THE ROLE OF ALDEHYDE DEHYDROGENASE 2 IN NITRATE TOLERANCE

by

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Abstract


Organic nitrates such as glyceryl trinitrate (GTN) are commonly used to treat myocardial
ischemia and congestive heart failure. GTN is proposed to act as a prodrug that requires
bioactivation for pharmacological activity. However, continuous administration results in
tolerance development, limiting its clinical usefulness. Aldehyde dehydrogenase 2
(ALDH2) has been proposed to be the primary enzyme responsible for GTN
bioactivation, and ALDH2 inactivation has been proposed as the sole basis of nitrate
tolerance. In the present study, we utilized an in vivo GTN tolerance model to investigate
the role of ALDH2 in GTN bioactivation and tolerance. We assessed changes in ALDH2
protein, mRNA and activity levels in rat blood vessels during chronic GTN exposure (0.4
mg/hr for 6, 12, 24 and 48 hr) in relation to changes in vasodilator responses to GTN. A
time-dependent decrease in both ALDH2 expression and activity occurred (80% in
tolerant veins and 30% in tolerant arteries after 48 hrs exposure to GTN), concomitant
with decreased vasodilator responses to GTN. However, after a 24 hr drug-free period
following 48 hr GTN exposure, the vasodilator responses to GTN had returned to control
values, whereas ALDH2 expression and activity were still markedly depressed. The
dissociation between reduced ALDH2 activity and expression, and the duration of the
impaired vasodilator responses to GTN in nitrate-tolerant blood vessels, suggest factors
other than changes in ALDH2-mediated GTN bioactivation contribute to nitrate
tolerance.
This thesis was based on research conducted by Yohan D’Souza under the supervision of Dr. Brian Bennett. All data was obtained and analyzed by Yohan D’Souza with the exception of the qRT-PCR, which was completed by Dr. Yanbin Ji.
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Dedication

To my family who offered me unconditional love and support throughout this thesis
# Table of Contents

ABSTRACT ................................................................................................................. ii
STATEMENT OF CO-AUTHORSHIP ........................................................................... iii
ACKNOWLEDGEMENTS ............................................................................................... iv
DEDICATION ..................................................................................................................... v
TABLE OF CONTENTS ................................................................................................. vi
LIST OF FIGURES .......................................................................................................... vii
LIST OF TABLES ............................................................................................................. viii
LIST OF ABBREVIATIONS ............................................................................................. ix

## CHAPTER 1: INTRODUCTION

1.1 Cardiovascular disease ....................................................................................... 1
1.2 Statement of the Research Problem ..................................................................... 2
1.3 Pharmacology of Organic Nitrates .................................................................... 5
  1.3.1 Structure and Chemistry ................................................................................. 5
  1.3.2 Clinical use of Organic Nitrates ................................................................. 6
  1.3.3 Pharmacological Effects of Organic Nitrates ............................................. 7
  1.3.4 Mechanisms of Action .................................................................................. 9
  1.3.5 Tolerance to Organic Nitrates .................................................................... 10
1.4 Biotransformation of Organic Nitrates ............................................................. 12
  1.4.1 Clearance-based and Mechanism-based Biotransformation ................. 12
  1.4.2 Enzymatic Biotransformation of GTN ...................................................... 13
1.5 Aldehyde Dehydrogenase 2 ............................................................................ 17
  1.5.1 Implications in Nitrate Tolerance ............................................................ 17
  1.5.2 Structure and Localization of ALDH2 .................................................... 18
  1.5.3 ALDH2*2 .................................................................................................. 19
  1.5.4 Proposed Role for ALDH2 in GTN Bioactivation and Tolerance .......... 21
  1.5.5 Past Studies ............................................................................................... 25
1.6 Rationale, Research Hypothesis and Objectives ............................................. 29

## CHAPTER 2: MATERIALS AND METHODS

2.1 Drugs and Solutions ............................................................................................. 31
2.2 Animals .................................................................................................................. 31
2.3 Induction of GTN Tolerance in Vivo .................................................................. 32
2.4 Isolated Blood Vessel Relaxation Responses .................................................. 33
  2.4.1 Preparation ................................................................................................ 33
  2.4.2 Relaxation Responses ................................................................................. 34
2.5 Biochemical Analysis .......................................................................................... 34
  2.5.1 Protein Determination ................................................................................ 34
  2.5.2 Immunoblot analysis of ALDH2 .............................................................. 35
  2.5.3 ALDH Activity .......................................................................................... 36
2.6 qRT-PCR .............................................................................................................. 36
2.7 Data Analysis ....................................................................................................... 37
CHAPTER 3: RESULTS

3.1 ALDH2 Protein Expression..........................38
3.2 ALDH2 Activity........................................46
3.3 Relaxation Responses to GTN........................39
  3.3.1 Aorta.................................................39
  3.3.2 Femoral Artery.................................46
  3.3.3 Femoral Vein.................................49
3.4 PCR Analysis........................................55

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS........57

REFERENCES..............................................66
List of Figures

**Figure 1-1:** Structure of GTN and some other organic nitrates

**Figure 1-2:** The mechanism of action of GTN detailing the clearance-based and mechanism-based pathways

**Figure 1-3:** Structure of ALDH2 tetramer

**Figure 1-4:** Structure of ALDH2 monomer

**Figure 1-5:** Proposed mechanism of bioactivation of GTN by ALDH2

**Figure 3-1:** Immunoblot analysis of vessel specific ALDH2 expression during chronic GTN-treatment and recovery

**Figure 3-2:** Changes in ALDH2 protein levels expressed as a percentage of sham in aorta from GTN-treated animals.

**Figure 3-3:** Changes in ALDH2 protein levels expressed as a percentage of sham in femoral arteries from GTN-treated animals.

**Figure 3-4:** Changes in ALDH2 protein levels expressed as a percentage of sham in vena cava from GTN-treated animals.

**Figure 3-5:** Changes in ALDH2 protein levels expressed as a percentage of sham in femoral veins from GTN-treated animals.

**Figure 3-6:** Changes in total ALDH activity during chronic GTN-treatment and recovery in rat aorta.

**Figure 3-7:** Effect of chronic GTN exposure on GTN-induced relaxation of isolated femoral arteries.

**Figure 3-8:** Effect of GTN-free period following a 48 hour GTN exposure on GTN-induced relaxation of isolated femoral arteries.

**Figure 3-9:** Effect of chronic GTN exposure on GTN-induced relaxation of isolated femoral veins.

**Figure 3-10:** Effect of GTN-free period following a 48 hour GTN exposure on GTN-induced relaxation of isolated femoral veins.

**Figure 3-11:** Effect of chronic GTN-exposure on ALDH2 mRNA expression.
**Figure 4-1** Proposed mechanism of GTN tolerance
List of Tables

**Table 3-1:** EC$_{50}$ values for GTN induced relaxation responses in femoral artery and vein.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>1,2-GDN</td>
<td>glyceryl-1,2-dinitrate</td>
</tr>
<tr>
<td>1,3-GDN</td>
<td>glyceryl-1,3-dinitrate</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>ALDH2</td>
<td>aldehyde dehydrogenase 2</td>
</tr>
<tr>
<td>ALDH2*1</td>
<td>Heterozygous for Glu504Lys mutation</td>
</tr>
<tr>
<td>ALDH2*2</td>
<td>Homozygous Glu504Lys mutation</td>
</tr>
<tr>
<td>cGMP</td>
<td>3’,5’-cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DEA/NO</td>
<td>Diethylamine-nonoate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>G&lt;sub&gt;q&lt;/sub&gt;</td>
<td>Gα subunit of the Gq class of G proteins</td>
</tr>
<tr>
<td>GTN</td>
<td>glyceryl trinitrate</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione transferase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>IRAG</td>
<td>inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate</td>
</tr>
<tr>
<td>ISDN</td>
<td>isosorbide dinitrate</td>
</tr>
<tr>
<td>ISMN</td>
<td>isosorbide mononitrate</td>
</tr>
<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Inorganic nitrite anion</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time Polymerase chain reaction</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RGS2</td>
<td>Regulator of G-protein signaling 2</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SH</td>
<td>sulphydryl</td>
</tr>
<tr>
<td>SNO</td>
<td>S-nitrosothiol</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscle</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
</tr>
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</table>
Chapter 1
Introduction

1.1 Cardiovascular Disease

Organic nitrates, such as glyceryl trinitrate (nitroglycerin, GTN), have been used in clinical practice for more than a century. Cardiovascular disease accounts for over 16.7 million deaths worldwide (one third of all deaths). In Canada, 41% of the economic burden of illness is attributed to cardiovascular disease (Canadian Institute for Health Information 2000). Ischemic heart disease is a primary contributor to the prevalence of cardiovascular disease, and angina pectoris is a principal symptom of ischemia (Katzung & Parmley, 1998). In Canada, angina pectoris affects 1 in 50 people. The primary cause of angina pectoris is an imbalance between myocardial oxygen supply and demand, and it is frequently caused by atherosclerotic coronary artery disease. Furthermore, in 2001, GTN was prescribed to treat angina more than 2 million times in the United States alone (Zaher et al., 2004). Thus, a reduction in the number of cases and optimized treatment of cardiovascular diseases could decrease the mortality rate as well as reduce the economic burden of illness.

When given acutely, GTN preferentially dilates the venous circulation and reduces preload, rendering it useful in the treatment of cardiovascular diseases such as congestive heart failure and angina pectoris (Abrams, 1996; Parker & Parker, 1998). However, chronic GTN therapy often leads to the onset of tolerance, as indicated by a reduced vasodilator response to GTN (Thandani & Lipicky, 1994). To counteract tolerance, patients are prescribed an elliptical dosing regimen, in which drug free periods
allow for the reversal of tolerance. This is nonetheless problematic, since during the drug-free periods, patients are susceptible to a rebound phenomenon characterized by an increased frequency of ischemic attacks (Thadani, 1997). Although the first account of tolerance was described in 1888 (Stewart), the mechanism underlying tolerance is still poorly defined. A more detailed understanding of the mechanisms underlying tolerance, the biotransformation of organic nitrates and the vasodilatory properties of nitrates could help prevent the onset of tolerance and may allow for the development of new therapeutic strategies.

