Title

Nitroglycerin metabolism and correlation to its biochemical responses and pharmacologic effects

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Nitroglycerin Metabolism and Correlation to its Biochemical Responses and Pharmacologic Effects

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Dedicated to my parents

Latabai and Rudraji Kenkare

for all the sacrifices they made,

the encouragement they provided,

and the efforts they put into the

advancement of my education.

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ABSTRACT

Metabolism of nitroglycerin (GTN) is believed to be important for pharmacologic activity. Glutathione S-transferases (GSTs) have been proposed to be involved in the regio selective denitration of GTN. The mu isozyme of GST, which exhibits a genetic polymorphism when measured in mononuclear leukocytes of caucasians, has highest activity to GTN. Tolerance, or a decrease in response upon continued dosing, develops to GTN and the involvement of GSTs in this process is not known. Thus the objective of this thesis is to investigate the role of the mu isozyme of GST in GTN metabolism, pharmacology and tolerance development.

A selective GST catalyzed C-1 denitration of 1,2-glyceryl dinitrate, in rabbit aortic homogenates was observed. Ethacrynic acid (ECA), a GST mu inhibitor, selectively inhibited this C-1 denitration. ECA also produced a significant decrease in cyclic guanosine 3', 4' monophosphate (cGMP) levels and relaxation responses to GTN in rabbit aortic strips. GST mu activity, but not total GST activity, measured in different rabbit aortas correlated very well with the increases in cGMP levels and relaxation responses to GTN. The mu isozyme activity in mononuclear leukocytes (RMLs) of different rabbits was found to correlate well with the activity in livers but not with the activity in aortas. If this is true in humans it may possibly explain why 50% of the population lacking in this isozyme (in the leukocytes) still responds to GTN. A decrease in the mu isozyme activity but not in the total GST activity was observed in rabbit aortas made tolerant to GTN *in vitro*. The decreased GST mu activity correlated very well with the decrease in

cGMP levels and relaxation responses to GTN. Tolerance to GTN could be reversed by washing and the mu isozyme activity also seemed to recover. Whether this recovery was due to synthesis of new protein or due to removal of inactive metabolite is unknown.

Overall, a good correlation was observed between the activity of the mu isozyme of GST and the responses to GTN in rabbit aorta studied *in vitro*.

GLOSSARY

1,2-GDN	1,2-Glyceryl dinitrate
1,3-GDN	1,3-Glyceryl dinitrate
1-GMN	1-Glyceryl mononitrate
2-GMN	2-Glyceryl mononitrate
ANOVA	Analysis of variance
AUC	Area under the concentration time curve
CDNB	1-Chloro-2,4-dinitrobenzene
cGMP	Cyclic 3',5'-guanosine monophosphate
CLp	Plasma clearance
CLb	Blood clearance
CO	Carbon monoxide
CV	Coefficient of variation
E_{max}	Maximum effect elicited by the drug
EC_{50}	Concentration required to elicit 50% of E_{max}
ECA	Ethacrynic acid
GC	Guanylate cyclase
GC-ECD	Gas chromatography with electron capture detection
GSH	Glutathione
GST	Glutathione S-transferase
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
HNO_2	Nitrous acid
MLC	Myosine light chain
MLCK	Myosine light chain kinase
NO	Nitric oxide

PE	Phenylephrine
PK-C	Phosphokinase C
RAH	Rabbit aortic homogenates
RML	Rabbit mononuclear leukocytes
RSNO	S-nitrosothiol
SBP	Sulfobromophthalein
SNAP	S-nitroso-acetyl penicillamine
SNP	Sodium nitroprusside
TCA	Trichloroacetic acid
TSO	Trans-stilbene oxide
γ	Hill coefficient

PREFACE

Many controversies exist with respect to the mechanism of action of glyceryl trinitrate (GTN) and other organic nitrates. The most accepted hypothesis describing the mechanism of action of GTN was put forth by Ignarro et al. (1981). According to this hypothesis, metabolism of GTN is essential for the production of its second messenger, cGMP, and the resulting vasodilation. However the exact route of metabolism of GTN that leads to this effect has not been elucidated. The site of action of this drug is the vasculature. Understanding the pathway that is involved in the metabolism of GTN leading to pharmacologic effects in the vasculature is important for an understanding of the mechanism of action. A number of possible metabolic pathways have been proposed by different investigators including: glutathione S-transferases, cytochrome P-450's, hemoproteins, guanylate cyclase and chemical degradation. It is possible that the metabolism of GTN results from one or all of these pathways.

Based on our previous metabolism studies in organs like liver and in vascular tissue, a role for glutathione S-transferases (GSTs) in the metabolism of GTN has been proposed. A distinct regio selectivity in denitration of GTN via GSTs has been observed in various tissues and in different species of animals. The term regio selectivity is used to describe the selective removal of the nitro group from either the terminal or middle carbon atoms of the glyceryl backbone of GTN. Such a regio selectivity indicates the possible involvement of several isozymes of GST. Isozymes of GSTs are known to exist in the vasculature. Previous studies have indicated that the GST mu isozyme in vasculature has high activity to

GTN while the pi isozyme does not have activity to GTN. The alpha isozyme was not detected in the vasculature of rabbits or humans. We propose that GSTs (especially the mu isozyme) may play a role in the metabolic step of GTN leading to cGMP formation and vasodilation. In humans the mu isozyme of GST, measured in livers and mononuclear leukocytes, is absent in 50% of the Caucasian population. If mu isozymes play a prominent role in cGMP formation, it would suggest that other isozymes not detected by current methods may play a role in humans not expressing the mu isozyme in liver and leukocytes. Alternatively, expression in the vasculature may be different from liver and leukocytes. It is not known if the isozyme/s in the vasculature, liver and mononuclear leukocytes are the product of a single gene. It is therefore of interest to study the relationship between the isozyme in these tissues to determine if there is a correlation between them. If GST (mu isozyme) is indeed involved in the mechanism of action of GTN, alteration of this enzyme/isozyme upon prolonged contact with the drug could be an explanation for the tolerance development commonly observed with GTN. It is also known that the tolerance to GTN can be reversed by a nitrate free interval *in vivo* and by washing *in vitro* tissues free of GTN. The processes have not been well characterized and possible changes in the metabolic enzymes have not been studied.

Although predominant effects of GTN are known to be on the peripheral and coronary blood vessels, obtaining sufficient quantities of these vessels is difficult. In addition to being an easily and readily accessible vessel, aortic tissues also respond to GTN in a manner similar to other vascular tissues in the body, they exhibit *in vitro* and *in vivo*

tolerance to GTN and also metabolize GTN like other vessels. Therefore aorta has been used as a model for vascular tissue in the studies described in this thesis.

The following will be investigated in subsequent chapters of this thesis which will describe the results of studies designed to:

(a) Examine the regio selectivity of metabolism of glyceryl nitrates via GSTs using 1,2-GDN as a model compound (Chapter 2).

(b) Explore the role of GST in the production of cGMP and relaxation in response to GTN in rabbit aorta (Chapter 3).

(c) Investigate the correlation between the activities of GST and its mu isozyme and vascular responses to GTN in rabbit aorta and furthermore to determine if there is a correlation between the isozyme in the vasculature, liver and mononuclear leukocytes (Chapter 4).

(d) Study the possible changes in GST and the mu isozyme during the development of tolerance to GTN and to investigate other possible mechanisms of tolerance development (Chapter 5).

(e) Demonstrate and characterize the reversal of GTN tolerance in the rabbit aortic tissue *in vitro* and study changes, if any, in the GST and mu isozyme in these tissues (Chapter 6).

CHAPTER 1

GLYCERYL TRINITRATE :

PHARMACOLOGY & PHARMACOKINETICS

1.1 Nitroglycerin: A historical perspective

The headache experienced by Ascanio Sobrero when he tasted nitroglycerin (GTN) triggered a chain of events which yielded one of our truly great drugs. It was 1846 when Sobrero synthesized GTN in Torino, and he described his "migraine" as well as his new reaction product in a letter to the editor of Comptes Rendus (Sobrero, 1847). Among the readers was Constantin Hering, a professor of materia medica at the Hahnemann Medical school in Philadelphia and a practitioner of Homeopathic medicine. Hering quickly obtained GTN and tested it on his students, and published his first paper on the new compound in 1849 (Hering, 1849) and in 1853, Hering authored an entire monograph of case reports suggesting the usefulness of GTN for treating angina pectoris, cardiac edema, epilepsy and headache (Hering, 1853; Munch and Petter, 1965). Lauder Brunton's finding in 1867 that isoamyl nitrite also aborts attacks of angina pectoris Brunton (1867) added a structural dimension to the clinical pharmacology.

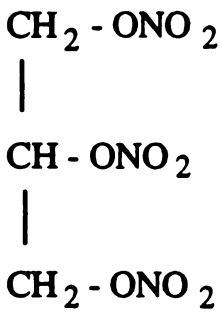
A more comprehensive report of the use of GTN appeared in a series of articles by William Murrel in the Lancet in the year 1879 (Murrel, 1879). In these articles the typical and stunning effects of GTN were described for the first time. Murrel described the effect of two drops of a one percent solution of GTN in alcohol as "About three minutes after the dose had been placed on his tongue he noticed a sensation of fullness in both sides of the neck, succeeded by nausea. For a moment or two there was a little mental confusion, accompanied by a loud rushing noise in the ears, like steam passing out of a kettle. He experienced a

feeling of constriction around the lower part of the neck, his forehead was wet with perspiration, and he yawned frequently. These sensations were succeeded by slight headache and a dull, heavy pain in the stomach, with a decided feeling of sickness, though without any apprehension that it would amount to vomiting. He felt languid and disinclined for exertion, either mental or physical. This condition lasted for half an hour with the exception of the headache, which continued till the next morning".

