THE ROLE OF ALDEHYDE DEHYDROGRENASE 2 IN NITRATE TOLERANCE: INVESTIGATION OF LOW POTENCY NITRATES AND CROSS TOLERANCE

by

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Abstract

Organic nitrates such as glyceryl trinitrate (GTN) are commonly used to treat cardiovascular disease. They are prodrugs that require bioactivation for pharmacological activity. Currently, there are two proposed pathways of organic nitrate activation: a high-affinity pathway for high-potency nitrates (GTN and pentaerythrityl tetranitate) and a low-affinity pathway for low-potency nitrates (isosorbide dinitrate (ISDN) and isosorbide mononitrate). A major limitation in the utility of organic nitrates is the rapid onset of tolerance during chronic treatment. Inhibition of the enzymes responsible for bioactivation has been put forward as the major cause of tolerance, and aldehyde dehydrogenase 2 (ALDH2) has been proposed as the primary enzyme responsible for bioactivation in the high-affinity pathway. ALDH2 activity is decreased in GTN-tolerant tissues and it has been suggested that ALDH2 inactivation is the underlying cause of GTN tolerance. However, several aspects of this hypothesis are problematic. My objective was to develop an *in vivo* model of ISDN tolerance and to use this model to examine the effects of chronic ISDN treatment on ALDH2 activity and on organic nitrate-induced vasodilation. The hypothesis of my research is that the reduced potency of GTN caused by ISDN tolerance is mediated by a mechanism independent of ALDH2 inactivation. ISDN tolerance was induced in rats using an *in vivo* model in which animals were exposed to 0.8 mg/hr ISDN for 24-96 hr. ALDH2 activity was measured in liver mitochondrial fractions and tolerance was assessed in isolated aortic preparations. There was no inhibition of mitochondrial ALDH2 activity in ISDN-treated animals. However, chronic treatment with ISDN resulted in parallel rightward shifts of both the ISDN and GTN concentration-response curves, which increased with increased duration of exposure

to ISDN. Concentration-response curves for acetylcholine (ACh), sodium nitroprusside (SNP), and diethylamine NONOate were unchanged in ISDN-treated animals. The model of chronic ISDN treatment results in cross tolerance to GTN but does not affect ALDH activity. The data presented in this study indicate no role for ALDH2 in the development of nitrate tolerance.

Co-Authorship

This thesis was based on research conducted by Adrian Cordova under the supervision of Dr. Brian Bennett. All data was obtained and analyzed by Adrian Cordova.

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Dedication

To my family: Mum, Dad, Tav, and Lar. Thank you for all your love and support.

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1,2-GDN	glyceryl-1,2-dinitrate
1,3-GDN	glyceryl-1,3-dinitrate
Ach	acetylcholine
ALDH	aldehyde dehydrogenase
ALDH2	aldehyde dehydrogenase 2
AT_1	angiotensin II receptor, type 1
BSA	bovine serum albumin
Ca/CaM	calcium/calmodulin
cGMP	3',5'-cyclic guanosine monophosphate
Cys	cysteine
DEA/NO	1,1-diethyl-2-hydroxy-2-nitrosohydrazine
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ETC	electron transport chain
GDN	glyceryl dinitrate
GTN	glyceryl trinitrate
GST	glutathione transferase
IP_3	inositol-1,4,5-triphosphate
IRAG	IP_3 receptor-associated cGMP kinase substrate
IIDN	isoidide dinitrate
IIMN	isoidide dinitrate
ISDN	isosorbide dinitrate
ISMN	isosorbide mononitrate
MLC	myosin light chain
MLCP	myosin light chain phosphatase
$NADH/NAD^+$	nicotinamide adenine dinucleotide
$NADPH/NADP^+$	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NO_2^-	inorganic nitrite anion
PETN	pentaerythritol tetranitrate
PKG	cGMP-dependent protein kinase
PMSF	phenylmethanesulfonylfluoride
RhoA	Ras homolog gene family, member A
RGS2	regulator of G-protein signalling 2
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
sGC	soluble guanylyl cyclase
SH	sulfhydryl
SNP	sodium nitroprusside
VSM	vascular smooth muscle

CHAPTER 1: GENERAL INTRODUCTION

1.1 Statement of the Research Problem

In 2005, cardiovascular disease killed more Canadians than any other disease, accounting for over 30% of the country's total deaths (Stats Can, 2009). Advances in treatment allow patients with damaged hearts to live longer, leading to an increased prevalence of heart failure and a growing challenge for physicians. The management of cardiovascular disease and its symptoms is multifaceted, and for decades has included the use of nitrovasodilator drugs. Organic nitrates such as glyceryl trinitrate (GTN) have been used to treat angina pectoris and congestive heart failure for well over a century (Abrams, 1996). The pharmacological properties of organic nitrates greatly affect the utility of such drugs and are the focus of research in our laboratory. Nitrates, such as GTN or isosorbide dinitrate (ISDN), preferentially dilate larger veins, reducing cardiac preload and decreasing the oxygen demand of the heart, making them key components in the treatment of angina pectoris and congestive heart failure (Abrams, 1996). A major limitation in the use of nitrates is the rapid onset of tolerance during constant exposure, characterized by a loss of the symptomatic and hemodynamic effects (Parker and Parker, 1998). A common method for circumventing tolerance is the use of a drug-free period, usually at night, allowing for the reversal of tolerance to occur. This solution, however, presents its own problems in the form of rebound effects causing increased susceptibility to anginal attacks during the nitrate-free period (Thadani, 1996).

Nitrates are considered to be prodrugs that require bioactivation to an active metabolite in order to produce their therapeutic effects. Specifically, denitration of the nitrate and production of nitric oxide (NO) or an NO-like compound (hereafter referred to

as ‘NO bioactivity’) at or near the site of action is required (Parker and Parker, 1998). As an example, the denitration of GTN produces glyceryl dinitrate (GDN) and inorganic nitrite anion (NO_2^-) (Kurz, 1993). However, the mechanism by which NO is produced from these (or other) products remains unclear. Evidence suggests that NO_2^- is an unlikely intermediate due to the small concentrations produced relative to the high endogenous concentrations present in the vasculature (Bennett and Marks, 1984; Bennett *et al*, 1994).

Although the mechanism leading to the production of NO bioactivity is unclear, the vasodilatory signalling pathway it initiates is well studied. NO activates soluble guanylyl cyclase (sGC) resulting in increased cyclic guanosine monophosphate (cGMP) (Katsuki *et al*, 1977a). This in turn leads to activation of cGMP-dependent protein kinase (PKG) (Fiscus *et al*, 1983). Through phosphorylation of various target proteins, PKG acts to both decrease intracellular calcium (Ca^{2+}) concentration and decrease the sensitivity of myosin to Ca^{2+}, leading to vascular smooth muscle (VSM) relaxation.

Whereas the development of tolerance is widely accepted, the mechanism by which it occurs is still an area of ongoing study. The number of proposed mechanisms of tolerance serve to highlight the complexity of the research question, and include intravascular volume expansion (Dupuis *et al*., 1990), thiol depletion (Boesgaard *et al.*, 1991), oxidative stress from increased superoxide formation (Munzel *et al*., 1995), increased endothelin-1 production (Munzel *et al*., 1995), neurohormonal counter-regulation (Kurz *et al*., 1999), desensitization of sGC to NO (Bennett *et al*., 1988; Bennett *et al*., 1989), reduced biotransformation to NO bioactivity (Difabio *et al*., 2003), and increased expression of phosphodiesterase 5 (MacPherson *et al*., 2005). Regardless

of the broad scope of these proposed mechanisms, nitrate tolerance is still only a partially understood phenomenon.

Although the mechanism of nitrate tolerance is not fully understood, there is evidence suggesting that reduced biotransformation to NO bioactivity plays a significant role. Of the enzymes with the potential to bioactivate nitrates, aldehyde dehydrogenase 2 (ALDH2) has drawn recent attention for its ability to selectively denitrate GTN. In addition, ALDH2 activity has been shown to be decreased in tolerant tissues, and ALDH2 inhibitors have been shown to cause a rightward shift in GTN concentration-response curves of isolated blood vessel preparations (Chen *et al*., 2002; Difabio *et al*, 2003). These observations suggest a role for ALDH2 in GTN biotransformation and that inactivation of this enzyme may have a role in tolerance development.

ALDH2 has been the subject of many studies of late, with findings often at odds with each other. As previously mentioned, Chen *et al* (2002) presented evidence for ALDH2 as a specific activator of GTN. In this study, the ALDH inhibitors cyanamide and chloral hydrate were shown to block the effects of GTN, including formation of 1,2-GDN, relaxation, and cGMP accumulation. These observations led them to propose ALDH2 inactivation as a mechanism of GTN tolerance. Several studies have since questioned these conclusions, and have suggested ALDH2 cannot be the only enzyme catalyzing the bioactivation of GTN. Difabio *et al* (2003) found that ALDH inhibitors were similarly effective in both tolerant and nontolerant aortas. That is, these inhibitors caused similar rightward shifts in the concentration-response curves of both tissues, suggesting that the non-specific activities of these inhibitors accounted for Chen's results. Clearly, Difabio *et al* (2003) showed that ALDH2 is not the only target in GTN tolerance.

Confirming these results, other research has compared nontolerant vessels exposed to inhibitors of GTN-induced vasodilation to GTN-tolerant vessels and concluded that reduced ALDH2 activity does not fully account for GTN-tolerance (de la Lande *et al*, 2004). Furthermore, in 2004, Kollau et al made use of the ALDH selective inhibitor daidzin to inhibit sGC activation by low concentrations of GTN, also concluding that ALDH2 is not solely responsible for bioactivation of GTN. Given the evidence for a multifactorial model of GTN tolerance, it is important to further address the specific role that ALDH2 plays in tolerance both with GTN and other nitrates.

An additional complexity in studying the underlying mechanisms of tolerance is the issue of cross-tolerance. That is, tolerance to one nitrate results in tolerance to other nitrates as well. The hemodynamic and vasodilatory effects of GTN in both arterial and venous circulation have been shown to be reduced in subjects pre-treated with other nitrates, such as isosorbide dinitrate (ISDN) (Manyari *et al*., 1985). Cross-tolerance complicates the clinical applications of organic nitrates as well, by precluding the substitution of other nitrates in tolerant patients. Therefore, it is reasonable to hypothesize that there are common factors contributing to the development of tolerance for all nitrates.

This thesis focuses on developing a model of ISDN tolerance for the purpose of studying cross-tolerance with GTN, and determining whether there is any effect on ALDH2 activity. An *in vivo* rat model of ISDN tolerance was designed to assess changes in vasodilator responses at various time points of ISDN exposure. In addition, ALDH2 activity was assayed in hepatic mitochondrial fractions from ISDN tolerant animals and compared to activities in control and GTN-tolerant animals. The goal of these studies was

to gain a better understanding of the mechanisms of nitrate tolerance, and in so doing, suggest new treatment strategies that may be able to avoid the onset of tolerance.

1.2 Organic Nitrates – General Introduction

1.2.1 Discovery and History of Organic Nitrates

The discovery and clinical use of organic nitrates date back more than 100 years. Ascanio Sobrero, an Italian chemist, first synthesized GTN in 1847 (Marsh and Marsh, 2000). Soon after, it was discovered that handling and exposure to the compound lead to sudden severe headache. Whereas this observation provided evidence of the vasodilatory properties of GTN, it was more than 30 years before the compound was used clinically. Following work that demonstrated the antianginal properties of amyl nitrite, Dr. William Murrell successfully experimented with GTN to treat angina and to lower blood pressure. Following the publication of his findings in 1879, GTN was adopted for widespread use in the treatment of angina pectoris.

1.2.2 Structure of Organic Nitrates

As the esters of nitric acid and various alcohols, organic nitrates all contain the same functional group (R-O-NO_2) that is responsible for the group's unique vasodilator properties. Because of this shared nitrate group, organic nitrates also share similar mechanisms of action and pharmacological properties. The structure of GTN (Figure 1.1) consists of three nitrate groups bonded to a glycerol backbone. Removal of the nitrate group from the 1- or 2- position forms the metabolites 1,2-GDN or 1,3-GDN, respectively. The structure of ISDN (Figure 1.2) contains nitrate groups at the 2- and 5-

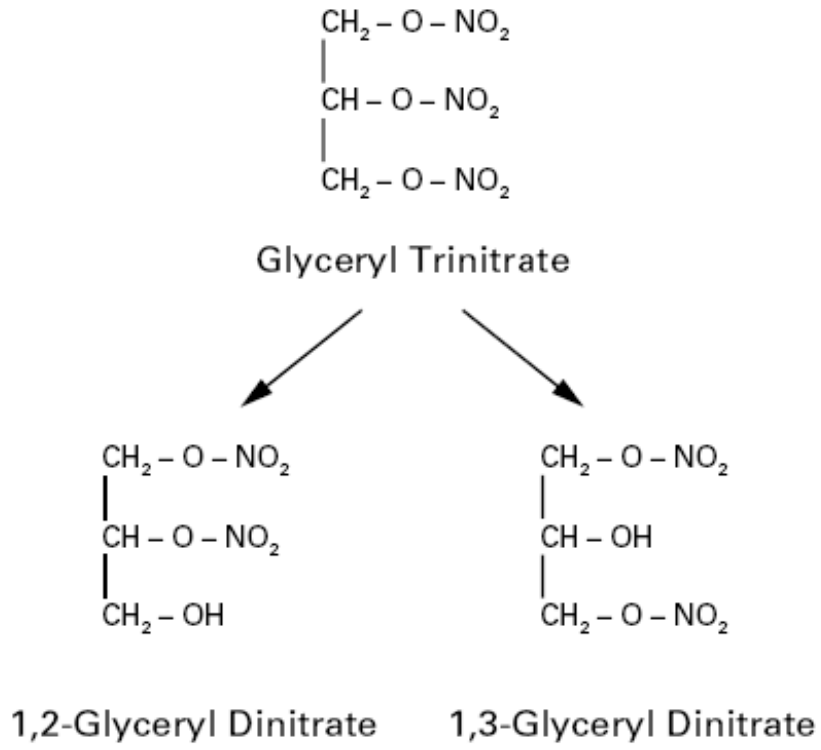

Figure 1.1. Structure of GTN and its metabolites

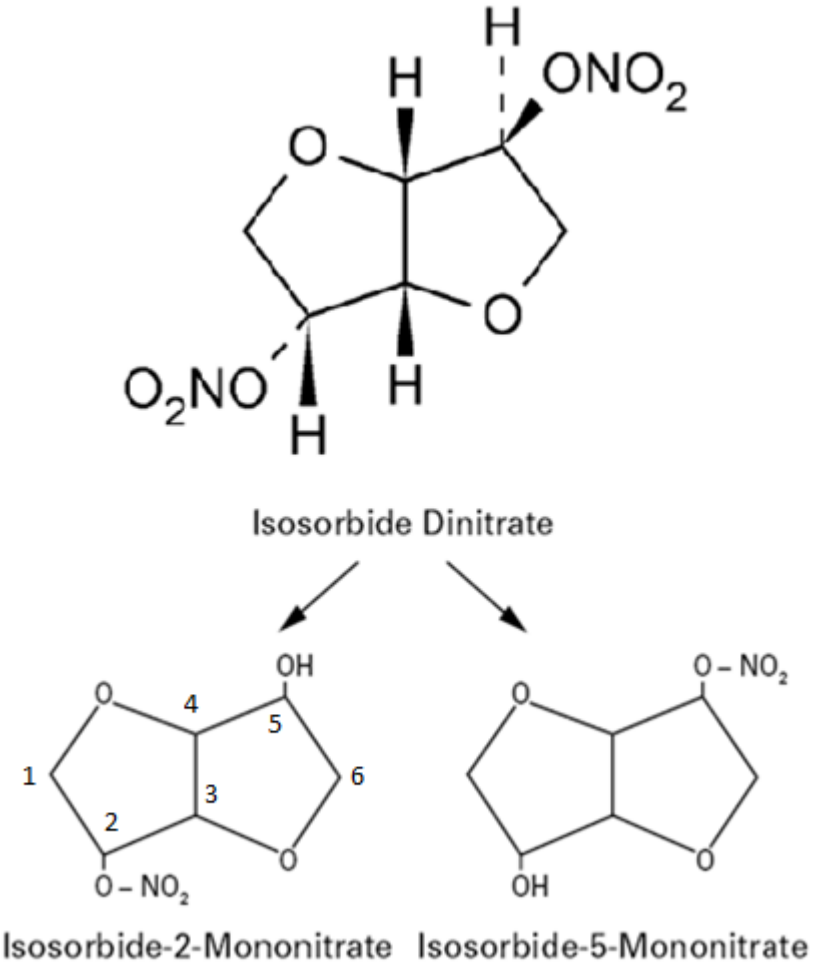

Figure 1.2. Structure of ISDN and its metabolites

positions of an isosorbide molecule. The metabolites of ISDN resulting from the removal of a nitrate group are isosorbide-2-mononitrate (2- ISMN) and isosorbide-5-mononitrate (5-ISMN). Both ISDN metabolites are pharmacologically active but only 5-ISMN is believed to contribute to the prolonged effect the drug provides (Abrams, 1995). As with all organic nitrates, GTN and ISDN are lipophilic and thus are readily absorbed into the body through skin and other mucus membranes (Parker and Parker, 1998).

1.2.3 Clinical Uses of Nitrates

The unique pharmacological effects of organic nitrates have lead to their prevalent use for the treatment of angina pectoris as well as congestive heart failure. The most commonly prescribed nitrovasodilators in North America are GTN, ISDN, and 5-ISMN (Parker and Parker, 1998; Abrams, 1995). GTN is the drug of choice for the treatment of acute angina attacks. The unique property of nitrates to selectively dilate venous capacitance vessels is a key factor for their antianginal effects. This selective venodilation redistributes blood away from the heart and lungs, resulting in reduced ventricular preload, and therefore reduced myocardial oxygen requirements. In patients with abnormal ventricular contractile performance, it is possible that stroke volume may even rise under these conditions, leading to increased cardiac oxygen supply and further symptomatic relief (Abrams, 1985). In addition to reducing myocardial oxygen demands, nitrates also provide vasodilator effects in the coronary circulation, thereby increasing myocardial oxygen supply and adding to their antianginal effects (Abrams, 1995). As a result of this unique hemodynamic profile, nitrates are the antianginal drugs of choice for patients with impaired cardiac contractile function.

In addition to their unique and diverse hemodynamic effects, nitrates also have an excellent therapeutic profile; they are generally fast acting, safe, free of serious side effects, and have a wide therapeutic index (Fung, 2004). All of these factors contribute to the widespread use of nitrates to treat a variety of cardiovascular diseases such as coronary artery disease, congestive heart failure, and acute myocardial infarction (Fung, 2004). The few adverse effects that nitrate therapy does cause are generally as a result of their vasodilator effects, and include headache, postural hypotension, and syncope (Parker and Parker, 1998).

Both ISDN and GTN undergo extensive first pass hepatic metabolism. The plasma half-life of GTN is approximately one to four minutes in humans (Armstrong *et al.*, 1982). It is metabolized in both the liver and the vasculature (and many other tissues) to form its biologically active metabolites, 1,2-GDN and 1,3-GDN, both of which have a half-life of about 40 minutes (Parker and Parker, 1998). Similarly, ISDN has a plasma half-life of approximately 40 minutes and forms two active metabolites, 2-ISMN and 5-ISMN, with half-lives of about two and four hours, respectively (Parker and Parker, 1998). Of the biologically active metabolites formed by GTN or ISDN, only 5-ISMN is believed to contribute significantly to the therapeutic response of either drug (Abrams, 1995). As such, 5-ISMN is used as a low-potency treatment of angina pectoris that provides a longer and more predictable anti-ischemic effect.

