

**NITROAROMATIC INDUCED METABOLIC IMPAIRMENT THROUGH  
CHEMICAL MODIFICATION OF  $\alpha$ -KETOACID DEHYDROGENASE  
COMPLEXES**

**By**

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## **DEDICATION**

**To my parents, whose continued love and support are undoubtedly responsible for the successes in my past as well as those in my future.**

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## LIST OF ABBREVIATIONS

1,3-DNB	1,3-Dinitrobenzene
3-NNB	3-Nitrosnitrobenzene
AcAc	Acetoacetate
AEDS	Acute Energy Deprivation Syndromes
ALCAR	Acetyl-Carnitine
AR	Adenosine Receptor
ATP	Adenosine Triphosphate
CNS	Central Nervous System
CoA	Coenzyme A
DCPIP	Dichlorophenolindophenol
DMPS	2,3-Dimercapto-1-propanesulfonic acid
DMSO	Dimethyl Sulfoxide
DNPH	2,4-Dinitrophenylhydrazine
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
DTT	1,4-Dithiothreitol
E3BP	E3 Binding Protein
EDTA	Ethylenedinitrilo Tetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid

ERK	Extracellular-Signal Regulated Kinases
FAD(H)	Flavin Adenine Dinucleotide
GSH	Glutathione
HEPES	(4-(2-Hydroxyethyl)-1-piperazinesulfonic Acid
HNE	4-Hydroxy-nonenal
HPLC	High Performance Liquid Chromatography
KGDHc	$\alpha$ -Ketoglutarate Dehydrogenase Complex
KSS	Kearns-Sayre Syndrome
LA	Lipoic Acid
LDH	Lactate Dehydrogenase
MD	Mitochondrial Diseases
MELAS	Mitochondrial Myopathy, Encephalopathy, Lactacidosis, and Stroke
MERRF	Myoclonic Epilepsy Associated with Ragged-Red Fibers
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide
NADP(H)	Nicotinamide Adenine Dinucleotide
NBT	Nitro Blue Tetrazolium
PDHc	Pyruvate Dehydrogenase Complex
PVDF	Polyvinylidene Difluoride
ROS	Reactive Oxygen Species
R-PIA	Phenylisopropyladenosine
TCA	Tricarboxylic Acid
TPP	Thiamine Pyrophosphate

# CHAPTER I

## INTRODUCTION

### **Background and Significance**

The human adult brain is highly metabolic, accounting for nearly a fourth of the body's total oxygen and glucose consumption, despite only representing 2% of total body weight. Additionally, the respiratory quotient ( $\text{CO}_2$  produced /  $\text{O}_2$  consumed) for the brain is nearly 1, indicating almost complete oxidation of glucose, and further suggesting that the brain is highly, if not exclusively, dependent on glucose as the sole energy producing fuel (Magistretti and Pellerin, 1996). With these characteristics, it is easy to see how structural and functional abnormalities of the central nervous system (CNS) are common consequences of aberrant energy metabolism, especially when due to dysfunctional mitochondrial respiration. The sensitivity of the CNS to energy deprivation is effectively demonstrated through examination of the various mitochondrial diseases (Brown and Squier, 1996).

Mitochondrial diseases are a heterogeneous collection of metabolic disorders resulting from genetic abnormalities of mitochondrial and nuclear DNA responsible for encoding components of both the respiratory chain and pyruvate metabolism. In general, the pathological responses of the brain to metabolic imbalance are relatively conserved with the most common outcomes being spongiform degeneration, neuronal loss, and extensive gliosis. Though most MD's present with one or more of these characteristics

their regional distribution and degree of severity may differ widely. These specific neuropathological changes are most commonly associated with Kearns-Sayre syndrome (KSS) and myoclonic epilepsy with ragged-red fibers (MERRF) in which defects in mitochondrial protein synthesis due to mutations of tRNA-encoding regions of mtDNA occur. (Lombes *et al.*, 1989).

Though there is considerable overlap in the pathology observed within the CNS for many of these metabolic disorders, some cases of MD's present with sufficiently distinctive changes in the pattern of neuropathology that allows for specific diagnosis. For example, focal necrosis followed by calcification, predominantly in cerebral cortex, is a hallmark of mitochondrial encephalopathy, lactic acidosis, and stroke like episode syndrome (MELAS). Like KSS and MERRF, MELAS is due to a point mutation in a tRNA gene of mtDNA. A second illustration of distinguishing neuropathology is Leigh's necrotizing encephalopathy. First described by Denis Leigh in 1951, Leigh's syndrome encompasses a variety of molecular defects in components of mitochondrial bioenergetics, including complexes I, II, and IV of the respiratory chain as well as the pyruvate dehydrogenase complex and ATPase. In Leigh's syndrome, the most striking feature is bilaterally symmetrical spongiform degeneration with the relative sparing of neurons.

The unique neuropathology encountered in Leigh's disease results in its inclusion with acute energy deprivation syndromes (AEDS) due to the striking similarities in lesion presentation (Cavanagh, 1993). Acute energy deprivation syndromes are a family of metabolic disorders with diverse etiologies, ranging from nutritional deficiencies, chemical intoxication to genetic abnormalities. Highly specific, regionally restricted



lesions within the neuraxis distinguish AEDS and like the analogous Leigh's syndrome, AEDS present with bilaterally symmetrical focal lesions, predominantly affecting the brainstem. The lesions are primarily gliovascular, with neuronal involvement being secondary to edema and loss of astrocytes. Regional metabolic demand seems to play a large role in selective vulnerability but areas with similar or even greater energy demands are spared, suggesting other factors may determine lesion restriction.

1,3-Dinitrobenzene (1,3-DNB) is one of several nitroaromatic compounds shown to produce the characteristic neuropathology of AEDS. Others include nitrobenzene, trinitrobenzene, and various nitroimidazoles such as metronidazole, and misonidazole. 1,3-DNB is an important chemical intermediate used in the production of azo and aniline dyes as well as a byproduct in the production of the most widely used explosive, trinitrotoluene (TNT), (Cody *et al.*, 1981). Like other nitroaromatic compounds, 1,3-DNB induces methemoglobinemia. Chemical oxidation of the heme iron renders hemoglobin incapable of transporting oxygen to tissues. Other clinical manifestations of 1,3-DNB exposure include headache, nausea, tachycardia, numbness in the distal portions of the limbs and hearing loss (Cody *et al.*, 1981; Philbert *et al.*, 1987). In addition to neurological defects incurred during exposure, animal studies have shown this compound to adversely affect male reproductive capacity. The primary target in the testis being the supportive Sertoli cells, with subsequent loss of germ cells.

Though the overall risk of exposure to the general population is relatively low, the ability of 1,3-DNB to mimic human metabolic diseases underscores its importance as a model to investigate fundamental mechanisms of acute energy deprivation syndromes. The molecular defects encountered in AEDS encompass multiple metabolic pathways,

including glycolysis, TCA cycle, mitochondrial respiration, and pentose phosphate pathway. Specific metabolic targets have been identified for a majority of these metabolic disrupters responsible in acute energy deprivation syndromes. 6-Aminonicotinamide, an anti-metabolite of nictotinamide, forms an inactive analogue of NADP, Which acts as a potent inhibitor of 6-phosphogluconate dehydrogenase, essential for the pentose phosphate pathway (Haghighat and McCandless, 1997).  $\alpha$ -Chlorohydrin, commonly used as a rodenticide, similarly to analogous 6-chlorosugars and 6-deoxysugars, disrupts glycolysis through the inhibition of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (Brown-Woodman *et al.*, 1978). In Leigh's Disease three specific gene products are affected due to mutations of both mitochondrial and nuclear DNA resulting in deficiencies in complex I, II and IV of the electron transport chain as well as the pyruvate dehydrogenase complex (PDHc) (Leigh, 1951; Rahman *et al.*, 1996). Acute thiamine deficiency greatly diminishes the activities of two key metabolic enzymes, pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase complexes, both of which require thiamine as a cofactor. At present, no direct targets of the nitroaromatic compound 1,3-dinitrobenzene have been determined.

The cellular presentation and regional distribution of lesions in nitroaromatic compound toxicity most strikingly resembles that of thiamine deficiency, suggesting that both cases of AEDS and 1,3-DNB toxicity may have a similar defect in energy metabolism, specifically, thiamine dependent enzymes. Thiamine, a precursor to the active coenzyme thiamine pyrophosphate, is a required cofactor for a family of mitochondrial metabolic complexes termed the  $\alpha$ -keto acid dehydrogenase complexes, which includes PDHc, KGDHc and the branched chain  $\alpha$ -ketoacid dehydrogenase

complex. These thiamine dependent enzyme complexes play crucial roles in oxidative energy metabolism and are essential to maintaining proper ATP production for cellular processes.

Both, PDHc and KGDHc occupy key sites in aerobic energy metabolism. Pyruvate dehydrogenase complex is the entry point of carbohydrates into the tricarboxylic acid (TCA) cycle, regulating the rate of glucose oxidation through the production of acetyl-CoA.  $\alpha$ -Ketoglutarate dehydrogenase complex is an essential component of the TCA cycle and is also involved in glutamate metabolism. Disturbances in either enzyme would greatly undermine cellular energy production and would ultimately result in cell demise.

There is considerable evidence that points to these mitochondrial dehydrogenase complexes as potential targets for 1,3-dinitrobenzene. As stated earlier, these complexes are affected during other instances of acute energy deprivation, including diet-induced thiamine deficiency and deficient enzyme activity due to genetic anomalies. Additionally, the nitroaromatic compound, metronidazole, has been shown to be converted to a thiamine analog capable of inhibiting thiamine pyrophosphokinase, the enzyme required for converting thiamine to the essential enzyme cofactor, thiamine pyrophosphate (Alston and Abeles, 1987). Thiamine and thiamine pyrophosphate co-administration with the nitroaromatic compound misonidazole affords some protection against the neurotoxic effects of this compound (Rose *et al.*, 1983).

The above evidence suggests that  $\alpha$ -ketoacid dehydrogenase complexes are potentially relevant in 1,3-DNB neurotoxicity and molecular characteristics of the complexes further support this hypothesis. The  $\alpha$ -ketoacid complexes are extremely

large (10 Mda) enzyme systems made up of multiple copies of three main catalytic enzymes and two associated regulatory enzymes. The complex geometry of these enzymes allow for sufficient substrate channeling of reaction intermediates from one active site to the next without release into the bulk media resulting in a “ping pong” mechanism of catalysis (Figure 1-1). The catalytic core of these complexes consists of a thiamine dependent  $\alpha$ -ketoacid dehydrogenase (E1), responsible for the rate limiting reaction of decarboxylation, which produces a 2-hydroxyethyl-TPP-carbanion intermediate. This carbanion then undergoes nucleophilic attack of the bound lipoic acid moiety of dihydrolipoyltransacetylase, the (E2) component. The lipoic acid bound to each of the two lipoyl domains of E2, through a flexible lysine chain linkage, facilitates transfer of the bound acyl group to coenzyme A. Lastly, dihydrolipoyl dehydrogenase, the (E3) component with a bound FAD<sup>+</sup>, re-oxidizes the reduced lipoic acid of E2 with concomitant production of NADH (Yeaman, 1989; Reed, 1998). The first two components are substrate specific depending on the complex, with the third being universally expressed. In addition to the catalytic components, PDHc contains a structural component, E3 binding protein (E3BP), which is responsible for binding the E3 to the E2 core. Like its E2 counterpart, this protein also contains a lipoyl domain with a bound lipoic acid though the function of this lipoic acid is not entirely understood since E3BP does not possess transacetylase activity. The activity of  $\alpha$ -keto acid dehydrogenase complexes is tightly controlled through a phosphorylation / dephosphorylation cycle that is mediated by a associated kinase and phosphatase bound to the lipoyl domains of the E2 component (Harris *et al.*, 1997). The structural

complexity of this enzyme complex matched with its strict cofactor requirements and tight metabolic regulation make it a prime target during many pathological conditions.

The vicinal dithiol group of lipoic acid is extremely sensitive to oxidative modification. As seen with arsenical compounds, protein adduction of this site is a common mechanism of inhibition. Akin to the classic arsenite induced dysfunction, other compounds such as strong electrophiles have been shown to disrupt the activity of  $\alpha$ -ketoacid dehydrogenase complexes in a similar manner. These inhibitors include the highly reactive  $\alpha,\beta$  unsaturated aldehydes, 4-hydroxynonenal and acrolein, both produced during lipid peroxidation, and the ubiquinone analogue coenzyme Q<sub>0</sub>. Additionally, lipoic acid can be directly affected by both superoxide generation as well as hydroxly radicals (Tabatabaie *et al.*, 1996). Direct modification by reactive oxygen species is suggested to underlie the observed loss of PDHc during the reperfusion stage of ischemia/reperfusion injury (Zaidan and Sims, 1993). The sensitivity of the essential cofactor, lipoic acid, to interference by either strong electrophiles or reactive oxygen species, further support the potential for PDHc and KGDHc activity to be disrupted by 1,3-DNB exposure.

During 1,3-DNB metabolism, both reactive oxygen species and strong electrophiles may be produced. 1,3-DNB undergoes a stepwise 6-electron reduction to 3-nitroaniline (Figure 1-2). Upon reduction, the highly reactive nitroxyl radical is formed and in the presence of molecular oxygen it is proposed that a futile redox cycle is established leading to the production of the reactive oxygen species, superoxide (Cossum and Rickert, 1985; Nystrom and Rickert, 1987; Jacobson and Miller, 1998). Evidence for the induction of oxidative stress upon metabolism of 1,3-DNB is supported by the

increase in superoxide levels as well as rapid GSH depletion observed during exposure of astrocytes to 1,3-DNB (Ray *et al.*, 1994; Tjalkens *et al.*, 2003). Following diminished cellular oxygen concentration, due to persistent redox cycling, the nitroxyl radical can be further reduced to the nitroso metabolite. Subsequent 2-electron reduction results in the production of 3-nitrophenylhydroxyl amine. Both nitrosoaromatics and hydroxylamines are strong electrophiles that have been shown to react with reduced sulfhydryls (Eyer, 1979; Eyer and Schneller, 1983; Umemoto *et al.*, 1988; Cribb *et al.*, 1991). It is plausible that either the induction of oxidative stress through the production of superoxide with subsequently formation of hydroxyl radicals or the presence of electrophilic reductive metabolites of 1,3-DNB may result in the oxidative modification of protein bound lipoic acid. Alternatively, lipid peroxidation may ensue post-oxidative stress induction resulting in the formation of reactive products that have already been shown to adversely effect  $\alpha$ -ketoacid dehydrogenase complexes through adduction of lipoic acid moieties.

The importance of metabolism in conferring neurotoxicity of 1,3-DNB is established through examination of the metabolic pathways for each of the three isomers. Only the 1,3 isomer is able to generate neurological dysfunction. Differences in isomer specific metabolism are cited to be potentially responsible for the inability of the 1,2 and 1,4 isomer to cause neurotoxicity. The primary route of 1,2-DNB metabolism is conjugation to GSH with a high percentage of the initial dose being excreted as S-(2-nitrophenyl)-N-acetylcysteine. 1,4-DNB appears to undergo both reduction as well as conjugation to GSH with the presence of both the reductive metabolites and the mercapturic acid conjugates being observed. Conversely, 1,3-DNB selectively undergoes reduction (Nystrom and Rickert, 1987). An interesting side note is that both the 1,3 and

1,4 isomers produce methemoglobinemia though only 1,3-DNB results in neuropathology, suggesting that diminished oxygen capacity is not the major mechanism of toxicity in 1,3-DNB induced encephalopathy.

Additional support for the inhibitory action of 1,3-DNB or its metabolites on  $\alpha$ -ketoacid dehydrogenase complexes, comes from the finding that mono-nitrosobenzene, a reductive metabolite of nitrobenzene, may undergo further metabolism by PDHc in the presence of 2-oxoacids including pyruvate. This interaction of nitrosoaromatic compounds with the enzyme prevents production of acetyl CoA due to transfer of the acetyl moiety to the highly reactive nitroso compound with subsequent formation of an N-hydroxy-N-arylacetylamide (Yoshioka and Uematsu, 1993; Yoshioka *et al.*, 1996). Investigations into the specific component responsible for the production of N-hydroxy-N-arylacetylamide by PDHc suggest that E1 catalyzed transacetylation is the step affected by nitrosoaromatic compounds due to apparent proper execution of the first step being the decarboxylation of the  $\alpha$ -ketoacid, pyruvate. In normal catalytic cycles of PDHc the pyruvate derived acetyl group is transferred to the E2 bound lipoyl moiety with subsequent acetyl transfer to coenzyme A, forming acetyl CoA. It is proposed that, in substitution for the lipoic acid group of E2, nitrosobenzene occupies the transacetylation site of E1 producing N-hydroxy-N-phenylacetamide upon reductive acetylation. PDHc catalyzed production of this highly reactive metabolite not only poses a threat due to its potential ability to form DNA adducts but it may also greatly compromise the enzyme's capacity to perform its natural function (Yoshioka *et al.*, 1993). Interference of acetyl-CoA production by nitrosoaromatic compounds would greatly hinder cellular energy production.

Formation of 3-nitrosanitrobenzene (3-NNB), a proposed toxic species of 1,3-DNB, as well as the possible inhibitor of PDHC, is an NADH / NAD(P)H dependent enzymatic process. The enzyme(s) responsible for the 2-electron reduction of the nitro group have not been definitively identified. Several enzyme systems have been reported to possess nitro reductase activity including xanthine oxidase, DT diaphorase and NADPH-cytochrome c reductase. These enzyme systems reside predominantly in either the endoplasmic reticulum or cytosol. Investigation into DNB metabolism by isolated seminiferous tubules, points to the mitochondria as the major site of reductive metabolism. Whether or not this is the case for the brain has not been addressed. Examination of mitochondrial NADH-dependent nitro reductase activity, in rat brain, demonstrated significant activity toward 1,3-dinitrobenzene that was more than 50 times higher than the microsome and cytosol fractions (Kochli *et al.*, 1980).

Interestingly, one mitochondrial enzyme that has been shown to possess nitroreductase activity is lipoamide dehydrogenase, which is a component of the  $\alpha$ -keto acid dehydrogenase enzyme complexes (Tsai, 1987). The unique organization of an enzyme complex allows for tightly regulated shuttling of intermediates between component enzymes. Lipoamide dehydrogenase, the E3 component of PDHC, may reduce 1,3-dinitrobenzene to the highly reactive nitroso intermediate allowing for this species to interact with the sulfhydryl groups of the lipoic acid moiety in the E2 component. The intrinsic flexibility of the lysine arm with bound lipoic acid, allows this element to swing back and forth between the E1 and E3 components of the complex. Upon interaction of the nitroso compound with the lipoic acid group it may be possible for the nitroso-nitrobenzene compound to bind the E2 complex allowing it to substitute



for lipoic acid at the transacetylation site. This substitution would prevent production of acetyl-CoA, greatly affecting its ability to produce adequate ATP.

The sensitivity of lipoic acid to oxidative modification, combined with the potential ability of lipoamide dehydrogenase to metabolize nitroaromatic compounds points to the  $\alpha$ -ketoacid dehydrogenase complexes as likely targets for direct modification by 1,3-DNB or its reductive metabolites. Alternatively, establishment of an oxidative environment through excess production of superoxide during 1,3-DNB metabolism may also result in dysfunctional enzyme activity either through direct interaction with reactive oxygen species or through induction of lipid peroxidation and production of reactive aldehydes capable of affecting the  $\alpha$ -ketoacid dehydrogenase complexes. The experiments described in this thesis aim to determine whether 1,3-DNB affect the  $\alpha$ -ketoacid dehydrogenase complexes.

In addition to investigating the potential biochemical targets of 1,3-DNB, this thesis also addresses the role of astrocyte / neuronal interactions in the initial sparring of neurons observed in the neuropathology of acute energy deprivation syndromes. Astrocytes perform numerous functions in the CNS including maintenance of the extracellular environment, formation and maintenance of the blood-brain barrier, as well as modulation of synaptic transmission. The functions of astrocytes are due to the formation of intimate relationships with both neuronal and endothelial components in the CNS. These relationships allow astrocytes to play an extremely important role in cerebral function, homeostasis, as well as in modulating toxicity. The ability of astrocytes to modulate physiological as well as pathological responses of neighbors cells in the CNS are accomplished through active signaling mediated by astrocyte-derived

neurotransmitters, termed gliotransmitters (Koizumi and Fujishita, 2007). One such gliotransmitter suggested to be involved in the intimate interaction between astrocyte and neurons as well as astrocytes and vascular endothelial cells is the purine, adenosine.

Adenosine, a hydrolysis product of ATP, is a potent modulator of many physiological functions in various systems, particularly those of excitable tissues such as heart, and brain. Under normal physiological conditions, adenosine concentrations in the extracellular space are in the nanomolar range but during times of energy deprivation, as seen in conditions of increased metabolic activity or in cases of hypoxia or ischemia, adenosine levels dramatically increase. Peak levels on the order of 3  $\mu$ M have been recorded in the striatum of rats following 24 hr of focal ischemia induced by occlusion of the middle cerebral artery (Melani *et al.*, 1999). Increased adenosine levels is followed by activation of suppressive signaling pathways resulting in compensatory actions such as vasodilation, cessation of neurotransmission, and diminution in cellular activity giving adenosine the distinction of a retaliatory signaling molecule (Yoon and Rothman, 1991). Interestingly, all these behaviors have been shown to occur in 1,3-DNB and other AEDS (Philbert *et al.*, 1987; Cavanagh, 1988). The induction of cellular signaling pathways by adenosine are mediated through activation of specific cell surface purinergic receptors aptly named the adenosine receptors (AR). The AR family of G-protein coupled receptors is subdivided into 4 known isotypes ( $A_1$ ,  $A_{2a}$ ,  $A_{2b}$ , and  $A_3$ ) based on their distinct pharmacological profiles, molecular structure and tissue distribution (Fredholm *et al.*, 2000). There has been a recent expansion of interest in adenosine receptors due to the therapeutic prospects these receptors may hold in various pathological conditions

including diabetes, epilepsy, cancer, neurodegeneration, cardiovascular disease and stroke (Williams and Jarvis, 2000; Stone, 2005).

Adenosine receptor activation results in the induction of numerous biochemical pathways. Like other G-protein coupled receptors, AR's modulate intracellular concentration of cAMP through their actions on adenylyl cyclase. In addition to cAMP dependent pathways other pathways are also activated including inositoltrisphosphate (IP3) and diacylglycerol (DAG) pathways. Adenosine receptors have also been shown to influence the activity of the mitogen activated protein kinase (MAPK) including ERK (1/2). Activation of MAPK leads to phosphorylation of numerous proteins including transcriptional factors as well as proteins involved in regulating cell death (e.g. Bcl-2; (Breitschopf *et al.*, 2000).

The Bcl-2 family proteins have been implicated in the control of cellular sensitivity to endogenous and exogenous stresses. They are believed to regulate mitochondrial permeability transition, a term used to describe an abrupt increase in mitochondrial inner membrane permeability to solutes smaller than 1500 Da, through intimate interactions with a multi-protein pore (Lemasters *et al.*, 1997). There are both pro (Bcl-2, Bcl-xl) and anti (Bax, Bid, Bad) members of the Bcl-2 family that interact with each other to regulate the fine line between life and death of a cell (Lindsten *et al.*, 2005).

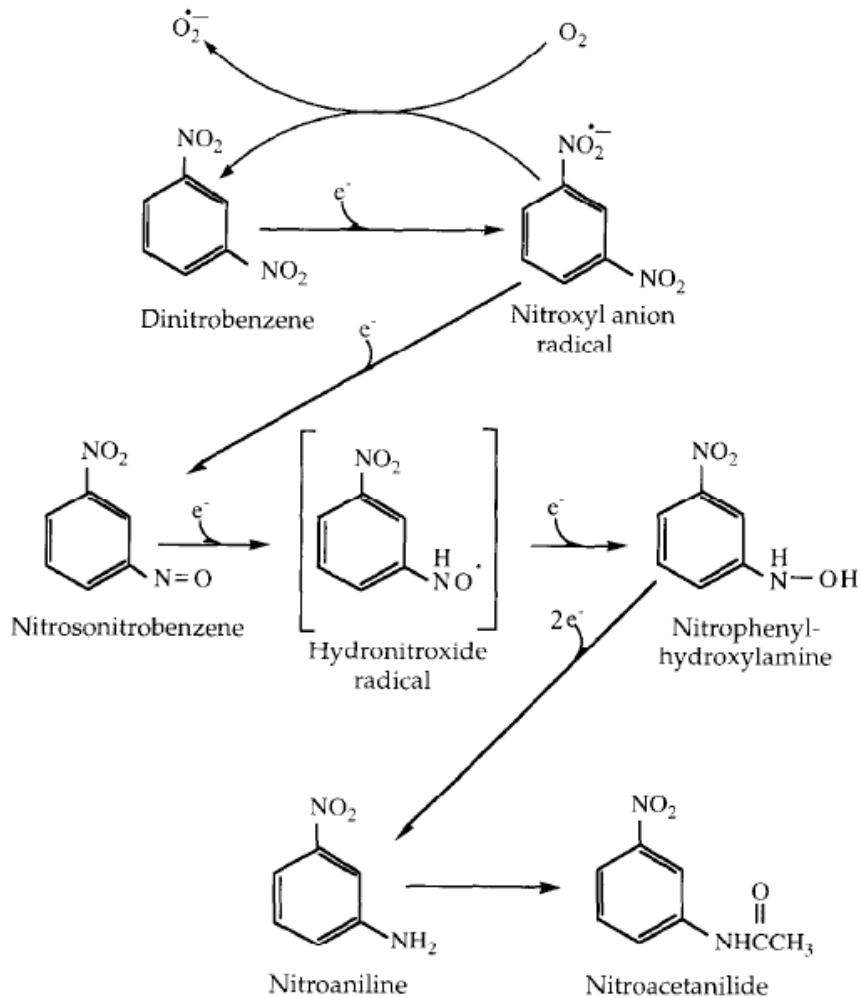
Control of apoptosis may be mediated through regulation of Bcl-2 family proteins via reversible phosphorylation. Phosphorylation of Bcl-2 family proteins regulates many aspects of these proteins including translocation, degradation and interactions with other members of the family (Breitschopf *et al.*, 2000). Adenosine has been shown to activate

ERK 1/2 in perfused rat heart through activation of all 4 adenosine receptors (Germack and Dickenson, 2005). Bcl-2 family proteins possess protein kinase consensus sites specific for ERK 1/2 suggesting that adenosine mediated regulation of apoptosis could be due to activation of ERK 1/2 and subsequent phosphorylation of Bcl-2 family proteins.

The ability of 1,3-DNB to induce mitochondrial permeability transition in a regionally selective manner supports the potential role for Bcl-2 family proteins in facilitating regional and cell specific neurotoxicity seen in 1,3-DNB and other AEDS (Tjalkens *et al.*, 2000; Tjalkens *et al.*, 2003). Consequently, regulation of Bcl-2 family proteins through induction of intracellular signaling pathways would also facilitate this selectivity. It is conceivable that intercellular signaling between astrocytes and neurons, mediated through adenosine receptor activation, may result in the induction of pro-survival mechanisms leading ultimately to cellular protection.

In the preceding sections of this Introduction, substantial evidence was outlined supporting the potential for  $\alpha$ -ketoacid dehydrogenase complexes as prime candidates responsible for the metabolic disruption induced by 1,3-DNB in the CNS. The studies presented in the following chapters of this thesis were designed to test the hypothesis that 1,3-DNB has the capability to elicit metabolic dysfunction through its actions on the  $\alpha$ -ketoacid dehydrogenase complexes and to examine the detailed mechanisms of this inhibition. In the final data chapter, the role of astrocyte-neuronal interactions in mediating the effects of 1,3-DNB is presented. Implications for the role of adenosine-mediated signaling pathways are discussed with respect to neuroprotection. Further investigations into the complex relationship between astrocytes and neurons during acute energy deprivation are required.





**Figure 1-2.** Proposed mechanism for 1,3-dinitrobenzene metabolism (Jacobson et al., 1998). The first step in reductive metabolism of 1,3-dinitrobenzene is a single-electron reduction to the reactive nitroxyl anion radical. In an oxygen rich environment, this radical species can redox cycle with molecular oxygen producing superoxide radical. Futile redox cycling results in reduced oxygen tension allowing for a second one electron reduction to 3-nitrosonitrobenzene. Further two electron reductions occur resulting in the final product, 3-nitroaniline.

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## CHAPTER 2

### SELECTIVE INACTIVATION OF THE PYRUVATE DEHYDROGENASE COMPLEX BY 1,3-DINITROBENZENE

#### ABSTRACT

Pyruvate dehydrogenase complex (PDHc), an essential multi-component enzyme system responsible for the production of acetyl-CoA from glycolytically derived pyruvate, links glycolysis to the TCA cycle. The adult brain relies almost exclusively on the complete oxidation of glucose, making PDHc function critical in maintaining cerebral energy metabolism. 1,3-Dinitrobenzene (1,3-DNB) selectively affects glio-vascular components in distinct brainstem regions, resembling the early effects of acute energy deprivation syndromes (AEDS). Both *in vivo* and *in vitro* studies point to metabolic disruption in 1,3-DNB neurotoxicity, though the specific site of impairment is not known. In the present study, the effects of 1,3-DNB and its reductive metabolite, 3-nitrosonitrobenzene (3-NNB) on PDHc and KGDHc activity were investigated using commercially available enzymes purified from porcine heart. Both compounds rapidly and dose-dependently inhibited PDHc. In contrast to PDHc, only 3-NNB showed significant inhibitory capacity toward KGDHc. The isomer-specificity and substrate requirements suggest that the E1 component enzyme is a primary target of 1,3-DNB. This hypothesis was confirmed

through inhibition studies on the separated E1 component. 3-Nitrosnitrobenzene was also shown to affect PDHc mediated metabolism of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) suggesting that this compound interferes with E2 bound lipoic acid. Though further investigation into the mechanism of inhibition is required, these data support the potential role of PDHc inhibition in the neurotoxic mechanism of 1,3-dinitrobenzene exposure.