Using a sphygmograph Murrel (1879) found that six or seven minutes after an oral dose the pulse pressure, heart rate and the rate of rise of systolic pressure increased and the dicrotic notch became more prominent. He wrote that "from a consideration of the physiological action of the drug and more especially from the similarity existing between its general action and that of nitrite of amyl, I concluded that it would probably prove of service in the treatment of angina pectoris, and I am happy to say that this anticipation has been realized".

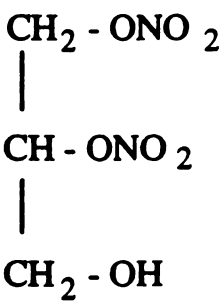
1.2 Physico-chemical properties of GTN

GTN (structure shown in Figure 1.1) is a simple ester of glycerol and nitric acid. It exists in liquid form at room temperature and crystallizes at 2.8^0C and 13.5^0C to form two different types of crystals. Upon heating it starts to decompose at 50^0C and rapidly vaporizes. GTN is also an explosive. Explosions can occur spontaneously above 218^0C (DiCarlo, 1975). GTN appears as a pale yellow, odorless, oily liquid having a sweet burning taste with a density of 1.601 at 15^0C. It has an



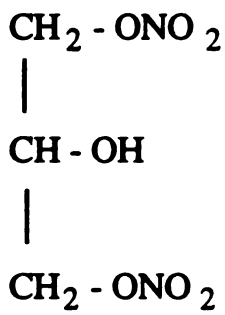
Glyceryl trinitrate

(GTN)



1, 2 - Glyceryl dinitrate

(1, 2 -GDN)



1, 3 - Glyceryl dinitrate

(1, 3-GDN)

Figure 1.1 Chemical structures of GTN and its metabolites 1,2-GDN and 1,3-GDN.

aqueous solubility of 1.73 and 2.46 mg/ml at 20 and 60^{0}C respectively; ethanol dissolves GTN at 375 mg/gm at 0^{0}C and 540 mg/gm at 20^{0}C. GTN is synthesized using equal volumes of nitric and sulfuric acids. A small volume of urea or urea nitrate is used as a scavenger for any excess nitrous acid present. Esterification is carried out by the slow addition of glycerol to the mixed acids. Below is shown a general alcohol being converted to its nitrate (R-O-NO_2) by the above reaction.

$$2\,H_2SO_4 + HNO_3 \longrightarrow NO_2^{+} + H_3O^{+} + 2HSO_4^{-}$$

$$NO_2^{+} + R\text{-}OH \longrightarrow R\text{-}O\text{-}NO_2 + H^{+}$$

GTN is relatively stable at neutral pH, but is susceptible to both hydrolytic and reductive reactions in the presence of acid and alkali. In extremely alkaline conditions the nitrate groups on GTN can be displaced by the hydroxyl moiety (OH^-) from the medium, either by attacking the C-atom or via a concerted mechanism (DiCarlo, 1975). Acid hydrolysis was shown to occur in the presence of 4N HCl to yield denitrated products as described by DiCarlo and Crew (1968). Both these reactions occur at unphysiologic pH and therefore should not contribute to the metabolism of this drug *in vivo*.

Heppel and Hilmoe (1950) studied the reduction of GTN in the presence of reduced glutathione. GTN can be degraded to yield inorganic nitrite ions (NO_2^-) accompanied by the formation of oxidized glutathione (GS-SG). The rate of this non-enzymatic reaction increased tremendously as the pH was increased from 7 to 9. An equation describing the above reaction was subsequently proposed by Needleman and Hunter (1965):

$$C_3H_5(ONO_2)_3 + 2GSH \longrightarrow C_3H_5\,OH(ONO_2)_2 + GS\text{-}SG + HNO_2$$

It is possible that this reductive reaction, in the presence of sulfhydryl moieties such as glutathione and cysteine can occur at an appreciable rate at physiologic pH. At pH 7.2 all organic nitrates tested underwent reductive degradation, as demonstrated by the consumption of sulfhydryl compounds and the appearance of inorganic nitrite (Yeates *et al.*, 1985).

1.3 Clinical indications for GTN

1.3.1 Angina Pectoris

GTN is primarily used in the treatment of angina. Table 1.1 shows the typical nitrate and nitrite drugs used in the treatment of this condition. Angina Pectoris was first described as a distinct clinical entity by William Heberden in the latter half of the 18th century. Angina or chest pain is caused by the accumulation of metabolites in striated muscle. Angina Pectoris is characterized by a sudden severe pressing substernal pain radiating to the left arm, that occurs when coronary blood flow is inadequate to supply the oxygen required by the heart.

The most frequent cause of angina is atheromatous obstruction of the large coronary vessels. This type of angina is called the *classic or atherosclerotic angina.* In this type of angina, the imbalance between the O_2 requirement and the O_2 supply to the heart via the coronary vessels is increased during exercise and is therefore sometimes referred to as "angina of effort". A transient spasm of localized portions of coronary vessels can also cause significant myocardial ischemia and

Drug	Dose	Duration of Action
"Short-acting"		
Nitroglycerin, sublingual	0.15 - 1.2 mg	10 - 30 mins
Isosorbide, sublingual	2.5 - 5 mg	10 - 60 mins
Amyl nitrite, inhalant	0.18 - 0.3 ml	3 - 5 mins
"Long-acting"		
Nitroglycerin, oral, sustained action	6.5 - 13 mg per 6 - 8 hours	6 - 8 hours
Nitroglycerin, 2 % ointment	1/2 - 2 inches per 4 hours	3 - 6 hours
Nitroglycerin, slow-release buccal	1 - 2 mg per 4 hours	3 - 6 hours
Nitroglycerin, slow-release transcutaneous	10 - 25 mg per 24 hours	24 hours or longer
Isosorbide dinitrate, sublingual	2.5 - 10 mg per 2 hours	1.5-2 hours
Isosorbide dinitrate, oral	10 - 60 mg per 4 - 6 hours	4 - 6 hours
Isosorbide dinitrate, chewable	5 - 10 mg per 2 - 4 hours	2 - 3 hours
Pentaerythritol tetranitrate	40 mg per 6 - 8 hours	6 - 8 hours
Erythrityl tetranitrate	10 - 40 mg per 6 - 8 hours	6 - 8 hours

Table 1.1 Typical nitrate and nitrite drugs used in the treatment of angina pectoris.

pain. This kind of transient anginal pain is called *variant or angiospastic angina*. During this type of angina, the O_2 delivery decreases as a result of reversible coronary vasospasms usually in the presence of atherosclerotic lesions in the coronary vessels affected. When there is a change in the character, frequency, duration and precipitating factors in patients with stable angina and when there are episodes of angina at rest, the condition is called *unstable angina*. Thus angina generally represents an imbalance of myocardial oxygen supply and demand.

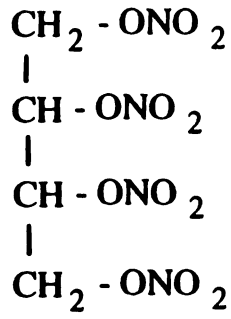
The major determinants of **myocardial oxygen demand** are wall stress, heart rate and contractility. Other minor determinants are activation energy and resting metabolism.

The primary objective of treatment in both forms of angina is to improve coronary blood flow. A secondary objective is reduction of the myocardial oxygen requirement. Traditional treatment achieves the second goal through the use of vasodilators, although all vasodilators are not effective in angina and some agents useful in angina (e.g., propranolol) are not vasodilators. Apart from GTN other anti-anginal drugs include:

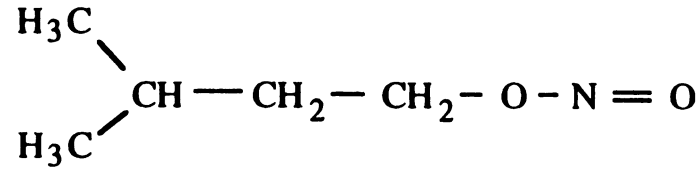
1. Other nitrates: Isosorbide dinitrate, amyl nitrite, erythrityl tetranitrate, pentaerythritol tetranitrate (Figure 1.2 A).

2. Ca^{++} channel blockers: Nifedipine, verapamil and diltiazem (Figure 1.2 B).

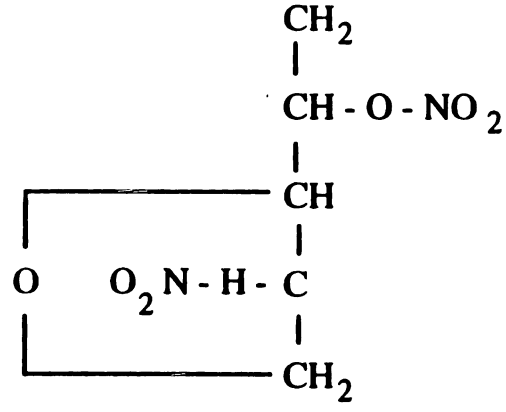
3. Beta blockers: Propranolol (Figure 1.2 B).



