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# The Effects of Berberine and Nitroxl (HNO) on the Glucose Uptake in L929 Fibroblast Cells

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# THE EFFECTS OF BERBERINE & NITROXYL (HNO) ON THE GLUCOSE UPTAKE IN L929 FIBROBLAST CELLS

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## DEDICATION

This work is dedicated to my Mentor; Professor Larry Louters, Parents; Mr & Mrs Richard and Mercy Chenge and my brother Augustine Chenge and sisters Mary and Justina Chenge and most importantly I dedicate it to my beloved Katherine Monday, all whose patience provided me with much of the courage and incentive to finish this journey.

## ACKNOWLEDGEMENTS

For Professor Larry Louters, without whom this Research mathmagic would not be possible. Thank you.

For my lab mates and lab neighbors past and present, including but not limited to Matt Salie, Alexander Cok, Dan Oram, Christina Plaisier, David Kuipers and Jared Scripture whose patience, conversations, and inspiration were invaluable. Thank you.

For all my teachers, especially my committee members: Patrick Thorpe, Mark Staves, Margaret Dietrich, Niraj Joshi and Osman Patel. You've given me the torch of knowledge, and I intend to pass it on. I hope I can emulate your successes. Thank you.

For my fellow students and friends, thank you, especially to Christina Plaisier, Matt Salie, and Jared Scripture. Without your patience, perspective, motivation, and therapeutic advice, I would be lost in oblivion. Thank you.

For my Dad and Mom, Richard and Mercy Chenge. You are wonderful, understanding, and supportive, for my Friend Doose Iornenge. Thank you.

Most importantly, my beloved Katherine A Monday. We dreamed this dream together. I am deeply proud to share this achievement with you. Thank you.

## ABSTRACT

Berberine, which has a long history of use in Chinese medicine, has recently been shown to have efficacy in the treatment of diabetes. While the hypoglycemic effect of berberine has been clearly documented in animal and cell line models, such as 3T3-L1 adipocytes and L6 myotube cells, the mechanism of action appears complex with data implicating activation of the insulin signaling pathway as well as activation of the exercise or AMP kinase-mediated pathway. There have been no reports of the acute affects of berberine on the transport activity of the insulin-insensitive glucose transporter, GLUT1. Therefore, we examined the acute effects of berberine on glucose uptake in L929 fibroblast cells, a cell line that express only GLUT1. Berberine- activated glucose uptake reaching maximum stimulation of five-fold at >40 μM. Significant activation ($P < 0.05$) was measured within 5 min reaching a maximum by 30 min. The berberine effect was not additive to the maximal stimulation by other known stimulants, azide, methylene blue or glucose deprivation, suggesting shared steps between berberine and these stimulants. Berberine significantly reduced the Km of glucose uptake from 6.7 ± 1.9 mM to 0.55 ± 0.08 mM, but had no effect on the Vmax of uptake. Compound C, an inhibitor of AMP kinase, did not affect berberine-stimulated glucose uptake, but inhibitors of downstream kinases partially blocked berberine stimulation. SB203580 (inhibitor of p38

MAP kinase) did not affect submaximal berberine activation, but did lower maximal berberine stimulation by 26%, while PD98059 (inhibitor of ERK kinase) completely blocked submaximal berberine activation and decreased the maximal stimulation by 55%. It appears from this study that a portion of the hypoglycemic effects of berberine can be attributed to its acute activation of the transport activity of GLUT1.

Nitroxyl (HNO) is a molecule of significant interest due to its unique pharmacological properties, particularly within the cardiovascular system. A large portion of HNO biological effects can be attributed to its reactivity with protein thiols, where it can generate disulfide bonds. Evidence from studies in erythrocytes suggests that the activity of GLUT1 is enhanced by the formation of an internal disulfide bond. However, there are no reports that document the effects of HNO on glucose uptake. Therefore, we examined the acute effects of Angeli's salt (AS), a HNO donor, on glucose uptake activity of GLUT1 in L929 fibroblast cells. We report that AS stimulates glucose uptake with a maximum effective concentration of 5.0 mM. An initial 7.2-fold increase occurs within 2 min, which decreases and plateaus to a 4.0-fold activation after 10 min. About 60% of the 4.0-fold activation recovers within 10 min, and 40% remains after an hour. The activation is blocked by the pretreatment of cells with thiol-reactive compounds, iodoacetamide (0.75 mM), cinnamaldehyde (2.0 mM), and phenylarsine oxide (10 μM). The effects of AS are not additive to the stimulatory effects of other acute activators of glucose uptake in L929 cells, such as azide (5 mM), berberine (50 μM), or

glucose deprivation. These data suggest that GLUT1 is acutely activated in L929 cells by the formation of a disulfide bond, likely within GLUT1 itself.

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## ABBREVIATIONS

AS (Angelis salt)

AMPK (Adenosine Monophosphate Protein Kinase)

CA (Cinnamaldehye)

DMEM (Dulbecco's Modified Eagle Medium)

FBS (Feotal Bovine Serum)

PAO (Phenarsine Oxide)

GLUT1 (Glucose transporter-1)

GLUT4 (Glucose transporter-4)

IRS (Insulin Receptor Substrate)

HNO (Nitroxyl)

*2DG (2-deoxy-D-glucose)

MB (Methylene Blue)

MAPK kinase (Mitogen Activator Protein Kinase Kinase)

ERK Kinase (Extracellular Receptor Kinase Kinase)

# CHAPTER I

## INTRODUCTION

Glucose is an important source of energy for a wide variety of living organisms. Body tissues, such as the brain, continually need constant supply of glucose. Low blood glucose can result in loss of consciousness, seizures and possibly death. However, prolonged increases in the concentration of blood glucose such as in poorly controlled diabetes results in renal failure, blindness, peripheral and cardiovascular disease, and neuropathy. Therefore, there is need to maintain the concentration of blood glucose within narrow limits. This can be achieved either by natural physiological processes or by introduction of safe pharmaceutical agents, which mimic the physiological processes and yield the same results. In the long run this manages hyperglycemia and more specifically, diabetes.

Diabetes mellitus is a heterogeneous disorder characterized by the derangement of carbohydrates, protein and lipid metabolism *(Yin et al., 2008; Rother., 2007; Muueckler., 1990)*. In the year 2005 there was an increase in the incidence of diabetes and at least 190 million people were diagnosed with the ailment worldwide *(Ravid and Rachmani., 2005)*. Presently, recent studies show a total of 300 million people with the ailment (IDF, 2011). Diabetes can be classified into two types; type-1 and type-2.

Diabetes mellitus type-1 is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas resulting to insulin deficiency. Type-1 diabetes can be further classified as immune-mediated or idiopathic. The majority of type-1 diabetes is of the immune-mediated nature, where beta cell loss is a T-cell mediated autoimmune attack *(Rother., 2007)*.

Diabetes mellitus type-2, which is the focus of this study is the more common type of diabetes and is characterized by cell insulin resistance, which may be combined with reduced insulin secretion *(Rother., 2007).*

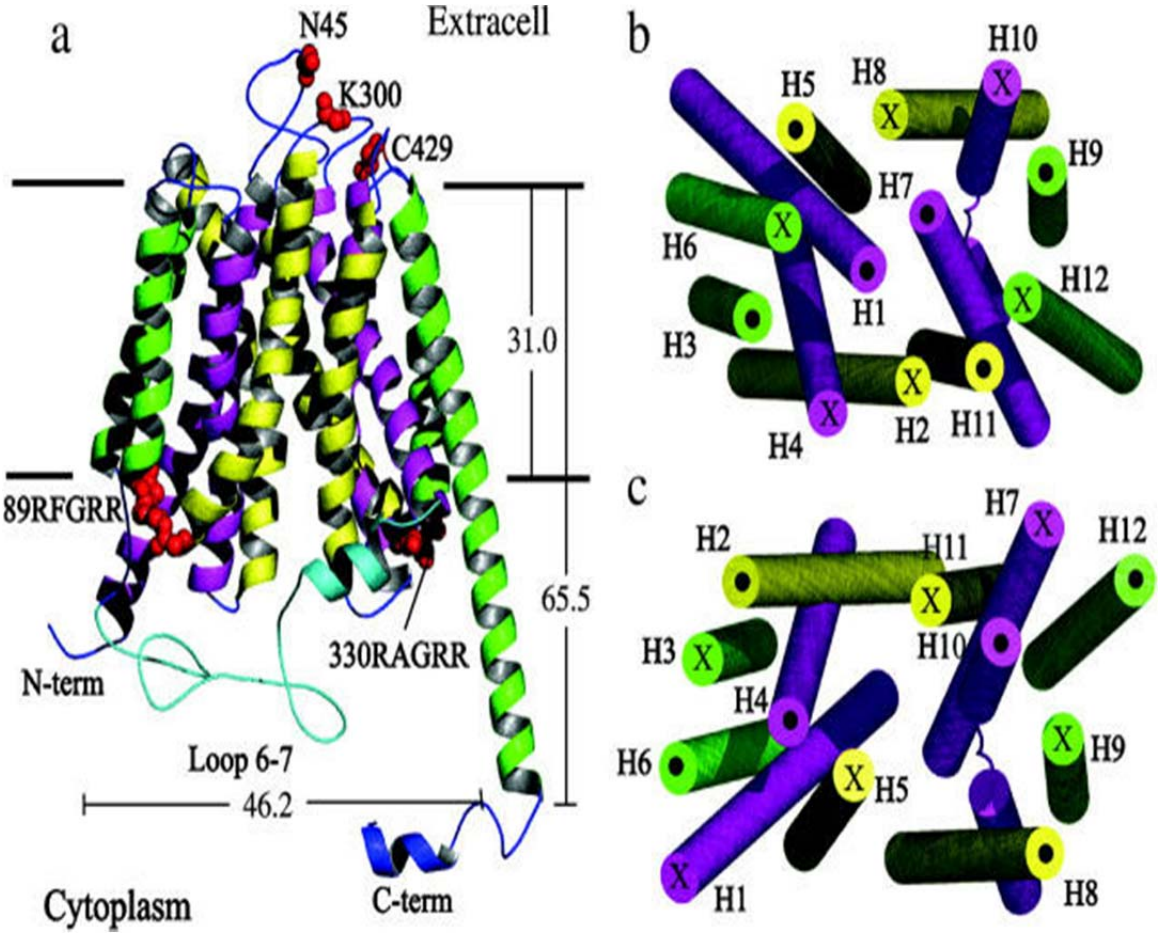
Hyperglycemia is the common effect of both of these abnormalities. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor *(Rother., 2007)*. Although the specific physiologic and genetic causes for insulin resistance are not fully understood; the predominant abnormality in the early stage of diabetes is reduced insulin sensitivity, which can be the result of genetic defects anywhere in the insulin-signaling pathway. At this stage hyperglycemia can be reversed by a variety of measures and medications. These treatments improve insulin sensitivity or reduce glucose production by the liver and enhance glucose uptake in the body tissues *(Rother., 2007).*

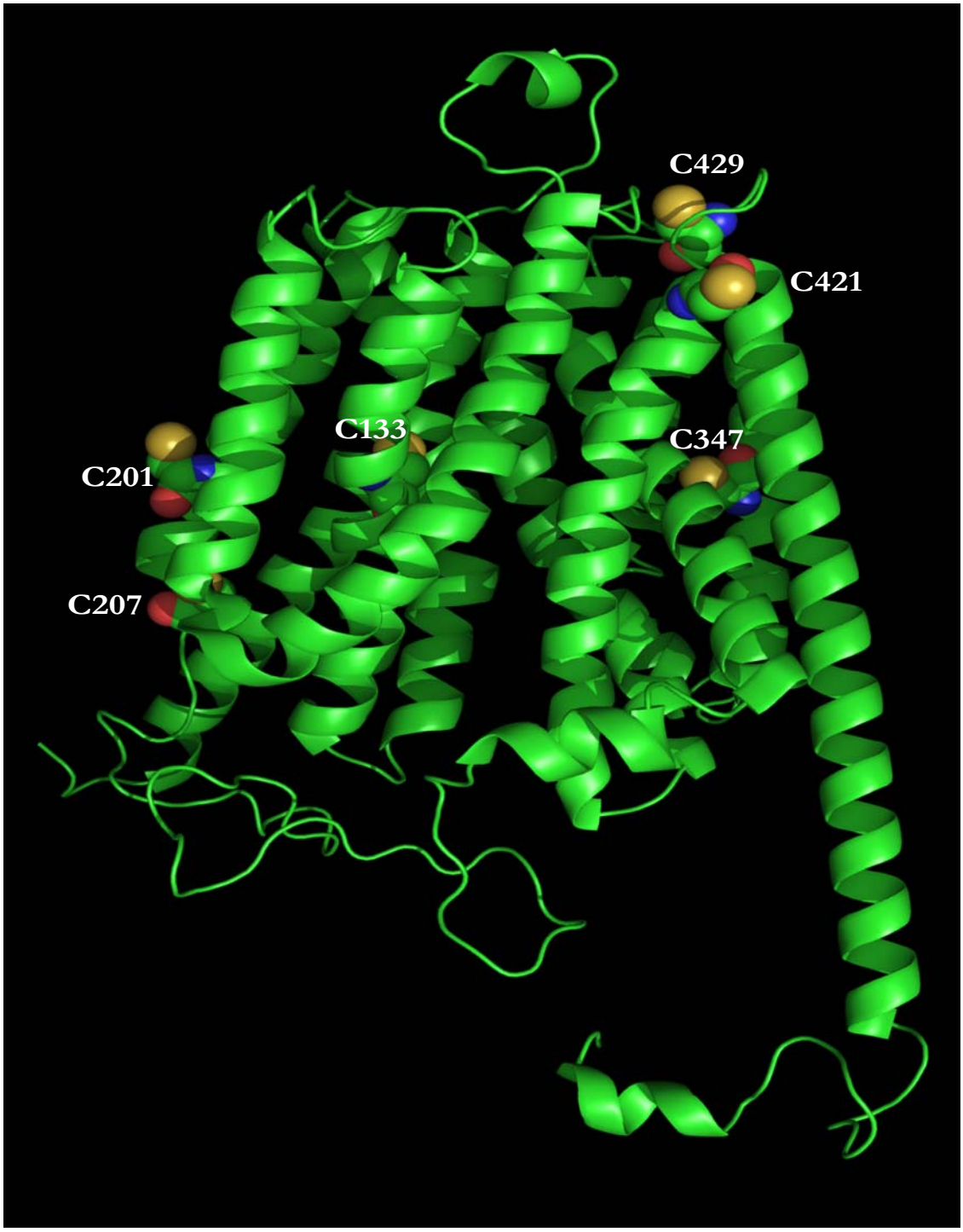
There is no absolute cure for diabetes type-1 and type-2, though significant success has been demonstrated with insulin administration either orally or intravenously *(Goldfine and Youngren.1998)*, but like all proteins, insulin is usually degraded which results in destruction of its activity *(Goldfine and Youngren., 1998 and Al-Awaidi etal., 1985)*. In addition, Oral hypoglycemic agents like sulphonylureas and biguanides *(Al-*