### **Introduction**

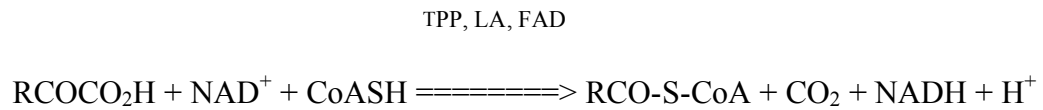
Nitroaromatic compounds are key intermediates used in the production of numerous industrial and commercial products including industrial solvents, dyes, plastics, and explosives (Harter, 1985). Though important for the chemical industry, due to their abundant use, nitroaromatics also pose many environmental issues and potential health risks. 1,3-Dinitrobenzene (1,3-DNB), important in azo and aniline dye synthesis, as and a byproduct in the production of the most widely used explosive, trinitrotoluene (TNT), is a multi-target toxicant affecting both the reproductive and nervous systems (Cody *et al.*, 1981). Accidental occupational exposure in humans produces methemoglobinemia, tachycardia, hearing loss, and various neurological deficits such as headaches, nausea, and vertigo. In animal studies, 1,3-DNB exposure results in significant testicular dysfunction, with Sertoli cells being the primary target (Foster, 1989). In addition to effects on male reproductive capacity, prolonged or repeated exposure to 1,3-DNB produces similar CNS lesions characteristic of acute energy deprivation syndromes (AEDS) (Philbert *et al.*, 1987; Romero *et al.*, 1991; Ray *et al.*, 1992). AEDS are a family of conditions, which include acute thiamine deficiency, the inherited neurometabolic

disorder Leigh's necrotizing encephalomyelopathy, and chemical intoxication by various compounds such as chlorosugars, and 6-aminonicotinamide (Cavanagh, 1993). All these conditions result in a distinctive bilaterally symmetrical lesion, with very similar regional distribution. While lesion location may vary slightly depending on stimulus and species afflicted, the auditory and vestibular centers of the brainstem appear to be most commonly affected. This is believed to be a result of high metabolic demand within these areas (Cavanagh, 1988). Though regional metabolic activity appears to play a significant role in determining patterns of CNS damage in AEDS, other factors such as site of biochemical defect may be involved in the observed selective vulnerability.

The commonality amongst all of these conditions is the disruption of key steps within the energy metabolism pathways. In the case of nitroaromatic compounds, the specific site of metabolic impairment is not known. Examination of the biochemical defects responsible for some AED syndromes including acute thiamine deficiency, metronidazole intoxication, and Leigh's syndrome suggests that thiamine status or more importantly the activity of thiamine dependent enzymes is the key factor linking these particular cases of AEDS. The compelling regional similarity in lesion presentation between these particular instances of AEDS and nitroaromatic compound exposure, and similar changes in biochemical parameters including increased glucose utilization and increased extracellular lactate, suggests that a possible target for nitroaromatic compounds may be thiamine dependent enzymes.

Thiamine, an essential precursor to thiamine pyrophosphate, is a required cofactor for  $\alpha$ -ketoacid dehydrogenase complexes. This family of multi-component complexes includes the branched chain  $\alpha$ -ketoacid dehydrogenase (BCKDC),  $\alpha$ -

ketoglutarate dehydrogenase (KGDHc), and the pyruvate dehydrogenase (PDHc) complexes (Yeaman, 1989). The  $\alpha$ -ketoacid dehydrogenase complexes are extremely large (10 MDa), multi-component enzyme systems that are made up of 3 catalytic enzymes, two regulatory enzymes and require 5 cofactors. The three constituent enzymes that make up the catalytic core of these complexes are a substrate specific  $\alpha$ -ketoacid decarboxylase (E1), a substrate specific acyltransacetylase (E2) and the shared dihydrolipoamide dehydrogenase (E3). All three component enzymes act in concert to facilitate the following overall reaction (Tsai *et al.*, 1973):



The individual steps that make of the overall reaction are described below using pyruvate as the  $\alpha$ -ketoacid:

- (1)  $\text{CH}_3\text{COCO}_2^- + \text{E1TPP} + \text{H}^+ \rightleftharpoons \text{CO}_2 + \text{E1CH}_3\text{C(OH)=TPP}$
- (2)  $\text{E1CH}_3\text{C(OH)=TPP} + \text{E2-LipS}_2 \rightleftharpoons \text{E1TPP} + \text{E2-Lip(SH)-S-COCH}_3$
- (3)  $\text{E2-Lip(SH)-S-COCH}_3 + \text{CoASH} \rightleftharpoons \text{E2-Lip(SH}_2) + \text{CH}_3\text{COSCoA}$
- (4)  $\text{E2-Lip(SH}_2) + \text{E3FAD} \rightleftharpoons \text{E2-LipS}_2$

The two complexes, PDHc and KGDHc, occupy critical sites in oxidative energy metabolism. PDHc plays a unique role in energy metabolism by acting as a critical gateway linking glycolysis to the TCA cycle, through the production of acetyl-CoA. This position assigns PDHc the important task of controlling the rate of complete oxidation of

carbohydrates (Wieland, 1983). KGDHc, being equally as important, catalyzes a key step in the tricarboxylic acid cycle (TCA) and is also responsible for linking the TCA cycle to glutamate metabolism with the help of glutamate dehydrogenase. Disturbances in the function of either of these two vital thiamine dependent enzyme systems frequently result in ensuing pathological states in those tissues that maintain a high aerobic rate, such as the brain. Normally, the adult brain maintains a high demand for energy with low energy reserves and is highly, if not exclusively, dependent on the complete oxidation of glucose as the sole energy producing fuel (Ritter and Robertson, 1994). These unique characteristics render the brain an extremely sensitive target to any disturbances in oxidative energy production and demonstrate the pivotal role both PDHc and KGDHc play in maintaining proper cerebral functioning. Disturbances in the activity of these enzyme complexes have been implicated as potentially relevant in various neurological disorders including Wernicke's encephalopathy (Butterworth *et al.*, 1993), cerebral ischemia/reperfusion (Martin *et al.*, 2005), and Alzheimer's and Parkinson's disease (Mizuno *et al.*, 1994; Kish, 1997). Similarly, these key metabolic enzymes may be relevant in the neurotoxicity observed during 1,3-DNB exposure.

In the present study, the effects of 1,3-dinitrobenzene and its highly reactive intermediate, 3-nitrosanitrobenzene, on PDHc and KGDHc were determined using commercially available purified enzymes from porcine heart.

### **Materials**

Purified pyruvate dehydrogenase complex and  $\alpha$ -ketoglutarate dehydrogenase complex from porcine heart were obtained from Sigma suspended in a 50% glycerol

solution containing 9 mg/ml bovine serum albumin, 30% sucrose, 1.5 mM EDTA, 1.5 mM EGTA, 1.5 mM 2-mercaptoethanol, 0.3% TRITON X-100, 0.003% sodium azide, and 15 mM potassium phosphate, pH 6.8. All other chemicals were obtained from Sigma unless otherwise stated.

## Methods

### Determination of PDHc and KGDHc activity

#### *Spectrophotometric assay*

A standard spectrophotometric assay was performed to determine the activities of PDHc and KGDHc as described previously (Hard *et al.*, 2001) with minor alterations. In brief, the formation of the reduced form of nicotinamide-adenine dinucleotide (NADH) was monitored at 340 nm at 30°C. Commercially available porcine heart PDHc (15 mg/ml) or KGDHc (10.2 mg/ml) were employed in this assay. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.4, 2.5 mM NAD<sup>+</sup>, 0.2 mM thiamine pyrophosphate (TPP), 1 mM MgCl<sub>2</sub>, 1 mM pyruvate or  $\alpha$ -ketoglutarate, PDHc (final concentration 75  $\mu$ g/ml) or KGDH (final concentration 50  $\mu$ g/ml). Reaction buffer combined with the appropriate enzyme were incubated for 2 min at 30°C. Varying concentrations of inhibitor were then added and incubated for an additional 3 min. At 5 min the reaction was started with addition of CoA (final concentration 0.2 mM). The assay was performed on a Spectramax microplate spectrophotometer (Molecular Devices, Inc). Absorbance data were fitted to equations that describe models of inhibition using Sigmaplot with the enzyme kinetics module (Systat). Best-fit models were determined



by comparing the sum of squares using non-linear regression and model selection was determined with the use of an F test.

To determine substrate requirements, as well as the potential for reversibility, PDHc was incubated with varying concentrations of 1,3-DNB in 50 mM potassium phosphate buffer, pH 7.4 containing various substrates as indicated in the figure legends. After exposure, samples of PDHc were taken and activity was determined as stated above. Through this method 1,3-DNB concentrations were diluted 50 fold. Commercially available purified PDHc has been shown to be almost completely saturated with bound TPP (Strumilo *et al.*, 2004). In order to investigate thiamine's role in inhibition by 1,3-DNB the removal of bound thiamine was necessary. It was found that due to the size of PDHc, the complex can be pelleted, resulting in the removal of TPP (Sumegi and Alkonyi, 1983). Briefly, PDHc diluted in 50 mM sodium phosphate buffer, pH 7.0 containing 5 mM EDTA was subjected to sedimentation by ultra-centrifugation at 150,000 x g at 4°C for 2 hrs. The pelleted complex was resuspended in reaction buffer minus substrates.

#### *HPLC method*

To determine PDHc activity, acetyl-CoA production was monitored using reverse phase high pressure liquid chromatography (HPLC) as described by Maehara *et al.*, 1990. The reaction buffer used was the same as that employed in the spectrophotometric assay with one exception. The concentration of pyruvate in the reaction buffer was increased to 2 mM. The reaction was initiated with the addition of CoA (final concentration 1 mM) and the reaction was allowed to proceed for 30 min, at which time, the reaction was

stopped by the addition of 70% perchloric acid (final concentration 6%). Protein precipitate was removed by centrifugation at 8000 X g for 10 min. Five  $\mu$ l of clear supernatant was injected into a Waters HPLC system equipped with a dual  $\lambda$ -absorbance UV detector and inline degasser. Acetyl-CoA separation was performed by isocratic elution on a Symmetry 300™ C-18 column (4.6 x 1.5 mm, 5  $\mu$ m) at a flow rate of 1 ml / min and peak absorbance was recorded at 254 nm. The mobile phase consisted of 0.1 M sodium phosphate buffer, pH 4.0 containing 0.1 mM EDTA and 20% methanol. The area under the curve was quantified and the concentration of acetyl-CoA was determined using a standard calibration graph.

#### **Separation of the E1 and E3 components of PDHc**

The individual components of the pyruvate dehydrogenase complex were separated by limited proteolysis and ammonium sulfate precipitations by a method described previously (Kresze *et al.*, 1980). Briefly, purified pyruvate dehydrogenase complex (5 mg/ml) was incubated with papain (35  $\mu$ g/ml) in 0.1 M phosphate buffer, pH 6.5, containing 2 mM cysteine, and 1 mM EDTA for 10 min at 25 °C with gentle stirring. The protein digestion was stopped with the addition of leupeptin (0.1 mg/ml) and the mixture was allowed to incubate for 15 min at 0°C. The solution was diluted in half with buffer A consisting of 20 mM potassium phosphate, pH 7.0, containing 1 mM MgCl<sub>2</sub>, 10  $\mu$ M thiamine pyrophosphate, 0.1 mM EDTA, 2 mM dithiothreitol, and 0.5 mM NAD. Proteolysis results in selective digestion of the E2 component, which causes dissociation of the two other components, the E1 and E3. The nicked E2 was precipitated out using 0.16 g of solid ammonium sulfate to produce a 28% saturated solution and incubated at

0°C for 15 min. To remove the precipitated nicked E2, the mixture was centrifuged at 10000 x g for 20 min at 4 °C. The clear supernatant was then made 59 % saturated by adding 0.19g of solid ammonium sulfate. The precipitated E1 component was pelleted by centrifugation and redissolved in buffer A containing 0.1 mg/ml leupeptin.

### **Determination of the activities of the separated E1 and E3 components**

#### *E1 activity*

The pyruvate dehydrogenase (E1) activity was determined spectrophotometrically by following the loss of absorbance of 2,6-dichlorophenolindophenol (DCPIP) (Alkonyi *et al.*, 1976). The reaction buffer consisted of 50 mM sodium phosphate buffer, pH 7.8, containing 2 mM MgCl<sub>2</sub>, 0.2 mM thiamine pyrophosphate and 0.2 mM DCPIP. The reaction was initiated by addition of pyruvate, 0.2 mM final concentration. The absorbance was monitored at 600 nm using a Spectramax microplate spectrophotometer (Molecular Devices, Inc).

#### *E3 activity*

The effects of 1,3-DNB on dihydrolipoamide dehydrogenase (E3) activity were determined using commercial preparations of purified E3 from porcine heart (13 mg/ml). The reaction buffer consisted of 50 mM sodium phosphate buffer, pH 7.4 containing 2.5 mM NADH, and 1mM MgCl<sub>2</sub>. The reaction was started with the addition of lipoamide, 3 mM final concentration. Loss of absorbance at 340 nm was recorded on a Spectramax microplate spectrophotometer (Molecular Devices, Inc).

### **Dithio-bis-nitrobenzoic acid (DTNB) assay**

To investigate the ability of 3-NNB to interfere with the lipoyl moieties of the E2 component, the DTNB assay as described previously was employed (Humphries and Szweda, 1998). It was necessary to remove  $\beta$ -mercaptoethanol present in the packaging solution of commercial PDHc, due to nonenzymatic reduction of DTNB. The complex was pelleted as described above and was resuspended in 50 mM sodium phosphate buffer, pH 7.4. The sulfhydryls of lipoic acid were reduced by the addition of 100  $\mu$ M NADH for 10 min. The reduced complex was then incubated with varying concentrations of 3-NNB for 10 min. The reaction was started with the addition of 0.5 mM DTNB and 100  $\mu$ M NADH and the rate of DTNB reduction was followed at 412 nm at 30 °C on a Spectramax microplate spectrophotometer.

## **Results**

### *1,3-DNB and 3-NNB impairment of PDHc and KGDHc*

Incubation of purified porcine heart PDHc with 1,3-DNB resulted in rapid inhibition as determined by a standard spectrophotometric assay (Figure 2-1). The degree of impairment responded in a dose-dependent manner, with approximately 12% at the lowest dose of 5  $\mu$ M and greater than 90% inhibition at 1 mM 1,3-DNB. The  $IC_{50}$  for 1,3-DNB impairment of PDHc was approximately 62  $\mu$ M. In contrast to PDHc, KGDHc showed dramatically less inhibition when incubated with 1,3-DNB with significant inhibition being observed only at 1,3-DNB concentrations above 500  $\mu$ M.  $IC_{50}$  for 1,3-DNB-mediated inhibition of KGDHc could not be accurately determined. Loss of total PDHc activity was confirmed using HPLC quantification of acetyl-CoA production

(Figure 2-2). 1,3-DNB impaired PDHc-mediated production of acetyl-CoA in a dose-dependent manner. Significant loss of acetyl-CoA production was observed at 50  $\mu$ M and greater than 70% at 1 mM 1,3-DNB. To further investigate the mode of inhibition, kinetic data were fitted to equations describing the different models of inhibition (figure 2-3). The best-fit model was determined through comparing the sum of squares obtained for each model and performing an F-test. The model describing mixed inhibition had the lowest sum of squares supporting the conclusion that inhibition of PDHc occurs through a mixed type mechanism.

Exposure to 3-NNB, a highly reactive reductive metabolite of 1,3-DNB, resulted in significant inhibition of both the PDHc and KGDHc complexes. Similar to the results obtained with 1,3-DNB, KGDHc appeared to be less sensitive to inhibition by 3-NNB ( $IC_{50}$  42.7  $\mu$ M for KGDHc versus 7.82  $\mu$ M for PDHc) though in contrast to 1,3-DNB, 3-NNB was able to elicit almost complete inhibition by the highest dose of 1 mM (figure 2-4).

#### *Isomer specificity*

Incubation of PDHc with each of the three isomer forms of dinitrobenzene revealed isomer specific inhibition (figure 2-5). Both, the meta- and ortho- isomers showed comparable inhibition with approximate  $IC_{50}$ 's of 46  $\mu$ M. In contrast, the para-substituted dinitrobenzene showed a significant reduction in inhibitory potential with an approximate  $IC_{50}$  of 325  $\mu$ M. The final nitroreduction product, 3-nitroaniline, showed very little inhibitory action with respect to PDHc activity.

### *Substrate requirements*

To determine the ability of 1,3-DNB to elicit inhibition through irreversible binding of PDHc, 1,3-DNB at 500 $\mu$ M was incubated with PDHc in buffer for 5 or 90 min (Figure 2-6). Enzyme with the inhibitor i.e. 1,3-DNB was diluted with an excess of reaction buffer (1:50 v/v) resulting in a final concentration of 10  $\mu$ M. Pre-incubation for both 5 and 90 min resulted in less than 20% inhibition. This corresponds to the amount of inhibition obtained with co-incubation of PDHc with 10  $\mu$ M 1,3-DNB. Whereas, co-incubation of PDHc with 500  $\mu$ M 1,3-DNB results in greater than 85% inhibition. Activation of the complex with the addition of thiamine pyrophosphate (TPP) prior to the addition of 1,3-DNB resulted in 17% loss of PDHc activity, which is slightly higher than that observed with 10  $\mu$ M co-incubation (figure 2-6b). Addition of the substrate pyruvate to the complex prior to incubation with 500  $\mu$ M 1,3-DNB resulted in slightly greater inhibition than that observed with TPP alone. In contrast, incubation with both TPP and pyruvate followed by 1,3-DNB resulted in approximately 80% inhibition which corresponded to the amount of inhibition that was obtained with co-incubation of 500  $\mu$ M 1,3-DNB.

### *DTNB reduction assay*

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) has previously been shown to bind reduced E2-bound lipoic acid with subsequent catalytic cleavage by lipoamide dehydrogenase, the E3 component of the complex ((Brown and Perham, 1976; Humphries and Szweda, 1998). Exploiting this reaction of DTNB with PDHc, it was possible to investigate the potential for 3-NNB to bind the E2 LA. As shown in figure 2-7, the ability of PDHc to metabolize DTNB was diminished by incubation of reduced

complex with increasing concentrations of 3-NNB suggesting that this strong electrophile is able to bind to reduced lipoic acid and may underlie the loss of activity of the complex.

#### *Component enzyme activities*

To further determine which component is affected by 1,3-DNB and its metabolite 3-NNB, PDHc was separated into its component enzymes through limited proteolysis by incubation with papain. This process affectively cleaves the E2 binding domains, releasing the bound E1 and E3 components without loss of component enzyme activity. No significant effects were observed with either 1,3-DNB or 3-NNB on the activity of E3 component (Figure 2-8a). Incubation of the E1 component with 1,3-DNB or 3-NNB resulted in significant inhibition. 3-NNB is a more potent inhibitor than its parent compound 1,3-DNB, with a calculated  $IC_{50}$  of 21  $\mu$ M versus 762  $\mu$ M for 1,3-DNB (Figure 2-8b).

### **Discussion**

Disturbance in the metabolic function of astrocytes is a key component in the pathological mechanism of 1,3-dinitrobenzene-mediated encephalopathy, though the specific metabolic target of this compound is still unknown. Astrocytes actively play an important role in neurological function requiring high amounts of available energy. These functions include maintenance of the brain microenvironment, such as regulation of excitatory neurotransmitters and ion homeostasis, as well as blood brain barrier facilitation and regulation of synaptic transmission (Rubin *et al.*, 1991; Shao and McCarthy, 1994; Chen and Swanson, 2003). Loss of available ATP pools through inhibition of essential metabolic enzymes would greatly compromise the ability of

astrocytes to maintain proper function and would result in neuropathology. The present study was undertaken to investigate the potential for 1,3-dinitrobenzene or its reductive metabolite 3-NNB to interfere with the activity of the key metabolic super complexes, PDHc or KGDHc.

1,3-DNB and the reductive metabolite, 3-NNB, both showed dose-dependent inhibition of PDHc activity with 3-NNB being greater than 5-fold more potent (Figure 2-4a). This disparity in effectiveness between 3-NNB and 1,3-DNB has also been observed in the ability of these compounds to elicit toxic outcomes in Sertoli cells, the primary target in 1,3-DNB-induced testicular toxicity (Cave and Foster, 1990). These studies suggest 1,3-DNB can be bio-activated through nitroreduction to 3-NNB and become a more cytotoxic species. Various nitroso-aromatic compounds can act as substrates for PDHc, as well as other  $\alpha$ -ketoacid dehydrogenase complexes, resulting in the production of the proximate carcinogens, N-hydroxy-N-arylamides (Yoshioka and Uematsu, 1993). The first step in normal catalytic function of PDHc is the E1-catalyzed decarboxylation of pyruvate with the formation of 2-(1-hydroxyethylidene)-TPP. This is followed by reductive acetylation of E2 bound lipoyl moieties (Jordan, 2003). It is thought that nitroso aromatic compounds replace the endogenous transacetylation site and are reductively acetylated in its place to form the corresponding N-arylhydroxamic acids. Due to this interaction with nitroso-aromatic compounds, pyruvate-derived acetyl-groups are diverted from their intended recipient, CoA, resulting in depletion of acetyl-CoA. This loss of the endogenous product of PDHc would cripple the cell's ability to produce ATP and would result in cellular demise. Metabolism of nitrosoaromatics to their respective N-hydroxy-N-arylamides has been shown to occur in numerous isolated



cells and perfused organs and is believed to occur in virtually all mammalian tissues (Yoshioka *et al.*, 1996). During this metabolism of nitrosoaromatics by PDHc, it was observed that bulky para-substituents on the aromatic ring reduced the enzymatic efficiency of the reaction. This reduction in efficiency was attributed to steric limitation of the E1 component active site (Yoshioka and Uematsu, 1993). Interestingly, examination of the three isomers of dinitrobenzene shows similar effects with respect to DNB inhibition of PDHc. The para-substituted DNB showed significantly lower ability to inhibit the complex, while both the ortho- and meta- isomers show comparable inhibition. The parallels between loss of catalytic efficiency and loss of inhibitory potency with para-substituted compounds support a common site of action. This is further reinforced by substrate activation requirements. Pre-activation of the complex by both, TPP and pyruvate, is required for substantial inactivation of PDHc by 1,3-DNB. This substrate requirement points to the involvement of the hydroxyethyl-TPP intermediate as key to the inhibition by 1,3-DNB. This intermediate is in equilibrium with the enamine or 2<sup>nd</sup> carbanion, which normally undergoes nucleophilic attack of the disulfide bond of E2 bound lipoic acid. In the production of N-hydroxy-N-arylacetamides the nitrosoaromatic, a strong electrophile, takes the place of LA. The ability of 3-NNB to inhibit the E1 component activity of PDHc supports the potential for direct nucleophilic attack by the carbanion. Conversely, the lowered capacity of 1,3-DNB to affect E1 activity again supports the need for bio-activation in order to disrupt PDHc activity.

In contrast to PDHc, 1,3-DNB does not significantly affect KGDHc activity, though 3-NNB demonstrated comparable inhibitory potency towards both complexes.

This suggests that there is potentially a bio-activation of 1,3-DNB with respect to PDHc, which is not present in KGDHc. The two enzyme complexes are very similar and accordingly would be expected to respond equally to the compounds. Examining the two complexes lends some insight into a potential explanation for this unexpected discrepancy. Both KGDHc and PDHc are made up of 3 catalytic components and 2 regulatory ones. In addition to these, PDHc also has an additional structural component, designated as the E3 binding protein (E3BP). E3BP is responsible for binding the E3 component to the E2 structural core and is essential for the catalytic activity of the overall complex. Its value is demonstrated by an 80 to 90 percent reduction in PDHc activity in those individuals who genetically lack E3BP (Aral *et al.*, 1997). The E3BP, like its E2 counterpart, possesses a subunit binding domain, a C-terminal domain and an N-terminal lipoyl domain with a covalently bound lipoic acid. In contrast, the C-terminal domain of E3BP lacks the conserved active site histidine essential for acetyltransferase activity (Harris *et al.*, 1997). Recent architectural studies on PDHc proposed that the E3BP and the E3 component form sub-complexes at a ratio of 2:1, respectively (Smolle *et al.*, 2006). This formation allows for cross bridging and also imparts a certain degree of restriction in the movement of the E2 and E3BP lipoyl-lysine flexible arms. Originally it was thought that the flexible lipoyl lysine arms of E2 and E3BP were able to visit all three active sites of the individual catalytic components. The new ultra-structural model suggests that the E2-lipoyl domains interact exclusively, or at least preferentially, with the E1 component while the E3BP-lipoyl domain interacts with the E3 component. If this model were correct, the electron flow from reduced lipoamide on the E2 to NAD<sup>+</sup> would have to go through the E3BP-lipoamide, supporting more than just a structural role

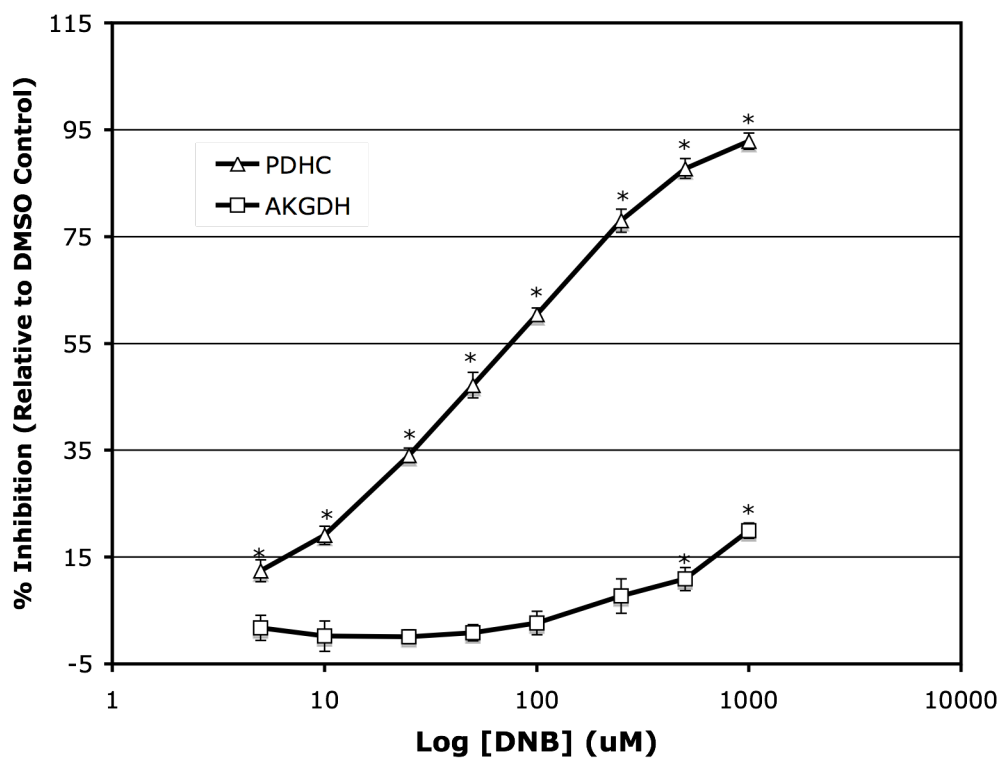
for this component. Lipoamide dehydrogenase, the E3 component of the complex, has been shown to reductively metabolize nitro-aromatic compounds to their corresponding reductive metabolites including the reactive nitroso intermediate (Tsai, 1987). The highly ordered arrangement of the catalytic components in the complex allows for efficient substrate channeling of reaction intermediates between the three active sites without release into the bulk media (Reed, 1974). It is conceivable that E3-mediated reductive metabolism, of the parent nitro-aromatic compound, would produce highly reactive intermediates including 3-NNB with direct access to the E3BP lipoic acid moiety and subsequently E2 and E1 active sites.

Lipoic acid, an essential coenzyme of the E2 component of PDC, consists of two vicinal sulfhydryl groups that are highly susceptible to oxidative modification. 3-NNB is extremely reactive towards sulfhydryls and interaction with the E2 bound lipoamide would directly position it at the transacylation site, where by, it could accept the acetyl group from the 2-(1-hydroxyethylidene)-TPP with subsequent formation of the N-aryhydroxamic acid. Inhibition of PDHc-mediated DTNB reduction by 3-NNB further supports the interaction of this reactive intermediate with the sulfhydryls of LA. DTNB was shown to bind reduced sulfhydryls of lipoic acid within the E2 component of PDHc and is subsequently cleaved by lipoamide dehydrogenase (E3) (Erfle and Sauer, 1968). This reaction was blocked by addition of N-ethylmaleimide, known to bind sulfhydryls (Brown and Perham, 1976). Incubation of 3-NNB with PDHc in the presence of NADH resulted in loss of DTNB reduction suggesting that 3-NNB interferes with binding of DTNB potentially by adducting lipoic acid sites. Further investigation into the ability of 3-NNB to bind to the sulfhydryl groups on the E2-bound lipoyl moieties are required.

If E3 in fact does metabolize DNB to the nitroso- with subsequent adduction to lipoamide on the E3BP, the E3BP protein could potentially facilitate transfer of the nitroso-compound to the E2 lipoamide and consequently result is disruption of transacetylation. The absence of E3BP in KGDHc may explain the greatly diminished impairment of this specific complex by 1,3-DNB. Additionally, modification of E3BP-lipoamide alone could disrupt transacetylation through interruption of lipoamide re-oxidation, which is required for the acceptance of subsequent acetyl groups. The involvement of E3BP in the mechanism of DNB-mediated inhibition of PDC is currently under investigation and potentially may shed some light on E3BP's role in overall complex activity.

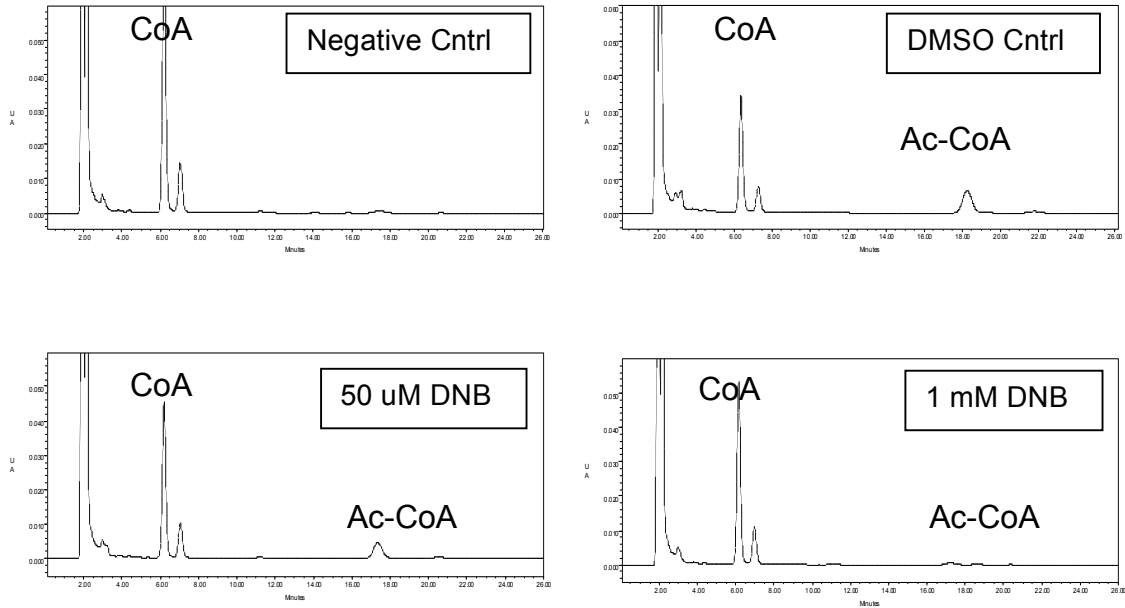
In conclusion, the present study establishes PDHc as a potential target of direct inhibition by 1,3-DNB and its highly reactive reductive intermediate, 3-NNB. The inhibition occurs through a mixed type mechanism and appears to require substrate activation prior to inhibition. The ability of 3-NNB to potently inhibit E1 component activity suggests that direct attack of the strongly electrophilic nitroso- group by the nucleophilic carbanion of 2-(1-hydroxyethylidene)-TPP may underlie its potent inhibition of the complex but does not rule out the potential of this thiol reactive compound from adducting the reduced sulfhydryls of E2 bound lipoic acid. The relatively lower capacity of 1,3-DNB to inhibit the E1 component suggests an alternative mechanism of inhibition and points to the potential need for bio-activation for inhibition. The differential effects observed between PDHc and KGDHc with respect to inhibition by 1,3-DNB further supports the need for bio-activation. Though, further studies into the direct mechanism of inhibition are required, it is apparent that these compounds have the potential to affect

the activity of these essential metabolic enzyme complexes and would ultimately result in metabolic dysfunction and subsequent neuropathology.

