

**BIOLOGICAL EFFECTS OF RESVERATROL ON
SKELETAL MUSCLE CELLS**

by

Danna M. Breen

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Acknowledgment

The best way out is always through. –*Robert Frost*

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Abstract

Resveratrol, a polyphenol found in red wine, has been reported to have antithrombotic, antiatherogenic, and anticancer properties both in vitro and in vivo. However, possible antidiabetic properties of resveratrol have not been examined. The objective of this study was to investigate the direct effects of resveratrol on basal and insulin-stimulated glucose uptake and to elucidate its mechanism of action in skeletal muscle cells. In addition, the effects of resveratrol on basal and insulin-stimulated amino acid transport and mitogenesis were also examined.

Fully differentiated L6 rat skeletal muscle cells were incubated with resveratrol concentrations ranging from 1 to 250 μM for 15 to 120 min. Maximum stimulation, $201 \pm 8.90\%$ of untreated control, ($p < 0.001$), of $2[{}^3\text{H}]$ deoxy- D- glucose (2DG) uptake was seen with 100 μM resveratrol after 120 min. Acute, 30 min, exposure of the cells to 100 nM insulin stimulated 2DG uptake to $226 \pm 12.52\%$ of untreated control ($p < 0.001$). This appears to be a specific property of resveratrol that is not shared by structurally similar antioxidants such as quercetin and rutin, both of which did not have any stimulatory effect. Resveratrol increased the response of the cells to submaximal insulin concentrations but did not alter the maximum insulin response. Resveratrol action did not require insulin and was not blocked by the protein synthesis inhibitor cycloheximide. LY294002 and wortmannin, inhibitors of PI3K, abolished both insulin and resveratrol-stimulated glucose uptake while phosphorylation of Akt/PKB, ERK1/2, JNK1/2, and p38 MAPK were not increased by resveratrol. Resveratrol did not stimulate GLUT4 transporter translocation in GLUT4*cmyc* overexpressing cells, in contrast to the significant translocation observed with insulin. Furthermore, resveratrol-stimulated

glucose transport was not blocked by the presence of the protein kinase C (PKC) inhibitors BIM1 and Go6983. Despite that, resveratrol- induced glucose transport required an intact actin network, similar to insulin.

In contrast to the stimulatory effect seen with resveratrol for glucose transport, [¹⁴C]methylaminoisobutyric acid (MeAIB) transport was inhibited. Significant reduction of MeAIB uptake was seen only with 100uM resveratrol ($74.2 \pm 6.55\%$ of untreated control, $p < 0.05$), which appeared to be maximum. In parallel experiments, insulin (100 nM, 30 min) increased MeAIB transport by $147 \pm 5.77\%$ ($p < 0.001$) compared to untreated control. In addition, resveratrol (100 μ M, 120 min) completely abolished insulin- stimulated amino acid transport ($103 \pm 7.35\%$ of untreated control, $p > 0.05$).

Resveratrol also inhibited cell proliferation in L6 myoblasts with maximal inhibition of [³H]thymidine incorporation observed with resveratrol at 50 μ M after 24 hours ($8 \pm 1.59\%$ of untreated control, $p < 0.001$). Insulin (100 nM, 24 h) significantly increased thymidine incorporation ($280 \pm 9.92\%$ of untreated control, $p < 0.001$) and media containing 10% FBS resulted in stimulation of thymidine incorporation to $691 \pm 36.92\%$ of untreated control, $p < 0.001$. Resveratrol (50 μ M) completely abolished both insulin- ($11 \pm 1.26\%$ of untreated control, $p < 0.001$) and FBS- stimulated ($36 \pm 5.16\%$ of untreated control, $p < 0.05$) cell proliferation.

These results suggest that resveratrol increases glucose transport in L6 skeletal muscle cells by a mechanism that is independent of insulin and protein synthesis. Resveratrol- stimulated glucose uptake may be PI3K and actin cytoskeleton- dependent and independent of Akt/PKB, PKC, ERK1/2, JNK1/2, p38 MAPK, and GLUT4

translocation. However, unlike glucose transport, resveratrol inhibits both basal and insulin- stimulated amino acid transport and mitogenesis.

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CHAPTER 1: BACKGROUND INFORMATION

1.1 INSULIN AND INSULIN SIGNALLING

1.1.1 Insulin

Insulin is a major anabolic protein hormone regulating numerous physiological processes in the liver, adipose tissue, and muscle (1). It is composed of two amino acid chains, an alpha (α) chain and a beta (β) chain, connected by disulfide bridges. Insulin is produced by the beta cells of the islets of Langerhans in the pancreas and is degraded by insulinase in the liver and kidney. Initially, insulin is secreted into the portal vein and is immediately exposed to liver insulinase. As a result, approximately one half of the insulin produced is degraded before entering the peripheral circulation.

Insulin is an important regulator of amino acid uptake, cell growth, development, and survival, lipid metabolism in muscle and adipose tissue, and transcription and translation rates as a mitogen, although it is best known for regulation of whole-body glucose homeostasis (Figure 1) (2). Plasma glucose elevations cause a rise in insulin secretion, which stimulates muscle and fat tissue glucose uptake and glycogen synthesis and inhibits liver glycogenolysis and gluconeogenesis, thereby maintaining glucose homeostasis (Figure 2). Very recent evidence indicates that insulin is also produced in nerve brain cells, although the function is not clear. The major target tissues of insulin action are the liver, adipose tissue, and skeletal muscle (Figure 2).

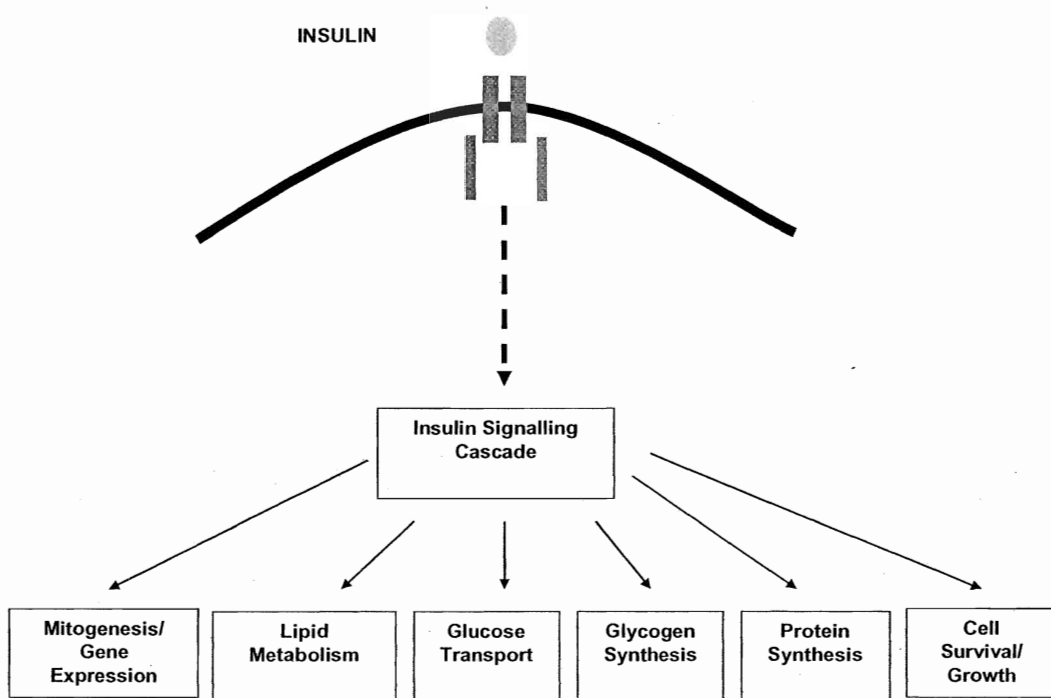


Figure 1: The pleiotropic effects of insulin.

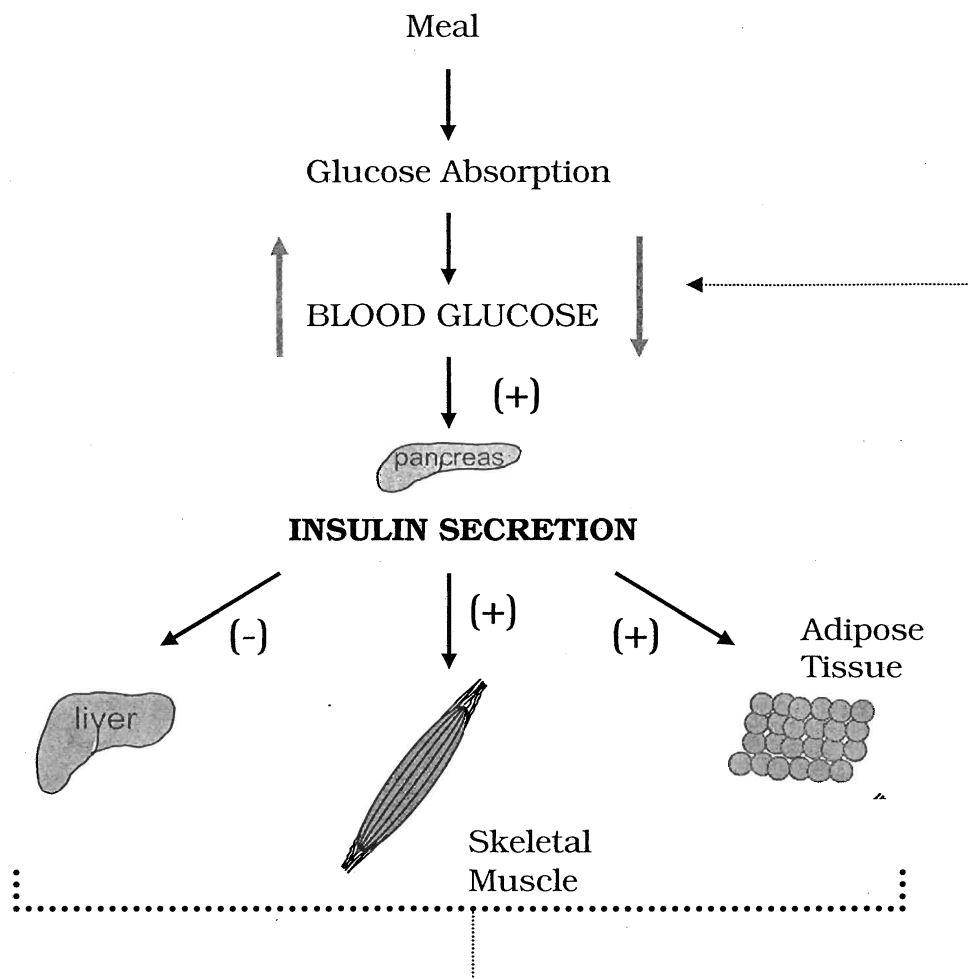


Figure 2: Major tissues involved in insulin action to maintain blood glucose homeostasis.

1.1.2 Glucose Transporters

The plasma membrane of cells is composed of a lipid bilayer that is impermeable to carbohydrates. Therefore, glucose does not freely travel across the plasma membrane and requires membrane-associated carrier proteins for transport. The glucose transporters are large integral proteins with 12 membrane-spanning domains. The structure of each transporter contains a cytoplasmic carboxyl (C)- and amino (N)- terminal. There are two known main groups of glucose transporters, including the sodium-glucose transporters (SGLTs) and facilitated diffusion transporters (GLUTs). The SGLT1- sodium dependent transporter is found in the intestinal epithelial and renal tubular cells and is responsible for sodium-dependent secondary active transport of glucose. When sodium is absent, these transporters have a higher affinity for fructose, however when sodium is present, the transporter is activated, which increases its affinity for glucose and therefore transports glucose against its concentration gradient.

Glucose is cleared from the bloodstream by a group of facilitative transporters known as the GLUTs. There are currently 12 known subtypes of this facilitative transporter, but GLUT1- 6 are most commonly discussed (3;4). In contrast to SGLTs, these proteins transport glucose down its concentration gradient. The GLUT transporters have distinct substrate specificities, tissue distribution, and kinetic properties that define their role. GLUT1 (46kDa) is ubiquitously expressed with high concentrations in human erythrocytes and in endothelial cells lining blood vessels in the brain. The GLUT1 transporter is essential since it has been characterized as the basal glucose transporter and is the major glucose transporter in adipocytes (5). GLUT1 has a high affinity (K_m) for

glucose at 20mM. GLUT2 is a low affinity transporter ($K_m = 42\text{mM}$) found in the liver, intestine, kidney, and pancreatic beta cells. This transporter is part of the glucose sensory system in beta cells and functions in the basolateral transport of intestinal epithelial cells. GLUT3 (45 kDa, $K_m = 10\text{mM}$) initially was thought to be expressed in fetal embryonic tissues like the placenta. It is also expressed in neurons, allowing glucose to cross the blood brain barrier (6). GLUT5, on the other hand, is a fructose transporter located in the jejunum of the small intestine with a very low affinity for glucose. The GLUT6 gene encodes a pseudogene with no corresponding protein (6;7).

GLUT4 (48 kDa, $K_m = 2\text{-}10\text{mM}$) is an insulin- responsive glucose transporter present in skeletal muscle, cardiac muscle, and adipose tissue. Insulin- stimulated glucose transporter translocation was initially demonstrated in 1980 by Cushman and Wardzala (8) and has been documented in several fat and muscle cell lines since then (9;10). The importance of GLUT4 in insulin- stimulated glucose transport was demonstrated in mice in which one allele encoding the *GLUT4* gene was disrupted. These mice exhibited severe insulin resistance (11). During the basal state, GLUT4 is continually cycled between the plasma membrane and several intracellular compartments, where the majority of the transporters are located inside the cell (~90%) (12). Rapid translocation of GLUT4 from an internal pool to the plasma membrane occurs in response to acute insulin stimulation in both fat cells and muscle fibres (13).

1.2 The Insulin Signalling Cascade

Signal transduction begins with an extracellular signal that produces an intracellular response. Molecules that are unable to penetrate the plasma membrane of the cell must bind to receptors, which then activates intrinsic enzymatic activity or modulates

transducing proteins. This stimulation eventually leads to either the activation or inhibition of effector proteins and subsequent alteration of gene expression. Signal transduction relies on signalling cascades to convey information to effectors within the cell, coordinate information from parallel pathways, and amplify signals (Figure 3).

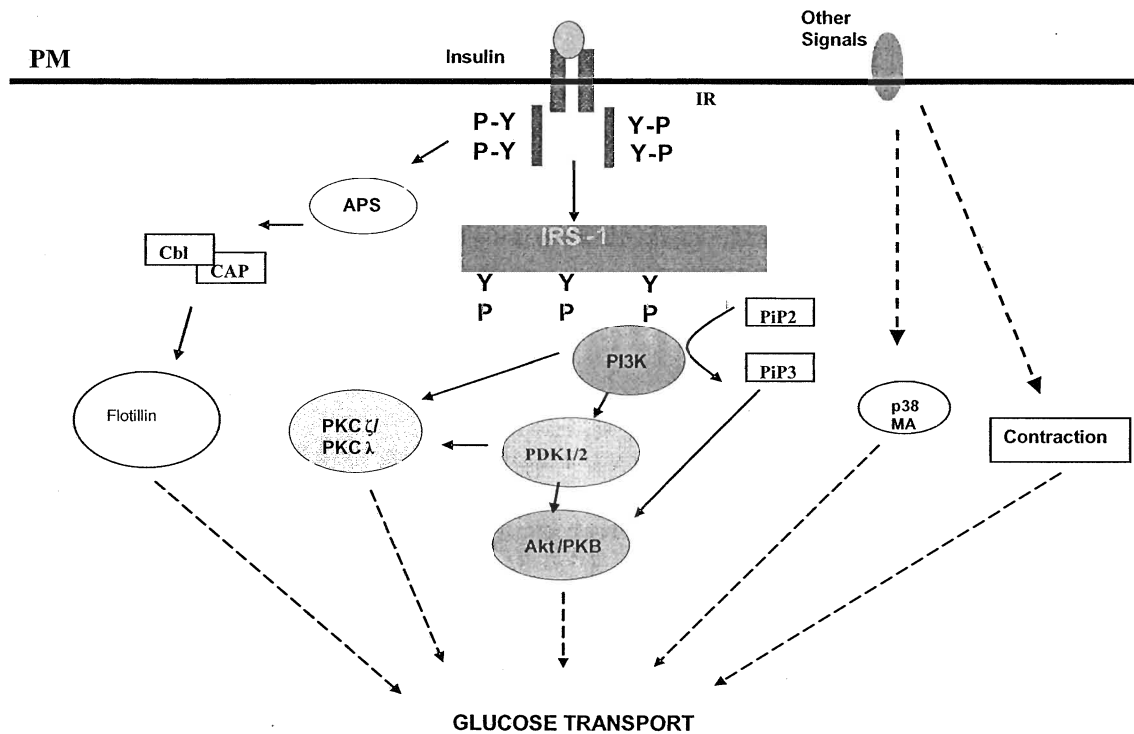


Figure 3: Insulin- stimulated signalling pathways leading to enhanced glucose uptake.

1.2.1 The Insulin Receptor

The insulin receptor (90kDa) is a heterotetrameric glycoprotein consisting of two extracellular alpha subunits containing the insulin binding sites and two beta subunits that span the plasma membrane containing cytoplasmic tyrosine kinase domains (14). The involvement of the receptor as an early event in insulin action was discovered when the occupied insulin receptor demonstrated by specific tyrosine phosphorylation of the beta subunit by insulin in a dose- dependent manner (15).

Insulin binding to the receptor causes a conformational change and leads to the autophosphorylation of the intrinsic tyrosine residues on the beta subunits to initiate the signalling cascade. Tyrosine autophosphorylation of the receptor is one of the earliest cellular responses to insulin stimulation. This causes an increase in tyrosine kinase activity leading to activation of insulin receptor substrate- 1 (IRS-1). However, insulin binding to its receptor is not essential for insulin receptor signalling since anti- insulin receptor antibodies may activate glucose transport (16). It is an active receptor tyrosine kinase that is essential for subsequent insulin signalling as evidenced by mutated human insulin receptors at the ATP- binding site of the tyrosine kinase domain that failed to mediate the post- receptor effects of insulin in both Chinese hamster ovary (CHO) (17) cells and NIH 3T3 fibroblasts (18).

1.2.2 Insulin Receptor Substrates

The insulin receptor substrates are cytoplasmic proteins that contain 22 potential tyrosine phosphorylation sites that serve as recognition sites for cellular substrates containing SH2 domains (19;20). Insulin receptor substrates 1 (180kDa) and 2 (185kDa) (IRS 1- 4), are phosphorylated on their tyrosine residues by receptor tyrosine kinase,

which creates new binding sites on the substrate (19). IRS-1 is the major substrate of the insulin receptor kinase (19). The amino acids surrounding the phosphotyrosine define the specificity of the binding site. IRS-1, in particular, contains multiple tyrosine phosphorylation motifs that serve as docking sites for proteins containing a Src homology 2 (SH2) domain.

IRS-1 and IRS-2 are best characterized for their role in insulin action and glucose transport as mediators between insulin receptors and the downstream effector molecules (21;22). The primary involvement of IRS-1 is in somatic cell growth and insulin action in muscle and adipose tissue. When IRS-1 is phosphorylated, it binds with proteins like the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), to mediate insulin action (23;24). Additionally, phosphorylation at Ser1101 of IRS-1 by PKC θ , results in an inhibition of the insulin signalling pathway and suggests a potential mechanism for insulin resistance (25). IRS-2 functions as a scaffolding protein to coordinate branches of insulin signalling cascades and is important in beta cell survival and growth, insulin action in the liver, brain growth, reproduction, and food intake. Therefore, IRS-2 knockout mice fail to maintain sufficient compensatory insulin secretion and develop diabetes (26). Glucose transport activity in IRS-1 knockout mice is still increased by insulin but with reduced maximum stimulation (27;28). It is suggested that IRS-2 compensates for IRS-1 by binding to α -p85 but it is possible that other substrates are upregulated as well.

Two other alternative insulin receptor substrates are IRS-3, which is expressed in adipose tissue, and IRS-4, which is expressed in neuroendocrine tissue. Both IRS-3 and IRS-4 are not essential in insulin signalling (29). Studies involving IRS-3 and IRS-4

knockout mice showed that in IRS-3 deficient mice plasma glucose and insulin levels were similar to the normal mice and did not display defects in growth (29) while the IRS-4 deficient mice had only minor problems with glucose homeostasis, growth, and reproduction (30).

1.2.3 Cbl

In addition to IRS-1 and IRS-2, the cytoplasmic adaptor protein Cbl (120 kDa) is activated through the insulin receptor (31). The structure of Cbl contains an amino-terminal phosphotyrosine binding domain (TKB) and a C3HC4 RING finger motif. The TKB domain recognizes both phosphorylated tyrosines on activated receptor tyrosine kinases and non-receptor tyrosine kinases. The RING finger recruits ubiquitin-conjugating enzymes.

Activation of Cbl begins with the association of the insulin receptor with the APS adaptor protein that contains both a pleckstrin homology (PH) and SH2 domain. This association is followed by subsequent phosphorylation of the associated protein substrate (APS) by the insulin receptor at tyrosine residue 618. Activated APS recruits Cbl to the insulin receptor for tyrosine phosphorylation, which is wortmannin-independent (32). Then Cbl-associated protein (CAP) binds to Cbl through the SH3 domain (33), allowing the complex to migrate to caveolae through interaction with the hydrophobic protein flotillin, which is also known as the lipid raft (32). At this point, it is able to bind to the SH2 domain of the adaptor protein CrKL (39kDa) leading to the activation of the small GTP-binding protein TC10 (32;34) to increase glucose transport through GLUT4 translocation and possible regulation of the actin cytoskeleton. Cbl activation is required but not sufficient for insulin-stimulated glucose transport since expression of mutant

CAP (CAP Δ SH3) that lacks the SH3 domain, reduced the stimulation of glucose transport (50%) by insulin without affecting PI3K- dependent signalling events in 3T3-L1 adipocytes (32;35).

1.3 THE PI3K/AKT/PKB PATHWAY

1.3.1 PI3K

Phosphoinositide 3 kinase (PI3K) is a lipid kinase that is a downstream effector protein in the insulin-signalling cascade (23). It is a heterodimer composed of a 110kDa catalytic subunit and an 85kDa regulatory subunit. Class 1_A and Class 1_B PI3K are activated by tyrosine kinase and G- protein coupled receptors respectively. Class 1_A PI3Ks form heterodimeric enzymes that consist of a catalytic and regulatory subunit. The catalytic subunit can be further divided into two subclasses, I_A and I_B. Subclass I_A contains 110 kDa catalytic subunits of p110 α and p110 β , whereas subclass I_B contains p110 γ . Each catalytic subunit contains an amino- terminal Ras- binding domain and a carboxyl- terminal phosphatidylinositol kinase domain.

Class 1_A enzymes possess an aminoterminal regulatory subunit- binding domain and may bind to five types of regulatory subunits; 85 α , p55 α , p50 α , p85 β , and p55 γ . Class 1_A PI3K enzymes contain SH2 domains in their amino- and carboxyl- terminals and bind PYXXM of phosphotyrosine motifs that exist in receptor tyrosine kinases themselves and their substrates, such as IRS- 1 (36). In contrast, the p110 γ and subclass of I_B isoforms of PI3K bind only one regulatory subunit, p101, which binds to a trimeric GTP- binding protein (G- protein), the target of G- protein coupled receptors (GPCRs).

The docking of PI3K near the plasma membrane through p85 recognition of the receptor phosphorylated YXXM activates the PI3K pathway. PI3K activation catalyzes

the phosphorylation of the inositol phospholipid phosphatidylinositol-4,5-bisphosphate (PI(4, 5)P₂) to generate phosphatidylinositol-3,4,5-triphosphate (PI(3, 4, 5)P₃), which attracts various kinases and regulators to the plasma membrane and eventually leads to cell growth and survival.

Involvement of PI3K in insulin- stimulated glucose uptake was initially indicated when activation was blocked by wortmannin to inhibit glucose uptake and transport in skeletal muscle (37). Wortmannin is a fungal metabolite that binds to the p110 catalytic subunit of type 1 PI3K to exert inhibition (38;39). Similar results were also obtained with LY294002 (40), a quercetin derivative that acts as a competitive inhibitor of the ATP binding site of PI3K (41). In addition, the mutant $\Delta p85$, which lacks a binding site for the p110 catalytic subunit, greatly inhibited insulin- stimulated glucose uptake in CHO cells (42), suggesting that PI3K is necessary for glucose transport. Therefore, PI3K activation is required for insulin- stimulated glucose transport but its' sufficiency is uncertain. Activation of PI3K by other growth factors such as platelet- derived growth factor (PDGF) are unable to stimulate glucose transport or GLUT4 translocation (43). However, active- PI3K overexpression increases both GLUT4 translocation and glucose uptake to 50- 100% of full insulin stimulation, yet insulin is able to further increase glucose transport (44;45). These studies suggest that an alternative insulin signalling pathway exists that is PI3K- independent.

1.3.2 Phosphoinositides

The inositol phospholipid, PIP₂, is phosphorylated on its 3- OH group by PI3K to generate the lipid products and second messengers phosphatidyl- inositol-3- phosphate (PI₃P), PI(3, 4)P₂, and PIP₃. PI₃P is localized on endosomes (46) and does not change in

response to cellular stimulation. However, recent studies in L6 cells and 3T3- L1 adipocytes with exogenous PI₃P suggest that PI₃P is produced in response to insulin-stimulated TC10 activation (a small GTP- binding protein) to promote plasma membrane translocation of GLUT4 (47). PI(3, 4)P₂ has been shown to be generated through integrin- dependent signalling in human platelets (48).

PIP₃ attracts signalling molecules, such as 3'- phosphoinositide- dependent kinase-1 (PDK1) (14), PDK2, Akt/PKB (49), mTOR (50), and atypical protein kinase Cs (PKCs) (51), to the plasma membrane by interacting with pleckstrin homology (PH) domains. PIP₃ is required for maximum PDK1 activity (52). PIP₃ does not directly alter Akt/PKB but recruits it to the plasma membrane (53) and alters its conformation to allow subsequent phosphorylation by PDK1.

PIP₂ and PIP₃ are the main substrates of phosphatase and tensin homologue deleted on chromosome ten (PTEN), which is a negative regulator of the PI3K/Akt pathway (54). PTEN removes a phosphate group from the 3- OH position of PIP₃ (55). Alternatively, SH2-containing inositol phosphatase 1 (SHIP-1) catalyzes the dephosphorylation of PIP₃ at the 5- OH position to regenerate PIP₂ in order to potentially promote Akt/PKB activation (56).

1.3.3 PDK1/PDK2

PDK1 is a 63-kDa serine/threonine kinase that contains a carboxyl terminal PH domain and was originally identified by two different groups (57;58). PDK1 localizes to the plasma membrane in response to PIP₃ in order to activate Akt/PKB. Colocalization, which is mediated by the phosphoinositides acting as scaffolding molecules (59), of PDK1 and Akt/PKB at the plasma membrane results in the phosphorylation of Akt/PKB

at the threonine residue by PDK1 (57) and Ser473 phosphorylation (60). It appears that PDK1 interacts with the Ser473- phosphorylated hydrophobic motif (HM) of Akt/PKB in order to bring PDK1 closer to the Thr308 residue to catalyze its phosphorylation (61).

The function of PDK1 was confirmed in a study by Williams et al. (2000)(62), where PDK1 was genetically disrupted and resulted in Akt/PKB not responding to mitogens because Thr308 was not phosphorylated. PDK1 is also substrate specific as evidenced by deletion of PDK1 in mouse embryonic cells, which demonstrated full inhibition of Akt/PKB (62).

1.3.4 Akt/PKB

Akt/PKB (56 kDa) is a serine/ threonine kinase and shares sequence homology with protein kinase A (PKA) and protein kinase C (PKC). Experiments with wortmannin and LY294002, both PI3K inhibitors, and by overexpression of a deletion mutant of the regulatory subunit of PI3K, demonstrate that Akt/PKB is a downstream target of PI3K (49;53). Identification of the Akt/PKB signalling pathway began with the isolation of the *Akt1* and *Akt2* genes, which are human homologues of the viral oncogene *v- akt* that causes a form of leukemia in mice (63). Subsequent studies revealed that *v- akt* and its mammalian homologues encoded a protein kinase that was similar to the already discovered PKC and PKA, which is why it was originally termed Related to A and C-Protein Kinase (RAC- PK) and then changed to PKB (64). The Akt/PKB kinase expresses three different isoforms, which include PKB α /Akt1 and PKB β /Akt2 that are broadly expressed, and PKB γ /Akt3 that has a more restricted tissue distribution (65;66). Akt3 is expressed mainly in the brain and testes while Akt2 is predominantly expressed in insulin target tissues like fat cells, the liver, and skeletal muscle.

There are three distinct functional regions on Akt/PKB; the N- terminal pleckstrin homology (PH) domain, the kinase domain, and the C- terminal hydrophobic motif (HM) (Figure 4). The PH domain mediates the binding of Akt/PKB to the 3- phosphoinositides, PI (3,4,5)P₃ and PI (3,4)P₂ that are generated by PI3K, and binds to both molecules with equal affinity (67). The PH domain is common to many signalling proteins and acts as the plasma membrane recruitment mechanism for Akt/PKB, which is the hallmark of Akt/PKB activation. The kinase domain is the second region of the PKB molecule and is located in the central catalytic domain, which is similar to that of PKA (68).

The third area is the C- terminal hydrophobic motif (HM) that contains the second regulatory phosphorylation site, Ser473 in PKB α /Akt1 for example. Specifically, the HM provides a docking site for the upstream activating kinase 3- phosphoinositide- dependent kinase- 1 (PDK1) (69). When this docking interaction is interrupted, phosphorylation of the activating loop (T- loop) is attenuated. The main phosphorylation site of Akt/PKB is within the activation T- loop at the Threonine 308 residue site (for PKB α). Phosphorylation of this residue causes a charge- induced change in conformation that allows substrate binding and increases the catalytic rate required for enzymatic activity (68).

The HM domain is also an allosteric regulator of catalytic activity (70). The catalytic core located in the kinase domain, is stabilized when the HM associates with hydrophobic and phosphate- binding pockets created by a cleft formed at the junction of the α B- helix, α C- helix, and the β 5- sheet in the N- lobe. This pocket is also referred to as the PRK2- interacting fragment (PIF)- pocket. Stabilization of the N- lobe increases the phosphotransfer rate by ten- fold.

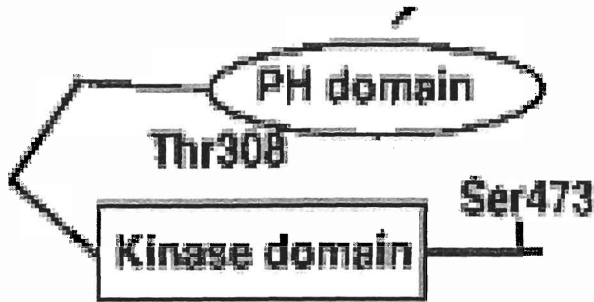


Figure 4: Structure of the Akt/PKB protein.

In order for full activation of Akt/PKB to occur, the Ser473 residue must be phosphorylated at the plasma membrane to increase the HM affinity for the PIF- pocket, unfortunately identification of this mechanism is still controversial (71). One theory suggests that PDK1 converts to a Ser473 kinase after Thr308 phosphorylation because it interacts with Akt/PKB and PRK-2, a protein fragment. Since Ser473 phosphorylation is dependent on PI3K and Thr308 activity, it was thought that PDK1 was the Ser473 kinase (72). However, ES PDK1 knockout mouse cells disproved this theory when Ser473 phosphorylation was similar to the wildtype and Thr308 phosphorylation was completely abolished. On the other hand, PDK1 overexpression in transfected cells increased Akt/PKB phosphorylation on Ser473, which indicated that PDK1 might have an indirect contribution to Ser473 phosphorylation (62).

Toker and Newton (2000) (73) argue that Ser473 is modified through autophosphorylation of Akt/PKB. Another possibility is that Ser473 may be phosphorylated by serine kinases like integrin- linked kinase- 1 (ILK- 1) (74). ILK- 1 is activated by insulin in a PI3K- dependent manner, which is in accordance with Akt/PKB

signalling. ILK- 1 could alternatively act as a facilitator instead of phosphorylating Akt/PKB directly since ILK- 1 kinase dead mutants were able to induce Ser473 phosphorylation (75). Persad et al. (2001) (76) also demonstrated that an ILK- inhibitor blocked Ser473 phosphorylation of Akt/PKB, suggesting that ILK- 1 could be an important upstream kinase for the regulation of Akt/PKB. It appears that PDK1 phosphorylates Thr308 and an unidentified PDK2 phosphorylates Ser473 on Akt/PKB. Recently, Hill et al. (2002) (77) isolated a protein kinase that specifically phosphorylated Ser473 but identification requires further investigation.

PI3K is also required for phosphorylation of Ser473 but the mechanism is distinct from Akt/PKB membrane localization. The role of PI3K has several possibilities such as PI3K- derived lipids activating a distinct Ser473 kinase, inhibiting a phosphatase, or even regulating the binding of a Ser473 regulatory protein (78). Scheid et al. (2002b) (79) also found that PI3K is important in Ser473 phosphorylation. In this study, mast cells derived from SHIP- knockout mice showed that Ser473 activity was sensitive to PI3K inhibition even though PIP₃ levels were high, Thr308 phosphorylation was normal, and Akt/PKB membrane localization was strong. PIP₂ may also have a role in promoting Ser473 phosphorylation because cells lacking SHIP pool PIP₃, which decreases PI3K activity and abolishes PIP₂. The addition of PIP₂ restored Ser473 phosphorylation and subsequent Akt/PKB activation (80).

Akt/PKB may also be phosphorylated on Ser124 and Thr450 residues but these sites do not regulate Akt/PKB and their phosphorylation is unchanged after stimulation (71). When PKB γ /Akt3 is spliced to PKB γ 1, which lacks the Ser472 phosphorylation site and only requires Threonine 305 phosphorylation for activation, Thr 305 phosphorylation

is decreased after pervanadate stimulation and insulin-induced PKB γ 1 translocation is reduced, suggesting that the second phosphorylation site limits its maximum potential for membrane translocation and catalytic activation (81). In summary, in the resting non-stimulated cell, the majority of Akt/PKB is located in the cytoplasm and when activated, it is translocated to the plasma membrane and then followed by translocation to the cytosol and the nucleus to elicit a response (61).

Phosphorylated Akt/PKB has many functions, which is partly attributed to the variation and specificity of its substrates. Mouse gene knockouts for each isoform of Akt/PKB provide significant insights into their specific functions. Akt/PKB is a regulator of several cellular processes, including apoptosis, proliferation, differentiation, and metabolism. For example, downstream of Akt/PKB, glycogen synthase kinase-3 (GSK-3), the pro-apoptotic protein Bad, caspase-9, Forkhead transcription factor (82), p70 ribosomal S6 kinase, I κ B kinase- α (IKK α) (83), and endothelial nitric oxide synthase (eNOS) (84) are activated.

Glycogen synthase kinase-3 (GSK-3) inactivates glycogen synthase in order to build up cellular glycogen stores in response to insulin stimulation and was the first substrate identified for Akt/PKB (49). The PI3K/PDK/Akt pathway enhances cyclin D1 protein stability by inactivating GSK-3 β through phosphorylation at Ser21, thereby preventing it from causing ubiquitin-mediated degradation of cyclin D1 (85).

Phosphodiesterase 3B is also phosphorylated by Akt/PKB to its active form and regulates intracellular cAMP and cGMP in response to insulin (86) while 6-phosphofructo-2-kinase is activated by Akt/PKB to promote glycolysis (87). The mammalian target of rapamycin (mTOR) regulates mRNA translation in signalling

pathways and is phosphorylated by Akt/PKB (88). However, p70 S6 kinase activity, a downstream target of mTOR, is not increased (89). Protein tyrosine phosphatase (PTP1B) negatively regulates insulin sensitivity by acting as a phosphatase to dephosphorylate the insulin receptor. Akt/PKB phosphorylates PTP1B to inhibit PTP1B phosphatase activity and enhances insulin signalling (90).

The most well known function of Akt/PKB is its involvement in insulin-stimulated glucose transport where Akt/PKB is translocated to the plasma membrane in response to insulin stimulation (91). For example, overexpression of Akt/PKB α in 3T3-L1 rat adipocytes increases glucose transport by stimulating GLUT4 translocation to the plasma membrane (92). Akt/PKB co-localization with GLUT4-containing vesicles in 3T3-L1 adipocytes is suggested to be involved in GLUT4 translocation (93). Additionally, Carvalho et al. (2000) (94) demonstrated that obese Zucker rats with impaired translocation of GLUT4 had defects in PI3K and Akt/PKB that reduced activation.