1.2 Statement of the Research Problem

Although GTN has been used for over 100 years, complete details of its mechanism of action still remain to be elucidated. GTN, and other organic nitrates, are considered to act as prodrugs, and bioactivation to an active metabolite (nitric oxide (NO) or NO-like species) is required before eliciting an effect (Bennett et al., 1994; Murad et al., 1978). GTN can be denitrated to its biologically active metabolites glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN) by acid or alkaline hydrolysis, or by an enzymatic process at the C-3 or C-2 positions, respectively. Enzymatic denitration can result in the production of NO₂ and ‘NO’ (or a similar species) (Figure 1-1). The bioactive molecule derived from GTN is thought to be NO, but attempts to measure or visualize NO formation at pharmacologically relevant concentrations of GTN have been unsuccessful (Kleschyov et al., 2003; Nunez et al., 2006). However, for the sake of simplicity, in this thesis, the active species generated from GTN will be referred
Figure 1-1. Structure of GTN and some other organic nitrates
to as “NO”. A number of enzymes have been shown to be capable of organic nitrate biotransformation, including glutathione transferases (GSTs) (Hill et al., 1992; Nigam et al., 1993; Tsuchida et al., 1990), aortic cytochrome P450s (CYP450) (McDonald & Bennett, 1993), and mitochondrial aldehyde dehydrogenase 2 (ALDH2) (Chen et al., 2002). However, the primary enzymatic pathway(s) responsible for nitrate action and tolerance have still not been identified. Once GTN is biotransformed to NO, the NO activates soluble guanylyl cyclase (sGC) which results in increased accumulation of cGMP (Katsuki et al., 1977a; Katsuki et al., 1977b). Increases in cGMP result in the activation of cGMP-dependent protein kinase (PKG). Phosphorylation of proteins by PKG leads to decreased intracellular Ca²⁺ levels and/or decreased sensitivity of the contractile apparatus, resulting in vascular smooth muscle relaxation (Fiscus et al., 1983; Petari et al., 2001). Previous research has suggested that 1,2-GDN is the predominant metabolite of GTN formed in vascular smooth muscle (VSM) and furthermore, the formation of 1,2-GDN is decreased in GTN-tolerant tissues (Brien et al., 1988; Ignarro et al., 1981; Kawamoto et al., 1990; Sage et al., 2000; Slack et al., 1989). These findings suggest that the pharmacological action of nitrates involves an enzyme that specifically catalyzes the formation of 1,2-GDN from GTN. Recently, the enzyme ALDH2 was discovered to specifically catalyze the formation of 1,2-GDN from GTN, and ALDH2 activity was found to be depressed in tolerant tissues (Chen et al., 2002). These data suggest that ALDH2 may play a significant role in GTN bioactivation. It is important to note that the formation of 1,2 GDN from GTN, and the link to GTN bioactivity, may be an epiphenomenon and does not necessarily indicate mechanism-based biotransformation. Furthermore, many studies performed investigating the role of
ALDH2 in GTN bioactivation, through the use of various ALDH2 inhibitors, have reported varying results and the role of ALDH2 in GTN bioactivation and tolerance is still questioned.

The aim of my thesis research was to evaluate the role of ALDH2 in GTN bioactivation. An in vivo GTN tolerance model was used to induce tolerance in order to examine ALDH2 protein and activity levels at various time points. Vascular ALDH2 protein and activity levels were correlated to vasodilator responses to GTN. Furthermore, the effects of GTN exposure on vascular ALDH2 mRNA levels were determined. The information obtained has contributed knowledge to the specific role of ALDH2 in GTN bioactivation and tolerance, as well as to the mechanism of action of GTN.

1.3 Pharmacology of Organic Nitrates

1.3.1 Structure and Chemistry

The organic nitrates, used in the treatment of angina pectoris, all contain the same nitrate ester functional group (R-O-NO₂) that confers their unique biological properties. As a result, these compounds share similar mechanisms of action and pharmacological properties. Furthermore, organic nitrates are lipophilic and thus readily cross cell membranes. Examples of classical organic nitrates include isosorbide dinitrate, pentaerythritol tetranitrate and GTN. Of these, the most commonly prescribed nitrate is GTN. GTN contains 3 nitro groups attached to a glycerol backbone through the hydroxyl groups of glycerol (R-O-NO₂) (Figure 1-1). An important feature of this molecule is the nitrate ester group, which differentiates organic nitrates from other nitrogen-oxygen containing species such as sodium nitroprusside (SNP). Furthermore,
the nitrate ester group is an essential component of the molecule and confers to organic nitrates their vasodilator properties (Ahlner et al., 1991).

A common feature of nitrovasodilators is the release of NO, a potent vasodilator. However, it is the way in which NO is released that differentiates nitrovasodilators from one another. Organic nitrates such as GTN contain the nitrate ester functional group (R-O-NO₂), which requires a 3-electron reduction and an oxygen atom transfer in order to be converted to NO. Nitroso compounds such as nitrosothiols only require a 1-electron reduction to yield NO (Thatcher et al., 2004). Spontaneous NO donors such as SNP or diazeniumdiolates (NONOates) are capable of releasing NO without biotransformation. GTN, on the other hand, has been termed a ‘NO-mimetic’. NO mimetics are compounds that can mimic the actions of NO without necessarily releasing NO. During GTN biotransformation, NO₂⁻ is the major nitrogen-oxygen species formed with NO being a minor product formed. Thus, GTN has the ability to mimic the actions of NO without the significant release of NO and is thus categorized as a NO mimetic rather than an NO donor (Thatcher et al., 2004). Furthermore, organic nitrates differ from spontaneous NO donors, nitro- and NO-containing compounds in potency and mechanism of action and should be considered a separate class of compounds.

1.3.2 Clinical use of Organic Nitrates

Organic nitrates have been used to treat cardiovascular disease for over a century and can be administered by several routes. Organic nitrates are lipophilic and this allows for sublingual or transdermal administration. GTN is rapidly metabolized by the liver, and oral administration is associated with a significant “first pass” effect. As a result,
GTN is rarely taken orally. Currently, transdermal GTN is a common route of administration, however the onset of action of transdermal GTN is not rapid enough to be useful in the setting of an acute anginal attack and thus the sublingual route of administration is preferred. GTN has a high therapeutic index and its use is associated with few adverse effects (Katzung & Parmley, 1998). This, in combination with GTN’s rapid onset of action makes it a versatile drug. Adverse effects of GTN include headache, dizziness, tachycardia and hypotension, but these can be controlled by decreasing the dosage. Although rare, nitrates can provoke an anginal attack by reducing blood pressure to the point where coronary flow is reduced to such an extent that it cannot meet myocardial demands. (Parker & Parker, 1998; Thadani, 1997).

1.3.3 Pharmacological Effects of Organic Nitrates

Organic nitrate therapy decreases myocardial oxygen demand as a result of decreased myocardial work. This mainly stems from their actions on the peripheral vasculature and, to a lesser extent, coronary blood flow. Organic nitrates are preferential venodilators and thus decrease left and right ventricular end-diastolic pressures (preload). Nitrates also decrease systemic arterial pressure (afterload), which may contribute to the antianginal effects of nitrates. However, this effect does not usually occur at clinically relevant concentrations of GTN. The reduction in preload results from the dilation of large veins; this promotes blood pooling, thus decreasing venous return to the heart and decreasing myocardial workload. In the setting of congestive heart failure, decreased preload provides a better perfusion pressure gradient during diastole, which results in increased endocardial perfusion and myocardial performance (Parker & Parker, 1998).
Thus, GTN treatment results in decreased myocardial work and decreased oxygen demand, and it is this effect that is the cornerstone behind the pain relief associated with typical angina (Ganz & Marcus, 1972). As mentioned above, GTN elicits a greater effect on large conductance vessels compared to resistance vessels. (Cohen & Kirk, 1973; Fam & McGregor, 1968; Macho & Vatner, 1981; Zhang et al., 1993). The underlying mechanism behind this vascular regioselectivity is still undefined, although several studies have suggested decreased bioactivation of organic nitrates in resistance vessels in the presence of NO as a major cause. Thus, a possible cause of a decreased vasodilator response in arteries compared to veins may be due to increased eNOS expression in endothelial cells of arteries. (de la Lande et al., 2004, Kojda et al., 1998). It has also been suggested that oxygen can act to inhibit biotransformation; Bennett et al. (1992) found that GTN biotransformation in aorta increased 3-fold when incubations were performed anaerobically. Other mechanisms proposed include counter-regulatory mechanisms that inhibit GTN-induced arterial dilation, decreased sGC activity in vascular smooth muscle of arteries, and reduced GTN biotransformation (Harrison & Bates, 1993; Sellke et al., 1990; Stewart et al., 1988). Furthermore, there are differences in responses to GTN amongst arteries; coronary arteries are more sensitive to GTN than mesenteric and femoral arteries, with all three being more sensitive than renal arteries. (Gharaibeh & Gross, 1984). Although the regioselectivity associated with GTN is well documented, the mechanism behind this effect still remains to be elucidated and furthermore, few studies have been aimed at testing a hypothesis for this observed effect. It is possible that the underlying mechanisms behind GTN bioactivation are different in venous tissue compared to arterial tissue and as a result, susceptibility to GTN tolerance differs.
Nevertheless, a better understanding of the venoselectivity associated with organic nitrates could lead to a better understanding of tolerance and could lead to improved treatment strategies.

1.3.4 Mechanisms of Action

Several mechanisms of action have been proposed for GTN; however it is generally accepted that GTN acts as a prodrug and undergoes bioactivation before vasodilation occurs (Bennett & Marks, 1984). Thus GTN must be transformed into an active metabolite, currently thought to be NO or a NO-like species, within vascular smooth muscle cells resulting in a sequence of events leading to vascular smooth muscle relaxation (Bennett et al., 1994). Once GTN is biotransformed to NO, the NO activates soluble guanylyl cyclase (sGC) (Ignarro et al., 1989; Murad, et al., 1979).

Guanylyl cyclase can be expressed as one of two isoforms in vascular smooth muscle: the particulate or membrane-bound form and soluble form (sGC), which is located in the cytosol. The soluble and particulate forms are distinct with respect to their structure and mode of regulation (Schulz, 1992). Particulate GC is monomeric and is regulated by a variety of ligands such as the atrial natriuretic peptides (Yuen & Garbers, 1992). Soluble GC is a heterodimer composed of α and β subunits and is regulated by diffusible compounds such as NO (Moncada et al., 1991). Several hypotheses exist detailing the activation and function of sGC in GTN action. However, it is widely held that NO binds to the heme moiety within the enzyme resulting in a conformational change and an increase in catalytic activity (Ignarro et al., 1982). One mechanism put forth involves several cysteine residues on sGC, which interact with nitrates to form NO
and subsequent activation of sGC by the formed NO (Artz et al., 2001). Regardless of the mechanism of NO formation from GTN, sGC activation results in the production and accumulation of cGMP (Ahlner et al., 1991 Katsuki et al., 1977a; Katsuki et al., 1977b). In vascular smooth muscle cells, increases in cGMP result in activation of cGMP-dependent protein kinase (PKG). PKG directly activates myosin light chain phosphotase (MLCP) and indirectly activates it through phosphorylation and inhibition of RhoA. This leads to myosin light chain dephosphorylation and inhibition of cross-bridge cycling. PKG also activates RGS2 (Regulator of G protein signaling 2), which inhibits $G_{q/11}$ signaling. Finally, PKG1/β activates IRAG (inositol 1,4,5-triphosphate (IP$_3$) receptor associated G kinase substrate), which inhibits Ca$^{2+}$ release from the sarcoplasmic reticulum via the IP$_3$ receptor (Surks, 2007). Thus, PKG alters cellular calcium handling, resulting in decreased intracellular calcium levels and decreased phosphorylation of myosin light chains (Ahlner et al., 1991).

1.3.5 Tolerance to Organic Nitrates

The phenomenon of organic nitrate tolerance has been acknowledged for over a century. GTN tolerance may be functionally characterized as a reduced vasodilator response to doses of GTN that normally elicit a response. The first account of nitrate tolerance was documented in 1888 (Stewart), however the mechanism underlying tolerance is still poorly defined. As mentioned previously, one of the major problems associated with the chronic use of GTN is the development of GTN tolerance. To remedy this, physicians employ an elliptical dosing regimen, which consists of treating patients with GTN for a 10-12 hour period followed by a drug-free period. This method is
successful in preventing the onset of tolerance. However, patients are left without the benefit of the drug during the withdrawal period. Also, there is suggested to be a rebound effect that occurs during this drug-free period in which there is a worsening of anginal symptoms. Many studies performed over the past century have investigated the phenomenon of tolerance, however a unifying hypothesis for the mechanism of nitrate tolerance still remains to be articulated (Parker & Gori, 2001). This situation is further complicated by the phenomenon of cross-tolerance between organic nitrates (Parker & Parker, 1998; Thadani, 2005). Cross tolerance is the phenomenon in which development of tolerance to one organic nitrate results in tolerance to other nitrates, thus preventing their use as a substitute in therapy. Due to the complex nature surrounding nitrate tolerance, there have been many hypotheses put forth in order to try to explain it.