*Awaidi etal., 1985)* and other currently available drugs pose significant side effects and high rate of secondary failure [8,10,11] *(Rother., 2007; Al-Awaidi etal., 1985 and Xie et al., 2005).* Thus, there is need for further research to find new oral medication for long-term control of blood glucose in diabetes type-2 patients. The hallmark characteristic of diabetes is compromised glucose uptake; thus, there is high interest in nutrichemicals or plant products and other potential biopharmaceutical agents that activate glucose uptake as potential treatments for diabetes. There has been wide use of certain plant products generally regarded as safe that manage diabetes due to their anti- oxidation, anti-obesity and anti-hyperglycemia properties *(Yin et al., 2008).* However, quality control in these plants is difficult as there are often several pharmacologically active components present in the extracts, each with unique medicinal effects. Consequently, for the purpose of scientific investigation, it is better to work with purified components. In this study, we have considered berberine, a purified isoquinoline compound, found in some medicinal plants such as coptis chinensis, and hydrastis canadensis. In addition, we have also considered nitroxyl (HNO), a potential biopharmaceutical that has recently received great attention because of its ability to form disulfide bonds in thio-proteins and hemeproteins which implicates HNO as a potential cell signaling molecule (Fukuto et al., 2005). We have investigated their potential medicinal properties, particularly the anti-hyperglycemia properties: activation of glucose uptake, and for HNO in particular; the effects on the structure of GLUT1, the ubiquitous glucose transporter focused in this study, which also contains cysteine groups in its structure (figure 1.0a & 1.0d).

GLUCOSE UPTAKE TRANSPORTERS

The fact that glucose, a monosaccharide, is an important energy source for a wide variety of cells cannot be over emphasized. The polarity of glucose prohibits simple diffusion across the membrane, and therefore, the transport of glucose into cells requires protein transporters. The most common mammalian glucose transporters are a family of passive transmembrane protein characterized of having twelve transmembrane spanning helices (Figure 1.0b)






**(d)**

**Figure 1.0** Structural representations of Glut1. (a) Side view showing relative positions of the helices. Residues in red represent topology constraints derived from experimental results involving N45, K300, and C429 for the extracellular side, and motifs 89RFGRR93 and 330RAGRR for the cytoplasmic side. Glut1 measures ~35.6 × 26.3 Å viewed from the top, and 46.2 Å × 27.2 Å from the bottom. Its height is ~61 Å. (b) View from the extracellular side showing the tilt of the 12 transmembrane helices. X marks loops entering, whereas dots mark loops exiting. (c) Cytoplasmic view; marks as above. (d) Structure showing cysteine (yellow balls) 133, 201, 207, 347, 421 & 429 residues. The helix colors are in concordance with the symmetry template found by Hirai et al. (2002). Figure drawn using PYMOL (http://pymol.sourceforge.net.)
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1304772/

Research has shown that five major mammalian glucose transporters (GLUT1, 2, 3, 4 &5) are selectively distributed on specific tissues *(Palfreyman et al., 1992)* (see table 1). Additionally, it has been suggested that the abundance of a particular isoform, in a particular tissue cell membrane, can be correlated to its properties of kinetic transport *(Palfreyman et al., 1992)*. This kinetic activity, further explains the responsiveness and sensitivity of the transporter to glucose, expressed in terms of Vmax and the Michelis - Menton constant Km, respectively *(Mueckler., 1990 and Pilch., 1990)*. For instance, In vitro cloned culture cells demonstrate high GLUT1 isomer concentrations *(Palfreyman et al., 1992)*. In addition, oncogene cell transformation, growth factors, and starvation *(Louters et al., 2006)* significantly increase the concentration, or the sensitivity and responsiveness of these transporters, resulting in enhancement of glucose transport across the plasma membrane *(Palfreyman et al., 1992).*

Arguably, the two most important members of the GLUT family are GLUT1 and GLUT4.

Table 1. **CHARACTERISTICS OF THE FIVE MAMMALIAN FACILATATIVE GLUCOSE TRANSPORTERS**

| TRANSPORTER | APPROXIMATE *Km FOR GLUCOSE (mmol/liter) | TISSUE DISTRIBUTION | CHARACTERISTICS |
|---|---|---|---|
| GLUT-1 | 20 | Widely expressed; high concentrations in the brain, erythrocytes and endothelial cells. | Constitutive glucose transporter |
| GLUT-2 | 42 | kidney, small intestine epithelia, liver, pancreatic beta cells. | Low-affinity glucose transporter; has a role in sensing glucose concentrations in islets |
| GLUT-3 | 10 | Neurons, Placenta | High affinity glucose transporter |
| GLUT-4 | 2 to 10 | Skeletal muscle, cardiac muscle, adipose cells | Insulin- responsive glucose transporter |
| GLUT-5 | NA | Small intestine, sperm, kidney, brain, adipose cells, muscle | Fructose transporter; very low affinity for glucose |

*Km denotes the Michaelis-Menten constant, and NA not applicable

http://escholarship.umassmed.edu/cgi/viewcontent.cgi?article=1393&context=gsbs_diss

## The Glucose Transporter-4 (GLUT4)

GLUT4 is the only insulin-responsive glucose transporter and is located primarily in muscles and adipocytes (table 1). The Michaelis-Menton constant (Km) of GLUT4 for glucose has been demonstrated to be (2-10 mM) *(Palfreyman et al., 1992)* and the translocation of this isoform to the cell surface of muscle and adipose cells is stimulated by both insulin and exercise *(Palfreyman et al., 1992).*

Studies on rat 3T3-L1 adipocytes show that exposure of these cells to insulin results in a large (in excess of 20- fold) increase in the rate of glucose uptake by these cells *(Gwyn., 1997 and James et al., 1986).* This increase is the result of an increased Vmax of transport with little or no change in Km.

Insulin stimulated glucose uptake into adipocytes and skeletal muscles, is primarily mediated by the translocation of the insulin responsive glucose transporter GLUT4, from an intracellular compartment to the plasma membrane, as well as other metabolic processes like glycogen synthesis and fatty acid metabolism as shown in figure 1.1, *(Birmbaum., 1992).* As a result of insulin response, GLUT4-containing vesicles move to and fuse with the plasma membrane of the cell, thereby resulting in increased total number of transporters on the cell membrane 10-40 fold *(Birmbaum., 1992)* as shown on Figure 1.2.

The insulin dependent intracellular signaling events, relies substantially on the mechanism of action of classical insulin receptors *(Gwyn., 1997).* These receptors are expressed fairly ubiquitously, but with significant variation in the number of receptors per cell, which determines responsiveness of the cell to insulin *(Gwyn., 1997).* These

receptors also have two substrates termed insulin receptor substrates (IRS1 and 2). The discovery of IRS1 and IRS2 as major substrates of the insulin receptor paves a key way in elucidating the early events in insulin signaling (figure 1.2) *(White et al., 1985 and Sun et al., 1995).* Furthermore, since the discovery of the IRS systems, considerable studies have been done to investigate which of these molecules, IRS1 or 2, mediate insulin's stimulation of glucose uptake.

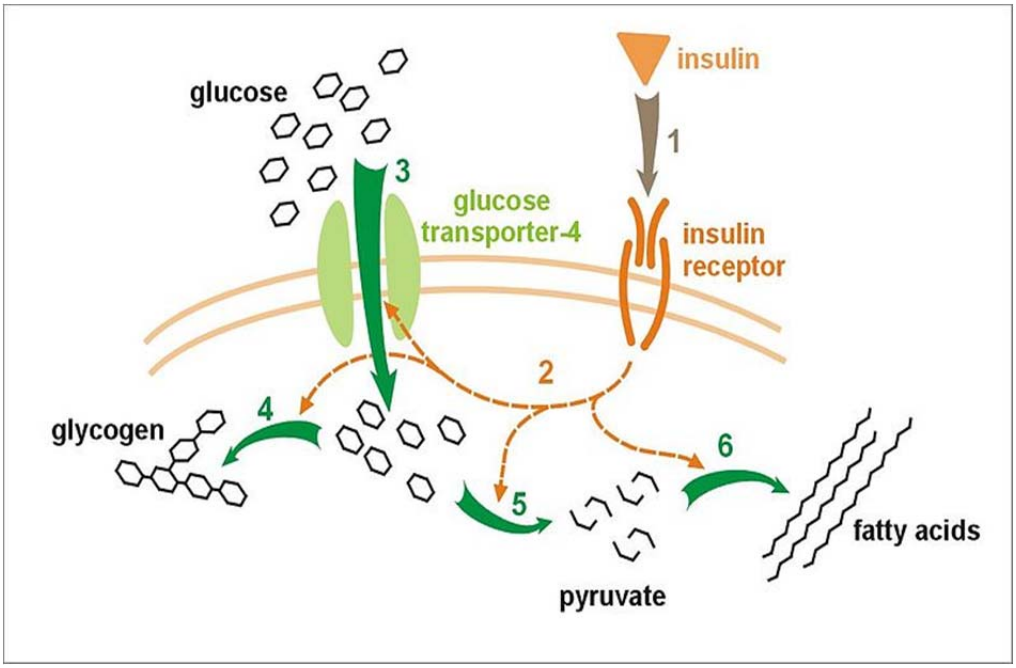



**Figure 1.1** Insulin triggers the uptake of glucose via GLUT4

Insulin initiates the uptake of glucose by binding to the insulin receptore (1), this step could lead to either of the four biological process; fatty acids metabolism-lipogenesis (6), metabolism of pyruvate (5) glycogenesis (4) and import of glucose through the formation of the glucose transporter-4 (3) leading to glycolysis.

(From, nutritionwonderland.com/2010/01/glucose-brain-alzheimers-diabetes)

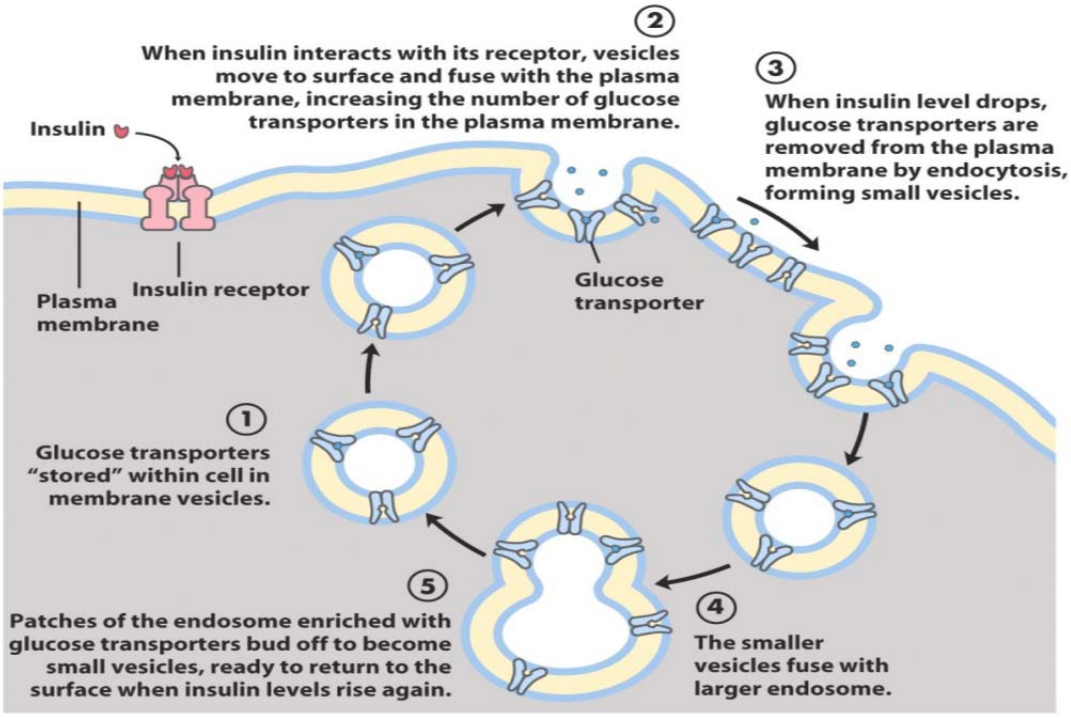



**Figure 1.2** IRS system conveys insulin signaling

The Insulin and Insulin receptor play a key role in insulin signaling, after insulin binds to the insulin receptor, a cascade of proteins including IRS1 & 2 participate in signaling the synthesis of GLUT4 which is transported to the plasma membrane and stimulates glucose transport. (Adapted from; Nelson D.L, Cox M.M (2001). *Lerhninger Principles of Biochemistry*. New York, WH Freeman and company, 11(394-395)

Rice et al showed that upon differentiation of 3T3-L1 fibroblasts into adipocytes, expression of IRS-1 increases 10-20 fold and correlated with the onset of GLUT4 and insulin receptor gene expression *(Klitzman et al., 1993)*. Thus, IRS1 presents a prime candidate for a proximal, obligate intermediate in the pathway mediating the metabolic actions of insulin (see figure 1.2) *(Gywn., 1997. and Klitzman et al., 1993)*. The metabolic function of the second substrate IRS2 is yet to be clarified *(Gwyn., 1997)*. Nevertheless, results from several studies have the general agreement that IRS1 is responsible for GLUT4 response to insulin.