The clinical preparations of nitrates used to treat angina pectoris can be separated into two categories; short-acting and long-acting. Short-acting forms consist mainly of sublingual preparations (tablet or spray) and are used to treat or prevent the onset of acute anginal attacks. Both GTN and ISDN are available in sublingual forms and provide rapid

onset of action (2-5 min for GTN, 5-20 min for ISDN) at the cost of a shorter duration of action (20-30 min for GTN, 45-120 min for ISDN) (Abrams, 1995). Long-acting preparations, on the other hand, are used for the chronic treatment of stable angina (Abrams, 1995). These include sustained release oral forms and transdermal patches and ointments that are effective at preventing symptoms for up to 12 hours. The main disadvantage of these long-acting preparations is the rapid onset of tolerance cause by prolonged exposure (Parker and Parker, 1998). This necessitates a 12 hour drug-free period for every 12 hour dosing period in order to prevent the loss of symptomatic benefits brought on by tolerance. Although tolerance is rapidly reversed during the drug-free period, patients are susceptible to rebound effects in the form of decreased anginal threshold (Parker *et al*., 1995).

1.3 Mechanisms of Action

1.3.1 Biotransformation of Organic Nitrates

Like most nitrates, GTN is considered to be a prodrug in that it requires bioactivation to an active metabolite before an effect is observed (Bennett *et al*., 1994). Formation of nitric oxide (NO) or an NO-like species is essential, although the identity of the compound(s) and details of their formation are unclear. GTN biotransformation has been demonstrated by several proteins, including myoglobin and hemoglobin (Bennett *et al*., 1986), old yellow enzyme (Meah *et al*., 2001), xanthine oxidoreductase (Doel *et al*., 2000), human and rat aortic glutathione transferases (Habig *et al*., 1975; Tsuchida *et al*., 1990; Nigam *et al*., 1993), rat aortic cytochrome P450 (McDonald and Bennett, 1990), and mitochondrial aldehyde dehydrogenase 2 (Chen *et al*., 2002). Despite their activity

with GTN, however, none have been shown to be the primary enzyme in the bioactivation pathway of GTN.

Regardless of the process that generates it, once formed, NO or the NO-like species binds to and activates sGC (Ahlner *et al*., 1991 Katsuki *et al.*, 1977a; Katsuki *et al.*, 1977b). This results in increased formation of cGMP from GTP, and subsequent activation of PKG. Two isoforms of PKG exist in the VSM; PKGα and PKGβ, both of which are activated by cGMP. PKG mediates relaxation by inhibiting both calcium-dependent and -independent contraction (Figure 1.3). PKG phosphorylates and inactivates phospholamban, which normally inhibits sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). Once uninhibited, SERCA sequesters Ca^{2+} in the sarcoplasmic reticulum at a greater rate, and decreases free intracellular Ca^{2+}. PKG also decreases intracellular Ca^{2+} by inhibiting the release of Ca^{2+} from the sarcoplasmic reticulum via the inositol 1,4,5-trisphosphate (IP_3) receptor (Surks, 2007). PKGα phosphorylates and activates regulator of G protein signalling 2 (RGS2) which inhibits IP_3 activation by inhibiting upstream $G_{\alpha q}$ signalling, thereby inhibiting the release of Ca^{2+} from the endoplasmic reticulum. Furthermore, PKGβ directly phosphorylates inositol 1,4,5-triphosphate (IP3) receptor associated G kinase substrate (IRAG), further inhibiting IP_3–mediated Ca^{2+} release (Surks, 2007). These mechanisms combine to lower intracellular Ca^{2+} concentrations and inhibit Ca^{2+}-dependent contraction in the smooth muscle cell. Free Ca^{2+} is required to bind with calmodulin in order to stimulate Ca^{2+}–calmodulin-dependent myosin light chain kinase (MLCK). When activated, Ca^{2+}–calmodulin-dependent MLCK phosphorylates the myosin light chain (MLC) allowing the formation of cross bridges with actin filaments, resulting in contraction (Ahlner *et al*., 1991).

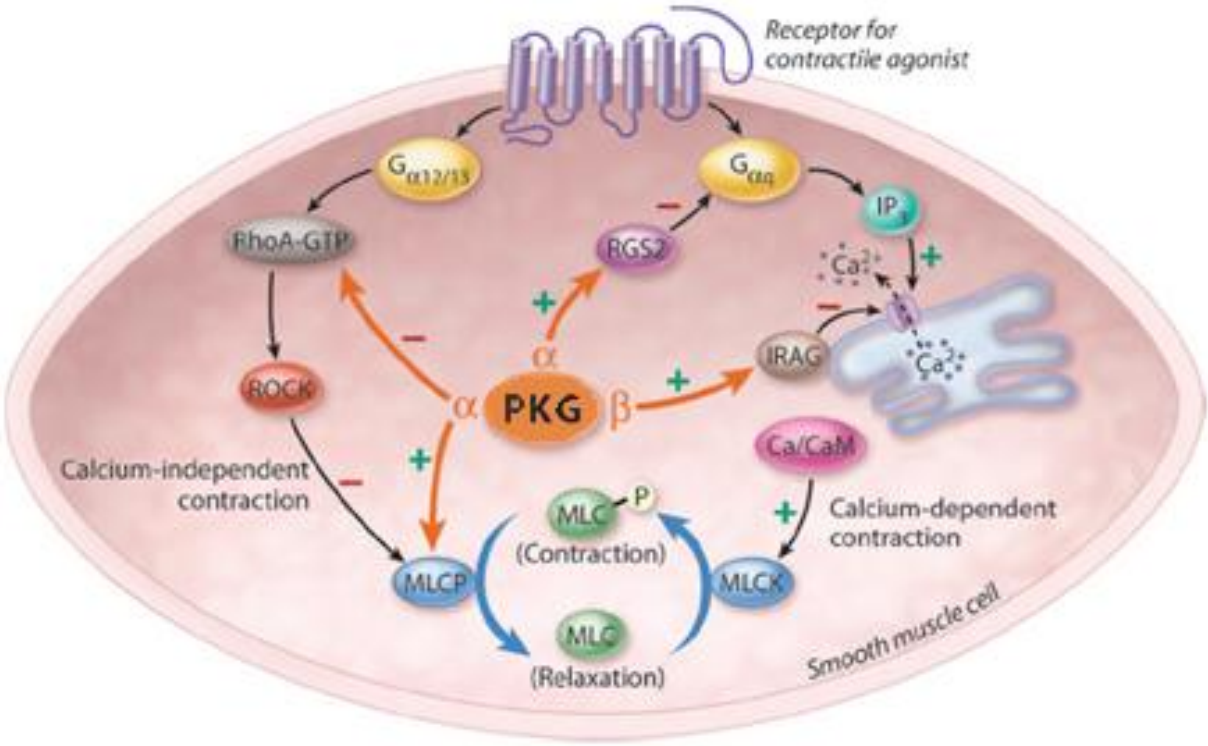

Figure 1.3. Calcium-dependent and –independent contraction in the smooth muscle and the effects of PKG. MLC phosphorylation determines smooth muscle cell contractility. Stimulation by a contractile agonist leads to both activation of RhoA (RhoA-GTP) and production of inositol 1,4,5 triphosphate (IP_3). IP_3 binds to its receptor on the sarcoplasmic reticulum, causing the release of Ca^{2+}. Upon binding to Ca^{2+}, calmodulin (Ca/CaM) activates MLCK which then phosphorylates MLC (calcium-dependent contraction). Activated RhoA activates ROCK which phosphorylates and inhibits MLCP, inhibiting MLC dephosphorylation (calcium-independent contraction). Both isoforms of PKG mediate relaxation by promoting MLC dephosphorylation. PKGα activates MLCP directly, inhibits RhoA activation, and activates RGS2, thereby inhibiting IP_3 production. PKGβ activates IRAG, which inhibits the release of Ca^{2+} by the IP_3 receptor on the sarcoplasmic reticulum. Modified from Surks (2007).

Therefore, decreased intracellular Ca^{2+} concentrations lead to MLCK inactivation and relaxation.

PKGα affects Ca^{2+}-independent contraction via regulation of myosin light chain phosphatase (MLCP). MLCP is activated both directly and indirectly by PKGα; directly through phosphorylation and indirectly through the phosphorylation and inhibition of RhoA (Surks, 2007). Activation of MLCP leads to dephosphorylation of MLC and inhibition of cross-bridge formation, thereby mediating smooth muscle relaxation.

1.3.2 Clearance- and Mechanism-based Biotransformation of Organic Nitrates

As prodrugs, organic nitrates require biotransformation to an active species before eliciting an effect at their site of action. Currently, NO bioactivity is believed to be the active species responsible for organic nitrate induced vasodilation. It is important to note, however, that inorganic nitrite anion (NO_2^-) is the main nitrogen-oxygen containing product of organic nitrate metabolism, with only a small amount of NO being produced. It is unclear which, if any, intermediate compounds are involved in NO formation from organic nitrates. Further complicating matters, the levels of NO produced by GTN are too low to account for observed VSM relaxation (Marks *et al*., 1995; Kleschyov *et al*., 2003). Thus, organic nitrates have the ability to mimic NO bioactivity without the significant release of NO and are thus categorized as NO mimetics rather than NO donors (Thatcher *et al*., 2004). Furthermore, the amount of NO_2^- produced by organic nitrate biotransformation is not significant relative to endogenous levels of NO_2^- found in VSM. As such, it is unlikely that NO_2^- is an intermediate in the production of NO (Bennett and Marks, 1984; Difabio *et al*., 2003). Additionally, NO_2^- is nearly 1000-fold less potent

than GTN in producing VSM relaxation (Romanin & Kukovetz, 1988). Consequently, the pathway responsible for producing NO_2^- has been dubbed "clearance-based" biotransformation (Figure 1.4). By definition, clearance-based metabolism does not result in sGC activation and does not produce VSM relaxation. In contrast, the "mechanism-based" pathway leads to the activation of sGC via NO bioactivity (Bennett *et al*., 1994).

1.4 Tolerance to Organic Nitrates

1.4.1 Clinical Impact of Organic Nitrate Tolerance

Drug tolerance is defined as a decrease in the observed effect of a drug following repeated exposure, thereby requiring a larger dose to achieve the same pharmacological/therapeutic effect. This is one of the main problems impacting the clinical use of nitrates, since prophylactic treatment with GTN can result in tolerance in as little as 24 hours. Such tolerance is characterized by a loss of symptomatic and hemodynamic effects, including vasodilatory action and relief of anginal symptoms. To combat the onset of tolerance, modern treatments with nitrates employ an elliptical dosing regimen. This most commonly consists of a 12 hour dosing period followed by a 12 hour drug-free period to allow for the reversal of tolerance. Although the drug-free period usually coincides with a period of decreased physical demand, such as during sleep, the major problem facing this strategy is that patients are without the benefit of the drug during the withdrawal period. Furthermore, evidence has been presented to suggest a rebound effect that occurs during this drug-free period in which there is a worsening of anginal symptoms (Thadani, 1996). While the phenomenon of nitrate tolerance has been documented for over 120 years, the underlying mechanism is not clear.

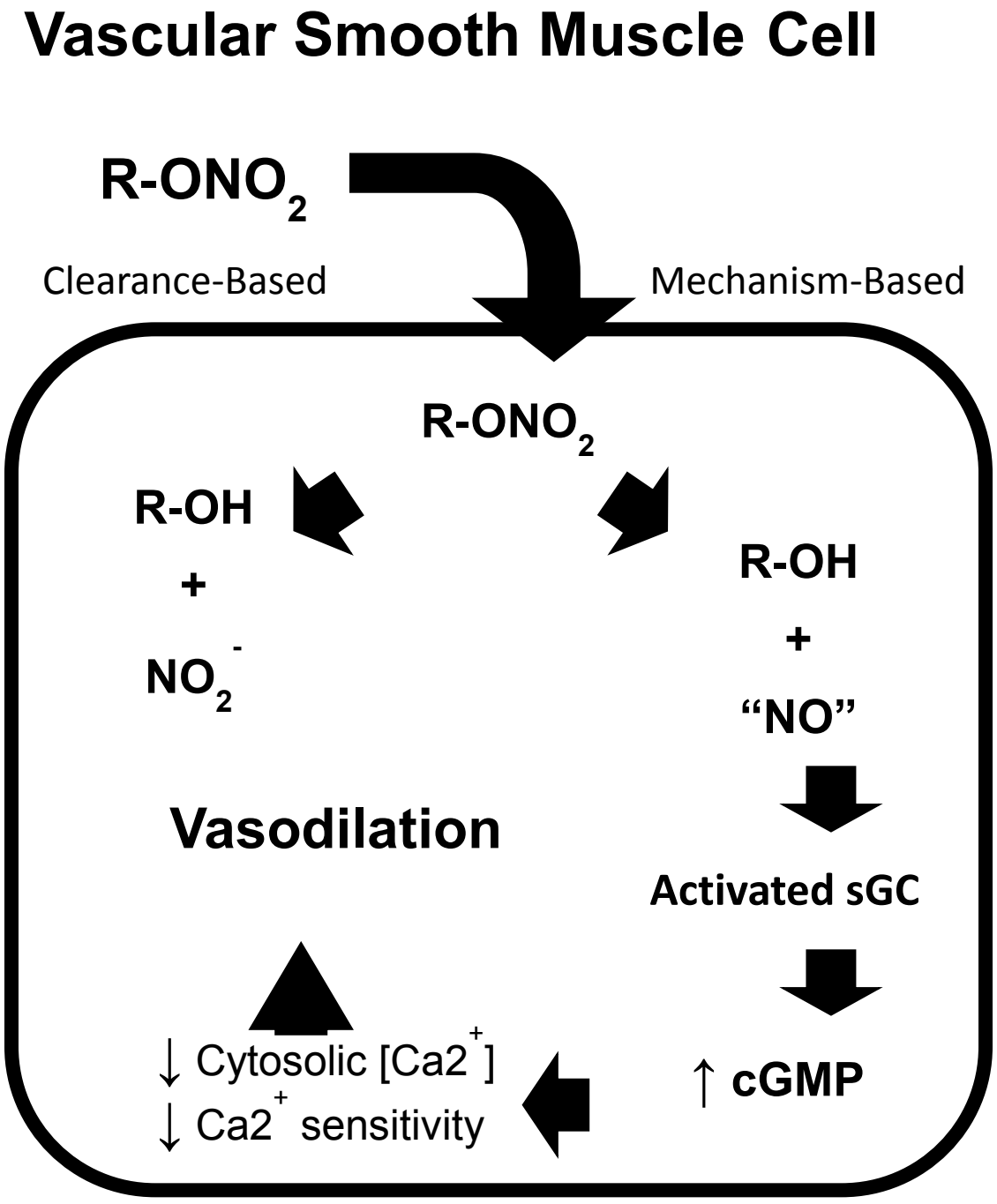

Figure 1.4. Scheme outlining the mechanism of action of organic nitrates with the mechanism-based and clearance-based pathways. Biotransformation occurs in the cytosol once the organic nitrate ($R\text{-}ONO_2$) enters the vascular smooth muscle cell. In the mechanism-based pathway, "NO" or some NO-like compound activates the soluble isoform of guanylyl cyclase (sGC), resulting in vasodilation. Clearance-based biotransformation results in the formation of inorganic nitrite ion (NO_2^-) and does not lead to vasodilation. Modified from Bennett *et al*. (1994).

1.4.2 Proposed Models of Nitrate Tolerance

More than a century's worth of study has not produced a unifying hypothesis of nitrate tolerance (Parker and Gori, 2001). Consequently, there are a number of models that attempt to explain the complex nature of tolerance. Proposed mechanisms of tolerance include: intravascular volume expansion (Lis *et al*., 1984; Dupuis *et al*., 1990), depletion of key sulfhydryl groups (Needleman and Johnson, Jr., 1973; Boesgaard *et al.*, 1991), oxidative stress from increased superoxide formation (Munzel *et al*., 1995; Munzel *et al*., 1999), increased endothelin-1 production (Munzel *et al*., 1995), neurohormonal counter-regulation (Parker *et al*., 1991; Jeserich *et al*., 1995; Kurz *et al*., 1999), reduced cGMP formation due to desensitization of sGC to NO (Bennett *et* al., 1988; Schröder *et al*., 1988; Bennett *et al*., 1989), reduced biotransformation to NO or an NO-like species (McGuire *et* al., 1994; Kenkare and Benet, 1996; Ratz *et al*., 2000; Difabio *et al*., 2003), and increased expression of phosphodiesterase (Axelsson and Andersson, 1983; Axelsson and Karlsson, 1984; Pagani *et* al., 1993; MacPherson *et al*., 2005). Despite the sheer number of hypotheses, no single model succeeds in explaining the exact cause of tolerance; most have evidence supporting and refuting them. As such, a multifactorial view of nitrate tolerance has emerged.

1.4.3 Prevention of Organic Nitrate Tolerance

Attempts to prevent tolerance through pharmacological means have met with limited success. To date, research has focused on targeting a specific hypothesized mechanism of tolerance in an effort to prevent or reverse tolerance (Gori and Parker, 2002). These studies include: repletion of sulfhydryl groups to combat thiol depletion

(Gruetter and Lemke, 1986; Horowitz *et al*., 1988; Boesgaard *et al*., 1991), use of angiotensin-converting enzyme inhibitors and AT_1-receptor antagonists to counter neurohormonal counter-regulation (Munzel and Bassenge, 1996; Longobardi *et al*., 2004), antioxidants such as vitamins E and C to prevent and reverse oxidative stress (Bassange *et al*., 1998; Munzel *et al*., 1999; Gori and Parker, 2002), and diuretics to counteract the observed increase in plasma volume (Sussex *et al*., 1994; Parker *et al*., 1996). None of these methods succeed in effectively preventing or reversing nitrate tolerance, thereby providing evidence for a multifactorial model of tolerance in which no single mechanism is responsible. Currently, the rapid reversal of tolerance is only possible through a drug-free period (Thadani, 2005).

1.5 Aldehyde Dehydrogenase 2

1.5.1 Identification of ALDH2

Efforts to identify the mechanism of bioactivation of organic nitrates have lead to the discovery of several enzymes able to catalyze the breakdown of GTN. Glutathione transferases (Yeates *et al.*, 1989; Tsuchida *et al*., 1990), xanthine oxidoreductase (Millar *et al*., 1998), cytochrome P450 (McDonald and Bennett, 1990; McDonald and Bennett, 1993), and cytochrome P450 reductase (McGuire *et al*., 1998) have each been shown to catalyze the reduction of GTN. Nevertheless, none of these enzymes show decreased activity in tolerant tissues and their role in generating NO bioactivity remains uncertain (Chen *et al*., 2002). ALDH2, however, has attracted recent attention for its potential role in GTN bioactivation. A 2002 study by Chen *et al*. implicated ALDH2 in GTN bioactivation by showing that the enzyme has decreased activity in GTN tolerant tissues.

Furthermore, ALDH2 is selective for the formation of 1,2-GDN from GTN. As discussed above, the main metabolites of GTN are NO_2^-, 1,2-GDN and 1,3-GDN. However, previous studies have shown that 1,2-GDN is the main metabolite formed in VSM and furthermore that this selective formation is attenuated in GTN tolerant tissues (Brien *et al*., 1988; Kawamoto *et al*., 1990). Given the association between 1,2-GDN and the mechanism-based pathway (Bennett *et al*., 1994), the selectivity of ALDH2 towards 1,2-GDN, and the decreased activity of the enzyme in tolerant tissues, it has been proposed that inactivation of ALDH2 is the sole basis for GTN tolerance (Chen *et al*., 2002).

The ALDH gene superfamily includes 19 enzymes that are critical for certain life processes and detoxification via the NAD(P)(+)-dependent oxidation of numerous endogenous and exogenous aldehyde substrates (Vasiliou & Nebert, 2005). ALDH2 is found in a range of tissues, with the highest levels found in the liver (Vasiliou *et al*., 2000). Also referred to as mitochondrial aldehyde dehydrogenase, ALDH2 is generally localized in the mitochondria. In humans, however, ALDH2 isoforms are expressed both in the mitochondria and the cytosol. Due to its low K_m value (<5μM) for acetaldehyde, the lowest of all ALDH enzymes, ALDH2 plays an important role in acetaldehyde oxidation and clearance. Specifically, ALDH2 facilitates the important second step in the ethanol clearance pathway by oxidizing acetaldehyde to acetic acid (Vasiliou *et al*., 2000). In addition to its dehydrogenase activity, ALDH2 also possesses esterase activity, which is thought to be responsible for mechanism-based GTN metabolism (Feldman & Weiner, 1972).

