\cdot}$ generating systems and $O_2^{\cdot}$ itself have been shown to cause a considerable amount of biological damage (50,64,65). Consequently, enzymes known as superoxide dismutases (SOD) have evolved to cause the dismutation reaction of $O_2^{\cdot}$ to hydrogen peroxide (H_2O_2) and dioxygen (59,65) (see fig. 5).

A.3.3. Hydrogen Peroxide

It would be expected that any system which produces $O_2^{\cdot}$ would also produce H_2O_2 due to the non-enzymatic or SOD-catalyzed dismutation of $O_2^{\cdot}$. Such H_2O_2 production has been observed in whole bacteria, phagocytic cells, mitochondria, and chloroplasts (64). However, several oxidase enzymes also produce H_2O_2 directly by the transfer of two electrons to oxygen (59). An example of such oxidases would be xanthine oxidase, glucose oxidase, and D-amino oxidase (59,64,158). Significantly, H_2O_2 is not a free radical in the strictest sense as it does not possess any unpaired electrons, but it does act as a relatively stable but weak oxidant and reducing agent with limited reactivity(59,65). Also of importance is the fact that H_2O_2 can readily pass through biological membranes which the charged $O_2^{\cdot}$ anion can only do very slowly (65,158). Perhaps the way in which H_2O_2 most contributes to the oxidative stress of a cell and brings about oxidative damage to biological molecules is through its ability to take part in the Fenton reaction or the Haber-Weiss reaction to ultimately produce the hydroxyl radical $^{\cdot}OH$ (65,158,59,64).

A.3.4. Hydroxyl Radical

Although the damaged targets of $O_2^{\cdot}$ and H_2O_2 have been identified, the moderate reactivity of these two ROS in aqueous solution would make it unlikely that the damage done by them is due to their direct action. Currently, it is thought that most molecular damage caused by either $O_2^{\cdot}$ or H_2O_2 is associated with their conversion into a more highly reactive species such as $^{\cdot}OH$ (64,65,158). The $^{\cdot}OH$ radical can be produced either through

the Fenton and Haber-Weiss reactions or when water is exposed to high-energy ionizing radiation (65).

·OH is perhaps the most highly reactive of any biological ROS and therefore reacts with any molecule which is close to its site of formation. In some instances, the reaction of ·OH with another biomolecule results in the production or conversion of this molecule into one with lower reactivity (65). This less reactive radical can then diffuse away from its site of formation to attack other more distal targets (65). The production of ·OH is mainly due to the presence of free metal ions which react with H_2O_2 as part of the Fenton reaction to form ·OH.

A.3.5. Fenton Reaction

In the 1890's a reaction between iron salts and H_2O_2 which caused oxidative damage to biological molecules was described by the chemist H.J.H. Fenton (46) (see fig. 5). Most of the ·OH generated *in vivo* is thought to occur through this metal-dependent breakdown of H_2O_2. The candidate metal ion which is thought to catalyze this reaction in most cells and organisms is iron(II) although the reaction can take place with other metal ions such as titanium(III) or copper(I), but reactions with these latter two ions are not thought to occur *in vivo* (65). In fact, arguments have arisen against the theory that the Fenton reaction does indeed occur *in vivo*. These arguments are based upon the criticisms that a) the Fenton reaction has a rate constant which is too low to have biological significance b) the Fenton reaction does not produce the ·OH radical c) no free metal ion catalysts such as iron(II) would be available *in vivo*, and d) any ·OH radicals present *in vivo* are produced in a reaction between organic molecules and H_2O_2 directly without the need for metal ion

Fig 5)

A) Reaction of Superoxide Dismutase

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

B)Fenton Reaction

$$Fe^{++} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + Fe^{+++}$$

C) Haber-Weiss Reaction

$$O_2^- + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2$$

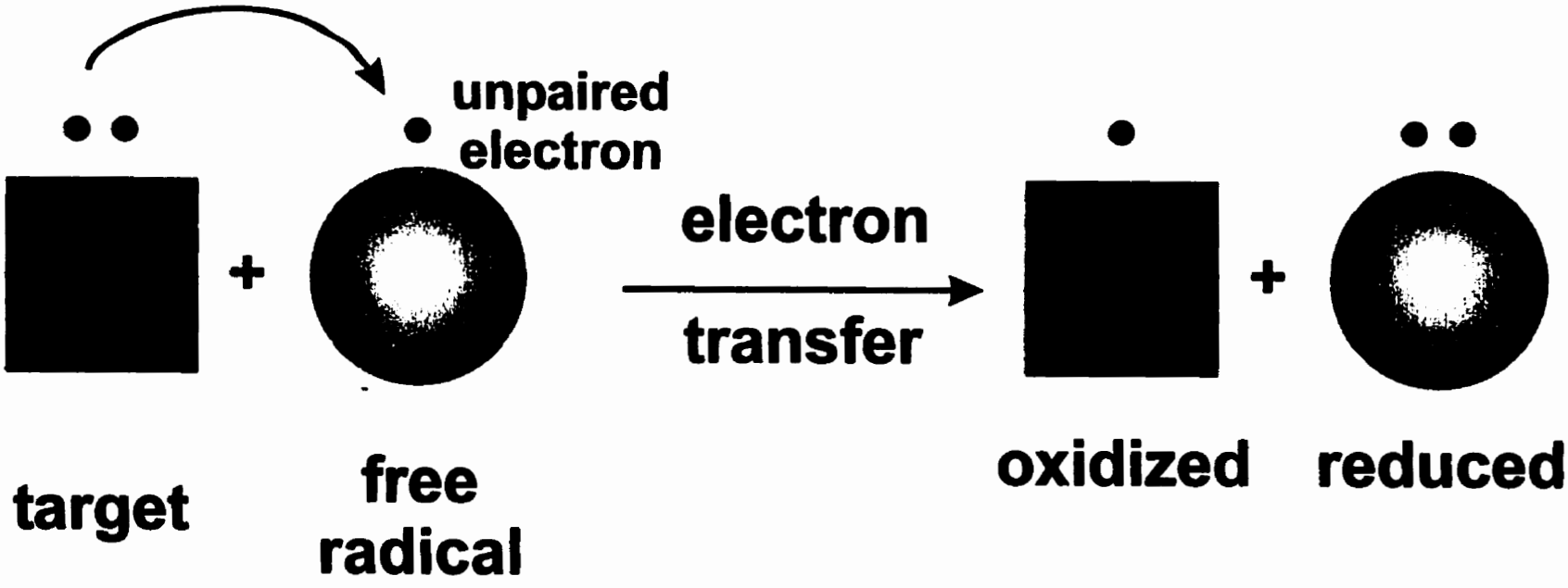
catalysts (1,12). However, these criticisms have yet to be substantiated and thus far, the evidence in support of the Fenton reaction taking place *in vivo* far outweighs any contradictory evidence.

An alternative way in which ·OH may be generated in vivo is through the Haber-Weiss reaction (65,109) (see fig. 5). In this reaction, no metal ion catalysts are needed as superoxide anion is capable of reacting directly with H_2O_2 to produce ·OH. Presently, evidence supporting the Haber-Weiss reaction taking place in vivo is both sparse and weak. In addition, methods to elucidate this mechanism remain difficult and elusive, therefore, any ·OH produced in vivo is normally attributed to the Fenton reaction while the Haber-Weiss reaction continues to play a small part in oxidative stress.

A.4. Damage by ROS

ROS cause damage to biological systems by stripping away electrons from major constituent molecules which may eventually lead to cell death (109). As the unpaired electron of the free radical is highly reactive, it will abstract an electron from a nonradical target. This electron transfer causes the radical to take on a stable conformation, but the target molecule which had a single electron removed has now become a radical as it is left with one unpaired electron (see fig. 6). At this point, any subsequent events will depend on the reactivity of the target radical. If the target radical possesses high reactivity, the opportunity exists for it to attack and oxidize other target molecules leading to the propagation of a free radical chain reaction. If the target radical has low reactivity, as in the case of antioxidants, the chain is broken. Therefore, free-radical chain propagation or

Figure 6
A) Free Radical Damage of a Target Molecule



autoxidation is usually divided into three steps: i) initiation, the original reaction which starts the chain ii) propagation, when many target molecules are oxidized as a result of initiation, and iii) termination, when the chain encounters an antioxidant or two radicals come together to give a nonradical (59,109,158). Furthermore, ROS are known to attack three important classes of biological molecules, namely lipids, protein, and DNA. Thus, the potential to damage these molecules indicates the ability of oxidative stress to play a role in many disease and pathological states.

A.4.1. Oxidation of lipids

The oxidation of polyunsaturated fatty acids by free radicals in biological systems is referred to as lipid peroxidation (59,65). The first-chain initiation of a peroxidation sequence begins with the attack by any species with sufficient reactivity to abstract a hydrogen atom (·H) from a methylene (-CH2-) group (59,65). $O_2^{\cdot}$ does not have sufficient reactivity to abstract a hydrogen atom from lipids, nor would it be capable of entering a membrane bilayer with its hydrophobic interior due to its charged nature (65). H_2O_2 also does not have sufficient reactivity to cause lipid peroxidation, but if H_2O_2 is catalyzed to ·OH, the hydroxyl radical will readily cause oxidation of lipids (59). Abstraction of .H from a -CH2- group will leave behind an unpaired electron on the carbon (65). The presence of a double bond in a fatty acid causes the C-H bond to weaken on the carbon atom adjacent to the double bond which more easily facilitates the abstraction of a ·H (59,65). This carbon radical will then try to stabilize itself by undergoing a molecular rearrangement to form a conjugated diene which will then follow one of two probable fates (65). If two of these conjugated dienes come into contact they will potentially cross-link the fatty acid molecules,

but it is more likely that the formation of a peroxyl radical will take place in the presence of O_2 (65). A peroxyl lipid radical is then capable of abstracting an ·H from another adjacent lipid molecule causing the propagation of a radical chain reaction (59,65). The peroxyl lipid which abstracted the ·H now becomes a lipid hydroperoxide (65).

If extensive lipid peroxidation takes place within biological membranes, loss of membrane fluidity, a decrease in membrane potential, an increased permeability to H^+ and other ions, and rupture of the membrane to release its contents can eventually occur (59,64). As well, particular end products of peroxide fragmentation can be cytotoxic (59).

A.4.2. Oxidation of Proteins

The ROS attack of proteins can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, or oxidation of the protein backbone which leads to protein fragmentation (9). Any of these protein modifications is capable of altering the structure and/or abolishing the function of the protein resulting in deleterious effects.

The polypeptide backbone of a protein undergoes oxidative attack when an ·OH radical abstracts the α-hydrogen atom of an amino acid residue to form a carbon-centered radical (9). Once this initial ·H abstraction has taken place, the carbon-centered radical can then undergo a series of intermediate reactions to form an alkylperoxyl radical, an alkylperoxide radical, an alkoxyl radical, and finally a hydroxyl protein derivative (9) (see fig. 7). Any of these intermediate radicals is capable of reacting with other amino acid residues in the same or different protein molecule to generate another carbon-centered radical and start a new chain of reactions (9). Two carbon-centered radicals can react with one another to form a protein-protein cross-linked derivative (9). Alternatively, once an

Fig 7) Chain Propagation of Protein Damage

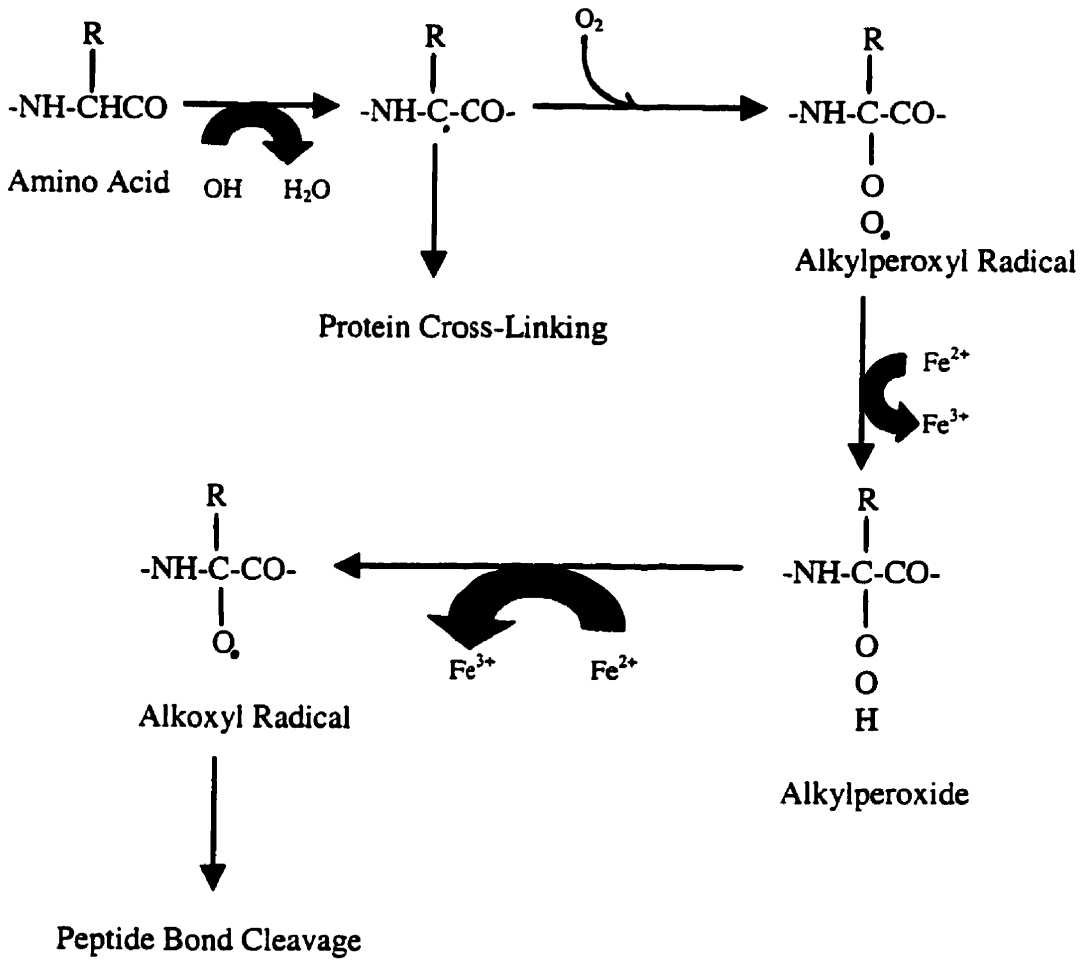
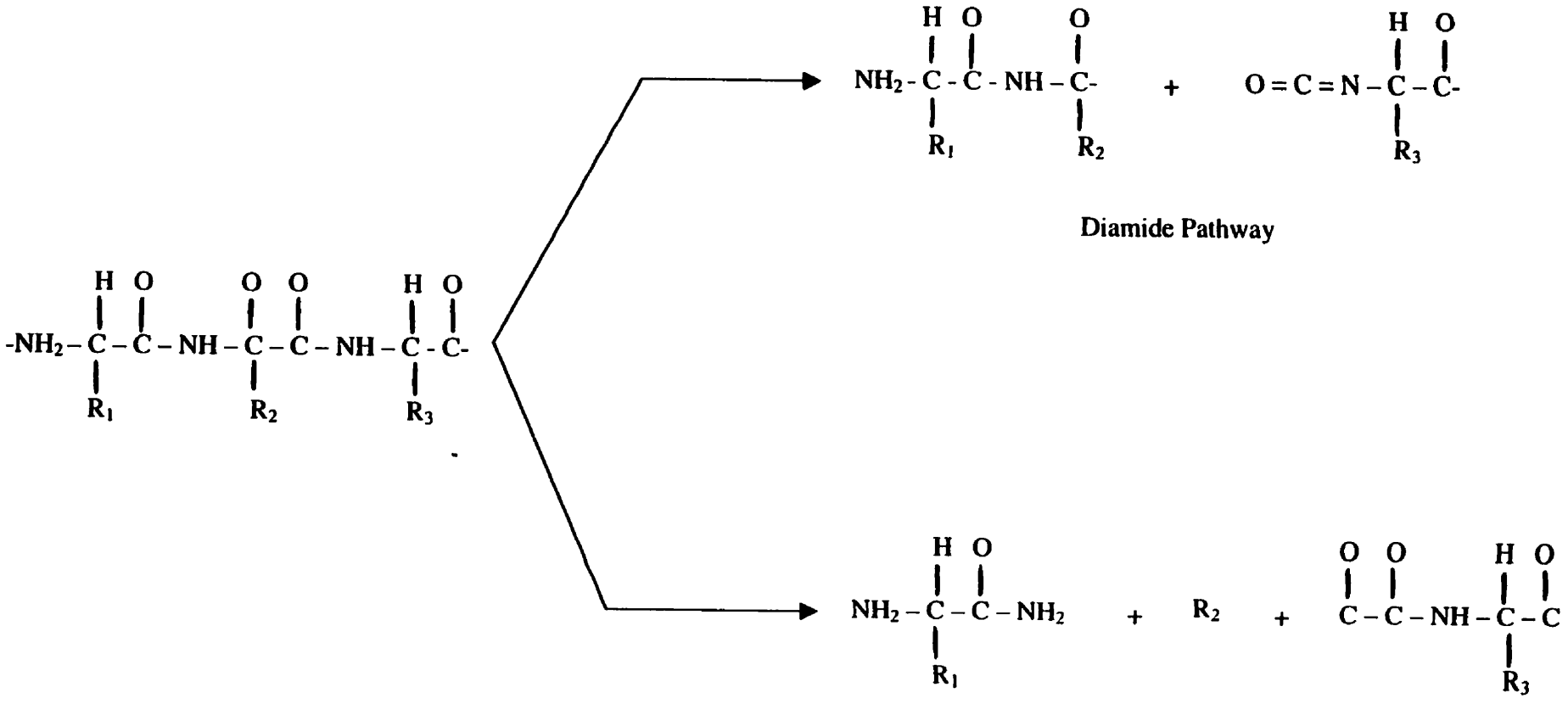


Fig 8) Diamide and α-Amidation Pathways of Peptide Bond Cleavage



alkoxyl radical has formed, cleavage of the peptide bond can occur by either the diamide or α-amidation pathways (9) (see fig. 8). Also, ROS attack of glutamyl, aspartyl, or prolyl side chains can lead to direct peptide bond cleavage (9,51).