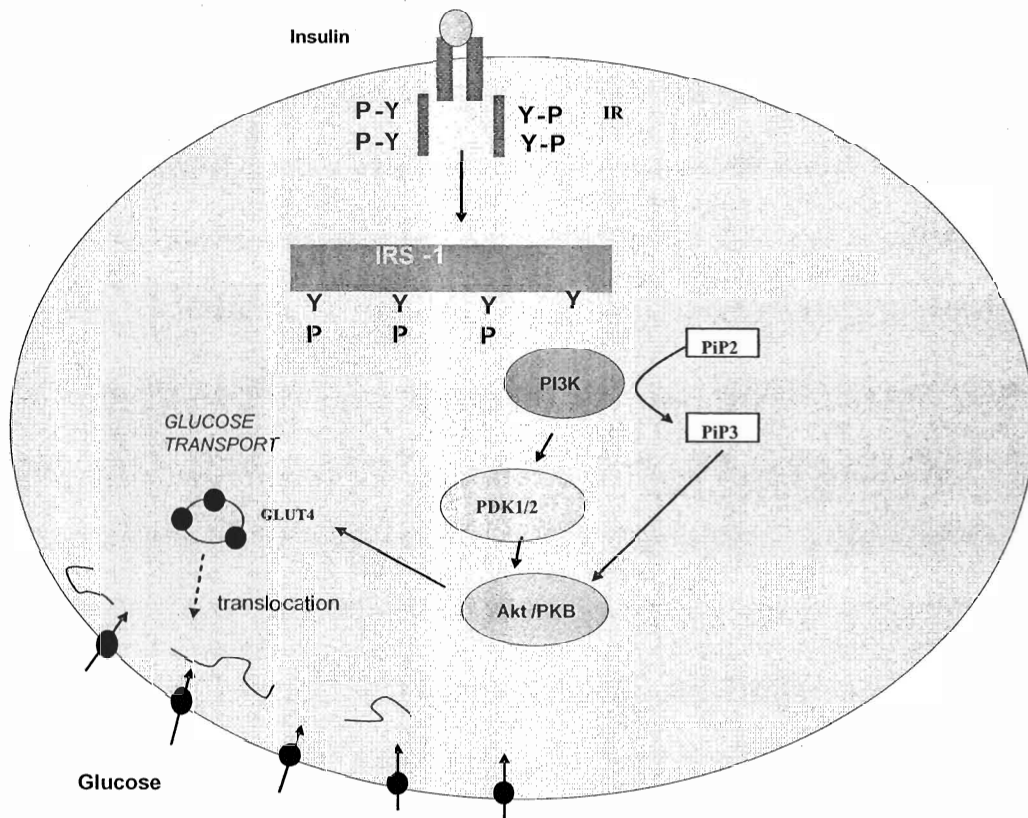


Figure 5: GLUT4 translocation in response to insulin stimulation via PI3K/ Akt.

1.4 THE RAS/MAPK PATHWAY

1.4.1 RAS

Ras (21kDa) is a guanine- nucleotide binding protein that is activated by receptor tyrosine kinases and G- protein coupled receptors. Activation of the insulin receptor results in the interaction of the adaptor protein growth factor receptor- binding protein 2 (Grb2)- Son of sevenless (Sos) complex with tyrosine phosphorylated IRS- 1, through the cytosolic scaffold protein Shc, to link the insulin receptor to the Ras- signalling pathway leading to ERK1/2 activation. The Grb2- Sos complex is formed by Sos, a guanine nucleotide exchange factor, binding to the SH3 domain of Grb2, which enhances GTP binding to Ras to make it active (Figure 6) (95;96).

1.4.2 RAF

The Ras- Raf physical interaction was a significant breakthrough in the ERK1/2 signalling cascade. GTP- Ras, which is the active form, binds to both sites of Raf (65-75kDa), which include the Ras binding domain (RBD) and the cysteine- rich domain (CRD). Activated Ras can bind to any one of the three isoforms of Raf; Raf- 1 (c- Raf), B- Raf, or A- Raf, to induce phosphorylation at multiple sites and then translocates to the plasma membrane where protein kinase activity is increased and the kinase cascade is activated (97). Different Ras isoforms activate Raf to varying degrees but only Raf- 1 and B- Raf phosphorylate MEK1/ 2 (98).

1.4.3 MEK1/2

Subsequently, Raf phosphorylates the serine residues (positions 217 and 221) of the dual specificity protein kinases, MEK1 (45 kDa) and MEK2 (99). This step involves amplification of the signalling cascade because Raf is present at much lower concentrations than MEK1 and MEK2. MEK1/2 activates ERK1/2 MAP kinase by

phosphorylating both threonine and tyrosine residues at sites located within the activation loop of kinase subdomain VIII (100).

1.4.4 ERK1/2 MAPK

Since ERK1 (44 kDa) and ERK2 (42 kDa) have overlapping signal capabilities, they are often listed as ERK1/2 and are the only well established substrates of MEK1/2. Other ERK isoforms have been identified by molecular cloning but are not as well understood and include ERK3 (62 kDa), ERK4, ERK5 (110 kDa), ERK6 (38 kDa), and ERK7 (61 kDa) (101). ERK3 is similar to ERK1/2 but is more highly concentrated in the nucleus (102) as opposed to the cytoplasm, while ERK5 is implicated in cell transformation and cell cycle control (103). In addition, ERK7 is involved in cell proliferation but requires further investigation (104).

ERKs are the most well studied MAPK family members and were the first signalling MAP kinase cascade to be recognized. ERK was originally termed “MAPK” and was first described in 1986 (105). Original identification of ERK was as a protein kinase that phosphorylated microtubule-associated protein 2 (MAP2), a cytoskeletal protein, in response to receptor protein tyrosine kinase activation by insulin (105). Additionally p42/44 MAPKs were found to regulate the protein kinase p90^{rsk} (106). Later studies identified the 44 kDa isoform of MAPK called ERK1 (107).

Mitogenic stimuli, cytokines, neurotrophic and growth factors, neurotransmitters, and hormones such as insulin activate ERK1/2. More recently, oxidative stress that induces apoptosis was found to mediate ERK1/2 phosphorylation (108). Phosphorylation of both tyrosine and threonine residues of ERK1 and ERK2 is catalyzed by MEK1 and MEK2. Dual phosphorylation is required in order to achieve high activity. No

amplification is needed in this step since the concentrations of both ERK1/2 and MEK1/2 are equivalent. The relatively high intracellular concentration of these enzymes is partly responsible for the rapid activation observed.

Downstream ERK1/2 activation leads to nuclear translocation from the cytoplasm and altered gene expression. ERK1/2 phosphorylates numerous enzymes and proteins that are involved in a variety of cellular processes. A specific example of ERK1/2 phosphorylation involves the induction of the *c-fos* gene via Elk-1. This results in the activation of a variety of transcriptional factors like activating protein-1 (AP-1) (109). ERK1/2 also phosphorylates the *c-myc* and *c-jun* transcription factors that control protein expression involved in cell growth (110). Additional substrates of ERK1 and ERK2 include factors involved in translation (PHAS-I), proteins involved in phosphorylation and dephosphorylation reactions (protein phosphatase 2C), and various signalling enzymes (cytoplasmic phospholipase A₂).

Specific ERK pathway inhibitors are commonly used to determine ERK requirements and functions. ERK1/2 plays a critical role in the regulation of cell growth and differentiation. Insulin is a pleiotropic hormone and therefore, in addition to glucose uptake, insulin stimulates mitogenesis to promote cell proliferation and differentiation. It has been previously documented that L6 myoblasts exhibit a proliferative response when stimulated with either insulin or insulin-like growth factor-1 (IGF-1) (111;112) and that the mechanism of insulin-stimulated cell proliferation involves the activation of the Ras-Raf-ERK1/2 pathway, which is similar to many other growth factors (96) (Figure 6). In order to promote DNA synthesis, insulin activates the Grb2/Ras/Raf/ERK1/2 pathway, which requires an intact cytoskeleton but is independent of PKC (113).

ERK1/2 activation also mediates cell cycle arrest (114). Constitutively activated MEK1, which is an upstream kinase of ERK1/2, has been shown to accelerate cell entry into the S phase of the cell cycle. In addition, 4-OH-tamoxifen-stimulated MEK1 down-regulated p27 and up-regulated cyclin D1 protein expression in NIH 3T3 cells, indicating a link between the ERK1/2 signalling pathway and the cell cycle (115). Another possible target of ERK is the pro-apoptotic Bcl-2 family protein Bad (116). Bad influences mitochondrial membrane integrity and the release of cytochrome c from the mitochondria indirectly by associating with Bcl-2 and Bcl-x_L and inhibiting their anti-apoptotic function.

ERK1/2 activation inhibits apoptosis induced by various stimuli including hypoxia (117), growth factor withdrawal (118), hydrogen peroxide (119), and chemotherapeutic agents and mediates non-apoptotic cell death as well (120). Xia et al. (1995) (121) observed that growth factor withdrawal-induced apoptosis inhibited ERK in rat PC12 cells. In Raf-1 knockout mice, B-Raf was discovered to compensate for Raf-1 concerning MEK1/2 activation however these cells were more susceptible to apoptotic stimuli despite the presence of A- and B-Raf (122).

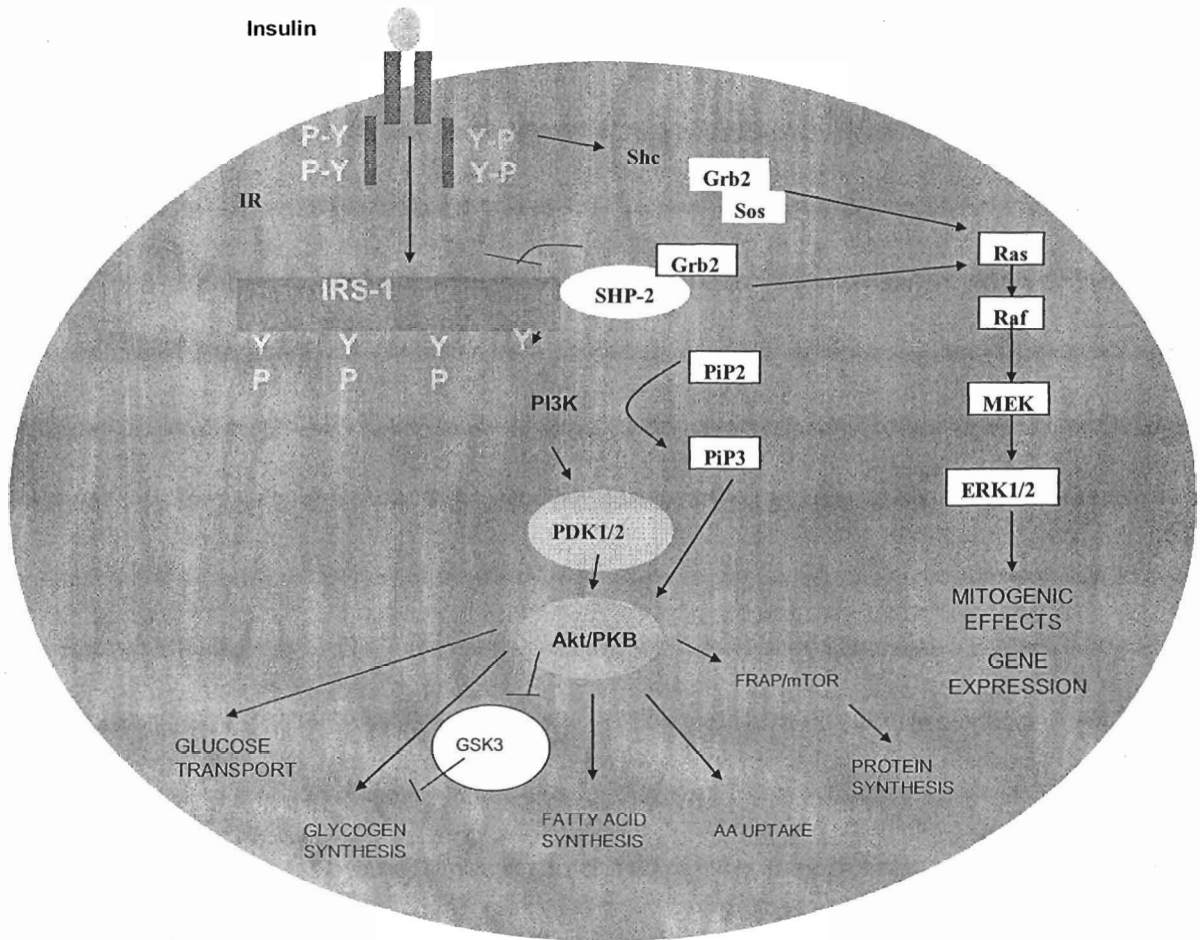


Figure 6: Insulin signalling- the Ras/Raf/MAPK pathway.

1.4.5 p38 MAPK

Another major subgroup of the MAPK family is p38 MAPK, which was initially discovered by several independent labs (123). p38 MAPK or p38 α MAPK (38 kDa), which is the mammalian homologue of the yeast HOG kinase, was first identified in budding yeast as a kinase activated by hyperosmolarity. Han et al., 1994 (124) determined p38 MAPK to be a MAPK family member through molecular cloning.

Homologues of p38 α MAPK, including p38 β MAPK (39 kDa) (125), p38 γ MAPK (43 kDa) (ERK6, SAPK3) (126), and p38 δ MAPK (40 kDa) (SAPK4) (127), were later cloned in mammals. p38 α MAPK and p38 β MAPK genes are ubiquitously expressed (127) while p38 γ MAPK expression is induced during muscle differentiation in skeletal muscle (126). In addition, p38 δ MAPK expression is developmentally regulated in the lung, kidney, testis, pancreas, and small intestine tissues (128). The most widely studied isoform is p38 α MAPK and still very little information exists on the activation of the other homologues.

p38 MAPK is activated by growth factors (129), muscle contraction (130) and numerous other environmental stresses like oxidative stress and pro-inflammatory cytokines such as interleukin- 1 (IL- 1), tumour necrosis factor- alpha (TNF α), TNF β , and CD40 ligand (131;132). Initiation of the p38 MAPK cascade begins with G- proteins like Rac1 and Cdc42 that phosphorylate and activate various MAPKKKs (133;134). p38 MAPK has divergent signalling pathways, which has been demonstrated through knockout mice of certain activators. MKK3 (135) and mitogen activated protein kinase- activated protein kinase- 2 (MAPKAPK2 or M2) (136) knockout mice were both viable but p38 α MAPK disruption proved to be lethal, indicating that the p38 MAPK cascade is

not a classical step- wise chain reaction. The upstream signalling pathway of p38 MAPK is very diverse, which partially explains why activation is stimulated by many stimuli like Ask- 1 (137), Msk- 1, Mlk- 3 (138), and Tak- 1 (139) for example. Overexpression of any of these enzymes may activate the p38 MAPK pathway.

The next step in this signalling cascade is phosphorylation of the group of MAPKKs by the MAPKKKs previously listed. The MAPKK family includes kinases like MKK3 (140), MKK4 (141), MKK6 (142), and MKK7. Stimulation of these MAPKKs activates p38 MAPK through phosphorylation at both the threonine (Thr 180) and tyrosine (Tyr 182) residues. These dual kinases have exhibited selective activation of the different isoforms of p38 MAPK. For example, MKK6 activates all four isoforms, MKK3 activates p38 α MAPK, p38 γ MAPK, and p38 δ MAPK (125), MKK4 activates p38 α MAPK and p38 δ MAPK (143), and MKK7 activates only p38 δ MAPK (144). Both MKK3 and MKK6 are regulated by dual serine and threonine phosphorylation.

Phosphorylated p38 MAPK may activate either transcription factor or non-transcription factor targets. Examples of non- transcription factor targets include MAPKAPKs -2, -3, -5, and MAP kinase interaction protein kinase (MNK1). MAPKAP kinase- 3 was identified as a substrate of p38 α MAPK in vitro and was then demonstrated in vivo when SB203580, a p38 α MAPK and p38 β MAPK inhibitor (145) blocked its activation. Another non- transcription factor target of p38 MAPK is MNK1, which is able to bind to both p38 MAPK and ERK1, was suggested to have a potential link with translational initiation (146). More recently, PRAK (p38 MAPK regulated/ activated kinase) was identified as a stress activated protein stimulated by p38 α MAPK and p38 β MAPK (147). MSK1/2 (mitogen- and stress- activated kinase), which activates

another transcription factor called CREB, is also activated by p38 α MAPK (148) and protects cells from growth factor deprivation- induced apoptosis.

p38 MAPK mediates the phosphorylation of numerous transcription factors and regulates downstream gene transcription (149). Phosphorylation of ATF- 2 and Sap- 1a by p38 α MAPK and p38 β MAPK stimulate growth arrest while the transcription factor 153 (GADD153) promotes DNA damage (150). p38 MAPK is primarily involved in the mediation of apoptosis and inflammation but recent evidence has shown biological importance in regulating other cellular processes such as cell proliferation, differentiation, inflammation, and developmental processes like actin cytoskeleton organization (151). Pro- apoptotic activity of p38 MAPK was shown by induction with NGF withdrawal and Fas ligation (152). However, overexpression of MKK6b induced caspase activation and cell death downstream of p38 MAPK. It appears that p38 MAPK functions both upstream and downstream of caspases in the apoptotic response.

Insulin also activates p38 MAPK in L6 myotubes to increase glucose transport through GLUT4 activation (10). Discrepancies have been observed between the magnitude of GLUT4 translocation and glucose transport, suggesting that in addition to GLUT4 translocation, there is also an increase in the intrinsic activity of GLUT4. Sweeney et al. (1999) (153) demonstrated that insulin- stimulated glucose uptake was reduced (60%) in both muscle and adipose tissue with the addition of p38 MAPK family inhibitor, SB203580. However, insulin- stimulated GLUT4myc translocation was unaffected. SB203580 is one of the most widely used inhibitors of p38 α MAPK and p38 β MAPK (154) and exerts its inhibition by acting as a competitive inhibitor of the ATP binding site of p38 MAPK. In addition, pre- incubation of L6 myotubes with the

azaazulene p38 MAPK inhibitors, A291077 and A304000, produced similar results and strengthened the concept that p38 MAPK inhibition reduces insulin-stimulated glucose transport (155). Mutant p38 MAPK (p38 MAPKAGF), where the regulatory serine and threonine residues were replaced with alanine and phenylalanine, in 3T3-L1 adipocytes also reduced insulin-stimulated glucose transport by 40% with no effect on GLUT4 translocation (155).

Further study revealed that insulin rapidly phosphorylates p38 MAPK to maximum (2.3-fold) within 5-10 minutes and then declines after 15 minutes (156) and that both MKK3 and MKK6 are also activated by insulin upstream of p38 MAPK (157). Similar results were also obtained in mature skeletal muscle (quadriceps and soleus) (130). It was suggested that p38 MAPK could activate GLUT4 intrinsic activity and may be involved in the activation of newly recruited transporters or be required to complete the fusion event. Since GLUT4 translocation precedes maximal glucose uptake, it was thought that GLUT4 activation occurs at the plasma membrane and that p38 MAPK migrates to the plasma membrane in response to insulin stimulation. Higher levels of immunodetectable p38 MAPK were detected at the plasma membrane after insulin stimulation in L6 myotubes (Sweeney et al. unpublished data reviewed in (158)), supporting the idea that p38 MAPK migrates to the plasma membrane after insulin stimulation.

Whether p38 MAPK directly interacts with GLUT4 still needs to be established but it is possible that the mechanism of GLUT4 activity regulation will involve phosphorylation of GLUT4 by p38 MAPK or association/dissociation of a ligand by p38 MAPK. On the other hand, several studies have demonstrated increases in glucose uptake

due to insulin, osmotic shock, or hyperosmolarity, which are unaffected by p38 MAPK inhibitor treatment (159;160). Fujishiro et al. (2001) (161) found that p38 MAPK activation was not necessary for glucose uptake induced by insulin and that it regulated glucose transporter expression. Therefore, the effect of p38 MAPK α and p38 MAPK β on stimulated glucose transport is still unresolved.

As in many other signalling cascades, it seems that the involvement of p38 MAPK in apoptosis is cell type and stimulus dependent. p38 MAPK regulation of apoptosis induction remains a controversial issue because both anti- apoptotic (162) and pro- apoptotic (163) results have been established.

Another essential role of p38 MAPK activation involves the inflammatory response. p38 MAPK is involved in the production of inflammatory cytokines such as IL-1 β , TNF- α , and IL-6 (164;165) and in the induction of enzymes like cyclooxygenase- 2 (COX-2) that control connective tissue remodelling. More specifically, p38 MAPK is suggested to be involved in the translation of both TNF α and IL- 1 β , since p38 MAPK inhibitors block protein synthesis but do not change the corresponding mRNA levels. Consequently, p38 MAPK demonstrated control of pro- inflammatory mRNA translation (166). p38 MAPK inhibitors at protein and mRNA levels, blocked expression of COX- 2, an additional pro- inflammatory mediator, suggesting that p38 MAPK functioned through more than translational regulation (165).

p38 MAPK is strongly activated by pro- inflammatory stimuli (123). p38 MAPK inhibitors also destabilized COX- 2 mRNA in IL- 1 stimulated HeLa cells (167) and in lipopolysaccharide (LPS)- stimulated primary human monocytes (168) where p38 MAPK inhibitors similarly blocked TNF α . As part of the inflammatory response, platelet

aggregation is also mediated by p38 MAPK (169). In addition, intracellular enzyme expression of iNOS that regulates oxidation and adherent protein induction of VCAM-1 is affected. Since initial studies of p38 MAPK focused on the involvement of p38 MAPK in inflammatory responses, it was also found to be involved in the proliferation and differentiation of cells in the immune system (170).

The involvement of p38 MAPK in cell cycle control was established in mammalian cells treated with SB203580. Activated p38 MAPK α was seen in mammalian cultured cells arrested in the M phase by spindle disruption with nocodazole (171). In the somatic cell cycle, p38 MAPK α activation was involved in the spindle assembly checkpoint. G1 arrest of NIH3T3 cells caused by microinjection of cdc42 was p38 MAPK α - dependent (172). As a result, it is possible that positive and inhibitory effects are exerted by p38 MAPK at different stages of the cell cycle. p38 MAPK is involved in the regulation of cell differentiation (173). Morooka et al. (1998) (174) and Engelman et al. (1999) (175) demonstrated that the differentiation of 3T3-L1 cells into adipocytes and PC12 cells into neurons, required p38 MAPK α and/ or p38 MAPK β .

1.4.6 JNK MAPK

The c- Jun N- terminal protein kinase (JNK) (54kDa) is a serine/ threonine protein kinase from the family of MAPKs. Kyriakis and Avruch first identified this protein kinase in 1990 (176), as a protein kinase activated in the liver of rodents exposed to the protein synthesis inhibitor cycloheximide through intraperitoneal injection. Cycloheximide activated JNK or stress activated protein kinase (SAPK), which it was originally termed, and demonstrated activity with microtubule- associated protein- 2. Subsequent studies lead to the isolation and molecular cloning of JNK and demonstration

that it belonged to the MAPK family where SAPK and JNK were identical enzymes (177). JNK has three encoding genes, *JNK1* (178), *JNK2* (179), and *JNK3* (180). *JNK1* and *JNK2* are ubiquitously expressed and *JNK3* is mainly located in the tissue of the brain and testes. Currently 10 different JNK isoforms in the 46 to 55 kDa range have been identified in the human brain through molecular cloning. The distinguishing factor of each of the isoforms is their varying substrate affinities (180).

There are 11 protein kinase subdomains of JNK (181) that bind adenosine triphosphate (ATP) and peptide substrates to maintain the three-dimensional folding structure. The N- terminal lobe binds ATP and aids in orientation while the C- terminal is involved in substrate recognition and anchoring the phosphate of Mg^{2+} ATP (182).

JNK may be activated by cytokines (TNF, IL- 1, other interleukins) and growth factors (insulin) through specific receptors and by environmental stresses such as heat shock (183), ultraviolet radiation (184), ischemia/ reperfusion, hypoxia, glucose deprivation, or hyper- osmolarity (185). The mechanism of activation by cellular stresses is still unknown, however a study by Rosette and Karin (1996) (186) demonstrated that ultraviolet light and osmotic shock stimulation of JNK1/2 activation involved clustering and internalisation of cell surface receptors for epidermal growth factor (EGF), tumour necrosis factor (TNF), and interleukin- 1 (IL- 1), playing an important role in the mediation of signal transduction.

The pathway of JNK activation is extremely complex and still incompletely understood. Initial receptor activation leads to the recruitment of adaptor molecules (Nck, Crk, Shc, or TRAFs) and activation of G- proteins or GTP- binding proteins like Ste20 homologues and low molecular weight GTP- binding proteins like Cdc42 (187), and

members of the Rho and Rac (Rac-1) family. It is still unclear how growth factor receptors activate JNK. One example is through the epidermal growth factor (EGF) receptor in order to activate the Rho family and subsequently the Ras proto- oncogene. Alternatively, PI3K and/ or PKC isoform activation could be involved (188). It is suggested that agonists acting through the TNF α receptor via sphingomyelinase enzymes could generate the lipid second messenger ceramide, which could then activate the JNK pathway by mechanisms through the Rho family of GTPases.

Stimulated MAPKKKKs (PAKs, Gck, and HPK) phosphorylate and activate MAPKKs (MLK1- 3, DLK, Tpl2, ASK- 1, and TAK- 1). Subsequently, the MAPKKs, MKK4 (SEK1) (189) and MKK7 (MAP2K7), which is an isoform of MKK4 (190), are phosphorylated and therefore activated on their tyrosine and threonine residues respectively, as a catalyst for JNK phosphorylation. MKK4 and MKK7 are analogous to MKK1/2 and mutually required for full activation of JNK MAPK through the phosphorylation of both threonine and tyrosine residues. JNK inactivation is controlled by serine and tyrosine phosphatases and dual specificity MAPK phosphatase.

JNK1/2 phosphorylates the transcription factor *c- Jun* at Serine 63 (Ser63) and Serine 73 (Ser73) and subsequently activates the *c- Jun* gene. Phosphorylation of *c- Jun* increases its ability to activate activator protein- 1 (AP- 1) enhancer elements in the promoters of many genes. JNK ultimately activates specific target proteins like members of the AP- 1 transcription factor family and other factors involved in regulating gene expression, cell survival, and proliferation. Alternative target substrates of activated JNKs include transcription factors such as Elk- 1, Sap- 1a, NFAT4, p53, ATF2, *c- Myc*, and non- nuclear targets like Bcl- 2, tau, and neurofilament H. Gene disruption studies in

mice showed JNK to be required for TNF α stimulated c- Jun phosphorylation and AP- 1 transcription factor activity (191). ATF2 and Elk- 1 may also be activated by p38 MAPK and in addition, ERK has been shown to activate Elk- 1. This demonstrates the possibility of cross- talk between the MAPK family pathways since more than one MAPK may activate several transcription factors.

Since there is more than one JNK activator, specificity is required in order to achieve the appropriate cellular response. One theory of specific activation involves scaffolding proteins that assemble functional signalling molecules. Scaffolding proteins lack enzymatic activity and instead act as organizers of specific pathways. An excellent example of this organizing ability involves JNK- interacting proteins (JIPs), specifically JIP- 1 (192), that are transported by the microtubule motor protein kinesin. Signal transduction components are concentrated in a specific area of the cell in order to regulate localized activation of JNK within cells and enhance activity.

Another alternative for signal transduction specificity of JNK is through protein-protein interaction mediation. A docking site on JNK, distant from the active site, is able to bind to the docking motifs, D- domain, and FXFP, located in the interacting proteins or substrates, MAPKKs and MAPK phosphatases (193). Irradiation was found to stimulate JNK activity in cultured human thyroid cells but not in WI- 38 fibroblasts, which indicates that there are distinct activation and regulation mechanisms involved in the JNK cascade (184). The types of cells involved, the context of the stimuli, and the duration of the activation signal are possible factors hypothesized as determinants of the nature of JNK- mediated signals.

A wide range of physiological functions is mediated via signalling through the JNK family, which include apoptosis, cell survival, and growth. More recently JNK has been shown to be involved in obesity- and TNF α - mediated insulin resistance by negative regulation of insulin- stimulated PI3K activity (194). Insulin has been shown to activate JNK in L6 myotubes, in addition to ERK1/2, p38 MAPK, and Akt/PKB. Originally JNK activation was thought to mediate glycogen synthase activation by insulin in skeletal muscle in vivo (195). In a later study, a JNK activity assay showed that RO 31-8220, a PKC inhibitor, also activates JNK but when added to insulin, JNK activity was not increased. However, this combination was additive for glycogen synthase activity, suggesting that JNK does not play a role in insulin- induced activation of glycogen synthase (196).

The involvement of JNK activation in apoptosis was first demonstrated in neuronal cells. Overexpression of the wild- type *c- Jun* induced apoptosis of cultured sympathetic neurons, while in the *c- Jun* mutant that lacked the transactivation domain; apoptosis was inhibited when induced by nerve growth factor (NGF) withdrawal (197). In rat PC- 12 pheochromocytoma cells following NGF withdrawal again, apoptosis was blocked in JNK mutants while MEKK1 overexpression stimulated apoptosis (121). The pro- apoptotic properties of JNK were further supported by mouse embryonic fibroblasts deficient in *jnk1* and *jnk2* when the cells resisted UV radiation induced apoptosis (198). The same results were reported in neuronal and melanoma cells (199). Previously, Xia et al. (1995) (121) and Brenner et al. (1997) (200) had also established the role of JNK in stress- induced programmed cell death.

In contrast, several studies have also demonstrated that JNK is involved in cell survival as opposed to apoptosis. Hilberg et al. (1993) (201) did not observe any defect in neuronal apoptosis in *c-jun*^{-/-} mice. Apoptosis was also shown to take place even in the absence of *c-Jun* (202). Similarly, Sabapathy et al. (1999) (203) established that *jnk-1*^{-/-} and *jnk-2*^{-/-} mice had increased apoptosis in both the hindbrain and forebrain, suggesting that JNK activation is required for survival. The evidence suggests that JNK acts as a modulator of apoptosis instead of as an intrinsic component, since both pro- and anti-apoptotic roles have been demonstrated extensively (204).

JNK control of cell proliferation by regulating gene expression via phosphorylating the transcription factor *c-Jun* was also established as a major role of the JNK signal transduction pathway (178). Recent research also suggested that JNK phosphorylation of the NH₂-terminus of *c-Myc* plays a role in cell proliferation and apoptosis (205). However, the primary consequence of gene disruption in the JNK pathway is apoptosis in a wide variety of cell types (206) where overexpression of ASK-1, MEKK-1, MLK, or TAK-1 induces cell death. Activation of AP-1, which is stimulated by JNK, has also been shown to lead to apoptosis and inflammation (207). A summary of the MAPK pathways is presented in Figure 7.

MAPK Family Signalling

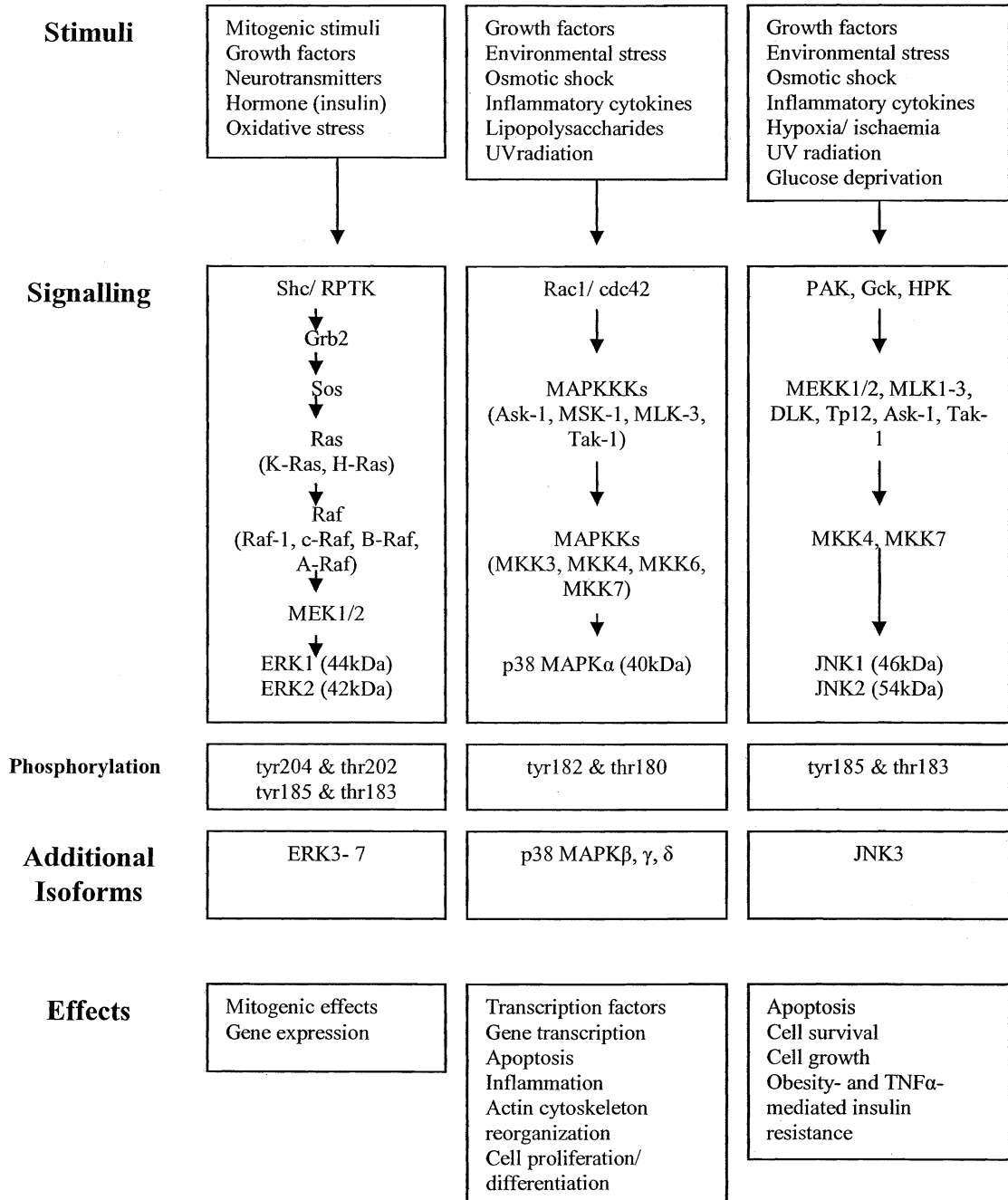


Figure 7: Summary of the MAPK family signalling pathway characteristics.

1.5 CROSSTALK

Crosstalk between signalling pathways has also been demonstrated with the Akt/PKB cascade. The first implication of crosstalk between the PI3K/ Akt/PKB- signalling pathway and ERK1/2 was observed after treatment with PI3K inhibitors, which decreased ERK1/2 activity (208). In addition, heterotrimeric guanosine triphosphate-binding protein- activated PI3K γ stimulates ERK1/2 activation through MEK phosphorylation (209). In the TNF α - signalling pathway, Rac was shown to be downstream of PI3K, leading to nuclear activation of JNK (210). In addition, a dominant negative mutant of the p85 subunit of PI3K reduced MEK1 and ERK1/2 activity with a subsequent decrease in cell proliferation (211). Akt/PKB has also been shown to inhibit apoptosis signal- regulating kinase-1 (ASK1) phosphorylation and its subsequent JNK activity, resulting in a reduction in apoptosis (212). Another implication of crosstalk activity was demonstrated when PI3K activated through the fibronectin receptor increased Raf activity (213).

As previously mentioned, ROS activate both the ERK1/2 and Akt/PKB pathways, which supports current crosstalk theories. It was discovered that the two pathways communicate via a Raf and Akt/PKB interaction, which might be dependent on the muscle cell differentiation stage (214). For example, Akt/PKB activation inhibits ERK1/2 in differentiated myotubes but not in myoblasts (215), which also demonstrates cross-regulation. On the other hand, Ras is phosphorylated by Akt/PKB to inactivate Raf signalling but is not a common occurrence because PI3K inhibition did not inactivate MAPK (216). The PI3K/ Akt/PKB pathway affects Raf through Akt/PKB interaction,

which was demonstrated when Raf- 1 activity was reduced by Akt/PKB- mediated S259 phosphorylation (215). Rommel et al. (1999) (216) found that Akt/PKB decreased Raf- 1 activity, which was specific to differentiation in skeletal muscle culture models. Therefore, it is important to realize that signalling cascades are not linear sequences but exist as components of an interactive branching network where not all components are equivalent in their effects.

1.6 PROTEIN KINASE C

The protein kinase C (PKC) family is a group of phospholipid- dependent serine- threonine protein kinases. The mammalian isoforms of PKC are divided into three groups according to activation, which include the conventional α , $\beta 1$, $\beta 2$, and γ , novel δ , ϵ , θ , and η , and atypical ζ , and ι / λ (217). Conventional PKCs require both calcium (Ca^{2+}) and diacylglycerol (DAG) for activation. However novel PKC isoforms require only DAG and the atypical isoforms are both DAG- and calcium- independent (218). Originally, atypical PKCs were known to be activated by phosphatidylserine or unsaturated fatty acids like arachidonic acid (219). In addition to Akt/PKB, PI3K regulates PKC δ , ϵ , ζ , and p70 S6 kinase, which play an important role in cell signalling leading to proliferation, survival, motility, and secretion in numerous cell types (69;220).

Phorbol- myristate- acetate (PMA) stimulates glucose transport in L6 myotubes independent of insulin, however the role of PKC activation in insulin- stimulated glucose transport remains controversial. In L6 myotubes, insulin has been shown to increase DAG (221) and DAG- sensitive PKCs (221;222). Insulin activates certain isoforms of PKC in a similar manner to Akt/PKB to increase glucose uptake, in particular, PKC ζ (78 kDa) (223;224). The lipid products of PI3K regulate the atypical PKC isoforms, PKC λ

and PKC ζ (225) through PKC activation loop phosphorylation, which modulate GLUT4 translocation. Expression of kinase- inactive forms of the conventional (α and β) and novel (δ and ϵ) isoforms had no effect on GLUT4 translocation (226) and have been shown to negatively regulate components of the insulin- signalling pathway (227). There is evidence to support that PKC- β has an inhibitory effect on GLUT4 translocation when PKC- β null mice displayed lowered blood glucose concentrations as a result of elevated basal and insulin- stimulated cell surface GLUT4 protein (228). Additionally, increased conventional and novel PKC expression and activity are involved in the pathogenesis of insulin resistance and type 2 diabetes (229). This issue is still debated and supports the need for caution when studying PKC where multiple approaches should be used.