1.5.2 Recent studies involving ALDH2

Needleman and Johnson first proposed a thiol requirement for nitrate-induced VSM relaxation in 1973. Despite conflicting evidence in the following years, support for Needleman and Johnson's model points to a role for sulfhydryl groups in nitrate biotransformation in at least some capacity. ALDH2 has drawn recent attention for its potential role in nitrate biotransformation as discussed above. Previous studies of ALDH2 have shown the presence of three thiol-containing cysteine groups at the active site of the enzyme (Vallari and Pietrusko *et al*., 1982). Furthermore, disulfide bridge formation has been observed to take place between these groups, thereby inhibiting ALDH2 (Shen *et al*., 2000). Both NO and GTN have been observed to inhibit ALDH2 activity (Towell *et al*., 1985; DeMaster *et al*., 1997), however it was Chen *et al* (2002) who hypothesized that disulfide bridge formation and the resultant ALDH2 inactivation are essential steps in mechanism-based nitrate biotransformation (Figure 1.5). In addition, according to the model of Chen *et al*., NO_2^- is an obligate intermediate in the ALDH2-mediated formation of NO or NO bioactivity from organic nitrates. Whereas evidence supports the hypothesized inhibition of ALDH2 via disulfide bridge formation, the proposed role for NO_2^- as an intermediate in the formation of NO is problematic for several reasons. As previously mentioned, NO_2^- is the main N,O-containing species in the *in vivo* biotransformation of organic nitrates. However, the low vasodilator potency and comparatively high endogenous concentrations of NO_2^- make it an unlikely intermediate in the activation of sGC (Bennett and Marks, 1984). The endogenous concentration of NO_2^- in rat aorta has been measured at 10μM (Rodriguez *et al*., 2003), and NO_2^- has been shown to be evenly distributed between plasma and erythrocytes, as well as between

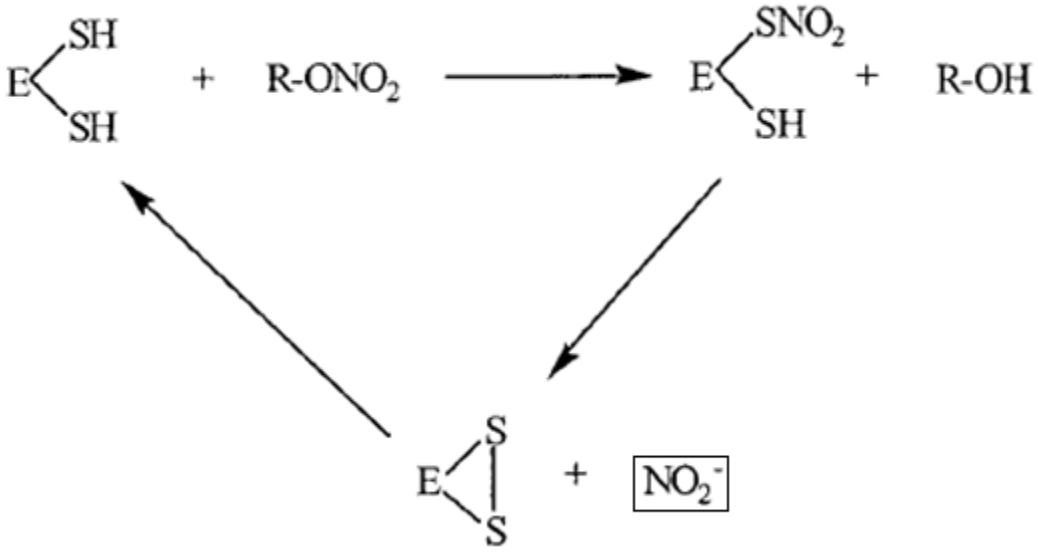

Figure 1.5. Proposed mechanism of bioactivation of organic nitrates by ALDH2. The sulfhydryl groups at the active site of ALDH2 react with the organic nitrate (R-ONO_2) resulting in the release of inorganic nitrite ion (NO_2^-). The subsequent disulfide bridge formation results in the inactivation of ALDH2. Modified from Chen *et al*. (2002).

intravascular and extravascular compartments (Parks *et al*., 1981). In comparison, pharmacologically relevant concentrations of GTN in VSM relaxation are in the nanomolar range; approximately 1000-fold less than endogenous NO_2^- concentrations (Difabio *et al*., 2003). Given the micromolar concentration and wide distribution of endogenous NO_2^-, any NO_2^- derived from pharmacologically active concentrations of GTN would be expected to have a negligible effect on sGC activation.

Further complicating the model put forth by the Stamler group (Chen *et al.*, 2005) is that the mechanism requires conversion of NO_2^- to NO in the mitochondrion. It is plausible that if the compartmentalized formation of NO from NO_2^- did occur in mitochondria, followed by the transport of the bioactive NO to its site of action, then NO_2^- could function as an intermediate in the mechanism-based biotransformation of organic nitrates (Difabio *et al*., 2003). Chen *et al* (2002) hypothesized two mechanisms by which this conversion may occur. The first proposed mechanism involves the reduction of NO_2^- to NO by components of the mitochondrial electron transport chain (ETC). Both the bc_1 complex of the ETC as well as cytochrome oxidase have been shown to reduce NO_2^- to NO (Brudvig *et al*., 1980; Kozlov *et al*., 1999). However, this reduction was only observed under anaerobic conditions and does not occur at physiological concentrations of oxygen. Furthermore, the formation of superoxide via the reduction of O_2 by the bc_1 complex under aerobic conditions indicates competition for available electrons that would likely lead to quenching of newly formed NO by associated superoxide formation. NO also binds to and inhibits cytochrome oxidase and under aerobic conditions will either dissociate unchanged or will be oxidized to NO_2^- depending on the rate of electron flux (Difabio *et al*., 2003; Sarti *et al*., 2003). Lastly,

NO_2^--cytochrome oxidase adducts only form when the protein is fully oxidized, and when reduced, NO_2^- is released and not reduced to NO (Giuffre *et al*., 2000). Therefore, under normal physiological conditions, cytochrome oxidase functions as a NO clearance pathway by facilitating its oxidation to NO_2^- rather than reducing NO_2^- to NO. The second mechanism put forward by Chen *et al.* (2002) relies on the disproportionation of nitrous acid (HNO_2) in the intermembrane space of the mitochondrion to form bioactive NO. However, the pH of 4 found in the intermembrane space leads to a predicted rate of formation of NO of 0.01% per second (Samouilov *et al*., 1998). Therefore, at a particular concentration of GTN, 10,000 fold less NO could be formed by this mechanism. Given pharmacologically active concentrations of GTN in the nanomolar range, this translates to subpicomolar concentrations of NO. In contrast, the NO donor DEA/NO has an EC_{50} of 9.2 nM for relaxation in isolated rat aorta, thousands of times greater than the concentrations of NO that could be produced by this mechanism (Difabio *et al*., 2003). Additionally, any NO formed is confined to the intermembrane space and would need to diffuse to its site of action outside the mitochondria.

Further complicating the model of ALDH2-dependent nitrate bioactivation and the mitochondrial conversion of NO_2^- to NO is the issue of the subcellular localization of the enzyme. Also known as mitochondrial ALDH, ALDH2 is found primarily in the mitochondria in rat liver (Difabio *et al*., 2003). This is in contrast to human liver, in which ALDH2 is distributed mainly in the cytosolic fraction (Tsutsumi *et al*., 1988). Furthermore, in both rabbit and rat aorta the majority of ALDH2 is found in the cytosol (Difabio *et al*., 2003). It is therefore reasonable to expect that any biotransformation

facilitated by ALDH2 leading to VSM relaxation would occur in the cytosol, and not the mitochondria.

Given the evidence, Chen *et al*'s (2002) model of ALDH2-dependent nitrate bioactivation does not seem plausible. Whereas it is possible that the enzyme plays a role in nitrate biotransformation, the evidence clearly does not support the assertion that NO_2^- is an obligate intermediate in the formation of NO bioactivity. Further research is needed to clarify the mechanisms of nitrate bioactivation and tolerance, as well as the function of ALDH2 in these processes.

1.6 Cross Tolerance

1.6.1 Cross Tolerance between Organic Nitrates

Causing added difficulties with identifying the underlying mechanism of nitrate tolerance is the phenomenon of cross tolerance. That is, tolerance to one nitrate will result in tolerance to other nitrates as well. The beneficial hemodynamic and vasodilator effects of GTN in both the arterial and venous circulation have been shown to be reduced in subjects pre-treated with other nitrates, such as ISDN (Manyari *et al*., 1985). Cross tolerance complicates the clinical applications of organic nitrates as well, by precluding the substitution of other nitrates in tolerant patients. Given the prodrug characteristics of organic nitrates, it is possible that a shared mechanism in the bioactivation pathway is responsible for the development of cross tolerance. Alternatively, cross tolerance may instead result from decreased sensitivity to the NO bioactivity produced by organic nitrates. Whether cross tolerance results from decreased production of NO bioactivity, decreased sensitivity to it, or some combination thereof is not currently understood and

warrants further investigation. Despite this lack of understanding, a model was put forward by Chen *et al* in 2005 attempting to explain nitrate cross tolerance as well as the role of ALDH2 in nitrate bioactivation and tolerance.

1.6.2 High and low affinity pathways

In 2005, Chen *et al* conducted a study examining the biotransformation of GTN in *Aldh2*$^{-/-}$ mice. They reported that GTN bioactivity could still be generated in *Aldh2*$^{-/-}$ mice, albeit at higher concentrations (10μM) of GTN. This suggests that there is an ALDH2-independent mechanism of GTN bioactivation with a low affinity for GTN. In addition, they also noted that the mechanism by which GTN was bioactivated in the *Aldh2*$^{-/-}$ mice did not seem to exhibit tolerance. That is, there was no shift in the concentration-response curve of aorta from *Aldh2*$^{-/-}$ mice between control and GTN tolerant (300 μM for 30 minutes) vessels. Based on this study`s findings, the Stamler group put forth two separate pathways for the bioactivation of GTN: 1) a high affinity ALDH2-dependent pathway specific to GTN and other high potency nitrates (e.g. PETN) and 2) a low affinity ALDH2-independent pathway, immune to tolerance and able to metabolize low potency nitrates as well as GTN, albeit at higher concentrations (Figure 1.6). Furthermore, they concluded that ALDH2 is the essential source of NO bioactivity at clinically relevant concentrations of GTN and that the enzyme`s inactivation is central to mechanism-based tolerance. While the proposed model does give an explanation for some observations, it does not account for the phenomenon of cross tolerance between organic nitrates. Firstly, *Aldh2*$^{-/-}$ mice exhibited unchanged vasodilator responses to ISDN compared to wild type mice, implying that ALDH2 is not involved in bioactivating

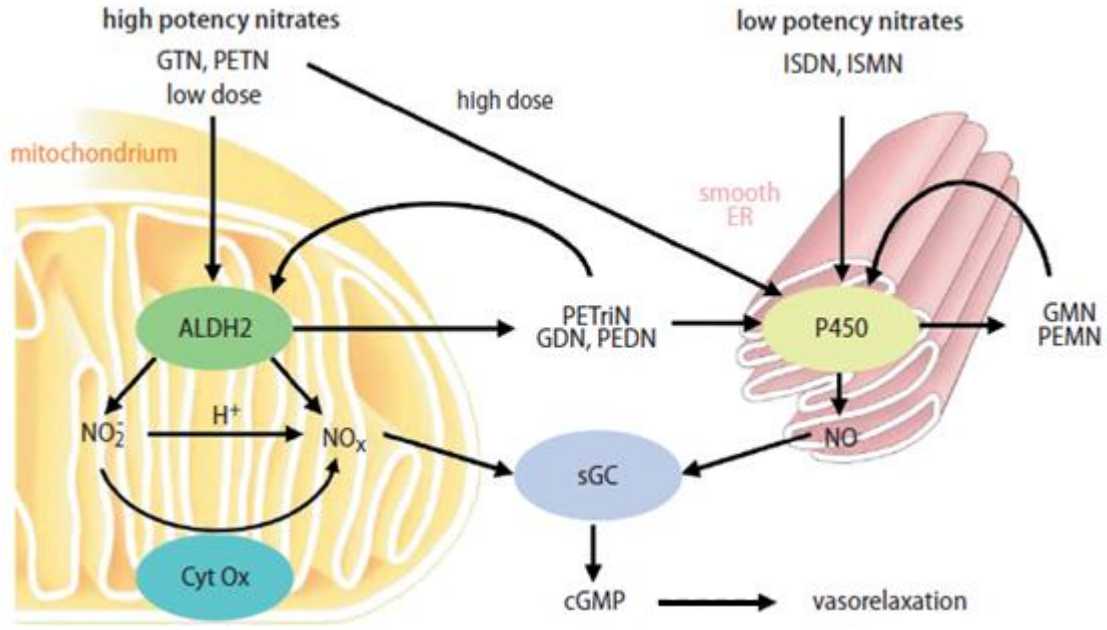

Figure 1.6. Proposed model of two pathway nitrate bioactivation. Two pathways of mechanism-based metabolism are proposed: A) The high affinity ALDH2-dependent pathway for low doses of high potency nitrates such as GTN and PETN. This takes place in the mitochondria and includes NO_2^- as an obligate intermediate for producing NO bioactivity. B) the low affinity ALDH2-independent pathway for low potency nitrates such as ISDN and ISMN as well as high doses of high potency nitrates. According to the model, ALDH2 is inactivated by cross bridge formation during bioactivation while the low potency pathway is not. Modified from Daiber *et al*. (2007).

this nitrate. However, previous studies have shown that not only does ALDH2 metabolize ISDN and other nitrate esters, but that it is also inactivated by them (Pietruszko *et al*., 1995). Additionally, cross tolerance to ISDN and other nitrates is known to occur in patients after chronic GTN exposure. This conflicts with several of Chen`s assertions; namely that ALDH2 is GTN-specific and that the low potency pathway is immune to tolerance. The proposed model also includes NO_2^- as an obligate intermediate of part of the ALDH2-dependent pathway. For reasons discussed previously, NO_2^- is an unlikely precursor to NO bioactivity inside the mitochondria. Furthermore, the proposed mechanism of low potency nitrate metabolism is unclear, both in which enzymes are active and in how NO bioactivity is generated. Clearly, the idea that ALDH2 is the principle enzyme responsible for GTN bioactivation and tolerance is problematic. Even so, the mechanism of GTN tolerance proposed by Chen states that inactivation of ALDH2 specifically affects the bioactivation of GTN. It is therefore necessary to further evaluate the effects of other organic nitrates on ALDH2 activity in addition to their effects on the vasodilator responses of GTN.

1.7 Rationale, Research Hypothesis and Objectives

The literature concerning the mechanisms of nitrate bioactivation and tolerance, specifically GTN, is fraught with inconsistencies. It has been established that, as prodrugs, organic nitrates require biotransformation to function as vasodilators and that tolerance is associated with a decrease in this biotransformation. Furthermore, the enzyme ALDH2, found to be capable of metabolizing GTN, has been shown to become inactivated during tolerance. As a result, several hypotheses have been formulated with

respect to the role of ALDH2 in GTN tolerance. Specifically, researchers have proposed that ALDH2 is the principle enzyme in the mechanism-based biotransformation of GTN and that inactivation of the enzyme is the sole basis for GTN tolerance. In addition, a second mechanism-based pathway, independent of ALDH2 and immune to tolerance, has been proposed to bioactivate GTN at high concentrations as well as other lower potency nitrates. In light of the extensive literature indicating multiple mechanisms of tolerance, this proposed model is problematic. By examining the functional and biochemical changes that occur in VSM as a result of cross tolerance from chronic ISDN exposure, whether or not ALDH2 inactivation is the sole basis for GTN tolerance will be examined.

The hypothesis of this research is that **the reduced potency of GTN caused by ISDN tolerance is mediated by a mechanism independent of ALDH2 inactivation.** As mentioned earlier, Chen *et al* (2005) proposed two pathways of mechanism-based nitrate metabolism; the ALDH2-dependent GTN-specific pathway and the ALDH2-independent pathway for low potency nitrates and higher concentrations of GTN. By inducing ISDN tolerance *in vivo* via chronic exposure to ISDN, we will be able to assess what if any effect there is on GTN potency and ALDH2 activity. If, according to Chen`s model, the ALDH2-dependent pathway is indeed GTN-specific, and the ALDH2-independent pathway is not susceptible to tolerance, then we would not expect to see decreased GTN potency or ALDH2 inactivation in an ISDN tolerant vessel. The specific objectives of the proposed research were: 1) to develop an *in vivo* rat model for ISDN tolerance and 2) to determine if chronic ISDN exposure affects ALDH2 activity or the potency of GTN, ACh, SNP, and DEA/NO as vasodilators. This study was undertaken in the hopes of clarifying the role of ALDH2 in GTN bioactivation and tolerance.

CHAPTER 2: MATERIALS AND METHODS

2.1 Drugs and Solutions

Transdermal ISDN patches were obtained from Toa Eiyo Ltd (Tokyo, Japan). A full transdermal patch (Frandol tape-S) contained 40 mg of ISDN with a release rate of 0.8 mg/hr over 24 hours. Drug-free patches were produced by soaking patches for a minimum of 48 hours in 95% ethanol (patches were allow to air dry for at least 15 minutes before implantation). Removal of ISDN from the patches was confirmed by the absence of ISDN or metabolites in the plasma of rats implanted with these sham patches. ISDN was obtained in a solution of ethanol. GTN was obtained as a solution (TRIDIL, 5mg/ml) in ethanol, propylene glycol and water (1:1:1:33) from Sabex (Boucherville, Quebec). Stock dilutions of ISDN and GTN were made up in Krebs' solution (NaCl 119mM; KCl 5.4mM; $CaCl_2$ 2.5mM; KH_2PO_4 0.6mM; $MgSO_4$ 1.2mM; $NaHCO_3$ 25mM; and glucose 11.7mM). The following items were purchased from Sigma-Aldrich Chemical Company (Oakville, Ontario); phenylephrine hydrochloride (PE); potassium chloride (KCl); potassium dihydrogen orthophosphate (KH_2PO_4); sodium chloride (NaCl); calcium chloride ($CaCl_2$); magnesium sulphate ($MgSO_4$); acetylcholine (ACh); sodium nitroprusside (SNP) and diethylamine NONOate (DEA/NO). Sodium hydrogen carbonate ($NaHCO_3$) and D-glucose were purchased from BDH Inc. (Toronto, Ontario). Halothane for inhalational anaesthesia was obtained from Halocarbon Laboratories (River Edge, New Jersey). All gases were purchased from Praxair (Mississauga, Ontario).

2.2 Animals

Male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec) weighing between 250 and 350g were used. The animals were allowed to acclimate to the new environment for at least one week prior to any experiments. The animals were housed in separate cages and maintained at a temperature of 22-24°C in a room with a 12 hour light/dark cycle. The rats had free access to standard rat chow and tap water. All experiments were carried out within the guidelines of the Canadian Council on Animal Care.

2.3 Induction of ISDN Tolerance in Vivo

Rats were randomly assigned into ISDN-treated or sham groups with each group consisting of 3-6 animals. ISDN-treated rats were exposed to a continuous source of ISDN for various time periods (24, 48, 72, 96 hours) via subdermal implantation of one 0.8mg/hr transdermal ISDN patch. Briefly, a 1cm transverse incision was made and the skin separated from the underlying fascia by blunt dissection. One transdermal patch was inserted folded in half back-to-back into the resulting subdermal space. The site was sutured closed and disinfected with 2.5% iodine solution. At 48 hours, the site was reopened and the patch was replaced. Animals in the sham group received similar treatment; however sham (drug-free) patches were used instead. Animals were sacrificed at various time points and the aortae or livers were removed and prepared. The sham animals for 24, 48, and 72 hours ISDN treatment were sacrificed after 24 hours. The sham animals for 96 hours ISDN treatment were kept for 96 hours and underwent surgery at 48 hours to replace the sham patch.