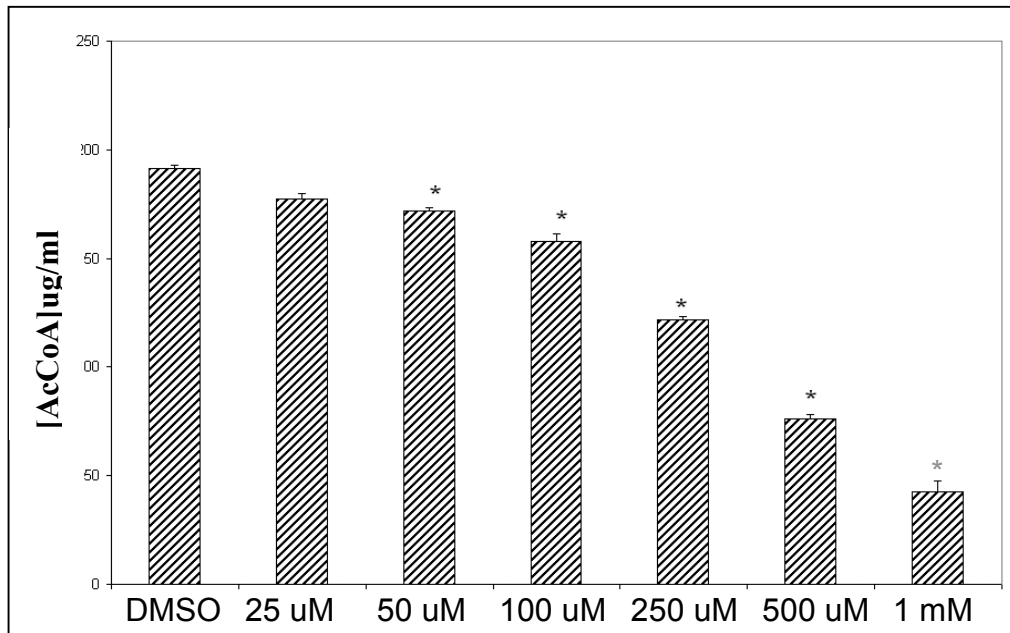


**Figure 2-1.** Differential effects of 1,3-DNB on the enzymatic activities of purified porcine heart 2-oxoacid dehydrogenases. Data expressed as percent inhibition (initial velocities) relative to vehicle control (n=6). \* denotes significant difference from control (P<0.01)

A) Representative chromatograms



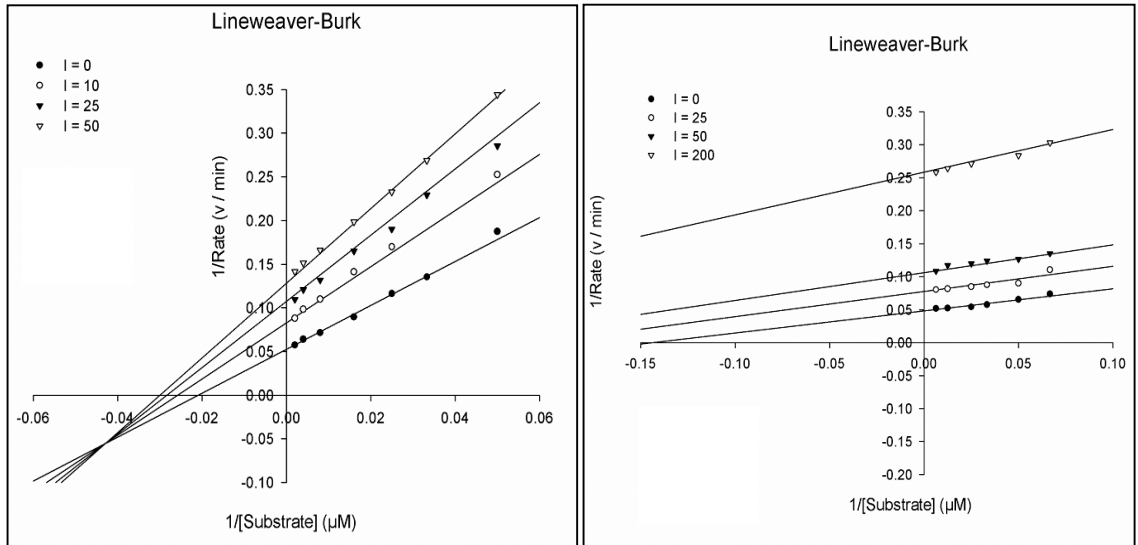
B) Graphical representation



**Figure 2-2.** Effects of 1,3-DNB of the production of Acetyl-CoA by porcine heart pyruvate dehydrogenase complex. A) Representative chromatograms of reaction supernatant after 60 min. B) Graphical representation of acetyl-CoA concentrations determined by reverse phase HPLC and expressed as  $\mu\text{g/ml}$  ( $n=6$ ). \* denotes significant difference from vehicle control ( $P < 0.01$ ).

A) Pyruvate

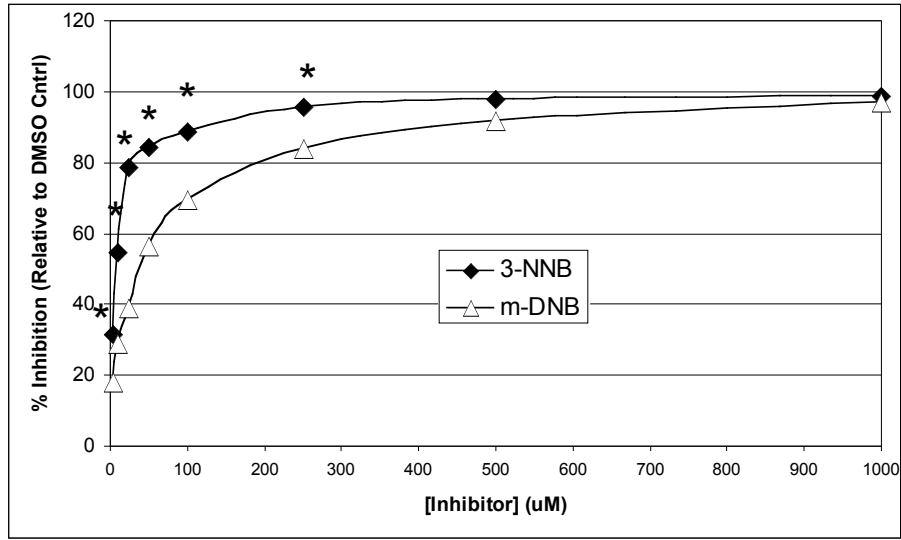
B) CoA



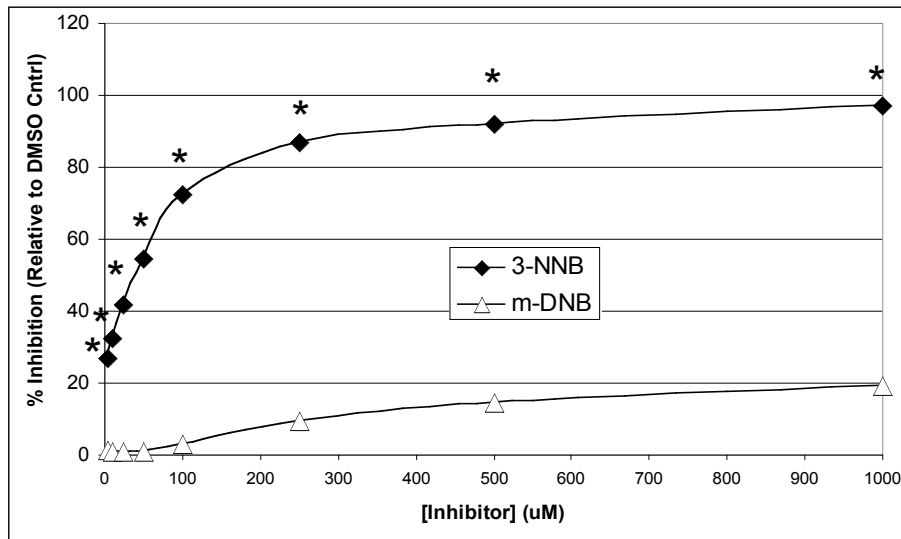
**Figure 2-3.** Double reciprocal plots of pyruvate dehydrogenase complex incubated with 1,3-DNB while varying substrate concentrations A) Pyruvate B) CoA. Employing Sigmaplot® with the enzyme kinetics module (Systat Inc.) the data was analyzed with non-linear regression and the results were fit to equations which describe the four types of inhibition. The predicted velocity values in the presence of inhibitor were fit to the different models of inhibition and goodness of fit was determined by the calculated sum of squares.



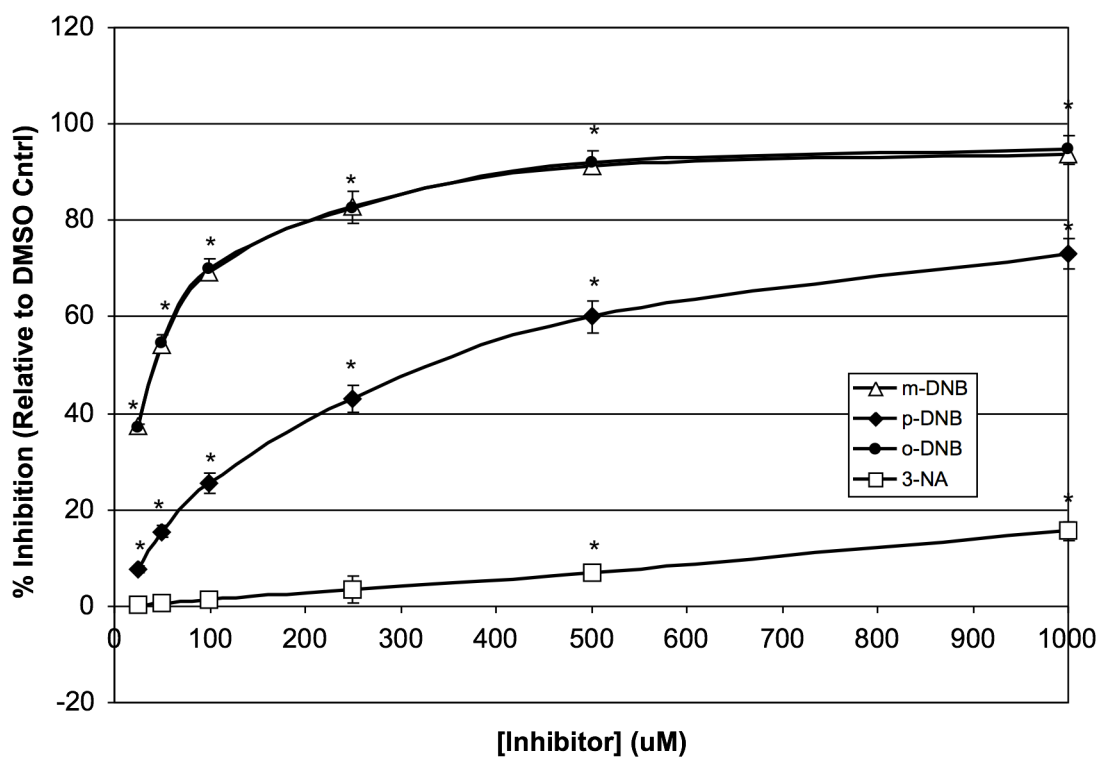
A) Pyruvate dehydrogenase complex



B)  $\alpha$ -Ketoglutarate dehydrogenase complex

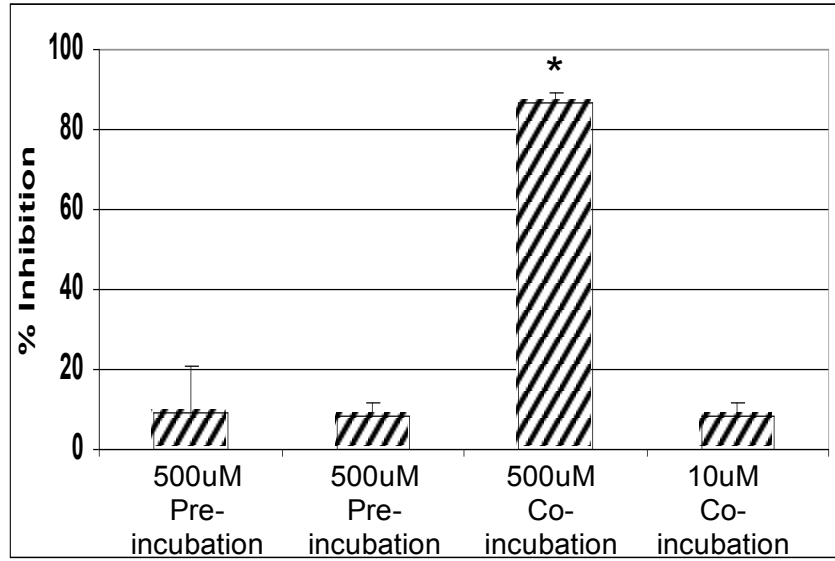


**Figure 2-4.** Comparison between the inhibitory effects of 1,3-dinitrobenzene and 3-nitrosonitrobenzene on the enzymatic activities of purified porcine heart 2-oxoacid dehydrogenases. Data expressed as percent inhibition (initial velocities) relative to vehicle control (n=6). \* denotes significant difference from m-DNB (P<0.01).

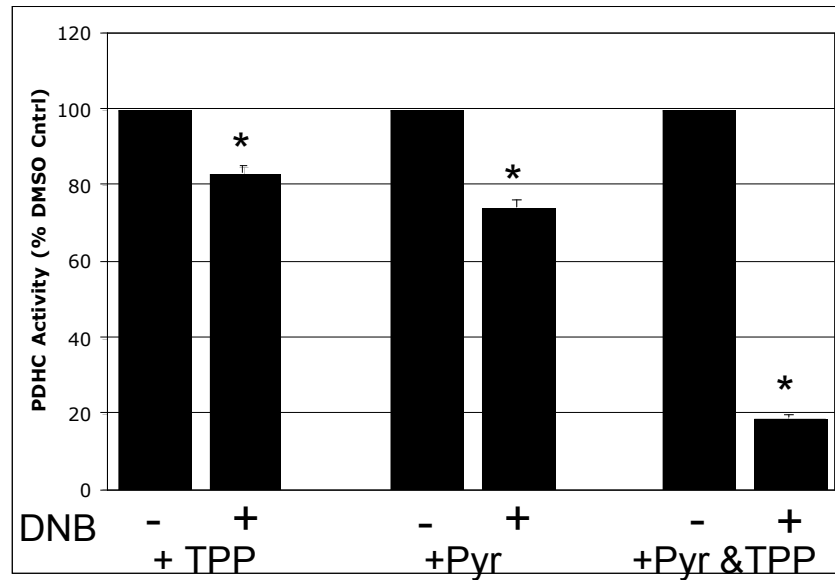


**Figure 2-5.** Comparison of the three isomers (meta, para, ortho) of DNB and the final reductive metabolite (3-NA) on the activity of porcine heart pyruvate dehydrogenase complex. Data expressed as percent inhibition (initial velocities) relative to vehicle control (n=6). \* denotes significant difference from m-DNB (P<0.01)

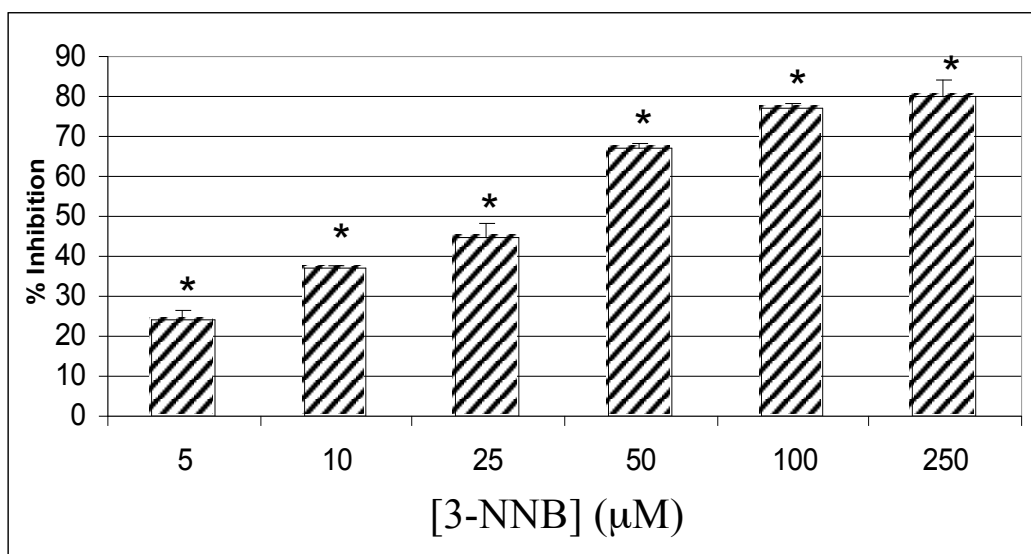
A) Pre-incubation versus Co-incubation



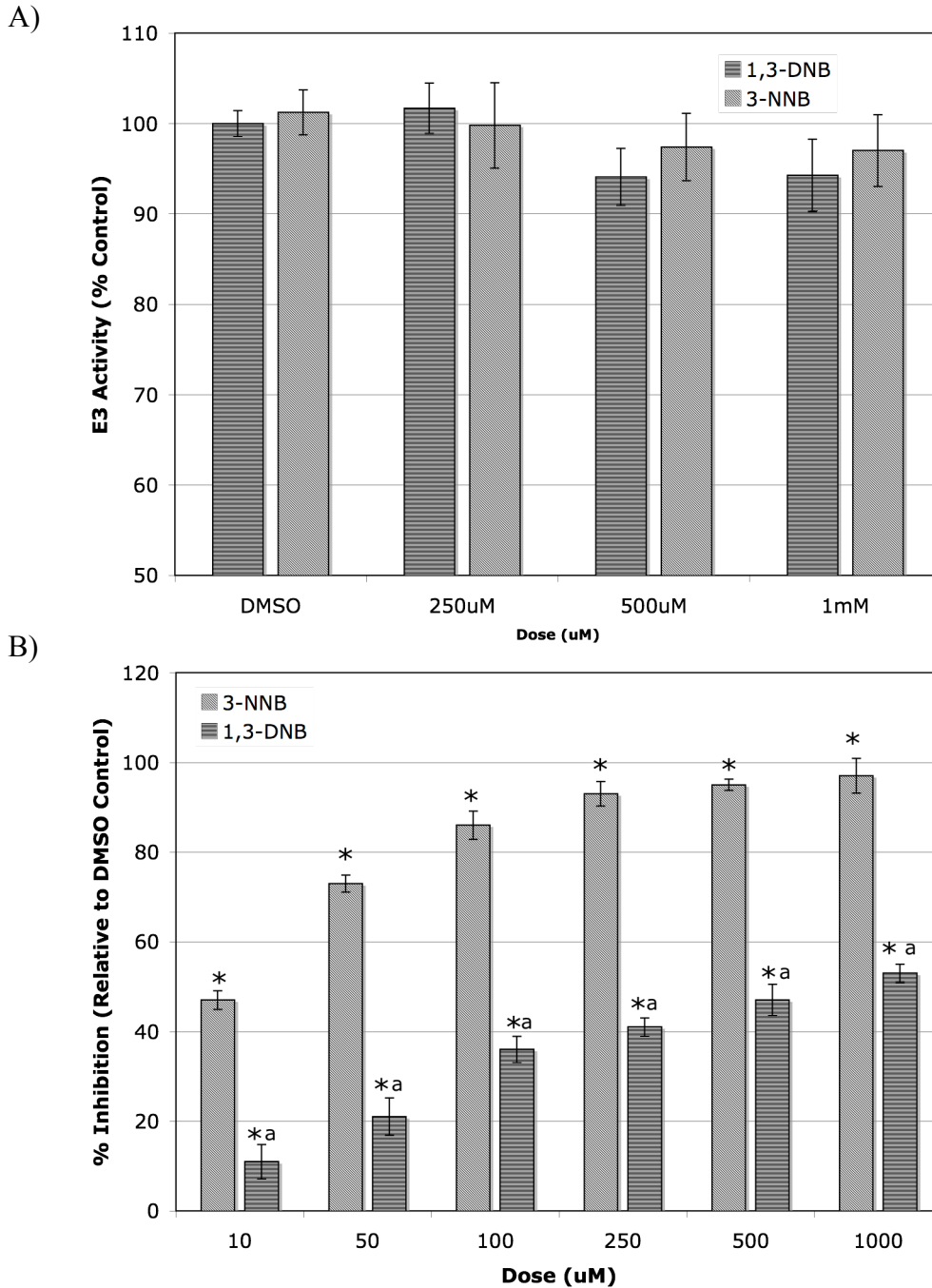
B) Substrate pre-activation requirements



**Figure 2-6.** (a) Pre-incubation versus co-incubation of varying concentrations of 1,3-DNB. Data expressed as % inhibition +/- SE, relative to vehicle control (n=4). (b) Substrate pre-activation requirements for inactivation by 1,3-DNB. Data expressed as % PDHC activity (initial velocity) relative to vehicle control +/- SE (n=4). \* denotes significant difference from control (P<0.01).



**Figure 2-7.** Effects of 3-NNB on porcine heart pyruvate dehydrogenase complex mediated reduction of dithio-bis-nitrobenzoic acid. Data expressed as percent inhibition  $\pm$  SE relative to vehicle control (n=4). (\*) denotes significant difference from DMSO controls ( $P < 0.01$ ) (n=6).



**Figure 2-8.** Effects of 1,3-DNB and 3-NNB on the E1 and E3 component enzymes of PDHc. (A) Both 1,3-DNB and 3-NNB show no significant effects on E3 activity. Data expressed as mean percent control  $\pm$  SE (n=6). (B) 3-NNB and 1,3-DNB show significant inhibition of the E1 component with 3-NNB being more potent.  $IC_{50}$  for 3-NNB and 1,3-DNB are approximately 21  $\mu$ M and 762  $\mu$ M, respectively. Data expressed as mean percent inhibition relative to DMSO control. (\*) denotes significant difference from DMSO control ( $P < 0.01$ ) (n=6). (a) denotes significant difference between 1,3-DNB and 3-NNB ( $P < 0.01$ ) (n=6).

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## **CHAPTER 3**

### **1,3-DINITROBENZENE INDUCES METABOLIC IMPAIRMENT IN C6 GLIOMAS THROUGH INHIBITION OF THE PYRUVATE DEHYDROGENASE COMPLEX**

#### **ABSTRACT**

Prolonged exposure to the chemical intermediate, 1,3-dinitrobenzene (1,3-DNB), produces neuropathology in the CNS of rodents analogous to those observed in various conditions of acute energy deprivation. Though the specific site of impairment is unknown, the metabolic status of astroglia, in susceptible regions, is compromised. The effects of 1,3-DNB on metabolic status and inhibition of pyruvate dehydrogenase complex were evaluated using rat C6 glioma cells in culture. Exposure of C6 gliomas to 1,3-DNB resulted in altered morphology and biochemical dysfunction consistent with disruption of oxidative energy metabolism. Both PDHc and KGDHc were affected by 1,3-DNB in a concentration-dependent manner, with PDHc being more sensitive to inhibition. Loss of lipoic acid (LA) immunoreactivity associated with protein carbonyl formation was a hallmark feature of in vitro exposure to 1,3-DNB. Addition of antioxidants and thiol containing compounds failed to attenuate the loss of LA immunoreactivity. These data support the hypothesis that 1,3-DNB impaired oxidative metabolism by the specific inhibition of the pyruvate dehydrogenase complex and that this impairment is due to perturbations in the function of protein-bound lipoic acid. The

inability of antioxidants to affect the loss of LA by 1,3-DNB points to the direct oxidative modification of PDHc by 1,3-DNB or one of its reactive intermediates, and presumably not to increased production of reactive oxygen species and subsequent protein oxidation.

### **Introduction**

Acute energy deprivation syndromes (AEDS; Cavanagh, 1988) are typified by gliovascular lesions in the auditory and vestibular regions of the brainstem. The regional distribution of lesions are similar to that produced by a broad spectrum of mutations, deletions and mis-arrangements of both mitochondrial and nuclear DNA encoding components of the mitochondrial respiratory chain and pyruvate metabolism (Brown and Squier, 1996). Consequently, perturbations in energy metabolism produce early CNS damage in conserved regions of the brainstem and result in human diseases such as Leigh's necrotizing encephalopathy. Rodent models of this mitochondrial disease are produced by a wide variety of metabolic disruptors including dietary induced vitamin B1 deficiency, anti-metabolites such as pyrithiamine and 6-aminonicotinamide, and chemical intoxication by nitrobenzenes, nitrotoluenes, nitroimidazoles and deoxy-chlorosugars (Cavanagh, 1993). Though topographic distribution of the lesion varies depending on the chemical and the animal species afflicted, auditory and vestibular centers in the brainstem and deep cerebellum are frequent targets, which is consistent with their high metabolic demand.

Acute energy deprivation syndromes are characterized by bilaterally symmetrical spongiform lesions, that involve swelling of astrocytes, followed by retraction of perivascular foot processes, and extensive cytoplasmic vacuolation (Philbert *et al.*, 1987).

The underlying cellular mechanisms responsible for the regional and cell-specific sensitivity in these conditions are not well understood, however, molecular targets have been identified. For example, 6-aminonicotinamide is a potent inhibitor of 6-phosphogluconate dehydrogenase, essential for the pentose phosphate pathway (Haghighat and McCandless, 1997). Similarly,  $\alpha$ -chlorohydrin disrupts the metabolism of sugars through the inhibition of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (Brown-Woodman *et al.*, 1978). In Leigh's disease, three specific gene products are affected due to mutation of both mitochondrial and nuclear DNA resulting in deficiencies in complex I, II and IV of the electron transport chain as well as the pyruvate dehydrogenase complex (PDHc) (Leigh, 1951; Rahman *et al.*, 1996). Acute thiamine deficiency greatly diminishes the activities of two key metabolic enzymes, PDHc and the  $\alpha$ -ketoglutarate dehydrogenase complex (KGDHc) both of which require thiamine as a cofactor. In the case of the nitroaromatics, mono- and di-nitrobenzene, the specific biochemical pathway that is affected is not completely known. Nevertheless, the regional distribution and severity of the lesions produced by nitroaromatic chemicals most closely resemble that of thiamine deficiency, suggesting that these AEDS may have similar defects in energy metabolism mediated by thiamine dependent enzymes.

Thiamine, a precursor to the active coenzyme thiamine pyrophosphate, is a required cofactor for a family of multi-component complexes termed the  $\alpha$ -keto acid dehydrogenase complexes, which includes PDHc, KGDHc and the branched chain  $\alpha$ -ketoacid dehydrogenase complex. PDHc and KGDHc occupy critical sites in energy metabolism. PDHc is responsible for linking glycolysis to the TCA cycle through irreversible oxidative decarboxylation of pyruvate and subsequent formation of acetyl

coenzyme A (Patel and Roche, 1990). KGDHc, being equally important, catalyzes a key step in the TCA cycle and is also responsible for linking the TCA cycle to glutamate metabolism with the help of glutamate dehydrogenase. The complexes are made up of multiple copies of three main catalytic enzymes and also contain two regulatory enzymes. The catalytic core of PDHc consists of the pyruvate dehydrogenase (E1) component, which is responsible for the rate limiting reaction of decarboxylation with the use of thiamine pyrophosphate. The second catalytic component, dihydrolipoyltransacetylase (E2) catalyzes the transfer of pyruvate derived acetyl groups to coenzyme A. A lipoic acid bound to each of the two lipoyl domains of E2, through a flexible lysine chain linkage, facilitates this transfer. Lastly, dihydrolipoyl dehydrogenase (E3) is charged with the re-oxidation of reduced lipoic acid of E2 with concomitant production of NADH (Yeaman, 1989; Reed, 1998). In addition to the catalytic components, PDHc contains a structural component designated as the E3 binding protein (E3BP), which is responsible for binding the E3 to the E2 core. This protein also contains a lipoyl domain with a bound lipoic acid though the function of this lipoic acid is not entirely understood since E3BP does not possess transacetylase activity (Harris et al., 1997). PDHc activity is tightly controlled through a phosphorylation / dephosphorylation cycle that is mediated by pyruvate dehydrogenase kinase (PDHK) and pyruvate dehydrogenase phosphatase (PDHP) bound to the lipoyl domains of the E2 component (Harris *et al.*, 1997). The structural complexity of this enzyme complex matched with its strict cofactor requirements and tight metabolic regulation make it an attractive target during many pathological conditions.

Chemical modification of protein bound lipoic acid is considered a primary mechanism for inhibition of PDHc. Classically, this is demonstrated by arsenical compounds, which have a high affinity for vicinal dithiols resulting in the formation of stable dithioarsinates (Samikkannu *et al.*, 2003). Additionally, various strong electrophiles such as the ubiquinone analog, coenzyme Q<sub>0</sub>, and the reactive lipid peroxidation products, 4-hydroxynonenal and acrolein, have shown a similar mechanism of action of forming stable adducts on lipoic acid (Humphries and Szveda, 1998; Pocernich and Butterfield, 2003; MacDonald *et al.*, 2004). Direct inactivation of PDHc by reactive oxygen species has also been reported (Tabatabaie *et al.*, 1996).

1,3-DNB undergoes nitroreduction to the final product 3-nitroaniline (Cossum and Rickert, 1985; Nystrom and Rickert, 1987). In the proposed mechanism, the reactive intermediate nitroxyl anion radical is formed, which can potentially react with molecular oxygen to produce the reactive oxygen species superoxide with regeneration of the parent compound (Mason and Josephy, 1985). The potential of nitroaromatic compounds to undergo futile redox cycling resulting in the increased production of superoxide suggests that like other neurological disorders, ROS production and subsequent oxidative damage of proteins may underlie the metabolic disruption induced by these compounds. Furthermore, during nitroreduction of 1,3-DNB, highly reactive electrophilic intermediates can also be produced offering an additional route to protein modification and subsequent functional disruption.