More recently, protein kinase D (PKD), which is also known as PKC μ , has been added to the PKC superfamily based on homology within the catalytic domain. However, PKD is distinguished by its transmembrane domain and PH domain and by its unique substrate recognition and Golgi localization. DAG also regulates PKD. New evidence suggests that PKD is responsible for insulin- independent increases in basal glucose uptake in L6 myotubes as demonstrated by siRNAs, dominant- negative PKD isoform expression, and PKD3 overexpression (230).

1.7 THE ACTIN CYTOSKELETON

The actin cytoskeleton also plays an important role in the metabolic effects of insulin (231). Rapid actin reorganization and plasma membrane ruffling induced by insulin, have been observed in both L6 myotubes (232;233) and in mouse 3T3-L1 adipocytes (234). PI3K is involved in the control of membrane ruffling as evidenced by the inhibition of insulin- induced actin reorganization (235) and membrane ruffling (236)

with the PI3K inhibitor wortmannin. Vesicles containing glucose transporters accumulate in these membrane ruffles for pinocytosis. Further investigation revealed that early insulin signalling events, including the activation of PI3K, do not require an intact cytoskeleton (231;232;234).

Downstream however, PI3K- signalling complex association with GLUT4-containing vesicles is actin- dependent. In addition, activation of p38 MAPK and ERK1/2 by insulin does appear to be cytoskeletal- dependent (113). Treatment with cytochalasin D, a compound that disassembles the actin cytoskeleton, demonstrated that translocation of the glucose transporters, GLUT1, GLUT3, and GLUT4, from the intracellular storage compartment to the plasma membrane is mediated by an intact actin cytoskeleton (232). Therefore, it is apparent that the actin cytoskeleton plays an important role in glucose transport for both the regulation of signalling molecules and transporter translocation.

1.8 DIABETES

Diabetes mellitus is a heterogeneous disease with many different forms and a variety of causes. The major types are type 1 diabetes (insulin- dependent diabetes mellitus) and type 2 diabetes (non- insulin- dependent diabetes mellitus). Type 1 diabetes is characterized by the lack of pancreatic insulin production caused by the destruction of pancreatic beta cells. Heredity is a risk factor for development and the majority of cases develop during childhood. It may be virally induced or caused by an autoimmune disorder. Autoimmune insulinitis with pancreatic mononuclear cell infiltration is characteristic at onset and islet cell autoantibodies are produced. Treatment for type 1 diabetes involves exogenous insulin injections in order to regulate and maintain glucose homeostasis.

The characteristics of type 2 diabetes are diminished insulin secretion and reduced insulin sensitivity or insulin resistance (237), which is defined as reduced target cell response to a given insulin concentration compared to normal. Beta cell destruction and dysfunction is responsible for the decrease in insulin secretion. In type 2 diabetes the glucose-consuming tissues, like skeletal muscle, become insensitive to insulin. The failure to increase glucose disposal into peripheral tissues in response to insulin is responsible for chronically elevated levels of glucose in the circulation followed by a compensatory rise in insulin (238). The combination of increased insulin and glucose levels promote exacerbated insulin resistance, contributing to the pathogenesis of the disease (239).

Obesity facilitates the development of this disease since it induces insulin resistance (240). As a result of chronically uncontrolled blood glucose levels, complications develop in approximately 40% of those with diabetes, including kidney failure, blindness, and lower- extremity amputations. Vascular damage also develops over time in response to hyperglycaemia, leading to cardiovascular disease.

Control of type 2 diabetes usually begins with diet and exercise modifications to achieve increased weight loss, reduce fat body mass, and increase insulin sensitivity. Additionally, pharmacological agents such as thiazolidinediones (TZDs) or metformin may be given to improve insulin sensitivity or sulfonylureas (SUs) may be administered to stimulate beta cell insulin secretion. The biguanide metformin is an insulin sensitizer that reverses insulin resistance and hyperglycaemia primarily by inhibiting gluconeogenesis and glycogenolysis in the liver, and by increasing insulin- stimulated glucose uptake in muscle and adipocytes (241). The sulfonylureas glyburide, glipizide,

and glimepiride interact with the sulfonylurea receptors on beta cells (242) to reduce blood glucose levels through stimulation of insulin secretion by the pancreas (243). Thiazolidinediones (TZDs) such as rosiglitazone, may be given to improve insulin sensitivity and also reduce insulin resistance and decrease hepatic glucose production by activating the nuclear receptor peroxisome- proliferators- activated receptor γ (PPAR γ) (reviewed in (244)). In addition, exogenous insulin administration is also used to control plasma glucose levels.

Diabetes mellitus is a disease with high prevalence in society. Type 2 diabetes is estimated to account for 90% to 95% of all cases of diabetes. It is projected that the total number of persons in the world with diagnosed diabetes will reach 300 million by the year 2025. In addition, the World Health Organization (WHO) estimates that between 2.5 and 15% of health budgets are depleted by diabetes related illnesses, depending on local prevalence. Specifically in Canada it is estimated that between five and six billion dollars is spent on diabetes treatment every year, while in the United States the total cost is approximately \$44 billion.

1.9 AMINO ACID TRANSPORT

1.9.1 Importance of Amino Acids

Amino acids circulate in the bloodstream and are absorbed by all tissues to support protein synthesis. Approximately one half of the required amino acids to build protein may be synthesized by the body. After a meal there is a compensatory rise in plasma amino acid levels, however when the amino acid concentration in the plasma decreases below normal physiological levels, amino acids are transported out of the cell to replenish plasma levels while intracellular proteins are degraded.

Skeletal muscle is the main storage tissue for both free amino acids and protein, while the liver regulates blood amino acid levels (35- 65 mg/dl) and is the most active site for amino acid catabolism to produce energy in the form of adenosine triphosphate (ATP). The liver also removes excess amino acids from the blood in order to synthesize proteins or convert them to lipids or glucose for additional storage. Deamination in the liver generates highly toxic ammonia molecules that are converted to the harmless water soluble compound urea. However in the presence of disease ammonia molecules accumulate in the bloodstream resulting in hepatic coma. Additionally, dysregulation of skeletal muscle amino acid balance may contribute to diseased states like diabetes mellitus and cancer (245;246).

Absorption of amino acids requires transport across the cell membrane and may be stimulated by adaptive regulation (247), cell stresses (248), growth factors (249), and several hormones, including androgens/ estrogens, and insulin (250), which is the primary hormone involved in the transport of amino acids into skeletal muscle. These

stimuli induce amino acid transport through an increase in maximum transport capacity (V_{\max}) without affecting transporter affinity (K_m) or the synthesis of new carriers (249).

There are multiple amino acid transport systems in humans; including Systems A, ASC, L, N^m , y^+ , $b^{0,+}$, $B^{0,+}$, y^+L , and X_{AG} , which differ by their substrate specificity, ion dependence, and kinetics. Furthermore, these systems may be categorized by the type of amino acids they transport and whether they are acidic, basic, or neutral (251).

System ASC, which is Na^+ - dependent, transports the small neutral amino acids alanine, cysteine, and serine (252). System N^m mediates glutamine transport, which is a neutral nonessential amino acid and is also Na^+ - dependent (253). System X_{AG} , again Na^+ - dependent, has a widespread tissue distribution but is localized in the brain and epithelial tissues (254). System $B^{0,+}$ is also Na^+ - dependent and transports cationic and neutral amino acids (253). System y^+L is a more recently discovered transporter that binds to both cationic and neutral amino acids, such as leucine and arginine, but with specificity that depends on ionic composition (255).

Unlike the previous transport systems, System L is Na^+ - independent and transports nonpolar, long chain, and aromatic amino acids like isoleucine, leucine, phenylalanine, and tyrosine (256;257). This system is also responsible for mediating the transport of essential neutral amino acids and is ubiquitously expressed. System T is functionally similar to System L but selects for benzenoid substrates and is expressed in human kidney (258). System y^+ transports basic cationic amino acids and arginine, lysine, and ornithine. System y^+ is Na^+ - independent as well and is ubiquitously expressed (259). The other basic amino acid transport system, System $b^{0,+}$, is Na^+ - independent and may be located in the kidney and intestine. It transports dibasic and

neutral amino acids like alanine, arginine, and cysteine (260). The neutral amino acid System A is most commonly studied in skeletal muscle tissue and cell culture and is predominantly regulated by insulin (261). Therefore, it is the focus of study in this thesis regarding questions on amino acid transport.

1.9.2 System A Amino Acid Transport

System A amino acid transport in mammalian cells involves the sodium-coupled neutral amino acid transporters (SNAT1, 2, and 4) of the SLC38 family, which are widely expressed (262). SNAT1 (GlnT), a neuronal Na⁺-dependent glutamine transporter was first cloned and proposed as a member of the System A transporter family but its expression is restricted to the brain and, given that System A activity is ubiquitously expressed, it seemed likely that there must be additional transporters (263;264). The SNAT2 isoform (also known as ATA2) is a membrane bound carrier and transports short polar straight- chain amino acids like alanine, proline, and the non-metabolizable amino acid analogue 2- methyl-aminoisobutyric acid (MeAIB) (263;265). MeAIB is specifically transported by this system, which makes it ideal for transport studies (253). System A transport is dependent on the Na⁺ electrochemical gradient, pH levels, amino acid availability, and exercise (266). For example, SNAT2 utilizes the Na⁺ electrochemical gradient to move amino acids into the cytosol.

Furthermore, insulin stimulates the translocation of SNAT2 from an endosomal compartment to the plasma membrane, which accounts for the increase in System A activity after acute insulin stimulation (267). Insulin stimulation of glucose and amino acid transport share certain characteristics, however there are also significant differences between the two. For example, both insulin- stimulated System A activity and glucose

transport are PI3K and Akt/PKB- dependent, which was demonstrated with pharmacological PI3K inhibition (248;268) and expression of a constitutively active form of Akt/PKB (269) that mimicked insulin action on amino acid transport. On the other hand, cytochalasin D, an actin disassembling drug, inhibits insulin- stimulated glucose uptake but not System A activity. See Table 1 for further details. However, the link between activation of these signalling molecules and System A activity is not yet fully understood.

Table 1: Comparison of glucose and System A transport in L6 cells.

	Glucose Transport	System A Amino Acid Transport
Similarities	a) wortmannin sensitive b) rapamycin insensitive c) PD098059 insensitive	a) wortmannin sensitive b) rapamycin insensitive c) PD098059 insensitive
Differences	a) constitutively active Akt maintains glucose transport in the presence of ceramide (270) b) CD blocks Akt activation and glucose transport (232;271) c) insulin signalling mimicked by phorbol esters	a) constitutively active Akt does not maintain System A in the presence of ceramide b) System A unaffected by CD (267) c) phorbol esters attenuate insulin stimulation in System A (272)

1.10 THE BIOLOGICAL EFFECTS OF RESVERATROL

1.10.1 The Chemical Properties of Resveratrol

Resveratrol (*trans*- 3, 4', 5- trihydroxystilbene) is a polyphenolic compound that has been discovered in 72 different plant species of mulberries, peanuts, and grapes (273). In grapes, resveratrol is concentrated in the skin and seed, where fresh grape skins contain 50- 100 mg of resveratrol per gram of grape skin (274). The dominant source of resveratrol is the root *Polygonum cuspidatum*, which was originally used in traditional Chinese and Japanese medicine as a treatment for heart disease, stroke, suppurative dermatitis, gonorrhea, favus, and athlete's foot (275;276). Resveratrol is known as a phytoalexin because it is produced in response to fungal infections and plays an important role in inhibiting fungal cell growth and proliferation.

Environmental stress is a significant factor contributing to plant resveratrol concentration where ultraviolet radiation and fungal attack are common stressors that stimulate resveratrol production in order to increase resistance to pathogens. Therefore plants located in colder climates that provide optimal conditions for fungal cell growth, often exhibit higher concentrations of resveratrol because of the increased incidence of fungal attack. As a result, the high content of resveratrol in grapes makes wine the leading source of resveratrol in the human diet. The average concentration of resveratrol in red wine ranges from 0.84 to 7.33mg/L (277).

The concentration of resveratrol is considerably higher in red wine than in white wine because of the different fermentation processes. During red wine fermentation, the seed and the skin remain in contact with the must for longer periods of time, which increases the absorption of resveratrol. In contrast, during white wine fermentation, the

must be separated from the skin and the seed much sooner. The significantly higher quantity of resveratrol in red wine has been speculated to be responsible for the positive health benefits associated with red wine consumption.

The structure of resveratrol is shown in Figure 8. The simplicity of the chemical structure allows it to interact with numerous receptors and enzymes so that it can be involved in many different pathways, which will be discussed later. Another advantage of the simple structure is that it is not difficult to synthesize, which makes it more readily available for experimentation (278). Both synthetic and plant-derived resveratrol demonstrate the same biological activity.

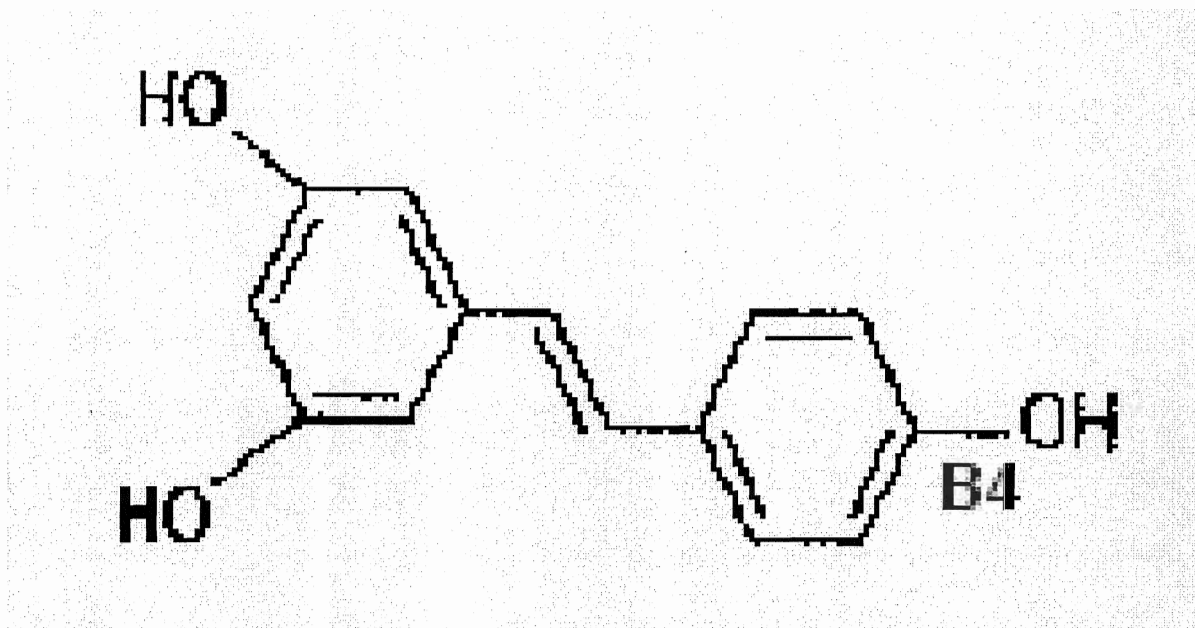


Figure 8: Chemical structure of *trans*-resveratrol (3, 4', 5-trihydroxystilbene).

There are two known isomers of the resveratrol molecule, the trans and cis forms, shown in Figure 9. The trans isoform is the primary isomer found in most resveratrol sources and is the biologically active form. Trans- resveratrol is readily isomerised to the cis state upon light exposure (279). Resveratrol is quite stable but when exposed to heat or extreme pH levels it is reactive. Both the cis and trans isomers are stable between pH 3- 8 but are completely degraded at a pH of ten (279).

Resveratrol contains 1, 2- diphenylethylene in its functional group and is classified as a stilbene (276). Phenylpropanoids are the precursors for stilbenes like resveratrol, flavonoids, and even furanocoumarins. Stilbenes, in particular, are synthesized by coumaryl- CoA and three molecules of malonyl- CoA under the cleavage of four carbon dioxide molecules (280).

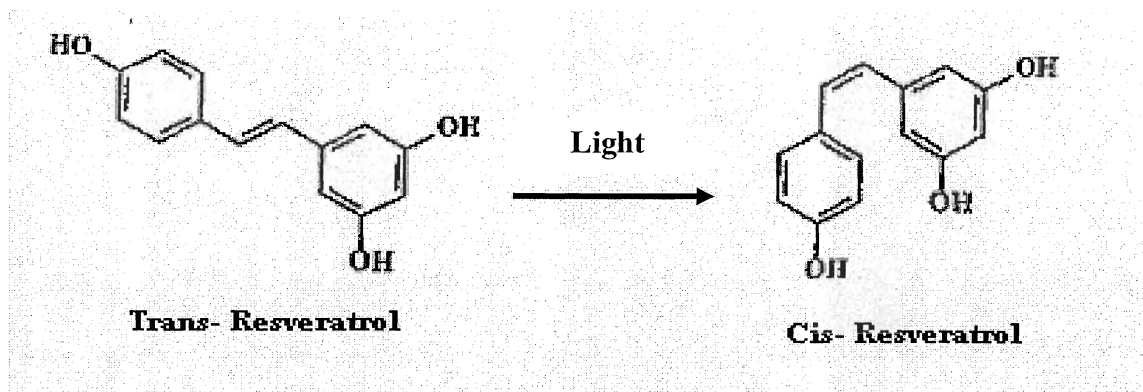


Figure 9: Isomerization of resveratrol that is induced by light exposure.

1.10.2 Bioavailability of Resveratrol

Resveratrol is a lipophilic molecule and can easily cross the blood brain barrier and affect the central nervous system. Studies in hepatocytes, HepG2 (281), and intestinal cells (282) have shown that resveratrol (30- 150 μ M) enters the cell by simple diffusion to significant levels within 5 minutes.

The biological effectiveness of resveratrol is dependent upon its bioavailability after it enters an in vivo system. Studies on the bioavailability of orally ingested resveratrol demonstrate that 50- 75% of the dose is absorbed in rats (283). Vitrac et al. (2003) (284) studied the distribution of radioactive resveratrol in Balb/c mice after oral administration via whole- body autoradiography. [14 C]trans-resveratrol was most concentrated in the digestive system, including the stomach, liver, kidney, intestine, bile, and urine, where the majority of resveratrol remained intact. It is possible that resveratrol will also be deposited in adipose tissue since it is lipid soluble or accumulate in skeletal muscle, both of which have not been examined.

High performance liquid chromatography (HPLC) analysis with diode array detection can be used to determine trans resveratrol levels in plasma. Oral administration of resveratrol (2mg/ kg) to male Sprague- Dawley rats was analyzed at 15, 30, and 45 minutes and after only 15 minutes, trans resveratrol was detected in the plasma at a concentration of 0.175mg/ L (285).

There are several natural and synthetic analogues of resveratrol with different isomers, derivatives, and conjugates/ metabolic products. The most common types are piceid (resveratrol glycosides), glucuronide, and sulphate conjugate (resveratrol-3-

sulphate) (286). Each of these conjugates/metabolic products seem to have distinct biological properties, however their specific effects have not been as extensively explored.

Despite the vast amount of resveratrol data available *in vitro* and *in vivo* (animals), few studies have examined any effects in humans. Oral doses of resveratrol (25 mg /70 kg) to healthy humans, in 100 ml of either white wine, grape juice, or vegetable juice, revealed high concentrations in the serum and urine predominantly as glucuronide and sulphate conjugates peaking after 30 minutes, whereas the serum concentration of free resveratrol was only 1.7- 1.9% of the total concentration (287). It was also determined that only 16.5% of the administered dose of resveratrol was seen after 24 hours in the urine. The transient increase of plasma resveratrol suggests that much larger oral doses of resveratrol would be required in order to sustain an adequate concentration to control disease processes.

Similarly, Walle et al. (2004) (288) administered [¹⁴C]resveratrol orally and through i.v. to six healthy humans. Absorption reached 70% with peak plasma levels of resveratrol and its metabolites at 491 ± 90 ng/ml ($\sim 2\mu\text{M}$), but again with only trace amounts of unconjugated resveratrol (<5 ng/ml) present. Sulfate conjugation by the intestine and liver seems to be the rate- limiting step in resveratrol bioavailability with considerable accumulation in the epithelial cells along the aerodigestive tract. These studies conclude that resveratrol is well absorbed and metabolized by the intestine.

1.10.3 The Antioxidant Properties of Resveratrol

Oxidizers produce free radicals, also called reactive oxygen species (ROS), which are electron-accepting molecules that have unpaired electrons and are extremely harmful

and highly reactive. Examples of ROS include the superoxide anion ($O_2^{\cdot-}$), the hydroxyl ion (OH^{\cdot}), nitric oxide (NO^{\cdot}), and molecules like hydrogen peroxide (H_2O_2). Antioxidants behave as reducers and donate electrons to these free radicals in order to make them harmless unreactive compounds. Increased ROS production or oxidative stress can cause deoxyribonucleic acid (DNA) damage, protein oxidation, and lipid peroxidation, which can lead to mutagenesis, carcinogenesis, membrane damage, atherosclerosis, and neoplasia (289). Other free radicals that cause oxidative damage in the body are produced by pollutants in the air, water, and food supply and from oxidation-reduction reactions of lipids and metal ions, and mitochondrial electron transport reactions in the body itself.

There are several methods used to measure antioxidant activity. For example, the antioxidant activity of resveratrol and other phenolic compounds may be examined via the spectrophotometric method of Rice-Evans and Miller, where cation radical absorption inhibition indicates antioxidant activity (290).

The most well known characteristic of resveratrol is its antioxidant properties. Polyphenolic compounds like resveratrol either donate hydrogen or react with superoxide anions, lipid peroxy radicals, and hydroxyl radicals. For example, deletion of the hydroxyl group at position B4 (Figure 8) reduces antioxidant activity (291). When the hydroxyl group is a free radical, it is a potent oxidizer but is unreactive when attached to a polyphenol like resveratrol. Resveratrol converts these radicals to unreactive compounds that are harmless to human tissues (292).

DNA damage is an indicator of oxidative damage from ROS. De Salvia et al. (2002) (293) examined the antioxidant capacity of resveratrol against ROS in Chinese hamster ovary cells. In this model, ROS activity was generated by H_2O_2 treatment.

Simultaneous administration of resveratrol and H₂O₂ produced a small significant reduction in intracellular oxidation, but when pre-treated with resveratrol (3 hours), increased oxidative damage was observed. Resveratrol had no effect on primary DNA damage when administered alone or simultaneously, but when applied for three hours before hydrogen peroxide, all damage was abolished. Additionally, resveratrol protected against fixed DNA damage during the combined treatment but enhanced oxidative damage with pre-treatment. Therefore, resveratrol exhibits both anti- and pro- oxidant activity, depending on the type of treatment.

Polyphenols containing a phenol ring, as opposed to a catechol ring, have been shown to generate reactive oxygen species (ROS) when metabolized by peroxidase as evidenced by NADH cooxidation (294). Compounds like resveratrol that act as antioxidants towards lipids, may also be pro- oxidant towards DNA and protein (295). For example, resveratrol increased ROS production in T- lymphoblastic leukemia CEM-C7H2 (296) and rat pheochromocytoma (PC12) (297) cells, playing a role in apoptosis induction. On the other hand, resveratrol decreased ROS production in MCF-7 breast cancer (298) and hepatoma cells (299;300).

Recently, several studies have focused on understanding the cancer chemopreventive and cardioprotective effects of resveratrol. It seems that the effects seen in both disease processes have a common aetiology that includes antioxidative, cell cycle control, and mitogen- activated protein kinase (MAPK) activation mechanisms. In addition, a possible role in estrogenic activity is also being explored where resveratrol has demonstrated both agonistic and antagonistic effects. Therefore, resveratrol is capable of affecting numerous biological activities involved in major disease processes.

1.10.4 The Anti- Cardiovascular Effects of Resveratrol

Research has demonstrated the cardiovascular benefits of resveratrol treatment in numerous ways, which include antioxidant, decreased smooth muscle cell proliferation, vasorelaxation, and anti- thrombotic theories. Each aspect will be considered in this section.

1.10.4a Antioxidant Properties

ROS are essential in cell signalling and regulation at low concentrations, affecting gene transcription, protein synthesis, and cell function. In the cardiovascular system, ROS play a role in maintaining cardiac and vascular integrity. However, an overabundance of ROS causes oxidative stress, which is one of the most important contributors to vascular damage. Therefore it is imperative that a pro-oxidant and anti-oxidant balance be maintained (289). An excellent example of oxidative stress is the production of superoxide anions ($O_2^{\cdot-}$) and nitric oxide (NO^{\cdot}) by endothelial cells, activated macrophages, and neutrophils during the inflammatory response. These two oxidants are the main sources for production of the oxidant peroxynitrite ($ONOO^{\cdot}$), which is involved in the development of atherosclerosis and rheumatoid arthritis.

Oxidative stress and associated oxidative damage are mediators of vascular injury and inflammation in many cardiovascular diseases including atherosclerosis. Under pathological conditions, increased ROS production leads to endothelial dysfunction, increased contractility, VSMC proliferation and migration, apoptosis, inflammation, and increased deposition of extracellular matrix proteins, all contributing to vascular damage and cardiovascular disease.

Several *in vitro* and *in vivo* studies have demonstrated that resveratrol is a potent antioxidant. The antioxidant and superoxide anion scavenging activity of resveratrol was demonstrated in rat cardiac muscle cells at 7.8 μ M and 245.1 μ M concentrations respectively. Conjugated dienes and TBARS (thiobarbituric acid- reactive substance) formation and electrophoretic mobility of oxidized LDL showed that resveratrol inhibited Cu^{2+} - induced LDL oxidation by 50% (291). Resveratrol (50 μ g/ml, 30 min) also significantly inhibited ROS and TBARS production in pig blood platelets stimulated by lipopolysaccharide (S1959) or thrombin (301).

As previously mentioned, lipid peroxidation may lead to a variety of disease processes. Therefore, inhibition of lipid peroxidation would be of great benefit and may be achieved with antioxidant treatment. Stojanovic et al. (2001) (302) compared the free radical scavenging activity of resveratrol and several other antioxidants via HPLC analysis and the iodometric method. Liposomes, in the absence of antioxidants, were used as a control and liposomes containing a particular antioxidant were γ - irradiated at various doses. As expected, the highest content of lipid hydroperoxide was seen in the control. HPLC analysis showed that resveratrol was a stronger inhibitor of lipid peroxidation (95% efficiency) than both Vitamin C and Vitamin E, which was consistent throughout the process. For confirmation, the procedure was repeated using the iodometric technique, which revealed that resveratrol was the most potent antioxidant (95%).

Low- density lipoprotein (LDL) oxidation is associated with the accumulation of LDLs in subendothelial cells of the heart and vessels and increased platelet adhesion to the epithelium (280). Ferrylmyoglobin and peroxynitrite are the main oxidants involved

in the pathophysiology of cardiovascular disease (278). Resveratrol protected human plasma isolated LDLs from oxidation by ferrylmyoglobin in vitro in a concentration-dependent manner and inhibited the protein modification effects of peroxynitrite.

Resveratrol (10 μ M, 10 min) infusion in Langendorff- perfused normoxic rat hearts, significantly increased coronary flow (46.3%) and adenosine release (95.5%). To confirm that the increase in coronary flow was mediated by adenosine, the adenosine receptor antagonist, 8- (p- sulfophenyl) theophylline (SPT), was administered in combination with resveratrol. The increase in blood flow was completely inhibited by the antagonist, which established the acute action of resveratrol. Chronic oral administration of resveratrol (25mg/ l in tap water, 15 days) to male Sprague- Dawley rats induced significant vasodilatation and improved heart functional recovery. Administration of N^G-nitro-L-arginine methyl ester (L-NAME) (30 μ M), a nitric oxide synthase inhibitor, reversed the resveratrol- induced effects. This inhibitor is not specific so the source of the nitric oxide (NO), whether intracellular nitric oxide synthase (iNOS) or endothelial nitric oxide synthase (eNOS), is uncertain. Resveratrol (10 μ M) has been shown in previous studies to have no significant effect on eNOS expression (303), however studies in iNOS knockout mice have shown that eNOS is upregulated (304). Therefore, resveratrol seems to improve cardiac function through a NO- mediated mechanism (305).

1.10.4b Anti- thrombotic Properties

The cyclooxygenase pathway promotes the formation of prostaglandins from unsaturated fatty acids. Prostaglandins initiate contraction of the smooth muscle of blood vessels and promote fever and pain as part of the inflammatory response to injury. Thromboxanes, which are produced by blood platelets, work with prostaglandins to

promote vasoconstriction and activate platelet coagulation in blood vessels that restricts blood flow to form a thrombus.

Resveratrol inhibits thrombus formation by inhibiting thromboxane B₂/ 12-hydroxyeicosatetraenoate, which is specifically responsible for platelet aggregation induction and contraction of arteries (306). In addition, when both synthetic and natural resveratrol (3.56µg/l) were administered to platelet- rich plasma for ten minutes, platelet aggregation was reduced by 50.3%, thereby reducing the risk of cardiovascular complications (307). Platelet aggregation inhibition was again demonstrated both in vivo and in vitro by resveratrol (10- 1000µM) in a concentration- dependent manner in platelets isolated from human plasma and in experimentally induced hypercholesterolemic rabbits (308).

Alternatively, thrombus formation may be prevented through an inhibition of cell adhesion to the endothelial lining of blood vessels. Resveratrol significantly inhibited U937 monocytoid cell adhesion to lipopolysaccharide- stimulated human saphenous vein endothelial cells and neutrophil adhesion to TNF- α -stimulated NIH/3T3 ICAM-1-transfected cells, resulting in decreased blood clotting activity (309).

Overnight treatment with resveratrol (100nM) in combination with TNF α , a pro-inflammatory mediator and coagulant, significantly decreased vascular cell adhesion molecule- 1 (VCAM- 1) expression but had no effect alone (310). This result would help decrease leukocyte adhesion to the vascular endothelium and perhaps reduce cardiovascular disease progression. The effects of TNF α are controlled by the NF-kB signalling pathway, which is mediated by I κ B α phosphorylation. Human umbilical vein endothelial cells stimulated with TNF α and resveratrol (100nM and 1µM) overnight

increased p50-NF κ B and p65-NF- κ B nuclear appearance. Acute (30 minute) resveratrol treatment increased I κ B α tyrosine phosphorylation of immunoprecipitated NF- κ B, which is indicative of an alteration in transcription (311).

Fulgenzi et al. (2001) (312) used TNF α to induce endothelial permeability in a Balb/c mouse model. Resveratrol (30 minutes) alone did not affect vascular permeability, but when given in combination with TNF α , vascular leakage values were significantly lower than TNF α alone. This confirmed previous in vitro data that suggested resveratrol affects endothelial function.

1.10.4c Vasodilation

Another way to prevent cardiovascular damage is through vessel dilatation to facilitate an increase in blood flow and oxygen transport to the tissues. Angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) inhibition decreases vasoconstriction. When there is a reduction in the concentration of these two compounds, vasodilatation and blood flow increases, peripheral vascular resistance and blood pressure are reduced, and the sodium- water balance is restored. When cells of the neuroblastoma line, SK- N- SH, were incubated with 10 μ M resveratrol for four days, there was weak inhibition of ACE and no effect on NEP, suggesting that resveratrol is a mild vasodilator (313).

To explore the mechanism behind the production of vasorelaxation by resveratrol, Li et al. (2000) (314) looked at possible actions on the ionic currents in endothelial cells. The effect of resveratrol on whole cell potassium outward currents in cultured human umbilical vein endothelial cells and calcium activated potassium channels in pancreatic endothelial cells (MS1) were examined. Resveratrol (30 μ M) increased the amplitude of the outward currents within only one minute of administration and increased the activity

of large- conductance calcium- activated potassium channels, which was explained by a lengthening of the open time of the channels. In MS1 endothelial cells, resveratrol stimulated membrane hyperpolarization in intermediate- conductance calcium- activated potassium channels, which subsequently affected endothelial function and induced vasorelaxation.

1.10.4d Cell Proliferation and Migration

Smooth muscle cell (SMC) proliferation is a known prerequisite for atherosclerosis and other cardiovascular disorders. Atherosclerosis is characterized by the thickening of arterial walls due to lipid deposits that obstruct the lumen and deteriorate the arterial wall. Arteriosclerosis is a characteristic of advanced atherosclerosis where the arterial walls lose elasticity and become rigid. It has also been documented that vascular smooth muscle cell migration, proliferation, and hypertrophy are involved in the pathogenesis of atherosclerosis. In particular, vascular smooth muscle cell proliferation is involved in the formation of atherosclerotic plaques that decrease the elasticity of the blood vessels (315).

Initial studies in newborn calf thoracic aorta in vitro (316) showed that resveratrol had the ability to control cell proliferation. Vascular smooth muscle cell proliferation and migration in vitro were reduced by 70- 90% after resveratrol administration in a dose-dependent manner, particularly at 50 and 100 μ M concentrations. The underlying mechanism was discovered to be a disruption in the cell cycle where the number of cells in the S phase (37%) was reduced, followed by a subsequent increase of the number of cells in the G1 phase (23%). From these results it was determined that resveratrol blocked the cell cycle at the G1/ S transition as opposed to inducing apoptosis. As a result, cell cycle control could potentially prevent the progression of atherosclerosis.

Subsequent studies also confirmed that vascular smooth muscle cell proliferation was inhibited by resveratrol treatment. Vascular smooth muscle cells from newborn calf thoracic aorta were incubated with resveratrol (6 hours) at concentrations of 10 and 100 μ m. [3 H]thymidine incorporation assays were used to measure smooth muscle cell proliferation. The results showed a 43.9% and 96.2% decrease in [3 H]thymidine incorporation, respectively, which is indicative of decreased DNA synthesis that normally occurs during the S phase of the cell cycle (317).

Vascular smooth muscle cell proliferation was also inhibited by resveratrol in human aortic vascular smooth muscle cells (HAVSMC) in a dose- and time- dependent manner. Concentrations as low as 1.1 μ M (120 h), demonstrated a reduction in the growth of HAVSMCs (6.5%). In addition, resveratrol inhibited DNA synthesis, as determined through thymidine uptake, by as much as 91.6% (12.5 μ M). It was discovered that resveratrol (6.25 μ M) was associated with increased intracellular p53 levels, which was dose- dependent. p53 was absent when resveratrol was not present, which confirmed that resveratrol induced p53 signalling. It is also important to note that in higher concentrations of resveratrol (25 μ M), apoptosis was induced in cells that were actively proliferating. Together, these results suggest that resveratrol exhibits a selective mechanism (318).

Endothelin- 1 is a known mediator of cardiovascular disease and is an upstream promoter of MAPK. MAPK induces smooth muscle cell proliferation and contraction in blood vessels, which leads to cardiovascular disease development. El- Mowafy et al. (1999) (319) examined the underlying mechanisms involved in resveratrol's protective effect on coronary heart disease and atherosclerosis. Resveratrol (37 μ M) down- regulated

endothelin-1 evoked tyrosine phosphorylation in endothelia removed from the left anterior descending artery of pig hearts. The subsequent effect of this inhibition was a significant reduction in MAPK activity. Endothelin-1 also augmented ERK1/2, JNK1, and p38 MAPK kinase phosphorylation, whereas resveratrol (50 μ M) counteracted these effects.

MAP kinase activation induced by collagen, thrombin, and ADP, was inhibited by resveratrol (10- 50 μ M) in washed platelets (320). Resveratrol (50 μ M) inhibited aggregation induced by collagen (91%), thrombin, and ADP. Exogenous fibrinogen added to the assay did not affect resveratrol, which indicated that the inactivation of MAPK was a possible mechanism causing reduced aggregation. The same effect was tested in whole blood to simulate *in vivo* conditions but more than 200 μ M of resveratrol was needed for a 30- 60% decrease in platelet aggregation.

It is well documented that angiotensin II induces smooth muscle cell hypertrophy. In a recent study in rat aortic smooth muscle cells it was shown that angiotensin II-induced [³H]leucine incorporation, which is an indicator of SMC hypertrophy, was inhibited by 71% by resveratrol (50 μ M). This result was associated with the inhibition of the PI3K- Akt/PKB signalling pathway. Angiotensin II- induced activation of both PI3K and Akt/PKB phosphorylation was significantly reduced with resveratrol at concentrations as low as 10 μ M. Additionally, ERK1/2 phosphorylation was dose-dependently inhibited by resveratrol with a maximum effect seen at 50 μ M (321).

1.10.5 The Anti- Cancer Effects of Resveratrol

1.10.5a Anti- Tumour Effects

The relationship between resveratrol and its role in cancer prevention is less debated and has been more extensively studied. There are three major stages documented in cancer progression, initiation, promotion, and progression. Chemoprevention involves the ingestion of chemical agents that are targeted at specific stages of tumour progression, some of which are harmful to more than just cancer cells. One of the landmark studies linking resveratrol administration to cancer chemoprevention is from Jang et al. (1997) (273). In this *in vivo* study, resveratrol inhibited preneoplastic lesions in carcinogen treated mammary glands of mice, and tumorigenesis in a mouse skin cancer model (88%), showing that resveratrol inhibited the initiation phase of carcinogenesis. Resveratrol inhibits carcinogenesis in human promyelocytic leukemia cells (HL-60) through a reduction in COX1/2 activity, which is responsible for the inhibition of tumour promotion and progression.

Later studies (322) demonstrated that resveratrol could decrease tumour incidence and multiplicity in female mice of specific pathogen- free ICR strain. Resveratrol (85nM) was applied topically one week before DMBA and TPA induced carcinogenesis and continually until week 20. In the control group there was 100% tumour incidence after 10 weeks, whereas the resveratrol group demonstrated an incidence of only 20%. After 20 weeks, resveratrol still showed a 30% inhibition of tumour growth. The number of tumours seen on each mouse was also evaluated, which showed that in the control group each mouse had an average of 10 tumours as opposed to the four-tumour average seen in those treated with resveratrol.