2.4 Isolated blood vessel relaxation responses

2.4.1 Preparation

Rats were treated with ISDN as described above and sacrificed by decapitation under anaesthesia. Aortae were removed and placed in ice-cold Krebs' solution previously bubbled with 95% O_2/5% CO_2. Extraneous fat and tissue were carefully excised within 10 minutes. While each vessel was being prepared, it was bathed in ice-cold Krebs' solution. A scalpel blade was used to cut 2-3mm rings from each blood vessel. The rings were then immediately mounted with minimal tension on a pair of stainless steel stirrups in a 5 ml tissue bath containing Krebs' solution at 37°C aerated with 95% O_2/5% CO_2. One of the stirrups was fixed, while the other was attached to an isometric force transducer coupled to a Myodaq data acquisition system. Four aortic rings were used in each experiment under an optimal resting tension of 9.8mN as previously reported (MacPherson *et al*., 2006) and allowed to equilibrate for one hour. The optimal PE concentration used was determined by performing a complete PE concentration-response curve (0.1µM-100µM). PE in the concentration range of 1-5µM resulted in contractions that were approximately 50-80% of the maximal contraction.

2.4.2 Relaxation Responses

Following equilibration as described above, tissues were sub-maximally contracted with PE (1µM-5µM). After PE-induced tone had stabilized (10 minutes), concentration-response curves were obtained for one of several drugs in each separate tissue. Cumulative ISDN concentration-response curves were obtained beginning at

10^{-9}M ISDN and increasing in half-log dose intervals (i.e. 10^{-9}M, 3×10^{-9}M, 10^{-8}M, etc.) until a concentration of 3×10^{-5}M ISDN was achieved in the tissue bath. Cumulative GTN concentration-response curves were obtained beginning at 10^{-9} M GTN and increasing in half-log dose intervals until a concentration of 3×10^{-5} M GTN was achieved in the tissue bath. Cumulative ACh concentration-response curves were obtained beginning at 10^{-9} M ACh and increasing in half-log dose intervals until a concentration of 2×10^{-4} M ACh was achieved in the tissue bath. Cumulative SNP concentration-response curves were obtained beginning at 10^{-9} M SNP and increasing in half-log dose intervals until a concentration of 3×10^{-5} M SNP was achieved in the tissue bath. Cumulative DEA/NO concentration-response curves were obtained beginning at 10^{-9} M DEA/NO and increasing in half-log dose intervals until a concentration of 3×10^{-5} M DEA/NO was achieved in the tissue bath. All dilutions were made in Krebs` solution.

2.5 Biochemical Analysis

2.5.1 Protein Determination

Briefly, livers were perfused via the vena cava using 60ml of ice-cold saline and excised from animals. Blood and extraneous tissue and fat were removed. Livers were cut into pieces and homogenized in 50ml of TES buffer (0.25M sucrose, 5mM tris-HCl, 0.5M EDTA, 0.1M PMSF, pH 7.2). Samples were then homogenized with a glass tube and Teflon pestle and centrifuged at 480*g* for 10min; the supernatant was centrifuged at 4,800*g* for 7 min to obtain the mitochondrial fraction (pellet). Bio-Rad (Brantford, Ontario) protein analysis was performed according to the manufacturer`s directions in order to determine protein concentration. Optical density was measured at 595nm in a

Cary UV-Visible spectrophotometer. Linear regression was performed using bovine serum albumin (BSA) as the standard.

2.5.2 ALDH activity

Mitochondria were isolated from liver homogenates by the procedure described above. Deoxycholic acid (2.5 mg/mg protein) was added to mitochondrial homogenates in order to solubilise membrane-bound proteins, and ALDH activity was measured as the change in absorbance at 340 nm during incubation with 1 mM NAD^+ in 50 mM sodium pyrophosphate buffer, pH 8.8, containing 2 µM rotenone (to inhibit NADH consumption by complex I of the electron transfer chain), 1 mM 4-methylpyrazone (to inhibit alcohol dehydrogenase) and substrate (50 µM or 5 mM propionaldehyde) (Tottmar *et al*., 1973; Loomis and Brien, 1983). Rate measurements were performed on a Cary UV-Visible spectrophotometer at 340 nm at 25°C for 5 min. The specific activity was calculated for each sample using the molar extinction coefficient for NADH of $6306 M^{-1} cm^{-1}$.

2.6 Gas Chromatography

Samples were extracted from 100 ul aliquots of plasma with hexanes and diethyl ether. Using isoidide dinitrate (IIDN) and isoidide mononitrate (IIMN) as internal standards (50 pmol), ISDN and its metabolites, 2-ISMN and 5-ISMN, were quantitated by gas chromatography with electron capture detection as described previously by McDonald and Bennett (1990). Briefly, aliquots (0.1 mL) of the collected plasma were added to tubes containing 50 pmol IIDN (internal standard) and extracted with isomeric hexanes (3 x 2 mL) to remove ISDN and IIDN. The extracts were then evaporated to dryness,

redissolved in benzene (50 µL), and frozen at -20°Cuntil analysis. Fifty picomoles of isoidide mononitrate (IIMN, internal standard for the ISMN assay) was then added to the plasma samples, which were then extracted with diethyl ether (3 x 2 mL) to remove 2- and 5-ISMN and IIMN. These diethyl ether extracts were dried with anhydrous $MgSO_4$, evaporated to dryness, and the residue redissolved in benzene (50 µL) and frozen at -20°C until analysis.

Gas chromatographic analysis was performed with a Hewlett-Packard 5890 instrument equipped with a 63Ni electron capture detector. Aliquots (2 µL) of each sample were injected into a 15 m X 0.53 mm inside diameter fused silica megabore capillary column coated with 2.85 µm HP-l (cross-linked methyl silicone gum). Carrier gas used was helium and make up gas was argon-methane (95:5). Chromatograms were analyzed using a Shimadzu CR501 Chromatopac integrator.

2.7 Data analysis

The relaxation data were analyzed using GraphPad Prism 5.0. Concentration-response curves were plotted for each of the treatment groups. Tissue responses to a drug were measured as the percent decrease in PE-induced tone. The mean ± standard deviation percentage relaxation at each drug concentration was calculated and plotted using a sigmoidal concentration-response curve-fitting algorithm. The EC_{50} (effective concentration producing 50% relaxation) for each curve was determined and compared to their respective controls using Student's t-test for unpaired data. The mean ± standard deviation specific activities were calculated for ALDH activity assays and compared

using a Student’s t-test for unpaired data. A p value of less than 0.05 was considered statistically significant.

CHAPTER 3: RESULTS

3.1 Chronic ISDN Exposure Study

After 24 hours *in vivo* ISDN treatment, there was a 3.7-fold rightward shift in the concentration-response curve to ISDN and a significant increase in the EC_{50} value: 210 ± 26 nM (sham) and 770 ± 140 nM (treated) ($p<0.01$) (Fig. 3.1). After 48 hours ISDN treatment, there was a 6-fold rightward shift in the concentration-response curve to ISDN and a significant increase in the EC_{50} value: 210 ± 26 nM (sham) and 1200 ± 200 nM (treated) ($p<0.01$) (Fig. 3.3). After 72 hours ISDN treatment, there was a 9.9-fold rightward shift in the concentration-response curve to ISDN (sham EC_{50} = 210 ± 26 nM, treated EC_{50} = 2000 ± 270 nM ($p<0.01$)) (Fig. 3.5). Finally, after 96 hours ISDN treatment, there was an 18.6-fold rightward shift in the concentration-response curve to ISDN (sham EC_{50} = 170 ± 32 nM, treated EC_{50} = 3900 ± 760 nM ($p<0.01$)) (Fig. 3.7). For 24, 48, 72, and 96 hour ISDN exposures, no significant differences were seen for the maximum relaxation responses.

3.2 GTN Relaxation Response in ISDN Tolerant Aortae

No significant differences were observed in the relaxation responses to GTN in the 24 and 48 hour ISDN treatment groups (Fig. 3.2 and 3.4). The EC_{50} values for each group were: 17 ± 1.5 nM (sham), 32 ± 6.9 nM (treated 24hr), 34 ± 6.5 nM (treated 48hr). After 72 hours *in vivo* ISDN treatment, there was a 3.6-fold rightward shift in the dose response curve to GTN and a significant increase in the EC_{50} value: 17 ± 1.5 nM (sham) and 61 ± 7.3 nM (treated) ($p<0.01$) (Fig 3.6). At 96 hours ISDN treatment, there was a 5.9-fold shift rightward shift in the concentration-response curve (sham EC_{50} = 16 ± 2.2

nM, treated EC_{50} = 92 ± 11 nM ($p<0.01$)) (Fig. 3.8). For all groups (24, 48, 72, and 96 hours ISDN exposure), there was no significant difference seen in the maximum relaxation responses compared to control groups.

3.3 Relaxation responses to other vasodilators

The relaxation response of aortae from ISDN-treated animals to other vasodilators was also tested. The 96 hour ISDN exposure time point was used after it was established that both ISDN and GTN relaxation response curves were significantly shifted rightward in this group. After 96 hours ISDN exposure, no significant difference was seen in the EC_{50} values of SNP and DEA/NO (SNP; sham EC_{50} = 19 ± 2.8 nM, treated EC_{50} = 16 ± 4.0 nM, DEA/NO; sham EC_{50} = 76 ± 9.8 nM, treated EC_{50} = 89 ± 12 nM) (Fig. 3.11 and 3.12). There was also no significant difference found in the EC_{50} for ACh after 96 hours ISDN exposure (sham EC_{50} = 30 ± 4.5 nM, treated EC_{50} = 35 ± 5.7 nM)(Fig 3.10).

3.4 ALDH activity

ALDH2 activity was assessed in mitochondria isolated from whole cell homogenates of the rat liver to determine if enzymatic activity changed during chronic ISDN treatment. ALDH2 specific activity was measured using a low substrate concentration. Previous data shows a significant decrease in specific activity at both low and high K_m substrate concentrations after 48 hours GTN exposure (Fig. 3.13 A) ($p<0.05$) (Difabio *et al*., 2003). Our study showed no significant difference in specific activity at either high or low K_m substrate concentrations after 96 hours ISDN treatment (Fig. 3.13 B).

3.5 ISDN and metabolite measurement

After the 96 hour ISDN treatment time point was established as showing GTN cross tolerance, ISDN and metabolite concentrations in the blood plasma were measured. These measurements were of interest to confirm drug delivery via the implanted subdermal patch as well as to compare to previous data of ISDN metabolite ratios. Gas chromatography was used to determine plasma the plasma concentration of ISDN, 2-ISMN, and 5-ISMN after 96 hours ISDN exposure. Plasma concentrations were found to be 41 ± 11 M (ISDN), 210 ± 16 nM (2-ISMN), and 600 ± 170 nM (5-ISMN) (Fig. 3.14). The ratio of 5-ISMN to 2-ISMN in plasma was about 3 to 1.

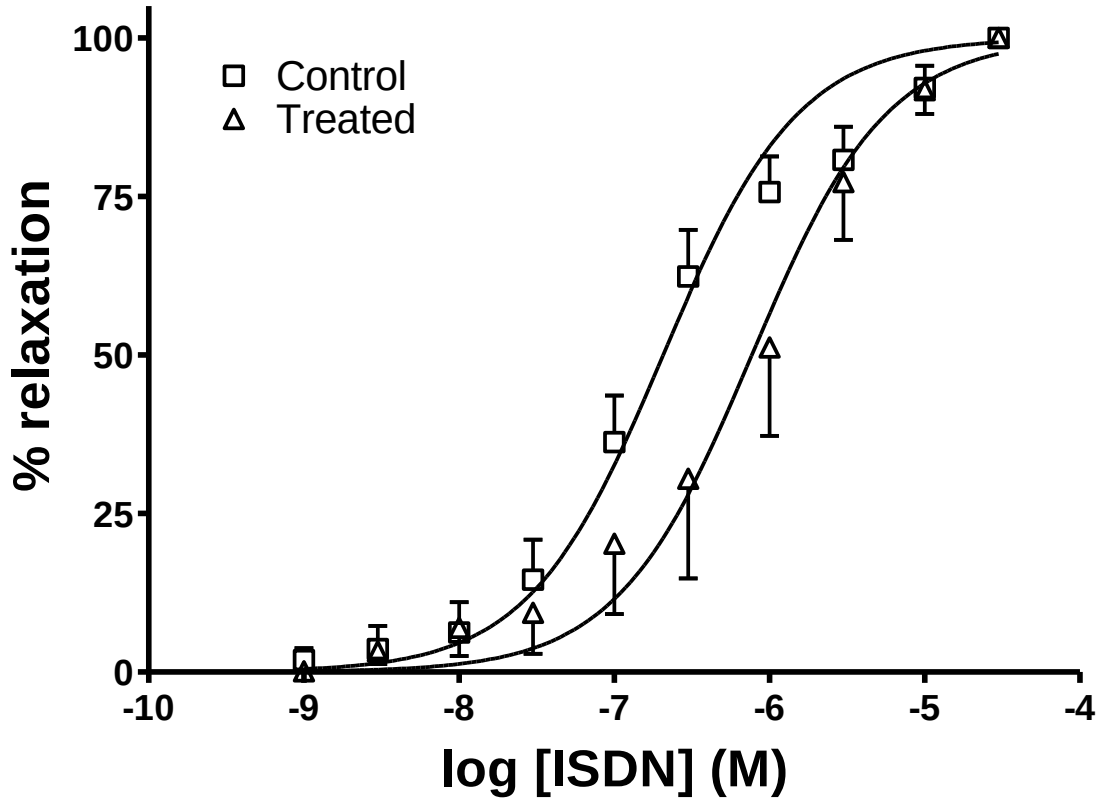

Figure 3.1 Effect of 24 hour ISDN exposure on ISDN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 24 hour ISDN exposure resulted in a significant increase in the EC_{50} value for ISDN-induced relaxation ($p<0.05$). No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

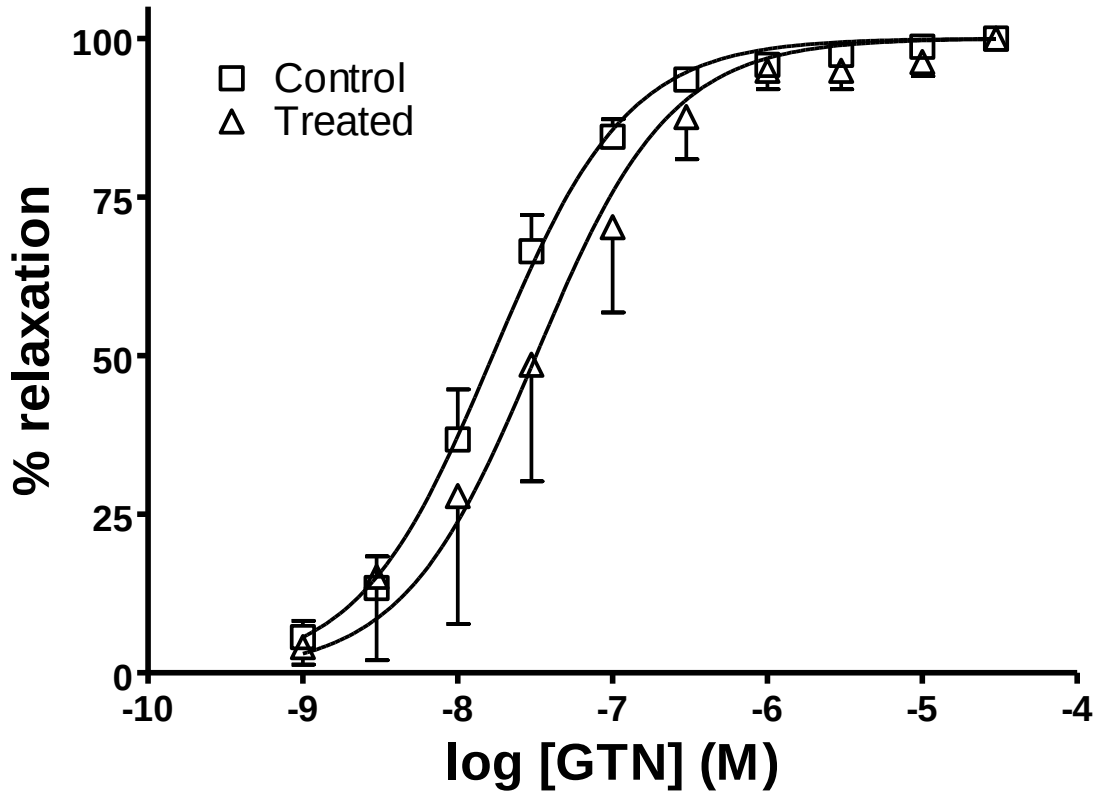

Figure 3.2 Effect of 24 hour ISDN exposure on GTN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 24 hour ISDN exposure resulted in no significant differences in the EC_{50} value for GTN-induced relaxation. No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

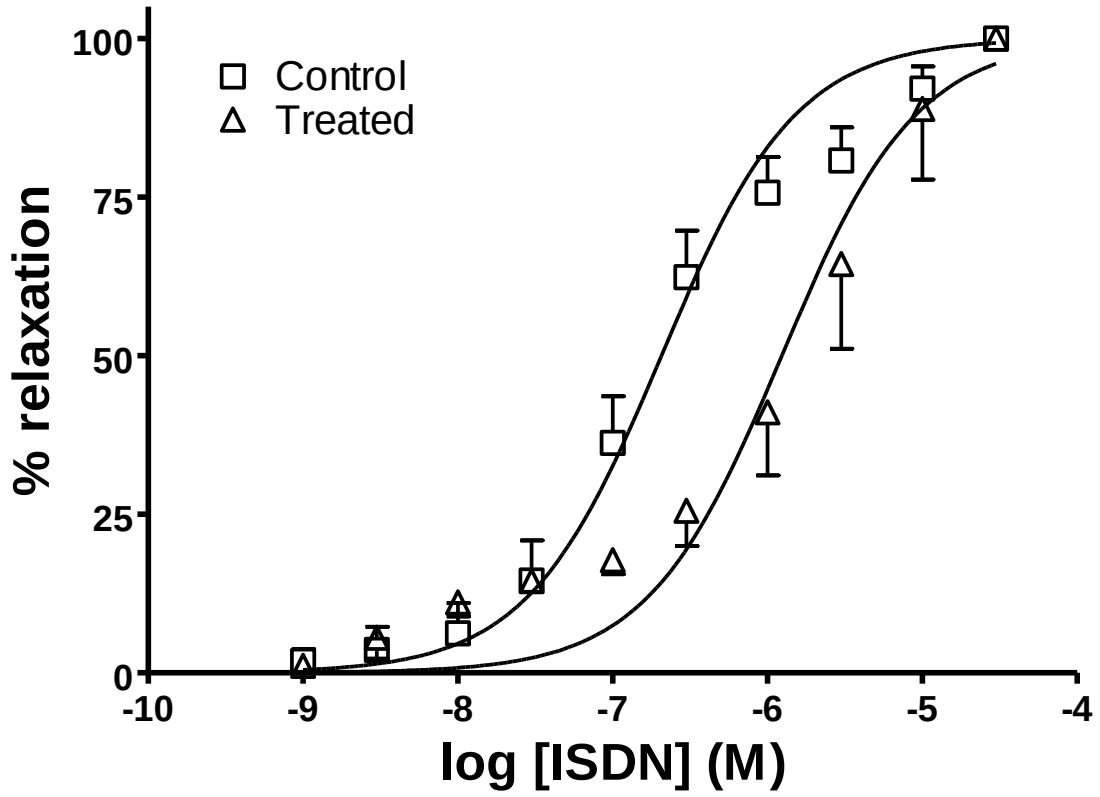

Figure 3.3 Effect of 48 hour ISDN exposure on ISDN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 48 hour ISDN exposure resulted in a significant increase in the EC_{50} value for ISDN-induced relaxation ($p<0.01$). No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