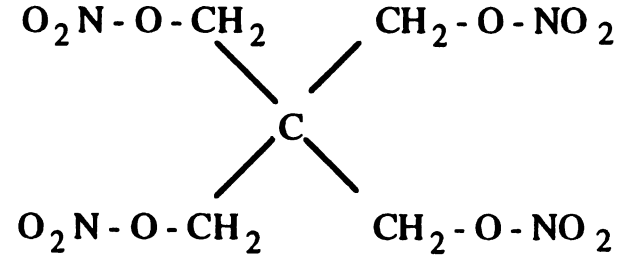
Erythrityl Tetranitrate



Amyl nitrite

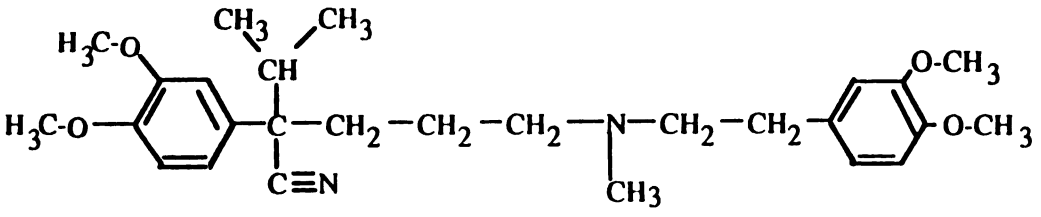


Isosorbide dinitrate

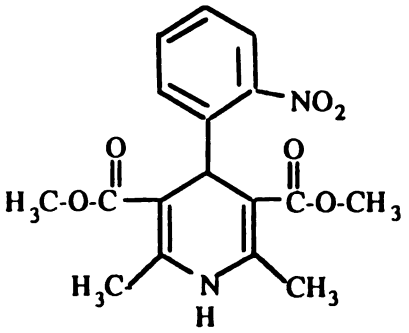


Pentaerythritol tetranitrate

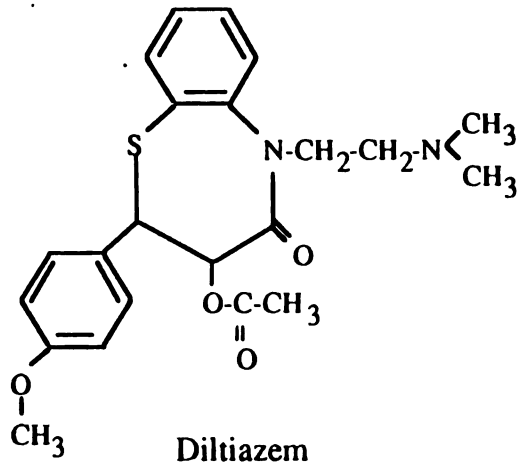
Figure 1.2A Other nitrate/nitrite drugs used as anti-anginal agents.



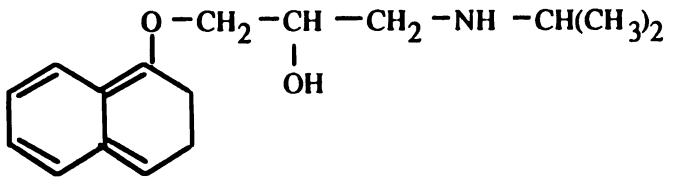
Verapamil



Nifedipine



Diltiazem



Propranolol

Figure 1.2B Calcium channel blockers and beta blockers used in the treatment of angina pectoris.

Sublingual GTN is the most frequently used agent for anginal treatment because of its rapid onset of action. However because its duration of action is short (not > than 20-30 minutes) it is not suitable for maintainance therapy. Intravenous GTN has a rapid onset too and its hemodynamic effects are quickly reversed by stopping its infusion. Intravenous GTN is therefore used clinically for the treatment of severe, recurrent unstable angina. Buccal, oral and transdermal dosage forms provide blood concentrations for longer periods.

1.3.2 Congestive heart failure

The goal of treatment of congestive heart failure is to increase cardiac output and decrease pulmonary and peripheral edema. Conventional therapy involves use of positive inotropic agents and diuretics. Nitrates can improve cardiovascular function in congestive heart failure, even in some patients who are unresponsive to conventional therapy. Nitrates are used in treatment of both acute and chronic heart failure.

1.3.3 Myocardial infarction

GTN is believed to favorably alter the O_2 balance to decrease the area of myocardial damage in an infarcted tissue, if given soon after infarction. Intravenous GTN infusion in patients with acute myocardial infarction at doses that maintain or improve stroke work can relieve pulmonary congestion by decreasing left ventricular filling pressure and decreasing myocardial O_2 demand.

1.4 Pharmacology of organic nitrates

The organic nitrates and nitrites are dilators of both arterial and venous smooth muscles. Low concentrations of GTN produce dilatation of the veins. This venodilation results in a decrease of left and right ventricular end-diastolic pressures, which on percent bases are more marked than the decrease in systemic arterial pressure. Net systemic vascular resistance is usually relatively unaffected, heart rate is unchanged or slightly increased and pulmonary vascular resistance is consistently decreased (Ferrer *et al.*, 1966). In normal individuals or those with coronary artery disease, sublingual administration of GTN decreases cardiac output. Doses of GTN that do not alter systemic arterial pressure often produce arteriolar dilatation in the face and neck, resulting in a flush. The same doses may also cause headache, presumably due to dilatation of meningeal arterial vessels.

Rapid administration of high doses of organic nitrates decreases the systolic and diastolic blood pressures and cardiac output, resulting in pallor, weakness, dizziness and activation of compensatory sympathetic reflexes. The resultant tachycardia and peripheral arteriolar vasoconstriction tend to maintain peripheral resistance; this is superimposed on sustained venous pooling. Coronary blood flow increases transiently due to coronary vasodilatation but subsequently falls as arterial blood pressure decreases and cardiac output falls.

Relief of angina is attained by either causing a reduction in oxygen demand on, or an increase in oxygen supply to, the myocardium. The original hypothesis was that nitrates cause relief of anginal pain by

dilating coronary blood vessels, thereby increasing coronary blood flow. This hypothesis was later questioned and the evidence now favors a decrease in the myocardial oxygen requirement as the major action. GTN and other organic nitrates probably act via both the above mentioned mechanisms. Their effects can be characterized within the following two categories of pharmacologic actions:

A. Organic nitrates decrease the requirement of the myocardium for oxygen by their effects on systemic circulation. The major determinants of myocardial oxygen consumption include the stress on the ventricular wall during systole, the state of contractility of the myocardium and the "preload" or the left ventricular end-diastolic pressure. As a consequence of the continuous activity of the heart, its oxygen needs are relatively high, and the heart extracts approximately 75% of the available oxygen even under conditions of no stress. The myocardial oxygen requirement increases during stress, particularly during physical exercise in patients with obstructive coronary artery disease.

Organic nitrates decrease venous resistance which decreases venous return to the heart, resulting in decreases in end diastolic pressure and volume or the "preload", thereby decreasing oxygen consumption . This cascade of effects increases the pressure gradient for perfusion across the ventricular wall favoring endocardial perfusion (Paratt, 1979). Similarly decreasing the peripheral arteriolar resistance decreases after load and therefore myocardial consumption of oxygen.

Organic nitrates do not directly alter the inotropic and chronotropic state of the heart. The drugs do decrease both preload and

afterload as a result of respective dilatation of venous capacitance and arteriolar resistance vessels. Since the primary determinants of oxygen demand are reduced by nitrates their net effect usually is to decrease myocardial consumption of oxygen.

B. Myocardial oxygen supply is a function of myocardial delivery and extraction. Since the myocardial oxygen extraction is nearly maximal at rest and the oxygen content of the blood cannot be significantly increased under normal atmospheric conditions, the increased myocardial demands for oxygen at a normal heart rate are met by augmenting coronary blood flow.

GTN can act on smooth muscle structures such as bronchi, biliary tract, gall bladder, biliary duct, spinchter of oddi, gastrointestinal tract, including the esophagus. Nitrates also relax the ureteral and uterine smooth muscles.

1.5 Pharmacokinetics of GTN

1.5.1 Absorption and Bioavailability

GTN is readily absorbed after sublingual or transdermal administration and, according to experimental studies in rats, also from the gastrointestinal tract (Hodgson and Lee, 1975). Although the clearance of GTN by the liver has been shown to be of minor importance following iv dosing (Blei *et al.*, 1984) since clearance greatly exceeds blood flow to the liver, the liver is the major organ for GTN metabolism when GTN is dosed orally. The bioavailability after oral administration is

therefore, very low because of extensive first pass metabolism in the liver and possibly in the gut wall. The availability of GTN through sheep liver is estimated to be less than 20% (Cossum *et al.*, 1986). In organ homogenates, the liver exhibited the highest GTN metabolic activity (Fung *et al.*, 1984; Cossum and Roberts, 1985). Furthermore Tam *et al.* (1988) reported that GTN degradation also occurs in the gastrointestinal tract, thus contributing to presystemic degradation of oral GTN preparations.

In studies carried out in humans (Ochs *et al.*, 1985; Noonan and Benet 1986; Laufen and Leitold 1988), GTN was not detected in the systemic circulation after oral administration of the substance, whereas high concentrations of metabolites were found. The absolute bioavailability following sublingual administration was studied by Noonan *et al.* (1985) and found to be 36.2 $\pm$ 24.9% (mean $\pm$ SD). The bioavailability varied from 2.6% to 113%. Times to peak concentration were also highly variable. The bioavailability after transdermal administration is about 70% (Nakashima *et al.*, 1987). As a result of the low bioavailability observed after oral dosing, questions have been raised with regard to the efficacy of oral GTN. Needleman *et al.* (1972) stated that oral dosing of GTN would be of no clinical benefit to the patient. However, other investigators have consistently observed clinical effectiveness of oral GTN (Krantz and Leake 1975; Winsor and Berger 1975). Saturable hepatic metabolism has been suggested by some investigators (Bashir *et al.*, 1982). Gumbleton and Benet (1991) have suggested that oral GTN preparations are active, but that most of their activity is not due to GTN concentrations. Rather the activity results from the high levels of GDN metabolites which occur due to GTN metabolism.

1.5.2. Distribution

McNiff *et al.* (1981) have reported a volume of distribution for GTN of about 3 L/Kg in humans. This is much greater than the plasma volume and therefore suggests significant uptake into the tissues. These authors calculated the amount of GTN in the plasma compartment to be approximately 1%. The distribution of GTN has not been widely studied in humans. However in a study by DiCarlo and Crew (1968) an aqueous solution of ^{14}C-GTN was administered by gavage to a group of rats and the distribution of the ^{14}C label was studied. The liver and the carcass took up majority of the label, while the heart, lung, kidney and the spleen also contained traces of the label. However no distinction was made as to whether the label was the drug or any of its metabolites. In studies carried out by Hodgson and Lee (1975) the concentrations of total radioactivity at 4 and 24 hours after oral administration of ^{14}C GTN were respectively 4.4 and 7.8 times greater in the liver and 2.4 and 2.8 times greater in the kidney than in the plasma.