An *in vivo* study on male Donryu rats implanted with AH109A hepatoma cells showed a positive effect with resveratrol treatment (323). In the untreated group, tumour size increased throughout the entire study (20 days). Resveratrol (50ppm) administered orally in the diet arrested tumour growth at day 12 and decreased hepatoma weight, the number of metastases, and metastases tumour weight. The control group expressed 60% incidence of metastases while the resveratrol treated group (50ppm) exhibited only 37.5% incidence. Serum TBARS and triglyceride levels also declined in the resveratrol group when compared to the hypercholesterolemia and hypertriglyceridemia expressed in the untreated group.

Resveratrol also inhibited tumour formation in a CD-1 mouse model, which was indicated by a decrease in the number of tumours developed and by the number of mice that developed tumours (324). In contrast, Bove et al. (2002) (325) did not find any inhibition by resveratrol of 4T1 breast cancer tumour growth in a BALB/c female mouse model. Daily injections were given at 1, 3, and 5mg/kg for 23 days and did not present any toxic effects as indicated by the absence of weight or behavioural changes. However, an *in vitro* model of 4T1 mammary carcinoma cells (E α / β positive) did show that resveratrol (30 μ M) completely inhibited cell growth in a dose-dependent manner after only 24 hours incubation. Therefore, caution should be used during extrapolation of data from an *in vitro* model to *in vivo*.

1.10.5b Cell Proliferation

Resveratrol reduces tumour formation through cell cycle control, which is paralleled with the effect on vascular smooth muscle cells in cardiovascular disease. Flow cytometry analysis is one of the most widely used methods for counting the number of cells in the various phases of the cell cycle. Ragione et al. (1998) (326) had confirmed

through flow cytometry analysis that HL-60 promyelocytic cells were completely inhibited by resveratrol (30 μ M) at the S/ G2 phase checkpoint, which was accompanied by an S phase elongation that prevented further cell proliferation (Figure 10).

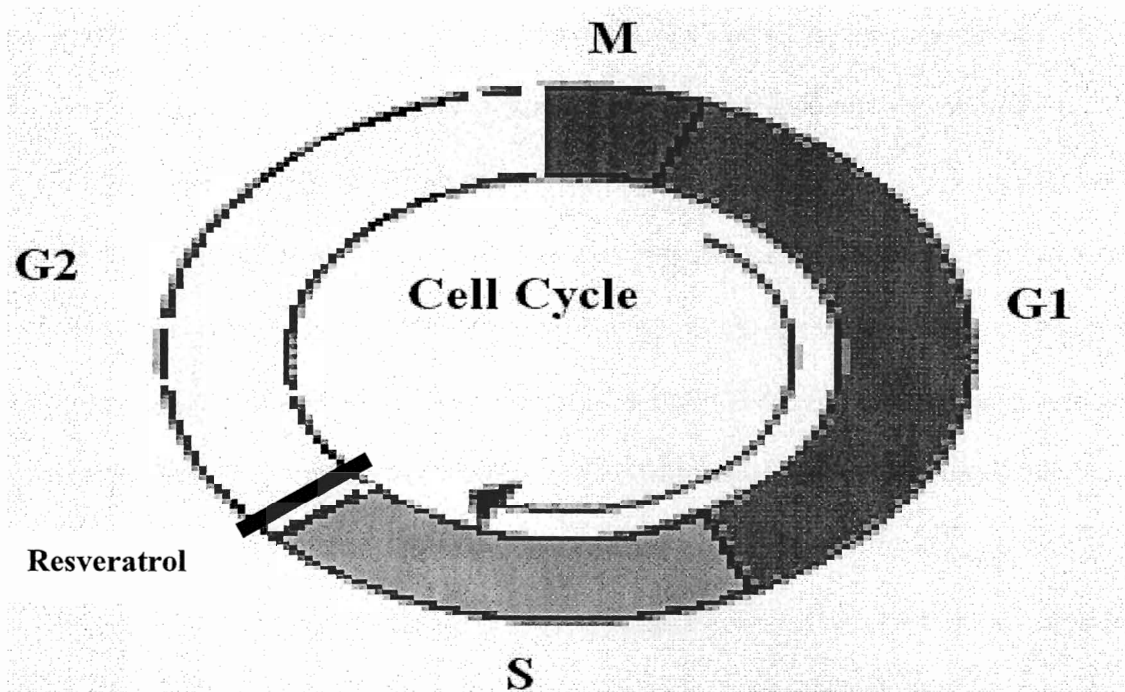


Figure 10: Cell cycle progression demonstrating the inhibition of resveratrol at S/ G2 phase.

One explanation of the disruptive activity on cell proliferation by resveratrol involves gap- junction intercellular communication (GJIC) (327). TPA (12-O-tetradecanoylphorbol-13-acetate) and DDT (1, 1-bis (p-chlorophenyl)-2, 2, 2-trichlorethane), which are tumour promoters, are known to block GJIC. These tumour promoters were added to WB- F344 rat liver epithelial cells along with resveratrol to examine GJIC through dye- coupling. As expected, pre- incubation with resveratrol (25 μ M) on cells treated with either TPA or DDT showed a remarkable increase in GJIC after only 30 minutes. Maximum dye- coupling was observed after 6 hours of pre- incubation with resveratrol. Resveratrol (5 h) also counteracted both TPA and DDT in a concentration- dependent manner. Resveratrol (35 μ M) enhanced TPA- inhibited GJIC activity to 71%, while DDT- inhibited GJIC activity was enhanced to 100% of the control cells by 17 μ M resveratrol. Resveratrol up- regulated GJIC through connexin43 phosphorylation control and reversed phosphorylation of connexin43 without affecting its expression. In conclusion, GJIC regulation by resveratrol may be an important anti- tumour mechanism.

Resveratrol blocked the cell cycle at the S/ G2 phase transition in SW 480 colorectal tumour cells (328), which is illustrated in Figure 10. When resveratrol (30 μ M, 24 h) was administered, cell growth was inhibited by 50% and the number of cells in the S phase increased. This effect was reversible and both dose- and time- dependent. The mechanism of action involved the inhibition of cdk1 and cdk2 that regulate proteins in the cell cycle to activate maturation-promoting factor and cyclin- dependent kinases. The effect was an elongation of the S phase, which therefore prevented cells from beginning the G2/ M phases where cell growth and mitosis occur.

Similarly Latruffe et al. (2002) (276) also found that resveratrol inhibited proliferation of human hepatoblastoma HepG2 and colonic cancer cells SW480. Resveratrol prevented cell cycle progression past the S phase, which was indicated by the large increase in cell number in the S phase and the increase in [³H]thymidine incorporation. Cell cycle inhibition prevented growth and decreased the number of viable cells. The mechanism of action was evaluated with Western blot analysis, where increased cdk1 and cdk2 levels were observed. Carbo et al. (1999) (329) also confirmed these results, where resveratrol decreased tumour growth by 25% in rats inoculated with Yoshida AH-130 ascites hepatoma. The effect was again attributed to cell cycle arrest.

Resveratrol (50µM, 12 h) also completely arrested MCF- 7 cell proliferation (330) through suppression of the cell cycle at the G₂/ M phase and stimulation of cells accumulating in the S phase (48% vs. 11% in control). A dose- dependent inhibition of cell proliferation by resveratrol (25- 100µM) was also found in human peripheral blood lymphocytes, where a 99% prevention of entry into the cell cycle was observed at 100µM. This effect was not attributed to apoptosis induction because fractional DNA content showed that the frequency of apoptosis was reduced with resveratrol. The mechanism of cell cycle control was recognized to be cyclin regulation where a significant inhibition of cyclin E was observed at 6 hours after cyclin D2 and D3 induction, which indicated cell cycle arrest at the G_{0/1} stage (331).

1.10.5c Apoptosis

A controversy remains with regard to apoptosis induction by resveratrol. Evidence exists that supports both the cell cycle control and apoptosis induction effect of

cell proliferation and growth. The action of resveratrol seems to be dependent on the cell line.

Surh et al. (1999) (332) examined cell proliferation in human promyelocytic leukemia (HL-60) cells. Resveratrol (50 μ M, 8 h) inhibited cell growth and reduced cell viability by 45% through apoptosis induction, which was confirmed by an increase in DNA fragmentation and morphological characteristics such as broken nuclei, which are associated with apoptosis. A 66% increase in DNA loss was also observed after 48-hour resveratrol (100 μ M) administration. Resveratrol increased Bcl-2 content, an anti-apoptotic oncoprotein commonly observed in cells undergoing cell death. Therefore, in this study, resveratrol inhibited cancer cell growth through apoptosis induction.

In the JB6P⁺ mouse epidermal cell line C1 41, resveratrol (2.5- 40 μ M) induced apoptosis through an increase in p53 activity (333). Apoptosis was not observed with resveratrol (24 h) treatment in a p53 deficient cell line (p53^{-/-}). It was only in the wild-type (p53^{+/+}) cells that apoptosis occurred. Therefore, the p53 pathway is involved in resveratrol-induced apoptosis and may help to explain why resveratrol exhibits anti-tumour activity.

Further investigation of the mechanism of apoptosis induction by resveratrol was completed in the JB6P⁺ mouse epidermal cell line C1 41 (334). Resveratrol (20 μ M) activated apoptosis and increased p53 phosphorylation at the serine 15 residue. MDM2, a feedback inhibitor of p53, was also induced by resveratrol but was interfered with by serine 15, which dissociated MDM2 and stabilized p53. ERK1/2 and p38 MAPK activation were required for p53 phosphorylation at serine 15, which again suggested that MAPKs are also affected by resveratrol.

To further elucidate the involvement of MAPK in resveratrol action, Niles et al. (2003) (335) examined cell proliferation and apoptosis in SK- me128 and A375 human melanoma cells. Resveratrol (50 μ M, 2 days) significantly inhibited proliferation in both cell lines and increased apoptosis. Increased phosphorylation of p38 MAPK or JNK was not observed in either cell line but ERK1/2 phosphorylation was induced in the A375 cell line. This data suggests that MAP kinase pathways are involved in apoptosis mediation, particularly through the p38 MAPK and JNK pathways.

Previous studies have demonstrated that ERK1/2 and p38 MAPK mediate p53 activation and the subsequent apoptosis that is responsible for antitumour activity. Current research now suggests that JNK is also involved in resveratrol- induced p53 activation. She et al. (2002) (336) found that resveratrol (10- 40 μ M, 1- 4 h) activated JNK isoforms in a JB6 C1 41 mouse epidermal cell line, resulting in p53 phosphorylation. Apoptosis was repressed in a knockout experiment with resveratrol administration to a negative mutant JNK1. Consequently, JNK is also an important mediator of resveratrol- induced apoptosis in addition to ERK and p38 MAPK.

When MAPK is activated, p53 and subsequent apoptosis is stimulated, as shown in human thyroid cancer cells (337). The same researchers found that short- term activation of MAPK was required for apoptosis to occur in normal cells and that normal p53 function is a requirement for apoptosis. Resveratrol (0.1 to 100 μ M) treatment, for 4 and 24 hours, activated MAPK and induced p53 accumulation leading to apoptosis of papillary thyroid carcinoma and follicular thyroid carcinoma cell lines. Resveratrol has also demonstrated apoptosis induction in prostate, breast, lymphoblast, and leukemia cancer cell lines.

Hsieh & Wu (1999) (338) compared androgen- responsive (LNCaP) and androgen- nonresponsive (DU-145, PC-3, and JCA-1) human prostate cancer cells concerning the effect of resveratrol on cell growth, cell cycle distribution, and apoptosis. Mitogenesis was negatively affected by resveratrol (25 μ M) treatment. Cell growth was decreased in LNCaP, PC-3, and DU-145 cells with only a mild effect on JCA-1 cells. Resveratrol also disrupted the G1/S phase transition in all cell lines except for LNCaP. Alternatively, resveratrol treatment induced apoptosis in the LNCaP cells. It is interesting to note that it was the androgen- responsive cells, LNCaP, that underwent apoptosis. Prostate- specific antigens (PSA) respond to androgens via the androgen receptor (AR) and are responsible for prostate cell growth. Resveratrol reduced PSA expression by almost 80% but did not change AR expression, which is upstream from PSA. Therefore, resveratrol regulated PSA gene expression independently of the androgen receptor, which may account for the decreased cell growth.

In another study on LNCaP cells, which are hormone sensitive, resveratrol was again found to induce apoptosis. Cell death was assessed via examination of cell morphology and caspase activity. In apoptotic cells, caspases are processed from their inactive to active state, which is widely accepted as an irreversible step in programmed cell death. After resveratrol administration, nuclear condensation and cell shrinkage were observed, which indicated that apoptosis had occurred. More evidence for apoptosis was shown through an increase in caspase- 3 activity (20nmol/min/mg) after 72-hour incubation with resveratrol (100 μ M) (339).

1.10.6 The Effect of Resveratrol on Estrogen Receptor Activity

Both alpha and beta estrogen receptors are overexpressed in breast cancer (340) and targeting their inhibition is a popular therapeutic approach to inhibit breast cancer progression. The structure of resveratrol is remarkably similar to that of the synthetic estrogen diethylstilbestrol, which is a non-steroidal estrogen used for treatment in menopausal disorders. It has been established that resveratrol is a phytoestrogen that mimics the effects of human estrogen through the binding and activation of alpha and beta estrogen receptors but is less reactive than endogenous estrogen (292). The modulatory effects of resveratrol on the estrogen receptor remain controversial since resveratrol activity has been shown to be both agonistic and antagonistic for both the alpha and beta-receptors, depending on the presence of endogenous estrogen (341). Additional factors have been documented to influence the activity of resveratrol, including cell type and the type of estrogen receptor expressed (342).

In the absence of estrogen, resveratrol promoted estrogen receptor (ER) - positive breast cancer cell proliferation. In the presence of estrogen however, resveratrol inhibited ER- positive MCF7 cell proliferation (342). Synthetic resveratrol (5- 40 μ g/ml, 24- 144 hours) tested in an estrogen receptor- positive breast cancer cell line (MCF- 7), an immortal estrogen receptor- negative breast epithelial cell line (MCF- 10F), and a malignant estrogen receptor- negative breast epithelial cell line (MDA- MB- 231), reduced the number of viable cells and prevented proliferation of each cell line.

In a study by Gehm et al. (1997) (340), resveratrol behaved as a superagonist in human breast adenocarcinoma cells (MCF-7) that expressed the estrogen receptor (ER+). Resveratrol (10 μ M) prevented estradiol from binding to the estrogen receptor and also

activated transcription. In addition, the proliferation of T47D breast cancer cells, which are estrogen- dependent, was promoted. It is also noteworthy that the combination of both resveratrol (5 μ M) and estradiol (100pM) demonstrated a synergistic effect in MCF-7 cells, which suggests that both compounds bind and activate the same receptor.

In contrast to the evidence of an agonistic effect of resveratrol, Turner et al. (1999) (343) found that resveratrol demonstrated antagonistic activity in a rat in vivo model. When uterine wet weight, epithelial cell height, and mRNA levels for IGF-I was examined after resveratrol administration, a statistically significant increase was not observed, which indicated that resveratrol is not an estrogen agonist. In addition, resveratrol (1000 μ g/ day) did not express any effect on serum cholesterol levels, unlike 17 β - estradiol (100 μ g/ day), which initiated a significant decrease after six days. These in vivo results suggest that resveratrol does not exhibit estrogen agonism when compared with previous in vitro studies.

Further uncertainty was added to this controversy in both in vitro and in vivo tumour models (300). MCF-7, T47D, LY2, and S30 mammary cancer cells were treated with resveratrol (1- 15 μ M) for 24 hours. A mild estrogenic response was observed in MCF-7 cells but changed to an antagonistic effect when combined with 17 β - estradiol. In the S30 cell line similar effects were seen, however in the T47D and LY2 cells a strong estrogen antagonistic effect was demonstrated. Up- regulation of estrogen- inducible genes is a classic agonistic effect, therefore expression of the estrogen- responsive proteins including the progesterone receptor (PR) and pS2 were also examined. In MCF-7 cells, resveratrol induced PR expression but a combination of resveratrol and estradiol inhibited PR expression. No change was seen in T47D cells but there was suppression of

the pS2 protein in LY2 and S30 cells regardless of estradiol presence. Similar results were observed in a mouse mammary organ culture model.

Bhat et al. (2001) (300) also studied this effect in mammary tumour- induced Sprague- Dawley rats at doses of 10 and 100mg/ kg of resveratrol (5 days/ week for 111 days). It was reported that resveratrol suppressed tumour multiplicity, reduced tumour incidence (50%), and increased the latency period by 27 days. These findings propose that resveratrol's activity, whether agonistic or antagonistic, is dependent on estradiol.

Henry and Witt (2002) (344) support the in vitro findings that resveratrol acts as a mixed agonist/ antagonist. Resveratrol (100 μ M, 7 days) significantly affected estrus cyclicity in female Sprague- Dawley rats. Only 41% of females exhibited the normal cycle (4-5 days), while 42% remained in constant estrus and 17% experienced a shortened 3-day cycle. In a second experiment, 1000 μ g subcutaneous injections of resveratrol in ovariectomized females decreased sexual receptivity to the male stimulus. Resveratrol did not have any other effects alone but did stimulate lordosis and increased plasma estrogen levels (238 vs. 37.8pg/ml in peanut oil) when combined with 17 β -estradiol benzoate (EB) (10 μ g). This study also showed that when the ovaries were removed, pre- exposure to resveratrol did not change lordosis behaviour when EB and progesterone were administered. Although in sexually receptive females, resveratrol pre- exposure caused a significant delay in rejection behaviour and decreased the number of interactions with the male stimulus (16.75 vs. 32 in water group). Hence, results from this study provide additional evidence that resveratrol is a mixed agonist/ antagonist of the estrogen receptor.

Additional studies suggested that the effect of resveratrol was unrelated to estrogen receptor status in Wistar rats (345). Scarlatti et al. (2003) (346) demonstrated growth inhibition and apoptosis in MDA- MB- 231 metastatic breast cancer cells. This cell line is ER- negative so it does not respond to estrogen or similar structures like resveratrol. A 24% increase in apoptosis was discovered with resveratrol (16 μ M) treatment after two days but the underlying mechanism was determined to be de novo ceramide synthesis and not p53 activation.

The alteration of autocrine growth modulators and their receptors is an additional mechanism through which resveratrol is capable of inhibiting breast cancer cell proliferation. Resveratrol was administered to ER- positive cells (MCF- 7) and ER- negative human breast carcinoma cells (MDA- MB- 468) for three days at 10⁻⁵ M and altered autocrine growth modulator pathways in breast cancer cells in order to inhibit proliferation. Autocrine growth stimulators transforming growth factor- alpha, PC cell-derived growth factor, and insulin- like growth factor I receptor mRNA reduction was determined as the mechanism (347).

In summary, resveratrol has been shown to have exceptional anti- oxidant, anti- thrombotic, and anti- cancer properties in several different cell lines. Resveratrol also has potential for treating other diseases. For example, resveratrol mimics calorie restriction in yeast by extending their lifespan by approximately 70% (348). However, even though resveratrol has been extensively examined in these critical health- related fields, studies investigating the possible anti- diabetic properties of resveratrol are lacking.

1.10.7 Resveratrol and Signal Transduction.

Resveratrol has been extensively studied in a variety of cell lines concerning its anti- cardiovascular disease properties, role as an anti- cancer agent, and an anti- oxidant. Several signalling molecules are affected by resveratrol, some of which are also involved in insulin signalling and glucose transport. However, resveratrol- stimulated signal transduction seems to be very much cell specific and concentration- dependent, as evidenced by both activation and inhibition of certain key signalling molecules and its biphasic effects in various cell lines, which is extensively reviewed in Aggarwal et al. (2004) (274).

Growth inhibition demonstrated by resveratrol is mediated through upregulation of p53, Bax, and p21^{Cip1/WAF1}, downregulation of cyclin D1, cyclin E, Bcl-2, Bcl-x_L, caspase activation, and cell cycle arrest. Resveratrol also inhibits the activation of transcription factors such as NFκB and AP-1, and protein kinases like IκBα kinase, ERK1/2, JNK, Akt, PKC, and PKD, and downregulates gene products such as COX-2, VEGF, and IL-1. Generally resveratrol inhibits the MAPK and NFκB pathway and activates the p53, Fas, and ceramide signalling pathways.

For example, DMBA- induced (7, 12- dimethylbenz (a) anthracene) protein synthesis of both COX2 and MMP- 9 is down- regulated by resveratrol (50μM) in MCF- 7 cells. Resveratrol (50μM, 4 h) also suppresses NF- κB activation, which is implicated in carcinogenesis and responsible for COX2 and MMP- 9 regulation. COX2 induction was not exhibited in any other cancer cell lines examined, which included Bic-1, HCE7, SW480, and HL60 cells (349). However, resveratrol (300μM, 48 h) did cause an increase in COX2 expression in Seg-1 cancer cells.

Phorbol ester- stimulated COX2 activity was inhibited by resveratrol (15 μ M, 30 min) in 184B5/ HER cells. Resveratrol inhibited COX2 through protein kinase C (PKC) inhibition where its translocation from the cytosol to the membrane was reduced. PKC is well established as a regulator in cell signalling and tumour promotion. As a result, suppression of prostaglandin synthesis, involved in the inflammatory response, was suppressed in addition to tumour progression (350).

Garcia- Garcia et al. (1999) (351) also found that resveratrol (30 μ M) inhibited PKC α enzymatic activity in vitro where membrane effects exerted near the lipid- water interface in the model membrane improved PKC α inhibition. It is possible that resveratrol action either directly inhibited PKC or disrupted the protein- phospholipid bilayer interaction. A summary of the effects of resveratrol on key signalling molecules is shown in Table 2.

Table 2: Effects of resveratrol on key signalling molecules.

Model	Resveratrol Treatment	Result/ Mechanism
GLUCOSE TRANSPORTERS		
U937 cells HL-60 cells CHO cells overexpressing GLUT1/3	73 uM 75 uM 100uM	50% inhibition of glucose and dehydroascorbic acid uptake since sodium independent transport blocked and R accumulated in cells (20- 1000uM) Glucose and dehydroascorbic acid uptake abolished with R in GLUT1/3 overexpressed cell (352)
INSULIN SECRETION		
Ins-1 cell assay (rat β pancreatic cells derived from parental RINm5f lines)	100ug/ml (60 min)	98% inhibition of COX-1 but no effect on COX-2 (involved in inflammation) No effect on insulin secretion by INS-1 cells (353)
PI3K AND Akt		
MCF-7 human breast epithelial cells	10uM (36 h) >50uM	Biphasic effect. 10uM R increased ER α - associated PI3K activity and Akt phosphorylation whereas >50uM R showed a decrease. There was no effect on the PI3K p85 subunit, >50uM R decreased ER α protein expression and phospho-Akt activity whereas 10uM resulted in increases (354)
Human embryonic lung fibroblasts infected with human cytomegalovirus	10- 20uM (10 min- 2h)	Decreased viral activity (replication) by blocking viral activation of phospho-Akt and phospho-PI3Kp85, and decreasing NF κ B transcription factor activation, and decreasing EGFR activation (355)
Cardiac fibroblasts	5- 25uM	Limits cell proliferation and differentiation involved in cardiac fibrosis by downregulating ERK1/2 activation, however Akt was unaffected (356)
VSMC	50uM (30 min)	71% inhibition of angiotensin II and inhibition of Akt, PI3K, and ERK1/2 phosphorylation thereby inhibiting angiotensin II induced VSMC hypertrophy (321)
PKC AND PKD		
Sf9 cells	100uM	Inhibition of Ca ²⁺ -dependent PKC α activity stimulated by phorbol esters or DAG. Additional inhibition of PKC β 1 but no effect on PKC ϵ and PKC ζ . Possible mechanism to enhance endothelium integrity (357)
COS-7 cells	100- 300uM (60 min) 800uM	Inhibition of PMA- stimulated PKD activity. 800uM R inhibited PKD phosphorylation (358)
Autophosphorylation reaction mixture	5-100uM (5 min)	80% PKD autophosphorylation inhibition. <20% inhibition on cPKC $\alpha,\beta,1,\gamma$, nPKC δ,ϵ , and aPKC ζ . Possible contribution to cancer chemoprevention action of R (359)
PC-3 prostate cancer cells	20- 120uM 2 h	Inhibits cell growth through downregulation of ERK1/2 and PKC α (360)
CYTOSKELETON		
Bovine pulmonary artery	10- 100uM 6, 48, 96h	Cardioprotection by R exerted from arterial shear stress by suppression of cell growth and induction of cell elongation found to be dependent on intracellular Ca ²⁺ , tyrosine kinase activity, actin microfilament and

endothelial cells (BPAEC)		microtubule assembly. No effect on PKC and R treated cells adhered more strongly to cover slips during arterial flow. ERK1/2 phosphorylation and NO synthase expression (6h- 4 days) were also increased (361)
NFkB/TNF/p53		
Endothelial cells	$\leq 1\mu\text{M}$ (30 min or overnight)	Overnight stimulation increased p50NFkB and p65NFkB nuclear appearance. Acute stimulation increased Ikb α tyrosine phosphorylation of NFkB. No change in cellular p21 or peroxisome proliferators-activated receptor α activity (311)
JB6 cells	15 and 20 μM	Radicals produced by cell exposure to metal (Cr(VI)) showed a decrease with R treatment indicated by a decrease in luciferase receptor activity indicating inhibition of NFkB signalling (362)
Murine microglial cells (N9) Primary rat microglia	0.01-10 $\mu\text{g/ml}$ (48 h)	Decreased LPS- stimulated NO production (37.6- 81.2%), TNF α production, iNOS expression (possible mechanism), degradation of Ikb α , and phosphorylation of p38 MAPK. Therefore NFkB role in resveratrol suppression of NO and TNF α release. Decreased LPS- stimulated NO production in primary cells (59.6-71.2%) (363)
MCF-7 cells	10, 25 and 50 μM (72h)	Cell growth and proliferation suppressed (cell accumulation in S phase). NF-kB activation suppressed (330)
Splenic lymphocytes	25- 50 μM	Inhibits proliferation of mitogen-, IL-2, or alloantigen- induced splenic lymphocytes through inhibition of NFkB, IL-2, and TNF (364)
Rat PC12 pheochromocytoma cells	25- 100 μM 30 min pre-incubation	Induces apoptosis by increasing DNA fragmentation and decreasing NFkB (297)
MAPK FAMILY (ERK1/2, JNK1/2, P38 MAPK)		
Mouse JB6 epidermal cells	20 μM (30 min, 2-4h)	R activated ERK1/2, JNK1/2, p38 MAPK, and serine 15 p53 phosphorylation. Dominant negative mutant of ERK1/2 or p38 MAPK or addition of PD98059/ SB202190 repressed p53 phosphorylation at ser 15 and repressed p53- dependent transcriptional activity and apoptosis. Overexpression of dominant negative mutant of JNK1 had no effect (334)
Porcine coronary arteries	10- 200 μM (20 min)	Basal and ET-1 stimulation of ERK1/2, JNK1, and p38 MAPK phosphorylation inhibited. ERK1/2 activity and nuclear translocation also inhibited by R (50 μM). No effect on cAMP/PKA (involved in MAPK inhibition). Estrogen receptor blockers (tamoxifen) did not block MAPK inhibition. ET-1 stimulation of protein tyrosine phosphorylation also decreased (319)
HL- 60 cells	20 μM (30 min-24h)	Increased FasL expression (involved in apoptosis induction) and JNK and p38 MAPK activity with no effect on ERK1/2. Dominant negative constitutively expressed JNK blocked R- stimulation of JNK and FasL upregulation (75%), DNA fragmentation, and reduced apoptosis, without p38 MAPK involvement. Kinase- inactive ASK1 mutant transfected cells showed ASK1 as an upstream regulator of R- induced apoptosis. Cdc42 also activated (30 min) and translocated in response to R. Cdc42/ASK1/JNK cascade (365)
HeLa cells (human cervical squamous carcinoma)	25, 50 μM (60 min)	PMA and UVC- stimulated AP-1 reporter gene transcription (involved in tumour formation) inhibited. ERK2, JNK1, and p38 MAPK activation inhibited but with no effect on Raf-1, MEKK1, or MKK6. Inhibition of c-Src tyrosine kinase activity and PKC activation (366)
HUVEC (human umbilical vein)	1-5 μM (12, 24h)	VEGF- mediated HUVEC migration and tube formation inhibited with no effect on cell proliferation (role in angiogenesis). No effect on VEGF receptor, ERK1/2, p38 MAPK or Akt. Tyrosine phosphorylation of VE-

endothelial cells)		cadherin and β -catenin abolished. ROS- dependent Src kinase activation also inhibited (367)
SK-mel28 and A375 human melanoma cells	30, 60 or 100 μ M (24-72 h)	Increased phos of ERK1/2 in A375 cells to contribute to apoptosis and inhibit cell proliferation. No effect in ERK1/2 (SK-mel28), p38 MAPK or JNK (335)
JB6 mouse epidermal cell line C141, stable transfectants CMV-neo and dominant negative mutant-JNK1	20 μ M (2, 16, 24h)	R activated JNKs phos p53 and are required for R-induced p53 dependent transcription and subsequent apoptosis induction (336)
SH-SY5Y neuroblastoma cells	1pM- 10uM 30 min	Apoptosis induction and increase in ERK1/2 and reduction in caspase-7 (368)
COX1/COX2		
Human promyelocytic leukemia cells, carcinogen-treated mouse mammary glands (HL-60)	0.01, 0.10, 1, 10, and 100 μ M (24 days)	Inhibition of DMBA-induced preneoplastic lesions by decreasing COX1/2 activity in HL-60 cells (273)
MCF7, SW480, HCE7, Seg-1, Bic-1, and HL60 human cancer cell lines	30-300uM (2- 48h)	Growth inhibition except in Bic-1 cells. Induction of apoptosis in all cell lines (only 19% in Bic-1) with S phase arrest. Cox-2 expressed in Seg-1 only after 48h. β -catechin decrease after 24/48h in SW480 (349)
184B5/HER human mammary, MSK Leuk1 oral epithelial cells	2.5- 100 μ M (30 min- 4.5h)	R suppresses prostaglandin synthesis via inhibition of COX-2 isoform of COX. R inhibits COX-2 induction by phorbol esters through inhibition of PKC translocation from cytosol to the membrane (350)
Female Sprague-Dawley rats	100 μ g/rat (12- 15g/rat/day) (120 days)	Reduced incidence (45%), multiplicity (55%) and extended latency period of tumour development (mammary). Suppressed DMBA induced ductal carcinoma. R suppressed DMBA- induced COX2 and matrix metalloprotease- 9 expression in breast tumour. R suppressed DMBA-induced NF-kB activation (NF-kB regulates COX2)(330)

1.11 HYPOTHESIS/ RATIONALE

Skeletal muscle is responsible for more than 75% of glucose disposal in the postprandial state and is therefore quantitatively, the most important insulin target tissue (369). Defects in the muscle glucose transport system may lead to reduced insulin sensitivity or insulin resistance, which characterize type 2 diabetes (369;370). An in depth understanding of the glucose transport mechanism in skeletal muscle cells and the action of different compounds that stimulate glucose transport may provide new therapeutic approaches to overcome insulin resistance and type 2 diabetes mellitus.

Substantial epidemiological data have shown that dietary sources containing resveratrol, including red wine, may protect against cardiovascular disease and cancer. Epidemiological evidence indicates that the French have the lowest incidence of cardiovascular disease compared to the Western world despite diets high in saturated fat. This is termed the French Paradox. Further study associated this phenomenon with increased red wine consumption (371-373). According to the WHO, the prevalence of type 2 diabetes in Canada and the United States in 2002 was approximately 6.41% whereas the prevalence in France was significantly lower at 2.86% (374). For these reasons resveratrol may have potential as an anti- diabetic compound.

Numerous studies have linked enhanced production of ROS and its subsequent oxidative stress, to certain vascular diseases. ROS may also contribute to type 2 diabetes. Several antioxidants, including tempol and alpha lipoic acid, have previously demonstrated an increased rate of glucose transport in vascular endothelial/ smooth muscle cells (375) and L6 skeletal muscle cells (376) respectively. Green tea polyphenols also increase glucose transport in adipocytes (377). Antioxidants have demonstrated a

positive effect on glucose homeostasis. For example, lipoic acid releases the IL-1 β -induced inhibition of glucose-stimulated insulin secretion from islet cells (378).

Antioxidants are valuable in reducing insulin resistance. The antioxidant tempol improved angiotensin II-induced insulin resistance as shown by decreased glucose infusion in the hyperinsulinemic-euglycemic clamp and enhanced insulin-stimulated PI3K activation in rat liver and adipose tissue (379). Another antioxidant, tranilast, also exhibits effects against insulin resistance through inhibition of inflammatory cytokines such as TGF β , IL-2, and monocyte chemoattractant protein 1 (MCP-1) that are involved in pathogenesis of insulin resistance by preventing TGF β release from fibroblasts and monocytes (380;381) and suppressing NF κ B in rat mesangial cells (382). Several studies show that resveratrol has exceptional anti-oxidant (278;279;291;292;323), anti-thrombotic (305;306;310;321;383), and anti-cancer (273;324;328;384-386) properties both in vitro and in vivo. **In the present study it is hypothesized that, similar to insulin, (1) resveratrol increases glucose transport into skeletal muscle cells and (2) employs signalling molecules that are involved in insulin-stimulated glucose uptake.**

1.12 OBJECTIVES

An in vitro skeletal muscle model was used. The primary objectives of this study were (1) *to examine the direct effects of resveratrol on basal and insulin-stimulated glucose transport*, (2) *to examine the effects of resveratrol on GLUT4 glucose transporter translocation*, and (3) *to attempt to elucidate the resveratrol signalling pathway and its mechanism of action*. The secondary objectives of this study were (1) *to examine the effects of resveratrol on basal and insulin-stimulated amino acid transport* and (2) *on basal and insulin-stimulated mitogenesis*.

To address the primary objectives, the effect of resveratrol on glucose uptake, transporter distribution, and the employment of key signalling molecules involved in the insulin signalling pathway, such as PI3K, Akt/PKB, and PKC, were examined. Furthermore, the amino acid analogue methyl-aminoisobutyric acid (MeAIB) was used to examine amino acid transport while thymidine incorporation was used to study cell proliferation. This study provides the basis for understanding the biological effects of resveratrol on the glucose transport system of skeletal muscle cells. The knowledge gained from these studies may be used to design in vivo experiments to understand the resveratrol effects on glucose homeostasis. It also provides evidence of the effects of resveratrol on amino acid transport and cell proliferation.

CHAPTER 2: METHODOLOGY

2.1 MATERIALS

Minimum essential medium (α -MEM), fetal bovine serum (FBS), goat serum, trypsin, and antibiotic were purchased from GIBCO Life Technologies (Burlington, ON, Canada). HRP- conjugated anti- rabbit secondary antibody, Akt, ERK1/2, JNK1/2, p38 MAPK, and tyrosine antibodies (Total and Phospho- specific) and LumiGLO reagents were purchased from New England Biolabs (Mississauga, ON, Canada). 9E10 anti-myc monoclonal antibody was purchased from Santa Cruz (Santa Cruz, CA) and HRP-conjugated donkey anti- mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). LY294002, wortmannin, cycloheximide (CHX), phorbol-12-myristate-13-acetate (PMA), and bovine serum albumin were purchased from Calbiochem. Polyvinylidene difluoride (PVDF) membranes, molecular weight protein standards, and electrophoresis reagents were purchased from BioRad. [^3H]2- deoxy-D-glucose, [^{14}C]methylaminoisobutyric acid, and [^3H]thymidine were purchased from PerkinElmer (Boston, MA). Paraformaldehyde was purchased from Canemco (St. Laurent, Quebec). All other chemicals, including cytochalasin D (CD), Go6983, bisindolylmaleimide 1 (BIM1), cytochalasin B (CB), cold methylaminoisobutyric acid, cold 2-deoxy-D-glucose, ammonium chloride, trichloroacetic acid (TCA), and orthophenyldiene reagent (OPD), were purchased from Sigma Chemicals (St. Louis, MO). Both the parental and GLUT4*myc* overexpressing L6 cells were a kind gift from Dr. A. Klip (Hospital for Sick Children, Toronto, ON).

2.2.1 PARENTAL L6 SKELETAL MUSCLE CELLS

L6 cells, which are derived from 3- day old rat hindlimb skeletal muscle, proliferate as mononucleated myoblasts in high serum (10% FBS) and spontaneously differentiate in low serum (2% FBS) through cellular fusion from myoblasts into multinucleated primary myotubes (387;388). The myotubes express the insulin receptor and insulin- like growth factor-I receptor (IGF-I) and several other proteins that are typical of skeletal muscle including the GLUT4 glucose transporter (389). As L6 myoblasts differentiate, the number of insulin receptors, GLUT4 transporter expression, and insulin responsiveness increases.

This cell line also expresses GLUT3 that is found in fetal muscle and neuronal tissue (390) and GLUT1 that is ubiquitously expressed in mature skeletal muscle but are not as abundant as the GLUT4 transporter (391). The ratio of GLUT1 to GLUT4 in the plasma membrane at rest is 1:0.8 (392;393). In this cell line, insulin treatment results in a two- fold increase in glucose transport within 15 minutes of exposure (394) and the translocation of glucose transporters from an intracellular pool to the plasma membrane (395-398).