Any and all amino acid residue side chains are susceptible to oxidation. Cysteine and methione are the only two residues that can be repaired once oxidized, although both residues are particularly sensitive to oxidation by all forms of ROS (9). The repair of these two residues is due to the actions of disulfide reductases and methionine sulfoxide reductases (9). Another class of amino acid which shows a high susceptibility to ROS attack are the aromatic amino acid residues (tryptophan and tyrosine) (9). Tyrosine is known to undergo a nitrosylation reaction in the presence of peroxynitrite. This irreversible nitration of tyrosine is important because such a modification would prevent tyrosine from taking part in any phosphorylation reactions (9,80). Preventing the phosphorylation of tyrosine would compromise a key mechanism of cellular regulation of enzyme activities and signal transduction networks (80,93).

ROS can cause the direct oxidation of lysine, arginine, proline, and threonine to yield a carbonyl derivative (102). The presence of carbonyl groups on proteins has now become a standard marker for evidence of ROS-mediated protein oxidation (102). The potential also exists that the alteration of these residues may alter the function of the protein which is oxidized. Thus, it becomes apparent that oxidation of proteins can alter the structure and function of proteins or result in protein breakdown, which ultimately culminates in the malfunction of the biological system.

A.4.3. Oxidation of DNA

The amount of steady state oxidative damage done to DNA would appear to be quite extensive with estimates at about $1.5X10^5$ oxidative adducts per human cell (7). Fortunately, most organisms have developed several ways in which to repair the DNA damage done by radical attack, the most common method being base excision through the use of endonuclease and glycosylase enzymes (7,40,73). The majority of DNA damage is thought to occur by ·OH as $O_2^{\cdot -}$ is relatively unreactive with DNA and H_2O_2 is likely to undergo the Fenton reaction (73,82). The Fenton oxidants of DNA would appear to fall into two classes where the type I oxidants seem to cleave DNA preferentially within the sequences R**T**GR, TA**T**TY, and CT**T**R, (where R represents the purine bases A and G and Y represents the pyrimadine bases C,T, and U, while N represents any base) and the type II oxidants cleave DNA in the sequence **NGG**G (the bold, underlined nucleotides being the sites of cleavage) (73). The damage done by these oxidants may occur at either the DNA base or the sugar. Damage to the sugar moiety is initiated when an ·H is abstracted from one of the deoxyribose carbons (73,163). This hydrogen abstraction will eventually lead to strand breakage and base release (163). If the DNA base is oxidized, this results in the addition of ·OH to the electron rich double bonds although radical attack on the base moieties of DNA does not usually result in strand breaks (73). However, radical attack of a single DNA base can lead by chain reaction propagation, to as many as 50 base alterations (74). Thus, any damage done to DNA by radical attack has the potential to alter the expression of various genes which could lead to such disease or pathological states as cancer.

A.5. Oxidants in Signal Transduction

Recently, evidence has emerged that ROS or oxidation-reduction reactions may play a role in regulating signal transduction. Oxidants have been shown to stimulate Ca^{2+} signaling by increasing the cytosolic Ca^{2+} concentration (155). In this manner, oxidants may play a physiological role in the regulation of Ca^{2+} signaling, but the exact source of Ca^{2+} release and the molecular targets of the oxidants have not yet been clearly defined. In addition, oxidants have been speculated to play a role in the regulation of protein phosphorylation. For example, it was discovered that H_2O_2 could cause tyrosine phosphorylation in different cell lines and cause stimulation of insulin receptor tyrosine kinase activity when present in conjunction with vanadate (71,45,95,179). The tyrosine phosphorylation which occurred was likely due to an inhibition of tyrosine phosphatases rather than a direct stimulation of the kinases. Oxidants have also been speculated to play a role in serine/threonine phosphorylation. Most of this speculation has revolved around the ability of oxidants to possibly activate protein kinase C (PKC) via an oxidation reaction in the enzyme's regulatory domain (155,56,57). Current opinion believes the zinc-thiolate structures in the C1 region of the PKC regulatory domain are quite susceptible to oxidative regulation as modification of the cysteine residues in this region leads to activation (155). However, modification of the cysteine residues in the catalytic domain are thought to lead to enzyme inactivation (155). Furthermore, H_2O_2 and ionizing radiation were also shown to stimulate threonine/tyrosine phosphorylation and kinase activity of mitogen-activated protein kinase (MAPK) (151). Also, phorbol ester activated MAPK activity was blocked by the antioxidant N-acetylcysteine, which would suggest the involvement of ROS in

activation of this enzyme (155). ROS are also thought to possibly play a role in the regulation of various transcription factors such as AP-1 and NF-κB. AP-1, a transcription factor which regulates expression of cell growth mediators, exists as a heterodimer of Fos and Jun proteins which themselves are products of the *c-fos* and *c-jun* proto-oncogenes (155). Previous studies have shown that free radicals produced by xanthine/xanthine oxidase and hydrogen peroxide can induce the expression of several early response genes including *c-fos* and *c-jun* (29,121,145). Further investigation has shown that mitogen-activated AP-1 activation is antioxidant inhibitable and therefore, ROS mediated (55). NF-kB is another transcription factor which is thought to be under oxidant regulation. Although the mechanism is unknown, H_2O_2 has been found to stimulate DNA binding of NF-kB in vivo while cells overexpressing catalase were unable to activate NF-κB in response to TNF-α and okadaic acid (141,142). The mechanism of oxidative NF-κB activation is thought to occur by an increase in tyrosine kinase activity and/or an increase in the intracellular Ca^{2+} concentration brought about by the ROS (57). High intracellular Ca_{2+} is thought to contribute to the proteolytic processing of IκB which serves as an inhibitory regulator of NF-κB (143). Thus, there is increasing evidence that, not only can ROS cause damage to biological molecules, but they may also play an important role in signal transduction as well as in the regulation of transcription factors.

A.6. Antioxidants

Almost all organisms have built up considerable defense mechanisms to destroy ROS as well as correct the damage as discussed above. Endogenous antioxidants prevent the

excessive accumulation of ROS and thus "oxidative stress". An antioxidant can be defined as a substance that can significantly inhibit the rate of oxidation of target molecules when present in low concentrations (109). Antioxidants tend to fall into one of three categories, i) enzymatic antioxidants, ii) nonenzymatic antioxidants and iii) preventative antioxidants. Antioxidants can also operate at different levels by preventing radical formation and intercepting radicals that have formed (59).

The enzymatic antioxidants consist of three main enzymes, namely superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Of the eukaryotic SOD family of enzymes, the best characterized are the cytosolic Cu,Zn-SOD, the mitochondrial Mn-SOD, and the extracellular Cu,Zn-SOD (143). SODs function as the primary defense by which O_2^- is eliminated and they act by dismutating O_2^- to hydrogen peroxide and O_2 (66,109). Catalases, which can be found in peroxisomes, function by converting the H_2O_2 generated by the action of oxidase enzymes, and SOD into water and oxygen (106,66). Therefore, catalases work to dispose of H_2O_2 (see fig. 9). In addition to catalase, glutathione peroxidase also functions to eliminate H_2O_2 and is perhaps the more important enzyme for this function (see fig. 9). GPx is a selenium containing enzyme which removes H_2O_2 by oxidizing two reduced glutathione (GSH) molecules to oxidized glutathione (GSSG) (66). The oxidized glutathione can then be regenerated back to GSH through the enzyme glutathione reductase which uses NADPH as a source of reducing power (18) (see fig. 9).

In addition to the enzymatic antioxidants, several non-enzymatic biological antioxidants exist of which Vitamin E and Vitamin C are perhaps the most common. Most often, these antioxidants function by serving as free radical scavengers or chain breaking

Fig. 9)

A) Catalase Reaction

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

B) Glutathione Peroxidase Reaction

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$$

C) Glutathione Reductase Reaction

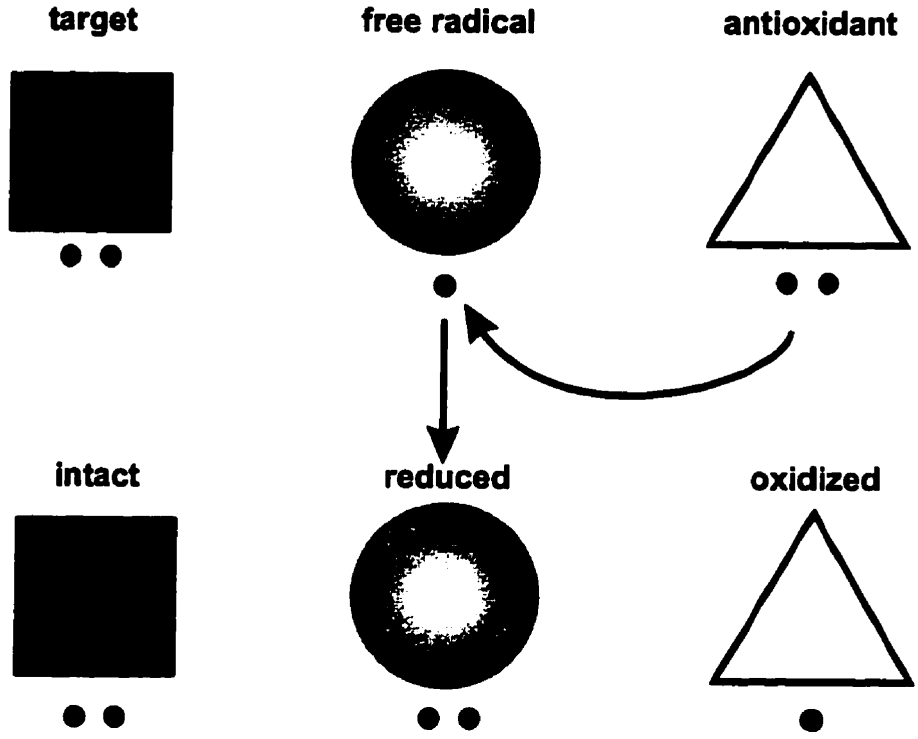
$$GSSG + NADPH \longrightarrow GSH + NADP^+$$

antioxidants. Essentially, they serve a sacrificial role by reacting preferentially with ROS before more vital molecules can become oxidized (see fig. 10). Once oxidized, these antioxidants are converted to products which have insufficient reactivity to propagate a chain reaction (109). Vitamin E, a lipid-soluble or hydrophobic antioxidant, is composed of four different tocopherol homologs (due to different side chains) of which α-tocopherol is the most relevant and potent (147). Within plasma and red blood cells, Vitamin E is known as the major lipid-soluble antioxidant protecting against lipid peroxidation (13). Unfortunately, little is known about the metabolism of this vitamin in humans. Vitamin C (L-ascorbic acid) is a water-soluble antioxidant which, under physiologic conditions, is present in its deprotonated state (147). Vitamin C is often considered the most important antioxidant in extracellular fluids as it has the ability to scavenge superoxide, hydrogen peroxide and the hydroxyl radical (8,10). Ascorbic acid is also known to play an important role in regenerating the other cellular antioxidants such as Vitamin E and GSH (125).

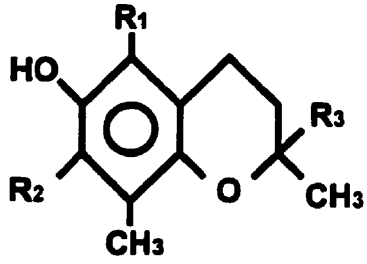
Certain molecules are thought to behave as antioxidants by serving a preventative role. Molecules such as transferrin (an iron transport protein) and lactoferrin fit this description for these molecules are responsible for keeping the concentration of free iron at a level which is negligible (606). The iron binding capacity of these molecules provide them with a powerful antioxidant property as they prevent any free iron from participating in free radical reactions such as the Fenton reaction (59,109). Therefore, it is apparent that biological organisms have developed an extensive and diverse array of antioxidants and methods by which to prevent the onset of oxidative damage.

Figure 10

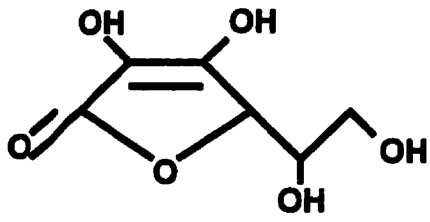
A) Sacrificed Role of Antioxidants



B) Vitamin E



C) Vitamin C



A.6.1. Glutathione Homeostasis

Another extremely vital and ubiquitous antioxidant which has diverse actions and is responsible for interacting with several other antioxidants is the tripeptide known as glutathione (GSH - γ-glutamylcysteinylglycine). GSH is the major cellular antioxidant found within mammalian cells with an intracellular concentration which ranges from 1 mM-10 mM (for a review see 1). From among its proposed functions, perhaps the most important one is that of protecting the cell from damage caused by free radicals. GSH has the ability to react nonenzymatically with ROS, removing them from the cellular milieu and preventing any damage which they may have incurred.

GSH is synthesized intracellularly by the consecutive actions of two enzymes known as γ-glutamylcysteine synthetase and GSH synthetase. The first enzyme (γ-glutamylcysteine synthetase) is the rate limiting enzyme and is responsible for forming L-γ-glutamyl-L-cynteine from L-cysteine and L-glutamate. However, the synthesis of GSH is not usually limited by this enzyme, but rather by the availability of its substrates (cysteine usually being the limiting substrate).

A.7. The Link Between Oxidative Stress and Insulin Resistance

Oxidative stress has long been associated with the complications of diabetes (nephropathy, retinopathy, vascular complications etc.) via the chronic hyperglycemia present within this condition. The prolonged hyperglycemia is known to deplete cellular

antioxidants and cause an increase in free radicals. For example, in cultured human endothelial cells incubated in a high glucose medium (20mM), there was an increase in the production of free radicals as seen by the markers malondialdehyde and conjugated dienes (34). Furthermore, addition of both SOD and glutathione could prevent the increase in both markers of oxidative stress (34). In a similar study done in umbilical vein and immortalized human endothelial cells, incubation with high glucose for 7 and 14 days caused an overexpression of Cu,Zn-SOD, catalase, and glutathione peroxidase (17). In rats made diabetic with streptozotocin, the renal mRNA levels of Cu,Zn-SOD and catalase were found to be significantly higher (146). The increased antioxidant mRNA levels were normalized upon intensive or high insulin treatment while moderate insulin treatment could only normalize catalase mRNA levels but not those of Cu,Zn-SOD (146). This suggested that hyperglycemia could indeed produce oxidative stress within cells which concomitantly could induce an increase in the intracellular antioxidant enzyme levels (17,146). Again, hyperglycemia was shown to produce an increase in ROS and lipid peroxidation while also increasing the accumulation of advaced glycation end (AGE) products in bovine endothelial cells (54). All of these parameters (ROS and AGEs) were decreased with the addition of α-tocopherol (54). Although most evidence linking hyperglycemia and oxidative stress has come from endothelial cells, high levels of glucose have also been shown to produce oxidative stress in human mesangial cells which could be prevented by the addition of α-tocopherol (156). Even under in vivo conditions, hyperglycemia has indirectly been demonstrated to increase oxidative stress as healthy and type II diabetic patients experienced a significant increase in plasma prothrombin fragments 1 and 2 during an oral glucose tolerance test (OGTT) which could be significantly decreased with the addition of GSH

(16). GSH administration alone could lower the prothrombin fragment levels in diabetic patients while no effects was seen in the controls (16). Thus, there is increasing evidence that hyperglycemia may mediate the induction of oxidative stress and that this may contribute to the pathology and conditions of diabetes.