Torfgard *et al.* (1989) administered GTN (50 mg/Kg) subcutaneously to rats and found that the highest concentration of the drug was in the adipose tissue, where the level was about forty times the level in plasma. The concentrations of GTN in the brain, heart and aortic tissue were about two to three fold greater than those in the plasma. It is believed that the high lipophilicity of GTN may facilitate its partition between these tissues. Adipose tissues may act as a reservoir of GTN, especially during sustained transdermal delivery of the drug.

The uptake of GTN into the smooth muscle cells of blood vessels is important, especially in light of the fact that these tissues represent the effector site for organic nitrates, which are potent vasodilators. Following intravascular injections in rats, GTN levels could be detected in various blood vessel segments in the vasculature (Fung *et al.*, 1984). The tissue to plasma ratios were found to vary as a function of time and as a function of the location of the blood vessels relative to the site of injection, with ratios as high as 40:1 observed. Furthermore, GTN was found to partition better into veins than arteries. This may be an important determinant of the relatively higher potency of GTN on the venous, as compared to the arterial, circulation.

1.5.3. Metabolism and elimination

The clearance of GTN in humans seems to be subject to high interindividual variation. As shown in Table 1.2, it is believed that the estimates of clearance depends on the mode of administration (bolus vs. slow infusion), sampling site (arterial vs venous) and health status of the subjects (normal vs. congestive heart failure patients). The reported values of plasma clearance (CL_p) for the arterial circulation ranged from 3.6 to 18.9 L/min while the plasma clearance for the venous circulation ranged from 15.8 to 54.5 L/min. Since the blood to plasma ratio is reported as 0.65 (Noonan and Benet, 1982), the blood clearance (CL_b) may be calculated:

$$CL_b=(C_p/C_b)CL_p = CL_p/0.65 = 1.54\ CL_p$$

Study	Infusion rate (µg/min)	Patients/ subjects	Arterial			Venous		
			CL	$t_{1/2}$	Vd	CL	$t_{1/2}$	Vd
Armstrong *et al.*,1982	15 - 440	CHF	12.0 (8.9)	1.4 (0.8)	23.0 (21.5)	39.1 (39.3)	--------	77.0 (79.2)
Armstrong *et al.*,1980b	15 - 94	CHF(I)*	13.8 (5.8)	-------	--------	--------	--------	--------
	59 - 440	CHF (II)*	3.6 (1.8)	1.7(0.7)	9.9 (6.7)	-------	--------	--------
Curfman *et al.*, 1983	10 - 200	Unstable angina	7.4 (3.9)	-------	--------	-------	--------	--------
McNiff *et al.*, 1981	18	Normal	--------	--------	--------	54.5 (21.2)	2.8 (0.9)	210 (80)!
Wei and Reid,1979	37.5 - 175	AMI	--------	--------	--------	82.3 (57.2)^	--------	--------
Idzu *et al.*, 1982	IV bolus 4 µg/Kg	Surgery	4.4 (3.1)**	5.3 (1.6)**	3.8 (3.3)**	-------	--------	--------
Armstrong *et al.*, 1980a	Sublingual (0.6 mg)	Normal	--------	--------	--------	28	4.4	180
Hill *et al.*,1981	Intranasal (0.8 mg)	Bypass surgery	18.9	5.6	153	15.8	5.4	123
Noonan and Benet, 1985	IV infusion (10 - 40 µg/min)	Healthy	--------	--------	--------	5.5 - 71	2.3	--------

CL= apparent clearance (L/min), $t_{1/2}$ = elimination half life (min), V_d = apparent volume of distribution (L), CHF= congestive heart failure, AMI = acute myocardial infarction.

* Plasma GTN concentration less than 11.1 ng/ml (Group I), greater that 11.1 ng/ml (Group II)

! Normalized to a 70 Kg man.

^ Estimated from plasma concentration data.

** Site of sampling not specified, listed under "arterial" because of magnitude of values.

Table 1.2 Summary of the reported pharmacokinetic parameters for GTN.

CL_b venous = 24.3 to 126 L/min

CL_b arterial = 6.76 to 29.1 L/min

Since the cardiac output is 5 L/min, it is clear that the venous blood clearance of GTN is much greater than the cardiac output whereas the arterial blood clearance is nearer to cardiac output.

GTN clearance is known to be nonlinear (Noonan *et al.*, 1985). When the infusion rate of GTN in human volunteers was increased from 10 to 40 μg/min the steady state plasma concentration increased disproportionately about 25 fold. This indicates that the clearance is not constant and that the kinetics are dose dependent. Furthermore, when the infusion rate was decreased to 10 μg/min, the steady state concentration did not decrease back to the original steady state concentration, but instead remained approximately 5 fold greater than the original level. These results indicate that GTN may also exhibit time dependencies or irreversible dose dependent changes in its kinetics.

Most metabolic studies of GTN have been carried out in animals, such as rats, rabbits and sheep. It is believed that the rapid and extensive elimination of GTN to its dinitrate metabolites 1,2- and 1,3-glyceryl dinitrates (GDNs) accounts for the extraordinarily high clearance, as the fraction excreted unchanged in the urine is very small (DiCarlo and Crew, 1968; Hodgson and Lee, 1975). The metabolic scheme of GTN was examined in detail using ^{14}C-labelled GTN, and the profile is shown in Figure 1.3.

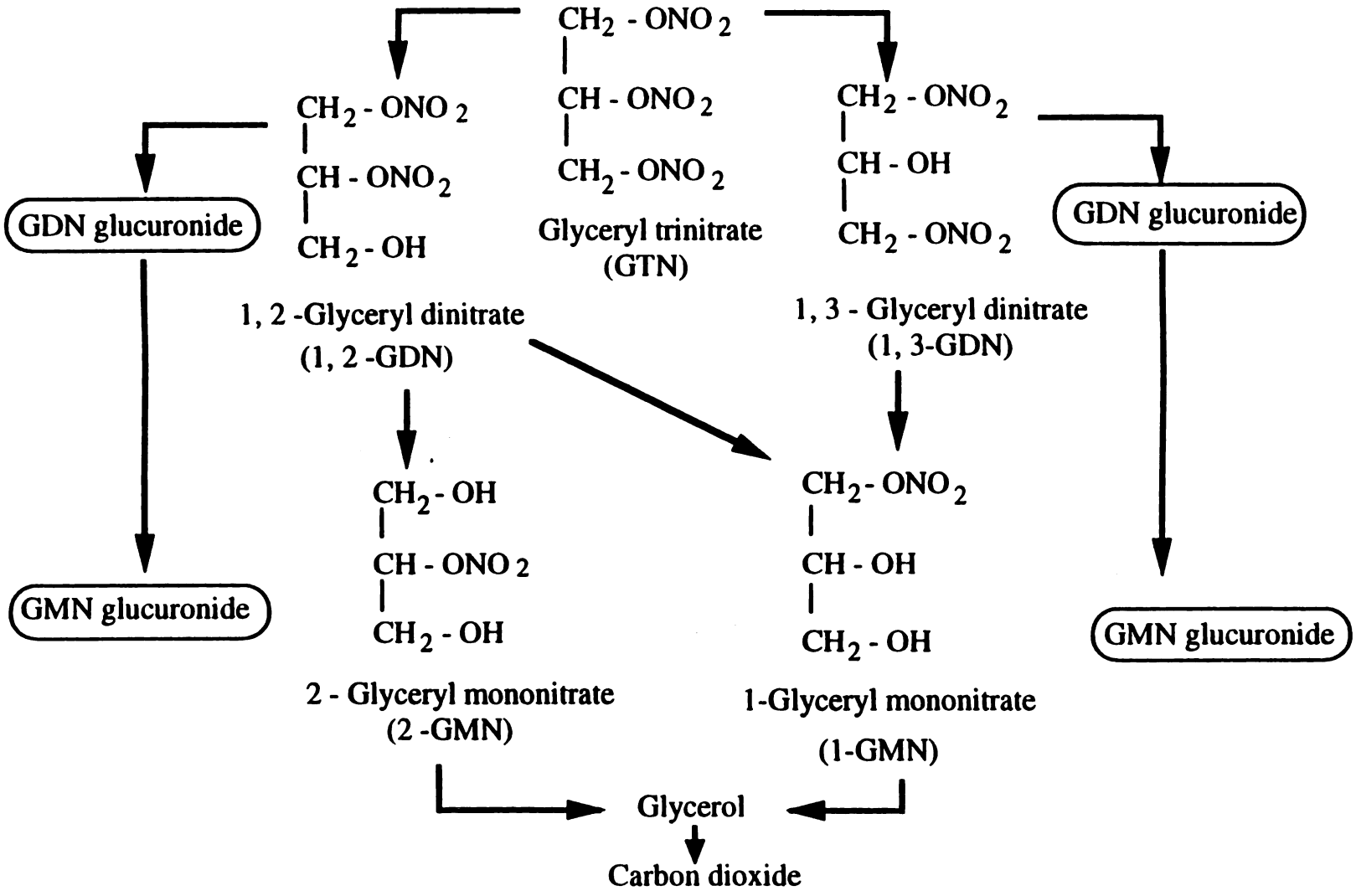


Figure 1.3 Metabolic scheme of glyceryl trinitrate (GTN).

Following administration GTN is metabolized to form the dinitrates 1,2- and 1,3-GDNs which are further metabolized to form the 1- and 2- glyceryl mononitrates (GMNs), and then further degraded to glycerol and carbon dioxide (Needleman *et al.*, 1971). Glucuronides of both GDNs and GMNs were detected in the urine (DiCarlo and Crew, 1968). However, administration of the GMNs did not result in formation of GMN-glucuronides, suggesting that GMN-glucuronides which appear following GTN administration result from denitration of GDN-glucuronides. It appears that denitration, followed by sub sequential glucuronidation of its metabolites, represent the two major metabolic pathways of GTN *in vivo*.