The present study was undertaken to determine if 1,3-dinitrobenzene mediated metabolic impairment is associated with loss of PDHc activity and if so, what mechanisms underlie the disruption of this key metabolic enzyme.

## **Materials**

Cell culture media and supplements were obtained from Invitrogen. BCA protein quantification reagents and bovine serum albumin (BSA) standards were obtained from Pierce. Precision plus molecular weight standards were purchased from Bio-Rad. PVDF membrane was purchased from Milli-Pore. Rabbit anti-lipoic acid antibody was obtained from Calbiochem. Chicken anti-E2 subunit of PDHc and goat anti-chicken-alkaline phosphatase antibodies were obtained from Abcam. All other reagents were purchased from Sigma unless otherwise stated.

## **Methods**

### **Cell Culture.**

Rat C6 glioma cells, obtained from the American Type Culture Collection, were maintained at 37°C under an atmosphere of 5% CO<sub>2</sub> / 95% air (v/v) in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum and 1x antibiotics (Penicillin, Streptomycin, and Neomycin). Cells were subcultured using 0.25% trypsin and used 48hrs after plating. Prior to treatment, cells were rinsed with Dulbecco's phosphate buffered saline (D-PBS) and all treatments were done in serum free DMEM.

### **Microscopy**

C6 glioma cells plated in 6 well plates were dosed with 1,3-DNB (250 μM, 500 μM and 1 mM) for 36 hr. Plates were then washed twice with D-PBS and phase-contrast light

microscopy was performed using an Olympus CKX41 inverted microscope equipped with a Spot Insight QE digital CCD camera.

## **Cytotoxicity**

### *Mitochondrial reducing potential*

Cytotoxic effects of 1,3-DNB on C6 glioma cells were determined by monitoring the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) as described (Mosmann, 1983). The yellow, soluble tetrazolium dye is metabolically reduced to a purple, water-insoluble formazan, which is detected by a spectrophotometer. Cells plated in a 96 well microtiter plate were dosed with increasing concentrations of 1,3-DNB. After exposure, wells were rinsed with D-PBS and MTT assay solution (1 mg/ml in DMEM) was added to each well and allowed to incubate for 2 h at 37°C. MTT solution was then aspirated and 100 µl of DMSO was added to dissolve the formazan precipitant. After 30 min incubation with vigorous mixing, the absorbance was determined at 550 nm. Mean absorbance values were compared to vehicle control and IC<sub>50</sub> values were determined by fitting data to a sigmoidal dose-response equation using Prism software (Graph Pad Software, Inc., San Diego, Ca).

### *LDH release*

Cell death was measured by monitoring the release of lactate dehydrogenase into the culture medium. Prior to exposure, cells in 24 well plates were rinsed with D-PBS and glucose free or glucose containing media was added to the cells for 1 hr. The cells were then incubated with varying concentrations of 1,3-DNB. After 24 hr exposure to 1,3-DNB in the presence or absence of glucose (4500 mg/l), culture medium was removed

and placed in microcentrifuge tubes. Samples were clarified by centrifugation (2000 x g, for 5 min at 4°C). Lactate dehydrogenase concentrations were determined using a COBAS FARA II centrifugal analyzer (Roche diagnostics, Indianapolis, IN) employing a commercial reagent (LD-L) from Sigma Diagnostics as described in provided manual.

### **Determination of Cellular ATP Content**

ATP levels were determined using an isocratic reverse-phase high-pressure liquid chromatography method described previously with slight modifications (Yang *et al.*, 2004). Briefly, cells plated in 10 cm tissue culture plates were exposed to 1 mM 1,3-DNB alone, or in the presence of 1 mM acetyl-carnitine (ALCAR) or 1 mM acetoacetate (AcAc). At the following time points (6, 12, 24, and 36hr), plates were rinsed in D-PBS and immediately snap frozen in liquid N<sub>2</sub>. ATP was acid-extracted with ice-cold perchloric acid (0.3M) containing EDTA (1 mM). The plates were scraped, acid extracts were transferred to microcentrifuge tubes, and protein was pelleted by centrifugation at 9000 x g for 5 min at 4 °C. Protein pellets were saved for protein quantification. Protein free extracts were neutralized with KOH and precipitated KClO<sub>4</sub> was removed by centrifugation (9000 x g, 4°C). Extracts were stored at -80°C until HPLC analysis was performed.

### **ATP Quantification by High Pressure Liquid Chromatography (HPLC)**

Adenine nucleotides were separated by HPLC using a Waters HPLC system equipped with a dual  $\lambda$ -absorbance UV detector and an inline degasser. Isocratic elution was performed on a Symmetry 300™ C-18 column (4.6 x 150 mm, 5  $\mu$ m) at a flow rate of 0.6



ml/min and peak absorbance recorded at 206 nm. The mobile phase consisted of 0.1 M ammonium dihydrogen phosphate with 1% methanol. ATP concentrations were calculated using peak height, which was determined to be directly proportional to ATP concentration when compared to ATP standards treated in the same manner as samples.

### **Measurement of Extracellular Lactate Concentrations**

Extracellular lactate accumulation was determined by the lactate dehydrogenase method through monitoring the conversion of NAD to NADH at 340 nm as previously detailed with slight modification (Yang and Balcarcel, 2004). Lactate assay reagent contained 0.2 M glycine buffer (pH 9.2), 0.15 M hydrazine, and 10 mM NAD. The reaction was started with the addition of lactate dehydrogenase (10 U) and the rate of conversion was monitored. The rate of conversion is directly proportional to lactate concentration, which was determined using lactate standards.

### **Cytochemical Determination of PDHc and KGDHc Activity.**

PDHc and KGDHc activity were determined by the quantitative cytochemical method as described by (Park *et al.*, 2000), with slight modifications. Briefly, cells treated in 6 well plates were rinsed with D-PBS twice and 1 ml of reaction buffer containing 50mM Tris-HCl (pH 7.6), 1 mM MgCl<sub>2</sub>, 0.1mM CaCl<sub>2</sub>, 0.05 mM EDTA, 0.3 mM thiamine pyrophosphate, 0.5 µg/ml rotenone (dissolved in 100% ethanol; final ethanol concentration, 0.1%), 0.2% Triton X-100, 3.5% polyvinyl alcohol, 3mM pyruvate or  $\alpha$ -ketoglutarate, 3 mM NAD<sup>+</sup>, 0.75 mg/ml coenzyme A, 0.75 mM nitroblue tetrazolium (NBT), and 0.05 mM phenazine methosulfate (PMS) was added to the cells. Both NBT

and PMS were added immediately before the reaction was initiated. The reaction was allowed to proceed for 1 hr and stopped by washing the plates twice with D-PBS. Solubilization of reduced NBT was accomplished by the method previously described (Rook *et al.*, 1985). Unreduced NBT was rinsed away with methanol twice and allowed to air dry. To the dried plates, 240  $\mu$ l of 2 M KOH was added followed by the addition of 280  $\mu$ l of DMSO. The contents of each well were mixed thoroughly by trituration and 200  $\mu$ l of solution from each well was transferred to a corresponding well of microtiter plate. Absorbance was read at 630 nm. Wells treated with reaction mixture minus pyruvate or  $\alpha$ -ketoglutarate were used as blanks to account for non-specific reduction of NBT.

### **SDS-PAGE and Western Blotting**

Total cellular protein was extracted with ice-cold cell lysis buffer consisting of Tris-HCl (50 mM, pH 7.4), NaCl (150mM), Triton X-100 (1% v/v), and protease and phosphatase inhibitor cocktails (0.1% v/v) (Sigma). Cell lysates were snap frozen in liquid N<sub>2</sub> and stored at -80 °C until protein quantification and SDS-PAGE. Cellular debris was removed by centrifugation and protein concentration was determined by the Bicinchoninic acid method (Smith *et al.*, 1985) using a commercial kit (Pierce) as described by the manufacturer. Approximately 20  $\mu$ g of protein was mixed with sample loading buffer containing  $\beta$ -mercaptoethanol and denatured at 100°C for 10 min. The proteins and molecular weight standards were resolved on 4-20% Tris-glycine gels (Lonza) and electroblotted to PVDF membrane. Membranes were blocked with non-fat milk (5% w/v) in Tris-buffered saline (pH 7.6) containing Tween-20 (1% v/v) (TBS-T).

After 30 min of blocking the membranes were probed with anti-lipoic acid (1:5000), and anti-E2 subunit of PDHc (1:2000) overnight at 4°C. The membranes were washed with TBS-T followed by probing with an appropriate alkaline phosphatase conjugated secondary antibodies (1:10000). The membranes were developed using an ECF detection system (Amersham Biosciences, Piscataway, NJ) and visualized on a FujiFilm laser imaging system.

### **Oxidized Protein Detection with OXYBLOT™**

Oxidative modification of proteins can result in carbonylation of amino acid side chains. Carbonylated proteins can be distinguished by derivatizing these carbonyls with 2,4-dinitrophenyl hydrazine (DNPH) and subsequent immunodetection with an antibody specific to the attached DNP moiety (Dalle-Donne *et al.*, 2003). Modified proteins were detected utilizing a commercial OXYBLOT™ kit (Chemicon, Temecula, CA) as detailed in the kit manual with slight modifications.

### *Mitochondrial Protein Isolation*

Mitochondria protein was isolated from treated C6 glioma cells plated in T-75 tissue culture flasks, following the commercial protocol MITOISO1 from Sigma (St. Louis, MO) with slight modification. Briefly, cells from 8 T-75 flasks were homogenized in 10 volumes of BSA (2mg/ml) containing extraction buffer with a dounce glass homogenizer and Teflon pestle. Extraction buffer consisted of 220 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, and 2 mM HEPES at a final pH of 7.4. Intact mitochondria were pelleted by centrifugation (11,000g, 4°C, 10 min). The pellet was then resuspended in extraction buffer and centrifuged again as before. This step was repeated a second time. The final

mitochondrial pellet was then resuspended in storage buffer consisting of 10 mM, HEPES, 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM  $K_2HPO_4$ , and 1mM DTT and protease inhibitor cocktail (Sigma, St. Louis, MO). Mitochondrial were lysed by repeated freeze thaw cycles and total mitochondrial protein was quantified by BCA as described earlier.

#### *Oxyblot Analysis*

Mitochondrial protein (20  $\mu$ g) was denatured with 12% SDS and derivatized with 1X solution of dinitrophenylhydrazine. The derivatized samples were separated by gel electrophoresis and electroblotted to PVDF membrane as described earlier. The membranes were probed with anti-DNP (1:150) overnight at 4°C. The membrane was then washed and probed with appropriate alkaline phosphatase conjugated secondary antibody (1:10000) (Amersham Biosciences) and they were developed and visualized as described earlier.

#### **Statistical Analysis**

LDH release, extracellular lactate concentrations, ATP concentrations, and enzyme activity levels in control and treated cells were compared using ANOVA with Student t-test for post hoc analysis (Prism version 2.0, Graph Pad Software, INC., San Diego, CA). Data is presented as mean +/- S.E. for a minimum of 4-6 independent experiments.

## Results

### *Morphology and Cytotoxicity*

Changes in the morphology of C6 glioma cells were dose-dependent and included extensive and increasing vacuolation with accompanying nuclear and cytoplasmic swelling at 36 hrs of exposure (Fig. 3-1). No morphological changes were observed in control cultures treated with DMSO. Higher concentrations of 500  $\mu$ M and 1 mM DNB produced significant cell shrinkage and debris suggesting cell death. This was confirmed by measuring release of the cytosolic marker LDH. Significant dose dependent increases in extracellular LDH occurred at both 500  $\mu$ M and 1 mM ( $P < 0.01$ ) (Figure 3-2). Removal of glucose through dosing in glucose free media resulted in exacerbation of cell death at both high doses. Additionally, The cytotoxic effects of 1,3-DNB were determined using the MTT reduction assay (Figure 3-3). Dose-dependent inhibition of mitochondrial reducing potential occurred upon treatment of C6 gliomas with 1,3-DNB (10  $\mu$ M – 2 mM) for 36 hours, with a calculated  $IC_{50} = 978 \mu$ M.

### *Metabolic Status.*

Significant accumulation of extracellular lactate was observed at 36 hours of exposure, beginning at the lowest dose employed of 100  $\mu$ M 1,3-DNB and increased in a dose dependent manner with nearly a 2-fold increase at the highest concentration of 1 mM (Figure 3-4). Co-treatment of cultures with either acetoacetate or acetyl-carnitine (potential alternative fuel sources) attenuated lactate accumulation in culture medium at all concentrations of 1,3-DNB. By contrast, equimolar levels of the non-acetylated analogue L-carnitine or free acetate did not affect lactate accumulation (Data not shown),

suggesting protective effects were attributable to the availability of the acetyl moiety. In addition to increased extracellular lactate concentrations, cellular ATP levels were significantly diminished by exposure to 1,3-DNB (Figure 3-5). Representative chromatographs of adenine nucleotides clearly show significant reduction in ATP levels with corresponding increases in both ADP and AMP concentrations at 24 hr of exposure to 1 mM 1,3-DNB and drastic depletion by 36 hr compared to vehicle control (Figure 3-5a). Figure 3-5b shows the time-dependent diminution of ATP concentrations, with significant reduction first detected after 12 hr of exposure. Similar to lactate accumulation, co-treatment with both acetoacetate and acetyl-carnitine preserved cellular ATP content.

*PDHc and KGDHc activity.*

Cytochemical determination of PDHc and KGDHc residual activity was achieved through quantifying substrate specific reduction of nitroblue tetrazolium dye. Prolonged exposure to 1,3-DNB (36hr) produced a concentration-dependent reduction in residual PDHc and KGDHc activities (Figure 3-6). At 50 $\mu$ M, the lowest concentration employed, there was greater than 60% reduction in PDHc activity and approximately 95% inhibition at 1 mM compared to vehicle control. KGDHc activity was less sensitive to the inhibitory effects of 1,3-DNB. IC<sub>50</sub> for PDHc and KGDHc were approximately 45  $\mu$ M and 250  $\mu$ M, respectively.

*Lipoic acid immunoreactivity.*

C6 glioma cells were exposed to either 1,3-DNB (50  $\mu$ M – 500  $\mu$ M) for 36 hours or to a constant concentration of 500  $\mu$ M 1,3-DNB from a period of 30 minutes to 48 hours. In order to determine if modification of protein bound lipoic acid results after exposure to 1,3-DNB, an antibody that specifically recognizes unmodified lipoic acid was used. Western blot analysis of total cellular protein, utilizing this lipoic acid antibody, reveals two distinct bands at approximately 70 and 55 kDa corresponding to the E2 components of the PDHc and KGDHc, respectively. Exposure to 1,3-DNB resulted in loss of LA immunoreactivity of PDHc in both a time- and dose-dependent manner (Figure 3-7). At 500  $\mu$ M 1,3-DNB, significant reduction in PDHC-LA by 6 hours and complete loss by 12 hrs was observed. Additionally, substantial reduction in LA immunoreactivity was observed at all concentrations of 1,3-DNB, at 36 hr of exposure with complete loss apparent at the dose of 50  $\mu$ M 1,3-DNB. Utilizing an antibody to the E2 subunit of PDHc, no significant loss in protein expression was observed (Figure 3-8a). 1,3-DNB also affects KGDHc-LA immunoreactivity with significant loss at 6 hrs of exposure. Residual immunoreactivity was observed at all time points following exposure to 1,3-DNB. Loss of KGDHc-LA was not observed at 36 hours less than 100  $\mu$ M 1,3-DNB and residual bands can be seen up through the 500  $\mu$ M dose. Co-treatment with various antioxidants ( $\alpha$ -tocopherol, deferoximine) and thiol containing compounds (L-cysteine, and DMSP) showed no significant attenuation of LA loss at 500  $\mu$ M 1,3-DNB following 6 hrs of exposure (Figure 3-8b). To further rule out the possibility of lipid peroxidation products being the cause for loss of LA immunoreactivity, total cellular protein was probed for 4-hydroxynonenal adducts (Figure 3-9a). No substantial increases

in 4HNE adducts were observed with exposure to increasing concentrations of 1,3-DNB. Interestingly, treatment of C6 glioma cells with the classical PDHc inhibitors, arsenic trioxide and phenyl arsine oxide, at concentrations shown to completely inhibit PDHc did not result in decreased LA immunoreactivity (Figure 3-9b).

#### *Protein Oxidation.*

To determine if loss of LA immunoreactivity is a consequence of increased protein oxidation, OxyBlot analysis of mitochondrial protein samples was performed (Figure 3-10). Exposure to 1 mM DNB for increasing time resulted in increased carbonylation of proteins corresponding to the molecular weights of both the PDHc and KGDHc E2 components with increases apparent by 45 minutes. Protein oxidation of PDHc appears to be greater than that of KGDHc, evident by 24 hrs.

### **Discussion**

Nitroaromatic induced CNS dysfunction is classified as part of a family of neurological disorders termed acute energy deprivation syndromes due to the similarities in lesion presentation as well as regional susceptibility differences (Cavanagh, 1993). The neuropathology observed in 1,3-DNB exposure most strikingly resembles that of diet induced thiamine deficiency resulting from a reduction in the activities of key thiamine dependent metabolic enzymes, the pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase complexes. The significant increases in lactate and increased cerebral glucose utilization observed in both *in vivo* and *in vitro* 1,3-DNB exposure models, suggests a dysfunction in oxidative energy metabolism (Romero *et al.*, 1991; Ray *et al.*,



1994; Romero *et al.*, 1995; Phelka *et al.*, 2003). The specific site where 1,3-DNB and other nitroaromatic compounds elicit their effects is not known, though studies have shown mitochondrial function to be adversely affected (Phelka *et al.*, 2003; Tjalkens *et al.*, 2003). We set out to investigate the ability of this neurotoxic compound to impair key metabolic enzymes in intact cells as well as examine potential mechanisms of this impairment.

In the present study, C6 glioma cells were used as an astrocyte model to investigate possible mechanisms underlying 1,3-DNB induced metabolic dysfunction. Exposure of C6 gliomas to 1,3-DNB resulted in similar morphological and biochemical changes that have been previously observed and reported in other models of 1,3-DNB susceptible CNS populations (Philbert *et al.*, 1987; Romero *et al.*, 1995). 1,3-DNB (500  $\mu$ M and 1 mM) elicited morphological changes including cellular swelling, and extensive vacuolization by 36 hours of exposure in cultures of C6 glioma cells (Figure 3-1) which is comparable to previous 1,3-DNB experiments performed in primary astrocyte cultures. Interestingly, similar structural changes were achieved at lower concentrations but this required a much longer time period, suggesting that the toxic mechanism potentially is initiated at lower dose levels but time is required to manifest into overt pathological changes. This delay may be due to the need for sufficient biochemical changes to occur before observable structural changes are noticeable.

During 1,3-DNB intoxication, the metabolic status of C6 gliomas is compromised evidenced by dose-dependent increases in extracellular lactate concentrations (figure 3-4), as well as depletion of ATP (figure 3-5). Increased extracellular lactate is a common outcome in conditions where mitochondrial oxidative metabolism is compromised such

as hypoxia/ischemia, traumatic brain injury (Ringel *et al.*, 2006), and genetic abnormalities of mitochondrial specific metabolic enzymes (Robinson, 2006). To maintain ATP levels in the absence of functional mitochondrial pathways, the cell must accelerate glycolytic production of ATP. This would account for the increased glucose utilization observed in some cases of AEDS including 1,3-DNB intoxication (Romero *et al.*, 1991; Ray *et al.*, 1994; Romero *et al.*, 1995). During exaggerated glycolytic activity NAD stores are potentially depleted. In order for glycolytically derived ATP production to proceed, NAD levels must be maintained due to the requirement for this cofactor by glyceraldehyde-3-phosphate dehydrogenase. Normally, NADH is re-oxidized in the mitochondria but if this process is disrupted, NAD may be formed during anaerobic glycolysis through lactate dehydrogenase, which converts pyruvate to lactate. This accumulation of lactate can have detrimental effects if not regulated quickly, mainly due to acidification of the extracellular and subsequently the intracellular environments. Acidosis-induced persistent cell swelling has been shown to occur in astrocytic C6 glioma cells (Nabekura *et al.*, 2003). In line with the previous thought, it may require sufficient accumulation of extracellular lactate with a corresponding drop in extracellular pH, to manifest the morphological changes observed.

The ability of both acetoacetate and acetyl-carnitine (ALCAR) to protect cells from structural and biochemical changes during 1,3-DNB intoxication (Figure 3-1, 3-4 and 3-5) further supports a defect in oxidative energy metabolism and more specifically PDHc. Both acetoacetate (AcAc) and ALCAR have the potential to be converted to acetyl-CoA and subsequently can enter the TCA cycle. The ketone body, acetoacetate, is an endogenously circulating substrate known to contribute to cerebral metabolism.

Under most normal conditions the primary fuel for the brain is glucose. However, during many physiologic and pathologic situations including specific periods during postnatal development, starvation and ischemia, cerebral adaptation to ketone metabolism can occur (Edmond, 1992; Morris, 2005). Direct administration of ketone bodies, as well as institution of ketogenic diets results in neuroprotection in numerous disorders, most relevant being pyruvate dehydrogenase complex deficiency. Similarly, ALCAR can be converted to acetyl-CoA through the actions of acetylcarnitine-CoA transferase. The protective effects of ALCAR have been demonstrated in models of ischemia and reperfusion in both cardiac and brain tissue (Rosenthal *et al.*, 1992; Lesnefsky *et al.*, 2006). The acetyl-CoA derived from acetoacetate and ALCAR potentially enter into oxidative energy metabolism effectively bypassing defective PDHc. This would allow for ATP levels to be sustained and result in the reduction of glycolytic activity and consequently lactate production. Both the reduction in extracellular lactate and maintenance of ATP level were observed upon co-administration of these metabolically active substrates with 1,3-DNB.

Though lactate accumulation and acidification of the extracellular environment maybe important in overall pathogenesis of 1,3-DNB neurotoxicity, it appears energetic status of the cell is the ultimate determinant of cell fate. This is apparent through the dramatic increase in cell death as determined by LDH release in glucose free cultures (Figure 3-2). Removal of glucose would prevent glycolytic ATP production and consequently reduce the amount of lactate produced. If lactate concentrations are most important then removal of glucose should show protective effects against 1,3-DNB. The 2-fold increase in extracellular LDH levels in the absence of glucose suggests that C6

gliomas maintain enough ATP through oxidation of glucose to prevent substantial cell loss. One alternative explanation is that C6 gliomas undergo apoptotic cell death in presence of glucose, which would not be quantifiable by LDH release. Apoptotic cell death is an energy demanding process and if ATP levels are not sustained at high enough levels throughout the process, necrotic cell death maybe triggered.

Exposure of C6 glioma cells to 1,3-DNB resulted in the loss of both PDHc and KGDHc activity in a dose dependent manner (Figure 3-6). Dramatic loss of PDHc activity was observed at the lowest concentration of 50  $\mu$ M. This dose level corresponds closely to the blood concentration of the threshold neuropathic dose of 25mg/kg in F344 rats which was calculated to be 43  $\mu$ M (Bailey *et al.*, 1988). This dose also was shown to be the threshold dose for increased glucose utilization in primary astrocytes (Romero *et al.*, 1995). There appears to be differential sensitivity, with PDHc being more susceptible to inhibition, demonstrated by a 5-fold difference in IC<sub>50</sub> concentrations. These complexes are very similar in structure and function and would be expected to respond similarly to 1,3-DNB. Further investigation on potential differences amongst the two complexes that confers the discriminating affects of 1,3-DNB is needed.

PDHc, like other  $\alpha$ -keto acid dehydrogenase complexes, possesses a covalently bound lipoic acid on the E2 component of the complex. The vicinal dithiol group of LA facilitates acetyl transfer from the hydroxyethyl-intermediate of the E1 bound thiamine pyrophosphate to coenzyme A. Covalent modification of LA is a major mechanism for PDHc inhibition. This is seen with mono- and bi-functional arsenicals, as well as strong electrophiles like the  $\alpha,\beta$ -unsaturated aldehydes produced during lipid peroxidation (Stevenson *et al.*, 1978; Patel and Korotchkina, 2002; Samikkannu *et al.*, 2003). Using

antibodies that specifically recognize the covalently bound lipoic acid moiety we investigated whether LA was potentially affected by 1,3-DNB. Western blot analysis of protein from C6 glioma cells exposed to increasing concentrations of 1,3-DNB showed dose-dependent decreases in LA immunoreactivity (Figure 3-7). In order to determine if reduced LA immunoreactivity was resultant from changes in protein expression of the E2 component of PDHc, membranes were probed with an anti-E2 antibody, which showed no changes in expression level (Figure 3-8). This data supports the conclusion that loss of LA immunoreactivity is due to modification of the antigenic site on lipoic acid and not because of reduction in protein expression of the E2 component. Modification of LA was also shown to be time dependent at the highest dose of 500  $\mu$ M DNB with changes in immunoreactivity evident at the earliest time point of 6 hr and complete loss by 12 hrs (Figure 3-7). Interestingly, when comparing the loss of LA with the activity of PDHc, we observe complete loss of LA immunoreactivity with 50  $\mu$ M at 36hrs though approximately 40% residual PDHc activity remains. This suggests that considerable loss of lipoic acid residues may be required in order for substantial reduction in PDHc activity to occur. Supporting this hypothesis is a study looking at the age-related increases in protein adduction of KGDHc by 4-hydroxynonenal (HNE) in rat heart (Moreau *et al.*, 2003). HNE is a highly reactive lipid peroxidation product believed to modify lipoyl moieties in both KGDHc and PDHc. The authors of this study found that a marked increase in adduction occurred in older rats without a corresponding drop in enzyme activity. In fact, activity in older rats increased 1 fold compared to young rats. The authors concluded that the amount of binding was at a low enough level not to significantly affect activity supporting the notion that these complexes may contain a

surplus of lipoic acid sites and that potentially a threshold level of sites must be modified before significant loss of activity is achieved. Interestingly, there also appears to be a compensatory increase in catalytic efficiency in response to increased protein oxidation, with adjustments to  $K_m$  towards the substrate  $\alpha$ -ketoglutarate.

Dysfunction of the pyruvate dehydrogenase complex has been reported in a host of neuropathological states including ischemia/reperfusion injury and neurodegenerative diseases (Sheu *et al.*, 1985; Butterworth and Besnard, 1990; Fukuchi *et al.*, 1998). In both situations, increased production of reactive oxygen species (ROS) have been implicated in disease progression. This increased oxidative stress is proposed to underlie the loss of PDHc activity. PDHc is highly sensitive to direct inactivation by hydroxyl radicals (Bogaert *et al.*, 1994). Indirectly, ROS can result in inactivation of PDHc through the initiation of lipid peroxidation and subsequent formation of highly reactive  $\alpha,\beta$ -unsaturated aldehydes like, 4-hydroxy-nonanal and 2-propen-1-al (acrolein) (Humphries and Szweda, 1998; Pocernich and Butterfield, 2003). Both compounds have been shown to adversely affect PDHc activity potentially through binding of protein bound lipoic acid.

Bioactivation of nitroaromatic compounds is believed to be a necessary step in the cytotoxic mechanisms of several of these chemicals (O'Brien *et al.*, 1990). Dinitrobenzenes, like many nitroaromatics, undergo nitroreduction as one of the main metabolizing pathways. 1,3-DNB undergoes 6-1e<sup>-</sup> reductions to the final product 3-nitroaniline. During this process, highly reactive intermediates may be formed which have the potential to further react with cellular macromolecules such as nucleic acids, lipids or proteins, effectively disrupting their functions. One electron reduction of the

nitro group of 1,3-DNB produces a radical anion that in the presence of an aerobic environment has the potential to redox cycle. This futile redox cycle results in increased production of superoxide anion and establishment of an oxidative stress environment. Evidence for this process has been shown in primary astrocyte cultures, where significant reduction in total glutathione (GSH) after 2 hours of exposure to 1,3-DNB, as well as an 8-fold increase in superoxide levels after 1 hour of exposure (Ray *et al.*, 1994). Alterations in GSH status were also shown to modulate susceptibility to 1,3-DNB induced neurotoxicity (Hu *et al.*, 1999). These studies support the hypothesis that oxidative stress is involved, at least in part, in the toxicity of 1,3-DNB.

To investigate whether 1,3-DNB-mediated oxidative stress maybe responsible for the loss of LA immunoreactivity, protein carbonylation, an indicator of protein oxidation, was determined. Time-dependent increases in carbonylation of proteins at the apparent molecular weights of the PDHc and KGDHc E2 components were observed (Figure 3-10). This suggests that oxidative modification of LA by carbonylation may be responsible for the apparent loss of LA immunoreactivity. However, the addition of antioxidants and thiol containing compounds (trolox, deferoximine, cysteine and DMPS) showed no attenuation of LA loss implying that the loss of lipoic acid immunoreactivity may not be due to either protein oxidation by reactive oxygen species or to adduction by lipid peroxidation products. Western blot analysis using an antibody specific to HNE showed no significant increases in HNE adducts of any proteins during 1,3-DNB exposure which strengthens the thought that lipid peroxidation products are not responsible for the loss of LA as well as the increased protein carbonylation observed (3-9).