In fact, the parameters of oxidative stress have been found to be significantly different in diabetic patients. NIDDM patients were found to have elevated levels of hydroperoxides (a measure of lipid peroxidation) and lowered levels of plasma α-tocopherol when compared to healthy controls (122). These differences between control and diabetic patients were seen regardless of whether the diabetic patients had complications or not suggesting that oxidative stress is an early stage in the pathology of NIDDM (122). In a separate study investigating the serum levels of lipid peroxides measured as thiobarbituric-acid-reactive substances (TBARS), diabetic patients were found to have significantly elevated levels in general while type II diabetics had levels significantly higher than those seen in type I diabetics (58). Interestingly, those patients with good metabolic control had TBARS levels significantly lower than those patients with poor metabolic control (58). A further point of interest was that the levels of free vitamin E did not differ between control and diabetic patients (58). In an alternate study, the plasma levels of H_2O_2 and malondialdehyde were higher in diabetic patients when compared to controls, but both parameters of oxidative stress decreased with two weeks of intensive insulin therapy although the levels still remained higher than that of controls (167). In erythrocytes from patients with type II diabetes and in K562 cells cultured under hyperglycemic conditions, the intracellular concentration of GSH was found to be depleted as was the activity of γ-glutamylcysteine synthetase (173). Thus, diabetic patients may experience an inactivation of glutathione

synthesis which increases their susceptibility to oxidative stress (173). Rats made diabetic with streptozotocin experienced higher levels of malondialdehyde and conjugated dienes while undergoing a concurrent decrease in ascorbate and α-tocopherol (174). Insulin treatment had the ability to return these parameters to normal as did treatment with ascorbate supplementation along with desferrioxamine treatment (174). However, treatment with either ascorbate or desferrioxamine alone could not reduce the oxidative stress which was seen with streptozotocin-induced diabetes (174). From such evidence, it can be concluded that diabetes (and/or hyperglycemia) is correlated with an increase in oxidative stress and a parallel decrease in antioxidants. However, the onset of oxidative stress may be ameliorated upon insulin treatment or good metabolic control.

An alternative approach to elucidate the relation between diabetes and oxidative stress is to see the effect of antioxidant treatment on diabetes and its various parameters. Thioctic acid (α-lipoic acid), a cofactor in several enzyme complexes which has antioxidant properties, can stimulate glucose utilization in rat hemidiaphragms in vitro and improve the insulin response of glucose uptake in muscles isolated from Zucker diabetic rats (an animal model of insulin resistance) treated with thioctic acid in vivo (68,84). Treatment with thioctic acid also increased insulin-stimulated glycogen synthesis and was associated with a marked increase in in vivo muscle glycogen concentration while decreasing plasma levels of insulin and free fatty acids (84). Protein levels of GLUT4 appeared to be unaffected by thioctic acid (84). When thioctic acid was used on L6 muscle cells and 3T3-L1 adipocytes, glucose uptake was rapidly increased (as a result of transporter translocation) to levels comparable to that of insulin (43). The glucose uptake elicited by thioctic acid was abolished by the inhibitory actions of wortmannin on PI-3 kinase which indicates that this

antioxidant may function through a similar pathway as insulin (43). In aged rats which experience insulin resistance or a decrease in insulin-stimulated glucose transport, ingestion of vitamin C or butylated hydroxytoluene (BHT) could significantly increase insulin-stimulated glucose transport in isolated adipocytes when compared to control rats of the same age (117). Furthermore, while insulin could not markedly stimulate glucose transport above basal activities in old (24 months) rats, rats fed vitamin C or BHT possessed significant stimulation of glucose uptake over basal levels with insulin (117). When type II patients were given a supplementation of ascorbic acid (2g/day), fasting blood glucose levels improved and beneficial effects on cholesterol and triglycerides were seen (42). From this evidence, the authors concluded that ascorbic acid supplementation may have beneficial effects on glycemic control and blood lipid levels in NIDDM patients (42). In a separate study, contradictory evidence was found in that intake of vitamin C and vitamin E was not associated with enhanced insulin-mediated glucose disposal (44). However, the study did find that a consumption of vitamin A of >10 000 IU/day could significantly lower plasma glucose and insulin responses to an oral glucose load (44). An explanation for this contradictory finding may be due to the fact that the intake of dietary constituents was self-reported within this study and may not accuately reflect the actual intake of the various vitamins (44). In a long term study in which NIDDM patients and control patients were given vitamin E over the course of four months, there appeared to be an increase in the plasma vitamin E levels as well as a decrease in the GSSG:GSH ratio in those patients receiving the vitamin E supplementation (15). Furthermore, vitamin E supplementation significantly increased insulin-mediated glucose disposal in both diabetic and control groups and it is proposed that the antioxidant properties of vitamin E are responsible for this

phenomenon (15). Further evidence that insulin resistance may be related to oxidative stress is the finding that elevated levels of plasma free fatty acid (FFA) levels (another hypothesized reason for the development of insulin resistance) is correlated with increased levels of TBARS (126). When lipid were infused, there was a significant elevation in plasma TBARS concentrations and an inhibition of insulin-stimulated whole body glucose disposal (126). Conversely, GSH infusion had the opposite effect of lowering plasma TBARS concentrations and increasing glucose disposal (126). Thus, a significant amount of evidence has been accumulated suggesting that supplementation with antioxidants may help to relieve the hazards of oxidative stress and prevent the onset of insulin resistance.

Curiously, in a study conducted within L6 myotubes and 3T3-L1 adipocytes, oxidative stress induced by glucose oxidase or xanthine oxidase (H_2O_2 generating systems) was able to elevate glucose uptake (98). There was an approximately two-fold increase in glucose consumption with an increased expression of GLUT1 mRNA and protein (98). Within this study however, the effects of oxidative stress on insulin-stimulated glucose uptake were not investigated. Fortunately, in a subsequent study these same investigators found that oxidative stress reduced insulin responsiveness in 3T3-L1 adipocytes while increasing basal glucose uptake (139). Again, oxidative stress was induced through the addition of glucose oxidase and an increase in GLUT1 was seen, but a decrease in insulin stimulatable GLUT4 mRNA and protein were also seen (139). Also observed was a reduction in insulin-stimulated lipogenesis and glycogen synthase activity (139). Thus, an increasing amount of evidence has been produced and more continues to arise which would denote that there is a close relationship between oxidative stress and the importance of its role in the onset of insulin resistance the the development of type II diabetes.

A.8. Previous Work Done In Laboratory

Previous work done within our laboratory has focussed upon the effect of oxidative stress on vanadate-stimulated glucose uptake in rat adipocytes. A paradoxical finding within the laboratory was that adipocytes made insulin resistant with hyperglycemia and hyperinsulinemia were more sensitive to the insulin mimetic effects of vanadate than were the control cells. It was then hypothesized that vanadyl (in its +4 oxidation state) was undergoing an oxidation reaction upon entering the cell to vanadate (+5 oxidation state). The +5 state of vanadate is hypothesized to be the active form of the molecule which is able to inhibit intracellular protein tyrosine phosphatases (PTPs). Incubation with L-buthionine-[S,R]-sulfoximine (BSO, an inducer of oxidative stress via the depletion of cellular GSH) was able to induce a similar paradoxical shift in the sensitivity of the cells to vanadate. Pretreatment of the cells with N-acetylcysteine (NAC, a precursor of GSH synthesis) prior to incubation with hyperglycemia and hyperinsulinemia was able to prevent the shift in vanadate sensitivity. When atomic absorption spectrometry was done to determine the relative vanadyl content, the hyperglycemia/hyperinsulinemia and BSO-treated cells were found to have less vanadyl than control cells. In total, this evidence seemed to indicate that the insulin resistance brought about by hyperglycemia and hyperinsulinemia was related to an increase in oxidative stress and that the paradoxical shift in vanadate sensitivity was due to the intracellular oxidative stress oxidizing the +4 vanadyl to the more biologically active +5 vanadate. BSO was also able to induce resistance to insulin-stimulated glucose uptake which was prevented by pretreatment with NAC.

A.9. Experimental Hypothesis or Objective

In light of the evidence presented thus far, the hypothesis and objective of the following studies was to further elucidate the link between oxidative stress and insulin resistance. A study of the glucosamine-induced model of insulin resistance predicts that the nature of this pathological state is related to and possibly the same as the hyperglycemia and hyperinsulinemia-induced model of insulin resistance. Therefore, if the condition of hyperglycemia and hyperinsulinemia is capable of inducing oxidative stress and subsequently insulin resistance, we would predict that glucosamine would have similar effects. In this instance, we hypothesize that incubation with glucosamine should cause a depletion in intracellular GSH as well as a reduction in insulin-stimulated glucose uptake. In addition, we further hypothesize that the use of cellular antioxidants such as NAC, vitamin E, and others should also prevent the induction of insulin resistance incurred by incubation with glucosamine by ameliorating the oxidative stress of the cells. Furthermore, while there is increasing evidence linking oxidative stress and the hyperglycemia-induced insulin resistance, the mechanisms of this model of resistance along with the role of glucosamine and the end-products of the hexosamine biosynthesis pathway are largely unknown, thus another objective has been to further elucidate the mechanisms associated with the etiology of this condition.

Chapter 2: Materials and Methods

B. Materials and Methods

B.1 Materials

Male Sprague Dawley Rats were supplied by Charles-River (Montreal, Que). Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, and fetal bovine serum were supplied by GIBCO/BRL (Grand Island, NY). Type I collagenase was from Worthington Biochemicals Corp. (Freehold, NJ). The following chemicals were obtained from Sigma (St. Louis, MO), bovine serum albumin (BSA, fraction V), L-buthionine-[S,R]-sulfoximine, α-tocopherol, β-nicotinamine adenine dinucleotide phosphate (reduced form), glutathione, glutathione ethyl ester (reduced form), glutathione reductase, phloretin, 5-sulfosalicylic acid (SSA), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), phthalic acid dinonyl ester, and all other chemicals used for KRBH and KR30H. Nitex nylon was purchased from Thompson (Sarborough, ON). 2-deoxy-D-[^{3}H] glucose (10 Ci/mmol) was obtained from DuPont – New England Nuclear (Mississauga, ON). Econofluor-2 was from Packard (Meriden, CT). SB203580, Ebselen, and PD98059 were supplied by Calbiochem-Novabiochem Corp. (La Jolla, CA). Human insulin was supplied by Eli Lilly Canada (Toronto, ON).

B.2. Preparation of Isolated Primary Adipocytes

Male Sprague Dawley rats were sacrificed by exposure to a 30% O_2, 70% CO_2 mixture followed by exposure to 100% CO_2. Subsequently, cervical dislocation was performed on the rats and the epididymal fat pads removed and placed into DMEM (pH 7.4) medium containing 3% BSA. Type I collagenase (2mg/ml) was then added to the medium

containing the fat pads and the mixture incubated in a shaking water bath at 37°C for approximately one hour. The digested fat pads were then removed from the water bath and filtered through Nitex nylon (pore size 1000μM) into a 250ml beaker. The filtrate was collected into 50ml conical centrifuge tubes and centrifuged at 500 rpm for 30 seconds at room temperature. After centrifugation, the infranatant was removed with a syringe (being careful not to disturb the upper layer of adipocytes) and fresh DMEM containing 3% BSA was added to wash the adipocytes. The adipocytes were then shaken extremely gently with the DMEM within the 50ml conical centrifuge tubes to wash the cells. This washing procedure was repeated once more with DMEM (pH 7.4) containing 3% BSA and twice with DMEM (pH 7.4) containing 1% BSA. After this repeated washing of the adipocytes, the adipocytes were resuspended in DMEM (pH 7.4) containing 1% BSA. Subsequently, the cells were aliquoted into 75cm^2 tissue culture flasks containing DMEM (pH 7.4) with 1% BSA (the adipocrit (cell volume) was kept at approximately 10% of the total cell suspension volume). The different treatments were then added to the adipocytes and the cells were incubated for 16-18 hours at 37°C in a humidified 5% CO_2/95% air mixture. The different treatments were as follows:

1) Control

2) High glucose (20mM), High insulin (600ng/ml)

3) Glucosamine (2.5mM and 5mM)

4) High glucose (20mM), High insulin (600ng/ml), NAC (5mM)

5) Glucosamine (2.5mM), NAC (5mM)

6) High glucose (20mM), High insulin (600ng/ml), GSH ethyl ester (10mM)

7) High glucose (20mM), High insulin (600ng/ml), Vitamin E (250μM)

8) Glucosamine (5mM), Ebselen (15μM)

9) High glucose (20mM), High insulin (600ng/ml), SB203580 (1μM)

10) Glucosamine (5mM), PD98059 (50μM)

B.3. Washing Procedure

Following the 16-18 hour incubation, the adipocytes were removed from the tissue culture flasks and transferred to 50ml conical centrifuge tubes. After centrifugation at 500rpm for 30 seconds, the infranatant was removed and replaced with Krebs Ringer 30mM Hepes (KR30H - pH 7.0) (137mM NaCl, 5mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.25mM $CaCl_2$, 30mM HEPES, 1mM pyruvate) solution containing 3% BSA and the mixture shaken with great care to wash the cells. The adipocytes were washed twice in this manner and then incubated in KR30H (pH7.0) containing 3% BSA for 30 minutes in a shaking water bath at 37°C. Following incubation, the adipocytes were washed twice more in Krebs Ringer Bicarbonate Hepes Buffer (KRBH – pH 7.4) (118mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 2.5mM $CaCl_2$, 1.2mM KH_2PO_4, 5mM $NaHCO_3$, 30mM HEPES, 1mM pyruvate) containing 3% BSA. The cells were then brought to an adipocrit of 12-15% with KRBH containg 3% BSA to be used for a 2-deoxyglucose uptake assay.

B.4. 2-Deoxyglucose Uptake Assay

The adipocytes were aliquoted into 12x75mm glass culture tubes containing 100μl-200μl KRBH with 3% BSA. Before each aliquot was taken, the cells were shaken gently to achieve a homogeneous mixture. At this point, the cells were stimulated with 100μl of various concentrations of insulin (final concentrations ranging from 0-25ng/ml), vortexed

gently, and placed into a shaking water bath at 37°C for 30 minutes. Subsequently, 100µl of 0.01µCi/µl 2-deoxy-D-[^{3}H]glucose (final concentration of 2-deoxyglucose of 0.1mM) was added to the adipocytes, vortexed gently, and further incubated for 3 minutes at 37°C in the shaking water bath. To stop the reaction, the adipocytes were removed from the water bath, placed on ice, 500µl of phloretin (final concentraion of 0.25mM) was added, the cells gently vortexed and placed back on ice. Non-specific uptake was determined by the addition of phloretin to unstimulated adipocytes prior to the addition of 2-deoxyglucose. 200µl aliquots of cell suspension were then aliquoted after gently vortexing the tissue culture tubes to achieve a homogeneous mixture, and placed into 400µl micro test tubes containing 200µl of phthalic acid dinonyl ester. The micro test tubes were then centrifuged at 1000rpm for 30 seconds. After centrifugation, the micro test tubes were cut with a scalpel through the phthalic acid dinonyl ester layer (the adipocytes being above this layer and the aqueous solution below) and the adipocytes (contained within the top portion of the micro test tubes) placed into 20ml scintillation vials. 10ml of Econoflour-2 scintillation solution was then added, the scintillation vials shaken, and the radioactivity measured in a Beckman LS6500 scintillation counter. The total counts were assessed by addition of 200µl of the whole mixture contained within two tissue culture tubes to two scintillation vials. Universol scintillation fluid was then added to these two scinillation vials and the vials counted along with the other vials. Within each experiment, the glucose uptake values were determined from the mean of duplicate determinations.

B.5. Intracellular GSH Measurement

The adipocytes were isolated, prepared and incubated as described above. After incubation of the adipocytes for 16-18 hours under the various conditions, the cells were transferred to 50ml conical centrifuge tubes. The adipocytes were centrifuged at 500rpm for 30 seconds and the infranatant was removed. KRBH (pH 7.4) was added to wash the cells and this washing procedure was repeated. The adipocytes were then placed on ice, an ice cold (0°C - 4°C) lysate solution of 5% sulfosalicylic acid was added, and the cells were homogenized using a glass mortar and pestle. The homogenate was then centrifuged at 4°C for 30 min at 3000xg. After centrifugation, the cell lysate (infranatant) was removed using a syringe and the upper lipid layer discarded. To assay for GSH, 100µl of lysate was added to 880µl of fresh assay buffer (0.2M Na_2HPO_4, 2mM EDTA, 0.1% SDS, 1U/ml of glutathione reductase, 0.2mM NADPH - pH 7.0) in a 12mmx75mm culture test tube. 20µl of 10mM DTNB in 0.05M NaH_2PO_4 (pH7.0) was then added and the mixture was vortexed. 200µl aliquots were then taken and added to the wells of a 96-well plate. After 10 minutes of incubation in the dark at 25°C, the 96-well plate was read at a wavelength of 414nm in a Titertek Plus MS2 Reader. Samples were assayed in triplicate and the values determined from the mean of the triplicate.