GTN can be metabolized by various organs. In studies carried out by Lau and Benet (1989) in subcellular fractions of rabbit liver, both cytosolic and microsomal fractions were found to be able to metabolize GTN, with the cytosol exhibiting a more rapid rate of GTN degradation. Both these pathways were glutathione dependent and enzymatic in nature. The affinity constant for 1,2-GDN formation was approximately three times lower than that for 1,3-GDN formation. In bovine coronary artery subcellular fractions (Lau *et al.*, 1991) unlike the liver, the majority of the GTN metabolism was shown to be carried out by the cytosolic fractions. Furthermore 1,3-GDN was the preferential metabolite.

1.6 Pharmacokinetics and pharmacodynamics of glyceryl dinitrates (GDNs)

Needleman *et al.* (1969) compared the potency in dogs of a number of organic nitrates as blood pressure depressants. The compounds were compared by evaluating the dosage required to produce a 10 to 20% vasodepression. GTN (25 μg/Kg) was at least 10-14 times more potent than GDNs. Bogaert *et al.* (1968) compared the cardiovascular effects of GTN and 1,3- and 1,2- GDNs. They found little difference in effectiveness between the GDNs. The GDN dose-response curve was parallel to the GTN dose-response curve. GDN was only 2% as active as GTN in lowering guinea pig blood pressure, 0.2% in relaxing rabbit aortic strips and 5% as active as GTN in decreasing dog hind leg resistance.

The GDNs exhibit longer half lives when compared to GTN. In dogs, GTN exhibited a half life of 4 min, while the half lives of 1,2- and 1,3- GDN were 43 and 46 min, respectively (Lee *et al.*, 1990) Similarly the half lives of the GDNs were much longer than that of GTN in humans (Noonan *et al.*, 1985). This suggests that the metabolites can reside in the body much longer and therefore may significantly contribute to the overall effects observed following GTN administration despite their lower potency. The bioavailabilities of 1,2- and 1,3-GDNs were estimated as 62% and 68% respectively in dogs (Lee *et al.*, 1990) compared to low or no bioavailability of GTN in the same species. These results suggest that the pharmacokinetic differences between GDNs and GTN may

compensate for the difference in potencies and that the contribution of GDNs to the overall effect should not be ignored.

Gumbleton and Benet (1991) administered the two GDNs orally to healthy volunteers at doses which would yield similar plasma concentrations of GDNs as compared to those observed following a 6.5 mg oral GTN solution dose. Significant blood pressure lowering effects were observed, further suggesting the possible contribution of GDNs to the overall GTN activity and the potential of these active metabolites as therapeutic agents.

1.7 Regio selectivity

The metabolism of GTN to its dinitrate metabolites has been found to be regio selective both *in vivo* and *in vitro*. When GTN is metabolized to GDNs, one might expect the ratio of 1,2-GDN/1,3-GDN to be 2:1, because there are two C-1 terminal nitrate groups to be removed, thus forming 1,2-GDN, versus only one C-2 nitrate, which upon denitration yields 1,3-GDN. However if the reaction is enzymatic, the pattern of metabolite formation would also depend upon other steric factors, such as orientation of the substrate at the active site of the enzyme. This may lead to regio selectivity of the denitration process.

Noonan and Benet (1987) reported that variable GDN formation resulted from dosing GTN via different routes of administration. The GDN ratios from administering GTN intravenously, sublingually, transdermally and orally were calculated as the ratios of the

steady state concentrations or areas under the curve of the metabolites and were found to be different following the various routes of administration. Intravenous infusion yielded the highest ratio of 7:1 (1,2 GDN/ 1,3-GDN), indicating a fairly specific 1,2-GDN formation. The ratios were lower for the transdermal and sublingual routes, approximately 4:1 in each case. Oral GTN dosing resulted in the lowest GDN ratio of 2:1. This suggests that different tissues may represent different site-specific "first pass organs" for GTN metabolism and that the various organs may contain enzymes which possess different regioselectivities for GDN formation.

To address the possibility that the observed differences were route and not dose related, GDN ratios were measured in healthy volunteers for a single route of GTN dosing, oral administration, with solution doses ranging from 0.4 mg to 13 mg (Nakashima *et al.*, 1990). A gradual decrease in the GDN ratio from 3.64 to 1.87 was observed as the dose was increased from 0.4 mg to 13 mg. This indicates the presence of a dose-dependent metabolic pathway that preferentially forms 1,2 GDN in the liver. This could also suggest the possibility of nonlinear systems existing in the body which lead to formation of GDN ratios which change with increasing doses of GTN. This may also suggest the possible involvement of different isozymes in the metabolism of GTN.

In vitro incubation experiments have also demonstrated that GDN formation is organ-dependent (Cossum *et al.*, 1986) as well as species dependent (Lee, 1973). Lau and Benet (1990) showed that the glutathione dependent metabolism of GTN lead to the predominant formation of 1,2-GDN in rabbit liver cytosol, while 1,3-GDN was the predominant metabolite from microsomal fractions. In bovine coronary

artery homogenates, Lau *et al.* (1992) reported that 1,3-GDN was the predominant metabolite. In a study by Short *et al.* (1977) a species dependence on the 1,2-GDN/1,3-GDN ratio was found. With ^{14}C GTN the authors showed that this ratio was less than one in rats and mice and greater than one in rabbits, dogs, monkeys and human livers.

1.8 Relationship between pharmacokinetics and the therapeutic effects of GTN

It is difficult or impossible to describe the therapeutic effects of nitrates as a dose-response or a concentration-response relationship. The plasma concentration of nitrates is not directly related to the therapeutic effect. This observation may be due to several factors:

1. The therapeutic action of nitrates is a secondary response resulting from different effects in various segments of the circulation. In each segment there is a different concentration-response curve. Morever the relative contribution of each action (coronary vasodilatation, arterial vasodilation, venodilation) is variable and differs between patients and conditions.

2. Nitrates are metabolized in the vascular wall and the process is associated with the mechanism of action of the drugs. It has been proposed that the pharmacodynamic effect of nitrates are better correlated with vascular wall concentrations than with plasma concentrations (Fung *et al.*, 1984).

3. The sensitivity of vascular walls to nitrates shows a marked intersubject variability (Eichler *et al.*, 1987)

4. Part of the pharmacodynamic response is exerted by the metabolites.

5. The concentration-response relationship is to a high degree complicated by development of tolerance. The concentration-response curve might differ between acute and chronic administration of nitrates. Tolerance might develop towards some effects but not to others.

6. The pharmacodynamics of nitrates might be more dependent on the rate and direction of changes in the plasma concentrations of drugs than on the absolute values of plasma concentration.

7. Initial effects may be attenuated by counter-regulatory mechanisms e.g., activation of adrenergic activity, the renin-angiotensin system or other mechanisms.

1.9 Cellular mechanisms of GTN action

The effects of GTN on the vasculature in the body has been well studied. However its exact mechanism of action can only be understood by examining its effects at a cellular level. Various hypotheses have been proposed to describe its cellular mechanism of action. They are discussed below.

1.9.1 S-Nitrosothiol / cGMP hypothesis (Figure 1.4)

This most commonly accepted hypothesis of organic nitrate action was proposed by Ignarro *et al.* (1981). The highly lipophilic organic nitrates easily penetrate vascular smooth muscle cells where they are metabolized, probably via an enzymatic process, to form denitrated product(s) [e.g., GDNs form from GTN, and an inorganic nitrite ion (NO_2^-)]. The inorganic nitrite ion produced then reacts with protons in the cellular medium to generate nitrous acid (HNO_2), and subsequently nitric oxide (NO). Nitric oxide reacts with -SH groups to form RSNO, which activates guanylate cyclase (GC), an enzyme responsible for converting guanosine triphosphate (GTP) to cyclic 3', 5' guanosine monophosphate (cGMP). cGMP accumulates in the smooth muscle cells and is believed to be the second messenger for nitroglycerin induced vasorelaxation, as it triggers a cascade of events which lead to vasorelaxation.

cGMP is believed to cause a decrease in the concentration of Ca^{++} ions in the cell. Although it is not completely clear, how it does this, various mechanisms have been proposed (Figure 1.5). cGMP stimulates the efflux of Ca^{++} ions out of the plasma membrane and uptake of Ca^{++} by intracellular calcium storing compartments (Kreye *et al.*, 1975; Lincoln, 1983; Twort and Van Breeman, 1988; Ahlner *et al.*, 1990), while inhibiting calcium influx into the plasma membrane (Kreye *et al.*, 1975; Zostér *et al.*, 1977; Hester *et al.*, 1979) and production of diacyl glycerol (DAG) and IP_3 (Ahlner *et al.*, 1986; Fujii *et al.*, 1986; Nakashima *et al.*, 1986; Rapaport, 1986;Takai *et al.*, 1981). This response