In addition to ROS production, highly reactive intermediates, including Nitrosonitrobenzene (NNB) and nitrophenylhydroylamine are formed during nitroaromatic metabolism. These compounds are strong electrophiles that can react with nucleophilic centers of proteins such as thiols, histidinyl secondary amines, and primary amines leading to protein modification. 3-NNB was shown to react with biological thiols, specifically GSH (Ellis *et al.*, 1992). Likewise, 3-NNB may potentially react with reduced thiols of LA resulting in modification and loss of immunoreactivity. The inability of both antioxidants and thiol containing compounds to protect against 1,3-DNB loss of LA may potentially be due to the inaccessibility of these compounds to the site of reactive intermediate production. The E3 component (Lipoamide dehydrogenase) of  $\alpha$ -ketoacid dehydrogenase complexes oxidizes the reduced lipoic acid of E2 using the cofactor FAD. This component was also shown to possess nitroreductase activity, catalyzing 1, 2 and 4 electron reductions of the nitro group (Tsai, 1987). Nitrofuran reduction by lipoamide dehydrogenase resulted in redox-cycling and production of superoxide (Sreider *et al.*, 1990). 1,3-DNB, which exclusively undergoes nitroreduction, is believed to be primarily metabolized in the mitochondria in seminiferous tubules resulting in depletion of mitochondrial GSH (Reeve *et al.*, 2002). The subcellular site of metabolism in other tissues has not been determined. These studies all point to the potential for the reductive metabolism of 1,3-DNB to occur in the mitochondria by lipoamide dehydrogenase with possible production of either superoxide or reactive electrophiles occurring within its active site. Complex geometry and consequential active site coupling within these enzyme systems could potentially allow for direct access of these reactive compounds to E2 bound lipoic acid and subsequent modification. Since

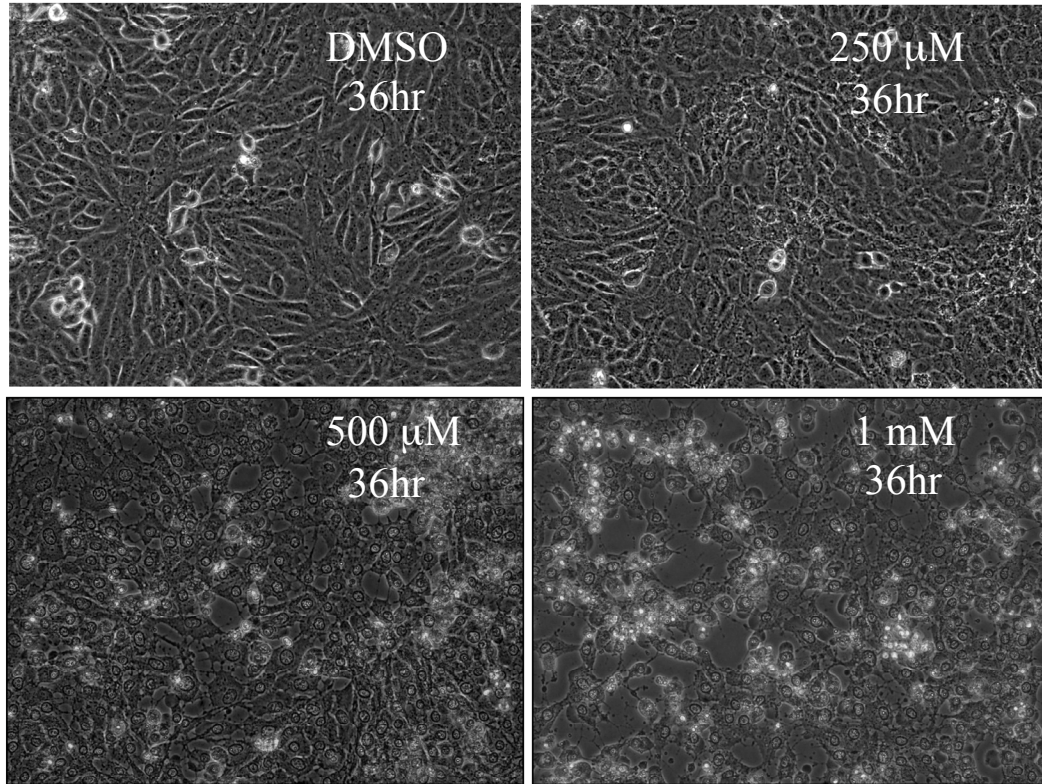


their production is within the complex, anti-oxidants and thiol containing compounds may not be able to sequester these intermediates, which preclude their protective function.

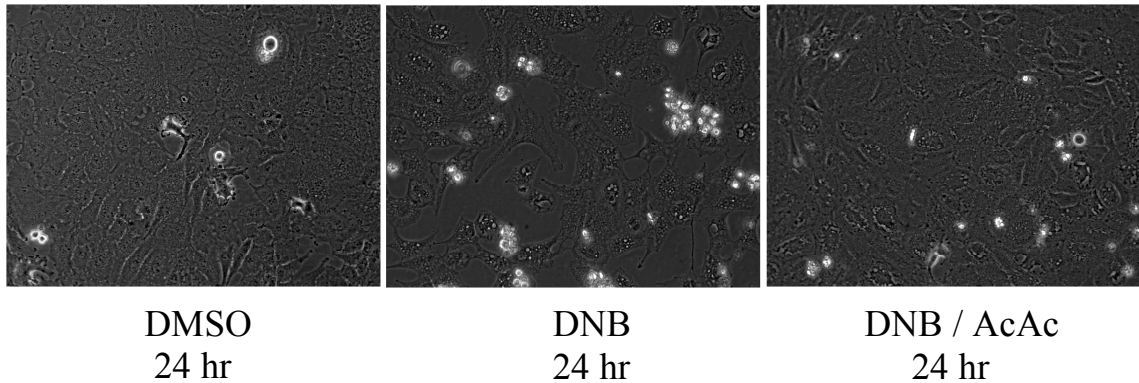
As with activity measurements, differential affects of 1,3-DNB on LA immunoreactivity and protein carbonylation were observed between the two complexes. PDHc was more sensitive than KGDHc with respect to both outcomes. The presence of the additional protein, E3BP, may explain why these structurally related compounds are differentially inhibited by 1,3-DNB exposure. E3BP, like the E2 component, possesses a lipoyl domain with a covalently bound LA. E3BP is responsible for binding the E3 component to the E2 structural core and is essential for the catalytic activity of the overall complex. Its value is demonstrated by an 80 to 90 percent reduction in PDHc activity in those individuals who genetically lack E3BP (Brown *et al.*, 2006). New ultra-structural models suggest that the E2 lipoyl domains interact exclusively or at least preferentially with the E1 component, while the E3BP lipoyl domain interacts with the E3 (Smolle *et al.*, 2006). If this model were correct, the electron flow from reduced LA on the E2 to NAD<sup>+</sup> would go through the E3BP-LA, supporting more than just a structural role for this component. E3BP may allow for the reactive intermediates produced by the E3 component to be efficiently transferred to the E2 bound lipoic acid. This component is not present in KGDHc, which potentially reduces the ability of these intermediates to access the E2 bound LA. The correlation between loss of LA, increases in protein oxidation and inhibition of activity all point to direct protein modification of PDHc as the most likely route for 1,3-DNB mediated inhibition.

In conclusion, 1,3-DNB results in metabolic impairment of the astrocyte-derived cell line, C6 glioma. This metabolic disruption appears to be mediated by inhibition of PDHc potentially through modification of protein bound LA. Though the data presented in this study suggests that reactive oxygen species, as well as lipid peroxidation products were not responsible for loss of LA immunoreactivity, the absence of evidence for direct adduction by 1,3-DNB or its reactive intermediates underscores the need for further investigation of the specific adduction species.

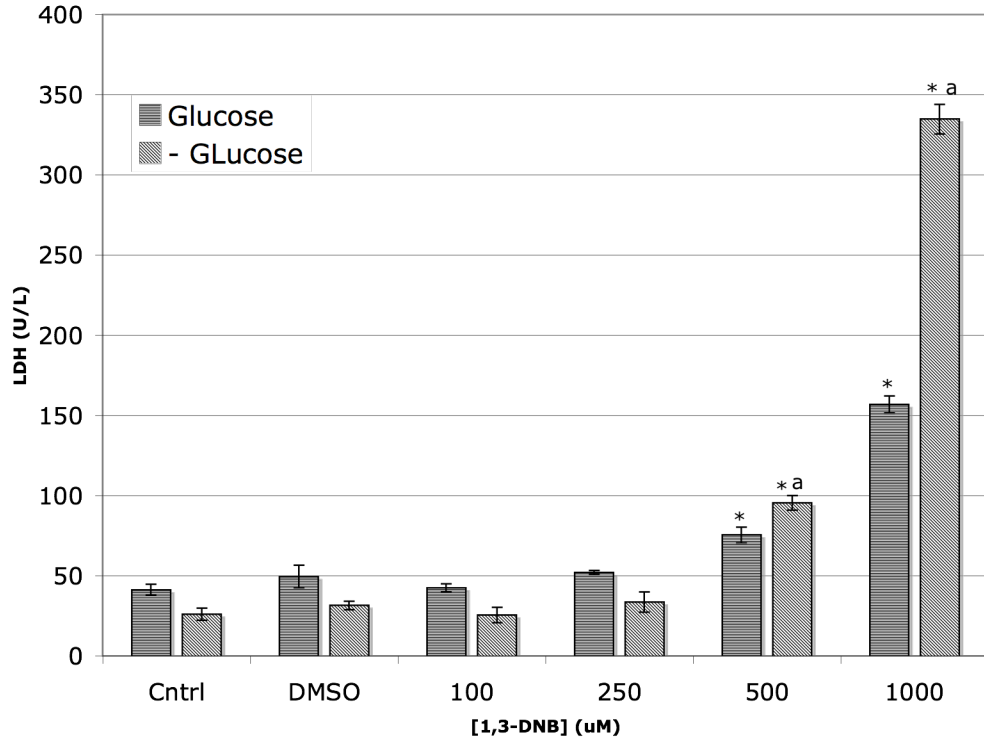
A)



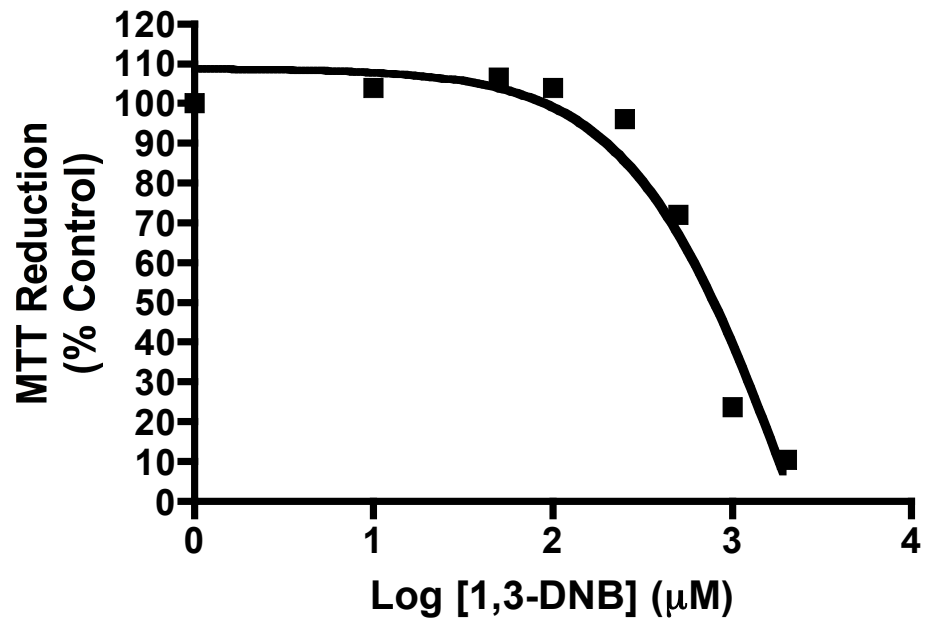
B)



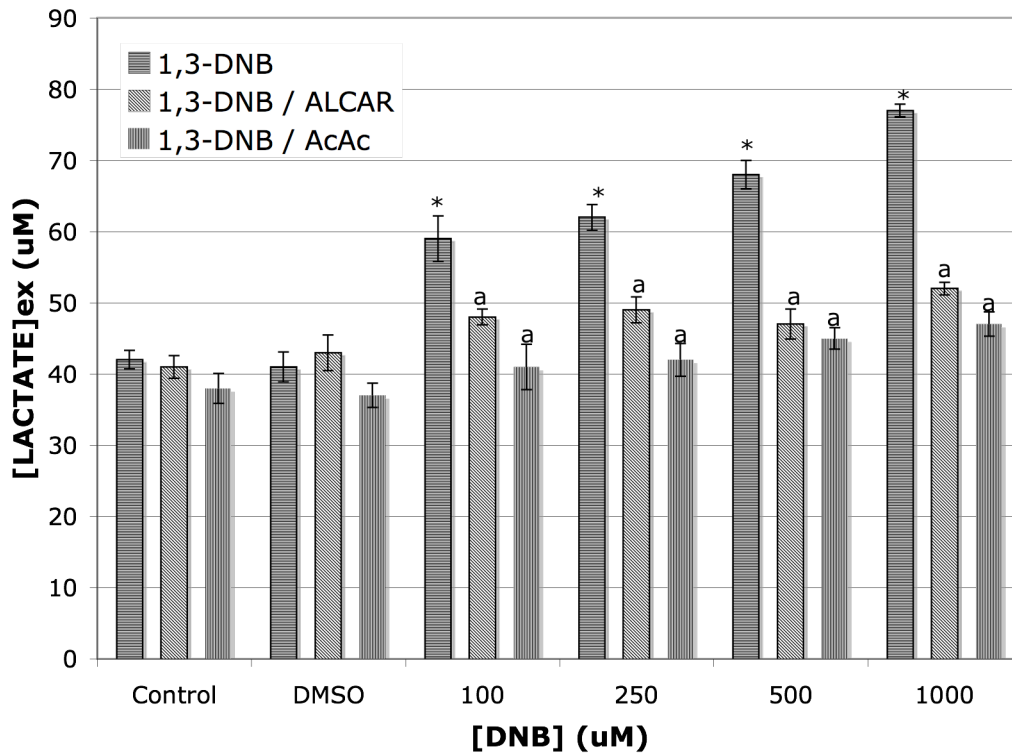
**Figure 3-1.** (A) Representative photomicrographs demonstrating dose dependent effects of 1,3-DNB (250  $\mu$ M – 1 mM) exposure on the morphology of C6 gliomas (10X). Cellular swelling, extensive vacuolation and loss of cell monolayer occurs at 500  $\mu$ M 1,3-DNB. The effects are greatly exaggerated at 1 mM with significant membrane blebbing and loss of cells. (B) Representative photomicrographs demonstrating the protective effects of acetoacetate (AcAc) (1 mM) on 1,3-DNB (500  $\mu$ M) mediate morphological changes at 24 hr of exposure (20X). Substantial reduction in cellular vacuolation and maintenance of cell monolayer is observed in co-treated cultures.



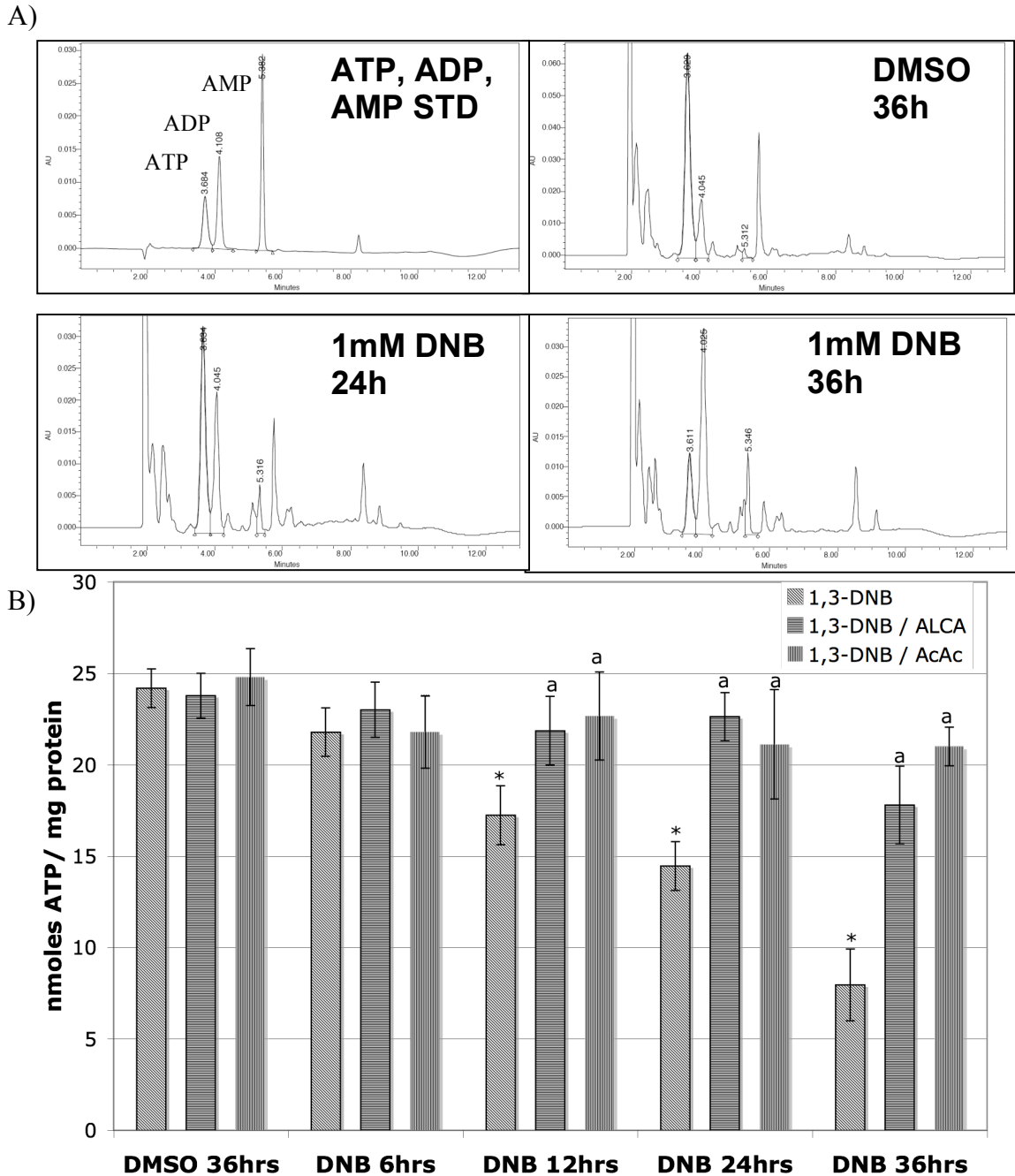
**Figure 3-2.** Effects of 1,3-DNB on C6 glioma cell viability. Cell death was assessed by measuring the release of the cytosolic marker lactate dehydrogenase (LDH). Exposure of C6 gliomas to 500  $\mu$ M and 1 mM 1,3-DNB results in significant increase in LDH release in the presence and absence of glucose. Removal of glucose results in greater than a 2-fold increase in LDH release. Data are expressed as mean concentration  $\pm$  SE (N=4) of extracellular LDH (U/L). (\*) denotes significant difference ( $P < 0.01$ ) from non-treated controls. (a) denotes significant difference ( $P < 0.01$ ) from +Glucose.



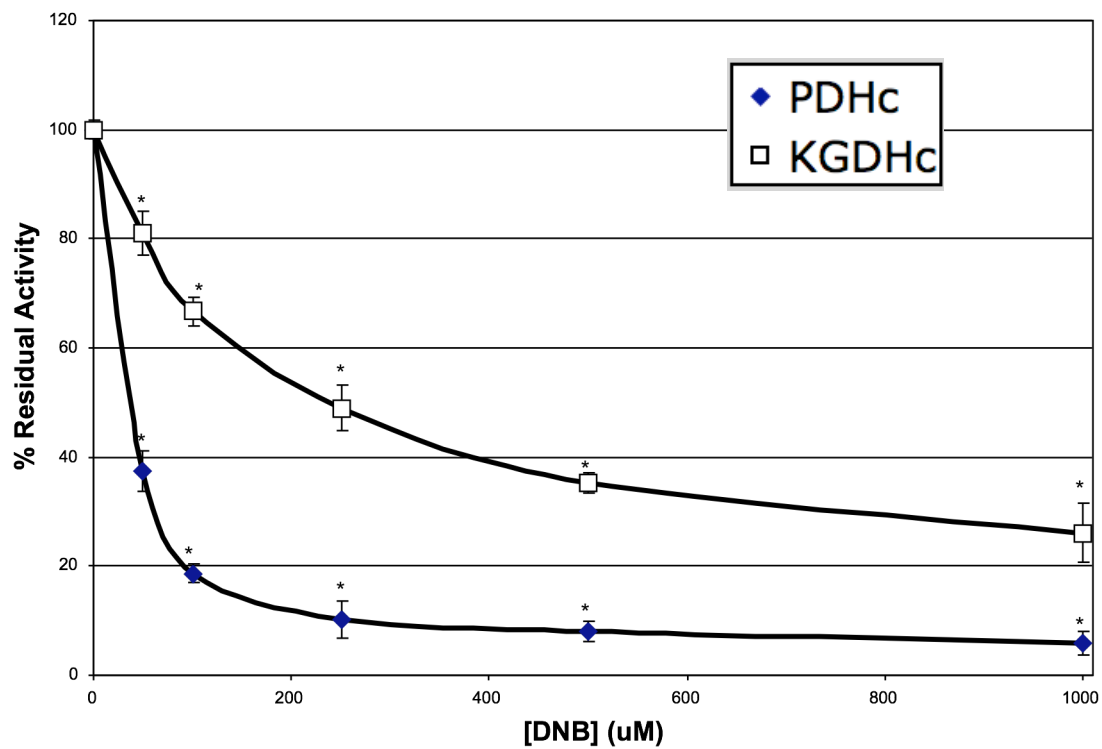
**Figure 3-3.** Effects 1,3-DNB on C6 glioma cell viability as assessed by MTT reduction. Dose dependent loss of mitochondrial reducing potential occurred at 36hrs after exposure to increasing concentration of 1,3-DNB (10 – 2000 µM). Data is presented as the mean +/- SE of percent reduction compared to DMSO treated controls. The IC<sub>50</sub> value for inhibition of MTT reduction was calculated to be 978 µM.



**Figure 3-4.** Determination of extracellular lactate accumulation in culture medium of C6 glioma cells exposed to increasing concentrations of 1,3-DNB alone or co-treated with 1,3-DNB and 1 mM acetyl-carnitine (ALCAR) or Acetoacetate (AcAc) for 36 hrs. 1,3-DNB exposure results in a significant increase in extracellular lactate in a dose dependent manner and this increase was attenuated with co-treatment of both ALCAR and AcAc. Results are expressed as means  $\pm$  SE (n=4) from 3 different experiments. (\*) denotes significant difference from Cntrl ( $P < 0.01$ ) and (a) denotes significant difference between 1,3-DNB alone and 1,3-DNB with AC ( $P < 0.01$ ).



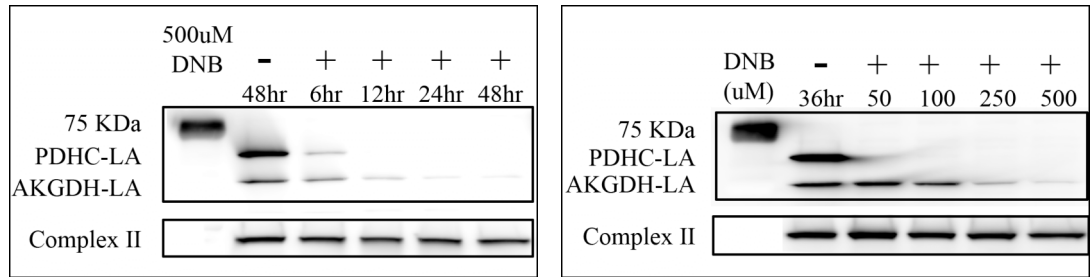
**Figure 3-5.** A) Representative chromatograms depicting time-dependent loss of intracellular ATP with concurrent increases in AMP. The HPLC retention times corresponding to the peaks of ATP, ADP, and AMP were 3.684, 4.108, and 5.382, respectively. B) ATP depletion in C6 glioma cells treated with either 1 mM 1,3-DNB alone or co-treated with 1,3-DNB and 1 mM acetyl-carnitine (ALCA) for up to 36hrs. Significant reduction in ATP was first observed at 12hrs and continued to decline in a time dependent manner. Results are expressed as means  $\pm$  SE (n=3). (\*) denotes significant difference from Cntrl ( $P < 0.05$ ) and (a) denotes significant difference between 1,3-DNB alone and 1,3-DNB with ALCA ( $P < 0.05$ ).



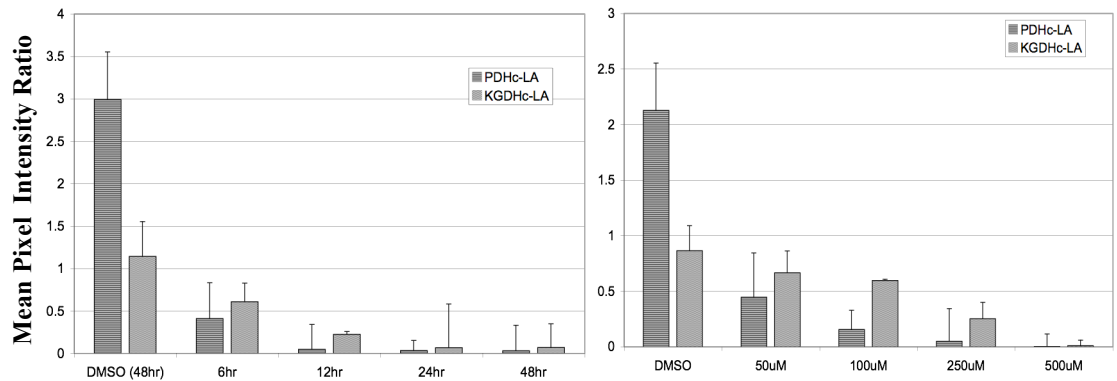
**Figure 3-6.** Dose dependent reduction in PDHc and KGDHc activities in C6 gliomas after 36hrs of exposure. Data expressed and % residual activity +/- SE as compared to sham treated cells. (\*) denotes significant difference from vehicle control (P<0.01).



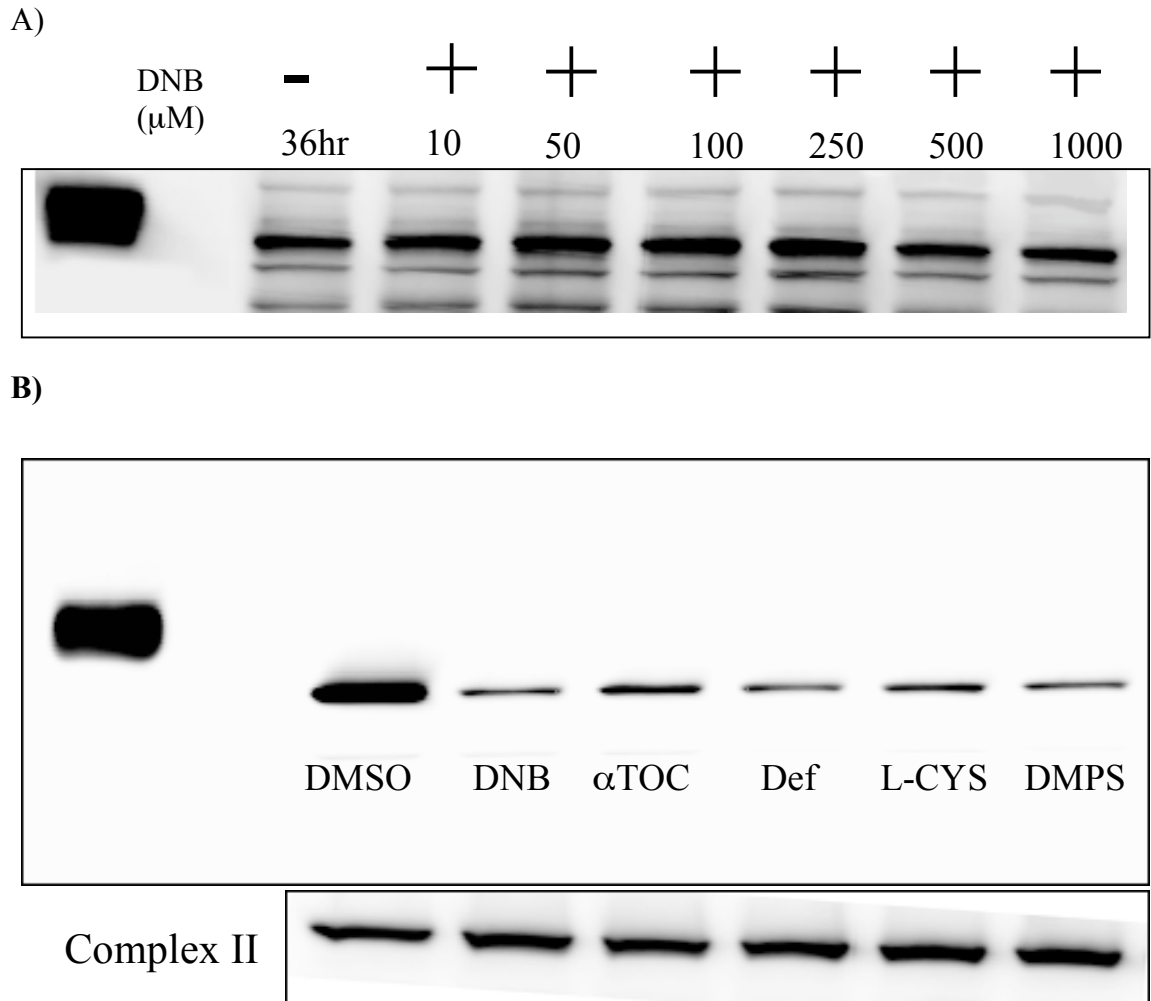
A)



B)

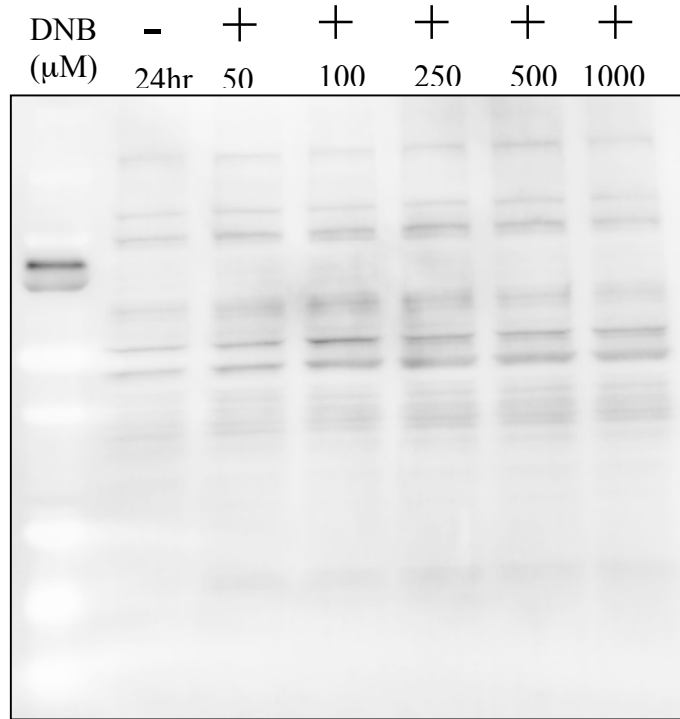


**Figure 3-7.** A) Lipoic acid (LA) immunoreactivity of total cellular protein from C6 gliomas exposed to either 500 μM 1,3-DNB for 6, 12, 24, and 48hr or 50, 100, 250, and 500 μM for 36 hr. B) Graphical representation of mean pixel intensity ratios normalized to complex II expression. 1,3-DNB exposure results in substantial loss of both PDHc and KGDHc LA immunoreactivity at 6hr and complete loss at 12hr. At 36 hr, dose dependent loss of LA immunoreactivity was observed. KGDHc-LA appears to be less sensitive to loss compared to PDHc-LA with substantial loss of KGDHc-LA only occurring at 250 μM and higher.