B.6. Statistical Analysis

All values are expressed as mean±SE. All data for glucose uptake assays were subject to analysis of variance while data for intracellular measurement of GSH was subject to Student's paired t-Test. Probabilities of 0.05 ($p<0.05$) were considered to be statistical significance.

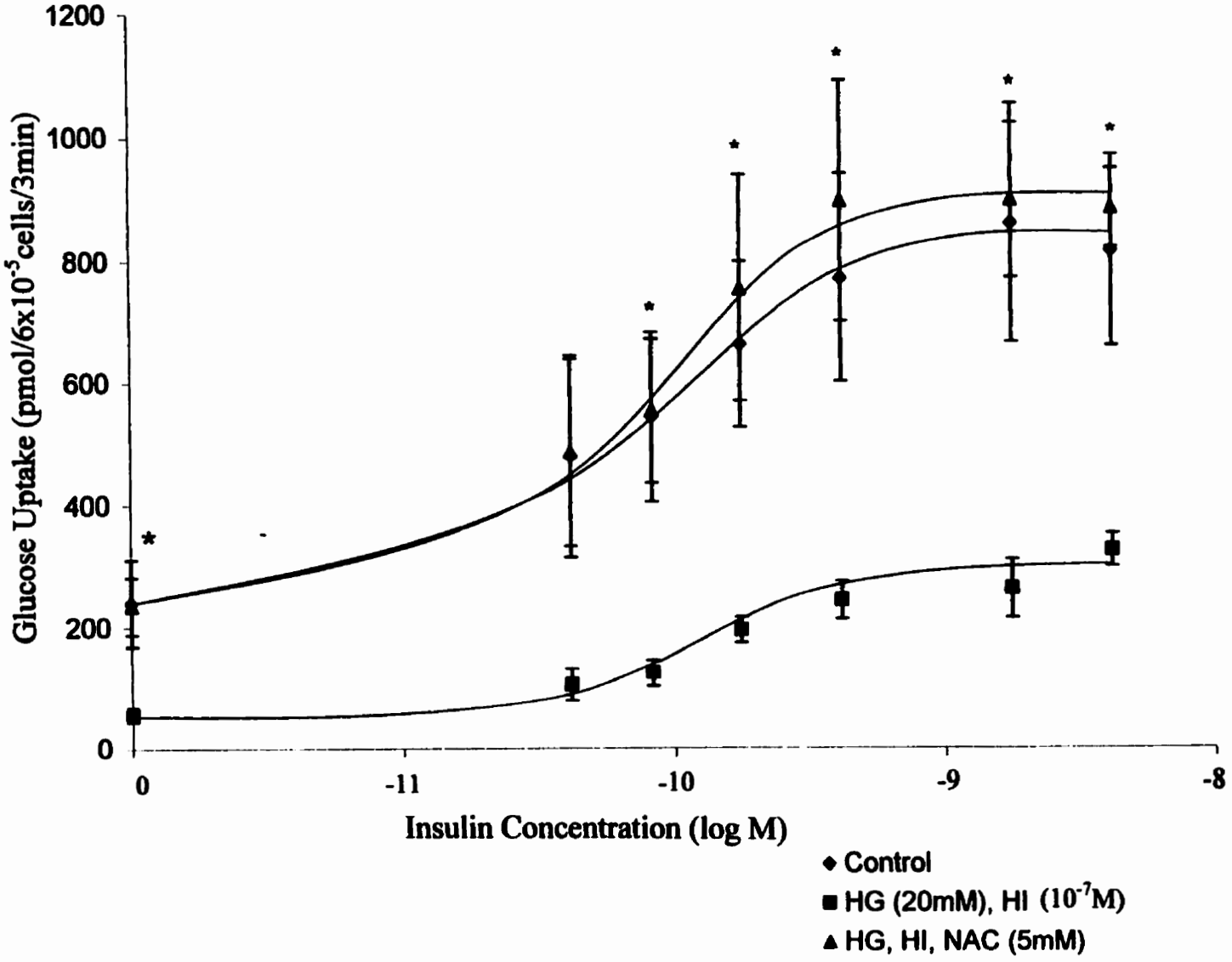
Chapter 3: Results

C. Results

C.1. Effect of N-Acetylcysteine on High Insulin and High Glucose Induced Resistance

Normal rat adipocytes freshly isolated and incubated for 18h at 37°C in DMEM in the presence of 5.5mM glucose show a dose-dependent marked increase in 2-deoxyglucose uptake when stimulated with insulin from $0.4x10^{-12}$mol/L to $417x10^{-12}$mol/L. Previous studies have shown that chronic exposure of rat adipocytes to high glucose in combination with insulin caused resistance to insulin-stimulated glucose transport. In these studies we have also found that adipocytes incubated for 18h under hyperglycemic (20mM) glucose and hyperinsulinemic (10^{-7}) conditions showed a marked decrease in the 2-deoxyglucose uptake response at all concentrations of insulin. At maximum stimulation by $417x10^{-12}$mol/L insulin there was a significantly lower glucose uptake in resistant cells (816±155.7 pmol/$6x10^5$cells/3min in control cells vs. 326±27.8 pmol/$6x10^5$cells/3min, $p<0.05$). The insulin resistant adipocytes also had a decrease in basal 2-deoxyglucose uptake when compared to control (control, 240±71.9 pmol/$6x10^5$cells/3min; resistant 55±11.7 pmol/$6.0x10^5$cells/3min, $p<0.05$). When the cells treated with high glucose and insulin were co-incubated with NAC, the basal activity of 2-deoxyglucose uptake was normalized as was the response to insulin when compared to resistant cells (basal, 235±47.4 pmol/$6x10^5$cells/3min; maximum insulin ($417x10^{-12}$mol/L) 885±63.9 pmol/$6.0x10^5$cells/3min). These values were not different from control but significantly greater than those observed in the presence of high glucose and insulin without NAC ($p<0.05$ for basal and $p<0.05$ for maximuminsulin, fig.1) Incubation with NAC alone had no significant effect. These data suggested that NAC had a preventative effect on the

Effect of NAC on HG/HI Induced Resistance



Effect of NAC on glucose uptake in control cells

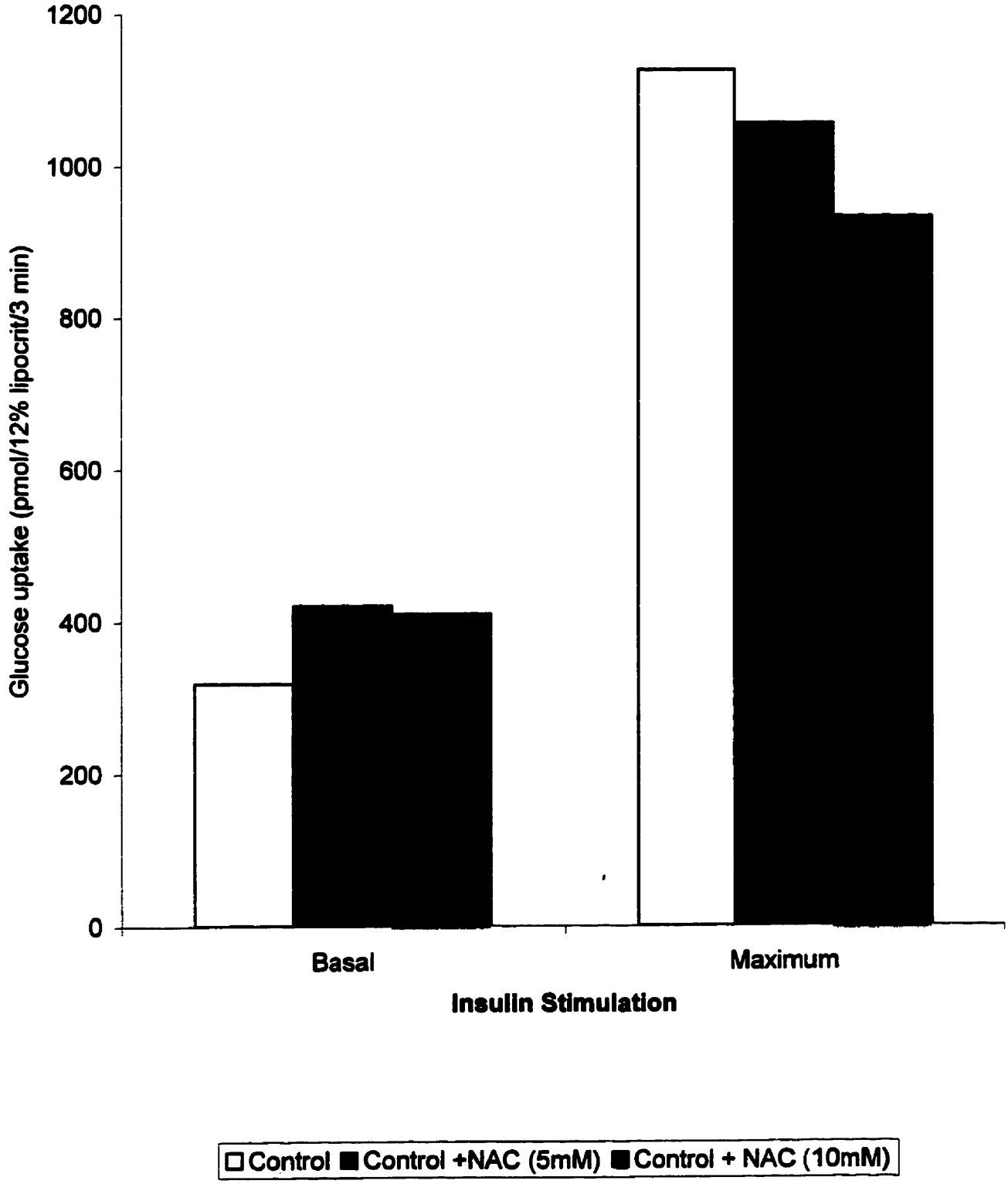


Figure 1. Effect of N-Acetylcysteine on high insulin and high glucose induced resistance in primary adipocytes and Effect of NAC alone.
Freshly isolated adipocytes were incubated at 37°C for 16-18 hrs in DMEM containing 1% BSA (control) with 600ng/ml insulin and 20mM glucose (insulin resistant), and with 600ng/ml insulin, 20mM glucose and 5mM NAC. After incubation, the cells were washed in KR30H (pH 7.0) containing 3% BSA for 30min at 37°C. The cells were then washed in KRBH (pH7.4) containing 3%BSA and a 2-deoxyglucose uptake was then performed. Adipocytes were stimulated with various concentrations of insulin (0.25 - 25ng/ml) for 30min at 37°C at which point 2-deoxyglucose was added for 3min. The reaction was stopped with the addition of 0.1M phloretin and the intracellular 2-deoxyglucose was determined. Incubation of NAC alone had no effect. Results represent mean±SE (n=3).
*$p<0.05$

hyperglycemia/hyperinsulinemia induced resistance as measured by the 2-deoxyglucose uptake response to insulin.

C.2. Effect of N-Acetylcysteine on Glucosamine Induced Resistance

Previous studies suggested that the mechanism by which high glucose causes insulin resistance in adipocytes involves an increased flux through the hexosamine biosynthesis pathway (see introduction section). The insulin resistance could thus be mimicked by exposure of adipocytes to glucosamine. In order to determine whether NAC could also affect glucosamine induced insulin resistance, adipocytes were incubated for 18h in the presence and absence of glucosamine in combination with or without NAC. Incubation with glucosamine alone produced a diminshed response of glucose uptake at all insulin concentrations. There was a slight but not significant decrease in basal 2-deoxyglucose uptake (307±28.5 pmol/6x10^5cells/3min in control cells vs. 254±53.5 pmol/6x10^5cells/3min in resistant cells), but a dramatic decrease in maximal insulin stimulated uptake (949±101.6 pmol/6x10^5cells/3min in control cells vs. 461±75.0 pmol/6x10^5cells/3min in resistant cells, $p<0.001$). Hence, incubation with glucosamine had a similar effect on insulin stimulated 2-deoxyglucose uptake as did incubation with high insulin and high glucose. Co-incubation with NAC was able to normalize the 2-deoxyglucose uptake response of the adipocytes to insulin stimulation (335±63.3 pmol/6x10^5cells/3min at basal and 1049±97.8 pmol/6x10^5cells/3min at maximal insulin stimulation). The response achieved with glucosamine plus NAC was again comparable to the response seen in control cells and significantly greater than that in the presence of glucosamine alone. This observation provided further evidence that NAC had a

Effect of NAC on Glucosamine Induced Resistance

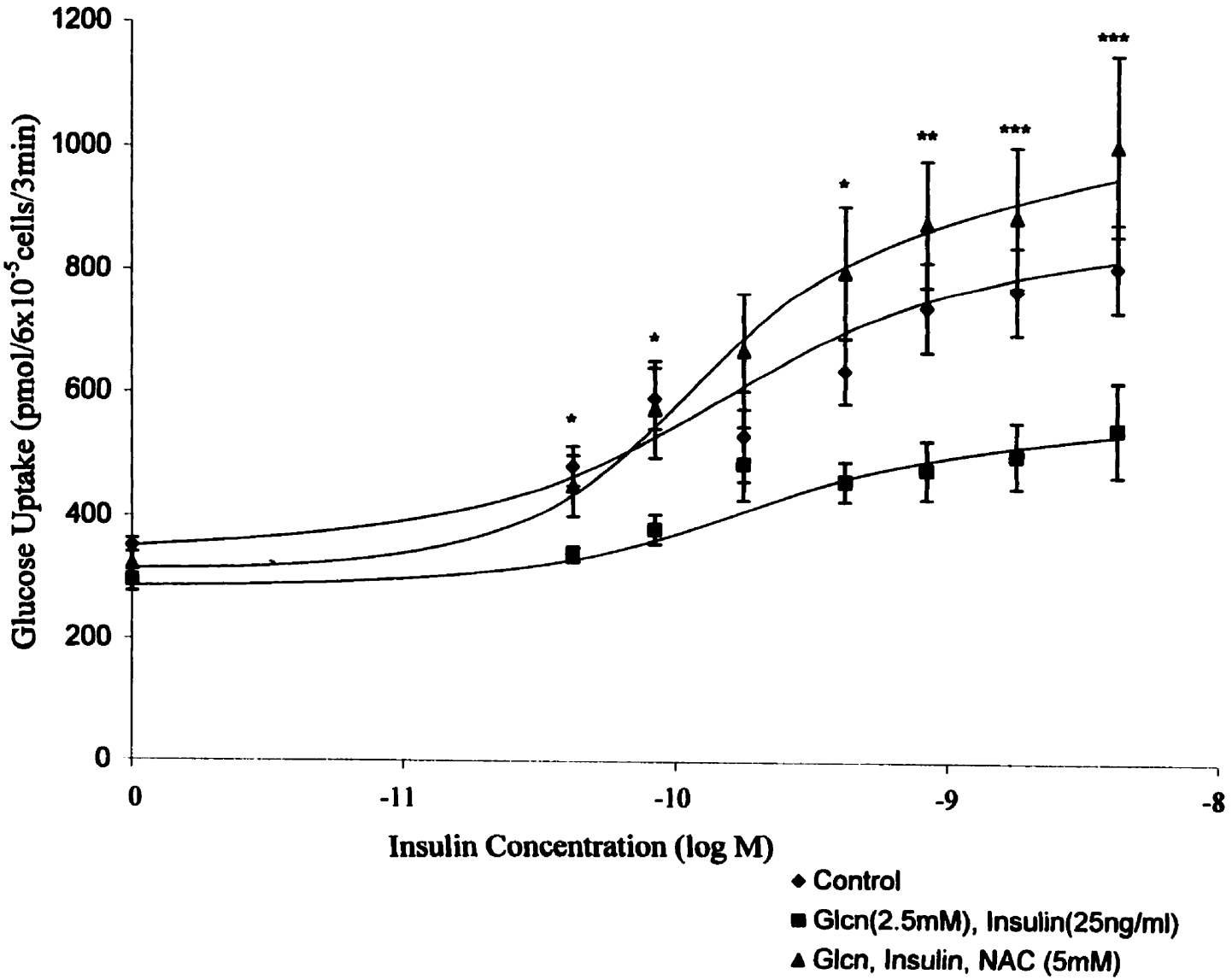


Figure 2. Effect of N-Acetylcysteine on glucosamine induced resitance in primary adipocytes

Freshly isolated adipocytes were incubated for 16-18 hrs at 37°C in DMEM containing 1% BSA (control) with 2.5mM glucosamine (insulin resistant), and with 2.5mM glucosamine and 5mM NAC. After incubation, the cells were washed in KR30H (pH 7.0) containing 3% BSA at 37°C for 30 min. The adipocytes were then washed in KRBH (pH 7.4) containing 3% BSA, brought to an adipocrit of 12%-15%, and a 2-deoxyglucose uptake assay was performed. Adipocytes were stimulated with various concentrations of insulin (0.25-25ng/ml) for 30min at 37°C and 2-deoxyglucose was subsequently added for 3 min. 0.1 M phloretin was added to stop the reaction and the intracellular content of 2-deoxyglucose was determined. Results represent mean±SE (n=7). *$p<0.05$, **$p<0.01$, ***$p<0.001$

preventative effect on insulin resistance by inhibiting the development of a defect common to the glucosamine-induced and high glucose-induced insulin resistance.