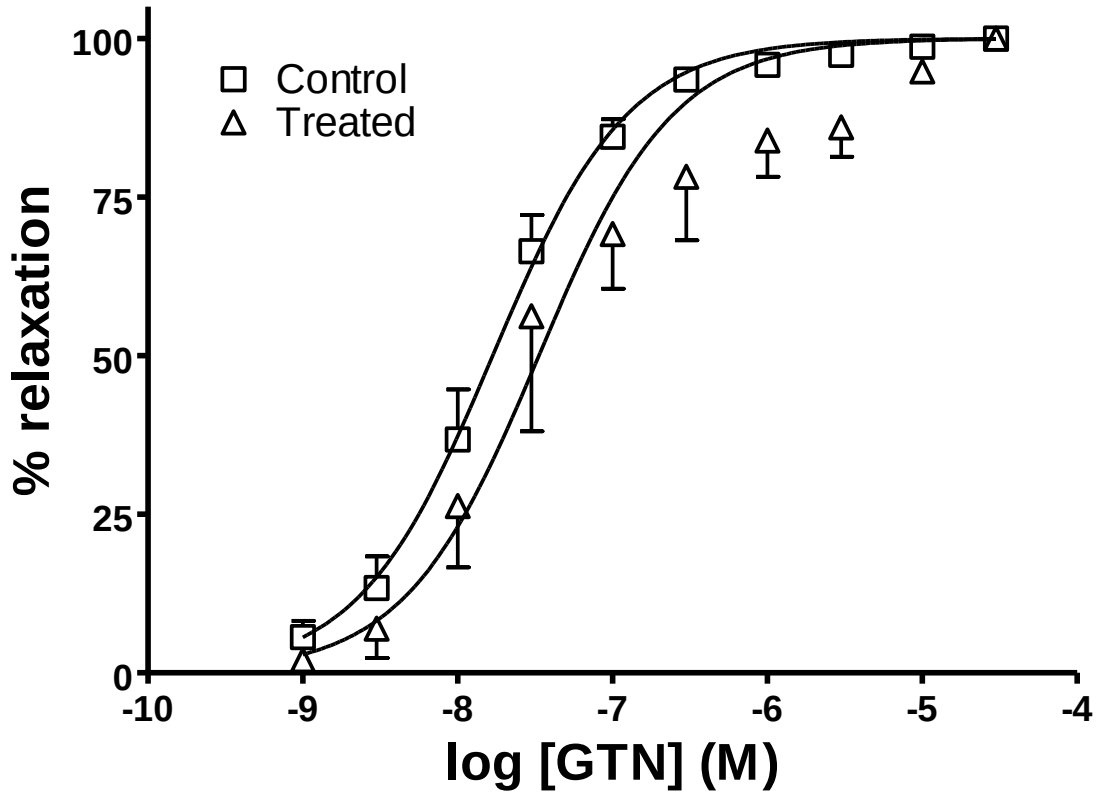

Figure 3.4 Effect of 48 hour ISDN exposure on GTN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 48 hour ISDN exposure resulted in no significant differences in the EC_{50} value for GTN-induced relaxation. No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

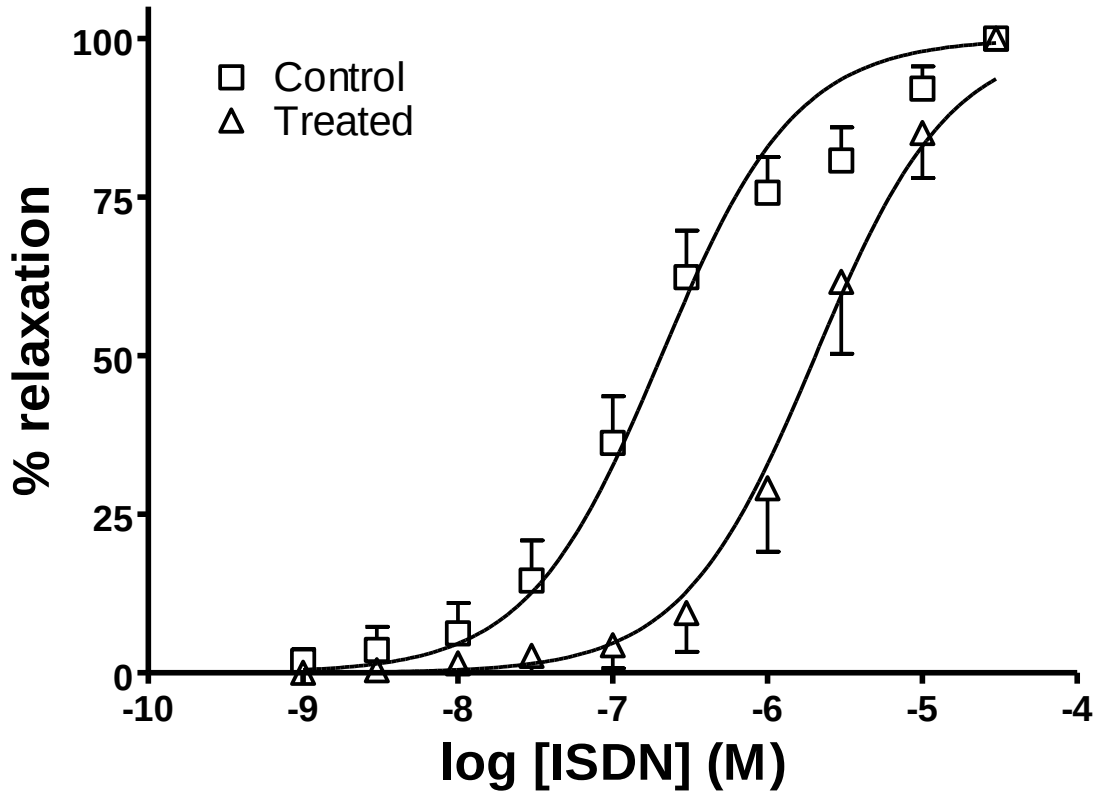

Figure 3.5 Effect of 72 hour ISDN exposure on ISDN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 72 hour ISDN exposure resulted in a significant increase in the EC_{50} value for ISDN-induced relaxation ($p<0.01$). No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

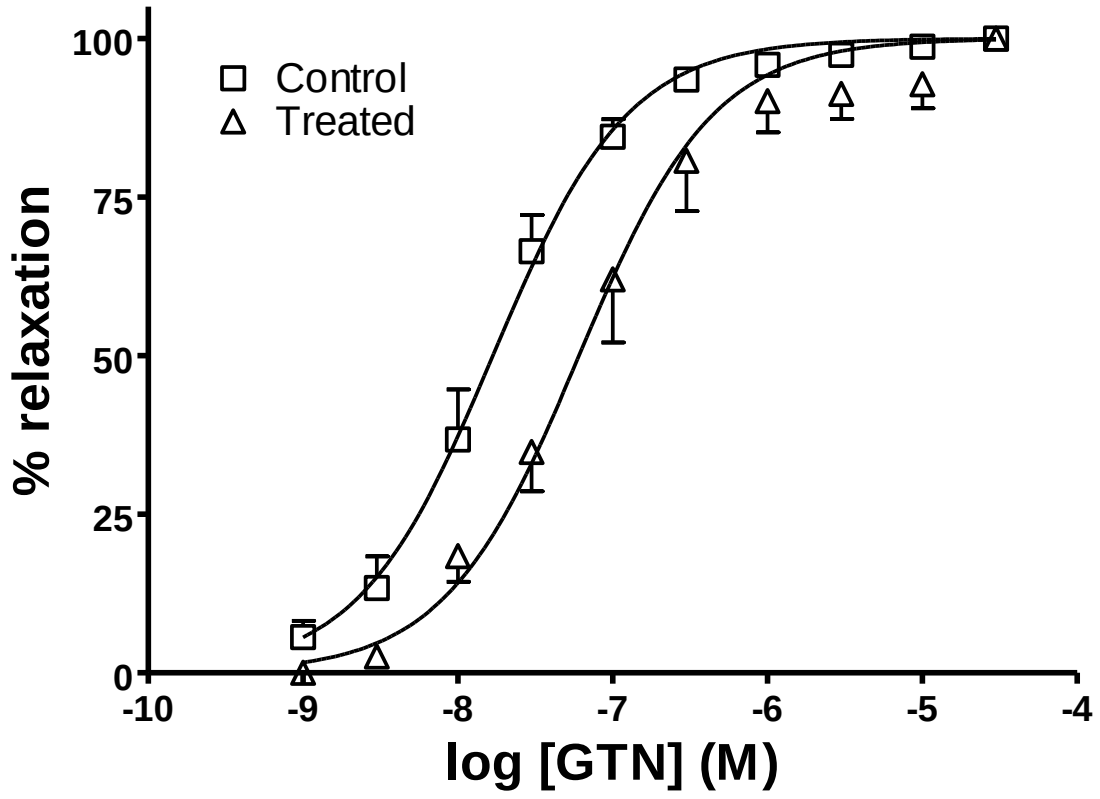

Figure 3.6 Effect of 72 hour ISDN exposure on GTN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 72 hour ISDN exposure resulted in a significant increase in the EC_{50} value for GTN-induced relaxation ($p<0.05$). No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

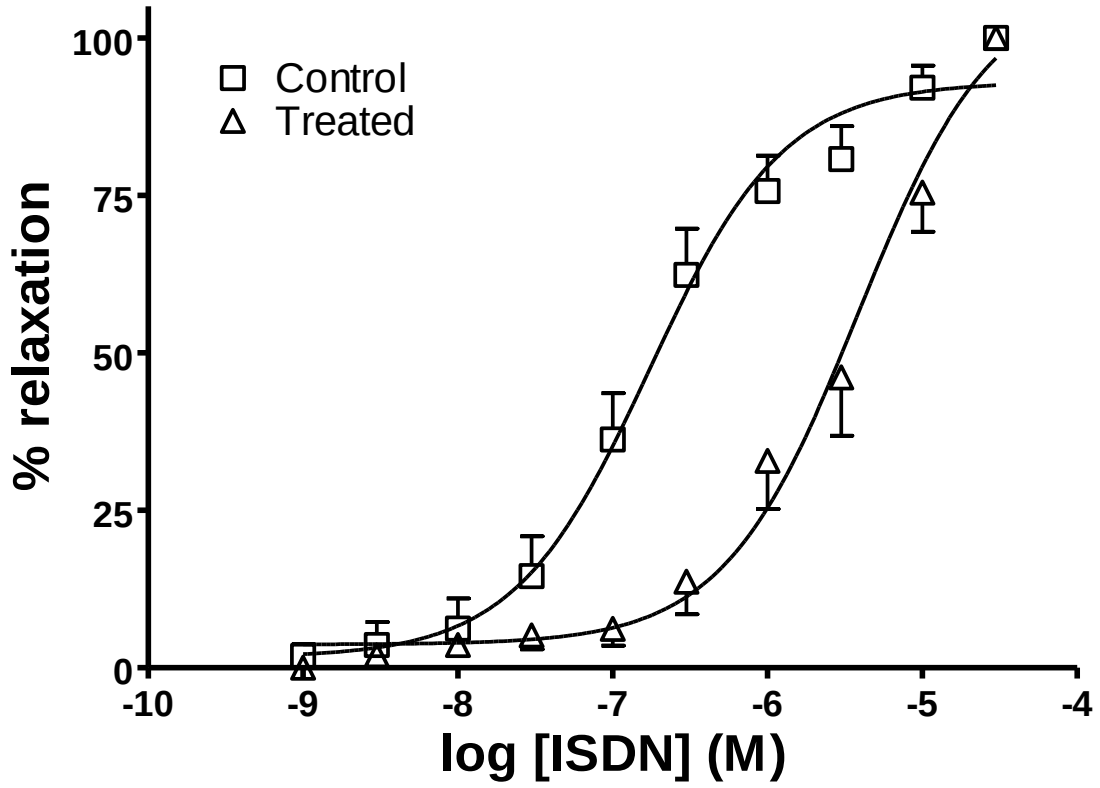

Figure 3.7 Effect of 96 hour ISDN exposure on ISDN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 96 hour ISDN exposure resulted in a significant increase in the EC_{50} value for ISDN-induced relaxation ($p<0.01$). No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

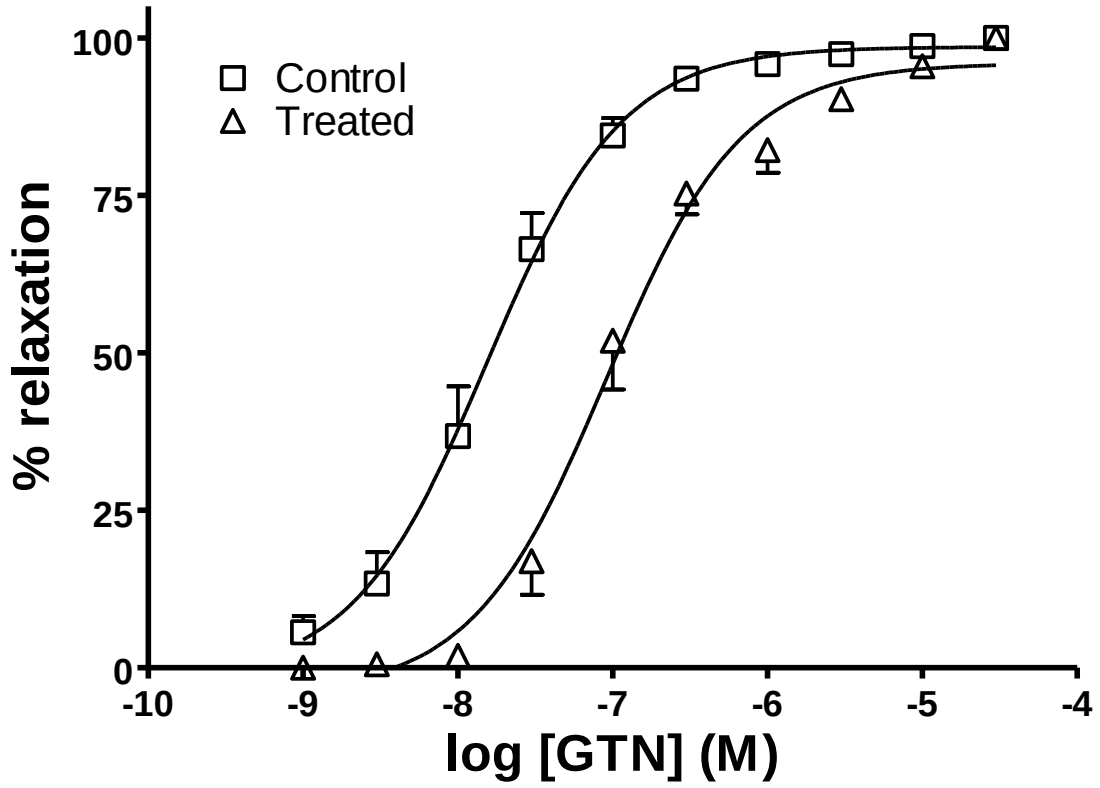

Figure 3.8 Effect of 96 hour ISDN exposure on GTN-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 96 hour ISDN exposure resulted in a significant increase in the EC_{50} value for GTN-induced relaxation ($p<0.01$). No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

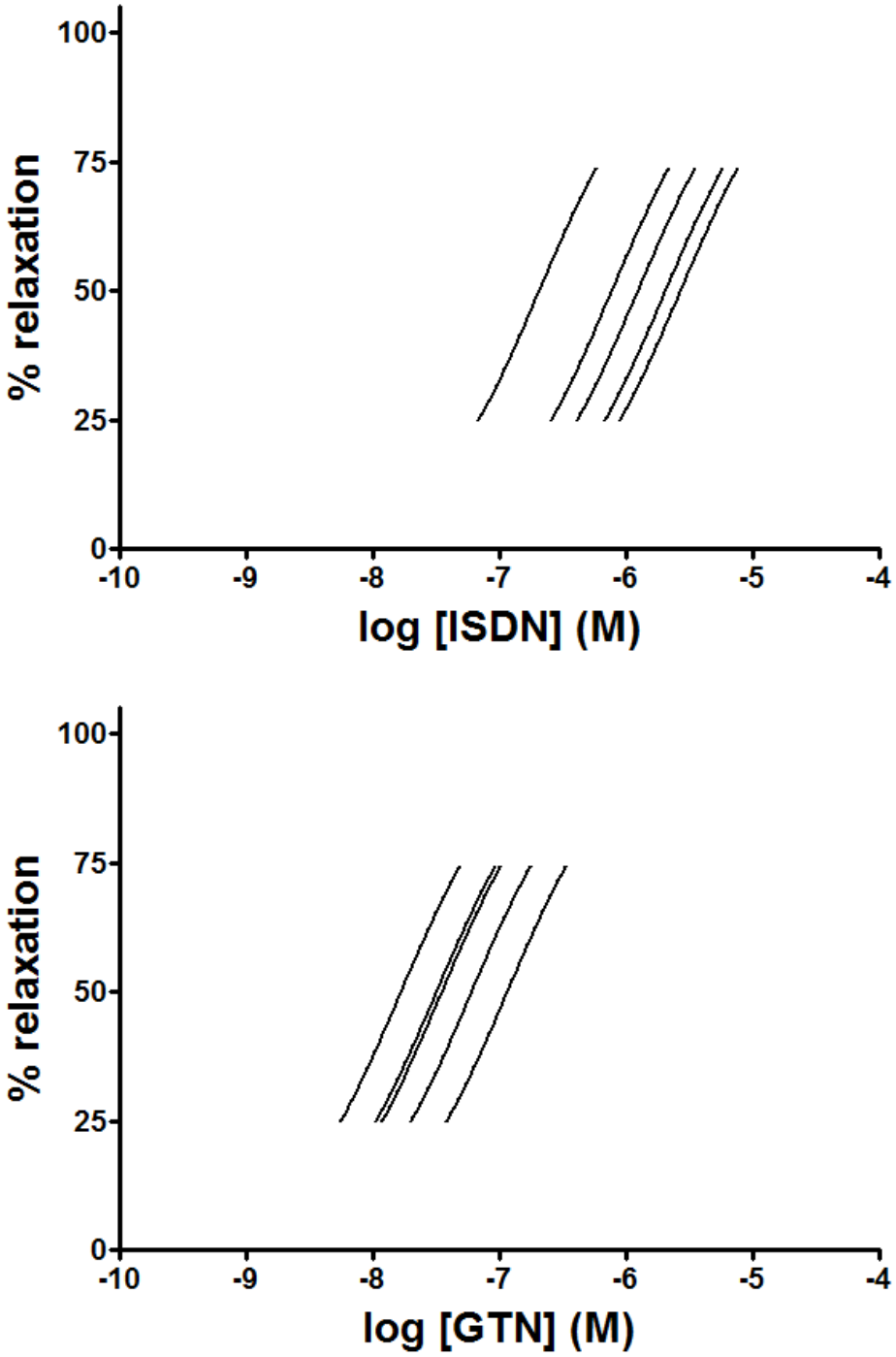

Figure 3.9 Composite highlighting the rightward shifts in concentration response curves of ISDN and GTN over 96 hours. Curves from left to right are; control, 24, 48, 72, and 96 hour ISDN exposures. The top graph shows the development of ISDN tolerance during chronic ISDN exposure. The bottom graph shows the development of cross tolerance to GTN during chronic ISDN exposure.