Proposed mechanisms include: intravascular volume expansion (Dupuis et al., 1990; Lis et al., 1984), neurohormonal counter-regulation (Jeserich et al., 1995; Kurz et al., 1999; Munzel et al., 1995a), increased superoxide formation and oxidative stress (Munzel et al., 1995; Munzel et al. 1999), thiol depletion (Boesgaard et al., 1991; Needleman & Johnson 1973) and reduced cGMP formation due to: desensitization of sGC to NO (Bennett et al., 1988; Bennett et al., 1989; Schröder et al., 1988) reduced biotransformation to ‘NO’ (DiFabio et al., 2003; Kenkare and Benet, 1996; McGuire et al., 1994) or increased phosphodiesterase activity (Axelsson and Andersson, 1983; Axelsson and Karlsson, 1984; MacPherson et al., 2006; Pagani et al., 1993). Although many potential mechanisms of GTN tolerance have been identified, none have proven to be the absolute cause of tolerance. Additionally, many of these mechanisms have been supported and refuted at various times, which has led researchers to conclude that tolerance is in fact a multi-
factorial phenomenon. Furthermore, many compounds such as antioxidants (vitamin C and E, thiols) protein synthesis inhibitors (cyclohexamide) (Schröder et al., 1998) vasodilators (hydralazine) and phosphodiesterase inhibitors affect tolerance to varying degrees; most show a reduction in the degree of tolerance (McAllister, 2000). Thus, it appears that nitrate tolerance is a complex phenomenon and to date there is yet to be a unifying theory to explain this phenomenon.

1.4 Biotransformation of Organic Nitrates

1.4.1 Clearance-based and Mechanism-based Biotransformation

As mentioned above, organic nitrates are prodrugs and must be bioactivated at their site of action to have an effect (Bennett & Marks, 1984; Bennett et al., 1994). It is currently believed that the active species generated through nitrate biotransformation is NO or a NO-like species. It should be noted that the only nitrogen-oxygen containing species that have been demonstrated to be formed in vascular tissues during incubation with GTN is the inorganic nitrite anion (NO$_2^-$) and a small amount of NO. A number of products and intermediates have been suggested through which NO is formed, including S-nitrosothiols, thionitrates, and sulphinyl nitrites. However, the role of NO in GTN-mediated vasodilation is under dispute, since the levels produced are too low to explain the smooth muscle relaxation observed at therapeutic levels of GTN (Kleschyov et al., 2003; Marks et al., 1995). Furthermore, NO$_2^-$ is the predominant species formed during biotransformation, but any pharmacological increase of NO$_2^-$ formed from GTN is minute compared to the high endogenous levels of NO$_2^-$ in vascular smooth muscle. Thus, it is highly unlikely that NO$_2^-$ is the intermediate species responsible for GTN-induced
relaxation (Bennett & Marks, 1984; Difabio et al., 2003). In addition, the vasodilator potency of NO$_2$ is 1000-fold less than that of GTN (Roman & Kukovetz, 1988). Due to these reasons the formation of NO$_2$ during GTN biotransformation has been termed clearance-based biotransformation and is associated with a lack of vasodilator response (Bennett et al., 1994). The term “mechanism-based” biotransformation has been used to describe the pathway involving the vascular formation of NO or vasoactive GTN metabolites that lead to the activation of sGC and vasorelaxation (Figure 1-2).

1.4.2 Enzymatic Biotransformation of GTN

Many enzymes or processes have been proposed as bioactivators of GTN including GSTs, xanthine oxidases, CYP450s and flavoenzymes. However, none of these enzymes have been shown to be capable of mediating the 3-electron reduction needed in order to form NO from GTN.

In 1973, Needleman & Johnson proposed a role for the depletion of sulfhydryl groups as a basis for nitrate tolerance. In their model, GTN binds to an organic nitrate receptor in VSM and oxidizes critical sulfhydryl groups, resulting in the formation of a disulfide bridge. This disulfide bridge inactivates the receptor leading to decreased cellular responses to GTN and the development of tolerance (Needleman & Johnson, 1973). This hypothesis also accounted for the phenomenon of cross-tolerance since the organic nitrate receptor was proposed to be a common nitrate receptor and thus inactivation would affect all nitrates. The authors were also able to show that addition of the thiol reducing agent dithiothreitol (DTT) resulted in the in vitro reversal of tolerance.
Figure 1-2. The mechanism of action of GTN detailing the clearance-based and mechanism-based pathways. A diagram detailing the mechanism of action of GTN in a vascular smooth muscle cell. GTN enters the cell and is biotransformed in the cytosol. There are two pathways: either NO is generated resulting in activation of sGC and subsequent vasodilation (mechanism-based pathway) or NO$_2^-$ is generated and does not lead to activation of sGC resulting in no effect (clearance based pathway). Modified from Bennett et al. (1994).
Since this hypothesis was proposed, many studies have been performed involving the use of sulfhydryl reducing agents aimed at preventing or reversing tolerance. The results so far indicate that these reducing agents inconsistently reverse or prevent tolerance (Parker & Parker, 1998). Furthermore, studies have refuted that nitrate tolerance is associated with the depletion of sulfhydryl stores. This hypothesis is continually revisited since many reduced thiols react with GTN resulting in the formation of 1,2-GDN and 1,3-GDN. In fact, just recently, ascorbate was shown to bioactivate GTN leading to the activation of purified sGC (Kollau et al. 2007). It is thought that any molecule with a thiol group can be involved in the biotransformation of GTN (Chong & Fung, 1991), but their specific involvement in the mechanism-based biotransformation of GTN needs to be clarified.

Another family of enzymes proposed to be involved in the bioactivation of GTN are the GSTs. In 1990, Tsuchida et al. found that the mu GST isoform isolated from human aorta was capable of catalyzing the denitrification of GTN, and this catalytic activity is inhibited using the GST inhibitor bromosulfophthalain (BSP). Other studies have shown that the GST inhibitors BSP and Cibachron blue, in tissues precontracted with K⁺, inhibited GTN-induced cGMP accumulation, GTN relaxation and GTN biotransformation (Nigam et al., 1993; Yeates et al., 1989). However, these GST inhibitors failed to inhibit the pharmacological effects of GTN when tissues were precontracted with phenylephrine (Chung & Fung, 1993, Nigam et al., 1996; Lau & Benet, 1992). Furthermore, Sokolowska et al. (2004) found that GST activity was inhibited by BSP to the same degree in tolerant and non-tolerant tissues and concluded
that GTN bioactivation was not dependant on GST activity. So far, studies examining the role of GSTs in the bioactivation of GTN are contradictory and the specific role of GSTs in the mechanism-based biotransformation of GTN remains to be clarified. Furthermore, many of the past studies examining GSTs in the biotransformation of GTN have focused on cytosolic GSTs. However, the microsomal GST1 (mGST1) has been shown to mediate GTN biotransformation although the role mGST1 plays in mechanism-based GTN biotransformation is still to be determined (Ji & Bennett, 2006).

Xanthine oxidase (XO) is a flavoenzyme that plays a variety of roles in mammalian cells. It is also the only enzyme known that can mediate organic and inorganic nitrate and nitrite reduction (Harrison et al., 2001; Zweier et al., 2001; Zweier et al., 2003; Zweier et al., 2004). Furthermore, XO has been shown to catalyze organic nitrate reduction forming NO₂⁻ under anaerobic conditions and it has been shown to stimulate the release of NO from GTN. However, allopurinol, a XO inhibitor does not inhibit GTN-induced vasorelaxation (Ratz et al., 2000). Thus, questions still exist regarding the role of XO in GTN bioactivation.

Hepatic and aortic biotransformation of GTN to 1,2 GDN and 1,3 GDN has been shown to be NADPH-dependant and as a result, many flavoenzymes have been proposed as possible mediators. Studies using hepatic and aortic microsomes have supported a role for the cytochrome P450-NADPH cytochrome P450 reductase system in the biotransformation of nitrates. Microsomal biotransformation of organic nitrates is NADPH-dependant, inhibited by CO and SKF 525A (2-diethylaminoethyl-2,2-diphenylvalerate), an inhibitor of cytochrome P450, and is increased in rats pretreated with phenobarbital (McDonald & Bennett, 1990; McDonald & Bennett, 1993; Servent et
Furthermore, the biotransformation of GTN in rat hepatic microsomes results in the formation of an activator of sGC (Bennett et al., 1992). Diphenyleneiodonium sulfate (DPI), an inhibitor of several flavoproteins, inhibits the metabolic activation of GTN in isolated rat aorta and in whole animals (McGuire et al., 1994), and this inhibition is thought to be a result of the inhibition of vascular NADPH-CYP450 reductase. Although these results suggest a role for CYP450 system in the biotransformation of GTN, it is important to note that this does not necessarily imply a role in the bioactivation of GTN. In 2001, Minamiyama et al. found that downregulation of CYP450 by IL-1β or through competitive inhibition of CYP450 using 7-ethoxyresorufin resulted in organic nitrate tolerance. However, the CYP450 inhibitors, SKF525A, cimetidine and metapyrone had no effect on GTN-induced vasorelaxation (Bennett et al., 1992; Bornfeldt et al., 1987; Liu et al., 1993). Thus the involvement of CYP450 in GTN tolerance has been inconsistent, and to date, there is no conclusive evidence for the involvement of CYP450 in the bioactivation of GTN and the development of tolerance.

1.5 Aldehyde Dehydrogenase 2

1.5.1 Implications in Nitrate Tolerance

Recently, aldehyde dehydrogenase 2 (ALDH2) has been suggested to play a role in the bioactivation of GTN (Chen et al., 2002). Furthermore, ALDH2 has been proposed to be the sole enzyme responsible for GTN tolerance (Chen et al., 2005). As mentioned previously, the biotransformation of GTN yields 1,2-GDN, 1,3-GDN, NO and NO−2. It has been shown that the predominant dinitrate metabolite formed in VSM is 1,2-GDN (Brien et al., 1988; Ignarro et al., 1981; Kawamoto et al., 1990). Furthermore, the
selective formation of 1,2-GDN is depressed in GTN-tolerant tissues. This suggests the involvement of an enzyme specific for the formation of 1,2-GDN in the bioactivation of GTN and the inhibition of the enzyme during GTN-tolerance. Studies have found that ALDH2 catalyzes the selective formation of 1,2-GDN from GTN, and that during GTN-tolerance, ALDH2 activity is inhibited (Chen et al., 2002). This study suggested a potential role for ALDH2 in GTN bioactivation and has led to the hypothesis that ALDH2 is the sole enzyme responsible for GTN tolerance.