## The Glucose Transporter-1 (GLUT1)

GLUT1 has also been recently studied extensively *(Wetheimer et al., 1991, and Bruckner et al 1999)*. This transporter is expressed ubiquitously and is responsible for the constitutive or basal level of glucose uptake *(Wetheimer et al., 1991 and Louters et al., 2006)*. Unlike GLUT4, GLUT1 is neither acutely responsive to insulin nor exercise. The primary mode of regulation of this transporter is the induction of GLUT1 protein synthesis by chronic exposure to cell stress *(Wetheimer et al., 1991)*. For instance, glucose deprivation for 12hr or more has been demonstrated to cause substantial increases in GLUT1 content and activity in 3T3-L1 adipocytes *(Klitzman et al., 1993)*. In neural cells the combined effect of glucose deprivation and oxygen deprivation resulted in increases in transport activity larger than 100-fold after 24h *(Bruckner et al., 1999)*. Chronic exposure to either hypoxia or oxidative phosphorylation inhibitors, such as azide, also up-regulated GLUT1 expression in clone 9 cells *(Behrooz et al., 1997)*. However, in addition to this chronic or long-term control, there is evidence that GLUT1 is also under acute regulation. 30min starvation in L6 skeletal muscle cells stimulated

glucose uptake independent of protein synthesis *(Mercado et al., 1989)*. Short term exposure to cell stressors, such as hyperosmolarity and azide also stimulated the glucose transport activity of GLUT1 in Clone 9 cells *(Mercado et al., 1989)*. This acute activation of GLUT1 increased the Vmax of transport without a change in the cell membrane concentration of GLUT1 *(Mercado et al., 1989)*. However, the location of the transporter on the plasma membrane may change due to this acute activation *(Barros et al., 2001).*

Research has also demonstrated that the transport of glucose by GLUT1 obeys the Michaelis- Menten kinetics *(Palfreyman et al., 1992 and Fukumoto et al., 1989)*. The transport of glucose is a facilitative process and is consistent with the alternating conformation model *(Gwyn., 1997).* This model predicts a single substrate (glucose) binding site, which is exposed either to the extracellular or intracellular space but not on both sides simultaneously *(Gwyn., 1997)*. Both sides, loaded or unloaded with glucose, can re-orientate via a conformational change within the protein, so as to face the opposite side of the plasma membrane (see figure 1.3). It should be noted that this mechanism can be equally applied to substrate influx or eflux and is driven purely by the chemical concentration gradient of glucose *(Gwyn., 1997)*. The rate constants governing substrate binding to GLUT1 can vary depending on which side of the membrane the transporter is facing, which gives rise to an observed kinetic asymmetry. Furthermore, the rate constants that govern the re-orientation of the empty GLUT1 are usually less than those that govern the re-orientation of the loaded GLUT1, accounting for the phenomenon of trans-acceleration *(Gwyn., 1997).*

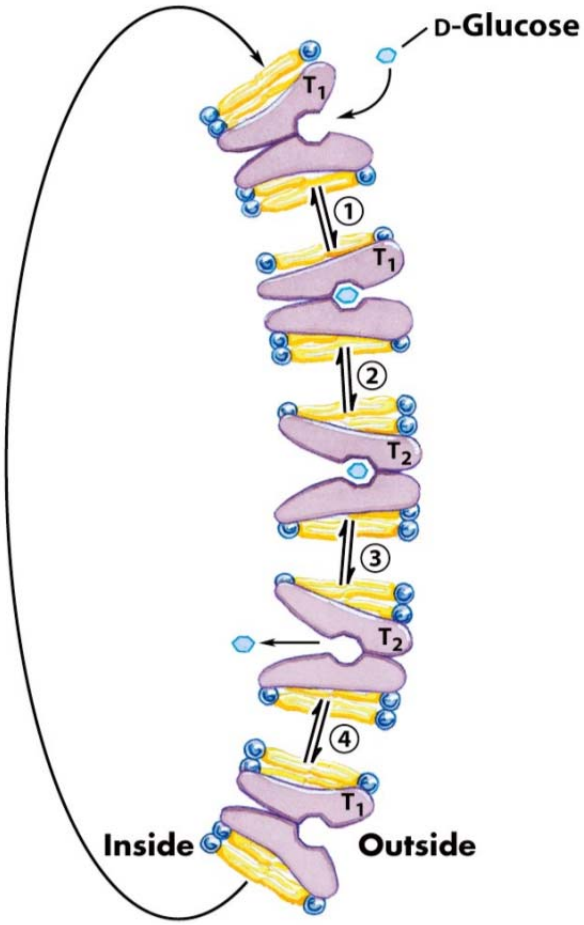



**Figure 1.3.** Alternate conformation mechanism of GLUT1

GLUT 1 in the (T1) state binds to glucose and changes conformation (T2) facing the opposite side of the plasma membrane and releases the glucose thereafter returns to its original conformation (T1) without undergoing any permanent irreversible structural changes**.** (Adapted from; Nelson D.L, Cox M.M (2001). *Lerhninger Principles of Biochemistry*. New York, WH Freeman and company, 11(392-393)

# MODEL SYSTEMS FOR THE STUDY OF GLUT1

In 1952, Widdas first theorized that sugar transport was a saturable, protein-mediated process using sheep blood-placental transport as his model system (Widdas., 1952). Since then, erythrocytes have provided an ideal model system for the study of GLUT1 kinetic properties: Red cells are readily available by donation, are uniform in size and volume, may be lysed and resealed with desired contents, and GLUT1 is thought to occupy 6% of the cell surface. Membrane-resident erythrocyte GLUT1 may be readily purified for biochemical study. GLUT1 isolated from red cells is more heterogeneously glycosylated than is observed in other tissues. While this impacts biochemical analysis, it is not thought to affect functional behavior (Carruthers., 1990). Nonetheless, erythrocyte GLUT1 provides a window into the kinetic properties of mammalian sugar flux.

The major disadvantage of erythrocyte GLUT1 is that it is not amenable to mutagenic analysis. While clinical cases of GLUT1 deficiency syndrome have provided some insight, they are resistant to systematic structural exploration. Some model systems have been successfully employed to address this; Xenopus laevis oocytes and mammalian cell lines such as 3T3L1 adipocytes, Chinese Hamster Ovary (CHO) cells, and Human Embryonic Kidney Cells (HEK).

## Xenopus Laevis

Mutant GLUT1 has been most commonly expressed via RNA injection in X. laevis oocytes (Wellner, 1992). Oocytes, with their consistent size and volume, provide a convenient vehicle for analysis of kinetic properties of mutant transporters by measuring the transport of radiolabelled sugars. However, oocyte expressed GLUT1 is not

uniformly gylcosylated, is not expressed at a sufficiently high density for purification, and it does not self-associate into dimers and tetramers as observed in red cells (Zottola, 1995). This results in differing kinetic behavior from the human erythrocyte model, notably the lack of reductant sensitivity (Levine et al, 2005). In other words, sugar uptake into control erythrocytes without extracellular reductant (DTT-) is almost twofold greater than erythrocytes treated with DTT (Zottola, 1995). Oocyte expressed GLUT1 is unaffected by the presence of extracellular reductant (Levine et al, 2005).

## Mammalian Cell Culture

Transient transfection of GLUT1 into Chinese Hamster Ovary (CHO) cells or Human Embryonic Kidney 293T (HEK 293) cells have also proved a convenient vehicle for studying the glucose fluxes of mutant GLUT1. Both cell types have a very low concentration of endogenous glucose transporters, thereby facilitating heterologous overexpression of mutant transporters. HEK 293 cells have the advantage that they retain erythrocyte-like glycosylation patterns on the protein (Levine et al, 2005). Their disadvantages lie in the cost of culture, and a lower protein density as compared to the erythrocyte, rendering mutant protein purification a more expensive and complex operation.

Furthermore, depending on the model system, to selectively measure the transport activity of GLUT1 in vitro maybe a difficult task. This is because it has been shown by immuno-precipitation using cell-surface transporter labeling that GLUT1 and GLUT4 are 80% present on the membrane of 3T3-L1 cells *(Palfreyman et al., 1992)*. Consequently, the presence of other transporters does not significantly contribute to total glucose uptake

*(Palfreyman et al., 1992 and Fukumoto et al., 1989).* In addition, the kinetics of glucose uptake that is modeled by the Michaelis-Menton equation is consequently the sum of the transport activity of two isoforms, GLUT1 and GLUT4 *(Palfreyman et al., 1992)*.

PROPOSED STUDY

To avoid the confusion of measuring the transport activity of both isoforms (GLUT1 and GLUT4) and other elements of the insulin signaling pathway such as IRS1 and IRS2, in order to understand the mechanism of berberine and HNO enhanced glucose uptake activation, we will measure the effects of berberine and HNO in L929 fibroblast cells, which only express GLUT1 and are non insulin responsive. Previous work has shown that this transport can be rapidly activated through starvation (Tinball et al., 2006) by a mechanism that has not yet been clarified.

## Objectives of the Study

(a) The objective of this study is to investigate the effect of berberine and nitroxyl on the transport activity of GLUT1 in L929 fibroblast cells: Does it increase or decrease the Michaelis- Menton constant ($K_m$) and maximum velocity ($V_{max}$)?

(b) To obtain the dose and time dependent effects of berberine and nitroxyl (HNO) on these cells.

(C) To obtain the recovery times of these cells from berberine and nitroxyl.

(d) To assay the effects of nitroxl on the structure of GLUT1, in order to understand the mechanism of activation of glucose uptake by GLUT1 in these cells.

Furthermore, we will measure the effects of specific pathway inhibitors in order to gain some insight into the mechanism of activation by berberine and nitroxyl. We will use the results to determine if the effects of berberine and nitroxl on GLUT1 transport activity,

might contribute to the improvement of glucose hemostasis in diabetic humans, upon consumption of berberine and or, nitroxyl containing biopharceuticals.

# CHAPTER II

# BERBERINE ACUTELY ACTIVATES THE GLUCOSE TRANSPORT ACTIVITY OF GLUT1

This chapter was published July 2011 to the journal Biochimie (Elsevier) and can be found using the following reference:

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(http://www.sciencedirect.com/science/article/pii/S0300908411001325)

Keywords: Berberine; Glucose uptake; GLUT1; ERK kinase; AMPK

Data presented in this chapter found in Tables 3.1 and 3.2, as well as Figures 3.1,3.2 and 3.4 were contributed to by myself, Jude Chenge; the remaining work was a collaboration with Alexandra Cok, Christina Plaisier, Mathew Salie and Daniel Oram, Under supervision by Larry Louters, Ph.D.

# ABSTRACT

Berberine, which has a long history of use in Chinese medicine, has recently been shown to have efficacy in the treatment of diabetes. While the hypoglycemic effect of berberine has been clearly documented in animal and cell line models, such as 3T3-L1 adipocytes and L6 myotube cells, the mechanism of action appears complex with data implicating activation of the insulin signaling pathway as well as activation of the exercise or AMP kinase-mediated pathway. There have been no reports of the acute affects of berberine on the transport activity of the insulin-insensitive glucose transporter, GLUT1. Therefore, we examined the acute effects of berberine on glucose uptake in L929 fibroblast cells, a cell line that express only GLUT1. Berberine- activated glucose uptake reaching maximum stimulation of five-fold at >40 μM. Significant activation ($P < 0.05$) was measured within 5 min reaching a maximum by 30 min. The berberine effect was not additive to the maximal stimulation by other known stimulants, azide, methylene blue or glucose deprivation, suggesting shared steps between berberine and these stimulants. Berberine significantly reduced the Km of glucose uptake from 6.7 ± 1.9 mM to 0.55 ± 0.08 mM, but had no effect on the Vmax of uptake. Compound C, an inhibitor of AMP kinase, did not affect berberine-stimulated glucose uptake, but inhibitors of downstream kinases partially blocked berberine stimulation. SB203580 (inhibitor of p38 MAP kinase) did not affect submaximal berberine activation, but did lower maximal berberine stimulation by 26%, while PD98059 (inhibitor of ERK kinase) completely blocked submaximal berberine activation and decreased the maximal stimulation by 55%. It appears from this study that a portion of the hypoglycemic effects of berberine can be attributed to its acute activation of the transport activity of GLUT1.

# INTRODUCTION

Berberine, an isoquinoline alkaloid isolated from several herbs including Rhizoma Coptidis, has a long history of use in Chinese medicine for the treatment of gastrointestinal infections, diarrhea, cardiovascular diseases, inflammation and hypercholesterolemia *(Yin et al., 2008 and Lau et al., 2001)*. More recently, berberine has been also proven to be efficacious for the treatment of type-2 diabetes *(Yin et al. 2008; Ni et al., 1988 and 1994)*. Studies using human patients *(Yin et al. 2008; Ni et al. 1988; 1994 and Lee et al., 2006)*, animal or cell models of insulin resistance *(Lee et al., 2006; Yin et al., 2008; Yi et al., 2008; Chen et al., 2009; Zhang et al., 2008; Leng et al., 2004 and Gao et al., 1997)*, or insulin sensitive cell lines *(Liu et al., 2010; Cheng et al., 2006; Kim et al., 2007 and Zhou et al., 2007)* that have all established a clear hypoglycemic effect of berberine.

In general, there are two distinct pathways to activate glucose uptake in peripheral tissues; one stimulated by insulin through the IRS-1/PI 3-kinase and the other by exercise or hypoxia via activation of AMPK as shown in figure 1.2. In muscle, which is the major tissue responsible for whole body glucose disposal, both pathways stimulate the translocation of GLUT4 to the cell membrane, which accounts for the enhanced glucose uptake *(Krook et al., 2004)*. Current data suggests that the effects of berberine are complex and may activate portions of both insulin and exercise-induced glucose uptake pathways *(Liu et al., 2010 and Kim et al., 2007)*. In addition, berberine inhibits intestinal absorption of glucose that also contributes to berberine's hypoglycemic effect *(Pan et al., 2003)*.