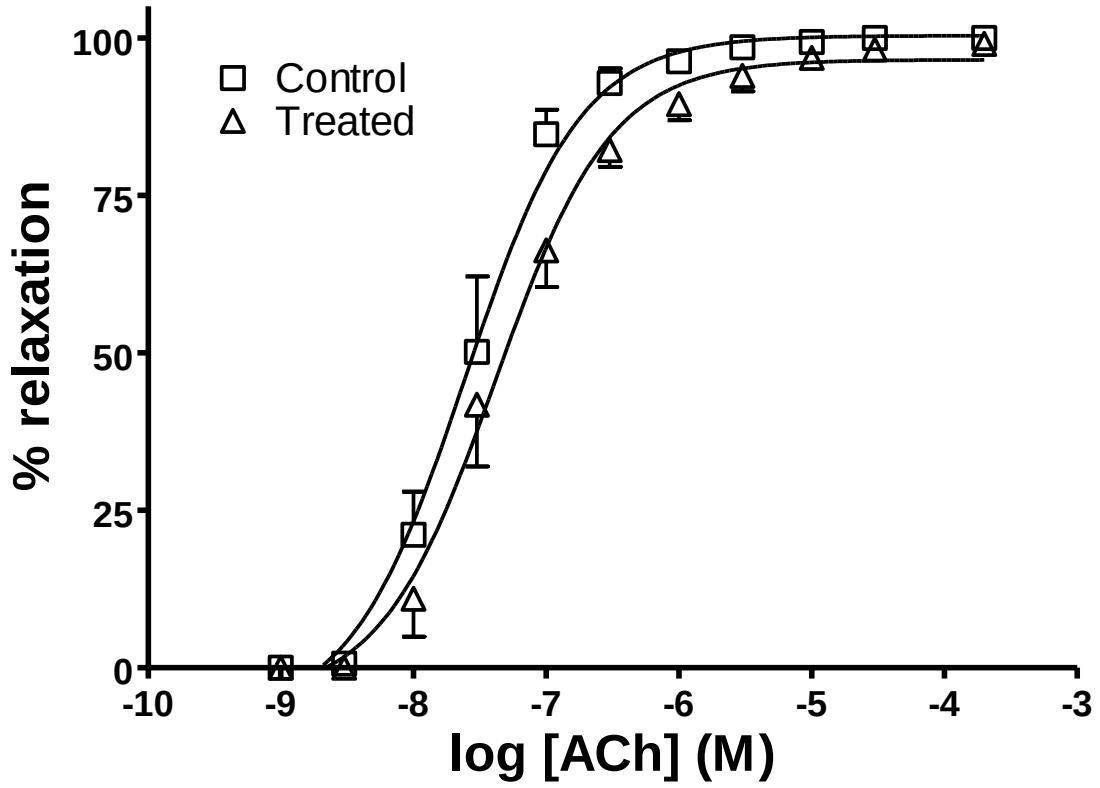

Figure 3.10 Effect of 96 hour ISDN exposure on ACh-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 96 hour ISDN exposure resulted in no significant differences in the EC_{50} value for ACh-induced relaxation. No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=5)

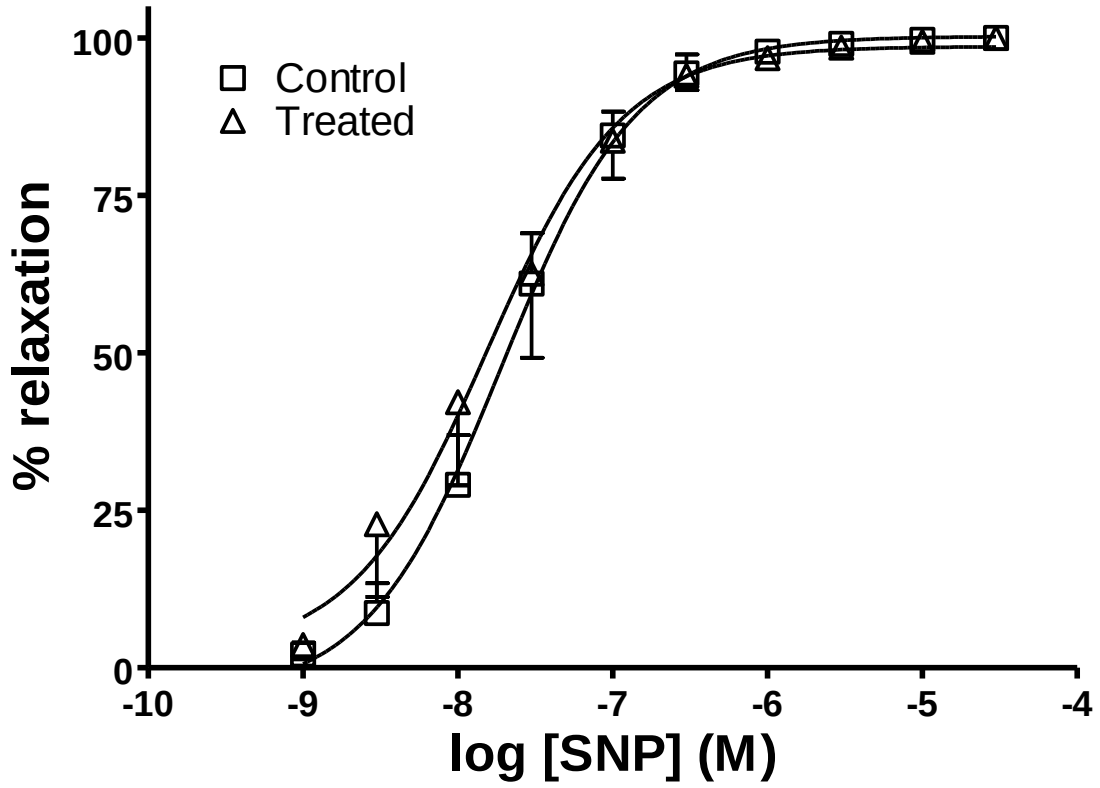

Figure 3.11 Effect of 96 hour ISDN exposure on SNP-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 96 hour ISDN exposure resulted in no significant differences in the EC_{50} value for SNP-induced relaxation. No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=6)

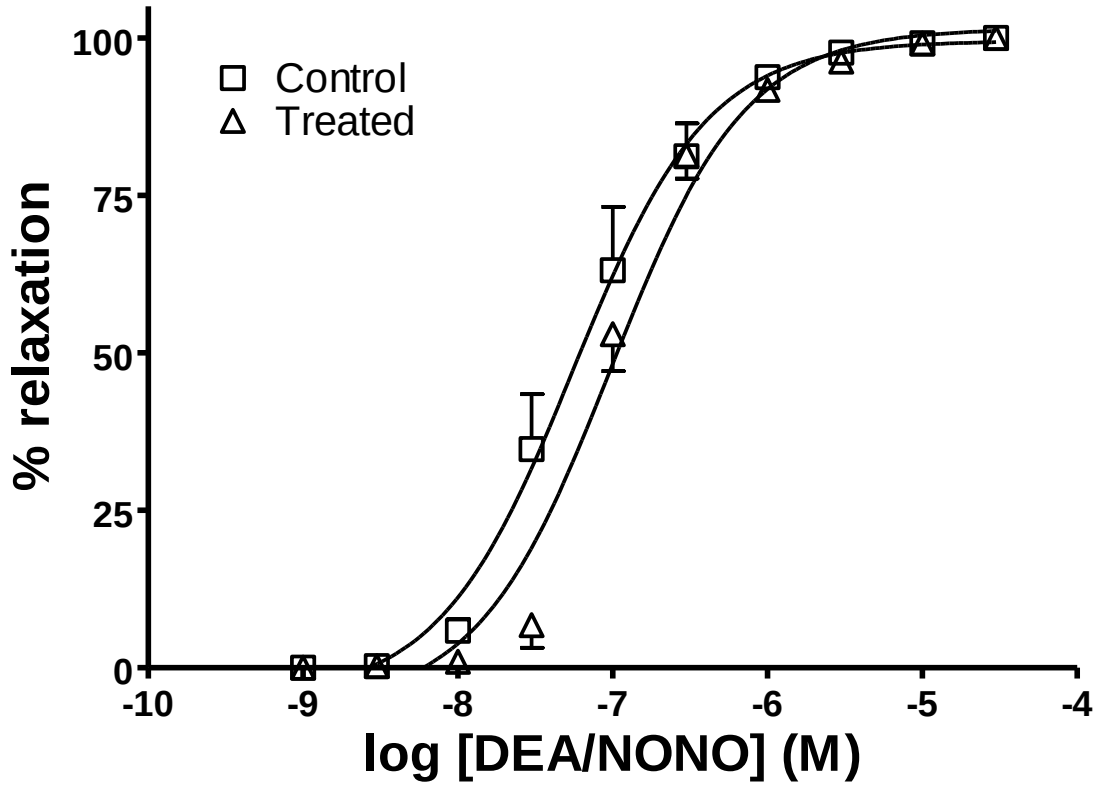

Figure 3.12 Effect of 96 hour ISDN exposure on DEA/NONO-induced relaxation of isolated rat aorta. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN. A 96 hour ISDN exposure resulted in no significant differences in the EC_{50} value for DEA/NONO-induced relaxation. No significant differences were observed in the maximal relaxation responses between sham and treated animals. Data is expressed as mean ± S.D. (n=4)

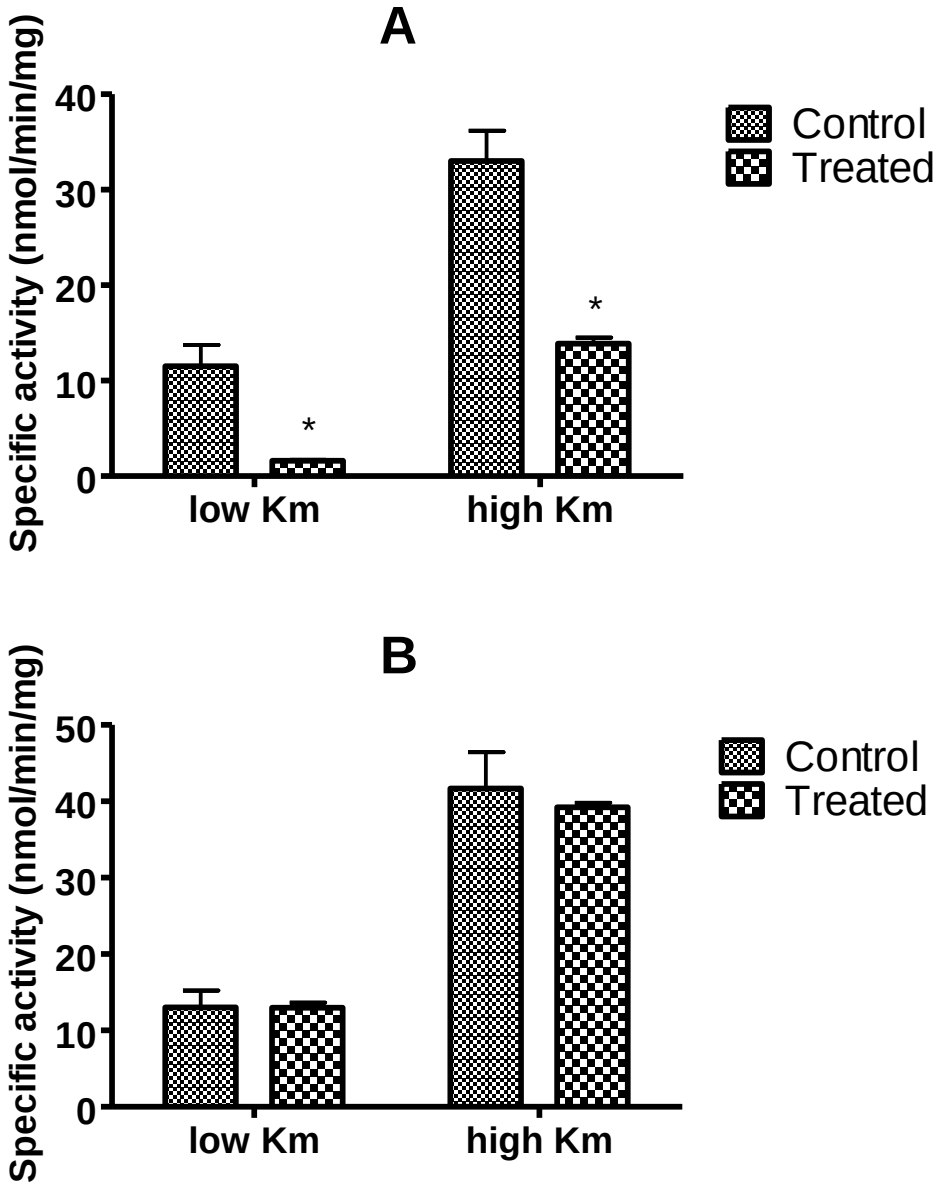

Figure 3.13 Hepatic mitochondrial ALDH activities during chronic nitrate treatment. Low K_m indicates substrate concentration of 0.05 mM propionaldehyde. High K_m indicates substrate concentration of 5 mM propionaldehyde. Letters indicate the following: A, Control indicates rats implanted with sham patches and Treated indicates rats administered 0.4 mg/hr GTN treatment for 48 hours (n=6); B, Control indicates rats implanted with sham patches and Treated indicates rats administered 0.8 mg/hr ISDN treatment for 96 hours (n=4). Bars represent the mean ±S.D. * indicates significant differences from control ($p< 0.05$). Results shown in Figure 13A modified from Difabio *et al*. (2003).

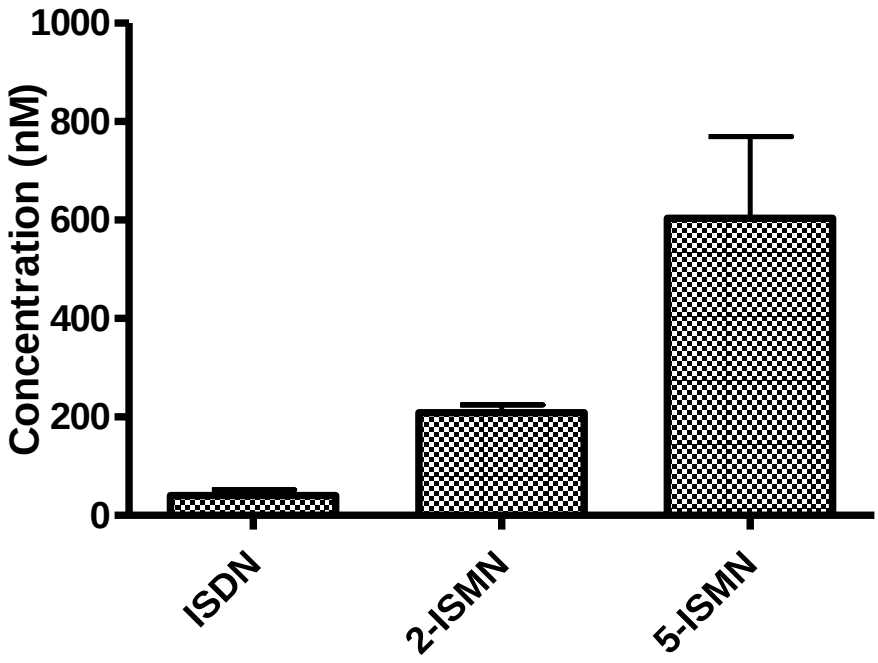

Figure 3.14 Plasma concentration of ISDN and metabolites after 96 hour ISDN exposure. Gas chromatography was used to determine plasma concentration of ISDN, 2-ISMN, and 5-ISMN in rats administered 0.8 mg/hr ISDN for 96 hours. Bars represent the mean ± S.D. (n=5)

To provide a clearer understanding of the shape of the rightward shift in the GTN concentration-response curves during ISDN-induced cross tolerance, the individual curves for each aortic ring were evaluated (Figure 3.15). This was done to ensure that the average curve generated from the individuals was an accurate reflection of the shift taking place after 96 hours of ISDN exposure. Furthermore, this allowed for the observation of interesting concentration-response curve shapes that may be otherwise blunted or obscured in the mean curve generated. Also included was ALDH activity data comparing the effects of several inhibiting compounds (Figure 3.16). Both ISDN and GTN were compared to the selective ALDH inhibitor, daidzin, for their ability to inhibit ALDH activity in this particular assay. GTN was found to effectively inhibit ALDH activity while ISDN had no effect.

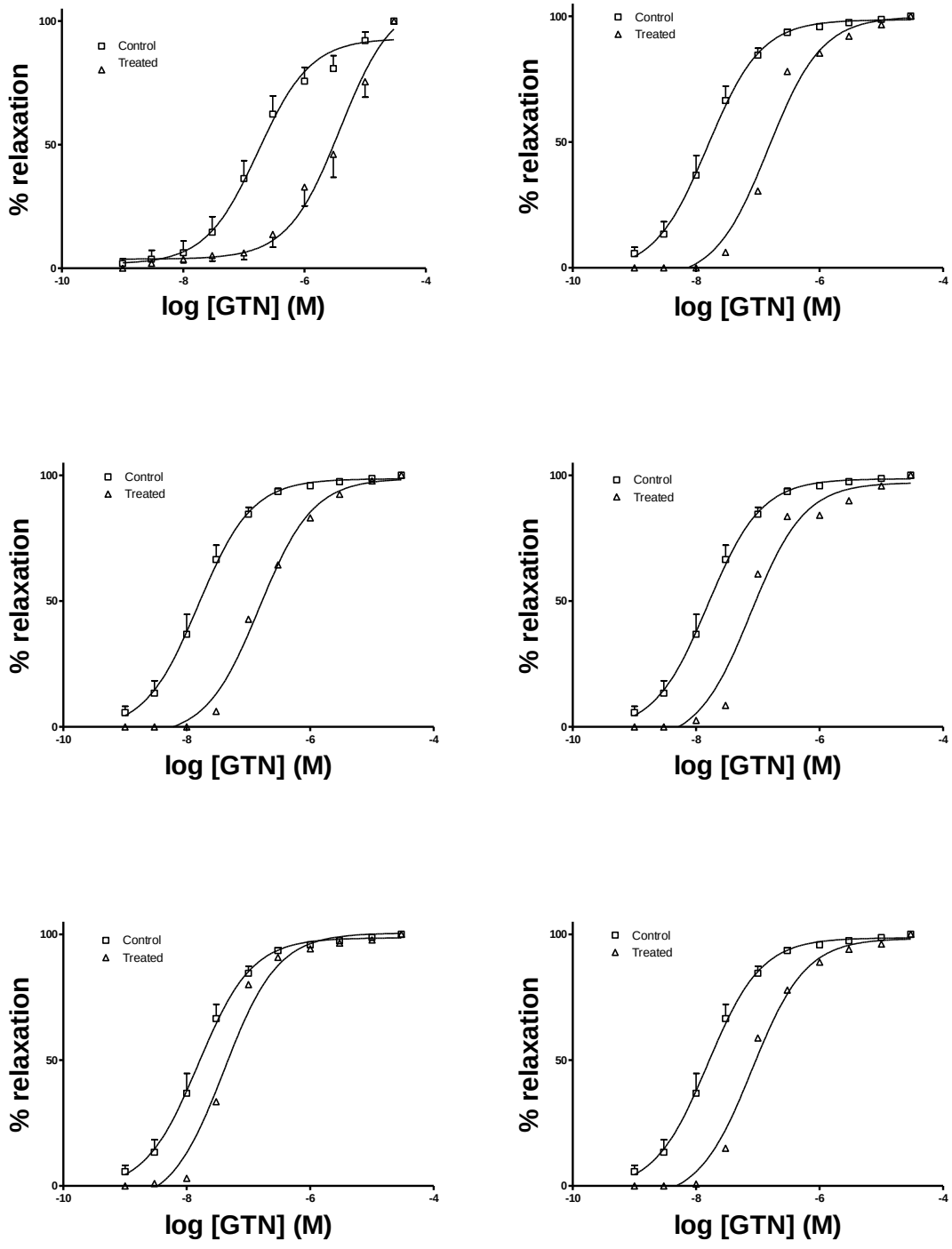

Figure 3.15 Individual GTN concentration response curves in aortae from rats treated with ISDN for 96 hours. The individual concentration response curves used to generate the mean concentration response curve for GTN in aortae from rats treated with ISDN for 96 hours (Fig 3.8).