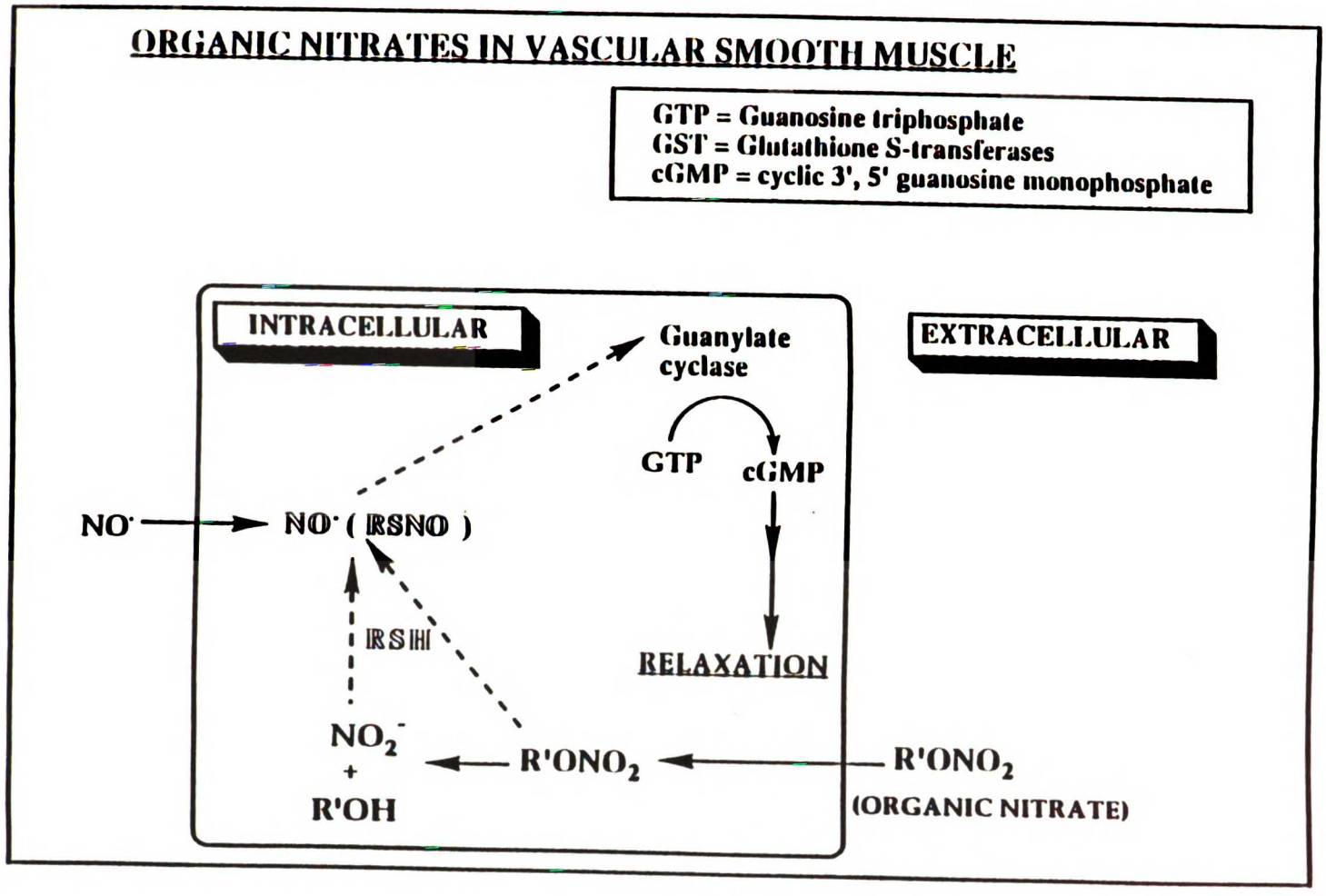


Figure 1.4 A simplified picture of the S-nitrosothiol /cGMP hypothesis as proposed originally by Ignarro *et al.* (1981).

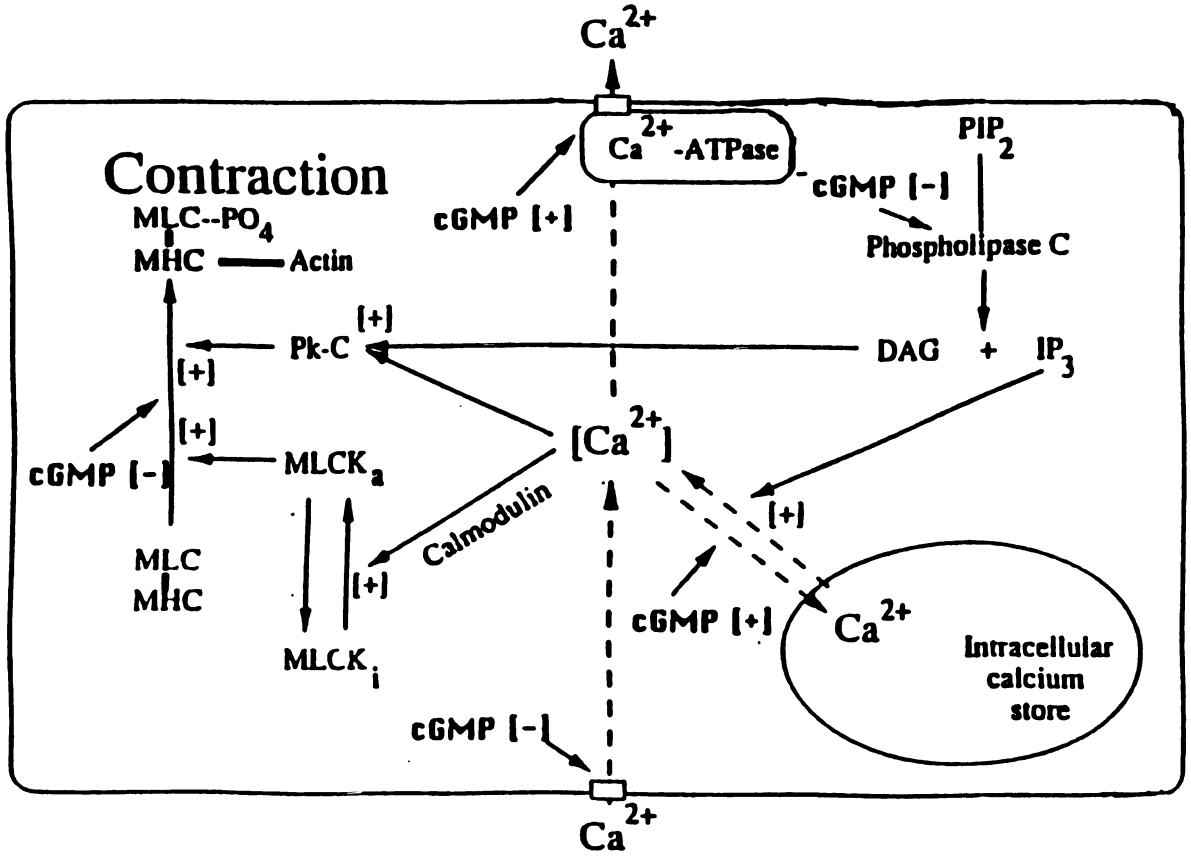


Figure 1.5 Schematic drawing showing some of the cellular mechanisms suggested to be targets for cGMP and mediating vascular smooth muscle relaxation. **Abbreviations:** (+) stimulation, (-) inhibition, diacyl glycerol (DAG), inositol triphosphate (IP3), protein kinase C (PK-C), myosin light chain kinase (MLCK), calcium-extrusion ATPase (Ca-ATPase), myosin heavy chain (MHC), phosphatidyl inositol bisphosphate (PIP2), inactive enzyme (i) and active enzyme (a).

to cGMP may result in decreased calcium liberation from intracellular stores and reduced myosin light chain (MLC) phosphorylation by protein kinase C (Pk-C) and calcium/calmodulin-activated myosin light chain kinase (MLCK). An inhibition of MLC phosphorylation by cGMP through other, as yet, unknown mechanisms is also indicated as a possibility. This would reduce actin-myosin interaction and, thus, reduce vascular smooth muscle contraction, causing relaxation.

That cGMP might be involved in the smooth muscle relaxation effect of certain nitrogen containing compounds was first suggested in 1975 when smooth muscle relaxants sodium azide, hydroxylamine and sodium nitrite were reported to elevate tissue cGMP levels and activate GC under specific conditions (Kimura *et al.*, 1975a; Kimura *et al.*, 1975b; Mittal *et al.*, 1975). Diamond and colleagues reported that GTN elevated cGMP levels in rat myometrium and canine femoral artery (Diamond and Holmes, 1975; Diamond and Blisard, 1976). Subsequently, other investigators showed that nitroprusside, GTN, $NaNO_2$ and related agents elevated cGMP levels in certain nonvascular and vascular smooth muscle tissues (Katsuki *et al.*, 1977; Schultz *et al.*, 1977; Bohme *et al.*, 1978; Axelsson *et al.*, 1979; Janis and Diamond, 1979; Kukovetz *et al.*, 1979). In studies done by DeRubertis and Craven (1976) and Arnold *et al.* (1977 a,b) nitric oxide gas and substances containing or releasing nitric oxide were reported to stimulate cGMP formation and these compounds were all found to be potent smooth muscle relaxants (Gruetter *et al.*, 1979, 1981). Therefore, it is now generally accepted that the action of organic nitrates is mediated via the stimulation of GC, which in turn leads to accumulation of cGMP. There is also evidence (Brien *et*

al., 1988) demonstrating a relationship between nitrate metabolism and cGMP elevation, in which the increase in cGMP levels was found to correlate with the extent of GTN metabolism, as measured by formation of GDN metabolites, in rabbit aortic strips.

Although the RSNO hypothesis was proposed more than ten years ago and many studies have been conducted to explain the various parts of this mechanism, a large portion of the chain of events that lead to the action of organic nitrates is still a black box. Several recent studies have provided information which is consistent with the idea that organic nitrates act as prodrugs which release a pharmacologically active intermediate; SH-containing molecules, such as glutathione or cysteine, are apparently of vital importance for this reaction. This concept was first introduced by Needleman and Krantz (1964) and Needleman *et al.* (1969). Ignarro and Gruetter (1980) showed that compounds like GTN, unlike nitroprusside and nitric oxide, required thiols to activate the enzyme, soluble guanylate cyclase (GC) from bovine coronary artery. Furthermore, S-nitrosothiols (RSNO) compounds formed by the reaction of thiols with NO_2^- or nitric oxide were potent activators of GC. Due to the similarity of the activation of GC by RSNO and nitric oxide, nitroprusside, etc. Ignarro *et al.* (1981) postulated that RSNO could probably act as an intermediate in the activation of GC by GTN, $NaNO_2$ and possibly sodium nitroprusside.