The effects of berberine on the insulin stimulated glucose uptake pathway are varied and sometimes conflicting *(Yin et al., 2008 and Liu et al., 2010)*, which can be, in part, attributed to the variety of cell types and treatment times utilized in these studies. However, there appears to be a general agreement from multiple studies that berberine activates AMPK *(Lee et al., 2006, Yin et al., 2008, Liu et al., 2010, Cheng et al., 2006, Kim et al., 2007 and Zhou et al., 2007, and Brusq et al., 2006)*. These studies have been done in insulin sensitive cells where a change in glucose transport is typically attributed to a change in GLUT4 activity. Some studies have specifically implicated GLUT1 as the primary transporter responsible for the enhanced glucose uptake, but these studies investigated the chronic effects of berberine (6 -12 hour treatments) *(Kim et al and Zhou et al., 2007)*. The acute or short-term effects of berberine on GLUT1 activity uptake have not been studied.

There is increasing evidence that the more widely expressed GLUT1, initially thought to be only responsible for basal glucose uptake, can be acutely activated by cell stressors such as azide *(Shetty et al., 1993 and Rubin., 2003),* osmotic stress *(Barnes et al., 2002 and Barros et al., 2001)*, methylene blue *(Louters et al., 2006)*, and glucose deprivation *(Kumar et al., 2004 and Roelofs et al., 2006)*. In addition, it has been recently shown that peptide C activates GLUT1 transport activity in erthryocytes, establishing a potential link between GLUT1 activity and diabetes *(Meyer et al., 2008).* In particular, the acute activation of GLUT1 by hypoxia or azide has been attributed to an activation of AMPK *(Barnes et al., 2002; Jing et al., 2007, 2008)*. Therefore, the specific purpose of this study was to systematically investigate the acute effects of berberine on glucose uptake in L929 fibroblast cells, a cell line that expresses only

GLUT1 *(Liong et al., 1999)* and has been shown to respond to acute cell stress by increasing glucose uptake *(Louters et al and Roelofs et al., 2006).*

# RESULTS

## Berberine Activates Glucose Uptake in a Dose Dependent Manner

A six-hour berberine treatment increased GLUT1 expression and subsequent glucose uptake in 3T3-L1 cells *(Kim et al., 2007)*. However, the acute effects of berberine on the activity of this transporter have not been measured. To investigate the acute effects of berberine on basal glucose uptake by GLUT1, L929 fibroblast cells were exposed to DMEM media containing 5.5 mM glucose and increasing concentrations of berberine for 30 minutes. The results, shown in Figure 2.1, show a dose dependent stimulation of glucose uptake that reached a maximum 5-fold stimulation at 100μM berberine. There was a statistical activation of uptake at 10 μM (1.55x) and uptakes at concentrations greater than 40 μM were not different from each other. Therefore in subsequent experiments, concentrations >40 μM berberine were considered to be maximally effective.

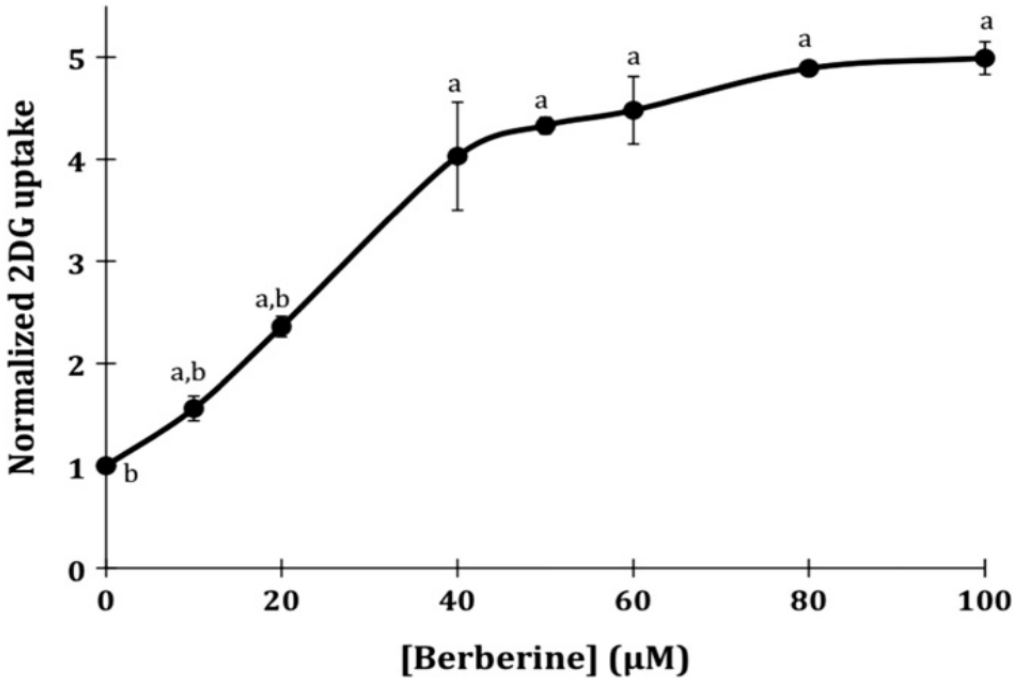


**Figure 2.1**. Effects of berberine on 2DG uptake.

L929 fibroblast cells were incubated at 37 °C for 30 min in DMEM media (5.5 mM glucose) supplemented with 0-100 mM berberine as indicated. Ten-minute 2DG uptakes were then measured as described in Materials and methods. Data are means± S.E. of normalized values from multiple experi- ments (samples size varied from 3-20). [a] significantly different than 0 mM berberine and [b]significantly different than 100 mM berberine at $P < 0.01$.

## Time Course of Activation and Recovery of Berberine Activated Glucose Uptake

To measure the effects of time, L929 cells were incubated in DMEM media (5.5 mM glucose) containing berberine for times ranging from 5-60 minutes. The results shown in Figure 2.2 indicate that a statistically activation was achieved within 5 minutes (from 1.79±0.09 to 2.97±0.18 nmol/10 min/well) and maximized by 30 minutes (4.97±0.17 nmol/10 min/well). The 0, 30, and 60 minute time points were also done in the presence of cyclohexamide , which had no effect on glucose uptake (data not shown). This indicates that the activation of glucose uptake by berberine is not dependent on protein synthesis.

In a separate experiment recovery from the effects of berberine were measured by exposing cells to berberine for 30 minutes, followed by a wash and resuspension in media lacking berberine for various times up to 60 minutes. As shown in Figure 2.3, the effects of berberine were largely maintained for a full 30 minutes post exposure (86.9%), and 50.7% of the activity remained after 60 minutes.

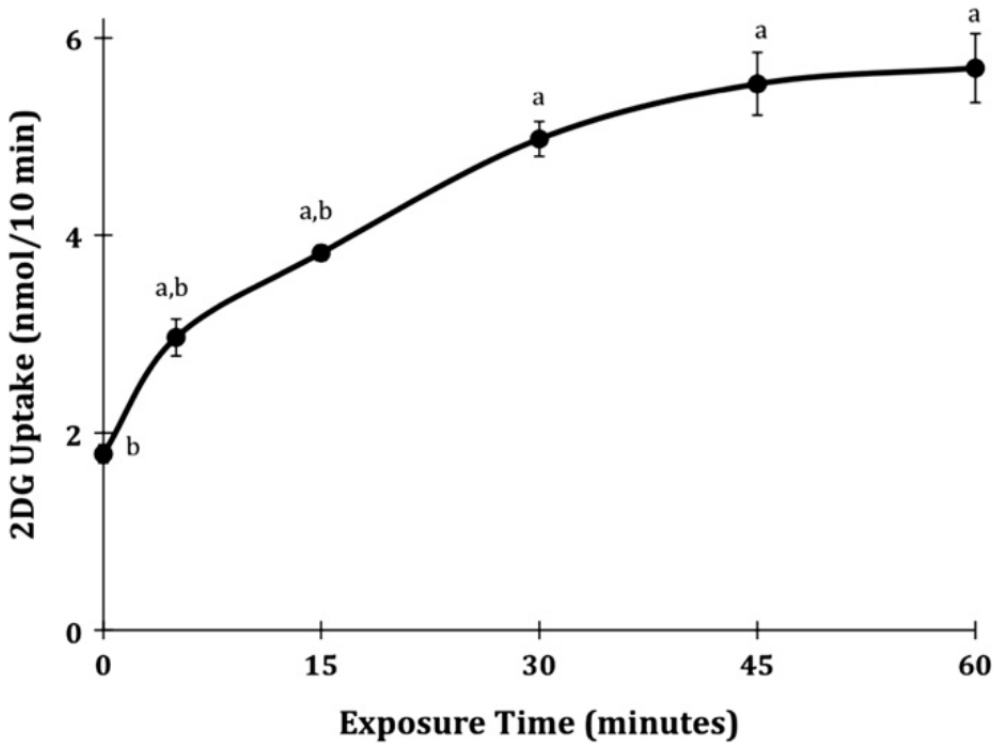


**Figure 2.2**. Effect of time of berberine exposure on 2DG uptake.

L929 fibroblast cells were incubated at 37 °C for 30 min in DMEM media (5.5 mM glucose) supplemented with 40 mM berberine for 0, 5, 10, 30, 45, and 60 min. Ten-minute 2DG uptakes were then measured as described in Materials and methods. Data are means ±S.E. of a representative experiment. [a]Significantly different than 0 min exposure to berberine and [b]significantly different than 1 h at $P < 0.01$.

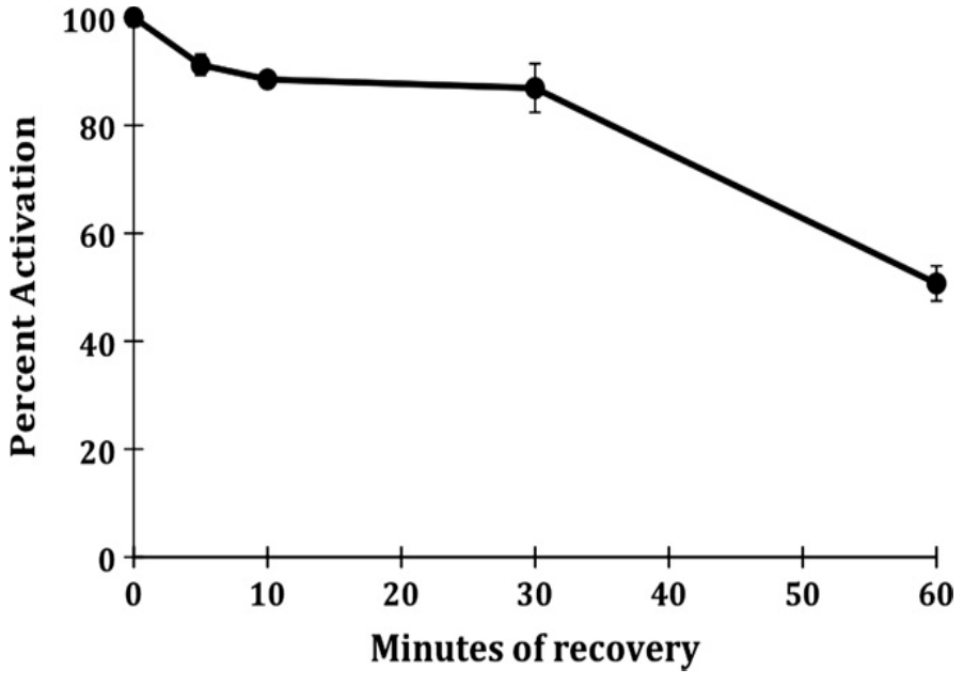


**Figure 2.3**. Effect of recovery time on berberine-activated 2DG uptake.
L929 fibroblast cells were incubated at 37 °C for 30 min in 5.5 mM glucose DMEM media supplemented with 40 mM berberine. Samples were washed and re-suspended in media without berberine for 0-60 min. Ten-minute 2DG uptakes were then measured as described in Materials and methods. Data are means ±S.E. of a representative experiment. 2DG uptake rates at all recovery times are statistically different from the control (0 min) at $P < 0.05$.

## Additive and Kinetics Effects Of Berberine Activation

In order to gain some insight into to the activation pathway of berberine, we conducted a series of additivity experiments as well as investigated the kinetics of the berberine effect. We had previously shown that glucose uptake in L929 fibroblast cells can be acutely activated by multiple agents including nitric oxide, troglitazone, azide, methylene blue and glucose deprivation *(Louters et al., 2006; Roelofs et al., 2006 and Van Dyke et al., 2003)*. In particular, acute activations by methylene blue and glucose deprivation were shown to be additive with different kinetic effects, suggesting that there is a least two distinct activation mechanisms of glucose uptake in L929 cells *(Louters et al., 2006 and Roelofs et al., 2006).*

L929 cells were exposed to maximally effective concentrations of either sodium azide (5 mM), methylene blue (50 μM), berberine (50 μM), or media lacking glucose for 30 minutes. Glucose uptake measurements are shown on Table 2.1 and as can be seen, each stimulant significantly activated glucose uptake. Additionally, other cells were exposed to a combination of maximally effective concentrations of berberine plus azide, or methylene blue, or glucose deprivation. As seen on Table 2.1, the effects of berberine were not additive to any of the stimulants, suggesting that berberine shares significant portions of the activation pathways with of all these stimulants.

The kinetics of glucose uptake were measured in the presence and absence of berberine and the results are shown on Figure 2.4. An best fit analysis of the data using Michaelis-Menton kinetics indicates that berberine does not affect the Vmax (both between 13-14

nmol/10 min/well) but dramatically lowers the Km of uptake from about 5.5 mM to 0.7 mM.