C.3. Effect of Glutathione Ethyl Monoester on Hyperglycemia and Hyperinsulinemia Induced Resistance

NAC is known to be a free radical scavenger as well as a precursor of glutathione. Providing additional cellular cysteine by NAC incubation has been demonstrated under some conditions to raise GSH levels. In order to determine whether alterations in GSH may be involved, adipocytes were rendered insulin resistant by exposure to high glucose in combination with insulin as described above, in the presence and absence of GSH monoethyl ester, a cell permeable form of reduced glutathione. As demonstrated above, high glucose and insulin treatment of adipocytes for 18h resulted in a significant decrease in both the basal levels of 2-deoxyglucose uptake (38±7 pmol/6x10^5cells/3min vs. control cells 298±36 pmol/6x10^5 cells/3min, $p<0.01$) as well as at maximal insulin (172±13 pmol/6x10^5/3min vs control cells 731±67 pmol/6x10^5/3min, $p<0.01$). This decreased response in the hyperglycemia/hyperinsulinemia-induced insulin resistant adipocytes was observed at all stimulatory concentrations of insulin. Upon co-incubation of the adipocytes with high glucose, insulin, and the GSH ethyl ester (10mM), there was a normalization of the 2-deoxyglucose uptake at both basal levels (263±38 pmol/6x10^5cells/3min) and in response to maximum insulin (838±39 pmol/6x10^5cells/3min). Thus, co-incubation with GSH ethyl ester had a similar effect to NAC to normalize the 2-deoxyglucose uptake of adipocytes incubated with high glucose and insulin.

Effect of GSH ethyl ester on HG/HI Induced Resistance

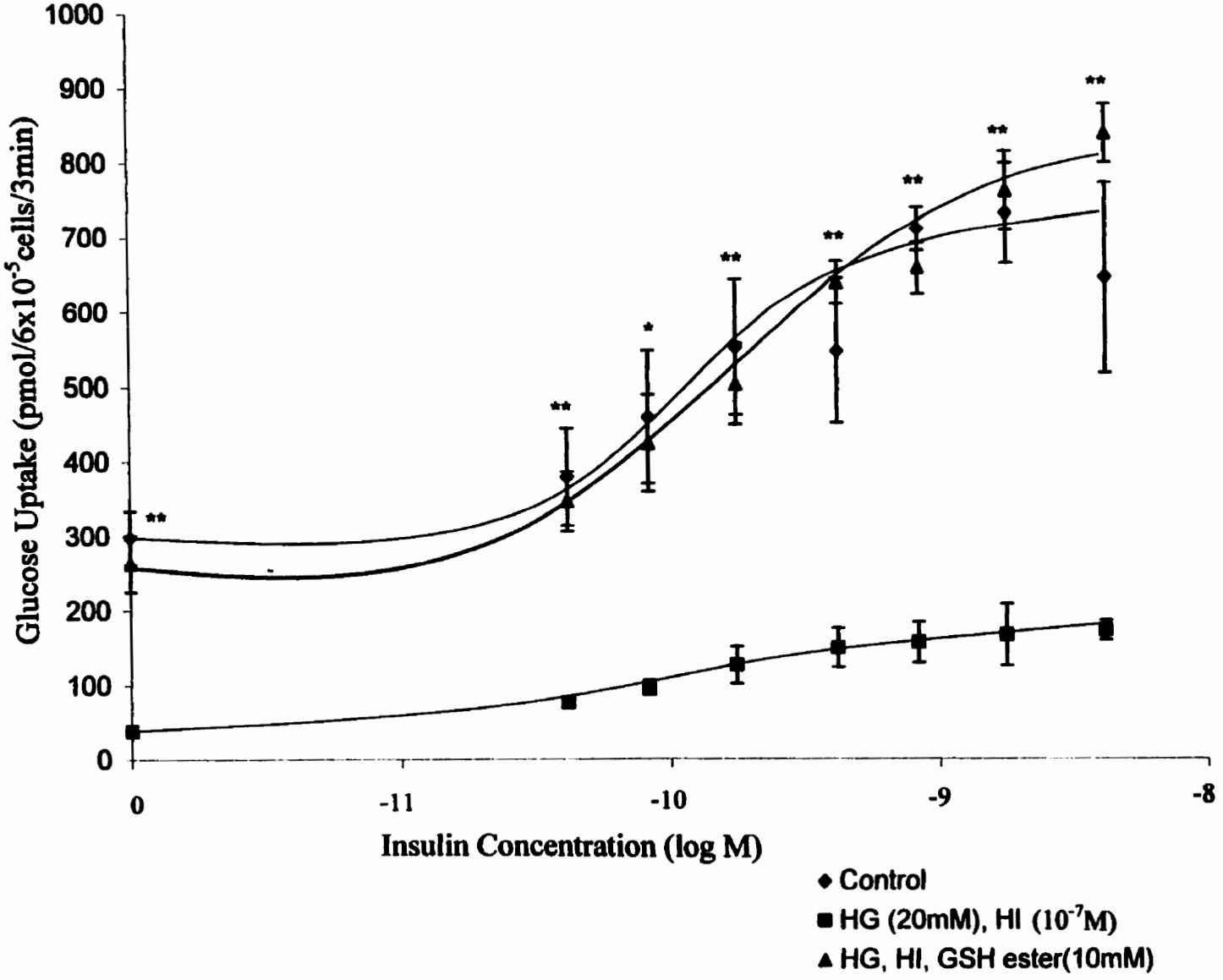


Figure 3. Effect of glutathione ethyl monoester on high glucose and high insulin induced insulin resistance in primary adipocytes.
Freshly isolated adipocytes were incubated for 16-18 hours in DMEM containing 1% BSA at 37°C. The adipocytes were either incubated alone (control), with 600ng/ml insulin and 20mM glucose (insulin resistant), or with 600ng/ml insulin, 20mM glucose, and 10mM glutathione ethyl monoester. Following the period of incubation, the adipocytes were washed in KR30H (pH 7.0) containing 3% BSA for 30 minutes. Following the 30 minute incubation, the adipocytes were washed in KRBH (pH 7.4) containing 3% BSA and an adipocrit of 12%-15% achieved. Subsequently, a 2-deoxyglucose uptake assay was performed. Adipocytes were stimulated at 37°C for 30 min with various concentrations of insulin (0.25-25ng/ml) and tritiated 2-deoxyglucose was subsequently added for 3min. The adipocytes were then placed on ice and the reaction stopped by the addition of 0.1M ice cold phloretin. The intracellular content of 2-deoxyglucose was then determined. Results represent the mean$\pm$SE (n=4). *$p<0.05$, **$p<0.01$

C.4. Effect of Vitamin E on High Insulin and High Glucose Induced Resistance

The effect of NAC and GSH ester to prevent insulin resistance indicates that intracellular GSH, a major determinant of redox state and a cellular anioxidant, is an important determinant of adipocyte insulin sensitivity. This also suggests that insulin resistance may be caused at least in part by oxidative stress, which is prevented by GSH. To further test wheter oxidative stress may be a mediator of the glucose and insulin induced insulin resistance, adipocytes were rendered insulin resistant as described above in the absence and presence of the lipid soluble antioxidant, α-tocopherol (Vitamin E). While incubation of adipocytes with high glucose and insulin without Vitamin E resulted in insulin resistance, there was a partial prevention in the presence of Vitamin E (250μM). However, the difference could not reach statistical significance. (Basal glucose uptake - control 235±41.0 pmol/6x10^5cells/3min; high glucose plus insulin 68±9.2 pmol/6x10^5cells/3min; high G/I plus Vitamin E 114±19.8 pmol/6x10^5cells/3min: maximum insulin - control 561±59.4 pmol/6x10^5cells/3min; high G/I 283±65.0 pmol/6x10^5cells/3min; high G/I plus Vitamin E 336±80.0 pmol/6x10^5cells/3min). Incubation with vitamin E alone appeared to have no effect on glucose uptake (data not shown). Hence, it appeared that vitamin E had a partial effect in preventing the insulin resistance induced by hyperglycemia and hyperinsulinemia.

Effect of Vitamin E on HG/HI Induced Resistance

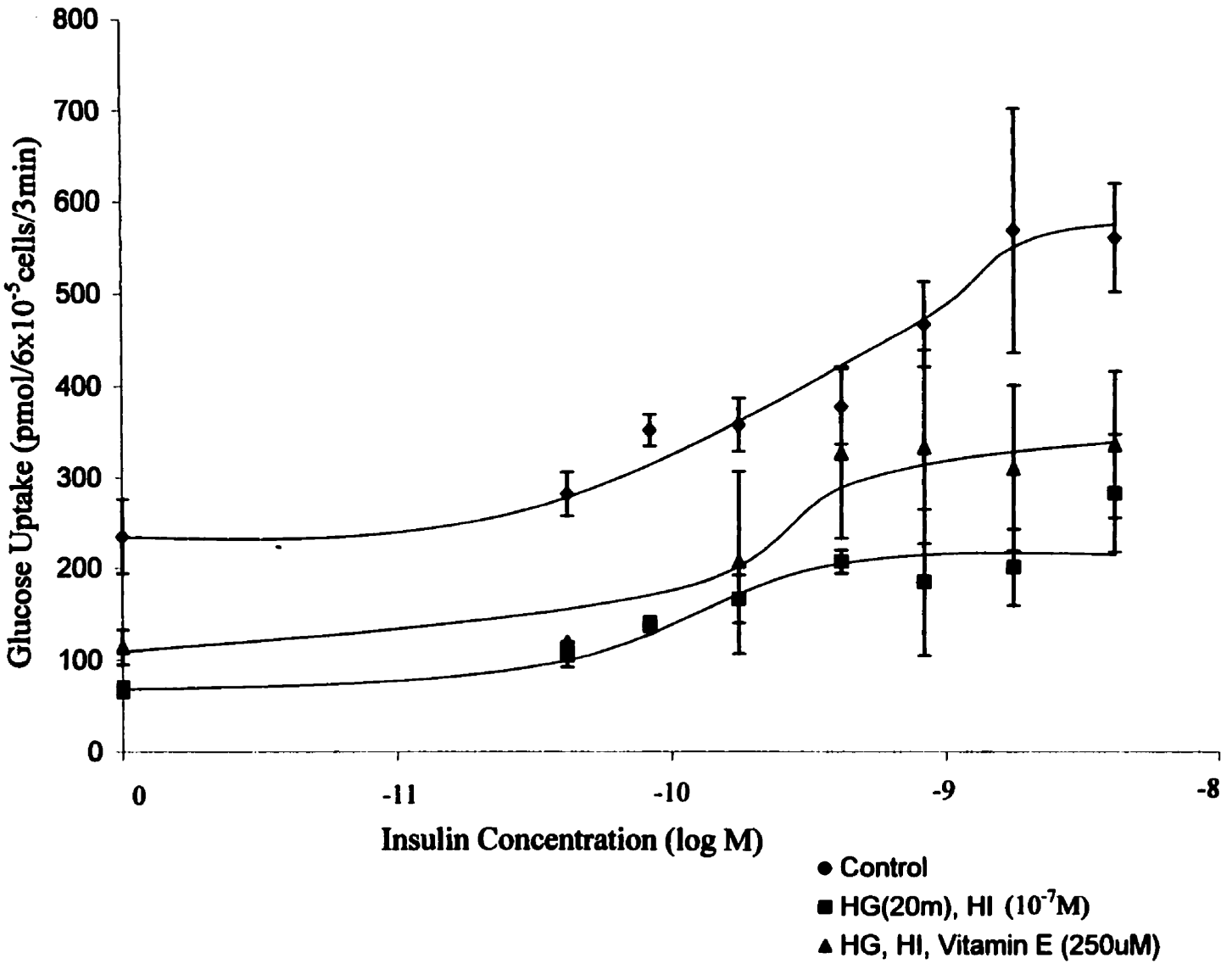


Figure 4. Effect of Vitamin E on high insulin and high glucose induced resistance in primary adipocytes

Freshly isolated adipocytes were incubated in DMEM containing 1% BSA for 16-18 hrs at 37°C. The adipocytes were incubated alone (control), with 600ng/ml insulin and 20mM glucose (insulin resistant), or with 600ng/ml insulin, 20mM glucose and 250μM vitamin E. Once the incubation was complete, the adipocytes were washed in KR30H (pH 7.0) containing 3% BSA for 30 min at 37°C. The cells were then washed in KRBH (pH 7.4) containing 3% BSA and brought to an adipocrit of 12%-15%. A 2-deoxyglucose uptake assay was then performed. Adipocytes were stimulated with various concentrations of insulin (0.25-25ng/ml) for 30 min at 37°C and 2-deoxyglucose was subsequently added for 3 min. The reaction was stopped by the addition of 0.1M phloretin and by placing the cells on ice. The intracellular content of 2-deoxyglucose was then determined. Results represent mean±SE (n=3).

C.5. Effect of Ebselen on Glucosamine Induced Resistance

Glutathione peroxidase is an antioxidant cellular enzyme which catalyzes the destruction of H_2O_2 in the presence of GSH to yield H_2O and oxidized glutathione (GSSG). Ebselen is a compound which mimics this enzyme. To test whether this enzyme activity may be a limiting factor in the putative induction of insulin resistance by oxidative stress, we incubated adipocytes with glucosamine in the presence and absence of ebselen (15μM). There was no effect of ebselen to prevent or modify the insulin resistance (Basal - control 136±15.5 pmol/6x10^5cells/3min; insulin resistant 75±18.2 pmol/6x10^5cells/3min; insulin resistant plus ebselen 69±12.4 pmol/6x10^5cells/3min: maximal insulin - control 716±112.3 pmol/6x10^5cells/3min; insulin resistant 460±85.1 pmol/6x10^5cells/3min; insulin resistant plus ebselen 423±73.6 pmol/6x10^5cells/3min). Thus, it appeared that the antioxidant properties of ebselen did not change the insulin resistance induced by glucosamine.

Effect of Ebselen on Glucosamine Induced Resistance

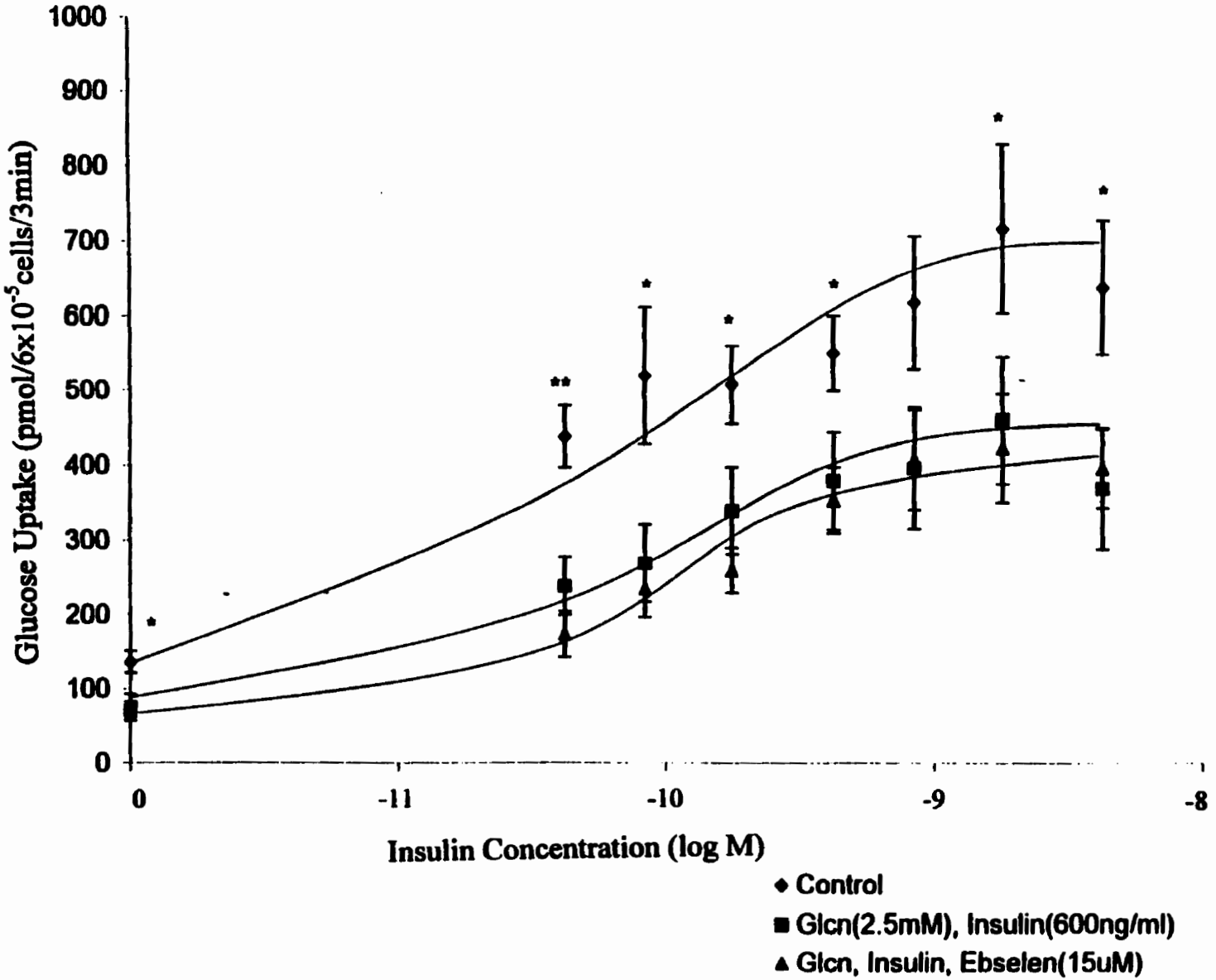


Figure 5. Effect of ebselen on glucosamine induced resistance in primary adipocytes Freshly isolated adipocytes were incubated for 16-18 hrs in DMEM containing 1% BSA at 37°C. The adipocytes were incubated alone (control), with 25ng/ml insulin and 2.5mM glucosamine (insulin resistant), or with 25ng/ml insulin, 2.5mM glucosamine, and 15μM ebselen. After the overnight incubation, the adipocytes were washed in KR30H (pH 7.0) containing 3% BSA for 30min at 37°C. After this initial washing, the adipocytes were washed in KRBH (pH 7.4), brought to an adipocrit of 12%-15%, and a 2-deoxyglucose uptake assay was performed. Adipocytes were stimulated for 30min at 37°C with various concentrations of insulin (0.25-25ng/ml) and 2-deoxyglucose was subsequently added for 3min. Addition of ice cold 0.1M phloretin was added to stop the reaction and the cells were also placed on ice. The intracellular content of 2-deoxyglucose was then determined. Results respresent mean±SE (n=5). *$p<0.05$, **$p<0.01$