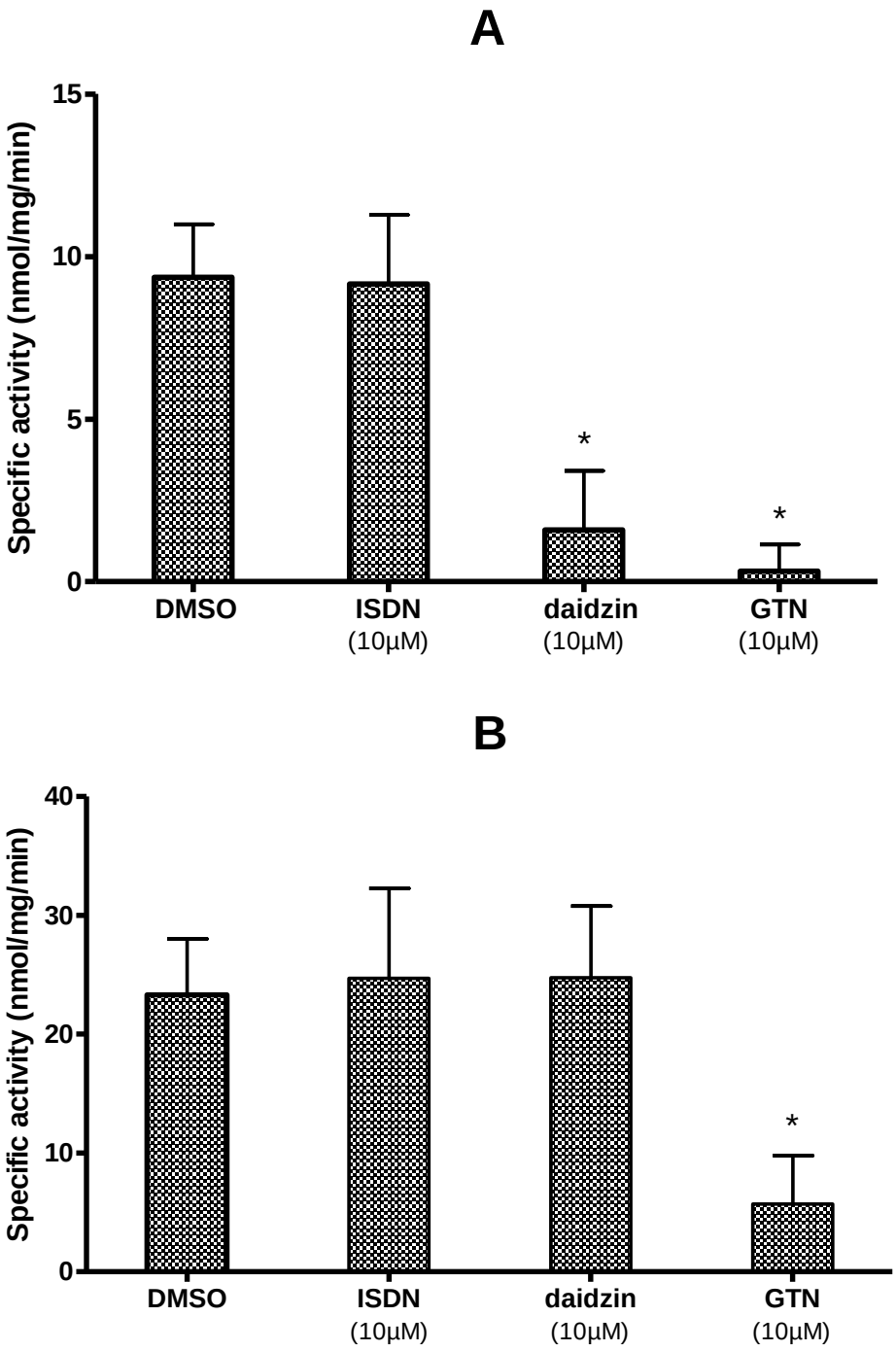

Figure 3.16 Mitochondrial ALDH inhibition by various compounds. 10 uM concentrations of ISDN, GTN and the selective ALDH2 inhibitor daidzin were used to inhibit the specific activity of ALDH in mitochondria isolated from hepatic tissue taken from untreated animals. DMSO was used as the control. A shows the low K_m activity while B illustrates the high K_m substrate concentration. Bars represent the mean ±S.D. * indicates significant differences from control ($p < 0.05$). N=4

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

The phenomenon of GTN tolerance has been observed for over 100 years. Recently, the role of ALDH2 in GTN biotransformation has been widely studied. To date, however, no study has examined the effects of ISDN exposure on ALDH2 activity and GTN responses. The present study examined the association between ALDH2 activity and *ex vivo* functional responses to GTN, during chronic ISDN exposure. This study allowed for the examination of the role of ALDH2 in GTN cross tolerance due to ISDN exposure. As previously stated, the objectives of the study were 1) to develop an *in vivo* model of ISDN tolerance and 2) to examine the changes in ALDH2 activity and GTN responses after chronic ISDN exposure. The results of this study indicate that cross tolerance to GTN does occur after chronic ISDN exposure *in vivo*. Furthermore, the significant rightward shift in ISDN and GTN concentration-response curves occurs with no significant decrease in hepatic mitochondrial ALDH2 activity, which was used a s a surrogate marker of ALDH2 (Fig 3.13B). To the extent that this surrogate marker reflects vascular ALDH2 activity, these results suggest that inactivation of ALDH2 is not the sole basis for GTN tolerance and that bioactivation of ISDN and GTN occurs at least partly via a shared mechanism that is susceptible to inactivation after chronic exposure to ISDN. Chen *et al* (2002 and 2005) have argued that inactivation of ALDH2 is in fact the sole basis for GTN tolerance. With some evidence supporting this hypothesis, the assertion still remains controversial. The first objective of the current study was to develop an *in vivo* model of ISDN tolerance that could be used to study cross tolerance with GTN. The method chosen for chronic ISDN exposure was subdermal implantation of transdermal drug patches, the same technique our laboratory uses for inducing GTN tolerance *in vivo*.

By using a similar methodology to the established model, it becomes easier to compare the effects of chronic ISDN exposure with chronic GTN exposure, thereby increasing the utility of the model. Furthermore, the parallels between the models allowed for a clear and quantifiable definition of cross tolerance. After 48 hours *in vivo* GTN exposure, animals are considered to be moderately tolerant to GTN. One measure of this tolerance is the rightward shift in the concentration-response curve of aortic rings isolated from GTN-treated animals. Previous studies have found approximately a 4.5-fold rightward shift in EC_{50} (30 ± 17 nM to 133 ± 86 nM) in GTN-induced relaxation in aortic rings from GTN-tolerant animals compared to control (Difabio *et al*., 2003). Using this degree of loss of function to define a state moderate GTN tolerance, a time course evaluation of GTN vasodilation in animals continually exposed to ISDN was performed, starting at 24 hours. It was unknown how quickly ISDN exposure would cause cross tolerance to GTN or if cross tolerance would even occur with the ISDN dosage used (0.8mg/hr). The 24 hour time point showed the rapid onset of ISDN tolerance and a slight rightward shift in the GTN concentration-response curve. Subsequent time points (48 and 72 hours) showed increasing rightward shifts in both the ISDN and GTN concentration-response curves. At the 96 hour time point, the magnitude of the rightward shift in the response was consistent with previous studies performed in our laboratory showing moderate GTN tolerance. The concentration-response curves for GTN produced after 96 hours ISDN exposure and 48 hours GTN exposure are nearly superimposable. It is for this reason (matching degrees of GTN tolerance) that further experiments in this study were performed using the 96 hour ISDN exposure time point. As a phenomenon, the degree of GTN tolerance can vary, as evident by the range of EC_{50} values obtained using different

dosage regimens. As a result, it is challenging to determine the relative quantitative role of ALDH2 in GTN tolerance. The methods used can vary markedly which may lead to varying results and conclusions, or results that cannot be directly compared due to differences in methods used. A more quantitative definition of certain characteristics of GTN tolerance may lead to a better understanding of the mechanisms involved in the complex research problem that is nitrate tolerance.

In order to further characterize the *in vivo* model of ISDN tolerance, the plasma concentration of ISDN and its metabolites was measured. This was performed for several reasons, the first being that subdermal patch implantation is a previously untested method of chronic ISDN exposure *in vivo*. Previous models of ISDN tolerance relied on interval doses of nitrate delivered to live animals or on using in vitro incubation with recently harvested tissues. Any model that attempts to induce tolerance once tissue has been harvested may exclude certain factors such as neurohormonal counter-regulation or changes in gene expression that may be relevant for the development of tolerance. Moreover, these models often resort to supraphamacological concentrations of the organic nitrate to induce tolerance in a short period of time. The method previously used by our laboratory and in this study has the advantages of both inducing tolerance *in vivo* and providing a continuous dose over a chosen time period. While the relaxation response of treated vessels is measured *ex vivo*, this method of inducing tolerance much more closely models the development of tolerance in a patient being treated prophylactically with a transdermal nitrate patch, and provides a more complete picture of nitrate tolerance. Even so, this method was untested with ISDN patches and thus it was important to measure the concentration of ISDN and its metabolites during treatment.

The second reason for measuring these plasma concentrations was to provide a measurement to compare to previous data gathered on ISDN metabolism. Specifically, the ratio of 5-ISMN formation to 2-ISMN formation is known to favour 5-ISMN formation. ISDN contains two nitrate groups; one endo oriented and one exo oriented in the molecule's structure. The energetically favoured removal of the more accessible exo nitrate group results in the formation of 5-ISMN. Gas chromatography experiments using the plasma from animals treated with ISDN for 96 hours found that formation of 5-ISMN was favoured nearly three to one over 2-ISMN. Previous studies have found similar ratios in favour of 5-ISMN, depending on the method used to administer the ISDN dose. Morrison and Fung (1984) reported a five to one ratio of 5-ISMN to 2-ISMN when ISDN was given intravenously and a nine to one ratio when ISDN was given orally. It is not possible to say whether the development of tolerance or the different methods of ISDN administration account for the different ratios observed. Further experiments measuring the ISDN metabolite concentrations in nontolerant animals dosed using subdermal patch implantation would be required to determine the effect of tolerance on the ratio of 5-ISMN to 2-ISMN.

The second objective of this study was to determine the effects of chronic ISDN treatment on the vasodilator response of GTN, and on the enzymatic activity of ALDH2. Furthermore, the vasodilator responses of several NO donors (SNP and DEA/NO) and an endothelium-dependent vasodilator (ACh) were assessed. As previously discussed, as little as 48 hours ISDN exposure caused a visible rightward shift in the GTN concentration-response curve. At 96 hours, the shift was equal to that caused by 48 hours of GTN exposure, as found in previous experiments (Difabio *et al*., 2003). This cross

tolerance contradicts the model set forth by Chen *et al* (2005) in which the low-affinity pathway responsible for bioactivating ISDN is not susceptible to tolerance. Clearly, ISDN treatment produces cross tolerance to GTN, and vice versa (eg. Schelling and Lasagna, 1967; Zelis and Mason, 1975; Manyari *et al*., 1985). Chen concluded that the low-affinity pathway for bioactivating nitrates does not exhibit tolerance based on the observation that no further shift in the concentration-response curve for GTN occurred after in vitro exposure of aortae from *Aldh2*$^{-/-}$ mice to high GTN concentration. It was therefore concluded that this pathway, responsible for bioactivating high concentrations of GTN and low potency nitrates like ISDN, was ALDH2-independent. This is a problem, however, because it fails to explain the development of tolerance to any nitrate other than GTN, in addition to the cross tolerance to other nitrates induced by GTN. The model also states that ALDH2 is essential in GTN bioactivation and that inactivation of the enzyme is solely responsible for the development of tolerance. In addition to significant cross tolerance to GTN, our study found that after 96 hours ISDN treatment, hepatic ALDH2 activity was not altered (Fig 3.13B). That is, tolerance equal to that produced by 48 hours continuous GTN treatment occurring with no significant change in ALDH2 activity. Clearly, this evidence directly contradicts the idea of ALDH2-dependent GTN tolerance. While many studies have linked ALDH2 inactivation with GTN tolerance, to my knowledge this is the first observation of significant GTN tolerance with no effect on ALDH2 activity. While there is evidence that ties ALDH2 to GTN tolerance, the conclusion that inactivation of the enzyme directly causes the phenomenon of tolerance does not appear to be valid. Combined with the findings of

previous studies (Chen *et al*., 2005; Difabio *et al*., 2003; D'Souza *et al*., in press) the presented data supports a multifactorial model of GTN and nitrate tolerance.

It is worthwhile to more closely examine the shape of the individual GTN concentration response curves in the 96 hour ISDN-treated animals. Specifically, whether the observed shift is parallel or happens to be greater at either high or low drug concentration. The results show a parallel shift in the curves, similar to that seen in GTN-induced tolerance (Figure 3.15). That is, both low and high concentrations of GTN were equally affected by the ISDN-induced cross tolerance. According to Chen's proposed model, ISDN treatment would only have an effect on the low affinity pathway for low potency nitrates while leaving the high affinity ALDH2-dependent pathway selective for GTN unaffected. If the model held true, we would expect to see a minimal shift in GTN potency at lower GTN concentrations, with tolerance being observed only at higher concentrations, not the observed parallel shift. Therefore, whatever the mechanism by which this cross tolerance occurs, it has a similar effect on both low and high concentrations of GTN. Perhaps the so-called "low-affinity" pathway is at work even at low GTN concentrations and has simply been overshadowed by bioactivation mediated by the "high affinity pathway". Further investigation is certainly necessary to properly understand this problem.

The study also examined the changes in vasodilator responses of several other drugs in ISDN tolerant aorta. The concentration-response curves of SNP and DEA/NO were both unchanged after 96 hours ISDN treatment. It was important to assess the effect of tolerance on the vasodilators to determine what, if any, effect there was on the NO signalling pathway downstream from nitrate bioactivation. SNP and DEA/NO both

produce NO bioactivity without requiring bioactivation as organic nitrates do. Thus, when ISDN/GTN tolerance is seen without a change in response to SNP or DEA/NO, it can be concluded that the mechanism responsible for such tolerance is likely occurring in the process of bioactivation, before NO bioactivity is produced. Additionally, concentration-response curves were also obtained for ACh. As with SNP and DEA/NO, no significant difference was seen in the EC_{50} for ACh-induced relaxation of aortae from control rats and rats treated for 96 hours with ISDN. As an endothelium-dependent vasodilator, an unchanged response to ACh between control and tolerant animals indicates that the VSM cell's relaxation response to direct NO stimulation is still intact. Furthermore, because ACh triggers the release of NO from the vascular endothelium, this result shows that the endothelium was undamaged. This is relevant because endothelial damage and GTN tolerance have been linked, most likely by oxidative stress (Gori and Parker, 2002). The results of these tissue bath experiments show that tolerance was specific to ISDN and GTN, and thus was unlikely to be caused by endothelial damage. This evidence supports the reduced bioactivation theory of nitrate tolerance, where inhibition of mechanism-based biotransformation is the principle cause of tolerance. As previously discussed, however, a multifactorial model of tolerance indicates that inactivation of several mechanisms is likely involved, and does not exclude the possibility of factors outside of bioactivation contributing to the development of a tolerant state.

In the present study, we have shown that a 96 hour exposure to ISDN results in GTN tolerance, but not ALDH2 inactivation, indicating that ALDH2 inactivation is not a prerequisite for GTN tolerance. Therefore, based on these results, we conclude that

ALDH2 inactivation is a consequence of chronic exposure to GTN and not the sole cause of nitrate tolerance. However, according to the study performed by Chen *et al* in 2005 using *Aldh2*$^{-/-}$ mice, the removal of ALDH2 does cause a rightward shift in the GTN concentration-response curve. One possible explanation for this could be that in the absence of ALDH2, an important detoxifying enzyme for a number of toxic aldehydes (eg. 4-hydoxynonenal), there is increased endogenous oxidative stress and increased susceptibility to exogenously triggered oxidative stress. Indeed, studies have shown that *Aldh2*$^{-/-}$ mice were more susceptible to nitroglycerin-, acetaldehyde- and doxorubicin-induced cardiovascular damage (Wenzel *et al*., 2008). Thus increased oxidative stress in the absence of ALDH2 could inactivate enzymes responsible for GTN bioactivation, or lead to quenching of NO and thus preventing its interaction with sGC (Gori and Parker, 2002). Another possibility is that *Aldh2*$^{-/-}$ mice are less susceptible to the vasodilatory effects of GTN simply because ALDH2 is missing and unable to bioactivate GTN. Future studies should focus in part on determining the cause and effect relationship between ALDH2 inactivation and GTN tolerance. Chronic GTN exposure is known to cause oxidative stress, but whether this occurs before or after ALDH2 inactivation is unclear. An experiment designed to measure the formation of reactive oxygen species in 96 hour ISDN-treated tissue would help shed light on this question. If reactive oxygen species formation similar to that seen in GTN treated tissue were found, then the cause of ALDH2 inactivation would presumably be specific to the interaction between GTN and the enzyme and not due to inactivation of ALDH2 by reactive oxygen species.

Previous work in our laboratory has found that GTN is a potent inhibitor of ALDH2 activity whereas ISDN had no effect (Fig 3.16). The inhibiting effect of GTN is

even greater than that seen by the selective ALDH2 inhibitor, daidzin. This data supports the findings in the current study regarding the lack of interaction between ISDN and ALDH2. Although ALDH2 inactivation does not appear to be the sole cause of GTN tolerance, the enzyme is clearly linked with the drug's biotransformation in some way. A model of GTN/ISDN bioactivation and tolerance consistent with the data obtained in this thesis is depicted in Fig 4.1. According to this model, all nitrates are bioactivated by a subset of sulfhydryl-containing or other enzymes. These enzymes are susceptible to oxidation and/or disulfide bridge formation which causes enzyme inactivation and typical nitrate tolerance. There is also a group of enzymes selective for high potency nitrates that may or may not include ALDH2 and are susceptible to inactivation. The results of this study suggest that it is more likely that ALDH2 is a clearance-based enzyme that is inactivated by GTN.

Future studies with the goal of constructing a more complete picture of nitrate tolerance might focus on differences in the potencies of nitrates and identification of which enzymes are responsible for bioactivation. It would be interesting to investigate the effect of ALDH inhibitors on GTN-induced relaxation in ISDN tolerant blood vessels, or to see if other high potency nitrates are affected by chronic ISDN treatment in a similar fashion.

In conclusion, the findings of this study further clarify the role of ALDH2 in nitrate tolerance. My results indicate that ALDH2 cannot be sole enzyme responsible for GTN tolerance and more importantly, that inactivation of ALDH2 is not required for the development of GTN tolerance. This study concludes that the inactivation of ALDH2 in GTN tolerance is most likely an epiphenomenon of the development of tolerance. These

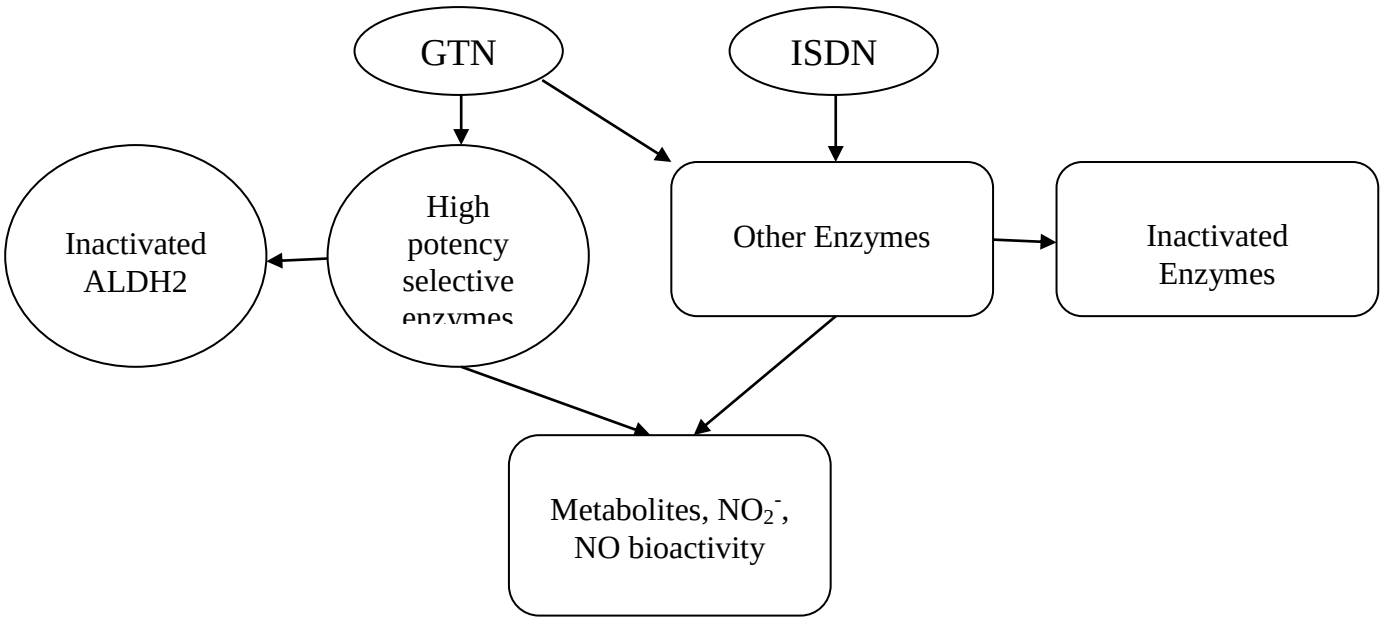

Figure 4.1 Proposed mechanism of ISDN/GTN tolerance. The proposed model includes multiple bioactivating enzymes. Some "Other Enzymes" activate all nitrates and are inactivated by all nitrates, thereby mediating cross tolerance. "High potency selective enzymes" can selectively bioactivate high potency nitrates and may include ALDH2. It is also possible that ALDH2 is just a clearance-based enzyme that is inactivated by GTN.

findings support a multifactorial theory of nitrate tolerance that challenges currently accepted models of GTN tolerance in which inactivation of ALDH2 is of central importance. The knowledge gained from this study has increased our understanding of the shared mechanisms of tolerance development to GTN and ISDN and may provide insights into novel nitrate therapies.