**Table 2.1.** Combined effects of various stimulants of glucose uptake

| [Berberine] | Controls | + Azide | + MB | +No glucose |
|---|---|---|---|---|
| 0 μM | 1.00□0.04 | 1.79□0.09 | 2.54□0.10 | 2.87□0.36 |
| 50 μM | 3.17□0.10 | 3.23□0.18 | 3.37□0.18 | 3.04□0.40 |

L929 fibroblast cells were incubated for 30 minutes at 37 □in DMEM media (5.5 mM glucose or 0 mM glucose) containing either 0 or 50 μM berberine alone or in combination with either 5.0 mM sodium azide, or 50 μM methylene blue. Glucose uptake was measured as described in Materials and Methods and normalized to control with no berberine. All uptakes are statistically higher than the control at P<.01, but all uptakes with 50 μM berberine were not different.

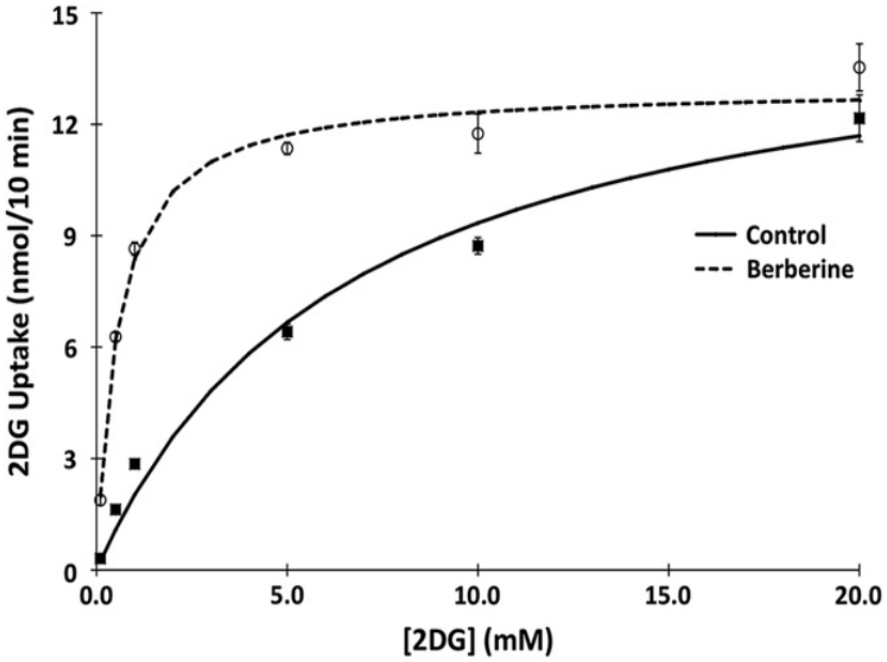


**Figure 2.4**. Kinetics of 2DG uptake in control and berberine-activated cells.
L929 fibroblast cells were incubated at 37 °C for 30 min in DMEM media (5.5 mM glucose) supple- mented with either 0 or 80 mM berberine. Ten-minute 2DG uptakes were then measured at varying concentrations of 2DG (0.1, 0.5, 1, 5, 10, or 20 mM). Data are means ±S.E. of a representative experiment.

## Effects of Inhibitors on Berberine Activated Glucose Uptake

Multiple studies have suggested that berberine activates AMPK *(Lee et al., 2006, Yin et al., 2008; Liu et al., 2010, Cheng et al., 2006; Kim et al., 2007, Zhou et al., 2007 and Brusq et al., 2006).* To explore this possibility, we pretreated cells with 0, 10, 20, or 50 μM compound C, an inhibitor of AMPK, for 45 minutes, followed by a 30 minute exposure to 50 μM berberine in the continued presence of compound C. The results, shown in Figure 2.5, again demonstrate a robust activation of glucose uptake from 2.39±0.17 to 7.74±0.36 nmol/10min/well, which was not altered by the pretreatment with compound C.

We also measured the effects of pretreatment of wortmannin (inhibitor of PI3K), SB203580 (inhibitor of p38 kinase), and PD98059 (inhibitor of ERK). As expected and previously shown *(Kim et al., 2007)*, wortmannin had no effect on either basal or berberine stimulated glucose uptake (data not shown). However, both SB203580 and PD98059 partially inhibited berberine stimulated glucose uptake. We pretreated cells with effective concentrations of either the p38 kinase inhibitor (10 μM SB203580) or the ERK inhibitor (50 μM PD98059) *(Kim et al., 2007)* followed by either a submaximum (10 μM) or maximum (50 μM) berberine activation in the continued presence of the inhibitor. As shown on Table 2.2, treatment with either inhibitor alone did not significantly altered basal glucose uptake. SB203580 did not affect submaximum berberine activation but did lower maximum berberine stimulation by 26% (decrease from 4.13 ± 0.17 to 3.31 ± 0.09 nmol/10min/well). In contrast PD98059 was a much more effective inhibitor. It completely blocked submax berberine activation and

decreased the maximum stimulation by 55% (decrease from 4.13 ± 0.17 to 2.41 ± 0.05 nmol/10min/well).

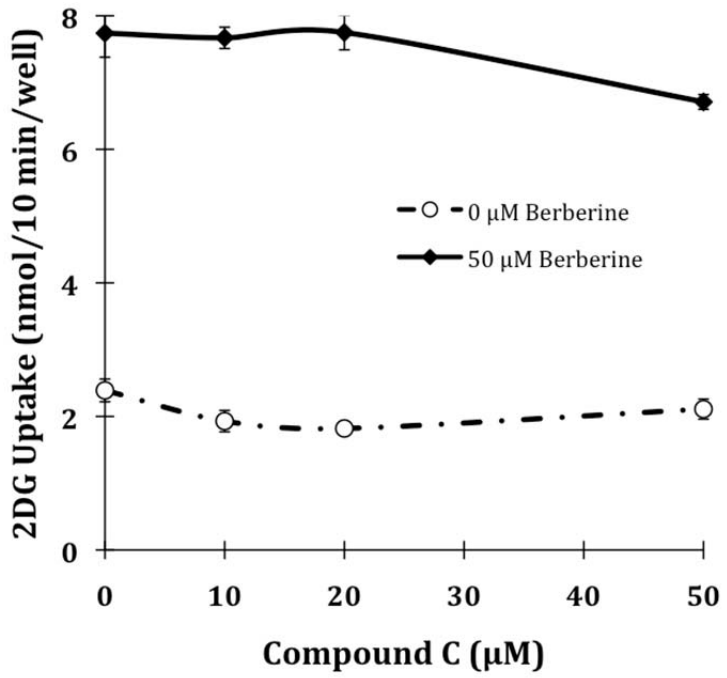


**Figure 2.5**. Effect of Compound C on berberine-activated 2DG uptake.

L929 fibroblast cells were pretreated for 45 min at 37 °C in DMEM media (5.5 mM glucose) supplemented with 0, 10, 20, or 50 mM compound C, then incubated for 30 min in 5.5 mM glucose DMEM media still supplemented with compound C and either 0 or 50 mM berberine. Ten-minute 2DG uptakes were then measured as described in Materials and methods. Data are means ±S.E. of a representative experiment.

**Table 2.2.** Effects of inhibitors on berberine activated glucose uptake

| **[Inhibitor]** | | **[Berberine]** | | |
|---|---|---|---|---|
| SB203580 | PD98059 | 0 μM | 10 μM | 50 μM |
| 0 μM | 0 μM | 1.00 □ 0.03 | 1.44 □ 0.08 | 4.13 □ 0.17 |
| 10 μM | 0 μM | 0.91 □ 0.02 | 1.46 □ 0.03 | 3.31 □ 0.09* |
| 0 μM | 50 μM | 0.91 □ 0.10 | 1.06 □ 0.08* | 2.41 □ 0.05# |

As controls, cells were incubated for 30 minutes at 37 □in DMEM media containing either 0 (basal), 10 μM (submax stimulation), or 50 μM (max stimulation). The inhibitors, SB203580 (at 10 μM) and PD98059 (at 50 μM) were incubated with cells for 45 minutes prior to a 30 minute incubation with either 0, 10, 50 μM berberine plus the inhibitor. Glucose uptake was measured as described in Materials and Methods and normalized to control with no berberine and no inhibitor. Significant effects of the inhibitor compare to its berberine control were at P<.05 (*) and P<.01 (#).

# DISCUSSION

Berberine, an isoquinoline alkaloid isolated from a number of plants, has a rich history of use in Chinese medicine. It seems clear, considering the wide spectrum of therapeutic applications such as treatments for parasitic infection, bacterial diarrhea, inflammation, cardiovascular disease, hypercholesteremia, and diabetes, that berberine must trigger a number of physiological responses in biological systems.

Recent studies have documented berberine's effectiveness in the treatment of both human diabetes patients *(Yin et al., 2008 and Ni et al., 1988 & 1994)* and animal models of diabetes *(Lee et al., 2006; Zhang et al., 2008; Leng et al., 2004 and Gao et al 1997).* This has lead to numerous studies designed to understand berberine's mechanism of action regarding its hypoglycemic effect. The results of these studies are somewhat confusing and sometimes conflicting. Some studies indicate that berberine works through the insulin pathway by enhancing either insulin secretion *(Leng et al., 2004)* or the signaling cascade *(Yi et al., 2008; Chen et al., 2009; Wang et al., 2012; Zhang et al 2010; Ko et al., 2005 and Chen et al., 2010)*. Others suggest berberine stimulates AMPK in an insulin pathway-independent fashion *(Lee et al., 2006, Cheng et al., 2006; Kim et al 2007; Zhou et al., 2007; and Turner et al., 2008)* while others suggest that berberine has effects on both pathways *(Yin et al., 2008 and Liu et al., 2010)*. In most studies the berberine-enhanced glucose uptake has been attributed to increased GLUT4 activity. Some studies indicated that berberine increases GLUT4 production *(Prabhakar et al., 2009)* or translocation (*Lee et al., 2006; Ko et al., 2005 and Prabhakar et al., 2009),*

however other studies were unable to detect changes in GLUT4 *(Kim et al., 2007 and Wang et al., 2008).*

The acute effects of berberine on the transport activity of GLUT1 remain largely unexplored. A study in 3T3-L1 adipocytes showed that a 6 hour exposure to berberine increases GLUT1 synthesis with no change in GLUT4 *(Kim et al., 2007)* however a second study reported that a 24 hour exposure to berberine did not change either GLUT1 or GLUT4 content and suggested in their conclusions that berberine increases GLUT1 activity, (*Zhou et al., 2007*). To explore the effects of berberine on GLUT1 activity, we measured glucose uptake in the presence of berberine in L929 fibroblast cells, a cell line that only expresses GLUT1 *(Liong et al., 1999)*. We report that berberine significantly activates glucose transport at 10 μM and reaches a maximum stimulation of about 5-fold at concentrations above 40 μM (see Figure 2.1). A significant activation occurs within 5 minutes and this acute effect platues at about 30 minutes. This activation was also not sensitive to cyclohexamide indicating that this berberine effect does not depend on new protein synthesis.

Recovery from berberine activation is slower than the activation with about 90% of the acti- vation remaining after 30 min and 50% after 60 min.

Previous studies have suggested two distinct mechanisms for the activation of glucose uptake in L929 cells, one mechanism illustrated by the effects of methylene blue or sodium azide and the other by glucose deprivation (*Louters et al., 2006 and Roelofs et al., 2006*). The kinetic analysis of the berberine activation indicates that the Km of glucose transport is decreased, but the Vmax is unaffected, suggesting that sensitivity of

the system is enhanced, but not the responsiveness. This kinetic behavior is similar to that observed upon glucose deprivation in this cell line (*Roelofs et al., 2006).* To further explore the mechanism of berberine- activated glucose uptake we explored the additivity of berberine's effects with maximally effective concentrations of methylene blue and azide or glucose deprivation. The results (Table 2.1) indicate that among these stimulants, berberine induces the most robust activation of glucose uptake and that its effects are not enhanced by the other stimulants. This suggests that the mechanism of berberine activation shares common steps with both pathways.

Key studies in other cell lines have strongly suggested that berberine activation of glucose uptake is mediated through activation of AMPK. To explore this possibility we measured the dose dependent effects of Compound C, an inhibitor of AMPK, on both basal and berberine-stimulated glucose uptake. Compound C had no effect on glucose uptake, suggesting AMPK is not involve in the stimulation of glucose uptake in L929 cells. This is consistent with previous work in L929 cells that reported that AICAR, an activator of AMPK, inhibited rather than activated glucose uptake (*Louters et al., 2006*). This result was somewhat surprising given that previous work in L6 myotube cells had shown that the activation of glucose uptake by berberine could be completely blocked by compound C (*Cheng et al., 2006*). We were able to repeat those results with L6 myotube cells in our laboratory as well (data not shown) suggesting that AMPK is not involved in the activation of glucose uptake in L929 cells. Also, since L6 myotube cells express both GLUT1 and GLUT4, this may suggest that AMPK activation is required for the activation of GLUT4, but not for GLUT1. The lack of involvement of AMPK in

regulating glucose uptake in L929 fibroblast cells indicate that additional studies need to be done investigate the expression levels and role of AMPK in this cell line.

Studies that support AMPK activation as a key step in berberine's effects are not all in agreement regarding which downstream kinases are involved. Some studies implicate activation of ERK kinase (*Zhou et al., 2007 and Kim et al., 2007)*, while others provide evidence of berberine's activation of p38 MAPK (*Lee et al., 2006 and Louters et al., 2006*). To investigate the involvement of these two kinases we measured the effects of SB203580 (inhibitor of p38 MAPK), and PD98059 (inhibitor of ERK kinase) on berberine- stimulated glucose uptake. SB203580 had no effect on sub- maximally effective concentrations of berberine, but reduced maximum stimulation by 26%, while PD98059 completely blocked submaximal berberine stimulation and reduced maximum stimulation by 55%. The combination of the two inhibitors did not further inhibit berberine stimulation of glucose uptake (data not shown). These data suggest that ERK kinase pathway may be more involved than p38 MAPK, but neither is able to account for the full stimulatory effect of berberine. It is important to note that these inhibitory studies should be considered to be just the initial steps in discerning the mechanism of berberine activation of GLUT1. One concern is a potential cross reactivity of the inhibitors. For example, recent work in striated muscle has demonstrated that SB203580 also significantly inhibits the activation of Akt, a serine/threonine protein kinase, activated in the insulin signaling system (*Kim et al., 2006*). While this may not be important in L929 fibroblast cells, since this cell line is not insulin sensitive, it does point to potential complications in interpreting the results. Thus, future studies should look for a berberine

stimulation of the phosphorylation of these kinases as well as identify other factors to account for the full activation of berberine.