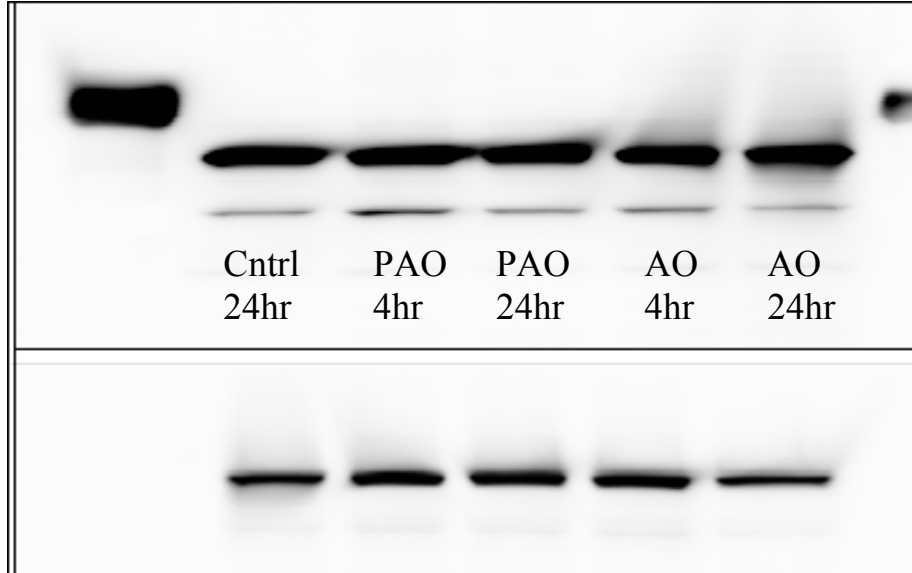


**Figure 3-8.** Western blot analysis of the E2 component of PDHc. No significant changes in E2 expression were observed with increasing concentrations of 1,3-DNB (10-1000 $\mu\text{M}$ ) (A). Effects of anti-oxidants,  $\alpha$ -tocopherol ( $\alpha$ -TOC), and deferoximine (Def) as well as thiol containing compounds, L-cysteine (L-CYS) and 2,3-dimercapto-1-propanesulfonic acid (DMPS) on 1,3-DNB induced loss of lipoic acid immunoreactivity. No significant protection was afforded by either type of compound.

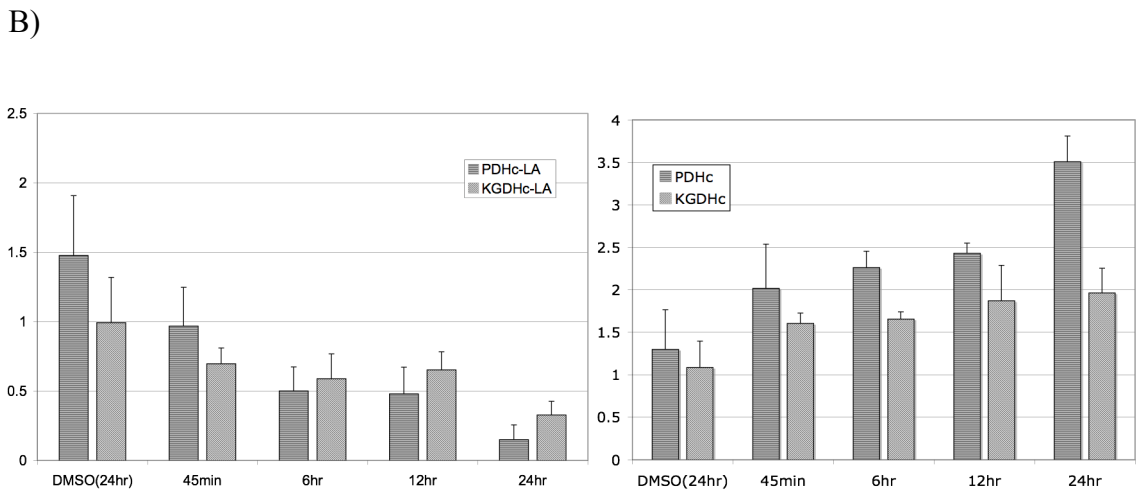
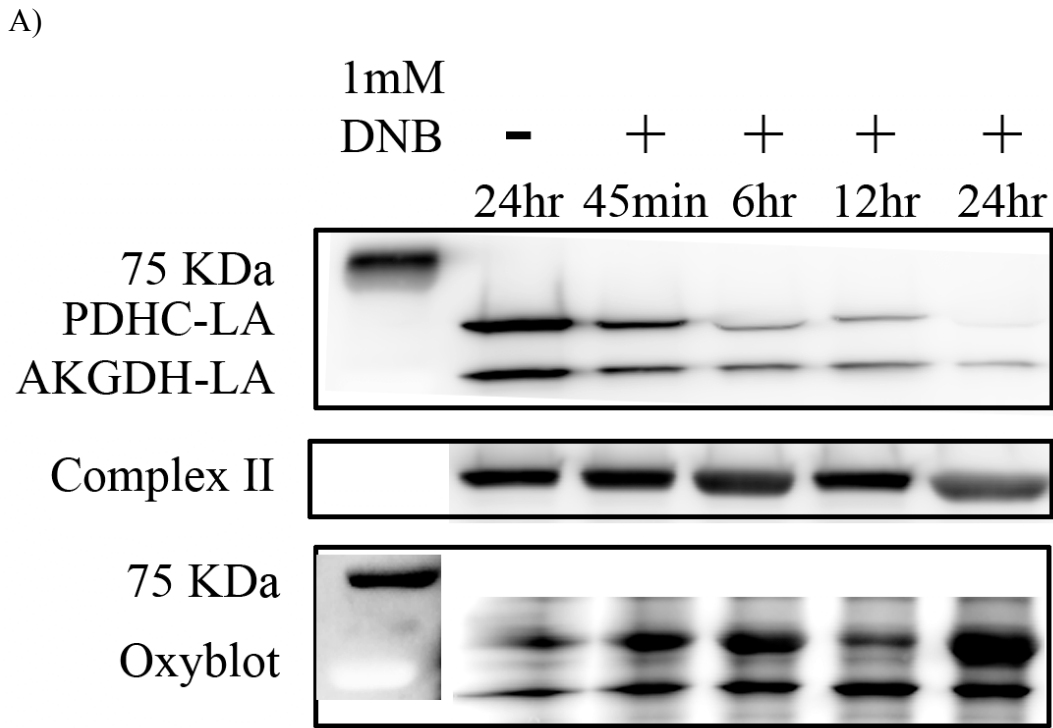
A)



B)



**Figure 3-9.** (A) Determination of 4-hydroxynonanal adducts of total cellular protein from C6 glioma cells dosed with increasing concentrations of 1,3-DNB (50 – 1000  $\mu$ M). No significant changes were observed. (B) Effects of the arsenical compounds, phenylarsine oxide (PAO) and arsenic trioxide (AO), on LA immunoreactivity in C6 glioma cells. Exposure to PAO (10  $\mu$ M) and AO (100  $\mu$ M) for 4 and 24 hr, showed no effects on PDHc LA immunoreactivity.



**Figure 3-10.** A) Lipoic acid immunoreactivity of total cellular protein from C6 gliomas dosed with 1,3-DNB (1 mM) for 45min, 6hr, 12hr, and 24hr. Loss of LA immunoreactivity was apparent at the earliest time point of 45 min and complete loss occurred by 24 hrs. Corresponding Oxyblot analysis showed time dependent increase in protein carbonylation of bands corresponding to the positions of PDHc and KGDHc E2 components. B) Graphical representation of mean pixel intensity ratios normalized to complex II expression.

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**CHAPTER 4**

**ASTROCYTE AND NEURONAL INTERACTIONS IN AN IN VITRO MODEL  
OF ACUTE ENERGY DEPRIVATION: IMPLICATIONS OF ADENOSINE  
RECEPTOR SIGNALING**

**Abstract**

1,3-Dinitrobenzene is one of several metabolic disruptors known to induce neuropathology characteristic of a class of diseases termed the acute energy deprivation syndromes (AEDS). A hallmark of AEDS is the initial sparing of neurons, with neuronal damage occurring secondary to edema and loss of astrocytes. The ability of astrocytes to modulate cytotoxic responses of neurons is well established. The current study was undertaken to investigate the ability of the astrocyte cell model DI TNC-1 to protect SH-SY5Y neuroblastomas during metabolic impairment induced by 1,3-DNB. SH-SY5Y cells, in single culture, showed substantial cytotoxicity upon exposure to 1,3-DNB (500  $\mu$ M, 24hr), which included morphological changes and loss of mitochondrial reducing potential. Both cytotoxic outcomes were attenuated in co-cultures. 1,3-DNB was also shown to induce cell death as assessed by caspase-3 activation. The amount of cleaved caspase-3 in SH-SY5Y cells was significantly reduced, in the presence of DI TNC-1. Interestingly, the pre-treatment with the A<sub>1</sub> adenosine receptor antagonist, DPCPX, resulted in increased caspase-3 activation even in the presence of DI TNC-1 cells, suggesting the involvement of purine signaling pathways in the cytoprotection observed.

Treatment of DI TNC-1 cells with 1,3-DNB resulted in increased extracellular adenosine, as well as AMP levels. This increase may activate adenosine receptors, which are known to mediate neuroprotection in the CNS during numerous neuropathological conditions (Dunwiddie and Masino, 2001; Kochanek *et al.*, 2005; Stone, 2005). Activation of adenosine receptors resulted in both reduction of SH-SY5Y excitability, determined through blockage of KCl-induced calcium waves, as well as induction of phospho-activation of ERK (1/2) and subsequent activation of the pro-survival protein BAD. Further investigation into the complex interaction between neurons and astrocytes is needed and may lend some insight into the mechanisms of regional and cell specific sensitivity in AEDS.

### **Introduction**

First being described as strictly structural scaffolding or glue, astrocytes have until recently been thought of as purely auxiliary components in the nervous system, the original belief being that astrocyte functions were restricted to passive maintenance of the extracellular environment. Recently, knowledge of the metabolic and trophic functions of astrocytes has evolved tremendously. One of the most important discoveries that reinvigorated interest in astrocytes was the observation of astrocytic  $Ca^{2+}$  excitability. This finding, coupled to release of “gliotransmitters” and the presence of numerous metabotropic receptors, suggested that astrocytes are in fact capable of active communication with their neighbors (Cornell-Bell *et al.*, 1990; Charles *et al.*, 1991). Astrocytes are found between neuronal synapses and the vasculature. This positioning, together with active signaling machinery, supports a potential role of astrocytes in

regulating both synaptic transmission, as well as neurovascular coupling (Haydon and Carmignoto, 2006). The continued uncovering of new and diverse functions of astroglia have led to the belief that these cells play critical roles in almost all aspects of neurophysiology, and maybe more importantly, are major determinants of neurological dysfunction.

1,3-Dinitrobenzene (1,3-DNB), an important industrial intermediate in the production of numerous commercial products, has been shown to be gliotoxic. Prolonged or repeated exposure of rats to this nitroaromatic compound produces a distinct bilaterally symmetrical lesion, which is gliovascular in origin with the astrocytic and vascular components primarily affected (Philbert *et al.*, 1987; Ray *et al.*, 1994; Romero *et al.*, 1995). Neuronal damage arises secondary to edema resultant from vascular compromise and loss of astrocytes. The increased sensitivity of astrocytes and the initial sparing of neurons, in 1,3-DNB exposures, is somewhat of a surprising outcome. It has been established, through various *in vitro* and *in vivo* studies, that this neurotoxic compound elicits CNS damage, at least in part, through disruption of energy metabolism (Ray *et al.*, 1992; Romero *et al.*, 1995; Romero *et al.*, 1996; Holton *et al.*, 1997). Active neurons are presumably more metabolically demanding compared to their glial counterparts and accordingly are expected to be more susceptible to metabolic impairment elicited by this chemical. Additionally, astroglia are exclusively responsible for formation of glycogen with enzymes involved in gluconeogenesis and glycogenolysis solely being expressed in astrocytes (Brown and Ransom, 2007). The availability of this repository energy substrate, though present at low levels, potentially allows for maintenance of ATP concentrations in astrocytes under conditions of diminished energy

production, such as aglycemia (Swanson and Choi, 1993; Brown and Ransom, 2007). Oxidative stress also appears to play a significant role in the pathogenesis of 1,3-DNB-mediated encephalopathy (Ray *et al.*, 1994; Hu *et al.*, 1999). Neurons are generally thought to have lower antioxidant capacity compared to astrocytes, again suggesting that this cell type should be more sensitive to 1,3-DNB cytotoxicity (Dringen *et al.*, 1999a). Though it may be possible that 1,3-DNB selectively targets astrocytes by a yet undetermined mechanism, one alternative explanation for cell specific differential sensitivity is the ability of astrocytes to potentially shield neurons through intercellular signaling or trophic support.

In addition to a trophic role, astrocytes are also able to influence both neuronal and endothelial cell function through active communication utilizing astrocyte derived intercellular signaling molecules, termed gliotransmitters (Newman, 2003; Zhang and Haydon, 2005). One potential signaling pathway believed to be involved in intercellular communication, in the CNS, is purinergic neurotransmission. The extracellular action of purines as potential signaling molecules was first proposed in the late 20' s, with the description of the potent actions of the purines, ATP and adenosine, in the heart (Drury and Szent-Gyorgyi, 1929). Their role in the CNS was later cemented by Burnstock and colleagues (Burnstock, 1972).

Adenosine, a ubiquitous purine nucleoside produced from the hydrolysis of ATP, is a potent modulator of many physiological functions through the activation of four specific adenosine receptors ( $A_1AR$ ,  $A_{2a}AR$ ,  $A_{2b}AR$ , and  $A_3AR$ ) (Fredholm *et al.*, 2000). The most abundant of these receptors, the  $A_1AR$ , have long been known to mediate neuroprotection in pathological conditions (Dunwiddie and Masino, 2001). Under

conditions of diminished energy production extracellular concentrations of adenosine dramatically increase resulting in activation of G-protein coupled metabotropic receptors in the efforts to reduce neuronal excitability through hyperpolarization and reduction of neurotransmitter release. The activation of this receptor, in effect, silences the neurons to shield them during times of energy deprivation.

In the present study, the astrocyte and neuronal cell lines, DI TNC-1 and SH-SY5Y neuroblastomas, were used as an *in vitro* model of 1,3-dinitrobenzene-induced encephalopathy, to investigate the cell specific sensitivity to this compound, as well as the potential for astrocytes to protect neurons from cytotoxicity. The role of adenosine receptor signaling in cellular communication between these cell types was also examined.

## **Materials and Methods**

### **Cell Culture**

The astrocytic cell line DI TNC-1 obtained from American Type Culture Collection (ATCC) was employed in this study as an astrocyte model. Originally established through targeted oncogenesis (Radany et al., 1992), these cells show similar characteristics to mature differentiated primary type 1 astrocytes, including substantial glial fibrillary acidic protein (GFAP) expression, high affinity uptake mechanism for GABA, and the ability to promote neurite outgrowth. The neuronal model used in this study was the human SH-SH-SY5Y neuroblastoma cell line also obtained from ATCC. Both cell lines were maintained at 37°C under an atmosphere of 5% CO<sub>2</sub> / 95% air (v/v) in Dulbecco's modified eagles medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and 1 x antibiotics (Penicillin, Streptomycin, and Neomycin; Gibco).

Cells were subcultured using 0.25% trypsin and used 48 hr after plating. Prior to treatment, cells were rinsed with Dulbecco's phosphate buffered saline (D-PBS) and all treatments were performed in serum free DMEM. For co-culturing experiments, transwell-clear permeable supports (Corning) were used, where SH-SY5Y neuroblastomas were plated in the wells and the DI TNC-1 were plated in the supports. At least 24 hours prior to treatment the two cell types were cultured together.

## **Cytotoxicity**

### *Microscopy*

SH-SY5Y and DI TNC-1 cells plated in 6 well plates were dosed with DMSO, 500  $\mu$ M 1,3-DNB, or 1 mM 1,3-DNB for 24 hr. For co-culture experiments, both the wells and the supports were dosed with equal amounts. After exposure, plates were then washed twice with D-PBS and phase-contrast light microscopy was performed using an Olympus CKX41 inverted microscope equipped with a Spot Insight QE digital CCD camera at 20X magnification.

### *Mitochondrial reducing potential*

Cytotoxic effects of 1,3-DNB on SH-SY5Y neuroblastomas alone and in co-culture were determined by monitoring the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) as described (Mosmann, 1983). The yellow, soluble tetrazolium dye is metabolically reduced to a purple, water-insoluble formazan, which is detected by a spectrophotometer. Cells plated in a 6 well plates were dosed with increasing concentrations of 1,3-DNB (10  $\mu$ M – 1000  $\mu$ M). After exposure, wells were rinsed with D-PBS and MTT assay solution (1 mg/ml in DMEM) was added to each well

and allowed to incubate for 2 h at 37°C. MTT solution was then aspirated and 500 µl of DMSO was added to dissolve the formazan dye. After 30 min incubation with vigorous mixing, absorbance was determined at 550 nm. Mean absorbance values were compared to vehicle control and IC<sub>50</sub> values were determined by fitting data to a sigmoidal dose-response equation using Prism software (Graph Pad Software, Inc., San Diego, Ca).

### **Intracellular Calcium Imaging by confocal microscopy**

Calcium wave images were acquired using an Olympus DSU confocal imaging system, consisting of an Olympus IX81 inverted microscope equipped with the disk scanning unit (DSU) and a ORCA-ER cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan). SH-SY5Y neuroblastoma cells were plated onto 22 mm glass round coverslips and allowed to attach and recover for 48 hrs. Cells were then incubated for 45 min with the Ca<sup>2+</sup> indicator dye FURA-2/AM (3 µM) (Molecular Probes, Eugene, OR, USA) in sodium-ringer's solution consisting of: NaCl (137 mM), KCl (5 mM), K<sub>2</sub>HPO<sub>4</sub> (0.44 mM), NaHCO<sub>3</sub> (4.2 mM), MgCl<sub>2</sub> (1.2 mM), HEPES (20 mM), CaCl<sub>2</sub> (1 mM), glucose (10 mM), and adjusted to pH 7.4 with NaOH. After incubation, cells were rinsed twice with sodium-ringer's solution and cells were then incubated for 1 hr in order for the dye to de-esterify. Coverslips were then mounted in an Atofluor® cell chamber (Invitrogen, Carlsbad, CA) and placed on a heated stage set at 37°C. Baseline images of Fura-2 fluorescence at 340 nm were recorded for 1 min (1 image / 10 sec) prior to induction of the calcium wave through depolarization by KCl (25 mM). Calcium wave propagation was monitored for 3 min.



## **Determination of Extracellular Adenyl Purines**

Extracellular concentrations of adenyl purines including; ATP, ADP, AMP, and adenosine, were determined through separation by high performance liquid chromatography with fluorescence derivatization as previously described (Katayama et al., 2001).

### *Derivatization Conditions*

Culture media from DI TNC-1 cells treated with 1,3-DNB, containing adenyl purines (100  $\mu$ l) were first clarified by centrifugation (3000 x g for 5 min at 4°C) followed by derivatization for 30 min at 80°C with 4.0 M 2-chloroacetaldehyde in 1.0 M acetate buffer (pH 4.5; 100  $\mu$ l). After derivatization, 10  $\mu$ l of the reaction solution was injected into the HPLC apparatus.

### *Chromatography conditions*

Adenyl purines were determined by separation on a Waters Alliance® HPLC. Isocratic elution was performed on an Xbridge™ C18 column (4.6mm x 100mm, 3.5 $\mu$ m) at a flow rate of 0.8 ml/min. Fluorescent detection of derivatized adenyl purines was performed with excitation wavelength at 280 nm and emission wavelength at 420 nm. The mobile phase consisted of 0.1M K<sub>2</sub>HPO<sub>4</sub>, 0.05 citric acid, pH: 4.5 with 3% methanol.

## **SDS-PAGE and Western Blotting**

SH-SY5Y cells plated in 6 well tissue culture dishes were exposed to 1,3-DNB (500  $\mu$ M) for 24 hr. The plates were washed with D-PBS twice and total cellular protein was extracted with ice-cold cell lysis buffer consisting of Tris-HCl (50 mM, pH 7.4), NaCl (150mM), Triton X-100 (1% v/v), and protease and phosphatase inhibitor cocktails (0.1%

v/v) (Sigma). Cell lysates were snap frozen in liquid N<sub>2</sub> and stored at -80 °C until protein quantification and SDS-PAGE. Cellular debris was removed by centrifugation and protein concentration was determined by the Bicinchoninic acid method (Smith et al., 1985) using a commercial kit (Pierce) as described by the manufacturer. Approximately 20 µg of protein was mixed with sample loading buffer containing β-mercaptoethanol and SDS and denatured at 100°C for 10 min. The proteins and molecular weight standards were resolved on 4-20% Tris-glycine gels (Lonza) and electroblotted to PVDF membrane. Membranes were blocked with non-fat milk (5% w/v) in Tris-buffered saline (pH 7.6) containing Tween-20 (1% v/v) (TBS-T). After 30 min of blocking, the membranes were probed with either anti-cleaved-CASPASE-3 (1:5000; Cell Signaling), anti-phospho-ERK1/2 (1:5000; Sigma), or anti-phospho-BAD (1:5000; Cell Signaling), at 4°C overnight. The membranes were washed with TBS-T followed by probing with an appropriate alkaline phosphatase conjugated secondary antibodies (1:10000). The membranes were developed using an ECF detection system (Amersham Biosciences) and visualized on a FujiFilm laser imaging system.

## RESULTS

### *Differential sensitivity of SH-SY5Y and DI TNC-1 cells with exposure to 1,3-DNB*

Exposure of both SH-SY5Y and DI TNC-1 cells to either 500 µM or 1 mM 1,3-DNB for 24 hr resulted in significant morphological changes with SH-SY5Y being more sensitive (Figure 4-1a). At 500 µM 1,3-DNB, DI TNC-1 cells showed classical changes in morphology including cellular swelling and extensive vacuolation. Loss of cell monolayer was also apparent at this dose. With the higher dose of 1 mM, exaggerated

swelling and vacuolation continued with the presence of cell debris indicating cell loss. SH-SY5Y cells showed substantial cytotoxicity with the balling of cells at the 500  $\mu$ M dose and at 1 mM cell loss was greatly increased.

*Protection of SH-SY5Y through co-culturing with the DI TNC-1 cell line*

Co-culturing of SH-SY5Y with DI TNC-1 cells greatly diminished the cytotoxicity incurred upon exposure to 1,3-DNB (500  $\mu$ M, and 1 mM) for 24 hr (figure 4-1b). The morphological changes that resulted with 500  $\mu$ M are completely attenuated. Though protection was noticeable at the 1 mM dose, cytotoxic changes were still apparent, including cell shrinkage and balling. The ability of DI TNC-1 cells to protect SH-SY5Y cells from toxicity was confirmed by monitoring mitochondrial reducing potential (Figure 4-2). 1,3-DNB (10  $\mu$ M – 1 mM) induced dose-dependent diminution of the cells reducing potential ( $P < 0.01$ ) as determined by MTT reduction assay. In contrast, co-culturing SH-SY5Y cells with the astrocytic DI TNC-1 cells showed significant ( $P < 0.01$ ) attenuation of reduction potential loss.

*Co-culturing reduces the induction of cell death by 1,3-DNB*

The ability of 1,3-DNB to induce cell death in SH-SY5Y neuroblastoma cells was determined through examining caspase-3 activation (Figure 4-3). Exposure to 500  $\mu$ M 1,3-DNB for 24 hr resulted in the cleavage of caspase-3 evident by detection of the 17 and 19 KDa cleavage products. Co-culturing SH-SY5Y cells with DI TNC-1 cells resulted in a significant reduction of detected cleaved caspase-3 fragments. Treatment of SH-SY5Y cells with the  $A_1$  adenosine receptor antagonist, 8-cyclopentyl-1,3-

dipropylxanthine (DPCPX), reversed the protection afforded by co-culturing, resulting in greater caspase-3 cleavage. DPCPX alone appeared to result in slight induction of caspase-3 activation.

*Induction of cell survival pathways in SH-SY5Ys through activation of A<sub>1</sub> adenosine receptors*

Activation of A<sub>1</sub> adenosine receptors by the specific agonist, R-phenylisopropyladenosine (R-PIA) resulted in the phosphorylation of ERK (1/2), with peak expression being observed at 1 hr. ERK (1/2) expression trailed off at 2 hours, and returned to basal levels by 4 hours (Figure 4-4). Subsequent to ERK phosphorylation, upregulation of phospho-BAD occurred at 2 hr with peak expression at 4 hr.

*Effects of adenosine on KCl induced Ca<sup>2+</sup> wave propagation.*

Addition of 25 mM KCl induced the propagation of an intense calcium wave (Figure 4-5). Treatment of SH-SY5Y cells with adenosine (5 nM, 20 min) prior to KCl depolarization delayed the induction of the calcium wave by approximately 30 sec, as well as suppressed the overall magnitude. Pre-treatment with either the A<sub>1</sub> specific adenosine receptor antagonist, DPCPX (250 nM, 30 min) or the broad-spectrum antagonist, theophylline (10 μM, 30 min) prior to adenosine treatment, prevented the calcium wave suppression. The A<sub>1</sub> specific antagonist appeared to be more efficient at blocking the effects of adenosine on calcium wave propagation.

*1,3-DNB mediated increases in extracellular AMP and adenosine.*

Treatment of DI TNC-1 cells with 1,3-DNB (500  $\mu$ M) for 24 hr resulted in increased extracellular concentrations of both adenosine and AMP.

## **Discussion**

1,3-Dinitrobenzene is one of several metabolic disruptors known to induce neuropathology characteristic of a class of diseases termed the acute energy deprivation syndromes (Cavanagh, 1993). AEDS produce focal, glio-vascular lesions, in highly conserved brainstem regions, consistent with their high metabolic activity. A hallmark of AEDS is the initial sparing of neurons, with neuronal damage occurring secondary to edema and loss of astrocytes (Philbert *et al.*, 1987). Metabolic impairment and induction of oxidative stress are key components to the pathogenesis of AEDS, making the observation that neurons are relatively insensitive to 1,3-DNB and other inducers of acute energy deprivation surprising. Compared to their glial counterparts, neurons are believed to be more metabolically dependent on available ATP pools and less competent in dealing with oxidative stress, two characteristics that should predispose neurons to injury (Dringen *et al.*, 1999a; Dienel and Hertz, 2005). Though there is the potential for 1,3-DNB to specifically target astrocytes over neurons, by a yet undetermined mechanism, it is more likely that the interrelationship of these two cell types determines their fate.

Astrocytes form intimate relationships with both neuronal and endothelial components in the CNS. These relationships allow astrocytes to play an extremely

important role in cerebral function, homeostasis, as well as modulate toxicity. Astrocyte functions include the regulation of extracellular environment through ion buffering and removal of the potentially toxic neurotransmitters, as well as facilitation and maintenance of both blood brain barrier (BBB) integrity and neuronal synaptic transmission (Hertz *et al.*, 1999; Haydon and Carmignoto, 2006). The demise of astrocytes would result in the breakdown of astrocyte/neuronal and astrocyte/endothelial interactions and ultimately produce both neuronal and vascular dysfunction.

In the present study, the ability of astrocytes, to modulate the toxic responses of neurons in 1,3-DNB exposure was investigated using the cell models, DI TNC-1 and SH-SY5Y neuroblastoma. Comparing the effects of 1,3-DNB on the two cell types reveals that the neuronal cell line, SH-SY5Y is more susceptible to 1,3-DNB toxicity compared to astrocyte-derived DI TNC-1 cells. This is contradictory to the *in vivo* situation where astrocytes are more sensitive to the pathological effects of 1,3-DNB. This difference between *in vivo* and *in vitro* outcomes supports a role for intercellular relationships in influencing susceptibility to 1,3-DNB.

Co-culturing DI TNC-1 cells with SH-SY5Y neuroblastoma cells significantly protected SH-SY5Y cells from both morphological and biochemical changes induced by 24 hr exposure to 500  $\mu$ M 1,3-DNB. Numerous studies have established the ability of astrocytes to modulate cytotoxic responses of neuronal cells, *in vitro*, upon either treatment with astrocyte-conditioned media or through co-culturing systems. In a study on rotenone toxicity, astrocyte-conditioned media protected MN9D neuronal cells from rotenone-induced oxidative stress and loss of cell viability (Cao *et al.*, 2007). Astrocytes were also shown to completely attenuate neuronal cell death resultant from thiamine

deficient culturing conditions (Park *et al.*, 2001). The ability of astrocytes to protect neurons potentially is due the supply of many factors including metabolic substrates, growth factors and cytokines, as well as the building blocks for neuronal oxidative stress defense. Since metabolic disruption and oxidative stress both occur in 1,3-DNB intoxication it is possible that DI TNC-1 cells elicit their protection by one these mechanisms.

Previous research has established that increased lactate production with release into the extracellular environment occurs during 1,3-DNB exposure in susceptible regions of the brain (Ray *et al.*, 1994; Romero *et al.*, 1995; Phelka *et al.*, 2003). This finding was also confirmed using C6 glioma cells as a model for astrocytes (Chapter 3). Though, lactic acidosis is a common event during disruption of aerobic energy metabolism and is believed to be related to disease progression, it has been proposed that increased lactate production is a compensatory action, by astrocytes, in order to supply metabolically deprived neurons with an alternative energy source (Schurr, 2006). This notion is some what controversial but recent evidence suggests that neurons are capable of metabolizing lactate as an alternative carbon source, allowing for the maintenance of energy charge (Deitmer, 2000).

Reductive metabolism of 1,3-DNB, in an aerobic environment, has the potential to produce reactive oxygen species through futile redox cycling. The establishment of an oxidative stress environment during 1,3-DNB exposures has been confirmed with the increased levels of superoxide and depletion of reduced glutathione (Ray *et al.*, 1994; Tjalkens *et al.*, 2000). The ability of astrocytes to regulate the antioxidant potential of neurons has occurs through release of ascorbate and subsequent uptake of

dehydroascorbate, as well as the supply of glutathione precursors, including cysteine and the dipeptides CysGly and  $\gamma$ GluCys (Dringen *et al.*, 1999b; Swanson *et al.*, 2004). The induction of neuronal antioxidant defense may also be a key mechanism underlying protection of SH-SY5Y in co-culturing with DI TNC-1

In addition to the nutritive responses, astrocytes may potentially limit neuronal cytotoxicity through regulation of neuronal excitability, and in doing so reduce neuronal metabolic demand during times of energy crisis. It is well established that adenosine, a ubiquitous purine nucleoside produced from the hydrolysis of ATP, acts as potent and critical modulator of several physiological functions in various systems, particularly those of excitable tissues such as cardiac and brain (Dunwiddie and Masino, 2001). This important signaling molecule mediates its actions through activation of specific cell surface purinergic receptors termed the adenosine receptors. There has been a recent expansion of interest in adenosine receptors due to the therapeutic prospect these receptors may hold in various pathological conditions including diabetes, epilepsy, cancer, neurodegeneration, cardiovascular disease and stroke (Williams and Jarvis, 2000; Stone, 2005).