1.5.2 Structure and Localization of ALDH2

ALDH2 is one of 19 members of the human ALDH family of NAD(P)⁺ dependant enzymes; they are differentiated on the basis of their physical properties, sub-cellular locations, substrate specificities and cofactor preference (Vasiliou & Nebert, 2005). In rat liver, ALDH2 is localized to the mitochondria and has been referred to as the mitochondrial ALDH. This is different in human liver, where ALDH2 is localized to both the mitochondria and cytosol. The function of ALDH2 in the liver is to catalyze the pyridine nucleotide-dependant oxidation of aldehydes to acids (Vasilou et al., 2000). ALDH2 has a high affinity for acetaldehyde such that it mediates almost all hepatic acetaldehyde oxidation. ALDH2 also possesses esterase activity, which is thought to be responsible for the metabolism of GTN (Feldman & Weiner, 1972). Studies involving site-directed mutagenesis have revealed that the thiolate moiety of Cys319 is essential for both the dehydrogenase and esterase activity of ALDH2 (Farres et al., 1995). ALDH2 is a homotetramer composed of 4 identical subunits consisting of 517 amino acid residues (Figure 1-3). However, the tetramer is regarded as a dimer of dimers, since the interface
Figure 1-3. Structure of the ALDH2 tetramer. Each subunit is represented by purple orange blue and red (modified from Hurley et al., 2004)

Figure 1-4 Structure of ALDH2 monomer. Each subunit is composed of 3 domains: a coenzyme binding domain, an oligomerization domain and a catalytic domain. (modified from Hurley et al., 2004)
between the 2 subunits and the interface between the 2 dimers varies drastically. Each subunit is composed of 3 domains: the oligomerization domain, the co-enzyme (NAD$^+$) domain and the catalytic domain (Steinmetz et al., 1997) (Figure 1-4). The active site of the enzyme is divided into two halves by the nicotinamide ring of NAD$^+$. Adjacent to the A-side of the nicotinamide ring is a cluster of three cysteines (Cys318, Cys319 and Cys320) at the active site, which are important for ALDH2 catalytic activity. Adjacent to the B-side are Thr261, Glu285 Glu504 and an ordered water molecule bound to Thr 261 and Glu493. In regards to ALDH2 dehydrogenase activity, the positions of residues near the nicotinamide ring of NAD$^+$ suggest a chemical reaction whereby Glu285 functions as a general base through a water molecule. The side chain amide nitrogen of Asn186 and the peptidyl nitrogen of Cys319 are in a position to stabilize the oxyanion formed in the tetrahedral transition state prior to hydride transfer (Steinmetz et al., 1997). Glu504 interacts with the enzyme’s active site by forming hydrogen bonds with Arg281 and Arg492 and is important for proper catalytic function (Larson et al., 2005).

### 1.5.3 ALDH2*2

A prevalent ALDH2 mutation exists in the Asian population; 30-50% of Asians possess a G1510A point mutation that results in the substitution of Glu for Lys at residue 504. The mutated protein has been termed ALDH2K and is essentially inactive. This mutation occurs in the oligomerization domain at the interface between the subunits located immediately below the active site of an ALDH2 monomer. This results in a large disordered region at the dimer interface and includes the coenzyme binding cleft and a loop of residues that form the base of the active site. As a consequence of these structural
changes, the enzyme’s catalytic and coenzyme-binding domains rotate relative to the oligomerization domain. These rigid perturbations are the direct result of the inability of Lys504 to form stabilizing hydrogen bonds with Arg281 and Arg492 (Larson et al., 2005). The variant enzyme is active, but it has a 200-fold increased $K_M$ for NAD$^+$ and a reduced $K_{cat}$. The 200-fold increase in $K_M$ exceeds the cells available concentration of NAD$^+$ by 15-fold and this in combination with the decreased $K_{cat}$ results in a 100-fold decrease in enzymatic activity (Larson et al., 2005). Because ALDH2 is a tetramer, one mutated subunit is enough to render the entire enzyme inactive. As a result, individuals that are heterozygous for the Glu504/Lys504 mutation (termed ALDH2*1) demonstrate only 8-15% of wild-type ALDH2 activity whereas individuals who are homozygous for the mutation (termed ALDH2*2) demonstrate negligible activity (Li et al., 2006).

Furthermore, because the Glu504/Lys504 mutation affects the NAD$^+$ binding site as well as the catalytic site, it is hypothesized that this mutation affects both dehydrogenase and esterase activity.

1.5.4 Proposed Role for ALDH2 in GTN Bioactivation and Tolerance

Needleman & Johnson (1973) were the first to propose a sulfhydryl requirement for GTN-induced VSM relaxation. Although their model was well characterized, it has never been fully explained and as a result has been challenged over the years. Boesgaard et al. (1994) failed to detect intracellular thiol depletion in GTN-tolerant vessels, other groups have implicated other enzymes in GTN bioactivation (McDonald & Bennett, 1990; Millar et al., 1998) and new mechanisms of GTN tolerance have been put forth (Munzel et al., 1995). Despite these arguments against Needleman & Johnson’s theory, it
is accepted that the sulfhydryl model still holds true in at least some form. The active site of ALDH2 contains 3 cysteine groups and it has been shown that a disulfide bridge forms between these cysteines (Vallari & Pietrusko, 1982; Shen et al., 2000). The formation of this disulfide bridge inactivates ALDH2 and has been implicated in the mechanism-based inhibition of ALDH2 by NO, SNO and GTN (DeMaster et al., 1997; DeMaster et al., 1998; Ikawa et al., 1983; Towell et al., 1985). Recently, Chen et al. (2002) have applied the above findings to their hypothesis suggesting that generation of the disulfide bridge occurs during GTN bioactivation and furthermore, that the inactivated form of ALDH2 is a necessary reaction intermediate in GTN bioactivation (Figure 1-5).

In the proposed reaction, put forth by the Stamler group, NO$_2$ is an obligate intermediate in the formation of NO or ‘NO bioactivity’ from GTN. This mechanism involves the conversion of NO$_2$ to NO. There are a number of problems with this hypothesis, one of which is that this mechanism requires the conversion of NO$_2$ to NO by the mitochondrion. Two mechanisms for this conversion have been proposed. The first involves the reduction of NO$_2$ to NO by the mitochondrial electron transport chain (ETC). It has been shown that the cytochrome bc 1 complex (complex III) and cytochrome c oxidase (complex IV) of the ETC can reduce NO$_2$ to NO under certain conditions (Brudvig et al., 1980; Kozlov et al., 1999). However, this has only been shown to occur under anaerobic conditions and does not occur at physiological oxygen concentrations. Furthermore, the reduction of O$_2$ to superoxide by the bc1 complex predicts that under aerobic conditions there would be competition between O$_2$ and NO$_2$ for available electrons, and that if NO were formed, there would be quenching of NO by concomitant superoxide formation. Furthermore, under aerobic conditions, NO binds and
GTN (R-ONO$_2$) reacts with the sulphydryl groups at the active site of ALDH2 followed by release of NO$_2^-$ and formation of a disulfide bridge resulting in inactivation of ALDH2. (Modified from Chen et al. 2002)
inhibits cytochrome oxidase. Depending on the rate of electron flux through the respiratory chain, bound NO either dissociates unchanged from the heme iron or is oxidized to NO$_2$ (Difabio et al., 2004; Sarti et al., 2003). Finally, NO$_2$-cytochrome oxidase adducts only occur when the protein is fully oxidized, and upon reduction, NO$_2$ is released and does not get reduced to NO (Giuffre et al., 2000). This suggests that under normal physiological conditions, cytochrome oxidase should be considered a NO clearance pathway. The second mechanism proposed involves the disproportionation of nitrous acid to NO. The issue here is that in the intermembranous space of the mitochondria at a pH of 4, the rate of NO formation is predicted to be 0.01% per second (Samouilov et al., 1998). Thus, the NO formed per second from a given concentration of GTN is 10,000 fold less than the concentration of GTN. At clinically relevant concentrations, this means only sub-picomolar concentrations of NO could be formed from nanomolar concentrations of GTN and this would be restricted to the small volume of the intramembranous space of the mitochondria. A spontaneous NO donor such as DEA/NO has an EC$_{50}$ of 9.2 nM for relaxation of isolated rat aorta, which is significantly higher than the picomolar concentrations of NO that could be formed from GTN (Ratz et al., 2000). Finally, the small amount of NO formed by this mechanism would occur in the intermembranous space of the mitochondrion and thus would need to diffuse from this site to the cytosol and interact with sGC in order to initiate a vasodilator response.

As mentioned previously, NO$_2$ is the predominant nitrogen-oxygen species formed during GTN biotransformation. However, NO$_2$ has very low vasodilator potency and the high endogenous levels of NO$_2$ make it highly unlikely that it is an intermediate in the formation of NO from GTN. NO$_2$ is evenly distributed in the body between
plasma and erythrocytes and between intracellular and extracellular compartments (Parks et al., 1981). The plasma concentrations of NO\textsubscript{2} are in the micromolar concentration range whereas the pharmacologically relevant concentrations of GTN in VSM are in the nanomolar range. Thus, NO\textsubscript{2} formed from GTN biotransformation would only make a minor contribution to the intracellular NO\textsubscript{2} pool and thus would not be expected to exert a pharmacological effect.

Given the evidence above, the proposed bioactivation of GTN by ALDH2 with NO\textsubscript{2} as an obligate intermediate seems implausible. More research is needed in order to modify the existing hypothesis and clarify the role of ALDH2 in GTN tolerance.

1.5.5 Past Studies

Recently, there have been numerous studies examining ALDH2 and its role in GTN bioactivation. In 2002, Chen et al. identified ALDH2 as an enzyme that bioactivates GTN resulting in vascular smooth muscle relaxation. Furthermore, using the ALDH2 inhibitors cyanamide and chloral hydrate (at millimolar concentrations), and the ALDH2 substrate acetaldehyde, they showed inhibition of specific 1,2-GDN formation from GTN, GTN-induced relaxation, and GTN-induced cGMP accumulation. They also found that ALDH2 activity and 1,2-GDN formation were reduced in GTN-tolerant tissues. Based on these results, they suggested that tolerance was due to inactivation of ALDH2.

In 2003, Difabio et al. criticized these findings showing that the inhibitors used in Chen’s study were equally effective in both tolerant and non-tolerant tissues, thus suggesting the non-specific effects of these inhibitors were responsible for the results.
observed by Chen et al. and that ALDH2 was not the only target affected during the development of GTN tolerance.

In 2004, Zhang et al., using cyanamide, observed significant decreases in the relaxation responses to GTN. They also found that cyanamide inhibited changes in coronary blood flow induced by GTN. However, the use of cyanamide did not result in the complete inhibition of GTN-induced responses and as a result, they suggested that ALDH2 mediates the cGMP-dependant component of vasorelaxation but also alluded to cGMP-independent effects, suggesting bioactivation is multifactorial and may involve more than one enzyme.

De la Lande et al. (2004) confirmed the results of Difabio et al. (2003) and suggested that inhibition of ALDH2 may not be the only mechanism of tolerance. Thus, once again a multifactorial characteristic for tolerance was suggested.

Kollau et al. (2004) used daidzin, a specific ALDH inhibitor to inhibit sGC activation at low GTN concentrations. However, based on their studies, they concluded that ALDH2 was not the sole enzyme catalyzing the bioactivation of GTN.

In 2004, Sydow et al. reported that GTN tolerance is associated with decreased ALDH2 activity. Furthermore, they reported no significant shifts in the GTN dose-response curve in GTN tolerant tissues when using the specific ALDH inhibitor daidzin. They concluded that GTN tolerance is mediated, at least in significant part, by inactivation of ALDH2 by oxidative stress. However, it should be noted that the results observed by this group directly contradict the observations of Difabio et al. (2003). This may be due to the greater degree of tolerance induced by Sydow et al. since they observed a 43-fold shift in the EC50 without a change in the Emax. This level of tolerance
is 10 times greater than that reported by Difabio et al. (2003). Furthermore, in a previous study using the same tolerance protocol, Sydow et al. found only a 3-fold shift in the EC$_{50}$ value for GTN (Munzel et al., 1996).