## CONCLUSSION

This study demonstrates for the first time that berberine acutely activates the transport activity of GLUT1. In addition, the mecha- nism of activation is complex, but likely involves, in part, the acti- vation of ERK kinase and to a lesser degree p38 MAPK. The activation of these kinases in L929 cells appears to occur without the activation of AMPK. Thus it is likely, that a portion of the hypoglycemic activity of berberine, that has been observed by others, can be attributed to its acute activation of the transport activity of GLUT1. GLUT1 is widely expressed and is the unique transporter responsible for glucose uptake into the central nervous system. Thus, the ability of berberine to acutely up regulate the activity this transporter may suggest a useful strategy to help counter the damaging effects of strokes caused by arterial blockage as well as damage to cardiomycytes that occurs during a cardiac infarction.

## ACKNOWLEDGEMENTS

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# CHAPTER III

# NITROXYL (HNO) ACUTELY ACTIVATES THE GLUCOSE UPTAKE ACTIVITY OF GLUT1

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(http://www.sciencedirect.com/science/article/pii/S0300908411004603)

Keywords: Angeli's salt; Nitroxyl (HNO); GLUT1; Glucose uptake; L929 fibroblast cells

Data presented in this chapter found in Table 3.1, as well as Figures 3.1,3.2 and 3.6 were contributed to by myself, Jude Chenge; the remaining work was a collaboration with Mathew Salie, Daniel Oram, Davis Kuipers, Jared Scripture and Griffin Macdonalds, under supervision by Larry Louters, Ph.D.

# ABSTRACT

Nitroxyl (HNO) is a molecule of significant interest due to its unique pharmacological properties, particularly within the cardiovascular system. A large portion of HNO biological effects can be attributed to its reactivity with protein thiols, where it can generate disulfide bonds. Evidence from studies in erythrocytes suggests that the activity of GLUT1 is enhanced by the formation of an internal disulfide bond. However, there are no reports that document the effects of HNO on glucose uptake. Therefore, we examined the acute effects of Angeli's salt (AS), a HNO donor, on glucose uptake activity of GLUT1 in L929 fibroblast cells. We report that AS stimulates glucose uptake with a maximum effective concentration of 5.0 mM. An initial 7.2-fold increase occurs within 2 min, which decreases and plateaus to a 4.0-fold activation after 10 min. About 60% of the 4.0-fold activation recovers within 10 min, and 40% remains after an hour. The activation is blocked by the pretreatment of cells with thiol-reactive compounds, iodoacetamide (0.75 mM), cinnamaldehyde (2.0 mM), and phenylarsine oxide (10 μM). The effects of AS are not additive to the stimulatory effects of other acute activators of glucose uptake in L929 cells, such as azide (5 mM), berberine (50 μM), or glucose deprivation. These data suggest that GLUT1 is acutely activated in L929 cells by the formation of a disulfide bond, likely within GLUT1 itself.

# INTRODUCTION

The biological effects of nitroxyl (HNO) are significantly different from nitric oxide (NO) and its distinctive properties and potential to be a cell-signaling molecule are receiving increasing attention *(Fukuto et al., 2009& 2011, Irvine et al., 2008, Switzer et al., 2009 and Paolocci et al., 2007).* In particular, HNO has documented efficacy for the treatment of heart failure *(Dai et al., 2007, Feelisch et al 2003, Froehlich et al 2008, Paolocci et al., 2001 and Tocchetti et al., 2007)* and alcoholism *(DeMaster et al., 1998).* The physiological effects of HNO appear to be mediated by its reactions with hemeproteins and with thiols *(Fukuto et al., 2009 & 2011, Kumar et al., 2010, and Miller et al., 2009).* In particular, the unique reaction of HNO with biological thiols is well established. It can react with one thiol to produce a sulfinamide as the endproduct, which often inhibits the protein. For example, HNO inhibits aldehyde dehydrogenase *(DeMaster et al., 1998, and Shoeman et al., 2000)* accounting for its use for treatment of alcoholism, glyceraldehyde-3-phosphate dehydrogenase *(Lopez et al., 2005 & 2007)* accounting for its toxicity in yeast, papain *(Vaananen et al., 2005 & 2008)*, and cathepsin B *(Vaananen et al., 2008).* In contrast, HNO can react with two thiols to produce a reversible disulfide end product plus hydroxylamine. This appears to account, in part, for the positive effects of HNO on cardiac muscle contractility. HNO has been shown to react with several sarcoplasmic proteins involved in $Ca^{2+}$ release and uptake, including the ryanodine receptor (Cheong et al 2005), $Ca^{2+}$-ATPase (SERCA) *(Tocchetti et al., 2007)* and phospholamban *(Froehlich et al., 2008),* a protein that regulates SERCA. It is not clear, at this time, if HNO is produced by cells as a physiological signal or if it is purely a pharmacological agent *(Fukuto et al., 2011).*

Long thought to be responsible only for basal glucose uptake, there is mounting evidence that the ubiquitously expressed GLUT1 can be acutely activated. Cell stressors such as azide *(Shetty et al., 1993 and Rubin et al., 2003),* osmotic stress *(Barnes et al., 2002 and Barros et al., 2001)*, methylene blue *(Louters et al., 2006),* C-peptide *(Meyer et al., 2008)* and glucose deprivation *(Kumar et al., 2004 and Roelofs et al., 2006)* all increase the transport activity of GLUT1. In contrast to GLUT4 activation, the activation of GLUT 1 occurs without a change the membrane concentration of the transporter. Currently it is not clear what alteration in GLUT1 structure accounts for its enhanced activity. One possible mechanism proposed by the Curruthers lab *(Graybill et al., 2006, Carruthers et al., 2009; Zottola et al., 1995; Hebert et al., 1992 and Pessino et al., 1991)* and based on their work with erythrocytes suggests that GLUT1 is activated by the formation of an internal disulfide bond. This triggers a conformational change in GLUT1 and leads the oligomerization (tetramer) and activation of the transporter. In support of this possible mechanism, we have found that two thiol active agents, phenylarsine oxide *(Scott et al., 2009)* and cinnamaldehyde *(Plaisier et al., 2011)*, both activate glucose uptake in L929 fibroblast cells, a cell line that express only the GLUT1 isoform (*Liong et al., 1999)*. Based on the Curruthers mechanism, we would predict that HNO would trigger a disulfide bond formation in GLUT1, which appears to be the preferred HNO-induced product in a hydrophobic environment *(Sherman et al., 2010)*, and thereby activate glucose uptake. Therefore, the specific purpose of this study was to systematically investigate the acute effects of Angeli's salt (AS), an in situ producer of HNO, on glucose uptake in L929 fibroblast cells.

# RESULTS

## Angeli's Salt Activates Glucose Uptake in a Dose Dependent Manner.

Data from erythrocytes suggests that GLUT1 can be activated by an internal disulfide bond formation, which leads to an oligomerization of the transporter *(Herbert et al., 1992 and Pessino et al., 1991).* Since AS stimulates disulfide bond formation via an in situ release of HNO *(Fukuto et al., 2009 & 2011),* we were curious to understand if AS could activate glucose uptake in L929 fibroblast cells, cells, which express only GLUT1 *(Liong et al., 1999)*. In our initial experiment, L929 fibroblast cells were exposed to 10 mM Angeli's salt either only during a 30-minute treatment phase, or during both the treatment and a 10-minute uptake phase, or only during the uptake phase. We choose this concentration because previous work had shown that AS in the range of 0.25-10 mM stimulated the dimerization of the cardiac sacroplasmic reticulum protein, phospholamban *(Froelich et al., 2008)*. The results, shown in Figure 3.1, reveal that the greatest activation is observed when AS is included only during the uptake phase (uptake increased 4.2x from 1.01±0.03 to 4.21±0.14 nmol/10 min/well). In a subsequent experiment, the dose dependency of glucose uptake was determined by exposing cells only during the 10-minute uptake to a concentration of AS ranging from 0-20 mM. The results are shown in Figure 3.2. There was a significant activation of uptake ($P<0.01$) at 1.0 mM (2.5x from 0.38±0.03 to 0.97±0.05 nmol/10 min/well), which maximized at 5.0 mM (uptake increased 3.8x from 0.38±0.03 to 1.46±0.14 nmol/10 min/well). There was a small, but significant decrease to 1.04±0.05 nmol/10 min/well in the activation at 20 mM AS. AS did not produce any visible toxic effects, as assessed by cell morphology and cell attachment, at any concentration tested.

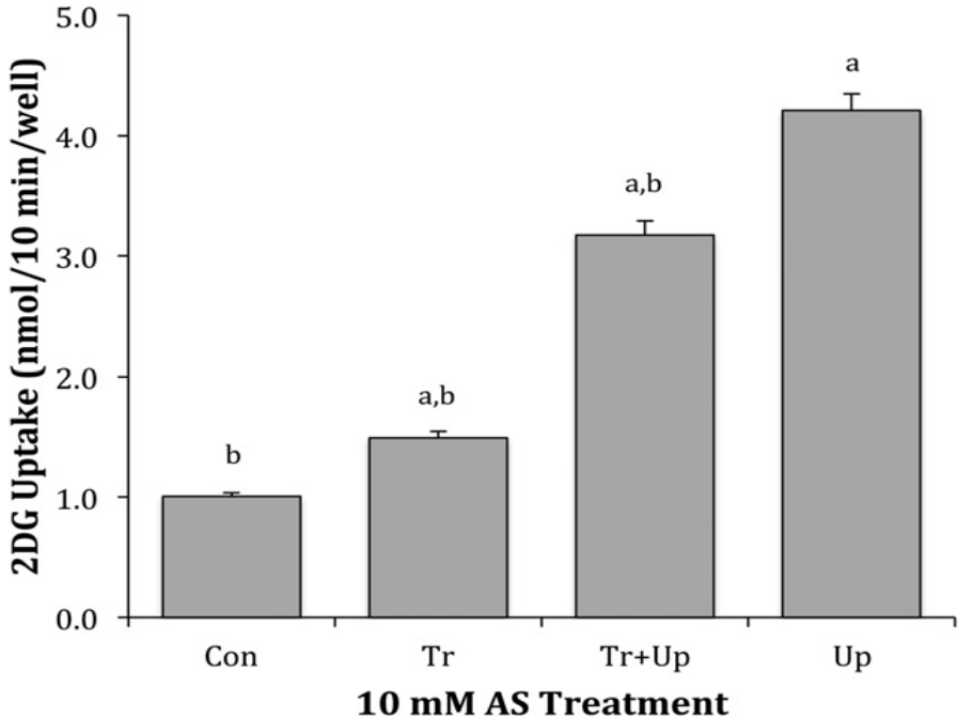


**Figure 3.1**. Effects of AS on 2DG uptake.

L929 fibroblast cells were incubated at 37 °C for 30 minutes in DMEM media (5.5 mM glucose) supplemented with or without 10.0 mM AS. Ten-minute 2DG uptakes were then measured as described in Materials and Methods in the presence and absence of 10 mM AS. Con (control) represent cells not exposed to AS, (Tr) represent cells exposed to AS during the 30-minute treatment phase, (Tr+Up) were cells treated to AS during both the treatment and uptake phase, and (Up) were cells exposed to AS only during the 10-minute uptake phase. Data are means ± S.E. All uptakes in AS-treated cells are significantly elevated at $P<0.01$.

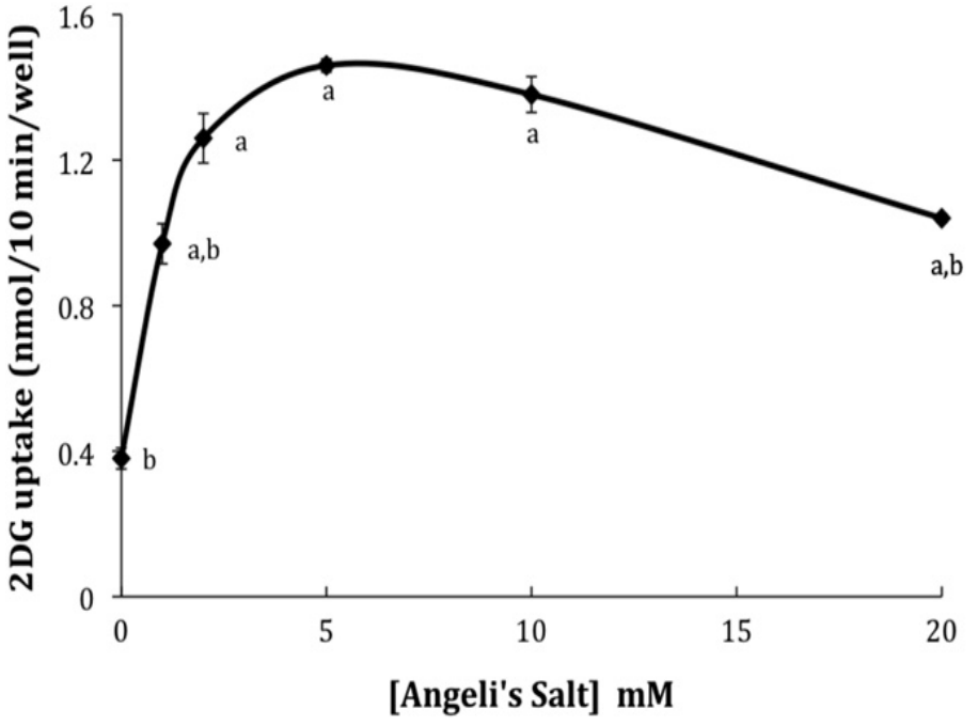


**Figure 3.2**. Dose dependent effects of AS on 2DG uptake.

Ten-minute 2DG uptakes were measured in the presence of AS concentrations ranging from 0-20 mM as described in Materials and Methods. Data are means ± S.E. of a representative experiment. [a]Significantly different than 0 mM exposure to AS and [b]significantly different than 5.0 mM AS at $P<0.01$.