It is plausible, that in the presence of 1,3-DNB, where ATP production is dramatically hampered, a condition reminiscent of ischemia/hypoxia, similar compensatory actions take place. The ability of the A<sub>1</sub> specific adenosine receptor antagonist, DPCPX, to reverse the protective effects of DI TNC-1 with respect to 1,3-DNB mediated induction of cell death in SH-SY5Y neuroblastomas, supports the potential role of adenosine receptors in the protection elicited by co-culturing. The observation that DPCPX alone increased caspase-3 cleavage suggests a basal tonic



activation of adenosine receptors may occur during physiological conditions. This is supported with the treatment of SH-SY5Y cells with adenosine deaminase, which breaks down adenosine. Treatment with adenosine deaminase resulted in the reduction of basal phospho-ERK expression in SH-SY5Y. Adenosine was also shown to reduce KCl mediated depolarization of SH-SY5Y excitability. Upon addition of adenosine, KCl-mediated calcium waves were not only delayed but overall magnitude was diminished. This response, mediated by adenosine, was due to activation of AR's since almost complete restoration of neuronal excitability was achieved by the non-specific AR antagonist theophylline. Use of the A<sub>1</sub>AR specific antagonist, DPCPX, indicates activation of the A<sub>1</sub>AR receptor as responsible for neuronal silencing. Depression of neuronal activity facilitates neuroprotection through counteracting release of excitotoxic neurotransmitters and continued membrane depolarization by activation ionotropic glutamate receptors (Rudolphi *et al.*, 1992).

Under basal conditions, extracellular concentrations of adenosine have been reported to be in the nanomolar range but upon establishment of a mismatch between energy expenditure and supply, as seen in conditions of increased metabolic activity or in cases of hypoxia or ischemia, the extracellular levels of adenosine dramatically increase (Phillis, 1989). Peak adenosine levels on the order of 3  $\mu$ M have been recorded in the striatum of rats following 24 hr of focal ischemia induced by middle cerebral artery occlusion (Melani *et al.*, 1999). Exposure of the astrocyte-derived cell line DI TNC-1 to 1,3-DNB resulted in significant increases in extracellular adenosine and AMP. Though only nanomolar levels of adenosine were achieved upon treatment with 1,3-DNB this level would still be sufficiently high in order for activation of the A<sub>1</sub>AR (Williams and

Jarvis, 2000; Stone, 2005). Extracellular adenosine can either be produced through direct release of intracellular adenosine or release of adenine nucleotides with subsequent metabolism by ecto-nucleotidases present on the plasma membrane (Martin *et al.*, 2007). The substantial increase in extracellular AMP levels upon treatment with 1,3-DNB may be due to a potential lack of ecto-nucleotidase expression in this cell type. C6 gliomas, another astrocyte-derived cell, do not release adenosine upon ischemic conditions due to the absence of AMP-preferring 5'-nucleotidase expression (Parkinson *et al.*, 2006). Expression profiles for adenine nucleotide metabolizing enzymes are needed for the DI TNC-1 cell line. Alternatively, adenosine is quickly metabolized by adenosine deaminase, which may also be why extracellular adenosine levels were not substantially increased.

Increased extracellular adenosine levels results in activation of AR, followed by induction of suppressive signaling pathways. These pathways result in compensatory actions such as vasodilation, cessation of neurotransmission, and diminution in cellular activity giving adenosine the distinction of a retaliatory signaling molecule (Yoon and Rothman, 1991). With the release of adenosine, the astrocyte, in a sense, strives to protect the functional neurological component, the neuron, through activation of complex pro-survival signaling cascades. The biochemical pathways involved in adenosine's ability to modulate cellular processes, in this perceived cell preservation, are vast, and not completely known. Like other G-protein coupled receptors, AR's regulate intracellular cyclic AMP levels through either activation ( $A_{2a}$  and  $A_{2b}$ ) or inhibition ( $A_1$  and  $A_3$ ) of adenylyl cyclase. Cyclic AMP is an important 2<sup>nd</sup> messenger involved in many aspects of cellular processes including glucose mobilization from glycogen, activation of

transcription factors, regulation of cAMP-gated ion channels and stimulation of specific protein kinases (i.e. protein kinase A) (Liu and Simon, 1996).

Adenosine receptor activation has also been shown to signal through the mitogen-activated protein kinases (MAPK) (Schulte and Fredholm, 2003). MAPKs are a diverse and well-conserved protein family of proline-directed serine/threonine kinases that have been described to control an extensive array of functions including, but not limited to, cell cycle progression, proliferation, and differentiation. The MAPK family is subdivided into three main groups, the stress activated protein kinases (SAPK), the c-jun N-terminal kinases (JNK) and the most widely studied, extracellular signal-regulated protein kinases (ERK). Activation of MAPK leads to phosphorylation of numerous transcriptional factors as well as various other proteins including those involved in regulating cell death (i.e. Bcl-2) (Breitschopf *et al.*, 2000).

The Bcl-2 family proteins have been implicated in the control of cellular sensitivity to endogenous and exogenous stresses. They are believed to regulate the mitochondrial permeability transition (MPT) through intimate interactions with a multi-protein pore (Lemasters *et al.*, 1997). MPT is one initiator of apoptotic cell death pathways. Both anti- (Bcl-2, Bcl-xl) and pro- (Bax, Bid, Bad) apoptotic members of the Bcl-2 family interact with each other to regulate the fine line between the life and death of a cell through modulating opening of the MPT pore. There is considerable evidence that the adenosine receptors may be involved in regulation of apoptosis and necrosis (Walker *et al.*, 1997; Huang *et al.*, 2001; Liu *et al.*, 2002; Lee *et al.*, 2003). Control of apoptosis potentially is through regulation of Bcl-2 family proteins via reversible phosphorylation. Phosphorylation of Bcl-2 family proteins regulates translocation,

degradation and interactions of these proteins with other members of the family (Breitschopf *et al.*, 2000). Bcl-2 family proteins possess protein kinase consensus sites specific for ERK 1/2 suggesting that adenosine-mediated regulation of apoptosis could be due to activation of ERK 1/2 and subsequent phosphorylation of Bcl-2 family proteins. Adenosine has been shown to activate ERK 1/2 in perfused rat heart through activation of all 4 adenosine receptors (Germack and Dickenson, 2004). Similarly A<sub>1</sub>AR activation by the specific agonist R-PIA resulted in the phosphorylation of ERK (1/2) in SH-SY5Y cells. Though a direct link between ERK (1/2) activation and BAD (Bcl-2 associated death protein phosphorylation was not made the temporal expression of phospho-BAD suggests that ERK(1/2) activation is responsible for phosphorylation of BAD. This is also supported by studies into hypoxia induced phosphorylation of BAD in glioblastoma cells where BAD phosphorylation was shown to be mediated by ERK (1/2) activation (Merighi *et al.*, 2007). Phosphorylation of BAD antagonizes its pro-apoptotic function resulting in cellular protection against apoptotic stimuli. Exposure to 1,3-DNB resulted in regional specific activation of ERK 1/2 in the brainstem of rats (unpublished data) supporting a potential role of adenosine signaling in 1,3-DNB mediated neurotoxicity.

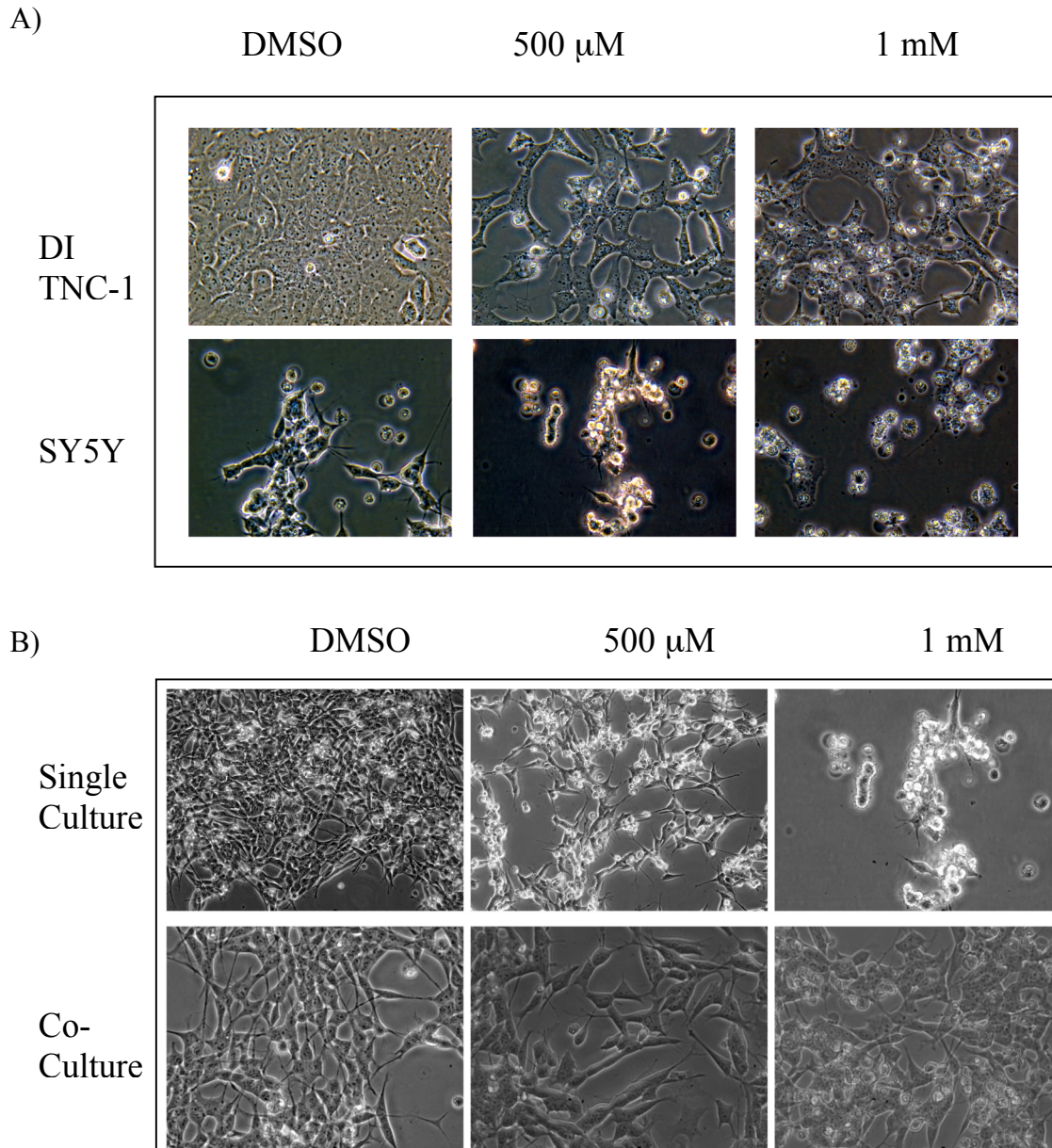
Additional evidence for adenosine receptor signaling playing a role in 1,3-DNB intoxication includes the observed increase in regional blood flow to areas affected by the toxin (Mulheran *et al.*, 1999). In addition to neuroprotection, astrocytes also have been shown to alter endothelial cell structure and function. Nitroaromatic-mediated neurotoxicity, like the related conditions of acute energy deprivation syndromes, appear to involve a strong vascular component, with increases in regional blood flow, reversible breakdown of blood brain barrier function and frequent occurrence of petechial

hemorrhaging. Astrocytes form intimate contact with cerebral vasculature through projections of cellular processes, terminating in the form of end-feet, which engulf the endothelial cells. Astrocyte–endothelial interactions have profound reciprocal effects on each cell type. Astrocytes show inductive function of many BBB features on endothelial cells such as establishment of tight junctions, and expression and polarization of specific transporters (i.e. glucose transporter ; GLUT1).

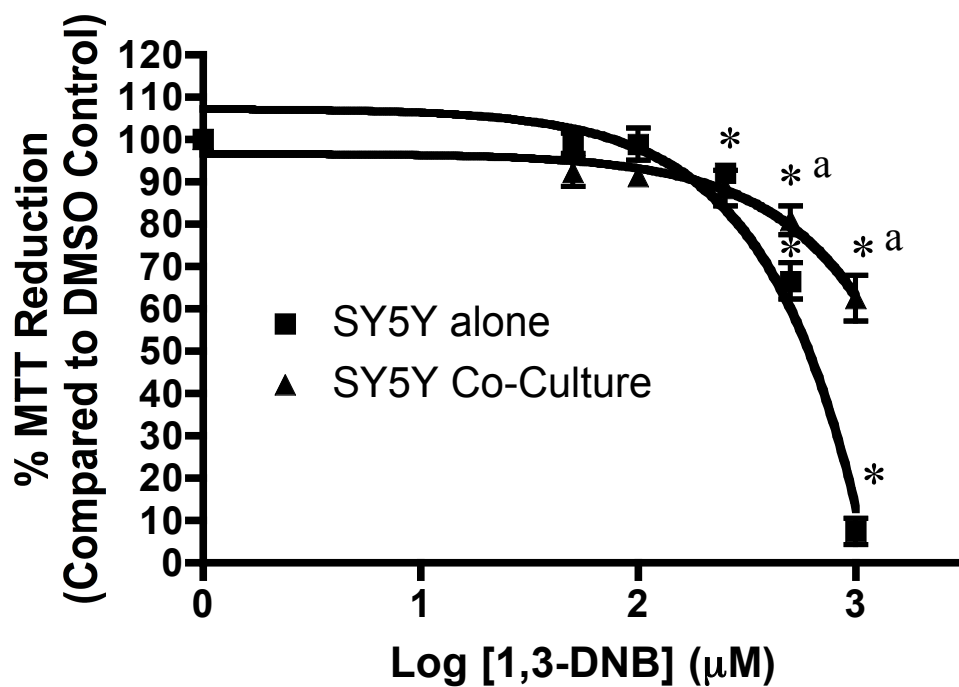
The vasodilatory effects of adenosine receptor activation are well documented and are believed to be mediated through  $A_2$  adenosine receptors. Intrahippocampal injections of a nonspecific agonist to adenosine receptors induce a marked, regional, and sustained increase in cerebral blood flow (CBF). Administration of a select  $A_{2a}$ AR agonist also causes a global increase in CBF (Kochanek *et al.*, 2005). This increase in CBF in 1,3-DNB neurotoxicity is most likely a compensatory action in order to increase the delivery of metabolic substrates to combat the decline in energy charge. In 1,3-DNB intoxication, one key structural change, which occurs as lesion progresses, is the retraction of astrocytic perivascular foot processes. This interruption of the relationship between astrocytes and vascular endothelial cells may be responsible for the BBB breakdown and loss of vascular control. The effects of astrocyte input on vascular endothelial cells were previously studied with respect to 1,3-DNB exposure (Romero *et al.*, 1996). In single cultures astrocytes appear to be substantially more sensitive to 1,3-DNB than endothelial cells. Endothelial cells showed increased severity in 1,3-DNB damage upon culturing with astrocytes. Alteration in basal metabolic rate of endothelial cells was one proposed mechanism. Additionally, activation of  $A_{2a}$ AR has also been shown to mediate astrocyte

proliferation (Hindley *et al.*, 1994), which is also seen upon removal of the toxic insult in 1,3-DNB toxicity.

In conclusion, astrocytes play major roles in both neurophysiology and neuropathology. In the present study the astrocyte cell model DI TNC-1 was able to rescue metabolically impaired SH-SY5Y cells from the cytotoxic effects of 1,3-DNB exposure. This protection maybe due to the nutritive role astrocytes play, either through the maintenance of neuronal ATP pools by shuttling metabolic substrates, or through the induction of increase antioxidant capacity of the neurons. The observation that the adenosine receptor antagonist, DPCPX, was able to block the protective effects of co-culturing suggests that adenosine signaling pathways play a role in the protective mechanism. The diminution of SH-SY5Y excitability, as well as the induction of both phospho-ERK (1/2) and phospho-BAD resulting from adenosine receptor activation are two potential mechanisms astrocyte derived adenosine may preclude cytotoxicity. Further investigation into the complex relationship between astrocytes and neurons in this model of AEDS is required and may lend some insight into the regional and cell specific selectivity of these conditions.

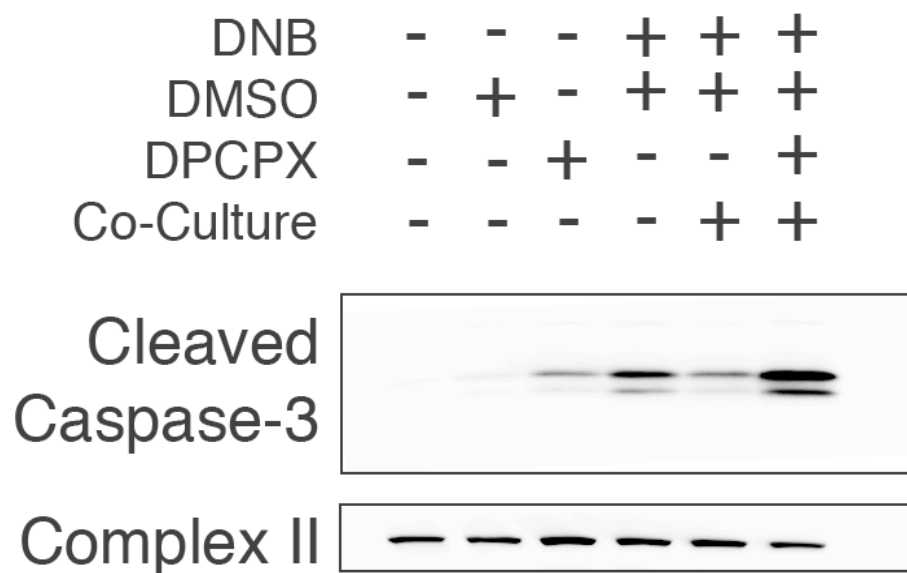


**Figure 4-1.** (A) Representative photomicrographs showing differential sensitivity between SY5Y neuroblastomas and DI TNC-1 astrocytes. Both cell types were dosed with either, DMSO, 500  $\mu$ M 1,3-DNB or 1 mM 1,3-DNB for 24 hr. DI TNC-1 showed the classical changes including cellular swelling and extensive vacuolation at 500  $\mu$ M and increased cell loss at 1 mM. at 500  $\mu$ M 1,3-DNB, substantial loss of SY5Y cells are apparent with almost complete loss at 1 mM 1,3-DNB. (B) Representative photomicrographs depicting the protective effects of co-culturing SY5Y cells with DI TNC-1 cells.



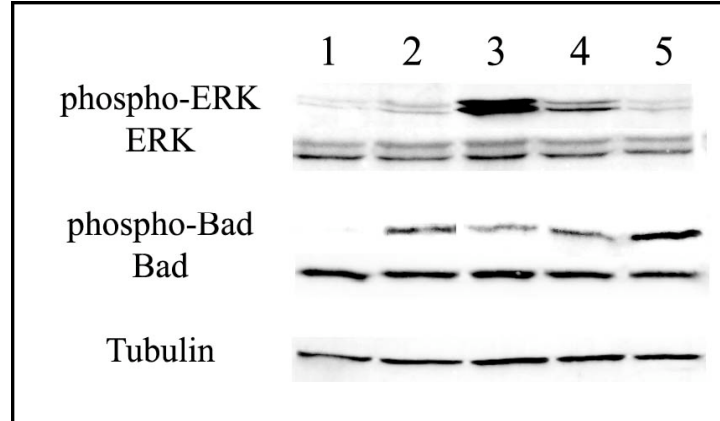
**Figure 4-2.** Co-culturing SY5Y neuroblastoma cells with the astrocyte cell line DI TNC-1 results in significant protection against 24 hr exposure to 1,3-DNB (10 µM – 1000 µM). Cell viability was assessed by MTT reduction. Data presented as mean percent MTT reduction +/- SE compared to DMSO control. (\*) denotes significant difference from DMSO control (P<0.01) (n=6). (a) denotes significant difference from SY5Y alone (P<0.01) (n=6).



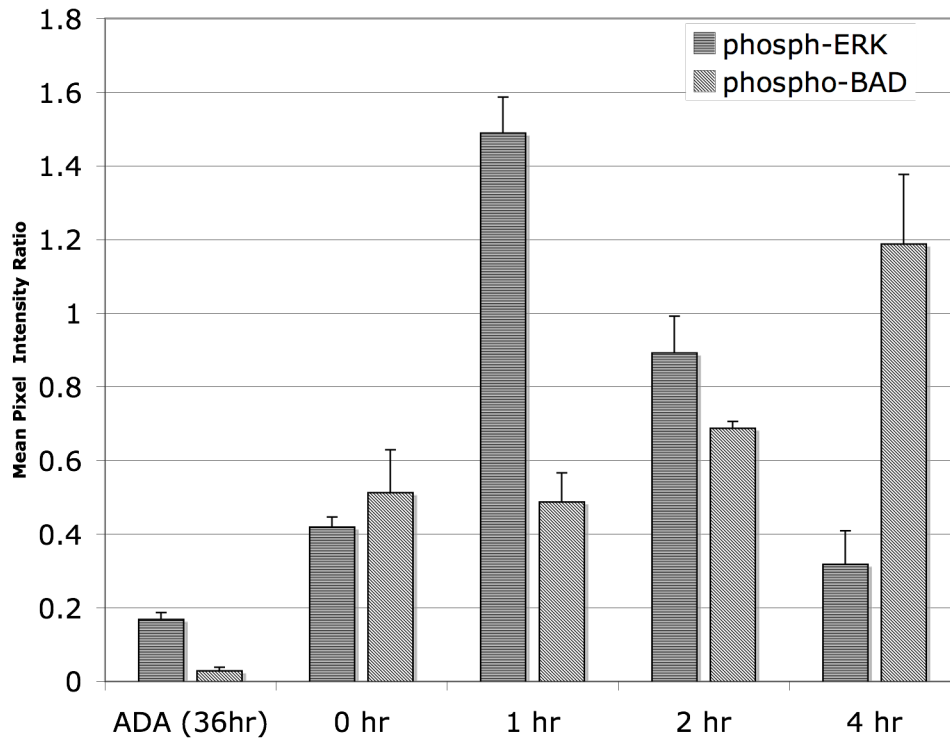


**Figure 4-3.** Co-culturing SY5Y neuroblastoma cells with the astrocyte cell line DI TNC-1 results in significant protection against 1,3-DNB (500  $\mu$ M) induced cell death at 24 hr. Cell death was determined by Western blotting for cleaved caspase-3. Pre-incubation of SY5Y with the specific  $A_1$  adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (250 nm) prevented the protection and resulted in an increased caspase activation.

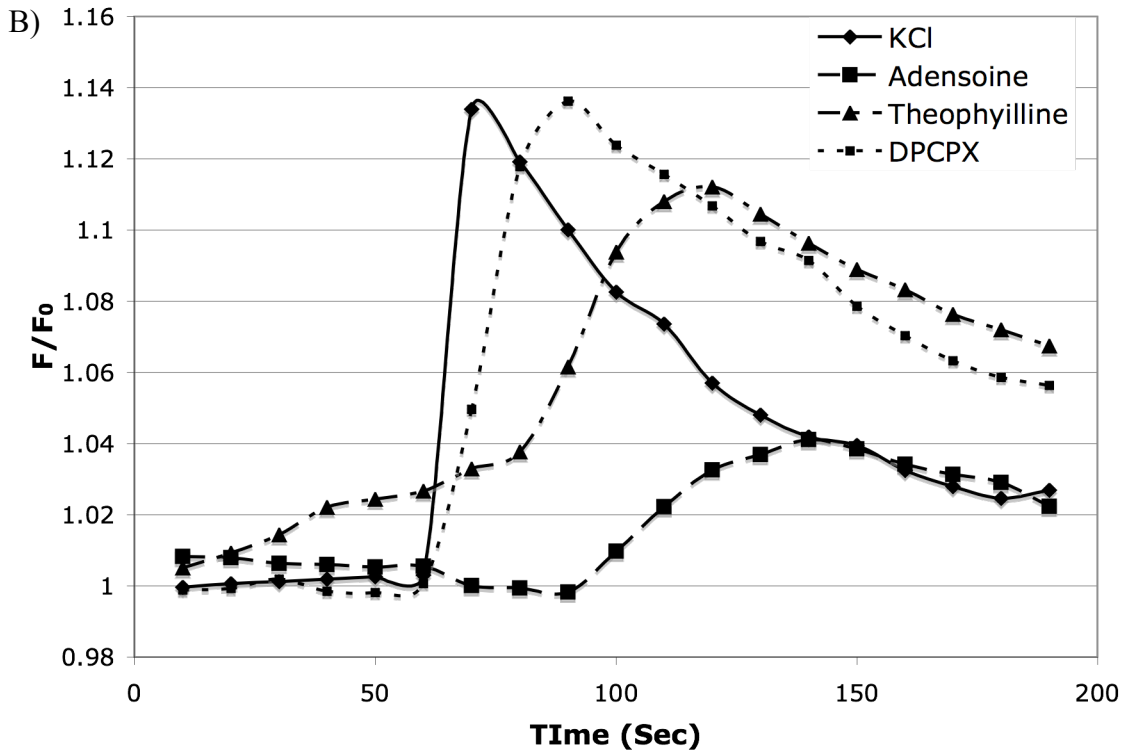
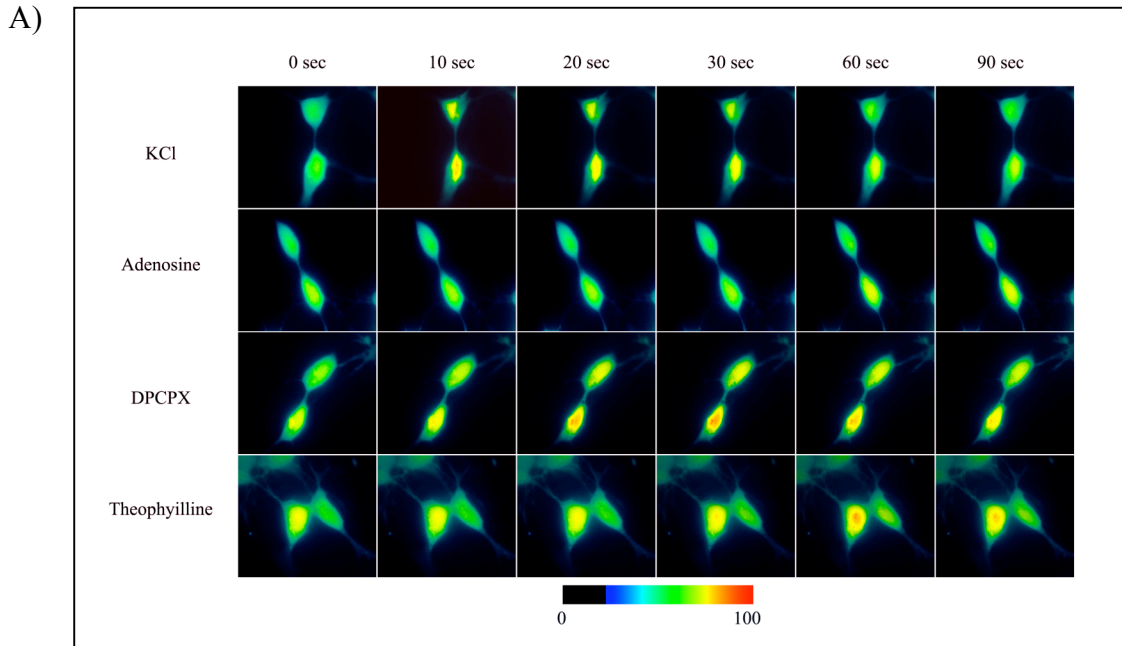
A)



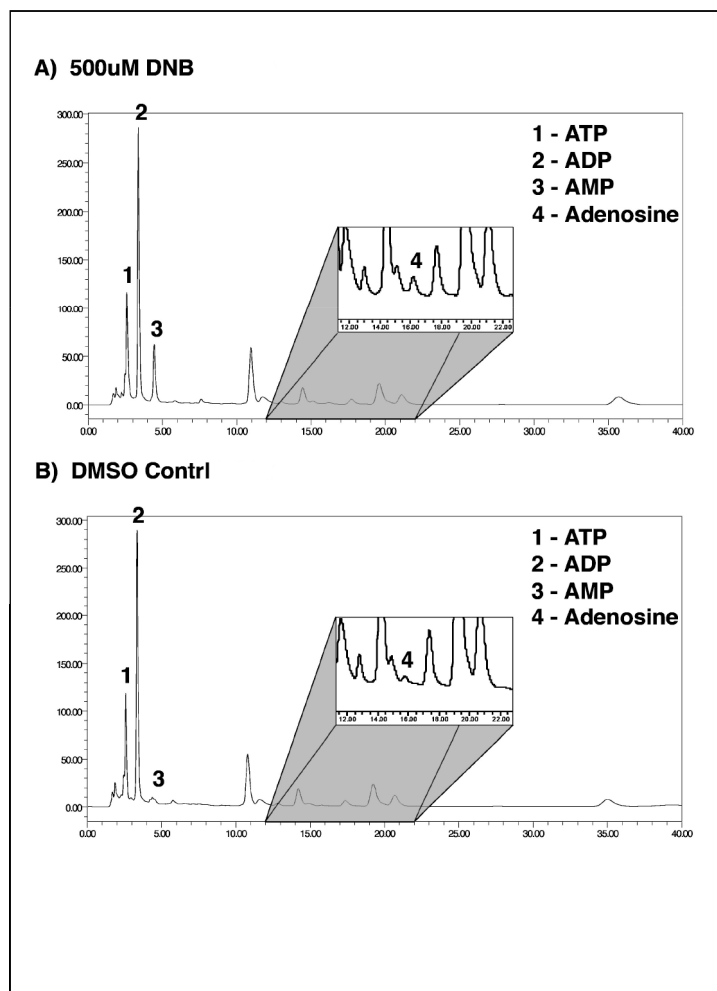
B)



**Figure 4-4.** Activation of A1R in SY5Y neuroblastomas Increases Phospho-ERK and Phospho-Bad Protein Expression (A). Graphical representation of mean pixel intensity ratios normalized to  $\beta$ -tubulin expression. Neurons were incubated with either adenosine deaminase (ADA) (10 U/mL) for 36h or R-PIA (100 nM) for various time points. Lane 1 = ADA; Lane 2 0h R-PIA; Lane 3 1h R-PIA; Lane 4 2h R-PIA; Lane 5 4h R-PIA. ADA = adenosine deaminase; R-PIA = R-Phenylisopropyladenosine



**Figure 4-5.** Adenosine pre-treatment of SY5Y neuroblastomas silences KCl-induced calcium waves. (A) representative, confocal images of calcium wave propagation induced by KCl (25mM). (B) Adenosine pre-incubation (5 nM , 20 min) resulted in the delay of calcium wave induction as well as a reduction in the overall magnitude. These effects were reverse by the adenosine receptor antagonists DPCPX (250 nM) and theophylline (10  $\mu$ M)



**Figure 4-6.** 1,3-DNB (500  $\mu$ M) treatment of the type 1 astrocyte cell line (DI TNC-1) results in increased extracellular concentrations of both AMP and adenosine. Representative HPLC chromatograms of acid extracted extracellular adenylyl purines from cells exposed to either 500  $\mu$ M 1,3-DNB in 0.5% DMSO (A) or 0.5% DMSO alone (B).