However, the intermediate steps between GTN metabolism and inhibition of GC activity have not been clarified. Two major questions exist in this part of the scheme:

1. What is the true intermediate (vasoactive species) of GTN? Is it RSNO or nitric oxide or some other closely related compound?

2. What is the nature of the metabolic step that leads to the formation of this vasoactive species?

Recently, investigators have discovered that nitric oxide is a very potent smooth muscle relaxant. In fact, nitric oxide is postulated to be (Ignarro *et al.*, 1988) or very closely associated with (Moncada *et al.*, 1988; Shikano *et al.*, 1988) endothelium-derived relaxation factor (EDRF), an endogenous substance believed to be responsible for maintaining the baseline vascular tone as well as mediating a number of cellular processes. Nitric oxide has been shown to stimulate GC (Ignarro, 1989). After incubation of GTN with blood vessel preparations, nitric oxide can be detected (Feelisch and Noack, 1987). Therefore, nitric oxide may represent the active species generated during organic nitrate metabolism. Furthermore, it is also possible that the vasoactive species could be an intermediate different from nitric oxide or RSNO. These observations have led to doubts as to the necessity for the generation of RSNOs as precursors for the expression of vasodilatory effects.

Secondly, the identity of the crucial enzyme involved in the biotransformation has yet to be unambiguously characterized. Candidates for this crucial enzyme have been proposed and will be discussed in the next section.

1.9.2 Other mechanisms

Various other mechanisms have been proposed for the mechanism of action of organic nitrate esters. The "nitrate receptor" theory was put forward by Needleman and coworkers (Needleman *et al.*, 1973; Needleman and Johnson, 1973). They suggested the existence of a specific nitrate receptor in the smooth muscle cell membrane. SH groups located on proteins constituted an integral part of the nitrate receptor. This putative receptor was not further characterized, nor was its mode of action. Since SH groups are important for modulating the activity of soluble GC, this enzyme may function as the receptor for organic nitrate esters because it interacts with nitric oxide released from these drugs.

A further biochemical pathway, hyperpolarization of vascular smooth muscle cells, has been proposed to be a mechanism for organic nitrates to produce effects (Haeusler and Thorens, 1976; Kuriyama *et al.*, 1982). Opening of potassium channels, stimulation of prostacyclin synthesis (Metha *et al.*, 1983), inhibition of ATPase activity (Krantz *et al.*, 1951) are other proposed mechanisms. All of these biochemical effects can modulate intracellular calcium homeostasis in vascular smooth muscle cells. However, none of these mechanisms can explain the tight relationship between GTN metabolism and effects; therefore, these mechanisms probably represent pharmacologically active pathways of minor importance.

1.10 The crucial enzyme in vascular GTN metabolism/ pharmacology

Cytochromes P-450 have been postulated as one of the possible candidates for the crucial enzyme. Servent *et al.* (1989) showed that the denitration of GTN to its dinitrate and mononitrate metabolites in rat liver microsomes occurred under anaerobic conditions, in the presence of NADPH as a cofactor and was inhibited by carbon monoxide (CO), metyrapone, and miconazole. Furthermore they could detect the formation of a cytochrome P450-Fe(II)-NO complex. Schröder and Schror (1990) showed that the cytochrome P-450 inhibitors cimetidine and miconazole decreased the cGMP stimulation by GTN in a kidney epithelial cell line (LLC-PK1). However, the P-450 inhibitors had no effect on the cGMP stimulation by sodium nitroprusside. McDonald and Bennett (1990) demonstrated in the microsomal fraction of rat liver, that NADPH dependent metabolism of GTN was first order and under anaerobic conditions, had a half life of 30 seconds. When Bennett *et al.* (1992) added the aortic supernatant from rat to rat hepatic microsomes, under anaerobic conditions and in the presence of NADPH, a concentration dependent increase in GC activity was observed. In phenobarbital treated rats, the increased GC activity was decreased by carbon monoxide, SKF 525A, metyrapone and cimetidine. Similar results were obtained using a rat lung fibroblast cell line (Schröder, 1992), although cultured porcine aortic endothelial cells were found to not produce cGMP in response to GTN.

In a recent study by Delaforge *et al.* (1993) the metabolism of GTN to its dinitrate and mononitrate metabolites in various rat hepatic

subcellular fractions was studied. The highest microsomal activity was seen in dexamethasone treated rats and in the presence of NADPH. The cytosolic activity was glutathione dependent and was a glutathione S-transferase (GST) mediated pathway. In hepatic 9000 x g supernatant that contained both the enzymes and their cofactors, the P-450 reaction accounted for 30-40 % of the total denitration activity observed under anaerobic conditions using 100 μM GTN, indicating that the majority of the metabolism is mediated by the GST pathway.

In blood vessels cytochrome P-450 mediated GTN metabolism was demonstrated by McDonald and Bennett (1993) who showed in microsomal fractions of rat aorta, that denitration of GTN to dinitrate was NADPH dependent and followed apparent first order kinetics ($t_{1/2}$ = 70.1 min) and was decreased by CO, oxygen and SKF 525A. The half life of GTN observed in this study is quite long considering the quick response to GTN found in blood vessels and also compared to the 30 second half life demonstrated in liver from their previous study (McDonald and Bennett, 1990). More recently GTN mediated relaxation of rat aorta was shown to be decreased with 7- ethoxyresorufin, a cytochrome P-450 inhibitor (Bennett *et al.*, 1992). However, Liu *et al.* (1993) showed that cytochrome P-450 inhibitors had no effect on relaxation by rabbit aortic strips. No significant effect of CO was observed on GTN metabolism to GDNs. In strips exposed to CO for 5 min or to NO for 10 min, there was no decrease in GDNs formed. These authors concluded that there was lack of evidence for the involvement of P-450's in the bioactivation of GTN.

As is clear from the above review of the evidence, most of the data implicating this P-450 enzyme was determined in tissues (liver) and cell lines (kidney epithelial cells, rat lung fibroblast cells) rather than in vascular cells. There seems to be no doubt that liver cytochrome P-450's can metabolize GTN under the appropriate conditions. However there is increasing evidence (Juchau *et al.*, 1976 and studies carried out in our laboratory) suggesting that the P-450 activity in blood vessels is negligible. Furthermore, it is clear from the data of Ignarro and Gruetter (1980), Gruetter *et al.* (1981) and Lau and Benet (1990) that the majority of the metabolism of GTN occurs in the cytosol while cytochrome P-450's exist in the plasma membrane and the endoplasmic reticulum. The reaction mechanism proposed involves a reduction of the nitrate moiety on the parent drug to a nitrite, which is subsequently cleaved to yield nitric oxide. However, the exact mechanism is not clear, especially for an oxidizing enzyme which would be required to carry out a reductive reaction in the highly oxygenated environment found in blood vessels.

Microsomal GSTs have been implicated in the metabolism of GTN. Chung and Fung (1990) observed that the activity of nitric oxide liberation following incubation of blood vessel preparations was associated with the plasma membrane fraction, suggesting that the crucial enzyme for the generation of pharmacologically active species may be located on the plasma membrane. Moreover, other investigators have demonstrated that the activation of GC can occur solely in the presence of the cytosolic fraction from blood vessels (Ignarro and Gruetter, 1980; Gruetter *et al.*, 1981), suggesting that GTN metabolism by the crucial enzyme may occur in the cytoplasm of vascular smooth muscles. Chung *et al.* (1992)

investigated the involvement of the microsomal enzyme that metabolizes GTN to NO. They found that the conversion of GTN to NO was decreased by 1-chloro-2,4-dinitrobenzene, a GST substrate, suggesting that this could be a GST mediated route. While known GST inhibitors did not decrease the NO formation (indomethacin and sulfobromophthalein), thiols such as glutathione and others could facilitate NO production. More recently Seth and Fung (1993) tried to purify the microsomal enzyme involved in the process and it seems very similar to GST, i.e. activity is thiol enhanced, decreased by GST substrate/inhibitors, a free thiol group is present on the enzyme and its MW is close to that of GST, 58 kDa.

Cytosolic GSTs have also been implicated as the crucial enzyme for organic nitrate activity. Because this enzyme is present in many different organs in the body, it is possible that the enzyme can be located in vascular smooth muscles and be responsible for the metabolism of organic nitrates. The sulfhydryl dependent nature of the metabolism of organic nitrates also supports the involvement of an enzyme which utilizes sulfhydryl compounds as co-factors. It was recently shown that the metabolism of GTN in the cytosolic fraction of rabbit liver was inhibited by inhibitors of GSTs (Lau and Benet, 1990). Similar results were also obtained in bovine coronary artery cytosolic fractions (Lau *et al.*, 1991). Furthermore inhibitors of GST were also shown to decrease the relaxation of GTN in rabbit aortic strips (Yeates *et al.*, 1989; Lau and Benet, 1990).

The majority of GSTs exist in the cytosol, although a microsomal form of the enzyme has also been recently isolated and characterized (Friedberg *et al.*, 1979; Morgenstern *et al.*, 1980, 1982). Cytosolic GST exists as dimers (Jakoby *et al.*, 1984) comprised of two

identical or different subunits, resulting in the formation of different isozymes. The various isozymes for GSTs exist in different tissues (Corrigall and Kirsch, 1988), and are known to exist in different subcellular locations (Morgenstern *et al.*, 1984). GSTs react with a wide spectrum of electrophilic and hydrophobic substrates. Different isozymes of GST possess selective, although usually not exclusively specific, substrate preferences (Habig *et al.*, 1974). These isozymes are classified into three major classes : the alpha, mu, pi GSTs, according to their pI's, molecular weights, immunoreactivities.