In 2005, Chen et al. reported a study using ALDH2$^{-/-}$ mice. They assessed the biotransformation of GTN by the mitochondria, GTN-induced vasodilation and development of tolerance to GTN. Relative to wild-type mice, exposure of ALDH2$^{-/-}$ mice to GTN resulted in decreased 1,2-GDN formation, cGMP accumulation and reduced vasorelaxation in isolated aorta at GTN concentrations of less than 0.5µM. These results implicated a role for ALDH2 in the bioactivation of GTN. However, the GTN-induced relaxation was only inhibited at low concentrations in the knockout mice, which suggest other mechanisms are involved for the bioactivation of GTN at concentrations greater than 1 µM. Isosorbide dinitrate (ISDN)-induced vasodilation was unaffected in the ALDH2 null mice, suggesting that ALDH2 is not involved in the bioactivation of this nitrate. Based on the findings in this study, Chen et al. proposed a high affinity ALDH2-dependant pathway for the bioactivation of GTN and a low affinity ALDH2-independent pathway involving GTN and other nitrates such as ISDN and isosorbide mononitrate (ISMN) that is unaffected in GTN tolerance. Although this model may be plausible, anomalies still exist with regards to cross-tolerance. First, vasodilator responses to ISDN in ALDH2 null mice were unaltered compared to wild-type mice suggesting ALDH2 is not involved in the bioactivation of this nitrate. This is puzzling since ALDH2 has been shown to metabolize ISDN and is inactivated by it (Pietrusko et al., 1995). Furthermore, ISDN and GTN exhibit cross-tolerance and since ALDH2 knockout mice show normal responses to ISDN, then the role of ALDH2 in GTN tolerance is questionable. The study
reported by Chen et al. (2004) did not perform any experiments to investigate this phenomenon.

A few clinical studies involving ALDH2 and GTN tolerance have been completed in recent years. First, Mackenzie et al. (2005) investigated forearm blood flow responses to GTN in subjects who were either heterozygous for the ALDH mutation (ALDH2*1) or were treated with the ALDH inhibitor, disulfuram. They found that the inhibitor disulfuram reduced the vasodilatory response to GTN, and that individuals with the inherited ALDH2*1 mutation showed a similar reduced response to GTN. They concluded that multiple enzymes are involved in GTN bioactivation and although ALDH2 is involved in the bioactivation of GTN in vivo, it accounts for less than half of the total bioactivation.

A second study investigated individuals who were homozygous and heterozygous for the ALDH2 mutation (Li et al., 2006). They found that subjects who were ALDH2*1 or ALDH2*2 showed decreased responses to sublingual GTN compared to subjects with the wild-type allele. This study also assessed enzyme kinetics using purified enzymes and found that ALDH2*1 had 8-15% of ALDH2 activity whereas, ALDH2*2 had about 6% of wild-type activity. This group concluded that the Lys504 mutation contributes in large part to the decreased efficacy of sublingual GTN. However, they noted that not all patients who were ALDH2*2 showed a decreased efficacy for GTN and not all patients who were wild-type for ALDH2 showed normal GTN efficacy. Thus, once again, multiple mechanisms are suggested and the role of ALDH2 in GTN tolerance is questioned.
A recent study investigated whether inhibition of ALDH2 contributes to GTN tolerance in human blood vessels (Hink et al., 2007). They found that long-term GTN treatment results in GTN tolerance and endothelial dysfunction. Furthermore, GTN tolerance was associated with the inhibition and down-regulation of vascular ALDH2.

A further note is that the inhibitors used in past studies are all non-specific, even daidzin, the most selective ALDH2 inhibitor, has non-specific effects. As a result the conclusions from studies involving these inhibitors are not concrete. Although it is widely accepted that ALDH2 is involved in the biotransformation of GTN but more evidence is needed to determine its specific role in the mechanism-based biotransformation of GTN and the development of tolerance.

The mechanisms leading to the development of in vivo and in vitro GTN tolerance are distinct from each other due to the involvement of different experimental conditions (Gruhn et al., 2002). In vitro studies often use supra-pharmacological concentrations of GTN for short periods of time to induce tolerance (Bennett et al., 1988; Brien et al., 1986; Needleman and Johnson, 1973). Since the production of NO may occur in many ways, high concentrations of GTN may not accurately reflect the production of NO during therapy (Bennett, 1988). Furthermore, in vitro models do not accurately mimic the effects of recovery from tolerance and lack neurohormonal changes or changes in gene expression (Azevedo et al., 2001; Münzel et al., 2000). Our lab has spent considerable effort developing an in vivo model of GTN tolerance using transdermal GTN patches. Ex vivo and in vivo measurements made from these animal models are more relevant to the clinical setting. In our rat model, ex vivo relaxation responses of isolated aorta to GTN and other nitrates are inhibited as well as GTN-induced cGMP production and GTN
biotransformation (Bennett et al., 2000). These effects can be reversed by a 2 day washout period. This suggests that tolerance is linked to the decreased mechanism-based biotransformation of GTN.

1.6 Rationale, research hypothesis and objectives

A review of the literature indicates that there are many inconsistencies with the mechanistic findings of GTN tolerance (Gori et al., 2004; Gruhn et al., 2002; Schulz et al., 2002). Furthermore, the specific biotransformation of GTN appears vital for its function as a vasodilator, and tolerance is associated with decreased biotransformation of nitrates. Thus, it is fitting that ALDH2, an enzyme capable of metabolizing GTN, becomes inactivated during tolerance and as a result causes a decrease in the mechanism-based biotransformation of GTN. It is this rationale that has lead researchers to propose a prominent role for ALDH2 in the mechanism-based biotransformation of GTN and that inactivation of this enzyme is the sole basis for GTN tolerance. We, amongst others, disagree, based on the extensive literature indicating multiple mechanisms of tolerance. Nevertheless significant questions still exist with regards to the relative role of ALDH2 in GTN tolerance, its inactivation, and whether its inactivation affects the vasodilator properties of other nitrates.

The hypothesis of this research is that the reduced potency of GTN during GTN tolerance is not solely mediated by inactivation of ALDH2. As mentioned earlier, Chen et al. (2002) proposed that GTN reacts with two free SH groups at the active site of ALDH2 resulting in the release of NO$_2^-$ and disulfide formation. This intramolecular disulfide bridge renders the enzyme inactive; the result being decreased
GTN biotransformation and thus decreased vasodilation. There is support for this model as mentioned earlier, and a time-course evaluation may provide further support. If inactivation of ALDH2 is the sole basis for GTN tolerance, then there should be an association between the decreased ALDH2 activity and the development of GTN tolerance. Furthermore, the decrease in ALDH2 activity and protein expression should correlate with functional responses in GTN-treated blood vessels. The specific objectives of the proposed research were: 1) to examine changes in ALDH2 activity after chronic GTN exposure (6, 12, 24, and 48 hrs) as well as recovery from tolerance (1, 3 and 5 days), and to correlate ALDH2 enzymatic activity to protein levels and vasodilator responses at each time point and 2) to determine if chronic GTN exposure affects ALDH2 mRNA expression. Through the knowledge gained here we hope to clarify the role of ALDH2 in GTN bioactivation and tolerance.
Chapter 2
Materials & Methods

2.1 Drugs and Solutions

Transdermal GTN patches were obtained as Transderm-Nitro (0.2mg/h) from Novartis Pharmaceuticals (Dorval, QC, Canada). Drug-free patches were produced by soaking patches for at least 2 days in 95% ethanol (patches were allowed to air dry for at least 15 minutes before implantation). Removal of GTN from the patches by this procedure was confirmed by the absence of GTN or metabolites in the plasma of rats implanted with these sham patches (Ratz et al., 2002). GTN was obtained as a solution (TRIDIL, 5 mg/ml) in ethanol, propylene glycol and water (1:1:1:33) from Sabex (Boucherville, Quebec). Stock dilutions of GTN were made up in Krebs’ solution (NaCl 119 mM; KCl 5.4 mM; CaCl2 2.5 mM; KH2PO4 0.6 mM; MgSO4 1.2 mM; NaHCO3 25 mM and glucose 11.7 mM). The following items were purchased from Sigma-Aldrich Chemical Company (Oakville Ontario); phenylephrine hydrochloride (PE); potassium chloride (KCl); potassium dihydrogen orthophosphate (KH2PO4); sodium chloride (NaCl); calcium chloride (CaCl2) and magnesium sulfate (MgSO4). Sodium hydrogen carbonate (NaHCO3) and D-Glucose were purchased from BDH Inc. (Toronto, Ontario). Halothane for inhalational anesthesia 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 to 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 rodent chow and tap water. All experiments were carried out within the guidelines of the Canadian Council on Animal Care.

2.3 Induction of GTN Tolerance in Vivo

Rats were randomly assigned into GTN-treated or sham groups with each group consisting of 3-6 animals. GTN-treated rats were exposed to a continuous source of GTN for various time periods (6, 12, 24, 48 hours on and 48 hours on with 1, 3 and 5 days off) via sub-dermal implantation of two 0.2 mg/hr transdermal GTN patches. Tolerance was assumed to occur after 48 hours GTN treatment based on previous work (Ratz et al., 2000). Briefly, a 1-cm transverse incision was made and the skin separated from the underlying fascia by blunt dissection. Two transdermal patches were inserted back-to-back into the resulting subdermal space. The site was sutured closed and disinfected with 2.5% iodine. At 24 h the site was reopened and both patches were replaced. Animals in the recovery group also received GTN patches for 48 hours. However, the patches were removed after the initial 48 hour GTN-treatment period to simulate a nitrate-free interval for 1, 3 or 5 days. Animals in the sham group received similar treatments, however sham (drug-free) patches were used instead. Animals were sacrificed at various time points and the aorta, vena cava, femoral arteries and femoral veins were removed and prepared.
2.4 Isolated Blood Vessel Relaxation Responses

2.4.1 Preparation

Rats were treated with GTN as described above and sacrificed by decapitation under ether anesthesia. Femoral arteries and veins from each hind limb were removed and placed in ice-cold Krebs’ solution previously bubbled with 95%O₂/5% CO₂. Blood vessels were placed under a microscope and extraneous fat and tissue were carefully excised within 10 min. 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 while viewed under a microscope. 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%O₂/5%CO₂. One of the stirrups was fixed, while the other was attached to an isometric force transducer coupled to a Myodaq data acquisition system. Two femoral arteries and two femoral veins were used in each experiment under an optimal resting tension of 2.5 mN for femoral veins and 5 mN for femoral arteries as reported previously (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. Femoral veins failing to generate >1mN (~.1g) of tension and femoral arteries failing to generate >5 mN (~0.5g) of tension with 5 µM PE were discarded.
2.4.2 Relaxation Responses

Following equilibration as described above, tissues were sub-maximally contracted with PE (1 μM-5 μM). After PE-induced tone had stabilized (10 min) cumulative GTN concentration-response curves were obtained beginning at $10^{-10}$M GTN and increasing in half-log dose intervals (i.e. $10^{-10}$ M, $3 \times 10^{-10}$ M, $10^{-9}$ M etc.) until a concentration of $3 \times 10^{-5}$M GTN was achieved in the tissue bath. Since there were two preparations of each vessel in each experiment, the average responses of the two were taken in order to provide a more accurate representation of the relaxation responses to GTN. All dilutions were made in Krebs’ solution.