Cultured cells have been used extensively to examine both hormonal and metabolic processes. The cell culture system has many advantages when studying molecular mechanisms of hormone action and transport function/ regulation. For example, it is a homogeneous population of muscle cells with limited intracellular space, unlike intact tissue preparation. Another advantage is that this model allows for isolated treatment, where the external environment is very tightly controlled and not influenced by other hormones/ factors found in in vivo studies. In addition, cell cultures have an

extended viability and may be used to examine acute as well as more chronic effects of a substance/ chemical under study.

Human studies are very invasive and the isolation of skeletal muscle strips is possible only during a surgical procedure, therefore greatly limiting availability. During removal of the muscle, there is a risk of compromising membrane integrity (399). Whole tissue cultures have limited viability (2-3 hrs) and significant diffusional limitations may influence hormone action or the effects of experimental chemicals/ drugs (400). Furthermore, physiological concentrations of insulin have been ineffective in stimulating glucose transport in primary cell culture and cultures of diverse origin (401;402). Primary muscle cell culture is not widely used and is difficult to establish.

Apart from L6 cells, rat pheochromocytoma cells (PC12) have also been used as a model of muscle in a limited number of studies, however these cells are derived from neural tissue and are not a good representation of skeletal muscle. PC12 cells are primarily used as a neuronal cell model system to study GLUT3 regulation (403). Mouse C₂C₁₂ cells are another model of skeletal muscle but these cells predominantly express the GLUT1 glucose transporter with low expression of GLUT4 (404;405) and most importantly lack insulin- stimulated glucose uptake (406).

Although L6 cells express higher ratios of GLUT1 and GLUT3 transporters and less GLUT4 than adult rat muscles, which is not an absolute representation of adult skeletal muscle (399;407), these cells have many skeletal muscle morphological and functional characteristics. The L6 cell line is one of few cell lines that differentiate in culture and is the only cell line that expresses GLUT1, GLUT3, and GLUT4 (389;408). L6 cells are widely used and the best available system representing skeletal muscle (387).

The cells grow in monolayers, which makes them readily and easily accessible to substrates and therefore a good model for transport studies.

2.2.2 GLUT4 OVEREXPRESSING L6 SKELETAL MUSCLE CELLS

The L6 GLUT4*myc* overexpressing cell line was constructed by inserting a human *myc* epitope tag within the first exofacial loop of the GLUT4 transporter (409). The *myc* tag does not interfere with GLUT4 activity (410) (Figure 11). These cells differentiate normally from myoblasts to fused myotubes, at which stage they respond to insulin with a two- fold stimulation of glucose uptake and a two- fold translocation of GLUT4*myc* (409). The K_m of glucose uptake is similar in L6 GLUT4*myc* cells to that of the parental L6 cells (156;394). Similar to L6 parental cells, L6 GLUT4*myc* cells respond to insulin by IRS-1 phosphorylation and subsequent PI3K and Akt/PKB activation, which are necessary for stimulation of glucose uptake (411).

GLUT1 expression is the same in L6 GLUT4*myc* cells as in parental L6 cells (392), however it is in low abundance compared to GLUT4*myc*. The ratio of GLUT4*myc* to GLUT4 and GLUT1 is 124:1:1.2 (392;412;413). GLUT4*myc* mediates both basal and insulin- stimulated glucose uptake predominantly, where the ratio of surface GLUT4*myc* to total cellular GLUT4*myc* is 1: 2.6 (409). This was established by almost complete inhibition of basal and insulin- stimulate glucose transport with indinavir, a selective inhibitor of GLUT4 mediated glucose influx (392;414). However, the possibility that GLUT1 may also contribute to glucose uptake cannot be fully eliminated. In the basal state, 90% of GLUT4*myc* is sequestered intracellularly and a significant portion is translocated to the cell surface in response to insulin (415). GLUT4*myc* recycling is the same as that of endogenous GLUT4 (416).

2.3 CELL CULTURE TECHNIQUE

L6 rat skeletal muscle cells (parental and GLUT4*cm*yc overexpressing) were grown in α -MEM (pH 7.4) containing 5mM glucose, 2% (v/v) FBS, and 1% (v/v) antibiotic- antimycotic solution (100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B) in a humidified atmosphere of 5% CO₂- 95% air at 37°C as previously described (417). Cells were grown in flasks and seeded to 12- well plates for glucose uptake measurements. The cells were allowed to reach confluence, align, and fuse into myotubes before being used for glucose and amino acid experiments. Undifferentiated myoblasts were used for the thymidine incorporation experiments. Prior to experiments, the cells were growth- arrested by incubation with serum- free α -MEM for 5 h. Serum deprivation decreases the rate of basal transport so that the cells are not at maximum capacity before stimulation, thereby making an increase more visible (418).

2.4 CELL TREATMENT

Resveratrol, CHX, PMA, and CD stock solutions were prepared using 100% ethanol, insulin stock solution, which was used as a positive control, was prepared in 2% FBS- containing media, while LY294002, wortmannin, BIM1, and Go6983 were all prepared in DMSO. The final concentration and the time of incubation for each compound are indicated in each figure. A vehicle- treated control group was used in parallel with the treated groups.

2.5 DETERMINATION OF 2- deoxy-D-glucose (2DG) UPTAKE.

At the end of the incubation period, the medium was removed, the cells were rinsed three times with HEPES- buffered saline solution (HBS; 140mM NaCl, 5mM KCl, 20mM HEPES, 2.5mM MgSO₄, and 1mM CaCl₂, pH 7.4), and subsequently, 2- deoxy-D-glucose uptake measurements were carried out for 10 minutes in HEPES- buffered

saline containing $10\mu\text{M}$ 2- ^3H]deoxy-D-glucose (417). Nonspecific uptake of glucose was determined in the presence of $10\mu\text{M}$ cytochalasin B and subtracted from total uptake (absence of CB) to determine specific carrier-mediated glucose transport. The transport assay was terminated by washing the cells three times with 1ml ice- cold 0.9% NaCl solution, followed by solubilization of the cells with 0.05N NaOH and radioactivity counting. All experiments were assayed in triplicate and performed 4- 8 times. Cellular protein content was measured by the BioRad Protein Assay method. Because of the structure of 2DG, it is not metabolized by the cell and remains intact for scintillation counting. The first 10 minutes of 2DG transport has been shown to be limited by transport alone.

2.6 DETERMINATION OF METHYLAMINOISOBUTYRIC ACID (MeAIB) UPTAKE.

At the end of the incubation period, the medium was removed, the cells were rinsed three times with HEPES- buffered saline solution (HBS; 140mM NaCl, 5mM KCl, 20mM HEPES, 2.5mM MgSO₄, and 1mM CaCl₂, pH 7.4), and subsequently, methylaminoisobutyric acid uptake measurements were carried out for 10 minutes in HEPES- buffered saline containing $10\mu\text{M}$ [^{14}C]methylaminoisobutyric acid. Nonspecific uptake of MeAIB was determined in the presence of 10mM cold MeAIB and subtracted from total uptake to determine specific carrier-mediated amino acid transport. The transport assay was terminated by washing the cells three times with 1ml ice- cold 0.9% NaCl solution, followed by solubilization of the cells with 0.05N NaOH and radioactivity counting. All experiments were assayed in triplicate and performed 4- 8 times. Cellular protein content was measured by the Bio Rad Protein Assay method.

2.7 WESTERN BLOT ANALYSIS

At the end of the treatment period, the cells were rinsed three times with HBS and then lysis buffer (20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM $\text{Na}_4\text{O}_7\text{P}_2$, 1mM β -glycerolphosphate, 1mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin, 1mM PMSF) was added and the lysate was scraped off and solubilized in electrophoresis sample buffer, followed by separation by sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis. The samples were subsequently transferred electrophoretically to PVDF membranes.

The membranes were incubated for 1 h at room temperature with 5% (w/v) nonfat dry milk in Tris- buffered saline and then overnight at 4°C with the primary antibody. The following primary antibodies were used: Phospho- Akt (Ser473) polyclonal antibody (1:1000), Total- Akt polyclonal antibody (1:1000), Phospho- p38 MAPK polyclonal antibody (1:1000), Total- p38 MAPK polyclonal antibody (1:1000), Phospho ERK1/2 polyclonal antibody (1:1000), Total- ERK1/2 polyclonal antibody (1:1000), Phospho-SAPK/JNK polyclonal antibody (1:1000), Total- SAPK/JNK polyclonal antibody (1:1000), and Phospho- Tyrosine monoclonal antibody (1:2000). The primary antibody was detected with either the HRP- conjugated anti- rabbit secondary antibody (1:2000) or the HRP- conjugated anti- mouse secondary antibody (1:2000), and LumiGLO reagent (NEB) and visualized by autoradiography using Kodak XAR-5 film.

2.8 MEASUREMENT OF GLUT4_{myc} TRANSLOCATION

The amount of myc- tagged GLUT4 at the surface of intact cells was measured by an antibody- coupled colorimetric assay as previously described (409) (Figure 11). Following the treatment period, the monolayer of myotubes was fixed with 3% paraformaldehyde for 3 min at room temperature, incubated with 1% glycine for 10 min

to reduce the remaining paraformaldehyde, blocked with 10% goat serum and 3% bovine serum albumin in PBS, and then exposed to anti-myc antibody (1:100) for 60 min, and followed by incubation with peroxidase- conjugated donkey anti- mouse IgG (1:1000) for 30 min, all at 4°C. Cells were washed extensively, and 1 ml of OPD reagent was added for 30 min at room temperature. The reaction was stopped by 0.25 ml of 3N HCl. The supernatant was collected, and the absorbance was measured at 492 nm. Nonspecific IgG binding, as measured by a peroxidase- conjugated anti- mouse IgG, was subtracted from experimental values.

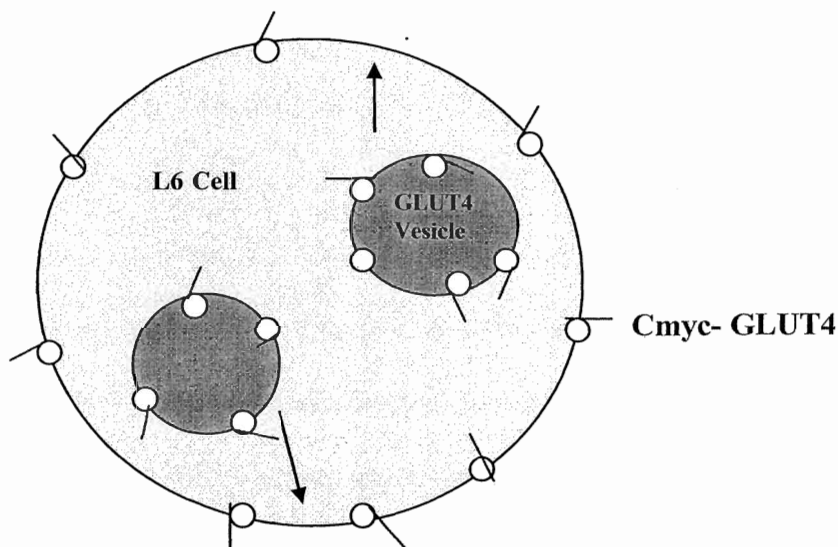


Figure 11: The GLUT4 myc transfected L6 cell.

2.9 MEASUREMENT OF THYMIDINE INCORPORATION

L6 myoblasts were used for experimentation. Subconfluent myoblasts (~50%) were serum deprived (0.1% FBS in α -MEM) for 24 h in order to bring the cells to a quiescent state. This was followed by cell treatment for 12 h in 0.1% FBS. 10 μ M [3 H]thymidine was then added to each well in parallel for an additional 12 hours. [3 H]thymidine is incorporated into the cell and remains intact. Total incubation time for all compounds was 24 hours.

At the end of the incubation period, the media was removed, the cells were rinsed three times with ice- cold HBS, and subsequently, the unincorporated [3 H]thymidine was precipitated out of the cells with 10% trichloro- acetic acid (TCA) for 10 minutes at 4 $^{\circ}$ C (317;356;360;419). The 10% TCA was aspirated and the cells were rinsed twice with ice- cold HBS, followed by solubilization of the cells with 0.05N NaOH and radioactivity

counting. All experiments were assayed in triplicate and performed 4- 6 times. Cellular protein content was measured by the BioRad Protein Assay method.

2.10 STATISTICAL ANALYSIS

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS). All data from several experiments were pooled and then presented as mean \pm SEM. The means of the groups were compared with either the student's independent samples t- test or the one- way analysis of variance (ANOVA).

CHAPTER 3: Section 1: RESULTS

3.1.1 Concentration and time- dependent effects of resveratrol on glucose uptake.

Antioxidants, such as tempol and alpha lipoic acid, and green tea polyphenols have previously demonstrated increased rates of glucose transport in various cell lines (375-377). Furthermore, resveratrol is a polyphenol with exceptional anti-oxidant (278;279;291;292;323) properties both in vitro and in vivo. We hypothesized that resveratrol may stimulate glucose transport in L6 skeletal muscle cells. To examine this hypothesis, L6 myotubes were incubated with resveratrol 10, 25, 50, 100, and 125 μ M for 120 min as indicated in Figure 12A and followed by glucose transport measurements.

Significant increases in glucose uptake were seen with 25 μ M resveratrol ($139 \pm 5.61\%$ of control, $p < 0.05$). Maximum stimulation by resveratrol ($201 \pm 8.90\%$ of control, $p < 0.001$) was reached at a concentration of 100 μ M. Higher concentrations, 125 μ M ($200 \pm 12.61\%$ of control, $p < 0.001$) and 150 μ M (data not shown), did not result in any greater stimulation of glucose uptake. Cell morphology was observed microscopically and was not affected by treatment. It is important to note that noncarrier- mediated glucose uptake, as determined by CB administration, was approximately 5% of the total uptake and was not significantly affected by cell treatment in all experiments presented in the results section.

To examine whether the response of the cells to resveratrol is time- dependent, the cells were incubated with 100 μ M resveratrol for 15, 30, 60, 90, and 120 minutes (Figure 12B). Resveratrol increased glucose uptake in L6 cells to significant levels within 30 minutes of incubation ($141 \pm 6.50\%$ of control, $p < 0.05$). Maximum stimulation by resveratrol was observed by 120 min ($201 \pm 8.90\%$ of control, $p < 0.001$), and in all

subsequent experiments 120 min incubation was used. In parallel experiments, insulin (10^{-7} M, 30 min) increased glucose transport by $208 \pm 10.87\%$ compared to control ($p < 0.001$), which is similar to that seen with the maximal resveratrol response (Figure 12C). These results suggest that resveratrol significantly stimulates glucose uptake in a dose- and time- dependent manner independent of insulin.

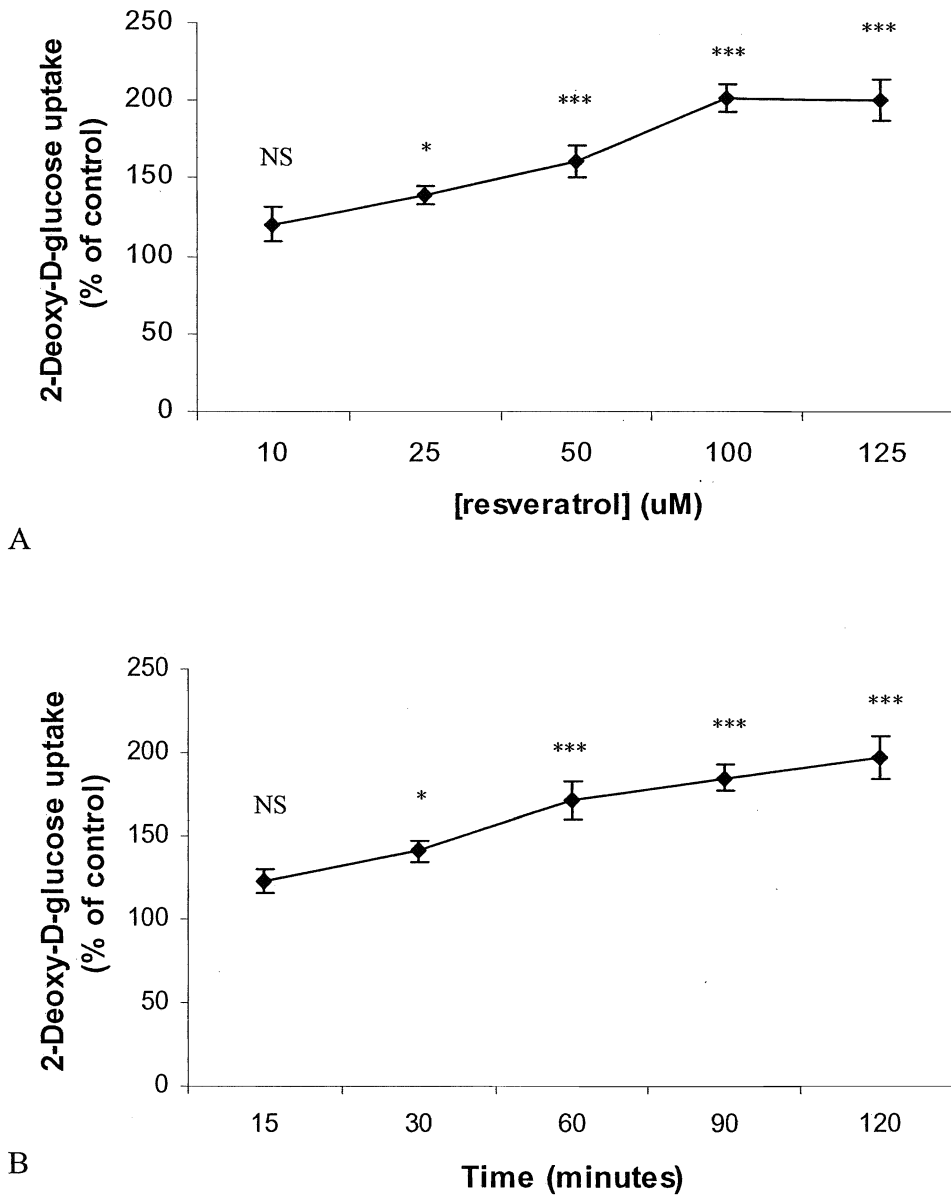


Figure 12: (A) Effects of resveratrol on 2DG uptake in L6 myotubes: dose response. L6 myotubes were incubated with the indicated concentrations of resveratrol for 120 min at 37°C. (B) Effects of resveratrol on 2DG uptake in L6 myotubes: time course. L6 myotubes were incubated for the indicated time periods with resveratrol (100 μ M) at 37°C. After treatment the cells were washed and [3 H]2DG uptake was measured. The results are the mean \pm SE of 4-7 independent experiments each performed in triplicate and expressed as a percent of control, * p <0.05, *** p <0.001, NS = not significant compared to untreated control.

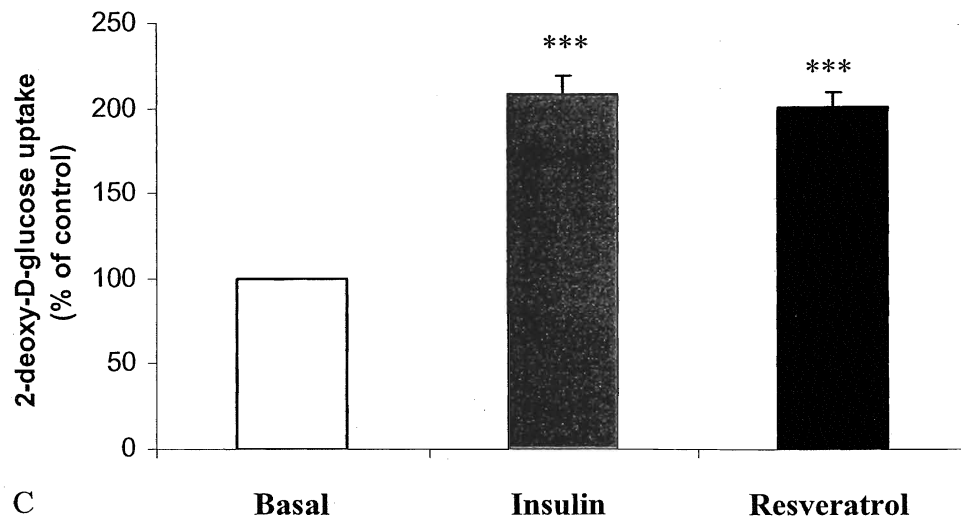


Figure 12: (C) Effects of insulin and resveratrol on 2DG uptake in L6 myotubes. L6 myotubes were incubated with either 100nM insulin (30 min) or 100 μ M resveratrol (120 min) at 37°C. Cells were washed and [3 H]2DG uptake was measured. The results are the mean \pm SE of 4-7 independent experiments each performed in triplicate and expressed as a percent of control, *** p <0.001 compared to untreated control.

3.1.2 Effect of resveratrol treatment on insulin- stimulated glucose uptake.

Several anti- diabetic agents, including metformin, result in an additivity in glucose transport when combined with insulin (420). To investigate whether the action of resveratrol to increase glucose uptake affects the acute insulin- induced increase in glucose transport, we examined the interaction between acute insulin exposure and resveratrol treatment (Figure 13). Insulin was added to the cells for 30 min before the measurement of glucose uptake in the control and resveratrol- pretreated cells. Insulin concentrations of 10^{-10} , 10^{-9} , 10^{-8} , and 10^{-7} M were used. Resveratrol treatment was continued in parallel with insulin treatment.

Insulin alone at 10^{-8} M and 10^{-7} M for 30 min resulted in a significant increase in glucose uptake ($209 \pm 9.96\%$; $227 \pm 12.52\%$ of control; $p < 0.001$). Treatment of myotubes for 120 min with resveratrol (100uM) and insulin added for the final 30 min, resulted in a significant increase in glucose uptake above basal values at all insulin concentrations used. No significant differences ($p > 0.05$) were observed between resveratrol- treated and non- treated cells at maximal insulin concentrations. However, a statistically significant difference ($p < 0.01$) was observed between the responses of the cells treated with insulin alone and those treated with both compounds at submaximal insulin levels (Figure 13). Thus, there is an apparent additivity of resveratrol with submaximal insulin concentrations.

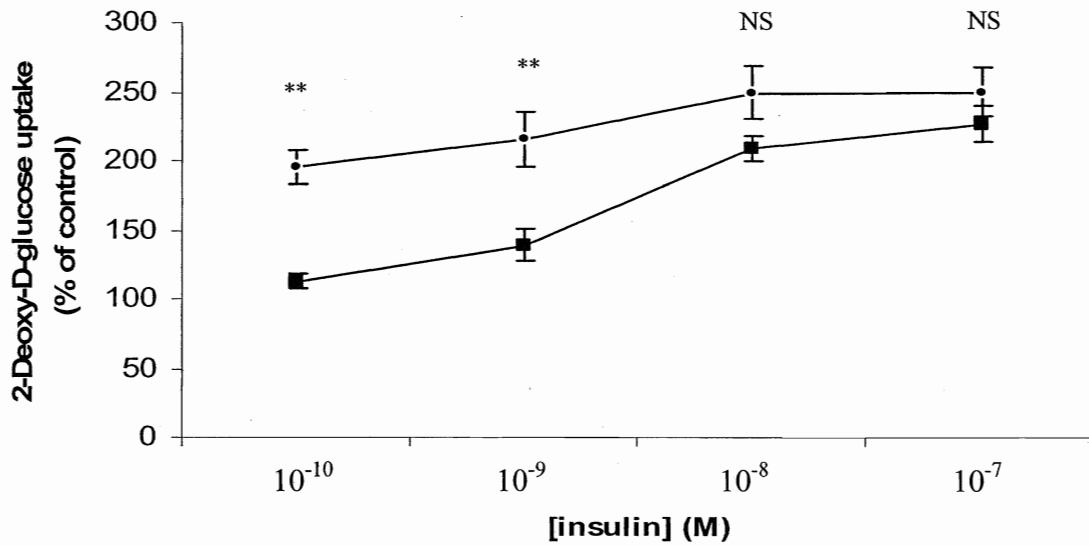


Figure 13: Effects of resveratrol treatment on insulin-stimulated 2DG uptake. L6 myotubes were incubated without (■) or with 100 μM resveratrol (90 min) (●), followed by the addition of insulin in parallel for 30 min at the indicated concentrations. This was followed by the [³H]2DG uptake assay. The results are the mean ± SE of 6-8 independent experiments performed in triplicate and expressed as a percent of control, ** $p < 0.01$, NS = not significant compared to control (resveratrol untreated cells).

3.1.3 Effect of cycloheximide (CHX) on resveratrol- stimulated glucose uptake.

Acute insulin stimulation in L6 myotubes is independent of protein synthesis and is CHX insensitive (394). The slightly increased response time observed with resveratrol treatment compared to insulin suggested that resveratrol action might induce the synthesis of a protein relevant for the stimulation of glucose uptake. In order to test this hypothesis, we examined the effects of resveratrol on glucose uptake in the presence of 1 µg/ml CHX. CHX inhibits translational elongation by competitively inhibiting peptidyl transferase, resulting in inhibition of protein synthesis. This concentration of CHX has previously been demonstrated to effectively block protein synthesis over 24 h in L6 cells, as measured by [³⁵S]methionine incorporation into protein (393).

Myotubes were incubated with 1 µg/ml CHX followed by resveratrol (100 µM, 120 min) or insulin (10⁻⁷ M, 30 min) treatment. Total incubation with CHX was 130 min in all groups. CHX did not demonstrate any significant effects on basal glucose uptake (101 ± 6.38% of untreated control, *p*>0.05). Acute insulin treatment stimulated glucose uptake to 205 ± 9.70% (*p*<0.001) of control and was not affected by CHX (211 ± 7.04% of control, *p*<0.001). The stimulation of glucose uptake was 182 ± 7.55% (*p*<0.001) of the control value in the presence of resveratrol alone (100 µM, 120 min) and stimulations of similar magnitude were observed with resveratrol in the presence of CHX (172 ± 8.54% of control, *p*<0.001) (Figure 14). The increase in glucose uptake induced by resveratrol was not prevented. These results suggest that resveratrol- induced glucose uptake is not dependent on protein synthesis.

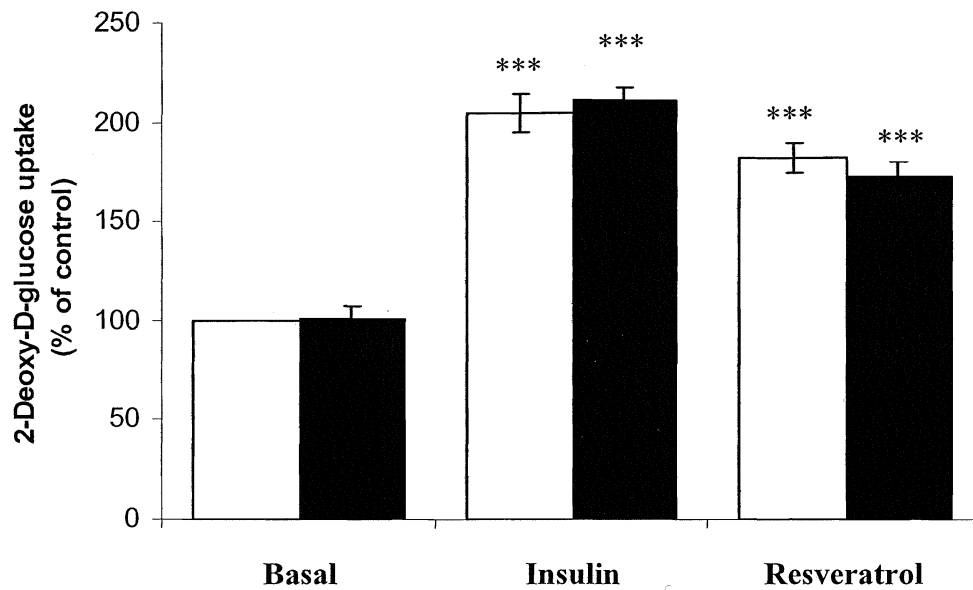


Figure 14: Effect of cycloheximide on resveratrol- and insulin- stimulated glucose transport. L6 myotubes incubated without (□) or with 1 μg/ml CHX (■) were stimulated with 100 μM resveratrol (120 min) or insulin (10^{-7} M, 30 min). Total treatment with CHX was 130 min. Results are the mean \pm SE of 7-8 independent experiments performed in triplicate and expressed as a percent of control, *** p < 0.001 compared to untreated control.

3.1.4 Effect of PI3K inhibitors on resveratrol- stimulated glucose uptake.

It is well known that stimulation of glucose uptake by insulin requires PI3K. Since resveratrol demonstrated a similar action to that of insulin, we hypothesized that PI3K may also contribute to the elevated levels of glucose uptake observed. To examine our hypothesis, we used specific PI3K inhibitors. LY294002 (100uM), a quercetin derivative that acts as a competitive inhibitor of the ATP binding site of PI3K (41), and wortmannin (1μM), a fungal metabolite that exerts its inhibition by binding to the p110 catalytic subunit of PI3K (38;39), were added to the cells 15 min prior to the addition of resveratrol (100uM, 60 min). Figure 15A shows that both insulin- ($221 \pm 7.24\%$ of control, $p < 0.001$) and resveratrol- ($167 \pm 14.13\%$ of control, $p < 0.001$) stimulated glucose uptake were abolished by LY294002 to $103 \pm 7.44\%$ and $69 \pm 17.39\%$ of control respectively. Similar results were obtained with wortmannin for both insulin and resveratrol, $126 \pm 9.93\%$ and $113 \pm 7.34\%$ of control.

To examine the effectiveness of wortmannin to inhibit PI3K, we analyzed Akt/PKB phosphorylation of insulin- stimulated cells pretreated with wortmannin through Western blot analysis. Under our experimental conditions, wortmannin effectively blocked insulin- stimulated Akt/PKB phosphorylation (C = 1.0; I = 5.5; W = 1.2; W + I = 1.2 arbitrary densitometry units compared to control) (Figure 15B), indicating an effective inhibition of PI3K activity. These data (Figure 15A, B) suggest that PI3K is involved in the regulation of glucose uptake by resveratrol.

A

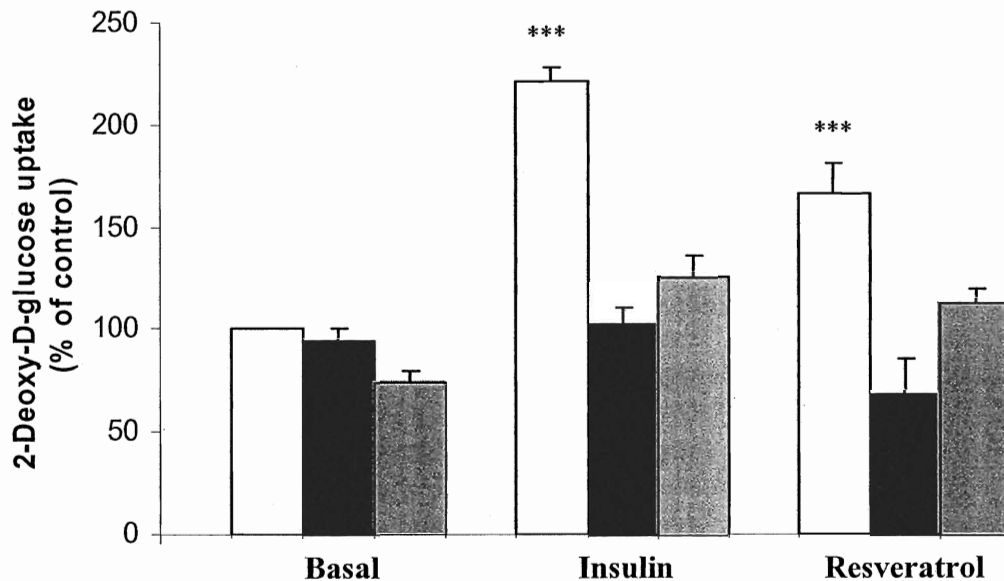


Figure 15A: Effect of LY294002 and wortmannin on insulin- and resveratrol-stimulated glucose transport. L6 cells were pre-incubated without (\square) or with 100 μ M LY294002 (\blacksquare) or 1 μ M wortmannin (\square), followed by the addition of resveratrol (100 μ M, 60 min) or insulin (10^{-7} M, 30 min). Total incubation with LY294002 and wortmannin was 75 minutes. The results are the mean \pm SE of 4 to 7 independent experiments performed in triplicate and expressed as a percent of control, *** p <0.001 compared to untreated control.

B

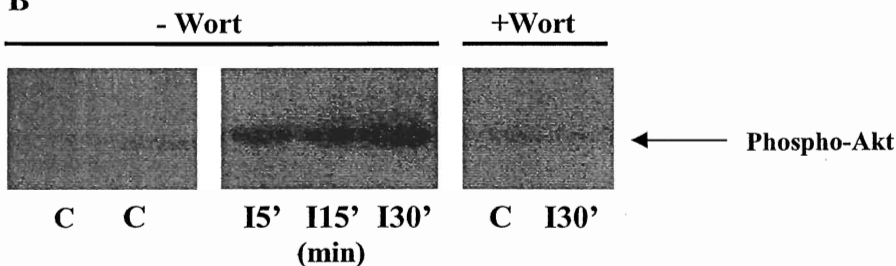


Figure 15B: Akt/PKB phosphorylation by insulin. Total cell lysates were prepared from L6 muscle cells that were treated for the indicated time with or without 10^{-7} M insulin or pre-treated with 1 μ M (15 min) wortmannin. Lysates (12.5 μ g) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-Akt/PKB. Representative immunoblot of 1 experiment. Immunoblot was scanned to quantitate the density of the bands where all values are arbitrary densitometric units expressed relative to untreated control cells. Note: Data is from the same original blot, which was modified to exclude extraneous information.

3.1.5 Effect of resveratrol on Akt/PKB phosphorylation.

In order to further delineate the mechanism by which resveratrol raises the rate of glucose uptake into the cell, we examined elements involved in the insulin- signalling pathway further downstream. Akt/PKB phosphorylation and subsequent activation has been shown in several studies to be a step downstream of PI3K in insulin- stimulated glucose transport (92;421), so we tested whether resveratrol employs Akt/PKB. We examined the effect of insulin and/ or resveratrol on Akt/PKB phosphorylation at the Ser473 residue. Western blot analysis revealed that stimulation of the cells with insulin for 5, 15 or 30 min resulted in significant Akt/PKB phosphorylation (5.84 ± 1.36 ; 5.36 ± 1.41 ; 5.14 ± 1.18 arbitrary densitometry units relative to untreated control, $p < 0.05$). Unlike insulin, stimulation of the cells with resveratrol (100 μ M) for 5, 15, 30, 60, and 120 min did not phosphorylate Akt/PKB ($p > 0.05$) (Figure 16A upper blot). In addition, resveratrol did not significantly affect insulin- induced Akt/PKB phosphorylation (4.31 ± 0.77 arbitrary densitometry units relative to untreated control, $p < 0.05$). The total levels of Akt/PKB were not affected by any treatment (Figure 16A lower blot). From the results presented here, it appears that the mechanism of action of resveratrol is PI3K- dependent but Akt/PKB- independent.

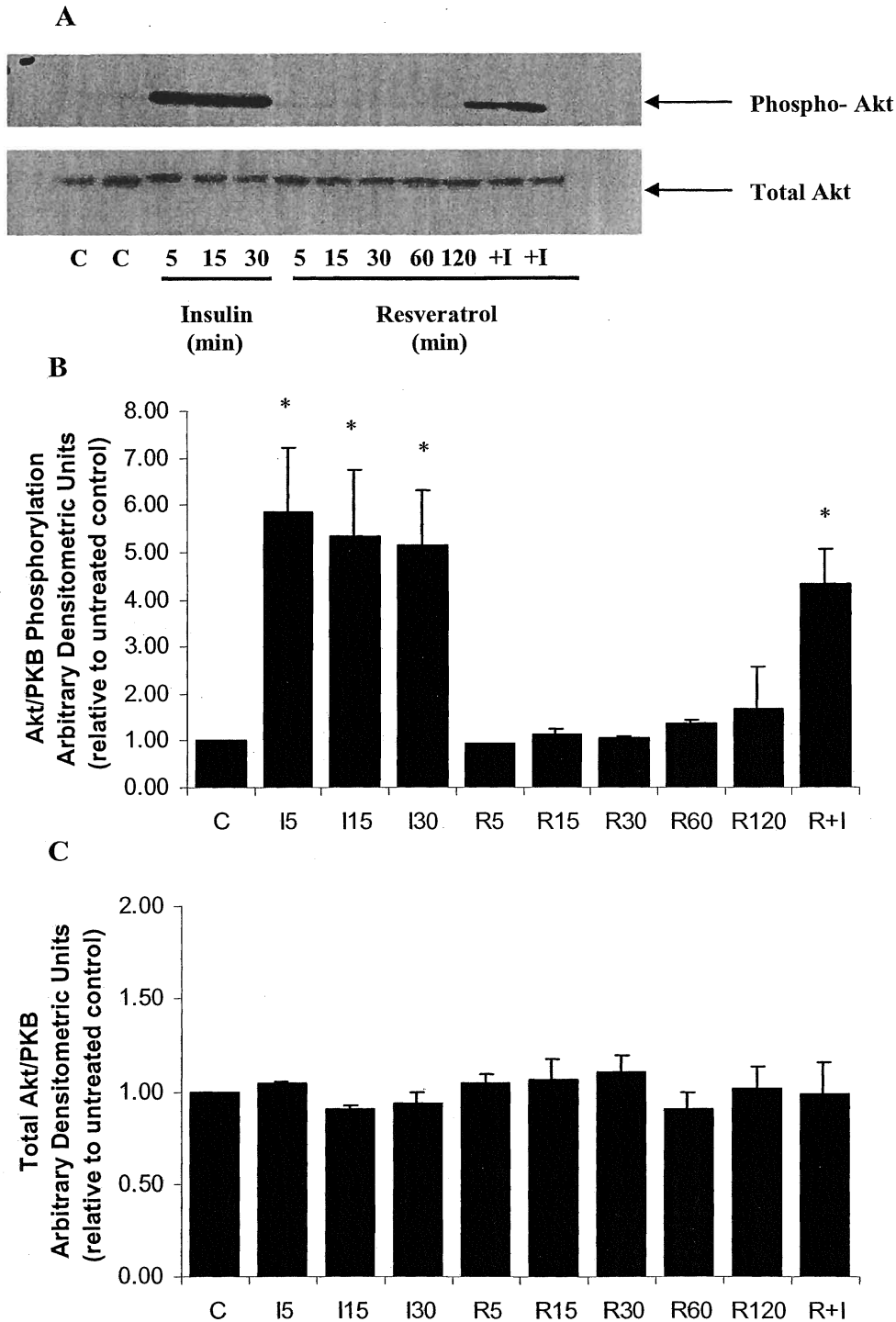


Figure 16: (A) Akt/PKB phosphorylation by insulin and/or resveratrol. Total cell lysates were prepared from L6 muscle cells that were treated for the indicated time (min) with or without 10^{-7} M insulin or 100μ M resveratrol or insulin and resveratrol combined. Lysates (12.5μ g) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-Akt or anti-total-Akt antibody. Representative immunoblot of 3 independent experiments. (B, C) Immunoblots were scanned to quantitate the density of the bands. Results are the mean \pm SE of 3 independent experiments. All values are arbitrary densitometric units expressed relative to untreated control cells, $*p < 0.05$ compared to untreated control.