C.6. Effect of p38 kinase inhibitor on high insulin and high glucose induced insulin resistance

p38 kinase (also known as HOG-1) is an enzyme belonging to the MAP kinase family of enzymes which is activated by different forms of cellular stress (164). Such activating cellular stresses would include osmotic stress, arsenite, and interestingly, oxidative stress (164,81,116). Furthermore, the antioxidant NAC has been shown to block the activation of this enzyme, indicating that p38 may be directly or indirectly regulated by the oxidative environment of a cell (103). Thus, the hypothesis was tested that activation of p38 by increased oxidative stress may be involved in the induction of insulin resistance and that NAC may function to improve or prevent this resistance by inhibiting the p38 enzyme or interfering with its function. A specific inhibitor of p38, [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole], otherwise known as SB203580 (3), is known to function by binding with high affinity to the ATP site of the enzyme in a 1:1 ratio, thereby preventing ATP from binding within the same pocket to catalyze the phosphorylation of particular substrates (175,159). In kinetic studies of active and phosphorylated p38, SB203580 was competitive with ATP with a Ki of 21nM (175). Thus, to determine whether p38 may have a role in mediating the hyperglycemia/hyperinsulinemia induced insulin resistance, adipocytes were exposed to the high glucose plus insulin in the presence and absence of 1μM SB203580.

As shown above, adipocytes were made insulin resistant under conditions of hyperglycemia and hyperinsulinemia, there was a significant decrease in the 2-deoxyglucose uptake response of these cells to all concentrations of insulin (control basal 160±9.3

Effect of SB203580 on HG/HI Induced Resistance

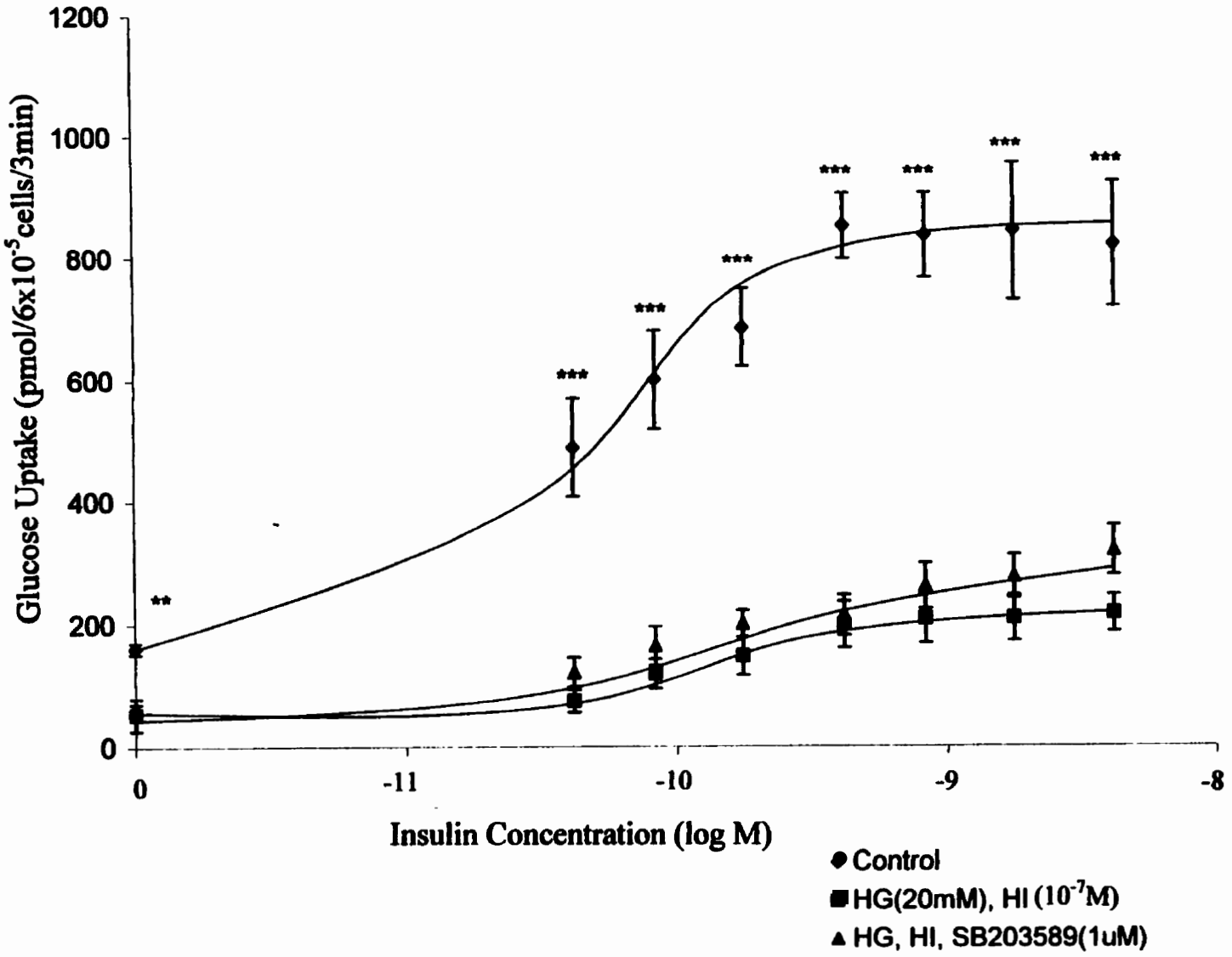


Figure 6. Effect of SB203580, a p38 kinase inhibitor, on high insulin and high glucose induced insulin resistance in primary adipocytes.
Freshly isolated adipocytes were incubated in DMEM containing 1% BSA for 16-18 hrs at 37°C. The adipocytes were incubated either alone (control), with 600ng/ml insulin and 20mM glucose (insulin resistant), or with 600ng/ml insulin, 20mM glucose, and 1μM SB203580. After the incubation, the adipocytes were washed for 30 min in KR30H (pH 7.0) containing 3% BSA at 37°C. After the wash in KR30H, the adipocytes were washed in KRBH (pH 7.4) containing 3% BSA, an adipocrit of 12%-15% was achieved, and a 2-deoxyglucose uptake assay was performed. Adipocytes were stimulated for 30 min at 37°C with various concentrations of insulin (0.25-25ng/ml) and tritiated 2-deoxyglucose was subsequently added for 3 min. The cells were placed on ice and 0.1M ice cold phloretin was added to stop the reaction. The intracellular content of 2-deoxyglucose was then determined. Results represent mean±SE (n=4). **$p<0.01$, ***$p<0.001$

pmol/6x10^5cells/3min vs. resistant basal 53±26.4 pmol/6x10^5cells/3min, $p<0.01$, and maximum insulin control 821±92.3 pmol/6x10^5cells/3min vs resistant maximum insulin 217±30.6 pmol/6.0x10^5cells/3min, $p<0.001$). Co-incubation with SB203580 in the insulin resistant cells to inhibit the p38 enzyme produced no improvement in the 2-deoxyglucose uptake of these adipocytes in response to insulin (63±7.1 pmol/6.0x10^5cells/3min at basal and 319±40.8 pmol/6.0x10^5cells/3min at maximal insulin concentration). Therefore, co-incubation with SB203580 did not significantly alter the insulin resistance in this model suggesting that p38 was not involved.

C.7.Effect of PD98059 MEK Inhibitor on Glucosamine Induced Insulin Resistance

Another attempt to delineate the mechanism of insulin resistance within the two models was made with the use of a compound known as PD98059. PD98059, otherwise known as [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one], is known to be a specific inhibitor of MAPK/ERK kinase 1 (MEK1). Therefore, PD98059 is commonly used as a MAP kinase inhibitor for it prohibits the activation of MAP kinase by inhibiting the immediate upstream activator of MAP kinase, the enzyme MEK1. Inhibition of MEK1 prevents phosphorylation of MAP kinase on the required threonine and tyrosine which is necessary for activation of this enzyme. Furthermore, along with p38, MAP kinase is another related protein which has been observed to be activated by oxidative stress - namely H_2O_2 (21,61). In fact, all three MAP kinase subfamilies (MAPK, SAP/JNK, and p38) were found to be potently activated by oxidative stress within cultured primary neonatal rat ventricular myocytes, HeLa, Rat1, PC12, primary aortic smooth muscle cells, and NIH 3T3 cells (21,61). Within these same studies, the activation of MAP kinase by H_2O_2 was blocked or inhibited by PD98059, the PKC inhibitor GF109203X, and NAC (21,61). Therefore, it

Effect of PD98059 on Glucosamine Induced Resistance

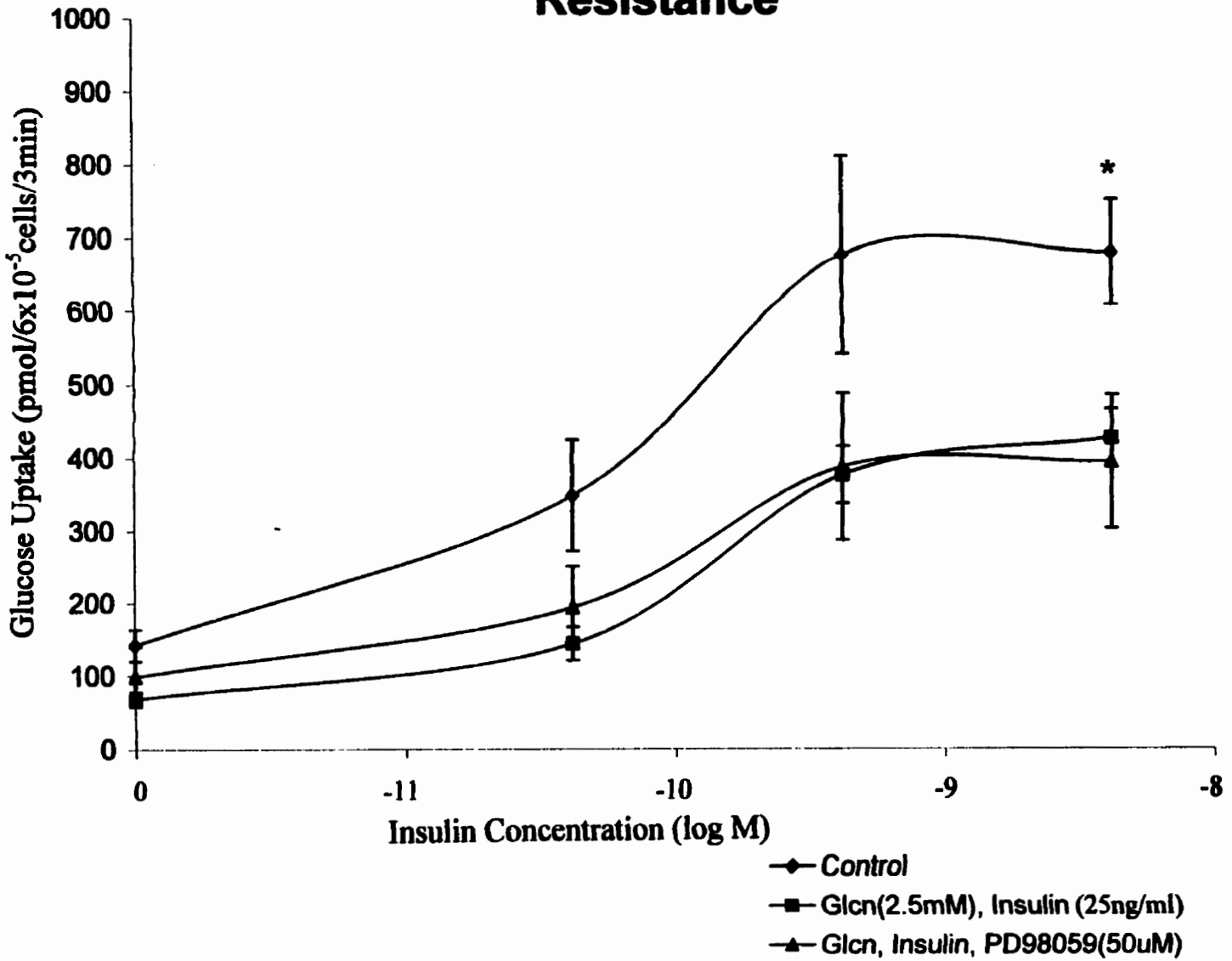


Figure 7. Effect of PD98059 on glucosamine induced insulin resistance in primary adipocytes.

Freshly isolated adipocytes were incubated in DMEM containing 1% BSA for 16-18 hours at 37°C in a 5% CO_2/95% air mixture. The incubation conditions of the adipocytes consisted of cells alone (control), glucosamine (2.5mM) and insulin (25ng/ml), or glucosamine (2.5mM), insulin (25ng/ml), and PD98059 (50μM). After the incubation, the adipocytes were washed with KR30H (pH 7.0) containing 3% BSA and incubated for 30 min in the same buffer. After incubation, the adipocytes were washed with KRBH (pH 7.4) containing 3% BSA and the cell volume adjusted to achieve an adipocrit of 12%-15%. A 2-deoxyglucose uptake assay was then performed. Adipocytes were stimulated for 30 min with various concentrations of insulin (0.25ng/ml. 2.5ng/ml, and 25ng/ml) at 37°C. Tritiated 2-deoxyglucose was subsequently added for 3 min at 37°C. The adipocytes were subsequently placed on ice and ice-cold 0.1M phloretin was added to stop the reaction. Intracellular 2-deoxyglucose content was then assessed. Results represent mean±SE. *$p<0.05$

became an interesting question whether inhibition of MAP kinase would have any effect upon insulin resistance.

Within the preliminary experiment performed, the control cells were stimulated with $0.4x10^{-12}$M, $4.2x10^{-12}$M, and $417x10^{-12}$M. Upon stimulation with insulin, the control adipocytes were observed to display a dose-dependent uptake of 2-deoxyglucose (142±22.1 pmol/$6x10^5$cells/3min basal uptake and 680±71.6 pmol/$6x10^5$cells/3min maximal uptake). Upon an 18h incubation with glucosamine, the adipocytes were rendered insulin resistant as they displayed decreased 2-deoxyglucose uptake in response to the concentrations of insulin used (68±10.8 pmol/$6x10^5$cells/3min basal uptake with 426±40.3 pmol/$6x10^5$cells/3min at maximal insulin concentrations, $p<0.05$). Co-incubation with the MEK inhibitor PD98059 appeared to have no effect over the glucosamine induced insulin resistance for no alteration in the 2-deoxyglucose uptake in response to insulin was seen (99±41.7 pmol/$6x10^5$cells/3min at basal levels and 394±91.7 pmol/$6x10^5$cells/3min at maximal insulin concentrations). Essentially, the adipocytes were still insulin resistant when co-incubated with PD98059 indicating that MAP kinase is perhaps not involved within the mechanism of insulin resistance.