2.5 Biochemical Analysis

2.5.1 Protein Determination

Briefly, blood vessels were excised from animals with blood and extraneous tissue removed. Tissues were then lysed with varying amounts of lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 1% Triton X-100 and protease inhibitors (protease inhibitor cocktail tablet, 1 tablet/10 mL)) depending on the vessel; 400 μL for aorta, 200 μL for vena cava, 100 μL for femoral artery and 80 μL for femoral vein. Samples were then homogenized with a glass tube and pestle and centrifuged in a 1.5 mL Eppendorf tube at 13,200 rpm (8500 g). Bio-Rad (Bradford) protein analysis was performed according to the manufacturer’s directions in order to determine protein concentration. Optical density was measured at 595 nm in a Cary UV-Visible spectrophotometer. Linear regression was performed using bovine serum albumin (BSA) as the standard.
2.5.2 Immunoblot Analysis of ALDH2

After tissues were lysed and protein concentration determined (as described above), samples were boiled in Laemmli sample buffer (0.5 M Tris-HCl, 9.5% glycerol, 10% SDS and 0.5% bromophenol blue) for 5 min. Next, 8 μg of each sample was loaded onto a 10% polyacrylamide separating gel and separated using a running buffer composed of 25 mM Tris-HCl pH 8.3, 192 mM glycine, and 5% SDS at 175 V for 1 hour in a Biorad Protean II mini-gel system. Purified ALDH2 (50 ng) obtained from Dr. Vasilis Vasiliou (University of Colorado Denver, CO), was loaded as a marker. After electrophoresis, proteins were transferred onto a polyvinylidenediflouride (PVDF) membrane (Millipore Co., Bedford, MA,) pre-soaked in methanol and then equilibrated in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine and 20% methanol) along with the gel and filter papers for 30 minutes. Semi-dry transfer was completed at 17 V for 16 mins. Subsequently, the membrane was blocked overnight in blocking solution containing 4% skim milk and Tris-buffered saline containing Tween (TBS-T) (50 mM Tris-HCl pH 7.5, 400 mM NaCl and 0.05% Tween). The membrane was then washed with TBS and incubated with a 1:1000 dilution of primary polyclonal ALDH2 antibody (obtained from Dr. Vasilis Vasiliou) for 1 hour at room temperature. Following this incubation, the membrane was washed 3 times with TBS-T and then incubated with a 1:3000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad Laboratories, Richmond CA) for 1 hour. The membrane was then washed once with TBS-T and four times with TBS and developed using a Lumi-Glo ECL chemiluminescence detection system (Kirkegaard & Perry Labs, Gaithersburg, MA) and
Hyperfilm-ECL imaging film (Interscience, Troy, NY). Immunoreactive bands were quantified by measuring their relative optical density (ROD) using Adobe Photoshop 6.

2.5.3 ALDH2 Activity

Whole cell homogenates were prepared from aorta by the procedure described above except TES buffer (0.25 M sucrose, 5 mM Tris-HCl, 0.5 M EDTA 0.1 PMSF, pH 7.2) was used instead of lysis buffer. Deoxycholic acid (2.5 mg/mg protein) was added to blood vessel homogenates in order to solublize 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 1 of the electron transfer chain), 1 mM 4-methylpyrazole (to inhibit alcohol dehydrogenase) and substrate (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 minutes. The specific activity was calculated for each sample using the molar extinction coefficient for NADH of 6306 M$^{-1}$cm$^{-1}$.

2.6 qRT-PCR

Total RNA was isolated from rat aortae using an RNeasy Mini Kit (QiaGen, Mississauga, On.) according to the manufacturers’ guidelines. Reverse transcription was performed first: 1 µg of oligo (dT)$_{12-18}$ primers and 5 µg of total RNA was heated in an Eppendorf tube at 70 °C for 10 min and then chilled on ice. 8 µl of 5X first strand buffer, 4 µl of 0.1 M DTT and 2 µl of dNTP mix (10mM) were then added to the mixture and
incubated at 42 °C for 2 min. 400 U of reverse transcriptase (SuperScript II Invitrogen Life Technology, Burlington, ON) was then added and the reaction carried out at 42 °C for 50 min. The reaction was stopped by incubation at 70 °C for 15 min. The qRT-PCR solution for ALDH2 or the GAPDH standard contained: 10µL of Qiagen Quantitect SYBR Green master mix, 1 µL of primers (ALDH2 forward primer: 5’-ATCCAGCCCACCGGTGTGAGAT-3’, ALDH2 reverse primer: 5’-CGGGCTGGCCGCAGCTTTC-3’), (GAPDH primers: F 5’-GACAACCTTTGCGATCGTGA-3’ R 5’-ATGAGGATGATGTTCTGGA-3’), 1 µL of 1:5 diluted reverse transcriptase solution and 7 µL of water. Amplification was performed with an initial denaturation step at 95 °C for 900 seconds, followed by 45 cycles of denaturation at 95 °C for 15 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 30 seconds.

2.7 Data Analysis

Tissue responses to GTN were measured as the percent decrease in PE-induced tone. EC\textsubscript{50} values for relaxation responses in isolated blood vessels were determined from the concentration-response curve obtained using a sigmoidal dose-response curve-fitting algorithm. The fold shift in the EC\textsubscript{50} values were compared using Student’s t tests for unpaired data. ALDH activity and qRT-PCR data was compared using a Student’s t-tests for unpaired data. All data was expressed as the mean ± standard deviation (S.D.). A \( P \) value of <0.05 was used to indicate statistical significance.
Chapter 3

Results

3.1 ALDH2 Protein Expression

ALDH2 protein expression was quantified by immunoblot analysis of each blood vessel type. Equivalent amounts of protein were loaded onto the gel in order to contrast vessel-specific differences in response to GTN treatment (Figure 3.1). Immunoreactive bands were observed with mobilities slightly less than the 50 kDa marker, with the same mobility as purified human ALDH2 and similar to the findings of Difabio et al. (2003) and Hink et al. (2007). Densitometric analysis using Adobe Photoshop software indicated a time-dependent decrease in ALDH2 protein expression with chronic GTN treatment. In the aorta and femoral artery, ALDH2 protein levels decreased in a time-dependent manner resulting in a 30% reduction at 48 hours compared to sham (Figure 3.2, Figure 3.3). In the vena cava and femoral vein, GTN-treatment resulted in an 80% reduction at 48 hours (Figure 3.4, Figure 3.5). A nitrate-free interval after chronic GTN-treatment resulted in ALDH2 protein levels returning to sham levels in the aorta and vena cava. However, in the femoral artery and femoral vein, even after a 5-day nitrate-free interval after 48 hrs of GTN exposure, ALDH2 protein levels did not return to sham levels. Furthermore, densitometric analysis indicated that ALDH2 expression was 2-3 fold greater in the aorta and femoral artery compared to the vena cava and femoral vein.
3.2 ALDH2 Activity

ALDH2 activity was assessed in whole cell homogenates of the rat aorta to determine if enzymatic activity changed during chronic GTN treatment. ALDH2 specific activity was measured using a low substrate concentration. However, this approach was unsuccessful due to low protein concentrations and low absorbance readings. Thus, total ALDH activity was measured to gain a sense of how ALDH activity changed (Figure 3.6). The protein yields obtained from the vena cava, femoral artery and femoral vein preparations were too low to assess ALDH activity and thus ALDH activity could only be measured in the aorta. GTN-treatment resulted in a time-dependant decrease in ALDH2 activity resulting in a 55% decrease at 24 hours. A nitrate-free period after chronic GTN exposure resulted in an increase in ALDH activity returning to sham levels after a 5-day drug-free period. Due to the low amounts of protein harvested from the vena cava, femoral artery and femoral vein, it was not possible to perform activity assays on these vessels. It is important to note, that total ALDH activity roughly correlated with ALDH2 protein levels in the aorta (Pearson’s test $r^2=0.5258$ p (two tailed) = 0.0652).

3.3 Relaxation responses to GTN

3.3.1 Aorta

GTN-relaxation responses in the aorta were previously performed in our laboratory (Dowlatshahi, MSc. Thesis) and therefore, the relaxation responses in aorta were not performed in the current study. In the previous study, there were no significant
Figure 3.1 Immunoblot analysis of vessel specific ALDH2 expression during chronic GTN-treatment and recovery. 8µg of protein was loaded in each well and resolved on a 10% SDS gel. The letters indicate the following: A, 6hr GTN treatment; B, 12hr GTN treatment; C, 24hr GTN treatment; D, 48hr GTN treatment; E, 1 Day GTN-free period after 48 hours GTN treatment; F, 3 day GTN-free period after 48 hours GTN treatment; G, 5 day GTN-free period after 48 hours GTN treatment.
Figure 3.2 Changes in ALDH2 protein levels expressed as a percentage of sham in aorta from GTN-treated animals. 6, 12, 24, and 48 refer to the duration of GTN treatment, whereas 1R, 3R and 5R refer to the duration of drug-free days after 48 hours of GTN-treatment. Bars represent the mean ± S.D. * indicates significant differences from sham (p< 0.05) (n=4)
Figure 3.3 Changes in ALDH2 protein levels expressed as a percentage of sham in femoral arteries from GTN-treated animals. 6, 12, 24, and 48 refer to the duration of GTN treatment, whereas 1R, 3R and 5R refer to the duration of drug-free days after 48 hours of GTN-treatment. Bars represent the mean ± S.D. * indicates significant differences from sham (p< 0.05) (n=4)
Figure 3.4 Changes in ALDH2 protein levels expressed as a percentage of sham in vena cava from GTN-treated animals. 6, 12, 24, and 48 refer to the duration of GTN treatment, whereas 1R, 3R and 5R refer to the duration of drug-free days after 48 hours of GTN-treatment. Bars represent the mean ± S.D. * indicates significant differences from sham (p< 0.05) (n=4)
Figure 3.5 Changes in ALDH2 protein levels expressed as a percentage of sham in femoral veins from GTN-treated animals. 6, 12, 24, and 48 refer to the duration of GTN treatment, whereas 1R, 3R and 5R refer to the duration of drug-free days after 48 hours of GTN-treatment. Bars represent the mean ± S.D. * indicates significant differences from sham (p< 0.05) (n=4)
Figure 3.6 Changes in total ALDH activity during chronic GTN-treatment and recovery in rat aorta. 6, 12, 24, and 48 refer to the duration of GTN treatment, whereas 1R, 3R and 5R refer to the duration of drug-free days after 48 hours of GTN-treatment. Bars represent the mean ± S.D. * indicates significant differences from sham (p< 0.05) and ** indicates p<0.01. (n=4)
differences in the relaxation responses between GTN-treated and sham treated animals in the 6 hour and 12 hour treatment groups. At 24 hours GTN-treatment, there was a significant rightward shift in the concentration response curve. This curve was shifted further to the right with 48 hours of GTN-treatment, however after a 24 hour nitrate-free interval there were no differences in the concentration response curves between the treated and sham animals.

3.3.2 Femoral Artery

No significant differences were observed in the relaxation responses to GTN in the 6 and 12 hour GTN treatment groups (Figure 3.7, Table 3.1). The EC$_{50}$ values for each group were 6 hr: 2.4 ± 1.3 nM (sham) and 2.7 ± 1.2 nM (treated) and 12 hr: 3.5 ± 1.2 nM (sham) and 3.1 ± 1.6 nM (treated). After 24 hours in vivo GTN treatment, there was a rightward shift in the dose response curve to GTN and a significant increase in the EC$_{50}$ value: 1.8 ± 0.8 nM (sham) and 4.5 ± 1.1 nM (treated) (p<0.05). At 48 hours GTN treatment, there was a 3.5 fold rightward shift in the concentration response curve (sham EC$_{50}$ = 1.9 ± 1.1 nM, treated 6.3 ± 1.0 nM (p<0.01)), however, no significant differences were seen for the maximum relaxation responses. The 3.5 fold increase in the EC$_{50}$ value is in agreement with previous studies and is accepted as an indication of tolerance (DiFabio et al., 2003; De La Lande et al., 2004).