## Control Experiments

AS decomposes with a half-life of about 2.5 minutes to release HNO and nitrite *(Irvine et al., 2008).* HNO itself is not stable for long term, but undergoes a self-dimerization reaction to produce nitrous oxide ($N_2O$) plus water *(Fukuto and Carrington 2011).* To insure that the activity we were observing was a result of HNO and not nitrite, we compared the effects of AS when is was added to uptake just before the measurement of uptake to the effects when AS was added to the uptake media and incubated at room temperature overnight. The results, normalized to control, are shown in Figure 3.3. The overnight incubation did reduce uptake from 3.04±0.01 times control to 2.19±0.06, but it remained significantly elevated. The activity could not be attributed to nitrite, because 5 mM nitrite had no effect of glucose uptake (see Figure 3.3). It may be that HNO dimerization is slow enough such that significant nitroxl remains after the overnight incubation. To test this, we incubated the 5.0 mM AS uptake media overnight in the presence of excess thiol (10.0 mM cysteine). As seen in Figure 3.3, this mixture no longer activated glucose uptake. In addition, cysteine by itself had no effect on glucose uptake.

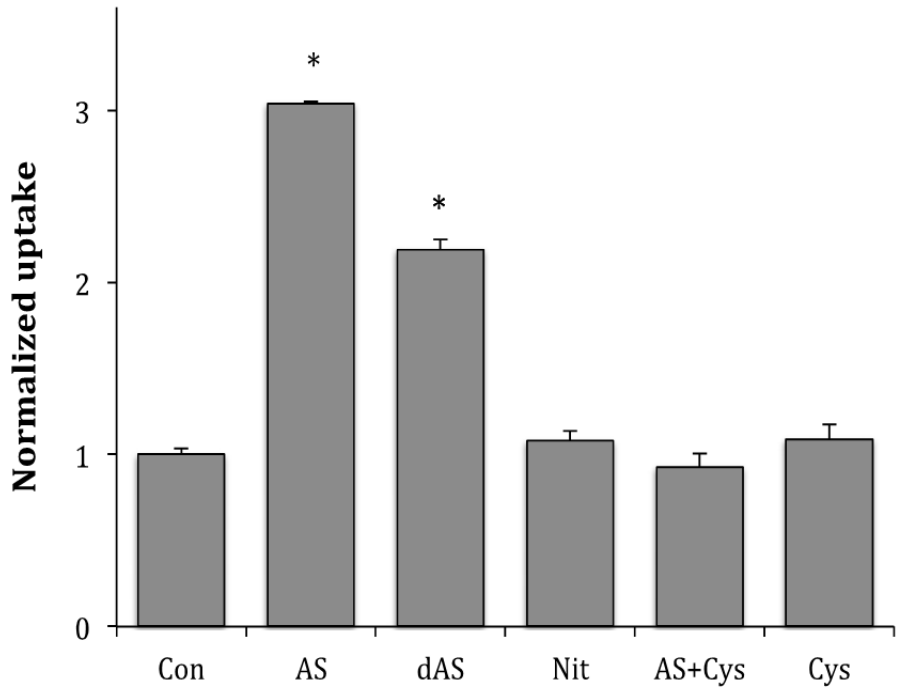


**Figure 3.3.** Control experiments.

2DG uptakes were measured as described in Materials and Methods in the presence of 5.0 mM AS added just before uptake (AS), 5.0 mM AS allowed to decompose overnight at room temperature (dAS), 5.0 mM sodium nitrite (Nit), 5.0 mM AS plus 10.0 mM cysteine incubated overnight, or 10.0 mM cysteine. Both AS and dAS were (*) significantly elevated at $P<0.01$.

## Time Course of Activation and Recovery of AS-activated Glucose Uptake

It seems possible that the much lower activation observed when AS was placed only in the 30-minute treatment phase (see Figure 3.1) could actually represent activation followed by a recovery as HNO is depleted. Therefore, to explore time course of activation, L929 cells were exposed to 5 mM AS in the radioactive uptake buffer for times ranging from 2-20 minutes. The results, expressed as nmols per minute, are shown in Figure 3.4. The zero time represents the uptake per minute for control cells that had been exposed to uptake media for 20 minutes without AS. The data shows a burst of activation in the first three minutes followed by a steady rate of uptake that remains elevated (4.0x) over control for the remainder of the 20 minutes. This burst of activity correlates nicely with the half-life for the release of HNO from AS.

To measure the recovery from the effects of AS, L929 cells were to AS for 10 minutes, then resuspended in DMEM media (5.5 mM glucose) without AS and uptakes were measured either immediately or after 5, 10, 25 and 50 minutes. As shown in Figure 3.5, about 60% of the activity recovered within 10 minutes, but about 40% remained even after 50 minutes. This experiment was repeated multiple times and all results revealed a fast recovery phase lasting between 5-10 minutes with a residual activation of 20-50% that remained after an hour.

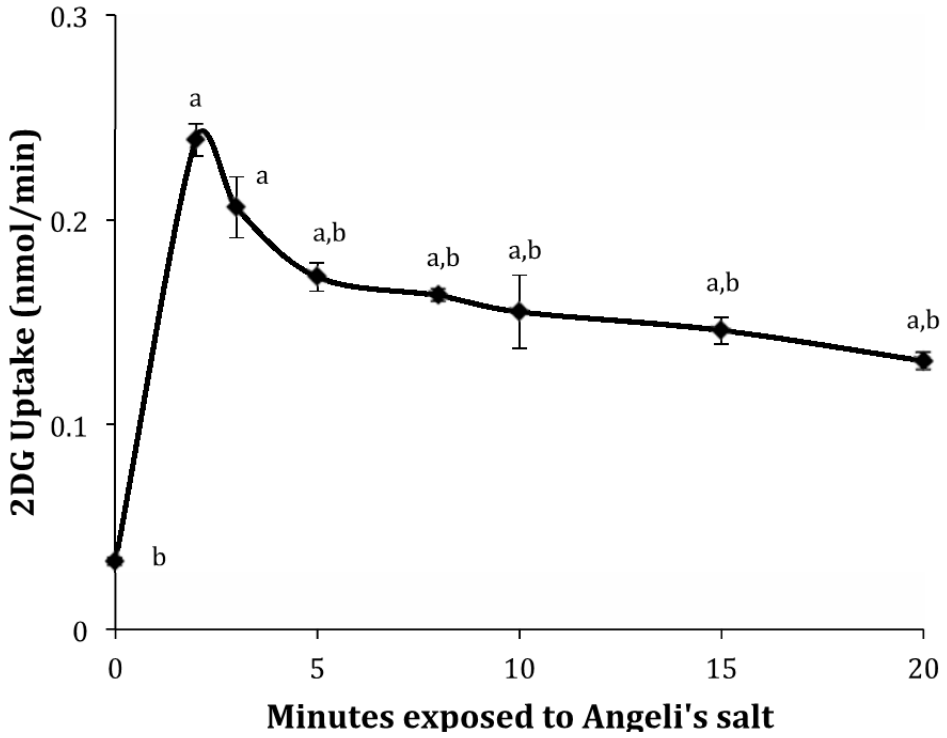


**Figure 3.4**. Time course for AS-activation of 2DG uptake.

2DG uptakes in the presence of 5.0 mM AS were measured for 2, 3, 5, 8, 10, 15, and 20 min as described in materials and methods and expressed as the average nmol 2DG/minute ` S.E from four wells of a representative experiment. The uptake at 0 min exposed to AS represents the per minute-uptake for a 20-min uptake for cells not exposed to AS. [a]Significantly different than zero time exposure to AS and [b]significantly different than maximum effect at 2 min exposure to AS at $P < 0.01$.

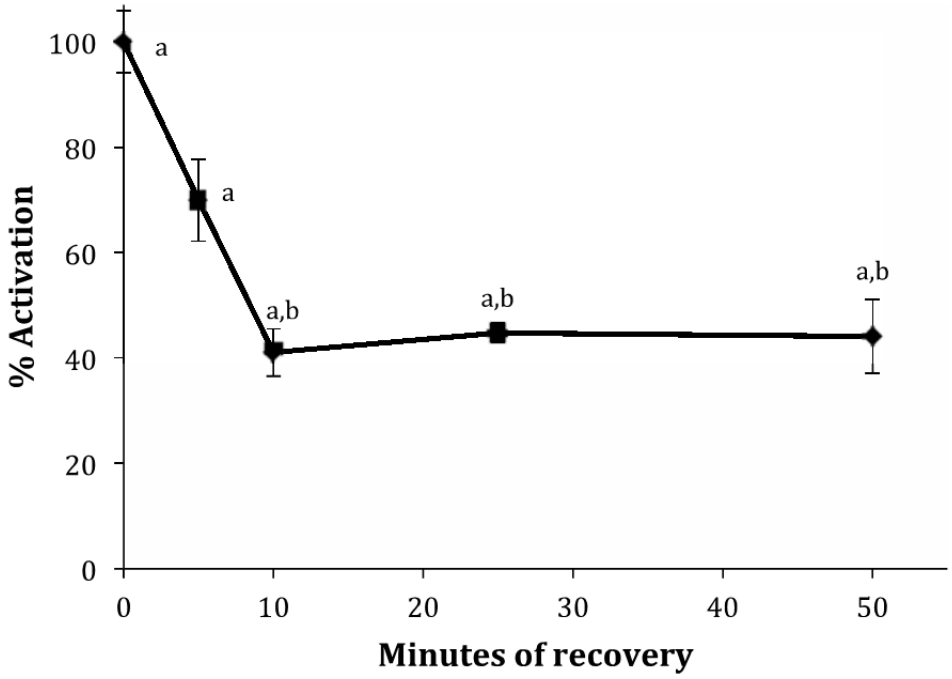


**Figure 3.5.** Effect of recovery time on AS-activated 2DG uptake.

L929 fibroblast cells were incubated at 37 C for 10 min in DMEM media (5.5 mM glucose) supplemented with 5.0 mM AS. Ten-minute 2DG uptakes were then measured immediately or media were replaced with AS-free media and uptakes were measured after 5, 10, 25, or 50 min. Data are expressed as a percentage of full activation (0 min recovery) ` S.E. from three wells of a representative experiment. [a]Significantly different than basal uptake (0% activation) and [b]significantly different than 100% activation at $P < 0.01$.

## Effects of Pretreatment with Thiol-reactive Compounds on AS Activation

If the activation of glucose uptake by AS is triggered by a reaction of HNO with thiols, we ought to be able to inhibit the activation by pretreating the cell with a thiol reactive compound. To test this, we pretreated L929 cells with 0.75 mM iodoacetamide for 20 minutes before activation with AS. The results are shown in Figure 3.6. Pretreatment with iodoacetamide by itself did not affect glucose uptake (0.97 ± 0.02 compared to 0.90 ± 0.04 nmol/10 min/well for control), but essentially completely inhibited activation by AS (reduced uptake from 3.78 ± 0.07 to 1.3 ± 0.05 nmol/10 min/well). When a stock iodoacetamide was incubated overnight (room temperature) with an equal concentration of cysteine, the iodoacetamide no longer inhibited AS-activation of glucose uptake. Cysteine itself did not alter glucose uptake (see Figure 3.3).

We also investigated the effects of two other thiol-reactive compounds, cinnamaldehyde (CA) and phenylarsine oxide (PAO). Previous work had shown that these two agents can activate glucose uptake in L929 cells, but they also inhibit subsequent activation by more robust activators of glucose uptake *(Scott et al., 2009 and Plaisier et al., 2011)*. We treated cells with either 2 mM CA or 10 μM PAO for 30 minutes and then measure glucose uptake in the presence and absence of 5 mM AS. The results are shown on Table 3.1. As previously reported, treatment with either CA or PAO activated glucose uptake *(Scott et al., 2009 and Plaisier et al., 2011),* but not to the same degree as AS. However, treatment with CA or PAO prior to treatment with AS blocked the activating effects of AS.

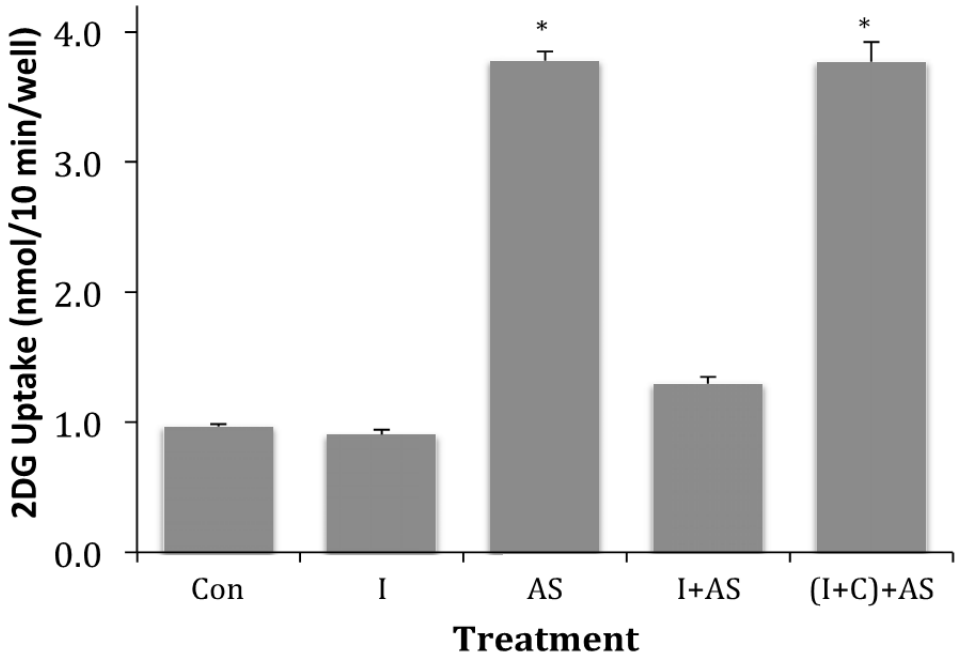


**Figure 3.6**. Effect of iodoacetamide on AS-activated 2DG uptake. L929 fibroblast cells were treated for 20 min at 37 C in DMEM media (5.5 mM glucose) alone or supplemented with 0.75 mM iodoacetamide or supplemented with a mixture of 0.75 mM iodoace- tamide and 0.75 mM cysteine that had been incubated overnight. Ten-minute 2DG uptakes were then measured in the presence and absence of 5 mM AS. (Con) repre- sents control cells not treated with iodoacetamide or AS, (I) represents cells exposed to iodoacetamide during the 20-min treatment, (AS) represents cells exposed to AS during the 10-min uptake, (I + AS) represents cells pre-treated with iodoacetamide and exposed to AS during the uptake phase, and ((I + C)+AS) represents cells pre- treated with an iodoacetamideecysteine mixture and then exposed to AS. Data are means ` S.E. from four wells of a representative experiment. *Significantly elevated from control at $P < 0.01$.