C.8. Measurement of Intracellular GSH Concentrations

The intracellular levels of GSH were determined after 18h incubations of the primary adipocytes under the different conditions described above. When the intracellular levels of GSH were determined for control cells, they were found to be 107±6.6μM. As expected, upon incubation of the adipocytes with the compound L-buthionine-[S,R]-sulfoximine, a known inhibitor of the enzyme γ-glutamylcysteine synthetase, the intracellular levels of

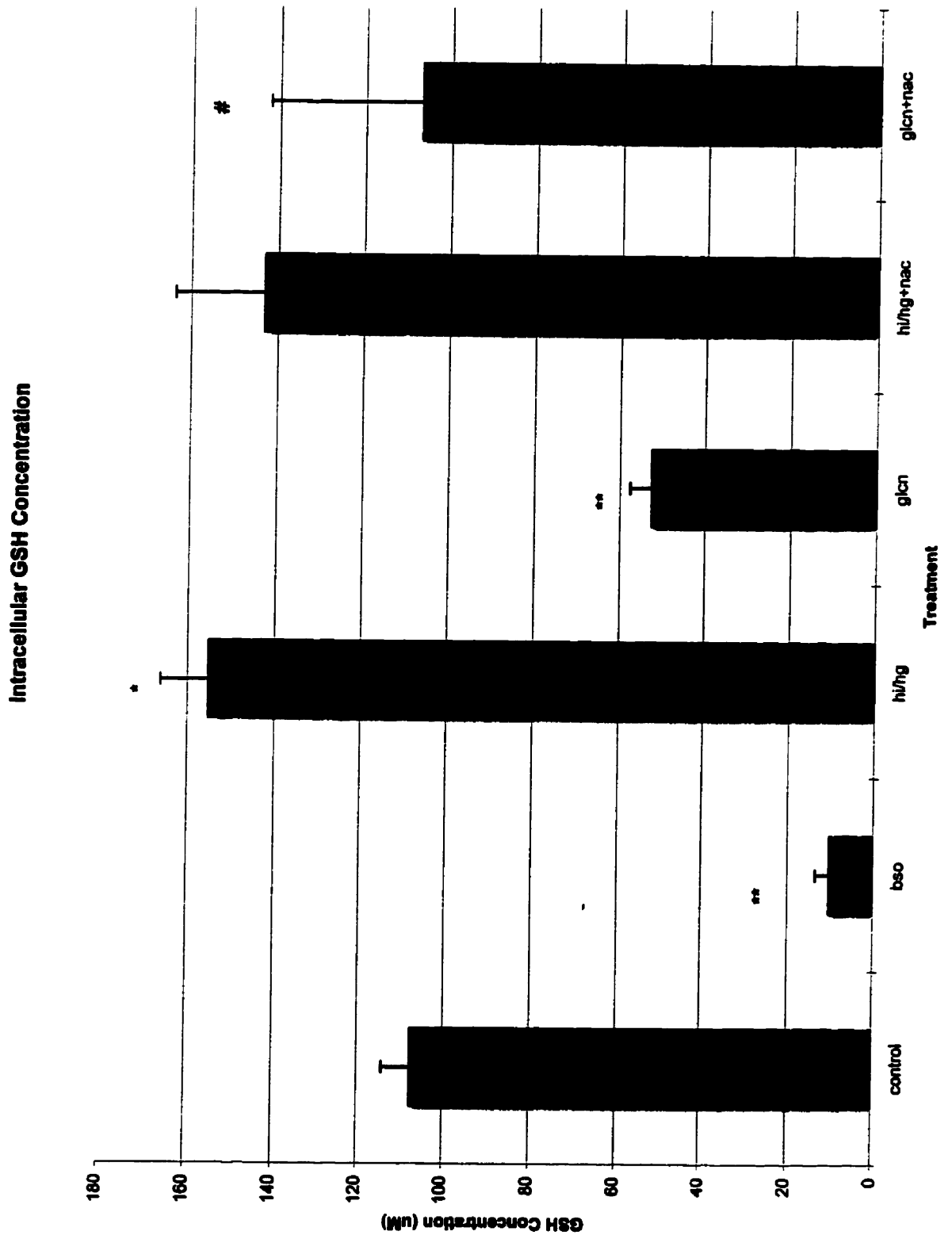

Intracellular GSH Concentration
GSH Concentration (uM)
0
20
40
60
80
100
120
140
160
180
control
bso
hi/hg
glcn
hi/hg+nac
glcn+nac
Treatment
*
**
**
#

Figure 8. Intracellular Measurement of reduced glutathione in primarary adipocytes. Freshly isolated primary adipocytes were incubated in DMEM containing 1% BSA for 16-18 hours at 37°C under various incubation conditions. Following incubation, the adipocytes were washed with KRBH (pH 7.4) twice, any excess buffer removed, and the cells placed on ice. Ice cold 5% sulfosalicylic acid was then added to the adipocytes and the adipocytes homogenized using a hand-held glass mortar and pestle. The cellular lysate was then centrifuged at 3000xg for 30 min at 4°C. Following centrifugation, the lysate (infranatant) was removed and used to assay for intracellular GSH. 100μl of cellular lysate was added to 880μl of assay buffer and 20μl of a DTNB solution in 0.05M NaH_2PO_4. 200μl of this mixture was then added to a microtiter plate and incubated within the dark at room temperature for 10 min. Subsequently, the absorbance of the microtiter plate was read at a wavelength of 414nm and the glutathione levels determined. Results represent mean±SE. *$p<0.05$ vs. control, **$p<0.01$ vs. control, #$p<0.05$ vs. glucosamine.

GSH decreased to 10±3.3μM ($p<0.01$ vs. control). As well, upon incubation with 5mM glucosamine there was also a resulting decrease in intracellular GSH levels to 52±5.2μM ($p<0.01$ vs. control). When adipocytes were co-incubated with 5mM glucosamine and 5mM NAC, the depletion of GSH found in the cells treated with glucosamine above was presented. Levels were comparable to the control condition, 93±35.4μM ($p<0.05$ vs. glucosamine above). In contrast, the adipocytes incubated under conditions of hyperglycemia (20mM) and hyperinsulinemia (10^{-7}M) did not show a decrease in intracellular GSH levels. Rather, there appeared to be a modest increase in the levels of intracellular GSH measured, 155±10.9μM ($p<0.05$ vs. control). Furthermore, when the hyperglycemia/hyperinsulinemia treated adipocytes were co-incubated with 5mM NAC, there was no effect on the levels of intracellular GSH, 143±20.5μM. Thus, there appeared to be a decrease in the levels of intracellular GSH within the adipocytes upon incubation with BSO, or with glucosamine. The glucosamine-depleted GSH levels could be corrected upon co-incubation with NAC. However, upon incubation of the adipocytes under conditions of hyperglycemia and hyperinsulinemia, the intracellular levels of GSH were found to be increased and co-incubation with NAC had no effect.

Chapter 4: Discussion and Future Directions

D. Discussion and Future Directions

The experiments performed within the scope of this thesis were undertaken to explore the relationship between oxidative stress and insulin resistance. The results observed thus far support the hypothesis that oxidative stress or free radicals do indeed play a role in the pathogenesis of insulin resistance, but the nature of this role or relationship would seem to be quite complex. Furthermore, the importance that antioxidants may play in altering the connection between oxidative stress and insulin resistance would also appear to be quite complex with glutathione playing a key role.

When NAC was used as a treatment in both models of insulin resistance, a prevention of the decrease in 2-deoxyglucose uptake in response to insulin was observed. NAC is an antioxidant which has been previously observed to directly reduce $^{\cdot}OH$, and H_2O_2, but it does not appear to interact with $O_2^{\cdot}$ to a major extent (114,2). Its interaction with $^{\cdot}OH$ is extremely rapid ($1.36x10^{10}$ M^{-1} sec^{-1}) while its reaction with H_2O_2 is fairly slow in comparison (2). However, not only does NAC act as a free radical scavenger, it can also exert its antioxidant effect indirectly by facilitating the biosynthesis of GSH for it acts as a precursor molecule for the synthesis of this peptide (28). GSH can then go on to function as an antioxidant itself or alternatively function as a cofactor for such antioxidant enzymes as glutathione peroxidase. Thus, the prevention of both hyperglycemia/hyperinsulinemia and glucosamine-induced insulin resistance through the treatment with NAC strongly suggests that oxidative stress does play a role in the pathology of insulin resistance, but the question

remains as to whether NAC is having this biological effect through its free radical scavenging activity or through its capacity to increase or maintain intracellular GSH.

One way in which to dissociate one effect from the other would be to use an alternative antioxidant which acts as only a free radical scavenger and to use GSH as a treatment directly to observe whether or not either of these treatments would have similar effects as NAC. If one treatment had similar effects as NAC but not the other, this would provide evidence as to the more important antioxidant function of NAC in preventing insulin resistance. One potential problem that could be encountered is the possibility that both of these potential treatments would have similar effects on 2-deoxyglucose uptake in response to insulin as NAC. This probability arises from the fact that most intracellular antioxidants have a complex and dynamic interaction with each other in that they possess the ability to regenerate each other. An example of this would be the ability of GSH and an enzyme known as dehydroascorbate reductase to reduce or convert the oxidized form of vitamin C (dehydroascorbate) back to its reduced form (ascorbic acid). In the process, two molecules of GSH are oxidized to GSSG, but GSSG is then reduced back to GSH by the activity of an enzyme known as glutathione reductase which uses NADPH as a cofactor or reducing agent. Thus, there is a dynamic interaction between most intracellular antioxidants which makes it extremely difficult to distinguish the specific actions or effect of one particular antioxidant apart from the rest as well as trying to determine the specific function of an antioxidant within an intricate cellular system.

An expanded hypothesis probing the manner in which the antioxidant properties of NAC may be functioning within the cell is the possibility that NAC may be modulating the activity of various transcription factors. Recently, there has been increasing evidence that

transcription factors such as nuclear factor kB (NF-kB) and activator protein 1 (AP-1) are redox-sensitive (155,55). It would appear that many of these transcription factors are activated by oxidative stress so that an increase in free radicals can cause translocation of these proteins to the nucleus with consequent binding to a consensus binding motif in the promoter of various genes (28). In many instances, NAC effectively inhibits the activation of these transcription factors while oxidants such as hydrogen peroxide could directly activate proteins such as NF-κB, but it is important to note that these effects elicited by NAC are not always specific as other thiol-containing compounds could elicit similar responses (28). In any case, it is important to note that NAC may function indirectly in cellular systems by modulating the function of various transcription factors and that this function is closely linked to the antioxidant properties of NAC. However, as mentioned previously, it is unknown whether the free radical scavenging activity or the ability of NAC to contribute to the synthesis of GSH is important for the potential of this compound to modulate the function of varous transcription factors. The possibility also exists that both functions of NAC may contribute to this particular phenomenon.

It is important to note at this point, as described above, that the ability of NAC to possibly function by modulating the activity of different transcription factors may prove to be of great importance since the induction of insulin resistance by glucose appeared to require the synthesis of mRNA (104). To reiterate, both actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (both inhibitors of mRNA synthesis), were able to prevent the desensitization of the glucose transport system in response to hyperglycemia (104). Current speculation states that mRNA synthesis is important to maintain GFAT turnover as it is a very short-lived protein. Therefore, by blocking mRNA synthesis the

functional activity of GFAT would disappear over time due to the rapid turnover of GFAT preventing flux down the hexosamine biosynthesis pathway. Another study has shown that glucosamine could stimulate GFAT mRNA accumulation alone as well as have additive effects with epidermal growth factor (EGF) on the accumulation of this mRNA in MDA468 cells (a human breast cancer cell line) (127). Interestingly, high levels of glucose (25mM) blocked the ability of EGF to stimulate GFAT gene transcription and accumulate its mRNA (127). A surprise of these observations was that glucose and glucosamine were not observed to have similar effects although it is currently thought that they evoke their effects on cellular metabolism through the same mechanism. It is possible that these compounds may exert similar effects on metabolism but have additional unique effects on gene regulation. Further work is needed to fully understand these effects and the relationships they may have with oxidative stress and antioxidants.

In trying to answer the question of the therapeutic possibilities of using NAC as a treatment for insulin resistance or type II diabetes, the question arises regarding the in vivo use of NAC. It has already been described that the infusion of glucosamine can mimic the insulin resistant state by causing a marked decrease in the glucose disposal rate during a euglycemic-hyperinsulinemic clamp (138,69). It would be interesting to see if the co-infusion of NAC within this system or the oral ingestion of NAC in different insulin resistant animal models could prevent the onset of in vivo insulin resistance. Normally, the bioavailability of NAC is quite low (approximately 5%) when ingested orally, but infusing the compound directly would overcome this problem (11,124). A problem arises however, when administering NAC orally for therapeutic use as concentrations of the compound used in cellular experiments such as the ones performed for this thesis would be difficult to

achieve within the plasma. With oral ingestion, free NAC was found to be undetectable with low levels of oxidized NAC detected for several hours, but when it was infused, NAC was observed to form disulfides in plasma which helped to prolong the existence of the drug in plasma (25). This evidence suggests that in response to an oral dose of NAC, the drug itself does not accumulate within the body, but rather there is an accumulation of its oxidized form and oxidized metabolites. As well, upon oral ingestion of NAC in rats, extensive intestinal deacetylation of NAC was found to take place such that the major metabolites entering the hepatic portal circulation were cysteine, cystine (an oxidized form consisting of two cysteine molecules linked by a disulfide bond), and sulfite (23). Human endothelial cells also have the ability to deacetylate NAC and use the liberated cysteine for intracellular GSH biosynthesis, indicating that NAC is likely to be deacetylated whether taken orally or infused within the systemic circulation (26). As NAC has been used in a therapeutic capacity to treat different pathologies of the lung, the question is raised as to whether these effects are due to NAC or to an increase in cysteine and/or GSH as both rat lung and liver homogenates have been observed to also deacetylate NAC (28). This also is an interesting question to ask regarding the use of NAC in treating insulin resistance. The possibility exists and it may even be expected that cysteine may have a similar ability to prevent insulin resistance induced by either hyperglycemia/hyperinsulinemia or glucosamine. If this hypothesis were observed to be true, it would prove interesting to see what effects this amino acid would have upon insulin resistance in vivo. Thus, pursuing the effects of NAC in an in vivo model of insulin resistance or in type II diabetic models would be an important step in trying to elucidate the therapeutic benefits of this compound.

The observation that co-incubation with GSH ethyl monoester could prevent the induction of insulin resistance by hyperglycemia and hyperinsulinemia within primary adipocytes was similar to the observations seen with NAC. This finding would seem to further implicate the importance of GSH in helping to prevent the induction of insulin resistance. A GSH ethyl monoester was chosen in these experiments as this form of GSH is known to be more readily and easily transported into many cells and tissues when compared to GSH. GSH, when administered to cells, is not readily transported intracellularly, but rather, becomes degraded extracellularly. These metabolized products are then transported into cells and used for GSH resynthesis. The diester form of GSH is known to be transported into cells at an even faster rate, but for the purposes of the experiments contained within this thesis, we found that the monoester form was sufficient. Use of the monoester form of GSH would have allowed for a slower but more prolonged administration of GSH (which would be desirable for incubation times of 16-18 hours). Furthermore, the diester form of GSH is known to be metabolized to the monoester form of GSH both intracellularly and extracellularly although the intracellular reaction occurs more rapidly than the extracellular breakdown of diester GSH (1). This discrepancy in reaction rates would tend to trap the monoester GSH intracellularly as the diester GSH would be readily transported within cells and metabolized to the monester GSH, which can then be transported out of the cell at a much slower rate or metabolized to GSH. Alternative methods of increasing cellular GSH would include addition of cysteine (which has been reported to be toxic in some cases), administration of L-2-Oxothiazolidine-4-Carboxylic Acid (OTC) (an alternative cysteine delivery compound), or administration of γ-glutamylcysteine (the first product of the GSH synthesis pathway). However,

administration of the monoester or diester form of GSH is currently thought to be the most effective way of increasing cellular GSH. Therefore, in light of the observed results with GSH ethyl monoester and NAC the probable mechanism by which NAC may be functioning is through its capacity to facilitate synthesis of GSH, although the importance of its radical scavenging activity cannot be discounted. Nevertheless, it is possible that as with NAC, GSH could also possibly prove to be a beneficial agent when preventing the onset or ameliorating the adverse effects of insulin resistance in vivo. GSH ethyl monoester can increase GSH levels in vivo whether it is administered intraperitoneally, subcutaneously, or orally (for review see 1). As with NAC, it is expected that the GSH ethyl monoester will also be able to prevent the onset of glucosamine induced insulin resistance, for the proposed mechanism is thought to be similar to hyperglycemia/hyperinsulinemia induced insulin resistance. However, this will have to be confirmed empirically and these experiments are currently underway.