3.1.6 Effect of PKC inhibitors on resveratrol- stimulated glucose transport.

Several PKC isoforms have been identified as downstream targets of PI3K activation, including PKC λ and PKC ζ , which have been shown to be involved in insulin-stimulated glucose transport (223-225). It has also been documented in skeletal muscle cells that acute activation of PKC by PMA, a DAG analogue, leads to increased glucose uptake (422;423). Since resveratrol employs PI3K but does not involve Akt/PKB activation, it was important to examine a possible alternative pathway that may involve PI3K and PKC to affect glucose transport. We hypothesized that members of the PKC family may be involved in resveratrol- stimulated glucose transport. To examine this hypothesis, we used the PKC inhibitors BIM1 (1 μ M, 60 min) and Go6983 (500nM, 20 min), followed by stimulation with PMA (1 μ M, 30 min) or resveratrol (100 μ M, 60 min). BIM1 and Go6983 are known inhibitors of the conventional, novel, and atypical isoforms of PKC. Both BIM1 and Go6983 act as competitive inhibitors for the ATP- binding site of PKC. BIM1 and Go6983 show high selectivity for different PKC isoforms, however at the concentrations used in this study they both effectively inhibit all PKC isoforms (423-426).

Acute exposure to PMA resulted in stimulation of glucose transport ($159 \pm 6.47\%$ of control, $p < 0.001$) to levels similar to those of resveratrol ($169 \pm 8.00\%$ of control, $p < 0.001$). BIM and Go6983 did not have a significant effect on basal glucose transport ($99 \pm 4.00\%$; $104 \pm 3.31\%$ of untreated control, $p > 0.05$). Both compounds significantly abolished the PMA- stimulated glucose transport ($109 \pm 3.99\%$; $106 \pm 2.65\%$ of control, $p > 0.05$), indicating that they are effective inhibitors of PKC. However resveratrol-stimulated glucose transport was not inhibited by BIM or Go6983 ($169 \pm 6.71\%$; $159 \pm$

9.63% of control ($p < 0.001$). The PKC inhibitors did not affect glucose transport stimulation by resveratrol (Figure 17) and these data indicate that PKC does not have a significant role in resveratrol-stimulated glucose transport.

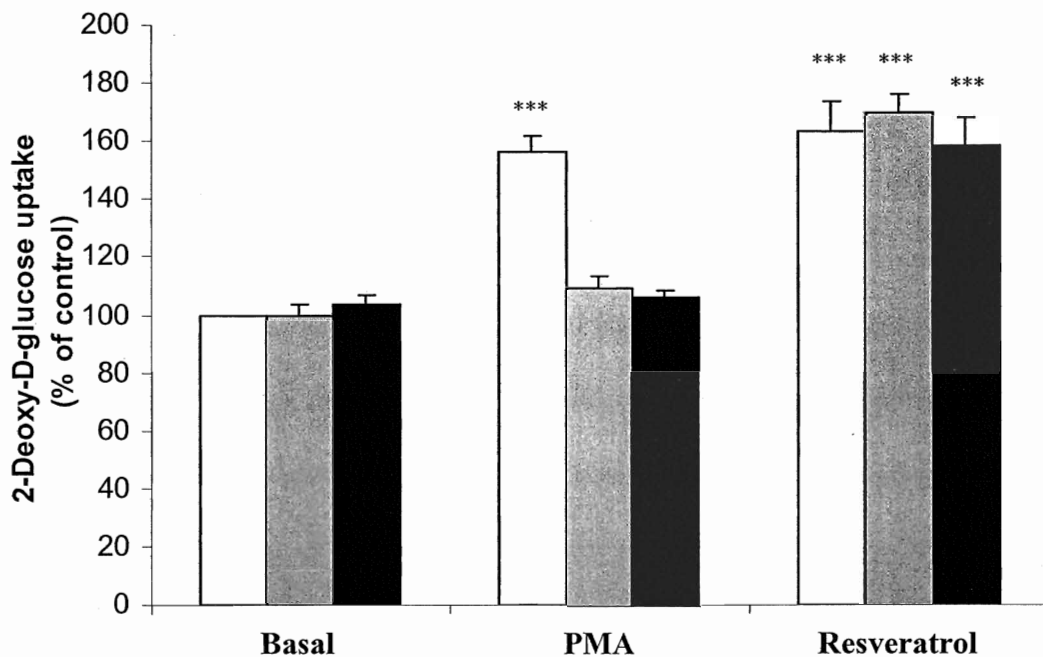


Figure 17: Effects of BIM1 and Go6983 on PMA- and resveratrol-stimulated glucose transport. L6 cells were pre-incubated without (□) or with 1 μM BIM1 (▒) or 500 nM Go6983 (■), followed by the addition of resveratrol (100 μM, 60 min) or PMA (1 μM, 30 min). Total incubation with BIM1 and Go6983 was 120 and 80 minutes respectively. The results are the mean \pm SE of 4-8 independent experiments performed in triplicate and expressed as a percent of control, *** $p < 0.001$ compared to untreated control.

3.1.7 Effect of resveratrol on glucose transporter translocation- GLUT4.

Insulin- stimulated glucose transport requires GLUT4 translocation. To elucidate the mechanism by which resveratrol altered glucose uptake, we measured surface GLUT4 levels under the various treatments. L6 GLUT myc cells stably express GLUT4 tagged with an exofacial myc epitope (GLUT myc) (409). GLUT4 myc segregates, cycles, and responds to insulin in a manner similar to endogenous GLUT4. The amount of GLUT myc incorporated into the plasma membrane was quantitated by immunologically labelling the myc epitope at the surface of intact cells.

Acute maximal insulin ($10^{-7}M$, 30 min) stimulation in the absence of resveratrol increased the amount of GLUT4 myc epitope at the surface of intact cells to $194 \pm 11.66\%$ of untreated control cells ($p < 0.001$) (Figure 18A). However, unlike that of insulin, resveratrol treatment (100uM, 120 min) did not cause a significant increase in recruitment of GLUT4 myc to the plasma membrane ($78 \pm 9.81\%$ of control, $p > 0.05$). In addition, resveratrol did not have any significant effect on insulin- stimulated GLUT4 translocation at both maximal and submaximal hormone concentrations (Figure 18B). These results suggest that resveratrol- stimulated glucose uptake is not due to GLUT4 translocation.

A

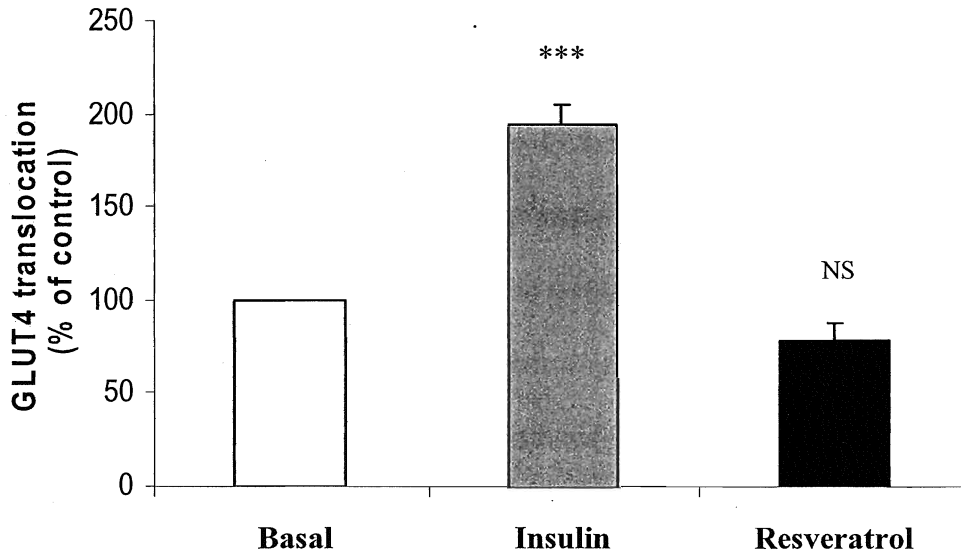


Figure 18A: Effect of insulin or resveratrol on plasma membrane GLUT4 levels in GLUT4*cmyc* overexpressing myotubes. Cells were treated with resveratrol (100 μ M, 120 min) (■) or insulin (10⁻⁷M, 30 min) (▨). GLUT4 transporter translocation was determined following incubation. The results are the mean \pm SE of 4-5 independent experiments performed in triplicate and expressed as a percent of control, *** p <0.001, NS = not significant compared to untreated control.

B

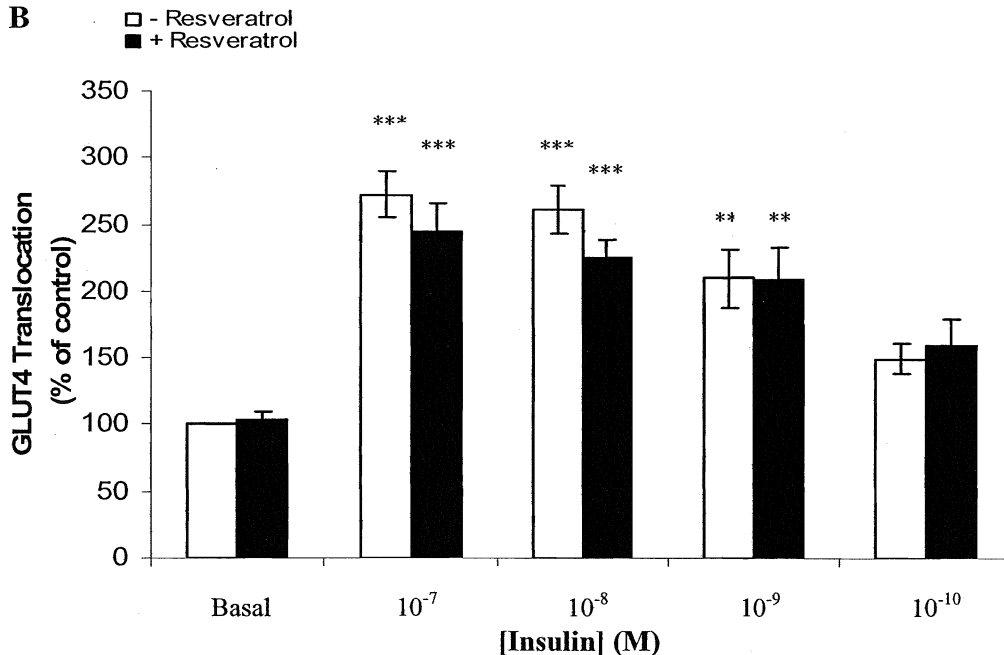


Figure 18B: Effect of resveratrol on insulin-mediated GLUT4 translocation in GLUT4*cmyc* overexpressing myotubes. Cells were treated with resveratrol (100 μ M, 120 min) (■), followed by treatment with insulin (30 min) at the indicated concentrations. GLUT4 transporter translocation was determined following incubation. The results are the mean \pm SE of 4 experiments performed in triplicate and expressed as a percent of control, ** p <0.01, *** p <0.001 compared to untreated control.

3.1.8 Effect of resveratrol on glucose uptake in GLUT4*myc* overexpressing cells.

Since resveratrol-stimulated glucose transport was observed in parental L6 cells and GLUT4 translocation was examined in GLUT4*myc* overexpressing cells, it was important to ensure that resveratrol also significantly stimulated glucose uptake in a similar fashion in the GLUT4*myc* tagged cell line. We measured insulin- and resveratrol-stimulated glucose transport in overexpressing GLUT4*myc* tagged cells. As previously seen in the parental L6 cell line, insulin (10^{-7} M, 30 min) induced a 2-fold increase in glucose transport ($192 \pm 9.43\%$ of control, $p < 0.001$) in GLUT4*myc* overexpressing L6 cells. Treatment with resveratrol (100 μ M, 120 min) did result in a significant increase in glucose uptake ($141 \pm 6.42\%$ of control, $p < 0.01$) (Figure 19) as well. Together these results (Figure 19 and Figure 18A) indicate that resveratrol significantly stimulates glucose uptake in L6 GLUT4*myc* cells by a mechanism that is independent of GLUT4 translocation.

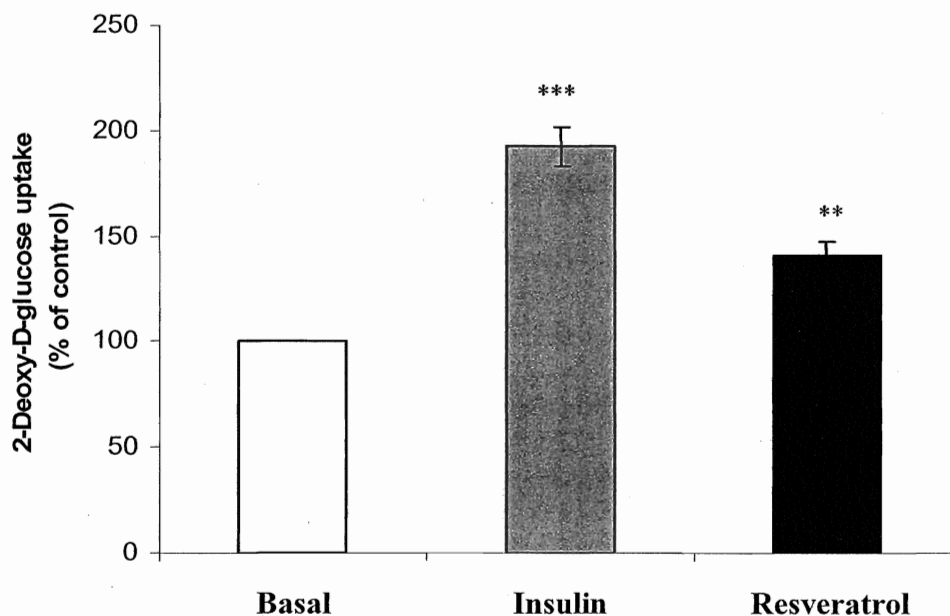


Figure 19: Effects of resveratrol on 2DG uptake in L6 GLUT4^{myc} tagged myotubes. Myotubes were incubated with either resveratrol (100 μ M, 120 min) or insulin (10⁻⁷M, 30 min) at 37°C. Cells were washed and [³H]2DG uptake was measured. The results are the mean \pm SE of 4-5 independent experiments performed in triplicate and expressed as a percent of control, ** p <0.01, *** p <0.001 compared to untreated control.

3.1.9 Effect of actin network disassembly on resveratrol-stimulated glucose transport.

Previous studies have shown that an intact actin cytoskeleton is required for insulin-stimulated glucose transport and glucose transporter translocation (232). Cytochalasin D (CD) is a fungal metabolite that disassembles the actin network and is widely used (427-430). The primary site of action of CD is actin where it binds to the barbed end of the actin monomer and filament and affects actin polymerization to decrease actin filament formation (429). Similar analogues such as cytochalasin B (CB) have a high affinity for facilitated diffusional glucose transporters (GLUTs) and inhibit glucose transport. However CD, at concentrations used to disassemble the actin cytoskeleton, has a very low affinity for the GLUTs and does not significantly affect basal glucose uptake (232;429;431).

An intact actin network may be required for transporter translocation (exocytosis) as well as for early insulin signalling. In bovine pulmonary artery endothelial cells, it was shown that resveratrol (100 μ M)-induced cell elongation was abolished by CD, indicating that an intact actin cytoskeleton is required for this response (361). In light of these data, we hypothesized that an intact actin network is required for resveratrol-stimulated glucose transport. Therefore, we treated L6 myotubes with CD (1 μ M) for 2 h, followed by stimulation with resveratrol (100 μ M, 120 min) or insulin (10^{-7} M, 30 min).

Exposure of the cells to insulin for 30 min or resveratrol for 60 or 120 min resulted in a significant increase in glucose uptake (223 ± 13.06 ; $163 \pm 4.73\%$, $p < 0.001$; $137 \pm 4.59\%$, $p < 0.01$) compared to control. Treatment with CD did not significantly affect basal glucose transport ($77 \pm 3.04\%$ of untreated control, $p > 0.05$). However, CD significantly reduced glucose transport stimulation by both insulin ($114 \pm 7.46\%$ of

control) and resveratrol at 120 min ($109 \pm 7.31\%$ of control) and 60 min ($87 \pm 4.58\%$ of control) (Figure 20). After approximately 10 minutes of exposure to cytochalasin D, the cells had undergone morphological changes (a star like appearance). The same morphological changes have been reported by others and are indicative of the effectiveness of CD to disassemble the actin network (232). These data indicate that the actin cytoskeleton appears to be required in resveratrol- stimulated glucose transport.

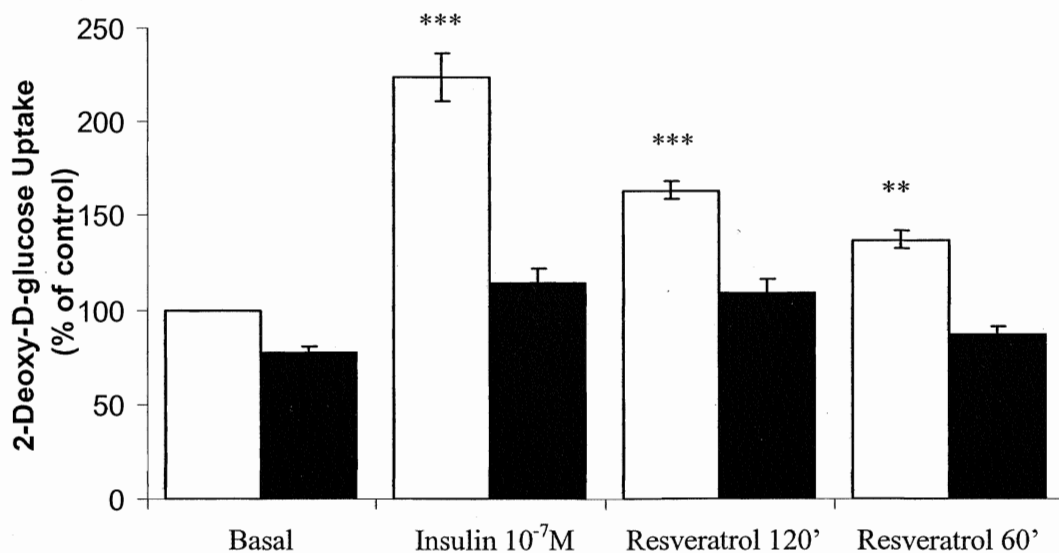


Figure 20: Effect of cytochalasin D (CD) on insulin- and resveratrol- stimulated glucose transport. L6 cells were pre- incubated in the absence (□) or presence of $1\mu M$ CD (■), followed by the addition of resveratrol ($100\mu M$, 120 or 60 min) or insulin ($10^{-7}M$, 30 min). Total incubation with CD was 120 minutes except for resveratrol (120 min), which was pre- incubated with CD for 30 minutes (150 min total CD treatment). The results are the mean \pm SE of 5 independent experiments performed in triplicate and expressed as a percent of control, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control.

3.2.1 Effect of resveratrol on ERK1/2 MAPK phosphorylation.

Resveratrol has been shown to activate ERK1/2 in different cell lines (334;335;368). Insulin has been demonstrated to activate the Ras- signalling pathway (96) and induce ERK1/2 activation, promoting cell proliferation, in several studies. In L6 cells, insulin rapidly activates ERK1/2 (111;112). Although the insulin- induced ERK1/2 activation is not required for insulin- stimulated glucose transport, the possibility that resveratrol acts via ERK1/2 exists. To examine this possibility we tested whether resveratrol, like insulin, would also have an effect on ERK1/2 phosphorylation. Insulin treated (5, 15, and 30 minutes at 10^{-7} M) cells were used as positive controls. Cells were exposed to 100 μ M resveratrol for 5, 15, 30, 60, or 120 minutes.

Western blot analysis revealed that insulin rapidly (within 5 min) stimulated ERK1/2 phosphorylation to significant levels (4.84 ± 1 arbitrary densitometry units relative to untreated control, $p < 0.001$) and the increased ERK1/2 phosphorylation was sustained after 30 min of exposure to the hormone. Resveratrol, in contrast to insulin, did not stimulate ERK1/2 phosphorylation at any time point (120 min = 0.68 ± 0.05 arbitrary densitometry units relative to untreated control, $p > 0.05$), as shown in Figure 21A (upper blot). The total levels of ERK1/2 expression were not significantly changed by any treatment (Figure 21A lower blot). These data indicate that the mechanism of action of resveratrol on glucose transport is ERK1/2- independent.

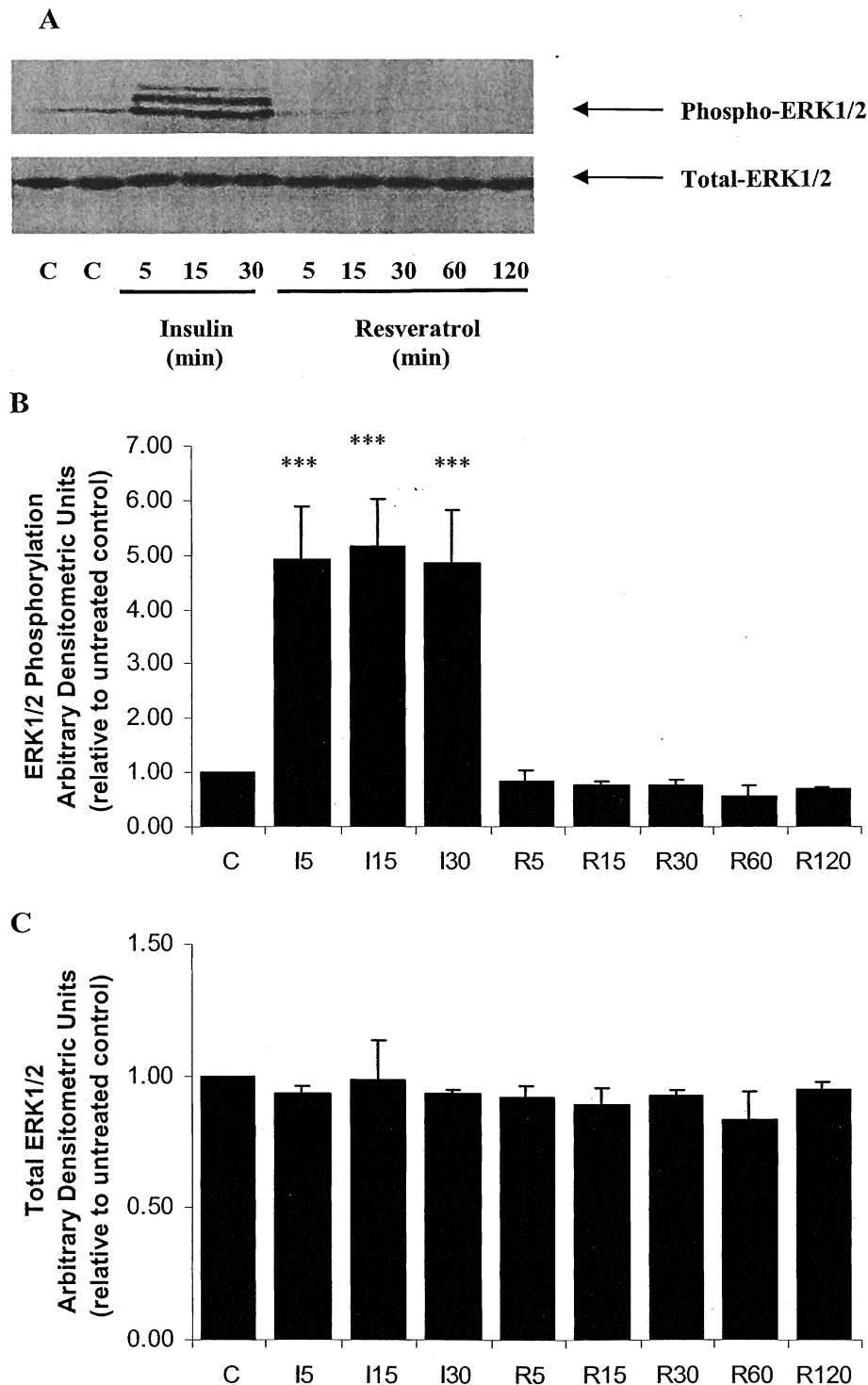


Figure 21: (A) ERK1/2 phosphorylation by insulin or resveratrol. Total cell lysates were prepared from L6 muscle cells that were treated for the indicated time (min) with or without 10^{-7} M insulin or $100\mu\text{M}$ resveratrol. Lysates ($12.5\mu\text{g}$) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-ERK1/2 or anti-total-ERK1/2 antibody. Representative immunoblot of 3 independent experiments. (B, C) Immunoblots were scanned to quantitate the density of the bands. Results are the mean \pm SE of 3 independent experiments. All values are arbitrary densitometric units expressed relative to untreated control, *** $p < 0.001$ compared to untreated control.

3.2.2 Effect of resveratrol on JNK1/2 MAPK phosphorylation.

JNK1/2 is another member of the MAPK superfamily that is activated by insulin in L6 myotubes (196). Previously, it was postulated that insulin-induced activation of glycogen synthase involved JNK phosphorylation in skeletal muscle (195), however further investigation demonstrated that insulin activation of glycogen synthase is JNK-independent in L6 myotubes (196). It is not clear whether JNK activation is required for insulin-stimulated glucose transport. Since resveratrol has been previously reported to activate JNK1/2 (334;336;365) we tested whether resveratrol, like insulin, would also phosphorylate JNK1/2 in the L6 cell line.

Figure 22A shows strong JNK1/2 phosphorylation with 5, 15, and 30 min insulin (10^{-7} M) treatment (3.91 ± 0.83 ; 3.82 ± 0.56 ; 3.37 ± 0.65 arbitrary densitometry units relative to untreated control, $p < 0.05$). Resveratrol ($100 \mu\text{M}$) did not phosphorylate JNK1/2 at any time point (5- 120 min = 0.83 ± 0.32 arbitrary densitometry units relative to untreated control, $p > 0.05$). These data suggest that resveratrol-stimulated glucose transport does not include JNK1/2 activation.

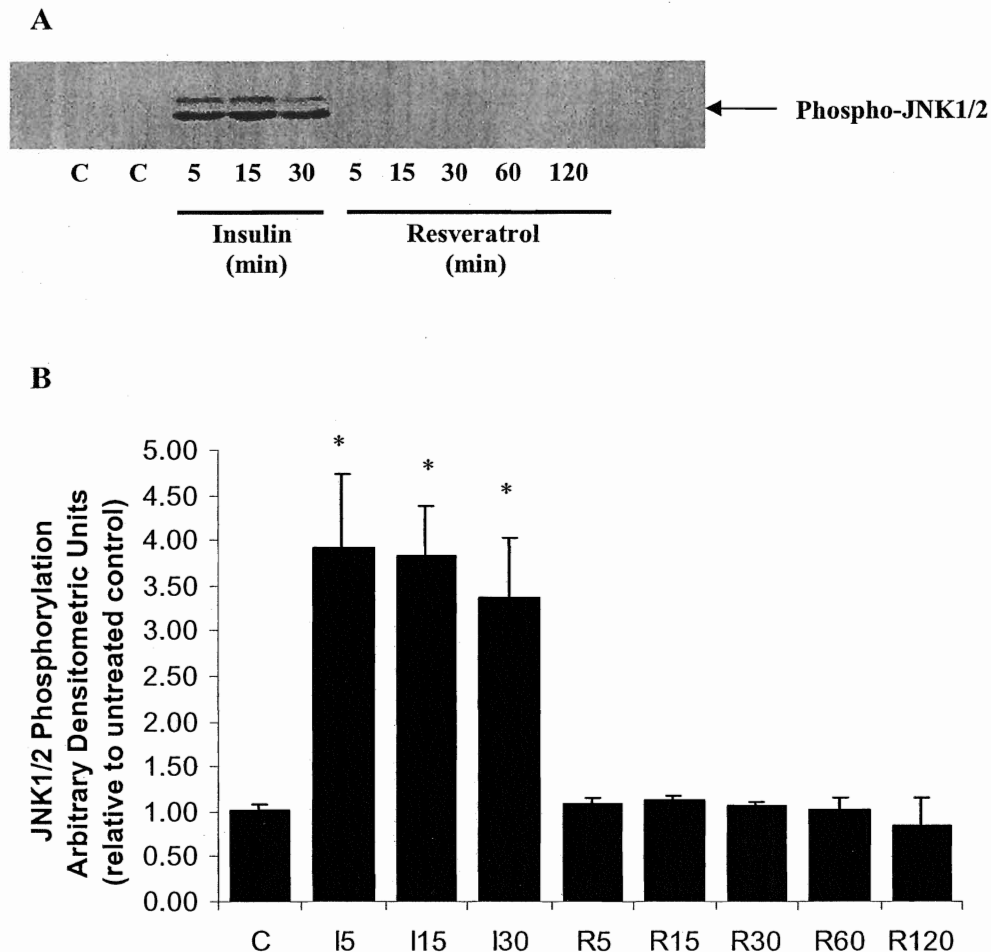


Figure 22: (A) JNK1/2 phosphorylation by insulin or resveratrol. Total cell lysates were prepared from L6 muscle cells that were treated for the indicated time (min) with or without 10^{-7} M insulin or $100\mu\text{M}$ resveratrol. Lysates ($12.5\mu\text{g}$) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-JNK1/2 antibody. Representative immunoblot of 3 independent experiments. (B) Immunoblots were scanned to quantitate the density of the bands. Results are the mean \pm SE of 3 independent experiments. All values are arbitrary densitometric units expressed relative to untreated control, $*p < 0.05$ compared to untreated control.

3.2.3 Effect of resveratrol on p38 MAPK phosphorylation.

p38 MAPK is a member of the MAPK family and is rapidly activated by insulin (10) through MKK3 and MKK6 activation in L6 myotubes (157). Inhibition of p38 MAPK with SB203580, a specific p38 MAPK inhibitor that does not affect other MAPK family members, reduced insulin-stimulated glucose transport by approximately 60%, suggesting that p38 MAPK phosphorylation is required for activation of the GLUT4 glucose transporter in order to achieve maximal glucose uptake (153). In addition, resveratrol has been reported to activate p38 MAPK in various cell lines (334;365). Therefore we tested whether resveratrol, like insulin, would also phosphorylate p38 MAPK in the L6 cell line.

Western blot analysis revealed that basal p38 MAPK phosphorylation levels were significantly elevated compared to ERK1/2 and JNK1/2 phosphorylation (Figure 21A and Figure 22A). Since the cells were serum deprived for 5h before treatment and cell lysis, we reasoned that the lack of serum may be acting as a stressor activating p38 MAPK phosphorylation. We therefore took a number of approaches to reduce basal p38 MAPK phosphorylation such as incubating the cells in media containing different FBS concentrations (0.1%, 2%) for different periods of time (data not shown). Unfortunately despite all efforts and approaches, we were not able to reduce basal p38 MAPK phosphorylation (Figure 29A), which is consistent with the data published by others (432-434). Insulin (10^{-7} M) stimulated p38 MAPK phosphorylation to maximum within 5 minutes (1.97 ± 0.20 arbitrary densitometry units relative to untreated control, $p < 0.05$) and began to decline at 15 (1.46 ± 0.08 arbitrary densitometry units relative to untreated control) and 30 (1.11 ± 0.05 arbitrary densitometry units relative to untreated control)

minutes. These results are in agreement with a previously published study (156) (Figure 23A). Unlike insulin, 100 μ M resveratrol for 5, 15, 30, 60, or 120 min did not increase p38 MAPK phosphorylation (120 min = 0.55 ± 0.12 arbitrary densitometry units relative to untreated control, $p > 0.05$). Total levels of p38 MAPK were not significantly altered by any treatment (Figure 23A lower blot). These data indicate that the mechanism of action of resveratrol on basal glucose transport is p38 MAPK- independent.

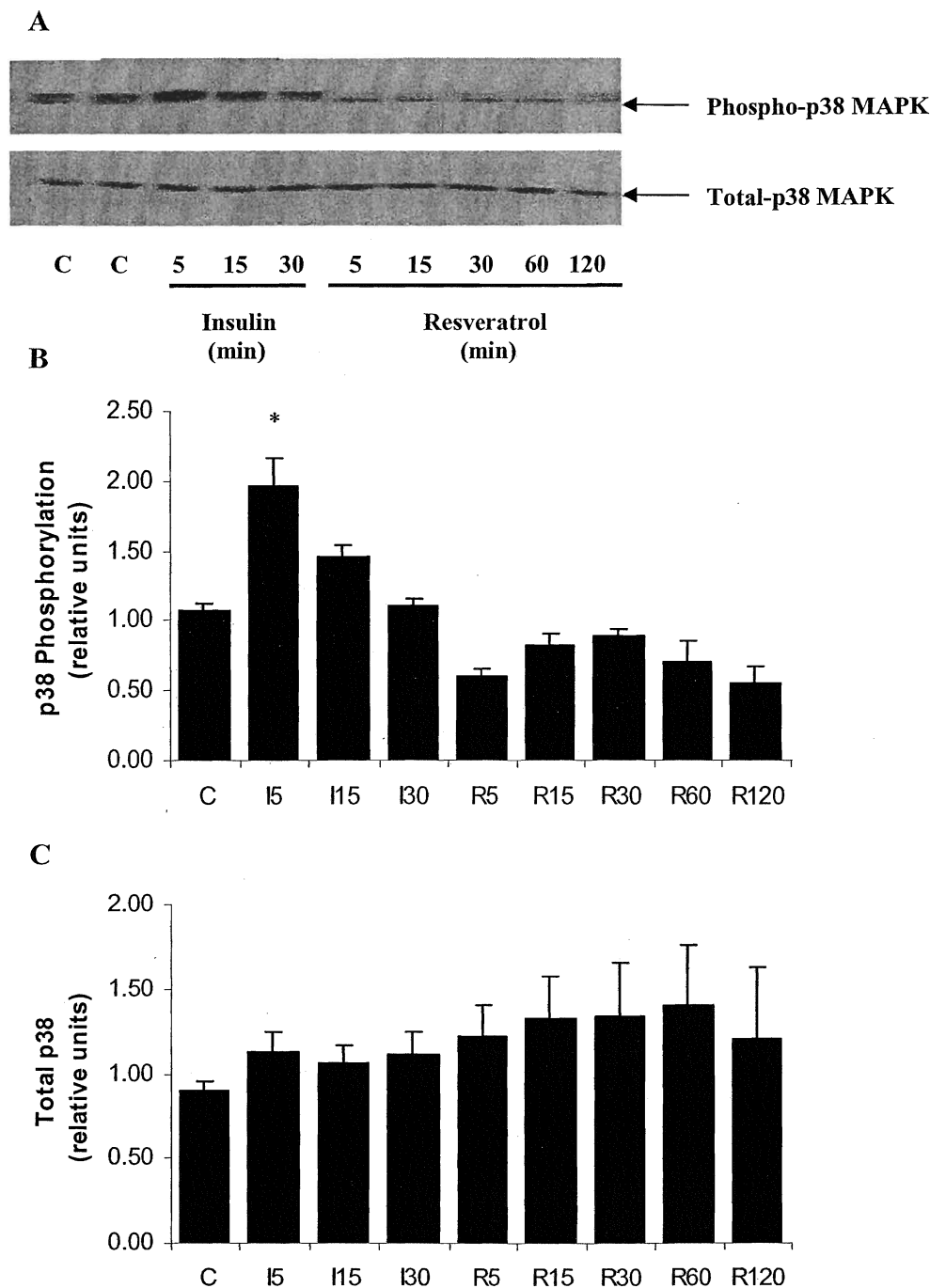


Figure 23: (A) p38 MAPK phosphorylation by insulin and/or resveratrol. Total cell lysates were prepared from L6 muscle cells that were treated for the indicated time (min) with or without 10^{-7} M insulin or $100\mu\text{M}$ resveratrol. Lysates ($12.5\mu\text{g}$) were resolved by 10% SDS-PAGE and immunoblotted with anti-phospho-p38 MAPK or anti-total-p38 MAPK antibody. Representative immunoblot of 3 independent experiments. (B, C) Immunoblots were scanned to quantitate the density of the bands. Results are the mean \pm SE of 3 independent experiments. All values are arbitrary densitometric units expressed relative to untreated control, * $p < 0.05$ compared to untreated control.