After 48 hours chronic GTN treatment, a nitrate-free period of only one day was required for vasodilator responses to return to sham levels, indicating a complete reversal of tolerance (Figure 3.8). The EC$_{50}$ values for 1 day recovery groups were: sham; 2.8 ±
Figure 3.7 Effect of chronic GTN exposure on GTN-induced relaxation of isolated femoral arteries. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.4 mg/hr GTN. Letters indicate the following: A) 6 hrs GTN treatment; B) 12 hrs GTN treatment; C) 24 hrs GTN treatment; D) 48 hrs GTN treatment. A 6 and 12 hour GTN exposure resulted in no significant differences in the EC$_{50}$ value for GTN-induced relaxation. A 24 hour GTN exposure resulted in a significant increase in the EC$_{50}$ value for GTN-induced relaxation (p<0.05). A 48 hour GTN 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=5)
Figure 3.8 Effect of a GTN-free period following a 48-hour GTN exposure on GTN-induced relaxation of isolated femoral arteries. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.4 mg/hr GTN for 6 hours. Letters indicate the following: A, 48 hrs GTN treatment; B, 1 day GTN-free after 48 hrs GTN treatment; C) 3 days GTN-free after 48 hrs GTN treatment; D) 5 days GTN-free after 48 hrs GTN treatment. A full recovery of the relaxation response to GTN was observed after a one day nitrate-free period. No significant differences were observed with regards to the relaxation responses to GTN between sham and three day GTN-free animals, or five day GTN-free animals and sham. (n=5)
0.9 nM and treated; 3.5 ± 1.3 nM. Similarly, the relaxation curves from 3 and 5-day nitrate-free periods were superimposable with sham curves. The EC\textsubscript{50} values were: 3-day sham; 2.0 ± 1.2 nM; treated 2.5 ± 1.7 nM and 5-day sham, 2.4 ± 1.8 nM and treated, 3.2 ± 1.7 nM.

3.3.3 Femoral Vein

Similar to the vasodilator responses in the femoral arteries, a 6 and 12 hour GTN treatment did not result in any significant differences in the concentration-response curves to GTN (Figure 3.9). The EC\textsubscript{50} values were: 6 hour, 9.0 ± 1.9 nM (sham) and 8.7 ± 2.1 nM (treated); and 12 hr; 6.1 ± 1.5 nM (sham) and 5.9 ± 1.2 nM (treated). At least 24 hours \textit{in vivo} GTN-treatment was required before a significant rightward shift in the GTN concentration-response curve was observed. The EC\textsubscript{50} values for the sham and treated groups were 5.0 ± 1.0 nM and 11 ± 1.7 nM respectively. 48 hours of GTN treatment resulted in an almost 5-fold shift in the concentration-response curve to GTN (EC\textsubscript{50} sham, 5.2 ± 1.1 nM and treated, 24 ± 7 nM) once again indicating that tolerance occurred after a 48 hour GTN exposure.

A nitrate-free interval after the onset of tolerance resulted in a leftward shift in the concentration-response curve to GTN. After a 24 hour nitrate-free period, the GTN-treated femoral vein mimicked the sham-treated vein with regards to GTN relaxation responses (Figure 3.10). The EC\textsubscript{50} values were: sham, 6.4 ± 2.2 nM and treated, 7.6 ± 2.3 nM. Similar to the femoral artery, the relaxation curves from 3 and 5-day nitrate-free
Figure 3.9 Effect of chronic GTN exposure on GTN-induced relaxation of isolated femoral veins. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.4 mg/hr GTN. Letters indicate the following: A, 6 hrs GTN treatment; B, 12 hrs GTN treatment; C) 24 hrs GTN treatment; D) 48 hrs GTN treatment. A 6 and 12 hour GTN exposure resulted in no significant differences in the EC$_{50}$ value for GTN-induced relaxation. A 24 hour GTN exposure resulted in a significant increase in the EC$_{50}$ value for GTN-induced relaxation (p<0.01). A 48 hour GTN 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=5)
Figure 3.10 Effect of a GTN-free period following a 48-hour GTN exposure on GTN-induced relaxation of isolated femoral veins. Control indicates rats implanted with sham patches and Treated indicates rats administered 0.4 mg/hr GTN for 6 hours. Letters indicate the following: A, 48 hrs GTN treatment; B, 1 day GTN-free after 48 hrs GTN treatment; C) 3 days GTN-free after 48 hrs GTN treatment; D) 5 days GTN-free after 48 hrs GTN treatment. A full recovery of the relaxation response to GTN was observed after a one day nitrate-free period. No significant differences were observed with regards to the relaxation responses to GTN between sham and three day GTN-free animals, or five day GTN-free animals and sham. (n=5)
Table 3.1 EC$_{50}$ values for GTN induced relaxation responses in femoral artery and vein. Values indicate EC$_{50}$ mean ± S.D for GTN induced relaxation. * indicates significant differences from sham (p< 0.05) and ** indicates p<0.01. (n=5)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Femoral artery</th>
<th>Femoral vein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Treated</td>
</tr>
<tr>
<td>6 hours</td>
<td>2.4 ± 1.3 nM</td>
<td>2.7 ± 1.2 nM</td>
</tr>
<tr>
<td>12 hours</td>
<td>3.5 ± 1.2 nM</td>
<td>3.1 ± 1.6 nM</td>
</tr>
<tr>
<td>24 hours</td>
<td>1.8 ± 0.8 nM</td>
<td>4.5 ± 1.1 nM*</td>
</tr>
<tr>
<td>48 hours</td>
<td>1.9 ± 1.1 nM</td>
<td>6.3 ± 1.0 nM**</td>
</tr>
<tr>
<td>1 day GTN-free</td>
<td>2.8 ± 0.9 nM</td>
<td>3.5 ± 1.3 nM</td>
</tr>
<tr>
<td>3 day GTN-free</td>
<td>2.0 ± 1.2 nM</td>
<td>2.5 ± 1.7 nM</td>
</tr>
<tr>
<td>5 day GTN-free</td>
<td>2.4 ± 1.8 nM</td>
<td>3.2 ± 1.7 nM</td>
</tr>
</tbody>
</table>
periods were superimposable with sham curves. The EC$_{50}$ values were, 3-day sham, 5.9 ± 1.7 nM; treated, 5.0 ± 1.9 nM and 5-day sham, 4.9 ± 1.8 nM and treated, 6.3 ± 1.3 nM.

3.4 PCR Analysis

Real-time quantitative RT-PCR analysis was performed using ALDH2 mRNA from GTN-treated aorta (Figure 3.11). GTN treatment had a marked effect on ALDH2 mRNA expression; after 24 hours of GTN treatment, ALDH2 mRNA expression was increased 3.5-fold compared to sham-treated animals, whereas after 48 hours GTN-treatment, ALDH2 mRNA decreased approximately 7-fold (15% of sham). A nitrate-free interval of 1 day after chronic exposure resulted ALDH2 mRNA levels of approximately 40% of sham (2.5 fold decrease from sham).
3.11 Effect of chronic GTN-exposure on ALDH2 mRNA expression. 24 and 48 refer to the duration of *in vivo* GTN exposure and 1R refers to a 1 day nitrate-free after a 48 hr *in vivo* GTN treatment period. Values are expressed as a ratio to sham. Bars represent the mean ± S.D. * indicates significant differences from sham (p< 0.05) and ** indicates p<0.01. (n=4)
Chapter 4
Discussion and Future Directions

The role of ALDH2 in GTN biotransformation has been widely studied in animal models as well as in humans (Chen et al., 2002; 2006; Munzel et al., 2006; Sydow et al., 2004). However, no study to date has examined the time-course effects of GTN exposure on ALDH2 activity and expression. The present study examined the association between ALDH2 activity and expression and *ex vivo* functional responses to GTN, during chronic GTN exposure. This study allowed for the examination of the role of ALDH2 in the onset of GTN tolerance and its reversal.

The objectives of the study were to: 1) to examine changes in ALDH2 activity after chronic GTN exposure (6, 12, 24, and 48 hours) as well as recovery from tolerance (48 hours on with 1, 3 and 5 days off), and to correlate ALDH2 enzymatic activity with protein levels and vasodilator responses at each time point and 2) to determine if chronic GTN exposure affects ALDH2 mRNA expression.

The results from this study indicated a lack of association between the functional responses of blood vessels to GTN, and ALDH2 activity and expression during chronic GTN treatment and recovery. These results indicate that the inactivation of ALDH2 is not the basis for GTN tolerance and suggests mechanisms other than ALDH2 mediated bioactivation play a role in GTN tolerance.

It has been proposed that ALDH2 is the enzyme responsible for GTN bioactivation and that inactivation of ALDH2 is the sole basis for GTN tolerance (Chen
et al., 2002; Chen et al., 2005). Although there is evidence to support this hypothesis, the ALDH2 theory remains controversial. Many of the studies performed to date testing the ALDH2 hypothesis involved the use of non-specific ALDH inhibitors such as acetaldehyde, chloral hydrate and cyanamide. Furthermore, the results from these studies have attributed findings such as an equivalent magnitude of inhibition of vasodilator responses in tolerant and non-tolerant aorta to the non-specific effects of these inhibitors and thus, there is yet to be conclusive evidence revealing the role of ALDH2 in GTN bioactivation and tolerance. The studies performed in this thesis demonstrate that ALDH2 inactivation does not parallel the time-course of GTN tolerance but rather that ALDH2 activity remains depressed long after GTN relaxation responses return to sham values. After 48 hours of GTN treatment, a 3.5-fold and 5-fold rightward shift was observed in the concentration-response curves for GTN-induced relaxation of femoral artery and femoral vein respectively. The magnitudes of the rightward shifts are in line with previous studies performed in our laboratory and are indicative of a modest degree of tolerance. ALDH2 activity in aorta and expression in all the blood vessels examined, decreased in a time-dependant manner in response to GTN treatment. After the induction of GTN tolerance, a nitrate-free interval of up to 5-days did not result in ALDH2 protein expression returning to sham levels despite the fact that the functional responses to GTN returned to sham values after a 1-day nitrate-free interval. This directly contradicts the ALDH2 inactivation model of GTN tolerance, which would predict a close association between ALDH2 activity and protein levels and vascular responses to GTN. The results of this study indicated that during modest tolerance, not only does ALDH2 activity and expression not correlate with the time-dependant onset of GTN tolerance and recovery
from tolerance, but ALDH2 expression also remains depressed in the femoral arteries and veins. There have been other studies indicating decreased ALDH2 expression during GTN tolerance (Munzel et al., 2007) but to my knowledge this is the first observation of depressed ALDH2 expression during a nitrate free interval. It appears that there may be long term changes associated with GTN tolerance, which may increase susceptibility to oxidative damage and endothelial dysfunction. As mentioned previously, ALDH2 plays an important role in detoxifying a number of toxic aldehydes in the body. Thus, GTN tolerance results in decreased ALDH2 activity and expression, and this may compromise the cell’s ability to deal with toxic aldehydes. However more studies are needed to confirm this hypothesis.