Further evidence implicating the importance of GSH within our two models of insulin resistance are the results of the intracellular GSH measurements under the various incubation conditions. BSO, an amino acid sulfoximine, is known to be a specific inhibitor of γ-glutamylcysteine synthetase (the first and rate-limiting enzyme in GSH biosynthesis), which subsequently causes a decrease in intracellular GSH. The drop in intracellular GSH seen with BSO however, is not caused by a depletion of GSH, but rather by an inhibition of resynthesis of this molecule. Therefore, as expected, upon incubation with BSO, there was a marked decrease in the intracellular levels of GSH. A similar decrease in GSH, but one not quite as drastic, was seen upon incubation of the adipocytes with glucosamine. Upon co-incubation of the adipocytes with glucosamine and NAC, the intracellular GSH levels

were found to be elevated when compared to glucosamine treated cells and comparable to the levels seen in control adipocytes. Thus, in the glucosamine induced insulin resistant condition, there appears to be a good correlation between induction of insulin resistance, prevention of insulin resistance with NAC, and the levels of intracellular GSH observed, whereby insulin resistance is correlated to lower levels of GSH and increased insulin sensitivity is correlated with increased levels of GSH. Although a correlational relationship does not necessarily signify a cause and effect relationship, it does provide evidence for a direct or perhaps indirect connection between the two correlatives.

However, when similar intracellular GSH measurements were performed on adipocytes incubated under conditions of hyperglycemia and hyperinsulinemia a similar correlation was not observed. Insulin resistance brought about by elevated glucose and insulin levels produced an increase in the total intracellular GSH levels. Furthermore, when these insulin resistant adipocytes were co-incubated with NAC, there was no alteration in the levels of intracellular GSH measured. The fact that the levels of intracellular GSH would be elevated in this model of insulin resistance is puzzling for it is inconsistent with the observations seen with glucosamine and also inconsistent with both NAC and GSH ethyl monoester being able to prevent the onset of insulin resistance in both models of insulin resistance. One possible explanation may be that much of the GSH within adipocytes incubated under conditions of hyperglycemia and hyperinsulinemia is present as the oxidized form glutathione disulfide (GSSG). It should be noted that the method used to assess intracellular GSH within these experiments is not sensitive to the ratio of GSH:GSSG as the methodology uses the enzyme glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) to reduce any GSSG to GSH. Therefore, the

measurements of GSH cited within this thesis are measurements of total cellular GSH. However, it is fully plausible that in the adipocytes incubated with high levels of glucose and insulin, the majority of the GSH exists as GSSG, but confirmation of this hypothesis is required. Perhaps the best and most accurate method of measuring intracellular GSH and GSSG levels would be to use high-performance liquid chromatography (HPLC). If, using HPLC, it was discovered that the ratio of GSH:GSSG was indeed lower in adipocytes incubated with elevated levels of glucose and insulin, it would be possible to conclude that glucosamine caused a depletion in total cellular GSH while hyperglycemia and hyperinsulinemia caused an elevation of GSH, but that this GSH was ineffective as an antioxidant as most of it existed in the form of GSSG. In this case, co-incubation of NAC in these adipocytes may have provided the reducing power to regenerate GSH rather than increase total glutathione (GSH and GSSG) levels. Clearly, further work will be needed to uncover the effect hyperglycemia/hyperinsulinemia and glucosamine have on intracellular GSH levels and ultimately how this may relate to the redox state of the cell.

Vitamin E, a nutrient antioxidant present in the lipid bilayer of most mammalian cells, appeared to cause a partial prevention of the insulin resistance induced by hyperglycemia and hyperinsulinemia although statistical significance was not achieved. The lack of statistical significance is likely due to biological variability and to the relative small sample size. It is expected that a larger sample size would produce a significant, but still incomplete effect. The observation that vitamin E could only produce a partial prevention of insulin resistance is interesting and merits further investigation. Previous studies have demonstrated that diabetic patients were found to have lower plasma vitamin E levels and GSH:GSSG ratios than controls (15). As well, within this same study, vitamin E

supplementation for four months could improve the action of insulin in patients with type II diabetes (15). This evidence, along with others (mentioned above in the introduction) indicated that vitamin E was one biological parameter which correlated with diabetes and insulin resistance, but also that alteration of this parameter could bring about alterations in insulin sensitivity. Thus, the possible importance of vitamin E in insulin action was established.

Vitamin E is a term given to a family of antioxidants termed tocopherols of which α-tocopherol is known to be the most biopotent. Within the experiments performed in this thesis an α-tocopherol acetate was used because this is the esterified tocopherol most commonly used in dietary supplements and pharmaceutical formulations. The esterified form, which prevents oxidation of the compound during preparation are much more resistant to oxidation than the non-esterified forms. However, the tocopherol esters display no antioxidant activity and must undergo enzymatic hydrolysis to release α-tocopherol. Thus, one explanation for the limited prevention of insulin resistance by vitamin E may be the insufficient hydrolysis of the α-tocopherol acetate within the primary adipocytes studied. Alternatively, the fact that vitamin E is a lipophilic antioxidant may also provide a clue as to its limited ability to prevent insulin resistance. It may be that cytosolic antioxidants such as NAC and GSH can serve as better antioxidants than vitamin E which is restricted to lipid membranes, or it may possibly be that NAC and GSH are functioning as molecules other than antioxidants - a function which is lacking in vitamin E. However, while these speculations remain valid possibilities, it remains a difficult task to determine which possibility is responsible for the empirical data observed. One compound which could help provide insight into the problem is a molecule named trolox. This compound is a water-

soluble or hydrophilic form of vitamin E. Thus, use of this molecule could potentially provide an answer as to whether cytosolic antioxidants are more important than lipid antioxidants in preventing the onset of insulin resistance within primary adipocytes. Once again, further investigation is needed in order to fully elucidate the effects vitamin E may be having on the prevention of insulin resistance and how it may differ from both NAC and GSH ethyl monoester in this respect.

Ebselen, a glutathione peroxidase enzyme mimetic also known as PZ-51, was unable to prevent glucosamine induced resistance within primary adipocytes. Ebselen functions primarily as a glutathione peroxidase mimetic although it does possess weak radical scavenging activity. Glutathione peroxidase and Ebselen are both thought to require the presence of two GSH molecules in their degradation of hydroperoxides. The two GSH molecules are subsequently oxidized to GSSG during the reaction. In the glucosamine induced insulin resistant condition, when the intracellular levels of GSH have been depleted, one possiblitiy for Ebselen having no effect is that there was an insufficient amount of GSH for this enzyme mimetic to function. For example, hepatocytes depleted of GSH could not be protected from iron-ADP-induced lipid peroxidation while normal cells were found to be protected by Ebselen (118). Furthermore, the generalization could be made that there was a general depletion or alteration of cellular thiols as Ebselen has been shown to use other thiols, such as dihydrolipoate and NAC, in addition to GSH to catalyse its reactions (27,62). Therefore, it is possible to conclude that not only have the intracellular levels of GSH been depleted, but that there could be an overall reduction or alteration of intracellular thiols which does not allow Ebselen to function in its capacity as a gluthione peroxidase mimetic.

Alternatively, there is also a possibility that Ebselen interferes with various enzymes due to the lack of intracellular GSH. For example, previous studies have shown that Ebselen has the ability to inhibit certain enzymes (such as NADPH oxidase and protein kinase C from human granulocytes (24) and nitric oxide synthases from bovine aortic endothelium (176)) at low concentrations. The mechanism of the inhibition of enzyme activity is thought to occur as a result of blockade of thiol groups essential for enzyme action such as those that may reside within the catalytic domain of some enzymes. Whether or not this ability of Ebselen will prove to be significant in our observation that Ebselen could not prevent the insulin resistance brought about by glucosamine remains to be determined. What is important to note however, is that once again, glutathione is perhaps playing a key role in determining the intracellular redox state of the cell.

p38, a protein belonging to the MAP kinase family of enzymes, has been observed to be activated by different cellular stressors including oxidative stress (116). At present, little is known about this particular enzyme and its specific biological or cellular functions except that it is thought to play a role in inflammation and in the production of proinflammatory cytokines like TNFα (100). The enzyme is activated upon phosphorylation by the dual specificity MAP kinase kinases 3 and 6 (MKK3 and MKK6) on threonine and tyrosine residues and has MAPKAPK2/3 (MAP kinase activated protein kinases 2 and 3) and the transcription factor ATF2 among its substrates (67,130,148,115,131,111). When looking at a potential mechanism of hyperglycemia or glucosamine induced insulin resistance, we hypothesized that p38 could be playing a possible role because of its activation by cellular stress and oxidative stress in particular. Therefore, we used the compound SB203580 to inhibit the activity of this enzyme. The results observed appear to indicate that this enzyme

is not involved in decreasing the uptake of glucose in response to insulin within the insulin resistant cells. However, an alternate explanation could be that the chosen inhibitor (SB203580) is not causing an effective inhibition of the p38 enzyme.

There are currently four known p38 enzymes - p38, p38β, p38γ, and p38δ. All four of these enzyme isoforms contain the TGY dual phosphorylation site which is characteristic of the MAP kinase family of enzymes. As well, there is approximately a 60% identity of the amino acid sequences among these proteins. With regard to tissue distribution, both p38 and p38β are known to be ubiquitously expressed, while p38γ is largely expressed within muscle only. To date, the particular tissue distribution of p38δ has not been examined and the relative levels of these enzymes within adipose tissue has not been documented. Furthermore, the functional significance of the different tissue specific expression of the various isoforms is currently unknown. The importance of recognizing the different isoforms of p38 lies in the possibility that all the isoforms discovered to date may not be equally inhibited by the pyridinyl imidazole SB203580. For example, in the study citing the first discovery of the fourth isoform of p38 (p38δ), the authors demonstrated that another pyridinyl imidazole compound known as SB202190 could effectively inhibit the activity of p38, p38β, and p38γ at concentrations of 0.1μM-1.0μM, but that similar concentrations of the inhibitor had no effect on the activation of p38δ (85). As well, the related compound SB203580 (used for the experiments within this thesis) was also shown to be ineffective in inhibiting p38γ, although others have observed contradictory results (33,86). Therefore, it is certainly a possible that SB203580 did not cause an inhibition of all the p38 isoforms found within rat primary adipocytes. Consequently, no effect of this inhibitor on insulin resistance would have been seen if the relevant p38 isoform of interest was not inhibited. In order to

validate the observations found in this thesis regarding the relationship between p38 and insulin resistance, it will be imperative to identify the isoforms of p38 expressed within adipocytes and to determine the ability of SB203580 and other compounds to inhibit these different isoforms.

MAP kinase, an enzyme which is closely related to the p38 family of kinases, is another protein which is known to be potently activated by oxidative stress (21,61). This activation of MAP kinase by oxidative stress has also been demonstrated to be inhibited through the use of the antioxidant NAC or the MEK inhibitor PD98059 (21,61). Such an observation raises the possibility that the mechanism through which NAC prevented the induction of insulin resistance in the glucosamine and hyperglycemia/hyperinsulinemia models is by inhibition of the MAP kinase enzyme. Increased levels of glucose have also been demonstrated to activate MAP kinase, although no studies have yet been performed with glucosamine (244). The potential role of MAP kinase activation in insulin resistance is augmented by the observation that IRS-1 contains four serines which fall into the consensus sequence recognized by the MAP kinase family of enzymes - Pro-X-Ser-Pro (91). Serine (Ser) and threonine (Thr) phosphorylation of IRS-1 is thought to be one possible mechanism of insulin resistance which can be observed upon PKC activation (or activation of tumor necrosis factor (TNF) signaling pathways (increased levels of TNF-α is one proposed cause for insulin resistance in type II diabetes and obesity) (78,39). Ser/Thr phosphorylation of IRS-1 is thought to inhibit the ability of the insulin receptor to tyrosine phosphorylate its downstream substrates including the IRS-1 protein (78). Furthermore, the serines thought to be phosphorylated on IRS-1 by MAP kinase are positioned exactly four amino acids downstream of potential tyrosine phosphorylation sites which would implicate serine

phosphorylation of IRS-1 as an important regulator of this protein (38). Use of the MEK inhibitor PD98059 has been demonstrated to prevent phosphorylation of IRS-1 by MAP kinase (38).

Co-incubation of adipocytes with the compound PD98059 was unable to prevent the insulin resistance induced by glucosamine. This implication would seem to indicate that MAP kinase is not involved in the mechanism of glucosamine induced insulin resistance. Furthermore, if IRS-1 serine phosphorylation is present within this model of insulin resistance, it is unlikely that the kinase responsible for this phosphorylation is MAP kinase. Therefore, evidence for the mechanism of the insulin resistance induced by glucosamine remains elusive and further experimentation is required to elucidate the cellular mechanisms involved and the point at which the defect appears within the insulin signaling pathway.

Having determined the ability of certain antioxidants to prevent the glucosamine and hyperglycemia/hyperinsulinemia induced insulin resistance in adipocytes, we have observed a definite link between oxidative stress and insulin resistance. An alternative approach to take in confirming this relationship between insulin resistance and free radicals would be to determine whether induction of oxidative stress has the ability to render the adipocytes insulin resistant. Bashan et al. demonstrated that induction of oxidative stress through the use of a H_2O_2 generating glucose/glucose oxidase system in 3T3-L1 adipocytes could indeed produce insulin resistance (139,140). The development of this oxidative stress mediated insulin resistance was evidenced by a decreased 2-deoxyglucose uptake response to insulin, reduced GLUT4 protein and mRNA content and in reduced translocation of GLUT4 to the cell surface upon insulin stimulation concomitant with a decreased IRS-1 associated PI3 kinase activity (139,140). In light of these results, it will be necessary to

confirm the induction of insulin resistance through oxidative stress in the isolated primary adipocytes and to assess whether antioxidants could also prevent (as one would expect) this form of insulin resistance. Such empirical data would serve as confirmation that insulin resistance can be mediated through oxidative stress and that antioxidants can prevent this resistance via their antioxidant properties rather than by any alternative properties they may have.

Another method by which to confirm the induction of insulin resistance through oxidative stress would be to assess the levels of free radicals within our two models of insulin resistance. Preliminary data from our laboratory using a fluorescence activated cell sorter (FACS analysis) has demonstrated an increase in free radicals within the adipocytes upon incubation with glucosamine and upon incubation with elevated levels of glucose and insulin (experiments performed by another member of our lab). Co-incubation with NAC was able to effectively decrease the levels of free radicals produced in both models of insulin resistance. Furthermore, western blot analysis of the adipocytes using an antibody specific for the protein carbonyl derivatives of oxidatively damages proteins has indicated that those adipocytes rendered insulin resistant by incubation with glucosamine or high levels of glucose and insulin demonstrate higher levels of protein damage when compared to control cells (experiments performed by others). Therefore, there appears to be a strong positive correlation between induction of insulin resistance and production of free radicals accompanied by biological damage to proteins. Once again however, correlation does not necessarily denote a cause and effect relationship, and thus, the exact nature of this correlation remains to be determined. As well, although diabetic patients have been observed to have increased levels of lipid oxidation (as mentioned above in the

introduction) as assessed by their levels of malondialdehyde and TBARS, it would be important to note the biological damage done to both lipids and DNA within these two models of insulin resistance.

Another important issue to resolve is whether the antioxidants which prevented the induction of insulin resistance could also cause a reversibility of insulin resistance. The experimental approach would entail the addition of antioxidants after the induction of insulin resistance with either glucosamine or hyperglycemia/hyperinsulinemia. If the insulin resistance were improved in such a situation, the conclusion could be drawn that not only could the antioxidants prevent the onset of insulin resistance, but that they could also cause a reversal of insulin resistance. Resolving this issue may become important in assessing the therapeutic value of various antioxidants for the treatment of type II diabetes mellitus.

On a final note, although it has been assumed by others and within this thesis that both models of insulin resistance (glucosamine induced and hyperglycemia/hyperinsulinemia induced) are produced through the same mechanism, there is evidence which has emerged recently that would indicate two different mechanisms of insulin resistance. If this discrepancy in the two models of insulin resistance is confirmed, then it is very significant that antioxidants such as NAC can prevent the induction of insulin resistance in both models. Also, it may suggest that while the two models of insulin resistance may have differences in some pathophysiological mechanisms, a common factor which is present and capable of being targeted in both models is the link between oxidative stress and insulin resistance. Thus, a closer examination of the mechanisms of resistance in both models is warranted.

In conclusion, we have reported the first instance of a possible therapeutic compound being able to prevent the insulin resistance induced both high glucose and insulin as well as by glucosamine. In addition, our data supports a definite link between oxidative stress and insulin resistance, but the relationship is a complex one requiring further work to delineate the role of oxidative stress as a common mechanism inducing insulin resistance. Furthermore, additional work is justified in determining the different roles of various antioxidants as preventative or restorative compounds of this resistance. It is hopeful that additional work to understand the pathophysiology of insulin resistance and the potential role of oxidative stress in the etiology of non-insulin dependent diabetes mellitus will give rise to possible new therapeutic treatments and/or preventative measures. As the prevalence of type II diabetes and obesity continues to rise and touch all of us, it becomes an absolute necessity to continue scientific research into the nature and treatment of this disease in order to improve the quality of life for these individuals.

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