3.2.4 Effect of quercetin, rutin, and alpha-tocopherol on glucose transport.

Resveratrol is a polyphenol that has demonstrated significant antioxidant activity.

The question arose if resveratrols' effect is dependent upon its structure or antioxidant capability. Since resveratrol stimulates glucose transport independent of insulin, we examined whether this is a general effect of antioxidants and/or polyphenols or an effect specific to resveratrol. Therefore, we incubated L6 myotubes with the polyphenols quercetin (100 μ M, 120 min) and rutin (100 μ M, 120 min), which possess antioxidant activity (435), and the antioxidant α -tocopherol (100 μ M, 120 min), alone and in combination with insulin (10⁻⁷M, 30 min), followed by glucose transport measurements (Figure 24).

No significant increases in basal glucose uptake were observed with any of the compounds. In addition, quercetin and α -tocopherol did not affect insulin- stimulated glucose transport (186%; 215% of control), while rutin did show a considerable decrease (130% of control). These data show that antioxidants and polyphenols with structural similarity to resveratrol do not increase or potentiate glucose transport and suggest that resveratrol- stimulated glucose transport is specific for trans- resveratrol.

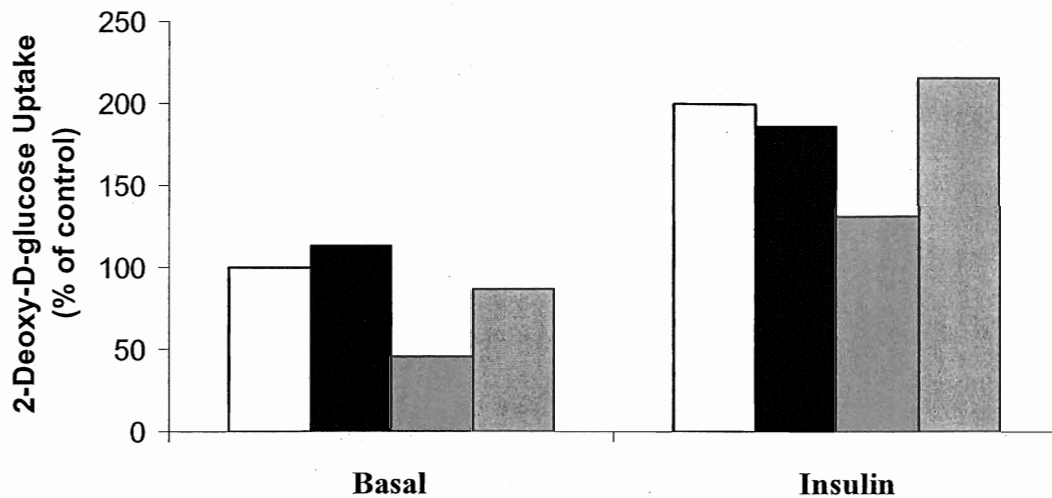


Figure 24: Effect of quercetin, rutin, and α -tocopherol on 2DG uptake in L6 myotubes. L6 myotubes were incubated with either quercetin (100 μ M, 120 min) (■), rutin (100 μ M, 120 min) (▨) or α -tocopherol (100 μ M, 120 min) (■) in the absence or presence of 10^{-7} M insulin (30 min) at 37°C. Cells were washed and [3 H]2DG uptake was measured. The results are the mean \pm SE of 2 independent experiments, each performed in triplicate and expressed as a percent of control.

3.3.1 Effect of resveratrol on amino acid uptake.

Insulin is a pleiotropic hormone. In skeletal muscle cells, apart from glucose transport, insulin also stimulates amino acid transport through the System A amino acid transport system. Since resveratrol stimulated glucose transport, we asked the question whether it also stimulated amino acid transport. MeAIB, the non-metabolizable amino acid analogue, is specifically transported by System A, which makes it ideal for transport studies (253). Myotubes were incubated with 10, 25, 50, 100, and 125 μ M concentrations of resveratrol as indicated (Figure 25A), followed by amino acid transport measurements.

In contrast to the stimulatory effect seen with resveratrol for glucose transport, amino acid transport was inhibited. Significant reduction of MeAIB uptake was seen only with 100 μ M resveratrol ($74.2 \pm 6.55\%$ of control, $p < 0.05$), which appeared to be maximum. Cell morphology was observed microscopically and was not affected by any treatment. In parallel experiments, insulin (10^{-7} M, 30 min) increased MeAIB transport by $147 \pm 5.77\%$ ($p < 0.001$) compared to control, which is in agreement to previously published data by other investigators (436). It is important to note that noncarrier-mediated MeAIB uptake, as determined by 10mM cold MeAIB administration, was approximately 5% of the total uptake and was not significantly affected by cell treatment.

Incubation of L6 myotubes with 100 μ M resveratrol (Figure 25B) for different periods of time resulted in a time-dependent inhibition of amino acid transport. Maximum inhibition by resveratrol was observed at 120 min ($74 \pm 6.55\%$ of control, $p < 0.05$).

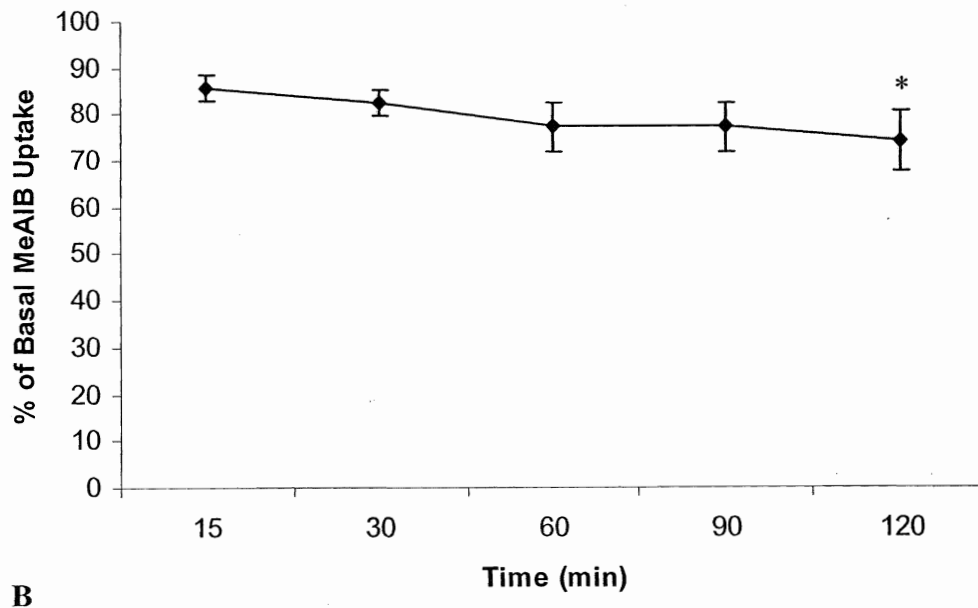
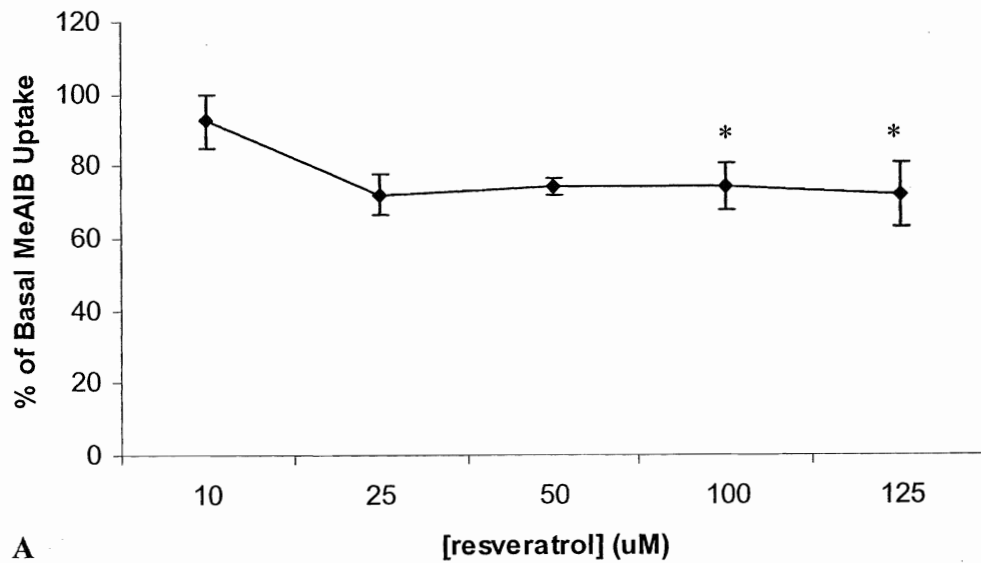


Figure 25: (A) Effects of resveratrol on MeAIB uptake in L6 myotubes: dose response. L6 myotubes were incubated with the indicated concentrations of resveratrol for 120 min at 37°C. (B) Effects of resveratrol on MeAIB uptake in L6 myotubes: time course. L6 myotubes were incubated for the indicated time periods with resveratrol (100µM) at 37°C. Cells were washed and [¹⁴C]MeAIB uptake was measured as discussed in the methods. The results are the mean ± SE of 3-5 independent experiments each performed in triplicate and expressed as a percent of control, **p*<0.05 compared to untreated control.

3.3.2 Effect of resveratrol on insulin- stimulated amino acid uptake.

To investigate whether, apart from inhibiting basal amino acid uptake, resveratrol affects the acute insulin- induced increase in amino acid transport, we examined the interaction between acute insulin exposure and resveratrol treatment (Figure 26). Insulin was added to the cells for 30 min before the measurement of MeAIB uptake in control- untreated and resveratrol- pretreated cells. Resveratrol treatment was continued in parallel with insulin treatment. Insulin alone at 10^{-7} M for 30 min resulted in a significant increase in MeAIB uptake ($147 \pm 5.77\%$ of control, $p < 0.001$). Treatment of myotubes for 120 min with resveratrol (100uM) alone resulted in a significant inhibition of basal amino acid uptake ($74 \pm 6.55\%$ of control, $p < 0.05$), which is in agreement with the previous dose- response and time- course experiments (Figure 25). Importantly, resveratrol completely abolished the insulin- stimulated amino acid transport ($103 \pm 7.35\%$ of control, $p > 0.05$).

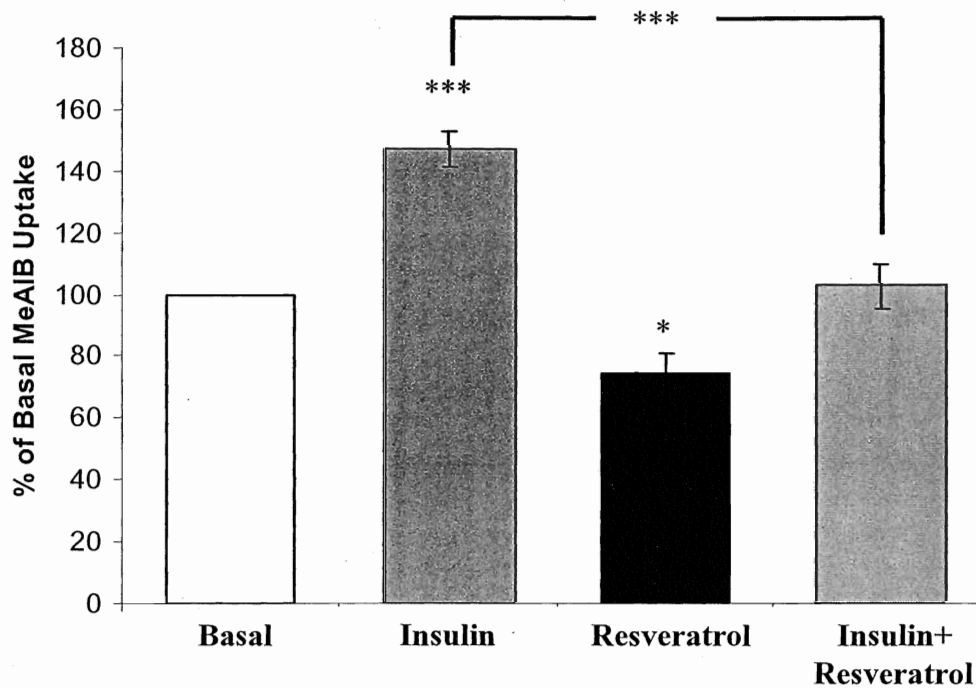


Figure 26: Effect of resveratrol on basal- and insulin- stimulated MeAIB uptake in L6 myotubes. L6 myotubes were incubated with resveratrol (100 μ M, 120 min), insulin (10^{-7} M, 30 min), or a combination of resveratrol and insulin at 37°C. Cells were washed and [14 C]MeAIB uptake was measured as indicated in the methods. The results are the mean \pm SE of 3-5 independent experiments performed in triplicate and expressed as a percent of control, * p <0.05, *** p <0.001 compared to untreated control.

3.4.1 Effect of resveratrol on thymidine incorporation in L6 myoblasts.

Insulin has a mitogenic effect and stimulates thymidine incorporation in L6 myoblasts (112;437). On the other hand, resveratrol has been shown to inhibit cell proliferation and thymidine incorporation in numerous cell lines (317;438;439). We wanted to determine whether resveratrol mimicked insulin's mitogenic effect. Subconfluent myoblasts (40- 55% confluent) were growth arrested by incubating them in medium without any serum for 24 h. The cells were then treated with 0.1, 1, 10, 50, or 100 μ M resveratrol and after 24h, thymidine incorporation was measured as indicated in the methods section (Figure 27A).

Resveratrol at 0.1 and 1 μ M did not have a significant effect on thymidine incorporation ($97 \pm 4.89\%$ and $100 \pm 2.72\%$ of control, $p > 0.05$ respectively). However, resveratrol at 10 μ M significantly inhibited thymidine incorporation ($36 \pm 8.04\%$ of control, $p < 0.05$). Maximum inhibition was observed at 50 μ M ($8 \pm 1.59\%$ of control, $p < 0.001$). The same resveratrol concentration (50 μ M) effectively inhibits thymidine incorporation in vascular smooth muscle cells and several cancer cell lines as reported by others (317;438;439). Visual microscopic inspection of all experimental groups revealed that resveratrol- treated cells were less confluent than control, indicating an inhibition of cell proliferation. Cell morphology, which was also observed microscopically, was not affected by any treatment.

We also examined the effect of resveratrol on the insulin- and FBS- stimulated mitogenic response (Figure 27B). Insulin (10^{-7} M, 24 h) significantly increased thymidine incorporation ($280 \pm 9.92\%$ of control, $p < 0.001$). Media containing 10% FBS resulted in stimulation of thymidine incorporation to $691 \pm 36.92\%$ of control, $p < 0.001$. Resveratrol

(50 μ M) completely abolished both insulin- ($11 \pm 1.26\%$ of control, $p < 0.001$) and FBS-stimulated ($36 \pm 5.16\%$ of control, $p < 0.05$) cell proliferation.

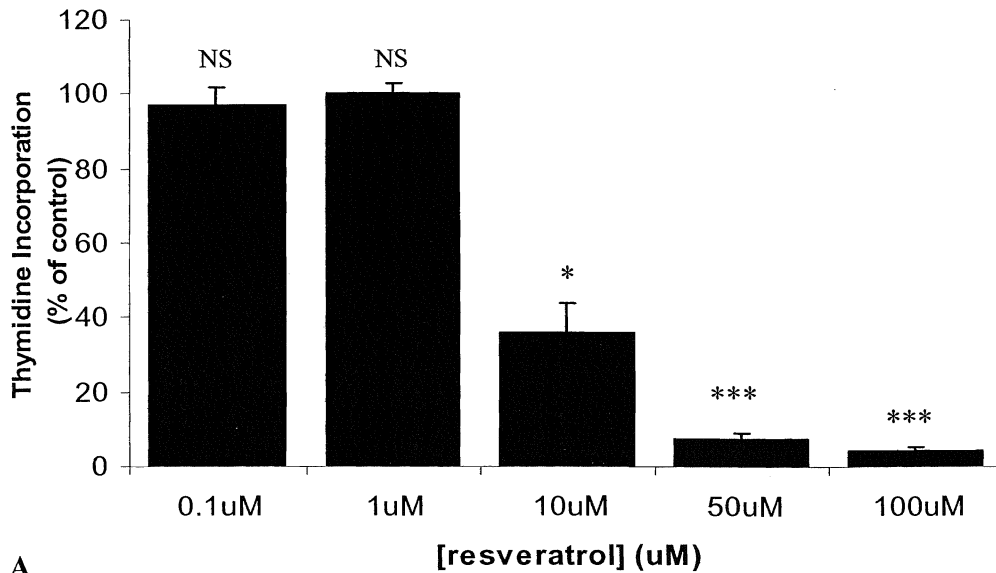


Figure 27A: Effects of resveratrol on thymidine incorporation in L6 myotubes: dose response. L6 myotubes were incubated with the indicated concentrations of resveratrol for 24 hours at 37°C. Cells were washed and [³H]thymidine incorporation was measured as discussed in the methods. The results are the mean ± SE of 4 independent experiments performed in triplicate and expressed as a percent of control, **p*<0.05, ****p*<0.001, NS = not significant compared to untreated control.

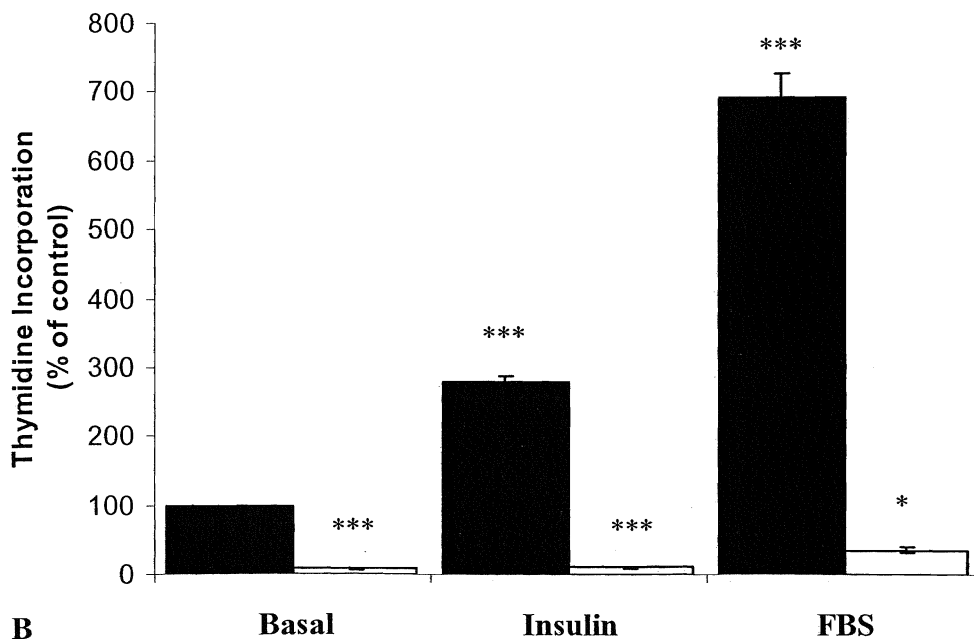


Figure 27B: Effects of resveratrol on insulin- and fetal bovine serum (FBS)-stimulated thymidine incorporation. L6 cells were incubated without (■) or with 50uM resveratrol (□), immediately followed by the addition of insulin (10⁻⁷M) or 10% FBS for 24 h at 37°C. Cells were washed and [³H]thymidine incorporation was measured as indicated in the methods. Results are the mean ± SE of 4 independent experiments performed in triplicate and expressed as a percent of control, **p*<0.05, ****p*<0.001 compared to untreated control.

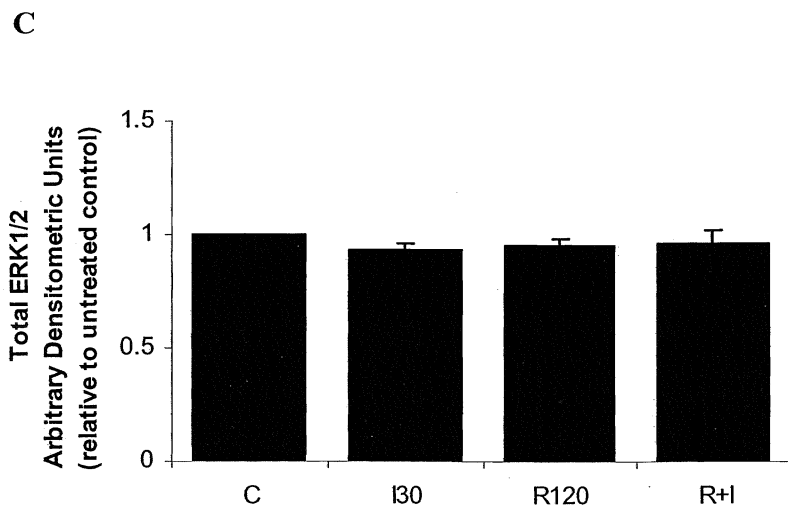
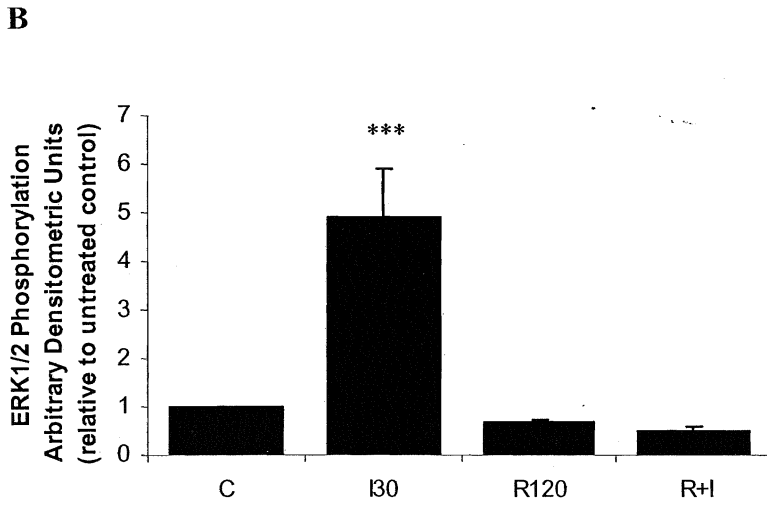
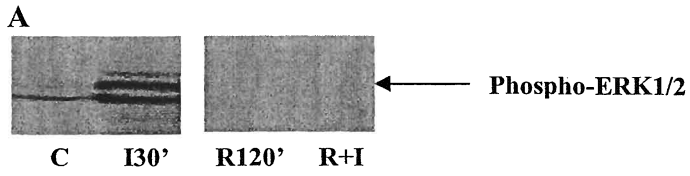
3.4.2 Effect of chronic resveratrol treatment on ERK1/2 phosphorylation.

ERK1/2 activation leads to cell proliferation (114). Several studies have shown that resveratrol inhibits ERK1/2 activation and cell proliferation (319;321;356;440). However, resveratrol has also been shown to activate ERK1/2 in many different cell lines while maintaining its inhibition of mitogenesis (334;335;368). Insulin also stimulates mitogenesis through the activation of the Ras- Raf- ERK1/2 pathway (96), to promote cell proliferation and differentiation. For example, L6 myoblasts exhibit a proliferative response when stimulated with either insulin or insulin- like growth factor- 1 (IGF-1) (111;112). We hypothesized that ERK1/2 activation may be involved in resveratrol inhibited thymidine incorporation. To examine this possibility we measured total and phosphorylated ERK1/2.

Acute treatment with insulin (10^{-7} M, 30 min) increased ERK1/2 phosphorylation as shown previously (Figure 21 and Figure 28A) (4.84 ± 1.00 arbitrary densitometry units relative to untreated control, $p > 0.001$). Resveratrol ($100 \mu\text{M}$, 120 min) had no effect (0.68 ± 0.05 arbitrary densitometry units relative to untreated control, $p > 0.05$) but did completely abolish the insulin response (0.51 ± 0.09 arbitrary densitometry units relative to untreated control, $p > 0.05$). This data shows that resveratrol very effectively blocks phosphorylation of ERK1/2 by insulin and suggests that the inhibitory effect of resveratrol on insulin- stimulated thymidine incorporation may be due to inhibition of ERK1/2 phosphorylation. All of the data presented up to now involve acute treatment and we also examined whether the same applies for chronic stimulation.

As shown in Figure 28D (upper blot), chronic (24 h) insulin (10^{-7} M) and 10% FBS treatment resulted in a significant phosphorylation of ERK1/2 (1.96 and 1.91 arbitrary densitometry units relative to untreated control). The chronic effect of insulin is

similar to that observed with acute (5- 30 min) treatment (Figure 21). In addition, chronic resveratrol treatment (50 μ M), which was maximum inhibition of thymidine incorporation (Figure 27A), did not affect ERK1/2 phosphorylation, which is also similar to the result seen with acute (5- 120 min) resveratrol treatment (0.85 arbitrary densitometry units relative to untreated control). Furthermore, resveratrol did not inhibit the insulin- or FBS-stimulated ERK1/2 phosphorylation (2.03 and 2.24 arbitrary densitometry units relative to untreated control). This effect with resveratrol is in opposition to that observed with acute treatment where preincubation with resveratrol completely abolished insulin-stimulated ERK1/2 phosphorylation (Figure 28).



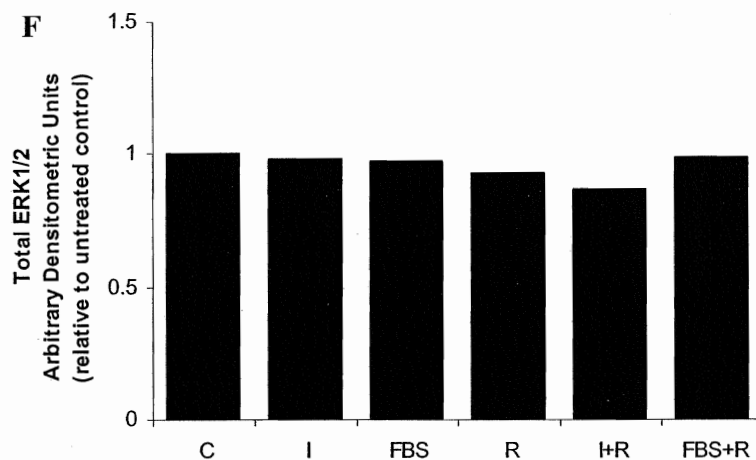
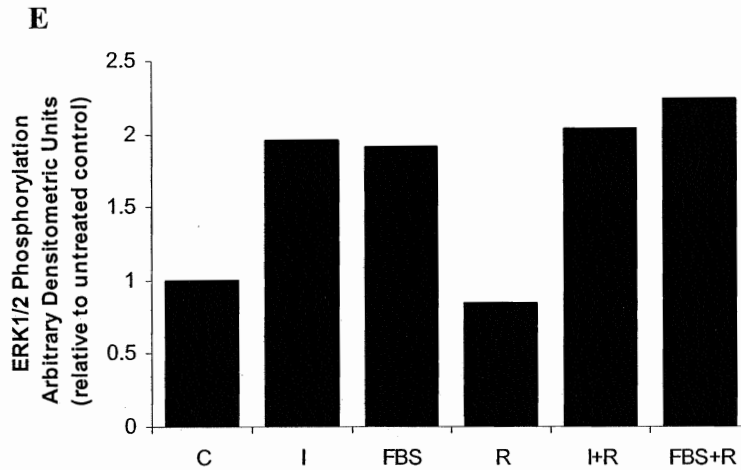
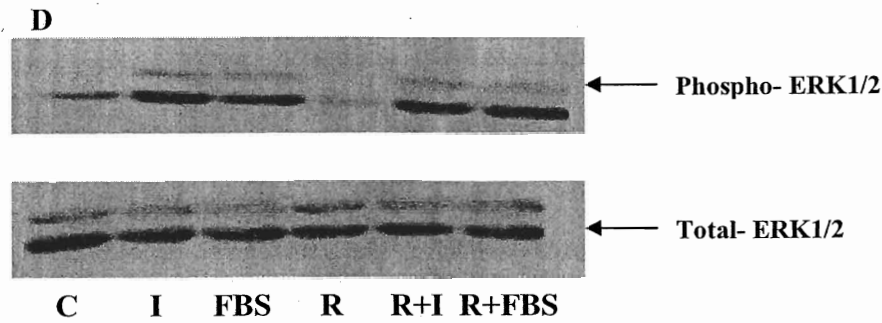


Figure 28: ERK1/2 phosphorylation by insulin, FBS or resveratrol. Total cell lysates (12.5 μ g) were prepared from treated L6 muscle cells, resolved by 10% SDS-PAGE, and immunoblotted with anti-phospho-ERK1/2 or anti-total-ERK1/2 antibody. (A) Cells were treated for the indicated time (min) with or without 10^{-7} M insulin or 100 μ M resveratrol or resveratrol + insulin. Representative immunoblot of 3 independent experiments. (D) Cells were treated for 24 h without or with either 10^{-7} M insulin, 10% FBS, or 50 μ M resveratrol or in combination with resveratrol. Representative immunoblot of 1 independent experiment. (B, C, E, F) Immunoblots were scanned to quantitate the density of the bands. Results are the mean \pm SE of 3 (B, C) and 1 (E, F) independent experiment. All values are arbitrary densitometric units expressed relative to untreated control cells, *** p <0.001 compared to untreated control.

3.4.3 Effect of chronic resveratrol treatment on p38 MAPK phosphorylation.

p38 MAPK activation has been shown to have biological importance in regulating cellular processes such as cell proliferation and differentiation (151). Resveratrol has been reported to activate p38 MAPK in various cell lines affecting mitogenesis (334;365). p38 MAPK can also be activated by acute insulin (30 min) stimulation in L6 myotubes (441), a process that involves MKK3 and MKK6 activation (157). Furthermore, chronic insulin treatment has been demonstrated to increase mitogenesis involving p38 MAPK activation in C₂C₁₂ cells (442). Therefore we hypothesized that p38 MAPK phosphorylation may be involved in FBS- and insulin- stimulated cell proliferation and that the inhibitory effect observed with resveratrol (Figure 27) could be due to inhibition of p38 MAPK.

Acute treatment with insulin (10^{-7} M, 5 min) rapidly increased p38 MAPK phosphorylation as previously shown (Figure 23A) (1.97 ± 0.20 arbitrary densitometry units relative to untreated control, $p < 0.05$). In contrast to insulin, resveratrol (100 μ M, 5-120 min) did not have any significant effect alone (120 min = 0.55 ± 0.12 arbitrary densitometry units relative to untreated control, $p > 0.05$) but did inhibit the insulin response (0.61 ± 0.10 arbitrary densitometry units relative to untreated control, $p > 0.05$) at 100 μ M (120 min) (Figure 29A). This data indicates that resveratrol is an effective inhibitor of insulin- stimulated p38 MAPK phosphorylation and suggests that perhaps the inhibitory effect of resveratrol on insulin- stimulated thymidine incorporation may be attributed to the acute inhibition of p38 MAPK phosphorylation. These data involve acute treatment only, so we also examined the chronic effect of resveratrol.

To address this question we treated L6 myoblasts in the exact same conditions as for thymidine incorporation experiments and after preparing a cell lysate we examined phosphorylated p38 MAPK. As shown in Figure 29C, chronic (24 h) insulin (10^{-7} M) and 10% FBS treatment resulted in increased phosphorylation of p38 MAPK (1.74 arbitrary densitometry units relative to untreated control). The chronic effect of insulin is similar to that seen with acute (5- 30 min) insulin treatment of the cells (Figure 23). Chronic resveratrol treatment (50 μ M) did not affect p38 MAPK phosphorylation (0.91 arbitrary densitometry units relative to untreated control). This result is similar to the effect observed with acute (5- 120 min) resveratrol treatment. Furthermore, resveratrol did not abolish the insulin or FBS stimulation of p38 MAPK phosphorylation (1.42 and 2.09 arbitrary densitometry units relative to untreated control). This effect of resveratrol is opposite to that seen with acute treatment where preincubation with resveratrol abolished insulin- stimulated p38 MAPK phosphorylation (Figure 29A).

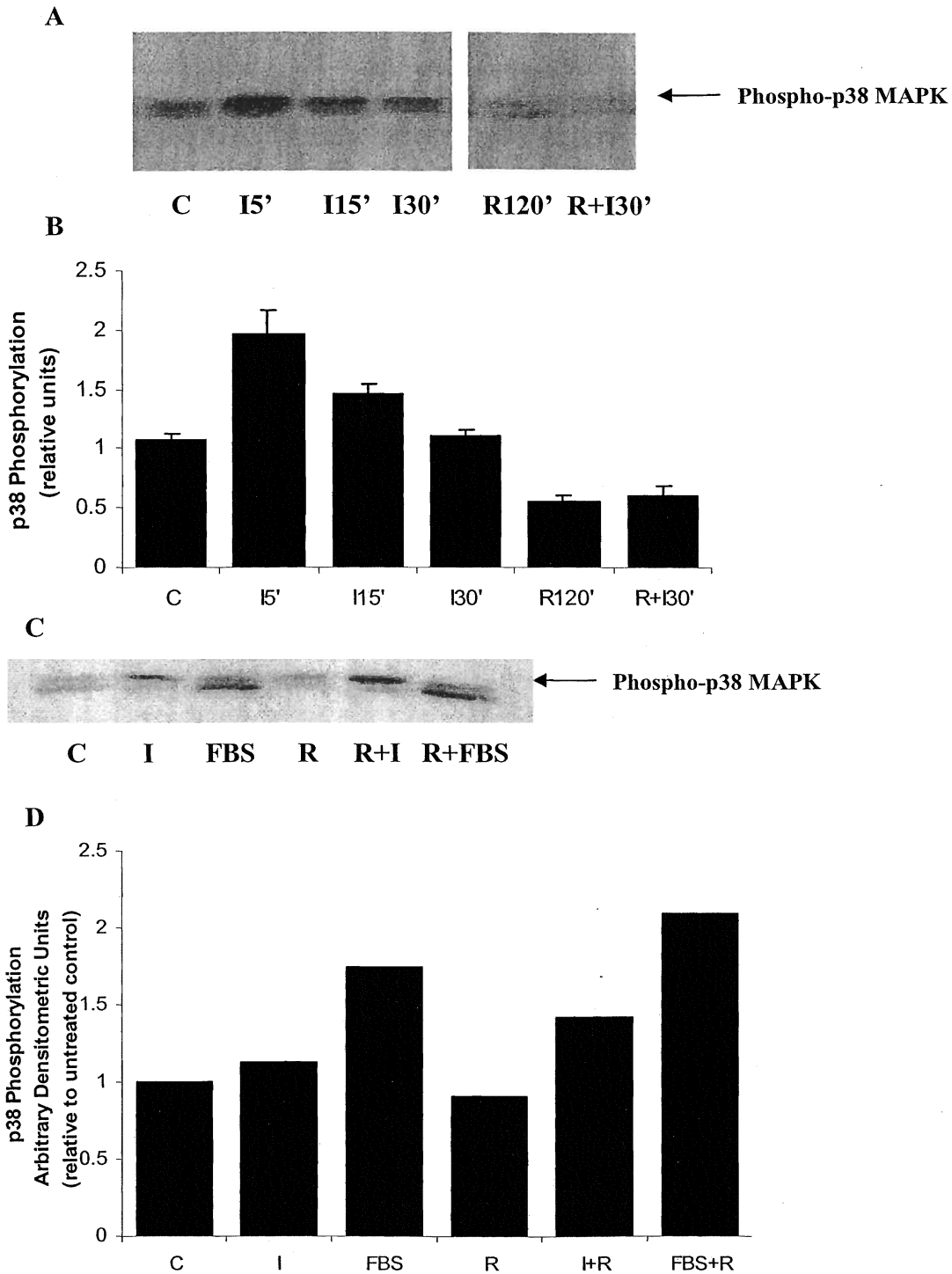


Figure 29: (A) p38 MAPK phosphorylation by insulin, FBS or resveratrol. Total cell lysates (12.5 μ g) were prepared from treated L6 muscle cells, resolved by 10% SDS-PAGE, and immunoblotted with anti-phospho-p38 MAPK. (A) Cells were treated for the indicated time (min) with or without 10^{-7} M insulin or 100μ M resveratrol or resveratrol + insulin. Representative immunoblot of 3 independent experiments. (C) Cells were treated for 24 h without or with either 10^{-7} M insulin, 10% FBS, or 50μ M resveratrol or in combination with resveratrol. Representative immunoblot of 1 independent experiment. (B, D) Immunoblots were scanned to quantitate the density of the bands. Results are the mean \pm SE of 3 (B) and 1 (D) independent experiment. All values are arbitrary densitometric units expressed relative to untreated control cells, * p <0.05 compared to untreated control.

3.4.4 Effect of chronic resveratrol treatment on JNK1/2 phosphorylation.

JNK1/2 activation is involved in the regulation of cell growth and mitogenesis (443) and is phosphorylated by acute insulin stimulation in L6 myotubes (196). Resveratrol also activates JNK1/2 in different cell lines leading to apoptosis (334;336), however resveratrol also inhibits JNK1/2 phosphorylation to decrease cell proliferation (319;444). We reasoned that JNK1/2 may be involved in resveratrol's inhibition of mitogenesis. To examine this possibility we used Western blot analysis to measure total and phosphorylated JNK1/2.