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## CHAPTER 5

### DISCUSSION

#### **Conclusions, Limitations, and Future Directions**

The research presented in this dissertation provides strong evidence for the involvement of  $\alpha$ -ketoacid dehydrogenase complexes, specifically the pyruvate dehydrogenase complex (PDHc), in the neurotoxic mechanism of the nitroaromatic compound, 1,3-dinitrobenzene (1,3-DNB). Much of the research surrounding the neurological deficits produced by this important industrial compound has focused mainly on determining the discriminating factors that ultimately are responsible for both the cell-specific and regional susceptibility differences encountered. A great deal of evidence has pointed to selective metabolic disruption compounded by regional metabolic demand but lacking from this body of research are detailed descriptions on the mechanisms by which this particular neurotoxicant may elicit metabolic impairment in the sensitive cells. To further expose, as well as understand what characteristics render one particular cell population prone to injury over another, it is necessary to identify what specific targets in the cell are compromised. By revealing these targets, therapeutic approaches may be devised not only for potential exposure to 1,3-DNB and other nitroaromatic compounds, but more importantly for analogous conditions of acute energy deprivation.

***1,3-Dinitrobenzene selectively inhibited purified pyruvate dehydrogenase complex but did not alter  $\alpha$ -ketoglutarate complex activity while the reductive metabolite 3-nitrosonitrobenzene inhibited both complexes.***

Mitochondrial dysfunction is a common occurrence in the pathogenesis of many diseases that predominantly affect the central nervous system (CNS). Loss of essential metabolic enzymes would greatly hamper the ability of the cell to maintain sufficiently high ATP levels to function properly. Pyruvate dehydrogenase complex (PDHc) is a gatekeeper for the TCA cycle, controlling entry of pyruvate into mitochondrial energy metabolism (Yeaman, 1989). Disruption at this site would result in cessation of oxidative energy metabolism and ultimately result in neuropathological responses. Defects in this enzyme have been implicated in numerous diseases of the CNS, most notably, cerebral ischemia/reperfusion and various neurodegenerative diseases (Mizuno *et al.*, 1994; Kish, 1997; Martin *et al.*, 2005).

1,3-Dinitrobenzene is one of many metabolic disruptors known to induce neuropathology reminiscent of acute energy deprivation syndromes. In purified enzyme preparations, this neurotoxic chemical resulted in the rapid and dose-dependent inhibition of PDHc (Chapter 2). Interestingly, 1,3-DNB did not substantially affect  $\alpha$ -ketoglutarate dehydrogenase complex (KGDHc) suggesting a fundamental difference between the two complexes. In contrast to 1,3-DNB, the reductive metabolite, 3-nitrosonitrobenzene (3-NNB) showed no discrimination between the two complexes with respect to its ability to inhibit them. 3-nitrosonitrobenzene maybe able to directly interact with the complex while 1,3-DNB needs to undergo metabolism prior to inhibition. The observation that 1,3-DNB did not effectively inhibit KGDHc precluded any non-specific effects of 1,3-DNB on components of the assay, such as cofactors, since all reaction components but

the specific  $\alpha$ -ketoacid were exactly the same. This conclusion points a direct interaction of 1,3-DNB with a component of the complex.

Future studies into the detailed mechanism of inhibition of the  $\alpha$ -ketoacid dehydrogenase complexes by both 1,3-DNB and 3-NNB are needed. Utilizing protein mass spectrometry, it may be possible to determine the specific site of interaction between the inhibitor and complex. Additionally, the differential effect of 1,3-DNB on the two enzymes raises interesting mechanistic questions and offers a powerful tool to probe differences between these two extremely similar enzyme complexes. One such potential difference is the absence of the E3 binding protein (E3BP) component in KGDHc. The lack of this component may render KGDHc insensitive to inhibition by 1,3-DNB. It would be of great interest to determine the effects of 1,3-DNB on the branch chain  $\alpha$ -ketoacid dehydrogenase complex, which, similarly to KGDHc, lacks an E3 binding protein (E3BP). This would allow for the further investigation into the role of the E3BP component in the reduction of PDHc activity by 1,3-DNB. Along the same lines, using PDHc from gram-negative bacterium (*Bacillus stearothermophilus*), which also does not possess an E3BP, would also lend insight into the mechanism of inhibition. The presence of the E3BP, in PDHc, may allow for shuttling of reductive metabolites including 3-NNB, if the E3 component (Lipoamide Dehydrogenase) is the enzyme responsible for reductive metabolism of 1,3-DNB. Lipoamide dehydrogenase has previously been shown to possess nitroreductase activity (Tsai, 1987) but whether this enzyme is responsible for reductive metabolism of 1,3-DNB or other nitroaromatic compounds remains to be shown. The potential involvement of the E3BP in the inhibitory function of 1,3-DNB on PDHc would also provide a means for studying the role of E3BP in PDHc

catalysis. The function of E3BP is believed to be purely structural despite the presence of a bound lipoic acid, due to the absence of detectable transacetylase activity (Harris *et al.*, 1997). The toxicant 1,3-DNB may potentially be used as a tool to probe the E3BP's role in catalytic function of the pyruvate dehydrogenase complex.

***Pyruvate dehydrogenase complex is inhibited in an isomer-specific manner by dinitrobenzene.***

A curious feature of 1,3-DNB is that it is the only isomer of dinitrobenzene that confers neurotoxicity. It has been proposed that the isomer specificity with respect to induction of neuropathology is mainly due to the preferred routes of metabolism each isomer undergoes (Nystrom and Rickert, 1987). The 1,2- isomer predominantly undergoes conjugation with GSH while 1,4-Dinitrobenzene undergoes rapid reduction followed by conjugation. In contrast to these two isomers, 1,3-DNB exclusively undergoes nitroreduction. Interestingly, both the 1,4- and 1,3- isomers induce methemoglobinemia with the 1,4- being more potent (Cossum and Rickert, 1985). This precludes the hypothesis that methemoglobinemia plays a role in 1,3-DNB induced neurotoxicity. Examination of the effects of the three different isomers on PDHc activity showed that both the 1,2- and 1,3- isomers inhibit the complex equally but that the 1,4- isomer showed greatly diminished inhibitory action towards this enzyme complex. Isomer specificity was also shown to play a role in PDHc mediated production of N-hydroxy-N-arylacetamides, in the presence of various nitroso-aromatics (Yoshioka and Uematsu, 1993; Yoshioka *et al.*, 1996). In these studies it was established that bulky para-substituents on the aromatic ring of nitrosoaromatics reduced the efficiency by which these compounds could be metabolized to their respective N-hydroxy-N-

arylacетamide products. This selectivity was attributed to the steric hindrance in the E1 active site of PDHc. This similarity between the metabolism of nitrosoaromatics and the inhibition by 1,3-DNB suggests a common mechanism of action and points to the E1 active site as the point of inhibition. This hypothesis is further supported by the ability of 3-NNB to potently inhibit the function of the E1 component when separated from the complex.

Future studies examining the ability of 1,3-DNB and other nitroaromatics to act as substrates in the production of N-hydroxy-N-arylacетamides are needed. This could be accomplished through quantification of the arylacетamide product by HPLC (Yoshioka and Uematsu, 1993; Yoshioka *et al.*, 1996). Additionally, it would be of interest to quantify their production in both cell culture and in whole animal as a potential marker to probe if regional metabolism of these nitroaromatic compounds occurs. There may also exist differential cellular capability to metabolize these compounds that could confer the cell specific sensitivity observed in 1,3-DNB neurotoxicity.

***Irreversible inhibition of PDHc by 1,3-dinitrobenzene required both the presence of thiamine pyrophosphate and pre-activation by the substrate pyruvate.***

Incubation of PDHc with high concentrations of 1,3-DNB followed by the effective dilution of the inhibitor resulted in the degree inhibition corresponding to the diluted concentration. This finding was initially assumed to mean that 1,3-DNB did not irreversibly inhibit the complex through covalent binding. Subsequent experiments, investigating the requirement of substrates for inhibition, confirmed that the initial assumption was wrong. In the presence of both thiamine pyrophosphate and pyruvate, the level of inhibition achieved corresponded to the initial concentration of inhibitor.

This supports the notion that 1,3-DNB effectively inhibits the complex through irreversible binding. It also suggests that the 2-hydroxyethylidene-TPP carbanion formed after decarboxylation of pyruvate maybe the predominant site affected by nitroaromatic compounds. The mechanism of how this may occur is not entirely clear. The carbanion is a potent nucleophile that can react strongly with an electrophile. This carbanion normally undergoes nucleophilic attack of the oxidized lipoic acid on the E2 component of the enzyme. The strong electrophilic nature of 3-NNB predisposes this compound to attack by the bound carbanion. This explains the ability of 3-NNB to potently inhibit the separated E1 component enzyme. Additionally it also explains why 1,3-DNB was less effective in inhibiting this reaction. The observation that the overall reactivity of the complex was inhibited to a greater extent by 1,3-DNB compared to the E1 component suggests a requirement of the other components, potentially metabolizing 1,3-DNB to a species capable of undergoing nucleophilic attack by the E1 bound carbanion.

Future studies into the involvement of the 2-hydroxyethylidene-TPP in inhibition of PDHc by 1,3-DNB maybe conducted using proton NMR ( $H^1$  NMR). Previous studies investigating the catalytic cycle of thiamine dependent enzymes showed that  $H^1$  NMR could be use to detect reaction intermediates during catalytic function of pyruvate oxidase (Wille *et al.*, 2006). Pyruvate oxidase catalyzes the same initial reaction that PDHc-E1 catalyzes, namely, decarboxylation of pyruvate to form a 2-hydroxyethylidene-TPP carbanion. The ability of 1,3-DNB or 3-NNB to interact with the carbanion should be able to be determined using  $H^1$  NMR with a change in chemical shift corresponding to the adduct.

***1,3-Dinitrobenzene induced metabolic impairment in the astrocyte-derived cell line C6 glioma.***

Lactic acidosis is a presenting feature in many metabolic disorders affecting mitochondrial aerobic energy metabolism. Increased concentrations of extracellular lactate in susceptible regions of CNS during 1,3-DNB induced encephalopathy have been previously observed (Phelka *et al.*, 2003), suggesting that disruption of mitochondrial metabolic enzymes underlie the resultant neuropathology. Similarly, using the astrocyte-derived cell model, C6 gliomas, 1,3-DNB exposure produced dose-dependent increases in lactate production and release (Chapter 3). Though *in vitro* models do not always mimic the responses seen in the whole animal, the ability of 1,3-DNB to elicit similar biochemical responses, in culture, support the notion that the initial biochemical defect responsible for cytotoxicity of astrocytes is most likely the same in both *in vivo* and *in vitro* situations. This conclusion allows for the use of this particular model to examine metabolic targets affected by 1,3-DNB. Though the initial target maybe the same, due to the intimate relationship of cell types within the CNS, and the ability of intercellular signaling to modulate cellular responses, especially with respect to energy metabolism, the progression of cytotoxicity potentially cannot be studied without proper inputs of neighboring cell types. An additional limitation is the use of a cancer cell line as a model for astrocytes. Though cancer cell lines offer many advantages over both primary cells and *in vivo* models, namely time and expense, there are some disadvantages that must also be considered. The most important concern when using cancer cell lines is loss of cellular characteristics that define the cell from which they were derived. One example of a characteristic change between cancer cell lines and primary cells is energy metabolism. Altered glycolytic rates and changes in glycogen utilization have both been



described in glioma cell lines (Galarraga *et al.*, 1986). The differences between cancer cell lines and primary cells must be carefully considered when interpreting data.

To further establish C6 gliomas as a representative model for astrocytes in 1,3-DNB neurotoxicity. Morphological changes were examined upon exposure to 1,3-DNB. Astrocytes in sensitive regions in the CNS undergo distinctive morphological changes that include edematous swellings, extensive vacuolation, and retraction of peri-vascular and peri-neuronal end foot processes (Philbert *et al.*, 1987). Similar morphological changes were observed in culture using C6 gliomas with extensive and progressive vacuolation being the most pronounced alteration. Cellular swelling occurred to some degree as cytotoxicity progressed, potentially due to the accumulation of lactic acid in the extracellular environment with subsequent acidification. Astrocytes are exquisitely sensitive to acidosis induced cellular swelling, specifically when mediated by lactic acid (Staub *et al.*, 1990). With similar changes in both morphology and biochemical parameters, C6 gliomas appeared to be a sufficient model to examine biochemical targets of 1,3-DNB. In addition to early biochemical changes, significant changes in the morphology of astrocytes occur in the CNS of 1,3-DNB and other AEDS.

***The alternative energy substrates acetoacetate and acetyl-carnitine protected C6 glioma from the cytotoxic effects of 1,3-DNB supporting PDHc as the site of interference.***

The ability of the alternative fuel sources, acetoacetate and acetyl-carnitine, to reverse many of the morphological and biochemical changes induced by 1,3-DNB points to the primary defect being prior to TCA cycle metabolism, due to the fact that both of these substrates can be converted to acetyl-CoA and subsequently enter metabolism at the point of citrate synthase (Edmond, 1992; Zanelli *et al.*, 2005). Under normal

physiological conditions, the human adult brain, exclusively, or at least preferentially oxidizes glucose to meet its energetic needs. During postnatal development or periods of starvation the brain can adapt to the utilization of ketone bodies to maintain energy charge. The ketone body acetoacetate is converted to two molecules of acetyl-CoA. Similarly, Acetyl-carnitine has also been shown to serve as an exogenous source of acetyl-CoA. This process has been exploited in order to deliver energy substrates during times of energy deprivation, including ischemia/reperfusion injury as well as pyruvate dehydrogenase deficiency (Weber *et al.*, 2001; Zanelli *et al.*, 2005). The inability of free acetate and L-carnitine to confer protection, points to the availability of the acetyl moiety for usage in energy metabolism as the neuroprotective mechanisms of these substrates. Though it is most likely the conversion of acetyl-carnitine to acetyl-CoA and further oxidation through the TCA cycle that cause the reduction in lactate concentrations and maintenance of ATP levels, other potential mechanism maybe in involved. Acetyl-carnitine has been shown to reduce oxidative stress in cerebral ischemia/reperfusion, as well as with treatment of A-beta amyloid through either direct reduction of mitochondrial redox state or indirectly through induction of antioxidant genes (Liu *et al.*, 1993; Calabrese *et al.*, 2005). There is the potential that acetyl-carnitine may protect against the effects of 1,3-DNB induced oxidative stress instead of through direct action as a metabolic substrate.

***Exposure of C6 glioma cells to 1,3-DNB resulted in loss of PDHc and KGDHc activity potentially through modification of protein bound lipoic acid.***

Cytochemical quantification of residual PDHc and KGDHc activity in the C6 glioma cell line showed dose dependent reductions in both PDHc and KGDHc activity. The differential sensitivity between the two complexes observed with purified enzymes mimics the results obtained from intact cells with PDHc again being more sensitive to inhibition. At high concentrations of 1,3-DNB, KGDHc in cells was inhibited to a greater extent than observed with the purified complex. Potentially, cell-mediated bioactivation of 1,3-DNB to its reductive metabolite, 3-nitrosanitrobenzene (3-NNB), resulted in the increased inhibition seen in culture, compared to the purified enzyme. This is supported by the significantly increased inhibitory effects of 3-NNB on purified enzyme preparations of KGDHc. Alternatively, oxidative stress due to redox cycling of 1,3-DNB, in intact cells, may underlie the increased inhibition of KGDHc.

Though 3-NNB showed strong inhibitory action toward the separated E1 component, the observation that 3-NNB was able to interfere with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) metabolism by PDHc also supports the interaction of 3-NNB with the reduced thiols of protein bound lipoic acid. Numerous nitrosoaromatics have been shown to react strongly with reduced thiols, such as those present in reduced glutathione (Eyer, 1979; Eyer and Schneller, 1983; Umemoto *et al.*, 1988; Cribb *et al.*, 1991). Adduction of reduced lipoic acid is a common mechanism of inhibition. This site is believed to be the point of impairment by many arsenical compounds, which are classic inhibitors of PDHc. The ability of 1,3-DNB exposure to result in changes in the lipoic acid site of PDHc was demonstrated through using an antibody that specifically recognizes native lipoic acid. This antibody has previously been used to explore the effects of 4-hydroxy-2-nonenal (4-HNE) in which modification of lipoic acid by 4-HNE

resulted in loss of immunoreactivity (Humphries and Szweda, 1998). Likewise, exposure of C6 glioma cells to 1,3-DNB resulted in loss of immunoreactivity in both a time and dose dependent manner indicating that modification of lipoic acid occurs during exposure to 1,3-DNB. Interestingly, the treatment of C6 gliomas with two classic arsenic containing compounds, phenyl arsine oxide, and arsenic trioxide, at concentrations that have previously been shown to completely inhibit the complex (Samikkannu *et al.*, 2003), showed no significant loss of lipoic acid immunoreactivity, even though significant loss of cell viability was apparent. The reason for this observation is unclear, but it does suggest that 1,3-DNB potently affects lipoic acid and further supports the hypothesis that 1,3-DNB renders it inhibitory action through modification of protein bound lipoic acid.

In Chapter 2, it was established that 1,3-DNB and its metabolite, 3-NNB, effectively inhibit the PDHc complex in purified enzyme preparations. The inhibitory actions of 1,3-DNB on PDHc were confirmed in intact cells. This inhibition appears to be a result of protein modification of E2 bound lipoic acid. Exposure of C6 gliomas to 1,3-dinitrobenzene resulted in a dose- and time-dependent reduction in lipoic acid immunoreactivity. This loss in immunoreactivity correlated with the increase in protein carbonylation of mitochondrial proteins that correspond to the molecular weights of the E2 components of both PDHc and KGDHc. Protein oxidation and subsequent protein dysfunction is a common occurrence during oxidative stress. Numerous proteins are predisposed to oxidation including those that possess critical cysteines due to the susceptible sulfur atom (Shacter, 2000). Protein oxidation can introduce carbonyl groups into the protein, which can be detected through derivatization by 2,4-

dinitrophenylhydrazine to the corresponding hydrazone. The proteins that are carbonylated are then probed using a specific antibody that recognizes the 2,4-dinitrophenyl (anti-2,3-DNP) group attached to the protein (Keller *et al.*, 1993). The increased carbonylation of proteins corresponding to the molecular weight of the E2 components suggests that loss of lipoic acid is potentially due to increased protein oxidation after induction of oxidative stress by 1,3-DNB. The inability of both antioxidants and thiol containing compounds to attenuate the loss of LA immunoreactivity contradicts this conclusion. Though protein not derivatized by 2,4-dinitrophenylhydrazine shows no reactivity with the anti-2,4-DNP antibody there may be the potential for 2,4-dinitrophenylhydrazine to react with the 1,3-DNB adduct resulting in detection by the antibody. In order to confirm that the loss of LA was not due to oxidation by lipid peroxidation products, total cellular protein was probed with an anti-4-hydroxy-2-nonenal antibody. No significant dose-dependent bands were detected. This does not rule out other lipid peroxidation products from being responsible for the LA loss or for direct oxidation by reactive oxygen species but it would be expected that if lipid peroxidation did occur, 4-hydroxy-2-nonenal adducts would be apparent.

Future studies are required in order to describe the exact mechanism of inhibition of PDHc by 1,3-DNB. Though the bands that possess carbonylated proteins correspond to the molecular weights of the E2 components of PDHc and KGDHc, 2D gel electrophoresis followed by protein mass spectrometry is needed to definitively identify these proteins. Additionally, mass spectral analysis may allow for the identification of the exact adduct responsible for loss of lipoic acid immunoreactivity. It would also be of interest to look for loss of lipoic acid immunoreactivity and carbonylated proteins using

Western blot analysis in brain tissue samples from rats exposed to 1,3-DNB. This could also be done using immunohistochemistry in order to correlate regional distribution of lipoic acid loss and carbonylation with lesion presentation.

***Co-culturing with DI TNC-1 cells protected against the cytotoxic effects of 1,3-DNB on SH-SY5Y.***

In addition to uncovering metabolic targets of 1,3-DNB, responsible for induction of acute energy deprivation, this thesis also examines the role astrocytes play in shielding neurons from the cytotoxic effects of metabolic disruption. A hallmark of acute energy deprivation syndromes is the relative sparing of neurons prior to substantial loss of the supportive astrocyte cells (Philbert *et al.*, 1987; Cavanagh, 1988; Cavanagh, 1993). Though astrocytes have traditionally been thought of as being less susceptible to cellular stresses than their neuronal counterparts there is growing evidence that astrocyte dysfunction may precede neuronal loss in numerous neurological disorders including focal ischemia and neurodegenerative diseases (Liu *et al.*, 1999; Maragakis and Rothstein, 2006). Several studies have shown the ability of astrocytes to modulate the toxic responses of both neuronal and endothelial cells (Chen *et al.*, 2001; Swanson *et al.*, 2004; Rao *et al.*, 2005; Cao *et al.*, 2007). Utilizing the type 1 astrocyte-derived model, DI TNC-1, and the neuronal SH-SY5Y neuroblastoma, the interactions of astrocytes and neurons during conditions of metabolic impairment were studied. In single culture, SH-SY5Y cells were highly sensitive to the cytotoxic effects of 1,3-DNB including loss of mitochondrial reducing potential, substantial morphological changes as well as the induction of cell death as determined by caspase-3 activation. Contrary to observations *in vivo*, the astrocyte-derived DI TNC-1 cells, in single culture, are relatively insensitive

compared to SH-SY5Y. Co-culturing SH-SY5Y cells with DI TNC-1 cells, protected SH-SY5Y cells from the deleterious effects of 1,3-DNB.

In the co-culture studies conducted, transwell inserts were used, which allowed for soluble factors to reach both cell type, but prevented direct contact between the cells. *In vivo*, astrocytes and neurons form intimate contacts and it would be interesting to determine if direct contact between the cell types altered the responses of each cell type to 1,3-DNB. In culture this would be somewhat difficult due to the inability to differentiate individual cell type responses but the use of acute brain slices may offer a better model to investigate intercellular communication in pathology progression during 1,3-DNB intoxication. In addition to using brain slices, primary cells may also offer a better understanding of the complex relationships between astrocytes and neurons. Cell lines offer many advantages over both primary cell culture and *in vivo* studies but a major concern when using cell lines is the loss of characteristics that define the cell they are supposed to model. An example of this that is relevant to studies conducted in this thesis is the observation that C6 glioma cells unlike primary astrocytes, do not release adenosine due to the absence of an AMP-preferring 5' nucleotidase expression (Parkinson *et al.*, 2006). Use of C6 gliomas to study intercellular adenosine signaling may have resulted in completely different results and altered conclusions. Future studies will employ either primary cells or brain slices to better mimic the response in the whole animal.

***Blockage of the A<sub>1</sub> adenosine receptor negated the protective effects of co-culturing SH-SY5Y cells with DI TNC-1***

The use of the A<sub>1</sub> adenosine receptor antagonist (DPCPX) reversed the protective mechanism of co-culturing SH-SY5Y cells with DI TNC-1 cells, suggesting that this potent intercellular signaling molecule plays a key role in the neuroprotection afforded by astrocytes. The retaliatory function of adenosine is well established (Kochanek *et al.*, 2005; Stone, 2005). During times of metabolic imbalance, such as seen in ischemia, extracellular adenosine concentrations dramatically increase resulting in the activation of corresponding adenosine receptors. Treatment with the metabolic disruptor, 1,3-DNB increased extracellular adenosine, as well as AMP concentrations. Though the increase in adenosine concentrations were only in the nanomolar range, this increase is significant enough to result in the activation of the A<sub>1</sub> receptor. Increases in extracellular adenosine levels are achieved either through release of intercellular adenosine or release of adenine nucleotides with subsequent metabolism by ectonucleotidases present on the plasma membrane. The substantial accumulation of AMP maybe a result of low expression of ecto-nucleotidases in DI TNC-1 cells that would normally metabolize AMP to adenosine. Alternatively the slight increase in adenosine maybe the result of rapid metabolism of adenosine by adenosine deaminase.

Further studies into the expression profile for enzymes and transporters involved in regulating adenosine levels are needed. It would also be of interest to see if activation of A<sub>1</sub> adenosine receptor in single cultures of SH-SY5Y cells is sufficient to protect these cells from 1,3-DNB toxicity.



***Activation of A<sub>1</sub> adenosine receptors in SH-SY5Y cells resulted in the induction of pro-survival mechanisms.***

Activation of the A<sub>1</sub> adenosine receptor acts to silence neuronal activity in the effort to reduce metabolic demand. This neuroprotection is mediated by many intracellular signaling pathways that result in hyperpolarization of neurons, decreased neurotransmitter release, as well as induction in the expression of pro-survival proteins (Drury and Szent-Gyorgyi, 1929; Dunwiddie and Masino, 2001; Kochanek *et al.*, 2005). All three pathways were shown to occur in SH-SY5Y neuroblastoma cells upon activation with either adenosine or the A<sub>1</sub> specific agonist R-PIA. Treatment of SH-SY5Y cells with R-PIA resulted in the phosphorylation of the extracellular signal-regulated protein kinase (ERK), a member of the mitogen activated protein kinase (MAPK) family. MAPK are involved in a wide array of cellular processes including cell cycle progression, proliferation, differentiation and apoptosis (Grewal *et al.*, 1999). Activation of MAPK results in the phosphorylation of numerous transcriptional factors as well as various other proteins most notably those involved in regulating cell death (i.e. Bcl-2 family proteins) (Breitschopf *et al.*, 2000). Interestingly, addition of adenosine deaminase resulted in loss of phospho-ERK suggesting a basal activation by adenosine of the ERK pathway. Subsequent to ERK activation, phosphorylation of the pro-apoptotic Bcl-2 family protein Bad (Bcl-2 associated death protein) occurs. The balance between anti-apoptotic (Bcl-2, Bcl-xl) and pro-apoptotic (Bax) members of the Bcl-2 family proteins is believed to regulate apoptosis. Active Bad binds to Bcl-xl, antagonizes the anti-apoptotic action of this protein. Upon induction of cell survival pathways Bad can be phosphorylated resulting in its sequestration by 14-3-3 in the cytosol (Zha *et al.*, 1996). Two known pathways for Bad phosphorylation are through the PI3K/AKT

pathway and ERK. Though a direct link between ERK activation and Bad phosphorylation was not made in the current study, the ability of ERK to phosphorylate Bad has been well established (Jin *et al.*, 2002). The temporal expression profile also supports the involvement of ERK in Bad phosphorylation.

Future studies are needed to uncover the detailed intracellular signaling pathways responsible for the neuroprotection observed with co-culturing astrocytes with neurons. Use of a MEK inhibitor, which is the upstream kinase responsible for activation of ERK, will determine if ERK activation is necessary for Bad phosphorylation. Modulating expression levels of Bad through knockdown as well as over-expression of the protein will also help determine Bad's role in controlling induction of cell death in 1,3-DNB exposure.

In addition to induction of anti-apoptotic signaling pathways, adenosine receptor activation results in cessation of neural transmission. In the present study, adenosine silenced KCl- induced calcium waves in SH-SY5Y cells. The action of adenosine was determined to be solely mediated through activation of the A<sub>1</sub> adenosine receptor due the restoration of neuronal excitability with treatment of the A<sub>1</sub> specific antagonist, DPCPX. Depression of neuronal activity mediates neuroprotection through counteracting release of potentially excitotoxic neurotransmitters as well as preventing persistent membrane depolarization through activation of ionotropic glutamate receptors. Though these studies show the potential involvement of purinergic signaling in the neuroprotection during 1,3-DNB exposure, further investigation into the role adenosine signaling may play in the cell and regional sensitivity of AEDS is required.

The use of brain slices maybe useful in determining if neuronal silencing mediated the observed neuroprotection in the co-culture studies. Measuring neuronal depolarization after 1,3-DNB treatment would show if neuronal activity was depressed and use of adenosine receptor antagonists would determine if the depression was mediated through adenosine.

### ***Overall Significance***

Most of the studies on 1,3-DNB have concentrated on determining the underlying factors of the regional and cell specific sensitivity observed with this neurotoxicant. This thesis is the first attempt at identifying direct targets of 1,3-DNB that are responsible for the metabolic disruption encountered in 1,3-DNB induced encephalopathy. The  $\alpha$ -ketoacid dehydrogenase complexes were demonstrated to be specific metabolic targets affected in 1,3-dinitrobenzene exposure. Through enzymatic analysis of purified protein, 1,3-DNB was shown to directly inhibit the pyruvate dehydrogenase complex, which was further confirmed in an astrocyte cell model, C6 glioma. In this cell type, both PDHc and KGDHc were significantly affected during acute exposure to 1,3-DNB. The inhibition of these complexes explains the biochemical changes that occur upon exposure to 1,3-DNB. The loss of lipoic acid immunoreactivity points to chemical modification of this essential cofactor. The inability of antioxidants and thiol containing compounds to attenuate this loss of LA suggests direct modification rather than resulting from induction of oxidative stress. Further investigations into the mechanism of inhibition within intact cells, as well as examination of these enzyme complexes *in vivo* during 1,3-DNB exposure are required.

Additionally, this thesis presents the first data investigating the role of astrocyte/neuronal relationships in the cell specific sensitivity to 1,3-DNB. The relative sparing of neurons in acute energy deprivation syndromes maybe due to the shielding of neurons from metabolic impairment rather than an innate insensitivity of neurons to the effects of energy deprivation. This hypothesis is supported by the exquisite sensitivity of the neuronal cell model, SH-SY5Y neuroblastomas, to the cytotoxic effects of 1,3-DNB when cultured alone and the extensive neuroprotection afforded through culturing with the astrocyte model, DI TNC-1. This protection was shown to be mediated, at least in part, through the activation of adenosine receptors. Indeed, further study on the intercellular relationship between neurons and astrocytes during acute energy deprivation is required, though the possibility for exploitation of purinergic signaling as a potential therapeutic route for treatment of AEDS is appealing.

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