REFERENCES

Abrams J (1985) Pharmacology of nitroglycerin and long-acting nitrates. *Am. J. Cardiol.* **56**:12A-18A.

Abrams J (1995) The role of nitrates in coronary heart disease. *Arch. Intern. Med.* **155**:357-364.

Abrams J (1996) Beneficial actions of nitrates in cardiovascular disease. *Am. J. Cardiol.* **77**:31C-37C.

Ahlner J, Andersson RGG, Torfgard K, Axelsson KL (1991) Organic nitrate esters: clinical use and mechanisms of actions. *Pharmacol. Rev.* **43**:351-423.

Armstrong PW, Moffat JA, and Marks GS (1982) Arterial-venous nitroglyceric gradient during intravenous infusion in man. *Circulation* **66**:1273-1276.

Axelsson KL and Andersson RGG (1983) Tolerance towards nitroglycerin, induced in vivo, is correlated to a reduced cGMP response and an alteration in cGMP turnover. *Eur. J. Pharmacol.* **88**:71-79.

Axelsson KL and Karlsson JOG. (1984) Nitroglycerin tolerance in vitro: Effect on cGMP turnover in vascular smooth muscle. *Acta Pharmacol. Toxicol.* **55**:203-210.

Bassenge E, Fink N, Slatchkov M, and Fink B (1998) Dietary supplement with vitamin C prevents nitrate tolerance. *J. Clin. Invest.* **102**:67-71.

Bennett BM and Marks G S (1984) How Does Nitroglycerin Induce Vascular Smooth Muscle Relaxation? *Trends Pharmacol.Sci.***5**: 329-332.

Bennett BM, Kobus SM, Brien JF, Nakatsu K, and Marks GS (1986) Requirement for reduced, unliganded hemoprotein for the hemoglobin- and myoglobin-mediated biotransformation of glyceryl trinitrate. *J. Pharmacol. Exp. Ther.* **237**:629-635.

Bennett BM, Leitman DC, Schröder H, Kawamoto JH, Nakatsu K, and Murad F (1989) Relationship between biotransformation of glyceryl trinitrate and cyclic GMP accumulation in various cultured cell lines. *J. Pharmacol. Exp. Ther.* **250**:316-323.

Bennett BM, McDonald BJ, Nigam R, and Simon WC (1994) Biotransformation of organic nitrates and vascular smooth muscle cell function. *Trends Pharmacol. Sci.* **15**:245-249.

Bennett BM, Schroder H, Hayward LD, Waldman SA and Murad F (1988) Effect of in vitro organic nitrate tolerance on relaxation, cyclic GMP accumulation, and guanylate cyclase activation by glyceryl trinitrate and the enantiomers of isoidide dinitrate. *Circ. Res.* **63***:693-701*.

Boesgaard S, Petersen JS, Aldershvile J, Poulsen HE, and Flachs H (1991) Nitrate tolerance: effect of thiol supplementation during prolonged nitroglycerin infusion in an in vivo rat model. *J. Pharmacol. Exp. Ther.* **258**:851-856.

Brien JF, McLaughlin BE, Kobus SM, Kawamoto JH, Nakatsu K, and Marks GS (1988) Mechanism of glyceryl trinitrate-induced vasodilation. I. Relationship between drug biotransformation, tissue cyclic GMP elevation and relaxation of rabbit aorta. *J. Pharmacol. Exp. Ther.* **244**:322-327.

Brudvig GW, Stevens TH, and Chan SI (1980) Reactions of nitric oxide with cytochrome c oxidase. *Biochem.* **19**:5275–5285.

Chen Z, Zhang J, and Stamler JS (2002) Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc. Natl. Acad. Sci. U.S.A.* **99**: 8306-8311.

Chen Z, Zhang J, Foster MW, and Stamler JS (2005) An essential role for mitochondrial aldehyde dehydrogenase in nitroglycerin bioactivation. *Proc Natl Acad Sci USA.* **102**:12159–12164.

Daiber A, Wenzel P, Oelze M, and Munzel T (2007) New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance. *Clin. Res. Cardiol.* **97**:12-20.

De la Lande IS, Stepien JM, Philpott AC, Hughes PA, Stafford I, and Horowitz JD (2004) Aldehyde dehydrogenase, nitric oxide synthase and superoxide in *ex vivo* nitrate tolerance in rat aorta. *Eur J. Pharmacol.* **496**:141-149.

DeMaster EG, Redfern B, and Quast BJ (1997) Mechanism for the inhibition of aldehyde dehydrogenase by nitric oxide. *Alcohol* **14**:181–189.

DiFabio J, Ji Y, Vasiliou V, Thatcher GR, and Bennett BM (2003) Role of mitochondrial aldehyde dehydrogenase in nitrate tolerance. *Mol. Pharmacol.* **64**:1109-1116.

Doel JJ, Godber BLJ, Gouly TA, Eisenthal R, and Harrison R (2000) Reduction of organic nitrites to nitric oxide catalyzed by xanthine oxidase: possible role in metabolism of nitrovasodilators. *Biochem. Biophys. Res. Commun.* **270**:880-885.

Dupuis J, Lalonde G, Lemieux R, and Rouleau JL (1990) Tolerance to intravenous nitroglycerin in patients with congestive heart failure: role of increased intravascular volume, neurohumoral activation and lack of prevention with N-acetylcysteine. *J. Am. Coll. Cardiol.* **16**:923-931.

D'Souza YP, Dowlatshahi S, and BennettBM (2011) Changes in aldehyde dehydrogenase 2 expression in rat blood vessels during glyceryl trinitrate tolerance development and reversal. Br. J. Pharmacol. In press.

Feldman RI and Weiner H (1972) Horse liver aldehyde dehydrogenase. *J.Biol. Chem.* **247**:260-272.

Fiscus RR, Rapoport RM, and Murad F (1983) Endothelium-dependent and nitrovasodilator-induced activation of cyclic GMP-dependent protein kinase in rat aorta. *J. Cyclic Nucleotide Protein Phosphor. Res.* **9**:415-425.

Fung H (2004) Biochemical mechanism of nitroglycerin action and tolerance: is this old mystery solved? *Annu. Rev. Pharmacol. Toxicol.* **44**:67-85.

Giuffre A, Barone MC, Mastronicola D, D'Itri E, Sarti P, and Brunori M (2000) Reaction of nitric oxide with the turnover intermediates of cytochrome c oxidase: reaction pathway and functional effects. *Biochemistry* **39**:15446-15453.

Gori T and Parker JD (2002) The puzzle of nitrate tolerance: pieces smaller than we thought? *Circulation* **106**:2404-2408.

Gruetter CA and Lemke SM (1986) Effects of sulfhydryl reagents on nitroglycerin-induced relaxation of bovine coronary artery. *Can. J. Physiol. Pharmacol.* **64**:1395-1401.

Habig WH, Keen JH, and Jakoby WB (1975) Glutathione S-transferase in the formation of cyanide from organic thiocyantes and as an organic nitrate reductase. *Biochem. Biophys. Res. Commun.* **64**:501-506

Horowitz JD, Henry CA, Syrjanen ML, Louis WJ, Fish RD, Antman EM, and Smith TW (1988) Nitroglycerin/N-acetylcysteine in the management of unstable angina pectoris. *Eur. Heart J.* Suppl **A**:95-100.

Katsuki S, Arnold W, Mittal C, and Murad F (1977a) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cyclic Nucleotide Res.* ***3****:*23-35.

Katsuki S, Arnold W P and Murad F (1977b) Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. *J.Cyclic Nucleotide Res.* ***3****:*239-247

Kawamoto JH, McLaughlin BE, Brien JF, Marks GS, and Nakatsu K (1990) Biotransformation of glyceryl trinitrate and elevation of cyclic GMP precede glyceryl trinitrate-induced vasodilation. *J. Cardiovasc. Pharmacol.* **15**:714-9.

Kenkare SR and Benet LZ (1996) Tolerance to nitroglycerin in rabbit aorta. Investigating the involvement of the mu isozyme of glutathione S-transferases. *Biochem. Pharmacol.* **51**:1357-1363.

Kleschyov AL, Oelze M, Daiber A, Huang Y, Mollnau H, Schulz E, Sydow K, Fichtlscherer B, Mulsch A, and Münzel T (2003) Does nitric oxide mediate the vasodilator activity of nitroglycerin? *Circ. Res.* **93**:E104-112.

Kollau A Hofer A, Russwurm M, Koesling D, Keung WM, Schmidt K, Brunner F, and Mayer B. (2004) Contribution of aldehyde dehydrogenase to mitochondrial bioactivation of nitroglycerin: evidence for the activation of purified soluble guanylate cyclase through direct formation of nitric oxide. *Biochem. J.* **385**:769-77.

Kozlov AV, Staniek K, and Nohl H (1999) Nitrate reductase activity is a novel function of mammalian mitochondria. *FEBS Lett*. **454**:127-130.

Kurz MA, Boyer TD, Whalen R, Peterson TE, and Harrison DG (1993) Nitroglycerin metabolism in vascular tissue: role of glutathione S-transferases and relationship between NO· and NO2- formation. *Biochem. J.* **292**:545-550.

Kurz S, Hink U, Nickenig G, Borthayre AB, Harrison DG and Munzel T (1999) Evidence for a causal role of the renin-angiotensin system in nitrate tolerance. *Circulation* **99**:3181-3187.

Lis Y, Bennett D, Lambert G, and Robson D (1984) A preliminary double-blind study of intravenous nitro in acute myocardial infarction. *Intensive Care Med*. **10:**179-184.

Longobardi G, Ferrara N, Leosco D, Abete P, Furgi G, Cacciatore F, Corbi G, Pescatore R, and Rengo F (2004) Angiotensin-II receptor antagonist losartan does not prevent nitroglycerin tolerance in patients with coronary artery disease. *Cardiovas. Drugs Ther.* **18**:363-370.

MacPherson JD, Gillespie T, Maurice DH, and Bennett BM (2005) Inhibition of phosphodiesterase type 5 selectively reverses nitrate tolerance in venous circulation. *J. Pharmacol. Expt. Ther*. **317**:188-95.

Manyari DE, Smith ER, and Spragg J (1985) Isosorbide dinitrate and glyceryl trinitrate: demonstration of cross tolerance in the capacitance vessels. *Am. J. Cardiol.* **55**:927-931.

Marks GS, McLaughlin BE, Jimmo SL, Poklewska-Koziell M, Brien JF, and Nakatsu K (1995) Time-dependent increase in nitric oxide formation concurrent with vasodilation induced by sodium nitroprusside, 3-morpholinosydnonimine, and S-nitroso-N-acetylpenicillamine but not by glyceryl trinitrate. *Drug Metab. Dispos.* **23**:1248-1252.

Marsh N and Marsh A (2000). A short history of nitroglycerine and nitric oxide in pharmacology and physiology. *Clin. Exp. Pharmacol.Physiol.***27**:313-319.

McDonald BJ and Bennett BM (1990) Cytochrome P-450 mediated biotransformation of organic nitrates. *Can. J. Physiol. Pharmacol.* **68**:1552-1557.

McDonald BJ and Bennett BM (1993) Biotransformation of glyceryl trinitrate by rat aortic cytochrome P450. *Biochem. Pharmacol.* **45**:268-270.

McGuire JJ, Anderson DJ, and Bennett BM (1994) Inhibition of the biotransformation and pharmacological actions of glyceryl trinitrate by the flavoprotein inhibitor, diphenyleneiodonium sulfate. *J. Pharmacol. Exp. Ther*. **271**:708-714.

McGuire JJ, Anderson DJ, McDonald BJ, Narayanasami R, and Bennett BM (1998) Inhibition of NADPH-cytochrome P450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodonium sulphate. *Biochem. Pharmacol* **56**:881-893.

Meah Y, Brown BJ, Chakraborty S, and Massey V (2001) Old yellow enzyme: reduction of nitrate esters, glycerine trinitrate, and propylene 1,2-dinitrate. *Proc. Natl. Acad. Sci. U.S.A.*. **98**8560-8565.

Millar FJ, Jr., Gutterman DD, Rios CD, Heistad DD, and Davidson BL (1998) Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ. Res*. **82**:1298-1305.

Münzel T and Bassenge E (1996) Long-term angiotensin-converting enzyme inhibition with high-dose enalapril retards nitrate tolerance in large epicardial arteries and prevents rebound coronary vasoconstriction in vivo. *Circulation.* **93**:2052-2058.

Münzel T, Hink U, Yigit H, Macharzina R, Harrison DG, and Mülsch A. (1999). Role of superoxide dismutase in *in vivo* and *in vitro* nitrate tolerance. *Br J Pharmacol*. **127**:1224-1230.

Münzel T, Mollnau H, Hartmann M, Geiger C, Oelze M, Warnholtz A, Yehia AH, Forstermann U, and Meinertz T (1999) Effects of a nitrate-free interval on tolerance, vasoconstrictor sensitivity and vascular superoxide production. *J. Am. Coll. Cardiol.* **36**:628-634.

Münzel T, Sayegh H, Freeman BA, Tarpey MM, and Harrison DG (1995) Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J. Clin. Invest.* **95**:187-194.

Needleman P and Johnson EM, Jr. (1973) Mechanism of tolerance development to organic nitrates. *J. Pharmacol. Exp. Ther.* **184**:709-715.

Nigam R, Whiting T, and Bennett BM (1993) Effect of inhibitors of glutathione S-transferase on glyceryl trinitrate activity in isolated rat aorta. *Can. J. Physiol. Pharmacol.* **71**:179-184.

Pagani ED, VanAller GS, O'Connor B, and Silver PJ (1993) Reversal of nitroglycerin tolerance in vitro by the cGMP-phosphodiesterase inhibitor zaprinast. *Eur. J. Pharmacol.* **243**:141-147

Parks NJ, Krohn KJ, Mathis CA, Chasko JH, Geiger KR, Gregor ME, and Peek NF (1981) Nitrogen-13-labeled nitrite and nitrate: distribution and metabolism after intratracheal administration. *Science* **212**:58-60.

Parker JD and Gori T (2001) Tolerance to the organic nitrates: New ideas, new mechanisms, continued mystery. *Circulation* **104**:2263-2265.

Parker JD and Parker JO (1998). Nitrate therapy for stable angina pectoris. *N. Engl. J. Med*. **338**:520-531.

Parker JD, Farrell B, Fenton T, Cohanim M, and Parker JO (1991) Counter-regulatory responses to continuous and intermittent therapy with nitroglycerin. *Circulation* **84**:2336-2345.

Parker JD, Parker AB, Farrell B, and Parker JO (1995) Intermittent transdermal nitroglycerin therapy: decreased anginal threshold during the nitrate-free interval. *Circulation* **91**:973.978.

Parker JD, Parker AB, Farrell B, and Parker JO (1996) Effects of diuretic therapy on the development of tolerance to nitroglycerin and exercise capacity in patients with chronic stable angina. *Circulation* **93**:691-696.

Pietruszko R, Mukerjee N, Blatter EE, and Lehmann T (1995) Nitrate esters as inhibitors and substrates of aldehyde dehydrogenase. *Adv. Exp. Med. Bilo.***372**:25–34.

Ratz JD, Fraser AB, Rees-Milton KJ, Adams MA, Bennett BM. (2000) Endothelin receptor antagonism does not prevent the development of in vivo glyceryl trinitrate tolerance in the rat. *J.Pharmacol. Exp. Ther.* **295**:578-585.

Rodriguez J, Maloney RE, Rassaf T, Bryan NS, and Feelisch M (2003) Chemical nature of nitric oxide storage forms in rat vascular tissue. *Proc. Natl. Acad. Sci. U.S.A.* **100**:336-41.

Romanin C and Kukovetz WR (1988) Guanylate cyclase activation by organic nitrates is ot mediated via nitrite. *J. Mol. Cell Cardiol.* **20**:389-396.

Samouilov A, Kuppusamy P, and Zweier JL (1998) Evaluation of the magnitude and rate of nitric oxide production from nitrite in biological systems. *Arch. Biochem. Biophys.* **357**:1-7.

Sarti P, Giuffre A, Barone MC, Forte E, Mastronicola D, and Brunori M (2003) Nitric oxide and cytochrome oxidase: reaction mechanisms from the enzyme to the cell. *Free Radic. Biol. Med.* **34**:509-520.

Schröder H, Leitman DC, Bennett BM, Waldman SA, and Murad F (1988) Glyceryl trinitrate-induced desensitization of guanylate cyclase in cultured rat lung fibroblasts. *J. Pharmacol. Exp. Ther.* **45**:413-418.

Shen ML, Lipsky JJ, and Naylor S (2000) Role of disulfiram in the in vitro inhibition of rat liver mitochondrial aldehyde dehydrogenase. *Biochem Pharmacol* **60**:947–953.

Surks HK (2007) cGMP-dependent protein kinase I and smooth muscle relaxation: a tale of two isoforms. *Circ. Res.* **101**:1078-1080.

Sussex BA, Campbell NR, Raju MK, and McKay DW (1994) The antianginal efficacy of isosorbide dinitrate therapy is maintained during diuretic treatment. *Clin. Pharmacol. Ther.* **56**:229-234.

Thadani U (1996) Nitrate tolerance, rebound, and their clinical relevance in stable angina pectoris, unstable angina and heart failure. *Cardiovasc. Drug Ther.* **10**:735-742.

Thandani U (2005) Prevention of nitrate tolerance with angiotensin II receptor type blocker in patients with stable angina: yet another failed strategy to prevent tolerance. *Cardiovasc. Drugs The.* **18**: 339-342.

Thatcher GR, Nicolescu AC, Bennett BM, and Toader V (2004) Nitrates and NO release: contemporary aspects in biological and medicinal chemistry. *Free Radic. Biol. Med.* **37**:1122-1143.

Towell J, Garthwaite T, and Wang R (1985) Erythrocyte aldehyde dehydrogenase and disulfiram-like side effects of hypoglycemics and antianginals. *Alcohol Clin. Exp. Res.* **9**:438-42.

Tsuchida S, Maki T, and Sato K (1990) Purification and characterization of glutathione transferases with an activity toward nitroglycerin from human aorta and heart. Multiplicity of the human class Mu forms. *J. Biol. Chem.* **265**:7150-7157.

Tsutsumi M, Takada A, Takase S, and Sugata K (1988) Hepatic aldehyde dehydrogenase isoenzymes: differences with respect to species. *Alcohol* **5**:33-8.

Vallari, R.C. & R. Pietruszko (1982) Human aldehyde dehydrogenase: mechanism of inhibition of disulfiram. *Science* **216**:637-639.

Vasiliou V and Nebert DW (2005) Analysis and update of the human aldehyde dehydrogenase (ALDH) gene family. *Hum. Genomics* **2**(2):138-43.

Vasiliou V, Pappa A, and Petersen DR (2000) Role of aldehyde dehydrogenase in endogenous and xenobiotic metabolism. *Chemico-Biological Interac.* **129**:1-19.

Wenzel P, Muller J, Zurmeyer S, SchuhmacherS, Schulz E, Oelze M *et al.* (2008). ALDH-2 deficiency increases cardiovascular oxidative stress-Evidence for indirect antioxidative properties. *Biochem. Biophys. Res. Commun.* **367:**137–143.

Yeates RA, Schmid M, and Leitold M (1989) Antagonism of glycerol trinitrate activity by an inhibitor of glutathione S-transferase. *Biochem. Pharmacol.* **38**:1749-1753.