Organic nitrates elicit a greater effect on large conductance vessels compared to resistance vessels (Cohen & Kirk, 1973; Fam & McGregor, 1968; Macho & Vatner, 1981; Zhang et al., 1993). Since a current hypothesis is that ALDH2 is the major enzyme responsible for GTN bioactivation, and several studies have shown greater GTN biotransformation in venous tissue, we expected to see a greater abundance of ALDH2 protein in venous tissue compared to arterial tissue. This would support a role of ALDH2 as a major bioactivating enzyme of GTN. Quite contrary to the expectations of this hypothesis, we observed 2-3 fold lower ALDH2 protein expression in venous tissue compared to arterial tissue. The study performed by Marks et al. (1990) showed that there is significantly greater GTN biotransformation and subsequent cGMP accumulation in venous tissue compared to arterial tissue. This, taken with the observation of a lower abundance of ALDH2 protein in veins compared to arteries, suggests that ALDH2 is not the sole enzyme responsible for GTN bioactivation. It is important to note that the
mechanism of GTN tolerance could be different in venous tissue compared to arterial
tissue and ALDH2 may have a more prominent role in arteries than in veins.

Chronic GTN treatment resulted in a decrease in ALDH2 protein expression in
all blood vessels tested. However, there was a greater reduction in venous tissue (80% 
reduction compared to sham) compared to arterial tissue (30% reduction compared to
sham). Past studies have indicated vessel specific differences such as increased sensitivity
to GTN and greater functional responses (Gharaibeh & Gross, 1984). As mentioned
previously, venoselectivity of vascular responses to GTN is well documented but the
mechanism responsible for this phenomenon has not been conclusively identified. The
current study revealed another venoselective action of GTN in that there was a greater
reduction of ALDH2 protein expression in venous tissue during chronic GTN treatment.
This finding does not support the ALDH2 inactivation model of tolerance since we
observed different degrees of ALDH2 depletion in veins compared with arteries but with
no significant change in the degree of tolerance. Furthermore, after a 1-day GTN-free
period after 48 hrs of GTN treatment, we observed different degrees of depletion in
ALDH2 (20% in aorta and femoral artery, 65% in vena cava and femoral vein), whereas
the GTN concentration-response curves were superimposable with the concentration-
response curves obtained using vascular preparations from sham animals. Based on this
finding, it is reasonable to assume that ALDH2 is not the sole enzyme responsible for
GTN bioactivation. In 2002, Chen et al. proposed inactivation of ALDH2 as the sole
basis of GTN tolerance. In their model, they proposed that interaction of GTN with
cysteine residues in the active site of ALDH2 results in the formation of a disulfide
bridge that inactivates ALDH2. This inactivation is thought to be irreversible in vivo. It
appears, based on the results presented in this study that during GTN treatment, ALDH2 is inactivated and that this inactivation is paralleled by the depletion of ALDH2 protein. Perhaps the cell recognizes the disulfide bridge formed and subsequently degrades the inactive ALDH2. More work is needed to test this hypothesis, however the study performed here suggests the possibility of a degradation pathway. In fact, qRT-PCR analysis was performed in order to determine whether GTN treatment altered ALDH2 mRNA expression in aorta and whether altered ALDH2 mRNA levels contributed to the decreased ALDH2 protein expression. At 24 hours, ALDH2 mRNA increased 3.5-fold relative to sham. However, at this time-point, ALDH2 protein levels had decreased by about 20%. Furthermore, after 48 hours of GTN treatment, ALDH2 mRNA expression decreased to about 15% of sham levels, whereas ALDH2 protein levels were only reduced by 30%. Given that the half-life of the ALDH2 protein is estimated to be 22 hours (Crab et al., 1996; Crab et al., 1998; Garver et al., 2001), the data in this study provides evidence for the degradation of ALDH2. If ALDH2 was not being degraded, we would expect an increase in ALDH2 protein levels between the 24 hour time-point and the 48 hour time-point. However the decrease in protein levels of ALDH2 indicates that the cell was degrading the protein.

The study performed by Chen et al. (2002) presented a number of findings, including decreased ALDH2 activity during tolerance, which supported inactivation of ALDH2 as a basis for tolerance. The time-course analysis in the current study indicated that ALDH activity decreased with GTN treatment and was in agreement with their findings. However, decreased ALDH activity did not correlate with the GTN relaxation responses in the aorta. We observed decreases in ALDH activity after 12 hours of GTN
treatment, with the greatest decrease occurring after 24 hours of GTN treatment. At least 24 hours of GTN treatment was required before a significant change in the vasodilator responses to GTN were observed. Furthermore, after 12 and 48 hours of GTN treatment, as well as in the 1-day recovery treatment group, ALDH activity was decreased by about 35 % of sham. The GTN concentration-response curves changed drastically during this time; at 12 hour and 1-day recovery groups, the GTN concentration-response curves were superimposable with their respective shams. However, at the 48 hour time-point, there was a significant rightward shift in the concentration-response curves for GTN-induced relaxation. The decreases in ALDH activity observed in this study thus do not correlate with the GTN vasodilator responses, and do support inactivation of ALDH2 as the sole basis for GTN tolerance.

There is evidence to support a role for reactive oxygen species as a basis for ALDH2 inactivation in GTN tolerance. One of the mechanisms put forth for GTN tolerance is oxidative damage and superoxide formation. Essentially, the concept entails increased superoxide formation in response to GTN treatment, which then decreases NO bioavailability and can result in peroxynitrite formation, NOS uncoupling and altered NO/cGMP signaling (Munzel et al., 2002). The evidence for oxidative stress in GTN tolerance, taken together with the findings in this study support a role for reactive oxygen species (ROS) generated in response to GTN treatment to inhibit ALDH2. This could account for the time-dependant decrease in ALDH2 activity and protein expression during chronic GTN treatment. A previous study indicated that 1 µM GTN was sufficient to inhibit the detoxification of 4-hydroxynonenal (4-HNE), an end product of lipid peroxidation generated through ROS (Petersen and Mitchell 1987; Peterson et al., 1995),
resulting in its accumulation in the cell. Furthermore, it has been demonstrated that 4-HNE at low concentrations (micromolar) is a reversible inhibitor of ALDH2 (Petersen and Mitchell, 1991). However at higher concentrations, it has been shown to cause irreversible covalent modifications of the enzyme (Peterson et al., 1999). Thus, a link exists between the formation of ROS, lipid peroxidation (Parker & Gori, 2001) and the generation of 4-HNE and inhibition of ALDH2. No cause and effect relationship has been determined, but the results in the study suggest the possibility of increased 4-HNE formation during chronic GTN treatment and subsequent inactivation of ALDH2.

Alternatively, chronic GTN treatment could directly inactivate ALDH2 through oxidation of critical SH groups at the active site. This would result in less toxic aldehyde metabolism by ALDH2 and a buildup of 4-HNE resulting in increased ROS formation.

A review of the literature indicates that there is a substantial lack of agreement with respect to the mechanisms of GTN tolerance (Gori et al., 2004; Gruhn et al., 2002; Schulz et al., 2002). As mentioned in Chapter 1, many enzymes have been proposed as bioactivators of GTN, however none have proven to be the sole enzyme involved in the development of tolerance. The study performed here was designed to assess the role of ALDH2 in the onset of GTN tolerance, and recovery from tolerance using a time-course analysis. The results of the time-course analysis suggest that ALDH2 is not the sole enzyme responsible for GTN bioactivation and tolerance. These data directly contradict the ALDH2 inactivation hypothesis and thus call for a new hypothesis. Based on the number of enzymes linked to GTN bioactivation and the number of complex mechanisms proposed, it has been suggested that GTN tolerance is a multi-factorial phenomenon. The results presented in this study are in agreement with a multi-factorial tolerance
mechanism. The results of this study do not rule out the involvement of ALDH2 in GTN tolerance, however they indicate the involvement of other factors. A link exists with regards to three proposed mechanisms of GTN tolerance: inactivation of ALDH2 (Chen et al., 2002), the sulfhydryl depletion model (Needleman and Johnson, 1973) and the superoxide and oxidative stress hypothesis (Munzel et al., 1995). Perhaps GTN tolerance occurs due to a combination of the three mechanisms proposed. It is possible that GTN is bioactivated by a number of sulfhydryl-containing enzymes, which in turn become oxidized during chronic GTN exposure. The oxidation of these enzymes could produce a cascade of effects such as decreased biotransformation, altered gene expression, increased superoxide formation and altered redox signaling (Figure 4-1). This hypothesis could be viewed as an extension of the Needleman and Johnson hypothesis proposed in 1973. Of interest will be to determine the sequence of events; whether the bioactivation of GTN results in the formation of ROS, which in turn oxidize a number of systems or whether bioactivation results in the inactivation of a number of detoxifying enzymes leading to the buildup of ROS.

The present study suggests that ALDH2 is not the sole enzyme responsible for GTN bioactivation and tolerance. However, the quantitative role of ALDH2 still needs to be elucidated in order to determine how much of a role this enzyme plays in GTN bioactivation. Past studies aimed at investigating the role ALDH2 plays in bioactivation and tolerance have involved the use of non-specific inhibitors, and thus the results drawn from these studies are equivocal. Using ALDH2-specific siRNA to inhibit the expression of ALDH2 could provide the specificity needed in order to fully investigate the role ALDH2 plays in tolerance. Furthermore, the long-term depression of ALDH2 and
Figure 4-1 Proposed mechanism of GTN tolerance. GTN is biotransformed by a number of sulfhydryl enzymes, which become oxidized. The oxidation inactivates the enzymes and leads to the accumulation of ROS and toxic aldehydes. ROS and 4-HNE accumulate in the cell resulting in altered signaling and gene expression. The ROS and 4-HNE also cascade back and inactivate sulfhydryl enzymes. The inactivated sulfhydryl enzymes are unable to metabolize GTN resulting in a lack of bioactivation and GTN tolerance.
potential long-term build up of toxic lipid aldehydes, such, as 4-HNE, requires further investigation. The PCR analysis in this study indicated that GTN treatment drastically altered the expression of ALDH2 mRNA. However, the effect of GTN on the expression of other sulfhydryl-containing enzymes is still not known. It is possible that GTN treatment affects the expression of a number of bioactivating enzymes to different extents, leading to decreased biotransformation.

Currently, there is no standardized way to quantify GTN tolerance and as a result it is hard to determine the relative quantitative role ALDH2 plays in GTN tolerance. Furthermore, there are many in vitro and in vivo methods of inducing GTN tolerance. These methods can vary markedly which may lead to varying results and conclusions. Perhaps a more specific definition of GTN tolerance and a universally accepted method of inducing GTN tolerance may lead to a better understanding of the mechanisms involved in nitrate tolerance.

In conclusion, the findings in this study shed light on the role of ALDH2 in GTN biotransformation. My findings indicate that ALDH2 is not the sole enzyme responsible for the bioactivation of GTN and furthermore, inactivation of ALDH2 is not the sole basis of GTN tolerance. These findings contradict the current model of GTN tolerance and call for a multifactorial theory on tolerance. These findings also suggest that GTN tolerance may result in long-term effects, and suggest that GTN may not be as safe as once thought. The knowledge gained from this study has increased our understanding of the mechanism behind GTN bioactivation and may aid in the optimization of GTN therapy.


Dowlatshahi, S., (2005). Correlation between endothelial cell apoptosis and glycercyl trinitrate-induced endothelial cell dysfunction. *MSc. Thesis*. Queen’s University