**Table 3.1** Effects of CA- and PAO-treatment on AS-activation of glucose uptake

| **Treatment** | **2DG uptake (w/o AS )** (nmol/10 min/well) | **2DG uptake (+ 5.0 mM AS)** (nmol/10 min/well) |
|---|---|---|
| *Control* | 1.41±0.03 | 2.84±0.08 |
| *2.0 mM CA* | 1.99±0.07* | 1.51±0.15* |
| *10.0 µM PAO* | 2.17±0.06* | 2.09±0.09# |

L929 fibroblast cells were incubated for 30 minutes at 37 °in DMEM media (5.5 mM glucose or 0 mM glucose) plus either no additions (control), 2.0 mM CA or 10.0 µM PAO. Glucose uptake was measured as described in Materials and Methods in the presence and absence of 5.0 mM AS. Significance from respective control at P<.01 (*), or at P<.05 (#).

## Combined Effects of Activators on Glucose Uptake

We had previously shown in L929 fibroblast cells that glucose uptake can be acutely activated by multiple agents *(Louters et al., 2006 and Roelofs et al., 2006)*. In order to gain some insight into to the activation pathway of AS, we conducted a series of additivity experiments with three of these activators, azide, berberine and glucose deprivation. L929 cells were incubated for 30 minutes with maximally effective concentrations of sodium azide (5 mM), berberine (50 μM), or media lacking glucose. Uptakes were then measured in the presence and absence of maximally effective concentration of AS (5.0 mM). The results are shown in Table 3.2. As expected, each stimulant significantly activated glucose uptake, however, AS did not increase the effects of the other stimulants. These results suggest that all stimulations, azide, berberine, glucose deprivation, and AS share a common, rate-determining step in their mechanisms of action.

**Table 3.2.** Combined effects of AS with other activators

| [AS] | Controls | + Azide | + Berb | +No glucose |
|---|---|---|---|---|
| 0 mM | 0.59±0.03 | 1.88±0.06 | 1.74±0.08 | 2.82±0.07 |
| 5.0 mM | 1.83±0.09 | 2.21±0.09 | 1.88±0.04 | 3.00±0.10 |

L929 fibroblast cells were incubated for 30 minutes at 37 °in DMEM media containing either 0 mM glucose (no glucose), or 5.5 mM glucose containing no additions (control), 5.0 mM sodium azide, or 50 μM berberine. Glucose uptake was measured as described in Materials and Methods in the presence and absence of 5.0 mM AS. Data are 2DG uptake in nmol/10min/well ± standard error. AS did not significantly increase the effects of azide, berberine, or glucose deprivation.

# DISCUSSION

The role of nitric oxide (NO) as a biological second messenger has been well established and this has led to an increased interest in the physiological action of other redox forms of NO *(Irvine et al., 2008)*. HNO, a one-electron reduction of NO, in particular has garnered a lot of attention because it has interesting pharmacological properties distinctly different from NO *(Fukuto et al., 2005, 2009 & 2011, Irvine et al., 2008; Switzer et al., 2009; paolocci et al., 2007, Miranda et al., 2003 and Wink et al., 2003)* . Of particular interest is the reaction of HNO with thiols. HNO can react with one thiol to produce a N-hydroxysulfenamide which can either rearrange to form a sulfinamide or react with a second thiol and produce a disulfide plus hydroxylamine *(Fukuto and Carington 2011).* This chemistry appears to account for much of the biological activity of HNO *(Irvine et al., 2008; Donzelli et al., 2006, and Shen et al., 2005).*

In this study we report for the first time that AS very quickly activates the glucose transport activity in L929 fibroblast cells, cells which only contain GLUT1 *(Liong et al., 1999)*. A 7.2-fold increase in activity occurs within 2 minutes of exposure to AS (see Figure 3.4), which averages to a 4.0-fold activation after 10 minutes (see Figure 3.1, 3.2, 3.4). The maximum effective concentration is about 5.0 mM and higher concentrations (20 mM) reduce this activation likely to additional reactions of HNO (see dose dependent data in Figure 3.2 and the twice-exposed data in Figure 3.1). The activation by AS can be blocked by three thiol active reagents, iodoacetamide, cinnamaldehye, and phenylarsine oxide (Figure 3.6, Table 3.1) suggesting the activation is caused by a reaction of HNO with cysteine residues. Possible targets for HNO are cysteine residues within GLUT1

itself. Previous work in erthyrocytes has shown that GLUT1 can be activated by disulfide bond formation within GLUT 1 *(Carruthers et al., 2009)* and computational studies have indicated that within a hydrophobic environment such as the cell membrane, the disulfide is the preferred product over the sulfinamide *(Sherman et al., 2010).* It is not known if HNO is the actual agent responsible for physiological activation of GLUT1 or if this is purely a pharmacological phenomenon. The mM concentrations required to activate GLUT1 are higher than μM concentrations needed to inhibit GAP *(Lopez et al., 2005 & 2007),* but are similar to the range concentrations shown to enhance cardiac function *(Tochetti et al., 2007).* Regardless, if HNO enhances glucose uptake via activation of GLUT1 in cardiac tissue as well as L929 fibroblast cells, that would be an added benefit in the use of HNO donors to treat heart failure. Future investigations should be done to determine if HNO does indeed activate glucose uptake in cardiac cells.

Angeli's salt can produce nitric oxide as well as HNO *(Fukuto et al., 2005).* This study does not directly test for the potential involvement of the nitric oxide signaling pathway in AS action. However, it seems unlikely that the causative agent for the activation of glucose uptake in these cells is nitric oxide. A previous study showed that the nitric oxide donor, sodium nitroprusside, did activate glucose uptake in L929 cells, but it was about half as effective as AS (2 activation rather than 4) and required a much longer activation time (30-45 min).

In addition to the interesting potential pharmacological effects of AS on glucose uptake, this study also sheds light on a potential mechanism for the acute activation of GLUT1 in L929 fibroblast cells. Once thought to be responsible only for basal glucose uptake, it has been shown by a number of studies that this ubiquitously expressed

transporter can be acutely activated by a variety of reagents *(Shetty et al., 1993; Rubin et al 2003; Barnes et al., 2002; Barros et al., 2001; Louters et al., 2006; Meyer et al., 2008; Kumar et al., 2004 and Roelofs et al., 2006).* It is known that the activation of GLUT1 occurs without a change in the concentration of the transporter in the plasma membrane, but the change in GLUT1 that accounts for increased activity is not known. One intriguing possible mechanism, based on Curruthers data in erythrocytes *(Graybill et al., 2006; Carruthers et al., 2009, Zottola et al., 1995; Herber et al., 1992, and Pessino et al., 1991)* and consistent with our data in L929 fibroblast cells, is outlined on Figure 3.7.


**Figure 3.7**. Mechanism for activation of GLUT1.

In this model for activation of GLUT1 in L929 fibroblast cells, GLUT1 exists is a number of states in the membrane ranging from a fully reduced monomer form which would have the lowest transport activity to an oligomer (dimers, tetramers) which have the highest activity.

This model proposes that GLUT1 can exist in multiple states ranging from monomers with reduced thiols to oligomers stabilized when GLUT1 is oxidized (internal disulfide). The reduced monomer would have the lowest activity and the oligomer the highest. Curruthers has demonstrated in erthryocytes that an internal disulfide bond forms between Cys 347 and Cys 421, which stabilize the more active GLUT1 oligomer *(Carruthers et al., 2009 and Zottola et al., 1995).* Furthermore, they have shown that Cys 421 can be modified by iodoacetamide. While this study in L929 cells does not provide direct evidence that GLUT1 is the target for HNO, the ability to block HNO activation with iodoacetamide suggest that activation requires a disulfide bond formation within some protein and the speed at which activation occurs makes GLUT1 itself the likely target. Also, cinnamaldehye, a known to Michael acceptor thiols, partially activates glucose uptake, but prevents the full, subsequent activation by AS, which can be explained by cinnamaldehye adding to a thiol on GLUT1 activating the transporter, but blocking the disulfide bond formation required to fully activate GLUT1 *(Plaisier et al., 2011).* In addition, phenylarsine oxide, a substance that reacts with vicinal thiols also activates, but prevents full activation by AS. Interestingly, PAO is typically a more robust activator than cinnamonaldehye, and like AS, activates glucose uptake within minutes *(Scott et al., 2009).* This suggests that the reaction of PAO with vicinal thiols mimics a disulfide bond, but it may be that the bulky phenyl ring prevents oligomerization and full activation of GLUT1.

We also show that AS does not further enhance the activation of other, slower acting stimulators such as sodium azide, berberine or glucose deprivation (see Table 3.2). A logical interpretation of this data is that these other methods of activation also stimulate

disulfide bond formation. Future studies should investigate if thiol reactive substances block activation by these agents.

## CONCLUSSION

This study demonstrates that AS, likely through the release of HNO and subsequent disulfide bond formation, very quickly activates the transport activity of GLUT1. The activation of glucose uptake by HNO has not been previously noted and may also contribute to the efficacy of using HNO as treatment for cardiac failure. These data are consistent with a proposed mechanism for GLUT1 activation that involves the formation of a disulfide bond within GLUT1.

## ACKNOWLEDGEMENT

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# CHAPTER IV

## MATERIALS AND METHODS

### CHEMICALS

AS was a generous gift of Dr. John P. Toscano (Johns Hopkins University). Phenylarsine oxide (PAO), cinnamaldehyde, cysteine, iodoacetamide, berberine, 2-deoxy-D-glucose-[1,2-$^{3}$H] (2DG) and D-mannitol-1-$^{14}$C were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

### CELL CULTURE

L929 mouse fibroblast cells were obtained from the American Type Culture Collection. To initiate each experiment, approximately 1.0 x $10^{5}$ L929 fibroblast cells were plated into each well of a 24-well culture-treated plate in 1.0 mL of low glucose (5.5 mM) DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were grown overnight at 37 °C in an incubator supplied with humidified room air with 5% CO2. L6 myoblast cells were grown in 24-well plates in DMEM (25.0 mM glucose) supplemented with 10% FBS. The cells were then differentiated for six days by reducing FBS to 2%.

### GENERAL EXPERIMENTAL DESIGN

In general, experiments had one or two phases, either a treatment phase followed by a glucose uptake phase, or just the glucose uptake phase. Times for each phase are indicated in the figure legends. To initiate an experiment with a treatment phase, the media from cells in 24-well plates were removed and then incubated in 0.8 mL of fresh

treatment media consisting of either low-glucose DMEM alone (0% FBS) (basal), or low-glucose DMEM plus the chemical of interest (see figure and table legends) or glucose-free DMEM (0% FBS) (activation by glucose deprivation). Cells were maintained at 37 °C for times indicated. If there was no treatment phase, cells were immediately incubated in glucose uptake media (see below). AS was stored at -4 °C under nitrogen. In all experiments using AS, the compound was weighed out and added to media at room temperature immediately prior to distribution to the cells in the 24-well plate. The cells were then returned to the incubator and the media allowed to warm to 37 °C.

In experiments designed to measure recovery from the effects of AS or Berberine, the treatment phase was followed by a recovery period in which cells were washed and returned to low-glucose media (0.8 mL) without AS or Berberine for varying times as indicated in figure legend. Glucose uptake was measured as described below.

## GLUCOSE UPTAKE ASSAY

Glucose uptake was measured using the radiolabeled glucose analog 2-deoxyglucose (2DG) as previously described [37]. Briefly, the media was replaced with 0.3 mL of glucose-free HEPES buffer (140 mM NaCl, 5 mM KCl, 20 mM HEPES/Na pH=7.4, 2.5 mM MgSO4, 1 mM $CaCl_2$, 2 mM NaPyruvate, 1 mM mannitol) supplemented with 1.0 mM (0.3 μCi/mL) 2-DG (1, 2-3H) and 1.0 mM (0.02 μCi/mL) mannitol (1-14C). Uptake media was supplemented with additional compounds, such as AS, as indicated in the figure and table legends. After a 10-minute incubation, cells were washed twice with cold glucose-free HEPES. The cells were lysed in 0.5 mL lysis buffer (10 mM Tris pH=7.4, 150 mM NaCl, 5 mM EDTA, 1.0% triton X-100, 0.4% SDS) and

the 3H-2 DG uptake with 14C-mannitol as the extracellular marker was measured using scintillation spectrometry. Uptake of 14C-mannitol only occurs if the cell membrane is compromised. Therefore, the use of a double-labeled uptake solution allows us to both measure surface binding and monitor potential toxic effects of the experimental treatments that would compromise the cell membrane.

## STATISTICAL ANALYSIS

Experimental conditions were repeated in triplicate or quadruplicate and glucose uptake was measured and reported as nmol/10 min/well ±standard error. Statistical significance was determined by either ANOVA followed by a post-hoc Dunnett test (dose and time dependent effects) or a two-tailed t-test. Statistical significance is reported at $P< 0.01$ or $P<0.05$. Experiments were repeated several times and results from representative experiments are reported.

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