Acute treatment with insulin (10^{-7} M, 30 min) increased JNK1/2 phosphorylation as shown previously (Figure 22 and Figure 30A) (3.37 ± 0.65 arbitrary densitometry units relative to untreated control, $p < 0.05$). In contrast to insulin, resveratrol ($100\mu\text{M}$, 5- 120 min) did not have any effect alone (0.83 ± 0.32 arbitrary densitometry units relative to untreated control, $p > 0.05$) but did completely abolish the insulin response (1.12 ± 0.21 arbitrary densitometry units relative to untreated control, $p > 0.05$) at $100\mu\text{M}$ (120 min). This data demonstrates that resveratrol is an effective inhibitor of insulin- stimulated JNK1/2 phosphorylation and suggests that the inhibitory effect of resveratrol on insulin-stimulated thymidine incorporation may be attributed to the inhibition of JNK1/2 phosphorylation. These data involve acute treatment and whether the same effect is observed with chronic treatment was also examined.

As shown in Figure 30C, chronic (24 h) insulin (10^{-7} M) and 10% FBS treatment resulted in a significant phosphorylation of JNK1/2 (2.96 and 2.76 arbitrary densitometry units relative to untreated control). The chronic effect of insulin is similar to that observed with acute (5- 30 min) treatment (Figure 22). In addition, chronic resveratrol

treatment (50 μ M) did not affect JNK1/2 phosphorylation, which is also similar to acute (5- 120 min) resveratrol treatment (0.98 arbitrary densitometry units relative to untreated control). Furthermore, resveratrol did not inhibit the insulin- or FBS- stimulated JNK1/2 phosphorylation (2.33 and 2.75 arbitrary densitometry units relative to untreated control). This effect observed with resveratrol is opposite to that observed with acute treatment where preincubation with resveratrol completely abolished insulin- stimulated JNK1/2 phosphorylation (Figure 30).

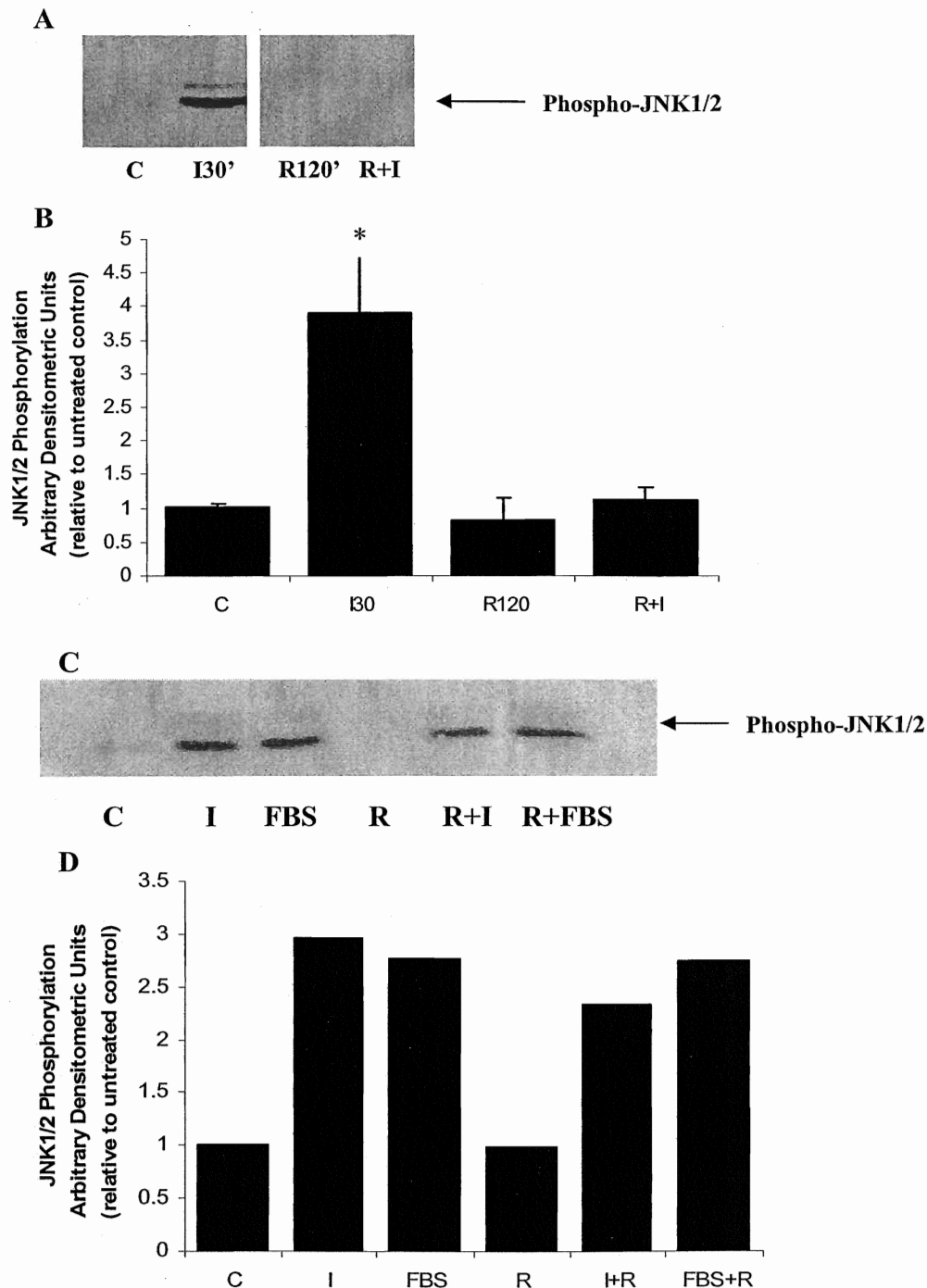


Figure 30: JNK1/2 phosphorylation by insulin, FBS or resveratrol. Total cell lysates (12.5 μ g) were prepared from treated L6 muscle cells, resolved by 10% SDS-PAGE, and immunoblotted with anti-phospho-JNK1/2. (A) Cells were treated for the indicated time (min) with or without 10^{-7} M insulin or 100 μ M resveratrol or resveratrol + insulin. Representative immunoblot of 3 independent experiments. (C) Cells were treated for 24 h without or with either 10^{-7} M insulin, 10% FBS, or 50 μ M resveratrol or in combination with resveratrol. Representative immunoblot of 1 independent experiment. (B, D) Immunoblots were scanned to quantitate the density of the bands. Results are the mean \pm SE of 3 (B) and 1 (D) independent experiment. All values are arbitrary densitometric units expressed relative to untreated control cells, * p <0.05 compared to untreated control.

CHAPTER 4: Section 1: DISCUSSION

4.1 Resveratrol and glucose transport.

4.1.1 Resveratrol significantly increases glucose uptake.

The incidence of type 2 diabetes is rapidly increasing on a global scale and has a significant impact on the Canadian health care system as a major cause of death in North America. A reduction in peripheral glucose uptake is observed in type 2 diabetic individuals (237). Although a number of different pharmacological agents are available and are clinically used to improve glycemic control in type 2 diabetes, a better understanding of glucose transport regulation is needed in order to develop new and more effective interventions.

Since skeletal muscle is the primary site for glucose disposal in the post-prandial state and is the major target of insulin action controlling glycaemia, it is expected that new interventions improving glucose transport in this tissue specifically would be beneficial. Resveratrol has beneficial effects as an anti-oxidant, anti-cardiovascular disease, and anti-cancer agent. Here we examined the effects of resveratrol on basal- and insulin-stimulated glucose transport.

The L6 skeletal muscle cell line is the most widely used and well-established model of skeletal muscle and is most suitable for studying glucose transport in vitro. In these cells, insulin causes a two-fold increase in glucose transport within 15 minutes of exposure, which is protein synthesis independent (394). L6 cells are currently the only cell line that expresses both the GLUT1 and GLUT4 glucose transporters (391). Therefore, we chose L6 muscle cells to examine the effects of resveratrol on glucose

transport. Currently, there are no previous studies examining the effects of resveratrol on glucose uptake in skeletal muscle cell lines, skeletal muscle in vitro or in vivo.

The results of the present study demonstrate for the first time that resveratrol stimulates glucose uptake in cultured L6 skeletal muscle cells, which confirms our initial hypothesis that resveratrol has insulin- like effects on skeletal muscle cells. This appears to be a specific property of resveratrol that is not shared by all antioxidants and structurally similar compounds since quercetin, rutin, and alpha- tocopherol did not have any effect. It is important to note that resveratrol- stimulated glucose transport is entirely independent of insulin since insulin was not present in the medium and the cells were serum deprived for 5 hours before treatment. This effect is time- dependent, requiring a relatively long time of exposure compared to insulin, for the development of the full response. Insulin (100nM) rapidly stimulates glucose transport activity to maximum within 15 minutes in L6 skeletal muscle cells (394).

In addition to the insulin receptor, L6 rat skeletal muscle cells also express functional estrogen receptors (α and β), which was demonstrated by increased myoblast proliferation and strong *c-fos* and *egr* gene induction after estrone or 17β - estradiol treatment (445). In addition, estrogens such as 17β - estradiol have been shown to promote insulin- mediated glucose uptake in mouse soleus skeletal muscle (446). Resveratrol has been shown in numerous studies to behave as both an estrogen receptor agonist and antagonist (292;340-343). Therefore we acknowledge the possibility that resveratrol- stimulated glucose uptake may be mediated by the estrogen receptors present in L6 cells. In the future this theory could be examined with specific estrogen receptor

inhibitors such as ICI- 182780 and tamoxifen or estrogen receptor knockouts, followed by glucose transport measurements after resveratrol treatment.

The delay in resveratrol action may be due to its permeation to an intracellular site and/or subsequent generation of a specific intracellular signal. Studies in both hepatocytes (281) and intestinal cells (282) have shown that resveratrol (30- 150 μ M) enters the cell by simple diffusion to significant levels within 5 minutes, suggesting that the delayed response observed in our study may be due to specific signalling events. Currently, resveratrol transport into skeletal muscle has not been examined, however future studies could use radioactive [14 C]resveratrol to study this mechanism. This study focused on the acute effects of resveratrol and insulin on glucose transport, however it is well known that chronic insulin treatment (24 h) also enhances glucose transport through a modification of protein synthesis. Therefore, future studies could focus on the chronic effects of resveratrol on glucose transport and glucose transporter expression.

The relatively long incubation period required to observe an effect with resveratrol suggested that there may be a need for synthesis of specific proteins, however experiments with cycloheximide showed that protein synthesis was not required for resveratrol- stimulated glucose uptake. It is well established by others that CHX at 1 μ g/ml, which is the concentration used in the present study, blocks protein synthesis. Although protein synthesis was not directly measured, to assess the effectiveness of CHX, [3 H]leucine incorporation into protein could be measured (447).

The dose of resveratrol required to achieve maximum stimulation is 100 μ M. This concentration of resveratrol is in accordance with previous studies where concentrations of less than 10 μ M generally do not result in any significant biological effect in various in

vitro systems (276;319;328;331;448). In addition, concentrations of resveratrol used in in vitro studies are usually 10- 1000 times greater than peak plasma concentrations found in humans after oral administration. For example, plasma resveratrol concentrations in humans after oral administration of resveratrol dissolved in either grape juice, vegetable juice or white wine, are approximately 2 μ M and may be as low as 20nM depending on the rate of metabolism, which is approximately 1000 times less than the concentrations used in our in vitro study (449). Lower plasma concentrations of a compound compared to in vitro studies does not indicate that the in vitro studies are irrelevant. Similar discrepancies between in vivo and in vitro concentrations have been observed for different agents used in the treatment of diabetes such as metformin, glyburide, and even insulin. Metformin is not metabolized in humans or rats (450) and its plasma concentration is approximately 537nM (451), which is 1000 times less than the commonly used 400 μ M concentration in in vitro studies (393). In addition, glyburide is frequently used in vitro at approximately 100 μ M (417) while the concentration found in human plasma is in the nanomolar range (105nM) (452). Similarly, the circulating human plasma insulin levels are 10pM (453) while the insulin concentration used in in vitro studies in cell cultures are 100nM. Therefore, the relatively high concentration of resveratrol required to see a biological effect in our in vitro system does not exclude in vivo relevance.

As a polyphenolic compound, resveratrol is structurally similar to several other compounds also found in red wine, including quercetin and rutin (454). Currently there are no studies concerning the effects of quercetin or rutin on glucose transport in skeletal muscle. However, quercetin (100 μ M) does inhibit insulin- stimulated glucose transport in

a dose- dependent manner in rat adipocytes (455). We found that at similar concentrations (100 μ M), quercetin did not affect basal or insulin- stimulated glucose transport in L6 myotubes whereas rutin showed a decrease. These data suggest that resveratrol- stimulated glucose uptake is specific to this compound. There are numerous other polyphenols present in red wine and future studies could examine their effects on glucose transport separately or in combination.

Alpha- tocopherol, which is a compound found in Vitamin E, contains only a single phenolic group and is an established antioxidant (456). Its antioxidant activity is similar to that of resveratrol, depending on the free radical stressor (457). Alpha- tocopherol has been shown to stimulate glucose transport in rat- hemidiaphragm in vivo (458) but did not increase glucose uptake in L6 myotubes in the present study (100 μ M, 120 min) or after 24 h (10 μ M) incubation (459). In our study, alpha- tocopherol did not alter basal or insulin- stimulated glucose transport, suggesting that only particular antioxidants are capable of stimulating glucose transport and that resveratrol- stimulated glucose uptake is specific.

It is not clear whether the effects of resveratrol are due to its antioxidant activity as ROS levels have not been measured. Generally ROS production is thought to decrease insulin sensitivity and inhibit glucose transport. However ROS, although diverse, also stimulate glucose transport in L6 cells and 3T3-L1 adipocytes through increased GLUT1 mRNA and protein expression (460). Antioxidants have also been shown to generate free radicals, for example the pro- oxidant role of antioxidants is supported by 4- hydroxyl tempol (375). Tempol (5mM, 12 h) increased the rate of glucose transport in both vascular endothelial and smooth muscle cells through an increase in GLUT1 mRNA and

protein content and plasma membrane abundance. These effects were related to ROS generation. Other antioxidants, such as alpha- lipoic acid (376), also stimulated glucose transport in L6 myotubes and 3T3-L1 adipocytes. Therefore, it is possible that resveratrol may stimulate glucose transport through modification of ROS levels. Future studies could focus on measuring the levels of ROS and the free radical scavenging activity of resveratrol in L6 myotubes.

Significant conjugation of trans- resveratrol has been demonstrated *in vivo*, which raises a question concerning the physiological relevance of unconjugated trans- resveratrol. The vast majority of *in vitro* studies pertaining to resveratrol also employ trans- resveratrol and despite substantial conjugation of resveratrol, studies on the bioavailability of orally ingested resveratrol demonstrate that a measurable portion of trans- resveratrol remains intact. This indicates that the present *in vivo* studies may have a significant *in vivo* relevance.

Several hypoglycaemic agents, including gliclazide, glyburide (417), troglitazone (459), vanadate, and pervanadate (436), enhance basal glucose transport but do not enhance the maximum insulin response. In our study, resveratrol did not potentiate maximal insulin but had a significant effect on the cell response to submaximal insulin levels, which is similar to the above-mentioned compounds. This finding indicates that resveratrol and submaximal insulin concentrations are additive. Our data suggest that resveratrol may be useful in states of insulin resistance and could enhance insulin action. Currently, several anti- diabetic agents used clinically, such as the thiazolidinediones rosiglitazone (461), and pioglitazone, and metformin (462), have been shown to play a role in reducing insulin resistance.

The lack of a significant change in maximal insulin responsiveness in the presence of resveratrol may be because resveratrol alone may nearly reach maximum before insulin is added, thereby reducing the requirement for insulin-stimulated glucose transport. A shorter incubation period (10- 15 min) with resveratrol combined with insulin may be sufficient to potentiate the submaximal insulin response and should be examined in the future.

4.1.2 Elucidating the mechanism of action of resveratrol.

Furthermore, the present study suggests that the signalling molecules involved in resveratrol action may be different than that of insulin. These findings are very important because certain signalling events or molecules involved in insulin action may be defective, leading to insulin resistance, and resveratrol may provide a tool of bypassing these defective-signalling events. PI3K is a downstream effector protein in the insulin-signalling cascade (23) that is composed of a 110kDa catalytic subunit and an 85kDa regulatory subunit. PI3K activation is necessary but not sufficient for insulin-stimulated glucose transport since mutant $\Delta p85$, which lacks a binding site for the p110 catalytic subunit, greatly inhibited insulin-stimulated glucose uptake in CHO cells (42) but active-PI3K overexpression required insulin to achieve maximal glucose uptake (44;45).

In this study PI3K involvement was examined with the pharmacological inhibitors, LY294002 and wortmannin. Wortmannin is a fungal metabolite that binds to the p110 catalytic subunit of type 1 PI3K to exert potent inhibition (38;39;463). Alternatively, LY294002, a quercetin derivative, acts as a competitive inhibitor of the ATP binding site of PI3K (41). Both of these inhibitors are widely used and shown to effectively inhibit PI3K in several different cell lines (37;268;423;432;464-467).

Specifically the insulin- induced PI3K activation is abolished by both LY294002 and wortmannin (468;469). In the present study, resveratrol- stimulated glucose transport was inhibited by both LY294002 and wortmannin as was the insulin- stimulated response. These data suggest that like insulin, the resveratrol response is PI3K- dependent. Although in the present study the activity of PI3K was not directly measured, insulin- dependent Akt/PKB phosphorylation in the presence of wortmannin was strongly inhibited, indicating that under our experimental design PI3K activity was effectively blocked by LY294002 and wortmannin. In the future, a direct approach to measure PI3K activation could be taken which is the measurement of the lipid products synthesized by PI3K, PIP₂ and PIP₃.

Akt/PKB exists as three different isoforms; PKB α , PKB β , and PKB γ , all of which are expressed in L6 skeletal muscle cells (470). Akt/PKB is located downstream of PI3K and is one of its major targets contributing to insulin- stimulated glucose transport (471). All three Akt/PKB isoforms are activated by insulin stimulation, however in L6 myotubes PKB α is the dominant isoform involved in insulin- stimulated glucose transport (269;470). Resveratrol has been reported previously to increase Akt/PKB phosphorylation in MCF-7 human breast epithelial cells (354). Activation of Akt/PKB α involves phosphorylation of two residues, Thr308 and Ser473. In order for full activation to occur the Ser473 residue must be phosphorylated. Akt/PKB may also be phosphorylated on Ser124 and Thr450 residues but these sites do not regulate Akt/PKB activity and their phosphorylation is unchanged after stimulation with insulin (71).

To determine the role of Akt/PKB activation in resveratrol- stimulated glucose transport, we examined Akt/PKB phosphorylation at Ser473 through Western blot

analysis (472-474). The antibody that recognizes Ser473 is the most widely used. It is generally accepted that phosphorylation of Akt/PKB represents activation and this technique has been employed in numerous studies. Although each Akt/PKB isoform was not examined separately in the present study, the antibody used recognizes all three isoforms when they are phosphorylated on their corresponding serine residues. This antibody does not recognize the Thr308 phosphorylation site.

The present results showing an increase in glucose transport by resveratrol without an effect on Akt/PKB phosphorylation/ activation, suggest a mechanism that is independent of Akt/PKB. Nitric oxide- stimulated glucose transport in 3T3- L1 adipocytes is also Akt/PKB independent (475). Similarly to our resveratrol data, neuregulin- stimulated glucose transport in L6E9 myotubes is PI3K- dependent but Akt/PKB- independent (476). The use of small interference RNA (siRNA) to silence proteins has been widely used in recent years. In siRNA knockouts, protein expression is inhibited via RNA interference where gene expression is selectively silenced through the transfection of double-stranded RNA molecules. Akt/PKB siRNA technology could be employed to further examine the involvement of Akt/PKB in resveratrol- stimulated transport. Additionally, an Akt/PKB activity assay could provide more information.

Insulin- stimulated GLUT4 glucose transporter translocation has been documented in several fat and muscle cell lines (9-11) and is well- established. Since resveratrol stimulated glucose transport to significant levels similar to insulin we reasoned that GLUT4 translocation may be involved. We measured surface GLUT4 levels with L6 GLUTmyc cells that stably express GLUT4 tagged with an exofacial myc epitope (GLUTmyc) (409). The amount of GLUTmyc incorporated into the plasma

membrane was quantitated by immunologically labelling the myc epitope at the surface of intact cells. Resveratrol- mediated glucose transport appears to be independent of GLUT4 translocation, despite a significant increase in glucose transport by resveratrol in the GLUT4 overexpressing cells. The non- involvement of GLUT4 translocation could suggest that resveratrol may have an effect or combined effect on 1) the intrinsic activity of the GLUT4 transporters, 2) the intrinsic activity of other glucose transporters expressed in the L6 cell line (GLUT1 or GLUT3), or 3) the translocation of GLUT1 and/or GLUT3. Interestingly, metformin action in L6 muscle cells had no effect on GLUT4 translocation but did significantly increase GLUT1 glucose transporter redistribution to the plasma membrane (420).

GLUT1 and GLUT3 glucose transporters are in low abundance compared to GLUT4 in L6- GLUT myc cells, where the ratio of GLUT4 myc to GLUT4 and GLUT1 is 124:1:1.2 (392;412;413). GLUT1 expression is the same in GLUT4 myc cells as in parental L6 cells (392). GLUT1 expression is the same in the parental and L6- GLUT4 myc cells but the ratio of GLUT1 to GLUT4 myc is significantly altered. Therefore, the overabundance of GLUT4 myc may interfere with the GLUT1 transporter translocation. It is possible that resveratrol- stimulated glucose uptake involves the GLUT1 transporter, which could explain why we observed slightly decreased glucose uptake with resveratrol in the L6- GLUT4 myc cells. Alternatively, resveratrol may prolong the half- life of glucose transporters by reducing degradation and stabilizing GLUTs at the plasma membrane.

Subcellular fractionation studies to examine the effect of resveratrol on GLUT1, 3, and 4 in both the intracellular and plasma membranes could be employed in the future

to gain a better understanding of the action of resveratrol on the different L6 glucose transporters. Translocation would be indicated if an increase in plasma membrane glucose transporters corresponded with a decrease in the intracellular membrane content of the transporters. Alternative methods of examining translocation include photolabelling and immunofluorescence but they are not as widely used.

p38 MAPK is rapidly activated by insulin (10) through MKK3 and MKK6 activation in L6 myotubes (157). Changes in the intrinsic activity of glucose transporters by insulin have been previously suggested through activation of p38 MAPK in both L6 myotubes (153) and 3T3-L1 adipocytes (153;155). Resveratrol has been reported to activate p38 MAPK in various cell lines as well (334;365). We tested whether resveratrol, like insulin, would also phosphorylate p38 MAPK in L6 myotubes through Western blot analysis.

We found that resveratrol did not phosphorylate p38 MAPK and even reduced acute insulin- stimulated p38 MAPK phosphorylation. These data suggest that resveratrol- stimulated glucose transport does not require p38 MAPK phosphorylation. Assuming that p38 MAPK is a major regulator of GLUT4 activity, since resveratrol did not phosphorylate p38 MAPK (Figure 23) it is likely that resveratrol- stimulated glucose transport does not involve an increase in p38 MAPK- mediated GLUT4 activity but could possibly stimulate an alternative regulator to increase GLUT4 activity.

Maximum insulin- stimulated glucose transport requires an increase in GLUT4 activity through p38 MAPK activation (153;155). The inhibition of insulin- stimulated p38 MAPK phosphorylation seen in our studies might explain the lack of additivity in glucose transport when maximum concentrations of resveratrol and insulin were

combined (Figure 13). Although in our studies p38 MAPK activity was not directly measured, p38 MAPK phosphorylation (Thr180 and Tyr182) correlates very highly with activation and is widely accepted as an indicator of activation (153;155;157). Basal p38 MAPK activity, as mentioned before (Section 3.2.3), was somehow elevated in our experiments. Although p38 MAPK is not phosphorylated/ activated by resveratrol, one may argue that the existing activated p38 MAPK may be sufficient for resveratrol signalling. The p38 MAPK inhibitor SB203580 or p38 MAPK siRNA approach could be employed to further examine the role of p38 MAPK in resveratrol- stimulated glucose transport. These approaches are widely used and accepted to effectively inhibit p38 MAPK activation (156;432;477).

Insulin activates ERK1/2, which is another member of the MAPK family, through the Ras/Raf/MEK pathway in L6 cells to stimulate mitogenesis, however this activation is not required for insulin- stimulated glucose transport (478). Still ERK1/2 activation may be involved in resveratrol- induced glucose uptake. Resveratrol has been shown to activate ERK1/2 in several cancer cell lines (334;335) and we used Western blot analysis to examine if resveratrol would also phosphorylate ERK1/2 in L6 myotubes. We discovered that, unlike insulin, resveratrol did not phosphorylate ERK1/2. These data indicate that resveratrol- induced glucose uptake does not involve ERK1/2 phosphorylation.

Insulin has been found to activate the MAPK JNK1/2 in L6 myotubes (196), however its' function is undefined and it is unclear whether it is required for insulin- stimulated glucose transport. Similar to the findings reported for the other MAPK family members, ERK1/2 and p38 MAPK, resveratrol has been reported to activate JNK1/2 in

various cell lines (334;336). We took the same approach as for p38 MAPK and ERK1/2 and examined if resveratrol induced JNK1/2 phosphorylation in L6 myotubes. Our results showed that insulin stimulated JNK1/2 phosphorylation as reported previously, however resveratrol did not have any effect. These results suggest that since resveratrol does not phosphorylate JNK1/2, it is unlikely that this kinase is required for resveratrol- stimulated glucose uptake.

An ERK1/2 and JNK1/2 activity assay was not used since phosphorylation is generally accepted as an indicator of activation of these kinases. Pharmacologic inhibition of MEK1/2, an upstream regulator of ERK1/2, with PD98059, a specific inhibitor of MEK1/2, and SP600125, a competitive inhibitor for the ATP- binding site of JNK1/2, would have provided additional information. However since we have no indication that resveratrol phosphorylates either ERK1/2 or JNK1/2, it is unlikely that the inhibitors would have any significant effect on resveratrol- stimulated glucose transport. As with Akt/PKB and p38 MAPK, ERK1/2 and JNK1/2 siRNAs could be employed to further examine their role in resveratrol- stimulated glucose uptake.

The role of PKC in insulin- stimulated glucose uptake is highly debated. Insulin increased the activity of PKC ζ , which was blocked by both wortmannin and LY294002. In addition, expression of inactive PKC ζ inhibited both basal and insulin- stimulated glucose transport in L6 myotubes (223), suggesting that PKC (λ , ζ) activation is required for insulin- stimulated glucose transport and is independent of Akt/PKB but requires PI3K (221-224). It has also been documented that acute activation of PKC by PMA, a DAG analogue, leads to increased glucose uptake (422). Since resveratrol employs PI3K but does not involve Akt/PKB activation, it was important to examine a possible

alternative pathway that involves PI3K and affects glucose transport. One of the difficulties in studying PKC is the large number of isoforms, which are divided into the conventional, novel, and atypical isoforms. Therefore, in our study we applied two inhibitors that are widely used in order to inhibit all isoforms of PKC, BIM1 and Go6983 and measured glucose transport after PMA or resveratrol treatment. BIM1 and Go6983 are selective inhibitors of all PKC isoforms that act as competitive inhibitors for the ATP-binding site of PKC.

Resveratrol- stimulated glucose uptake was not affected by either inhibitor, suggesting that resveratrol action is PKC- independent. If we had an indication of PKC involvement in resveratrol action, we could have taken a multiple approach to elucidate the isoforms employed and their roles. For example, additional inhibitors could have been used, siRNA knockouts, and even dominant negative mutants for specific PKC isoforms could have been employed.

It was shown previously that an intact actin cytoskeleton is required for the translocation of glucose transporters (GLUT1, 3, and 4) and early signalling steps activated by insulin (232). In bovine pulmonary artery endothelial cells, it was shown that resveratrol (100 μ M)- induced cell elongation was abolished by cytochalasin D (CD), indicating that an intact actin cytoskeleton is required (361). As mentioned previously (Section 3.1.9), the fungal metabolite CD binds to the barbed end of the actin monomer or filament and affects actin polymerization to decrease actin filament formation (429). We used CD to examine the role of an intact actin network in resveratrol- stimulated glucose transport. CD treatment effectively inhibited both insulin- and resveratrol- stimulated glucose transport, indicating that an intact actin network is required for both

stimuli. The fact that the response of the cells to resveratrol is sensitive to CD and the lack of potentiation of the maximal insulin response by resveratrol suggest that the resveratrol signalling pathway may converge with that of insulin.

4.2 Resveratrol and amino acid transport.

Insulin is a pleiotropic hormone and apart from stimulating glucose transport, it also stimulates amino acid transport in skeletal muscle (261). Amino acid transport is essential for protein synthesis and adaptive regulation in response to chronic amino acid deprivation (479) and is most commonly measured with the amino acid analogue MeAIB, which is specifically transported by the insulin-sensitive System A amino acid carrier (253). Currently the effect of resveratrol on amino acid uptake in skeletal muscle has not been examined. Since resveratrol stimulates glucose uptake similar to that of insulin, we wanted to examine whether resveratrol would mimic additional metabolic effects of insulin and examined MeAIB uptake in L6 myotubes.

Insulin significantly increased MeAIB uptake, however resveratrol reduced both basal- and insulin-stimulated MeAIB transport. These results are similar to the vanadium compounds sodium orthovanadate and pervanadate, all of which increased glucose transport but decreased amino acid uptake (436). Future studies could focus on the mechanism of inhibition of amino acid transport by resveratrol, the mechanism may involve the reduction of the rate of amino acid transport through a decrease in the number of SNAT2 transporters (V_{\max}) and subsequently decreasing SNAT2 translocation or activity and/ or an increase in K_m indicating a lower affinity for the amino acids to be transported into the cell. The reduction in insulin-stimulated amino acid transport by resveratrol may be due to inhibition of key signalling molecules required for insulin-

stimulated amino acid transport, which would most likely occur downstream of PI3K and Akt/PKB since insulin-stimulated Akt/PKB phosphorylation was not modified by resveratrol, suggesting that the PI3K/ Akt/PKB pathway is not influenced.

4.3 Resveratrol and mitogenesis.

Insulin is well known for its metabolic effects, however it also has mitogenic properties. More specifically, insulin stimulates cell proliferation and differentiation (111;112). FBS contains many growth factors (480) and it is widely used to stimulate cell growth. Numerous studies have shown that resveratrol has an inhibitory effect on cell growth and proliferation (328;331;335;346). Therefore we wanted to examine if resveratrol mimicked insulin action beyond its metabolic effects. We studied the effect of resveratrol on mitogenesis through measurement of tritiated thymidine incorporation into L6 myoblasts. We found that resveratrol inhibited mitogenesis as demonstrated by a decrease in thymidine incorporation, which is consistent with previous studies with resveratrol in various cancer cell lines (317;438;439). In addition, resveratrol completely abolished insulin- and FBS- stimulated cell proliferation. In the current study we used thymidine incorporation to measure cell proliferation but if we used bromodeoxyuridine (BrdU) staining where BrdU incorporation in cells in the S phase causes fluorescence (328), we would expect a similar result. Although cell viability was not directly measured, cell morphology was assessed with microscopic observation and was unaffected after chronic resveratrol treatment.

Insulin stimulates ERK1/2 phosphorylation through the Ras- signalling pathway in order to promote cell proliferation and mitogenesis (96). Studies in several cancer and vascular smooth muscle cell lines have demonstrated that chronic resveratrol treatment

decreases ERK1/2 phosphorylation/ activation and subsequently inhibits cell proliferation and mitogenesis (319;321;356;440). However resveratrol has also been shown to activate ERK1/2 (334;335;368). We previously found that acute resveratrol treatment did not phosphorylate ERK1/2 (Figure 21). Therefore, we attempted to elucidate the mechanism of resveratrol- inhibited mitogenesis by examining ERK1/2 phosphorylation through Western blot analysis.

Our data show that acute resveratrol treatment abolished insulin- stimulated ERK1/2 phosphorylation but chronic resveratrol (24 h) did not affect ERK1/2 phosphorylation and had no effect on insulin- or FBS- induced ERK1/2 phosphorylation. Perhaps the acute inhibition of insulin- and FBS- stimulated ERK1/2 phosphorylation by resveratrol is sufficient to block mitogenesis and ERK1/2 inhibition is reversed as a compensatory mechanism. In addition, even though the cell compensates by increasing ERK1/2 phosphorylation, this may not result in increased cell proliferation if resveratrol inhibits alternate signalling pathways involved in the regulation of cell proliferation. Alternatively, it is possible that resveratrol may restrict the phase of the cell cycle. Future experiments could address these possibilities by flow cytometry analysis or Western blot analysis of the cyclins and cdks responsible for regulating cell cycle progression.

p38 MAPK and JNK1/2 are also activated by growth factors such as insulin and although they are primarily involved in the mediation of apoptosis and inflammation, recent evidence has shown biological importance in regulating cell proliferation (151;201-203). Resveratrol has been shown to activate both p38 MAPK and JNK1/2 in different cell lines (334;336;365). Insulin also stimulates p38 MAPK activation in L6 myotubes (441) through MKK3 and MKK6 activation (157) where prolonged insulin

treatment has been demonstrated to increase mitogenesis involving p38 MAPK activation in C₂C₁₂ cells (442). Insulin stimulates JNK1/2 activation in L6 myotubes as well, however its exact role is unknown (196).

We examined the effect of acute and chronic (24 h) insulin, FBS, and resveratrol treatment on p38 MAPK and JNK1/2 phosphorylation through Western blot analysis. Our results indicate that acute resveratrol treatment did not increase p38 MAPK or JNK1/2 phosphorylation but inhibited insulin- stimulated phosphorylation of the two kinases. Chronic resveratrol treatment did not affect insulin- or FBS- induced p38 MAPK or JNK1/2 phosphorylation. These data suggest that p38 MAPK and JNK1/2 phosphorylation is present after chronic resveratrol treatment even though it is inhibited during acute stimulation. Similar to ERK1/2 activation, it is possible that acute inhibition of insulin- and FBS- stimulated p38 MAPK and JNK1/2 phosphorylation by resveratrol is sufficient to block mitogenesis and inhibition of these MAPK members is reversed as a compensatory mechanism to promote cell survival. This compensation does not appear to be sufficient to increase cell proliferation, which may be explained if resveratrol inhibits additional signalling pathways involved in mitogenic regulation. JNK1/2 and p38 MAPK activation is often associated with apoptosis induction (334;336). The lack of inhibition of insulin- and FBS- mediated MAPK phosphorylation by resveratrol may be an indicator of apoptosis induction. Apoptosis was not examined in this study however future experiments could explore this possibility.

In summary, the present study provides important insights into the role of resveratrol in glucose transport regulation in L6 skeletal muscle cells. Our results showed that resveratrol increases glucose uptake in a dose- and time- dependent fashion that is

independent of protein synthesis and does not require the presence of insulin. Resveratrol may act as an insulin sensitizer, however further studies are needed to establish this concept. Investigation into the mechanism of resveratrol-stimulated glucose uptake revealed that PI3K and an intact actin network are involved in signalling. However, Akt/PKB, PKC, ERK1/2, p38 MAPK or JNK1/2 activation and GLUT4 translocation cannot explain the increase in glucose transport. Further investigation of this novel action is required.

4.4 Significance of the Present Study

Type 2 diabetes has a high prevalence worldwide. The increasing prevalence of type 2 diabetes may also increase the frequency of cardiovascular disease and other complications of diabetes. Insulin resistance or insulin insensitivity characterizes the disease where defects in the skeletal muscle glucose transport system are present (369;370). Therefore an in depth understanding of the glucose transport mechanism in skeletal muscle cells and the action of different compounds that stimulate glucose transport may provide new therapeutic approaches to overcome insulin resistance and type 2 diabetes mellitus. As previously mentioned, resveratrol has demonstrated significant positive health benefits as an antioxidant and anti-cardiovascular disease and anti-cancer agent. Recent studies have shown that resveratrol activates sirtuins, which have been implicated in longevity and improving insulin sensitivity in 3T3-L1 adipocytes (481).

This study is the first study to examine the effects of resveratrol on glucose transport in one of the major insulin target tissues. Furthermore, our findings are novel since we discovered that resveratrol did in fact stimulate glucose uptake into L6 skeletal

muscle cells to levels similar to that of insulin independent of protein synthesis and the presence of insulin. The mechanism of resveratrol- stimulated glucose uptake is PI3K- dependent and requires an intact actin network. However Akt/PKB, PKC, ERK1/2, JNK1/2, p38 MAPK, and GLUT4 translocation do not appear to be employed in resveratrol's action. Our results suggest that resveratrol may have potential for use against insulin resistance. Currently, several oral anti- diabetic drugs are used to manage type 2 diabetes, however it would be beneficial if this drugs had a positive pleiotropic effect on health. Resveratrol has demonstrated positive health benefits in several major diseases, including cancer and cardiovascular disease. In addition, our study is the first step in this exciting new area of research and indicates that resveratrol's actions/ effects should be considered for further studies in animal models as a possible anti- diabetic drug.

4.5 Future Directions

Our research concentrated on the in vitro effects of resveratrol in L6 skeletal muscle cells and therefore may be extended to new in vitro models examining alternate insulin target tissues and in vivo models to study glucose transport. Furthermore, significant study is still required to further delineate the mechanism of action of resveratrol- stimulated glucose uptake in L6 skeletal muscle cells. This analysis could include examination of the GLUT1 and GLUT3 glucose transporters or the AMPK and cbl- signalling pathways. Future studies could also examine:

- 1) The effects of resveratrol on glucose transport in intact skeletal muscle.
- 2) The effects of resveratrol on glucose transport and glucose homeostasis in a rat in vivo model of insulin resistance.

- 3) The effects of resveratrol on glucose transport in adipocytes.
- 4) The effects of resveratrol on gluconeogenesis, glycogenolysis, and glucose release in hepatocytes, another insulin target tissue.
- 5) The effects of resveratrol on insulin secretion in pancreatic beta cells.

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