More recently Chern *et al.* (1991) showed that immunoprecipitation of rabbit aortic cytosol with antiserum against the pi isozyme did not decrease the metabolism of GTN, whereas antiserum against the mu isozyme seems to decrease the metabolism of GTN by about 80%. The alpha isozyme was found to exist in undetectably small amounts in the rabbit aortic cytosol. Similar results were obtained by Tsuchida *et al.* (1990) who purified the different GSTs from human aorta and heart. These investigators found that in human aorta there were no detectable alpha forms, while the majority of the GSTs were in pi form. However, the mu isozymes had the highest activity to GTN while the pi isozyme(s) had no detectable activity to GTN. The GSTs metabolize GTN in vascular tissue via a thiol reaction as has been proposed for a long time. Furthermore these enzymes exist in the cytosol where majority of the GTN metabolism was found to occur. Among the GSTs in the aorta, the mu isozymes predominantly metabolizes GTN, although they only consists of 15% of total GST. The potential significance of GSTs and the mu

isozyme in the metabolism and pharmacology of GTN and its metabolites will be investigated in Chapters 3 through 6 of this thesis.

1. 11 Tolerance development and dependency

Nitrate tolerance may be defined as that condition in which increasing doses of nitrates are required to induce a given hemodynamic or anti-anginal effect. Tolerance to organic nitrate esters has been recognized as a pharmacological phenomenom for more than a hundred years. The first description of tolerance to organic nitrate esters was by Stewart (1888) who reported tolerance to GTN in humans. Subsequently, several reports showed the existence of tolerance in humans and in experimental animals *in vivo* (Ebright, 1914; Myers and Austin, 1929; Crandall *et al.*, 1931; Bogaert, 1968; Bogaert *et al.*, 1968; Clark, 1969). These findings prompted investigation of the possible existence of tolerance in isolated vascular smooth muscle taken from animals made tolerant to organic nitrate esters *in vivo* (Needleman, 1970; Herman and Bogaert, 1971)

When transdermal patches were first used in the early 80's, many cases of decreased or complete loss of drug activity following daily application of GTN patches were reported (Parker and Fung, 1984; Nabel *et al.*, 1989) Continous IV therapy also has been found to lead to tolerance development (Elkayam *et al.*, 1987; Horowitz *et al.*, 1988) Similarly other formulations of GTN have been shown to produce tolerance (Parker *et al.*, 1985 and 1987).

There are many different proposed mechanisms for nitrate tolerance development as reviewed by Fung *et al.* (1989a) and Katz (1990). These hypothesis can be divided into three broad categories:

1.11.1 Pharmacokinetic alterations

Nitrate tolerance could potentially be due to a decrease in the level of parent drug at the site of action (vasculature), which could occur via a decreased absorption, decreased tissue uptake, or increased elimination of the drug. The persistence of tolerance in vascular smooth muscle prepared from animals made tolerant *in vivo* and the fact that tolerance can be readily induced in vascular smooth muscle *in vitro* suggest that pharmacokinetic factors may be less important than pharmacodynamic factors.

1.11.2 Physiologic alterations

The body's neurohormonal response may be elevated by prolonged use of vasodilators like GTN and the compensatory neurohormonal response counteracts the action of GTN upon prolonged administration. An increased renin activity was observed by Packer *et al.* (1987) during a 48 hour infusion, which was decreased to normal 24 hours post GTN withdrawal. In another study Dupuis *et al.* (1990) found an elevation of plasma epinephrine levels upon GTN tolerance. This increase was accompanied by an increase in the levels of atrial natriuretic peptide. Furthermore it is known that such changes can induce sodium and water retention with an increase in plasma volume. In a recent study, Parker and Parker (1993) showed that plasma volume expansion plays a more important role than neurohormonal responses in the loss of nitrate

effects during sustained therapy. That therapy with an angiotensin converting enzyme inhibitor did not modify the response to continous GTN therapy supported this conclusion. Although a good correlation exists between these physiologic changes and nitrate tolerance, the exact cause and effect relationship has yet to be determined.

1.11.3 Biochemical alteration

Tolerance to GTN has been proposed to be due to an alteration at a molecular level of the machinery that is involved in the cellular effects of GTN. Three major hypotheses have been proposed:

1.11.3.1 Reduced guanylate cyclase activity

Axelsson and Karlsson (1984) suggested that guanylate cyclase (GC) activity was decreased, in bovine mesenteric arteries made tolerant to GTN. Similar results were obtained by others (Romanin and Kukovetz, 1989). Waldman *et al.* (1986) proposed that there is a stable alteration of the GC upon prolonged contact with GTN. Furthermore they found that purified GC from tolerant tissues exhibited reduced activities towards GTN, sodium nitroprusside and nitric oxide.

However, there is also a lot of data which is in conflict with this hypothesis. For example, Mulsch *et al.* (1989) reported that a decrease in GC activity occurred in homogenate preparations while no decrease in cGMP accumulation in intact tissues was found. Prior to these studies, investigators had reported that in intact tolerant vessels, GC was still responsive to activation by nitric oxide (Mulsch *et al.*, 1988). Berkenboom *et al.* (1988) also showed that vessels (canine and human

coronary arteries) made tolerant to GTN, were still responsive to SIN-1, a compound that releases nitric oxide spontaneously. Similar results were also found by Fung and Poliszczuk (1986) and Kowaluk *et al.* (1987).

1.11.3.2 Vascular GTN biotransformation

Although the metabolizing enzyme for GTN biotransformation has not been identified, various investigators have observed that in nitrate tolerant tissues (Slack *et al.* 1989), homogenates (Fung and Poliszczuk, 1986) and cells (Bennett *et al.*, 1989) a decrease in the extent of nitrate metabolism occurred. Brien *et al.* (1986) found that *in vitro* tolerance to GTN resulted in a significant reduction in the level of dinitrates in the aortic wall, suggesting a decreased biotransformation of GTN in the vascular wall. This decrease could be either due to a decrease in enzyme activity, enzyme levels or cofactors. It is interesting to note that along with the decrease in metabolism, there was a decrease in the regio selectivity for GTN metabolism. This was first reported by Fung and Poliszczuk (1986) who found that in rat aortic homogenates, the preference for 1,2-GDN formation during GTN degradation dissipated when incubations of GTN were performed with tolerant tissue homogenates. Similarly a more pronounced decrease in the formation of 1,2-GDN versus 1,3-GDN was noted by Bennett *et al.* (1989) and Slack *et al.* (1989). However the molecular basis for this metabolic selectivity, as well as the relationship between the formation of the two metabolites and NO/RSNO remains to be investigated.

1.11.3.3 Reduction in the sulfhydryl groups

This factor could go hand in hand with the reduced metabolism of GTN in tolerant tissues, if thiols are indeed important for the metabolism of GTN. This concept was first introduced by Needleman and Johnson (1973), who demonstrated that tolerance to GTN in rat aorta was accompanied by a net loss of titratable SH groups and that tolerance to GTN can be reversed by sulfhydryl reducing agents. Clinical evidence has shown that administration of sulfhydryl compounds such as N-acetyl cysteine (Torresi *et al.*, 1985; May *et al.*, 1987) and methionine (Levy *et al.*, 1991) can reverse GTN tolerance. However these results were not reproducible in all reported *in vitro* (Fung *et al.*, 1988) and *in vivo* (Parker *et al.*, 1987; Hogan *et al.*, 1989; Munzel *et al.*, 1989) experiments. It was shown that the benefecial effect of thiol compound co-administration may arise from a extracellular reaction (Fung *et al.*, 1988) which can form RSNO which penetrates the cell membrane and activates GC. Gruetter and Lemke (1985) found that the tissue content of cysteine and glutathione were also decreased in bovine coronary arteries, but that responsiveness could not be restored by incubation with cysteine or glutathione. Similarly Abdollah *et al.* (1987) were unable to show an effect of N-acetyl cysteine on *in vitro* tolerance to GTN. Therefore there seems to be conflicting evidence for the depletion of sulfhydryl groups as a mechanism for tolerance development.

Of all the hypotheses described above, those based on biochemical mechanisms are generally believed to be the most crucial factor in causing nitrate tolerance. Moreover, neurohormonal alteration upon repeated nitrate dosing have been observed in clinical studies. The

CHAPTER 2

SELECTIVE DENITRATION OF GLYCERYL NITRATES

2.1 Background

GTN is metabolized to its dinitrate metabolites 1,2-GDN and 1,3-GDN. *In vitro* studies have indicated that a major part of this metabolism is catalyzed by GSTs and a striking regio selectivity of denitration has been observed both *in vivo* and *in vitro*. When an oral dose of sustained release GTN was administered to healthy human subjects, 1,2-GDN, a product resulting from terminal denitration of GTN (Figure 2.1) and 2-GMN, a product resulting from terminal denitration of 1,2-GDN (Figure 2.2) were present in significantly higher concentrations than 1,3-GDN and 1-GMN, which are products of C-2 denitration of GTN (Laufen and Leitold, 1988).

Noonan and Benet (1987) found that with different dosage forms, IV vs. sublingual vs. topical vs. oral, the 1,2 : 1,3 ratio was greater than one. These ratios varied from 7.36 : 4.6 : 3.86 : 1.99, respectively, indicating a route dependency in the selectivity of denitration. Later Nakashima *et al.* (1990), in other studies carried out in humans, showed that this ratio of metabolites was also dose-dependent, i.e., higher ratios of 1, 2-GDN : 1, 3-GDN were observed with lower doses of GTN.

In an in-situ study, rats were infused IV with GTN and the metabolism in different tissues quantitated. The leg and veins were found to form 1,2-GDN preferentially, while the liver preferentially formed 1,3-GDN (Nakashima *et al.*, 1991) indicating that there was a difference in the denitration preference in different tissues. In a study by Short *et al.* (1977) a species dependency of the 1,2-GDN : 1,3-GDN ratio was found.

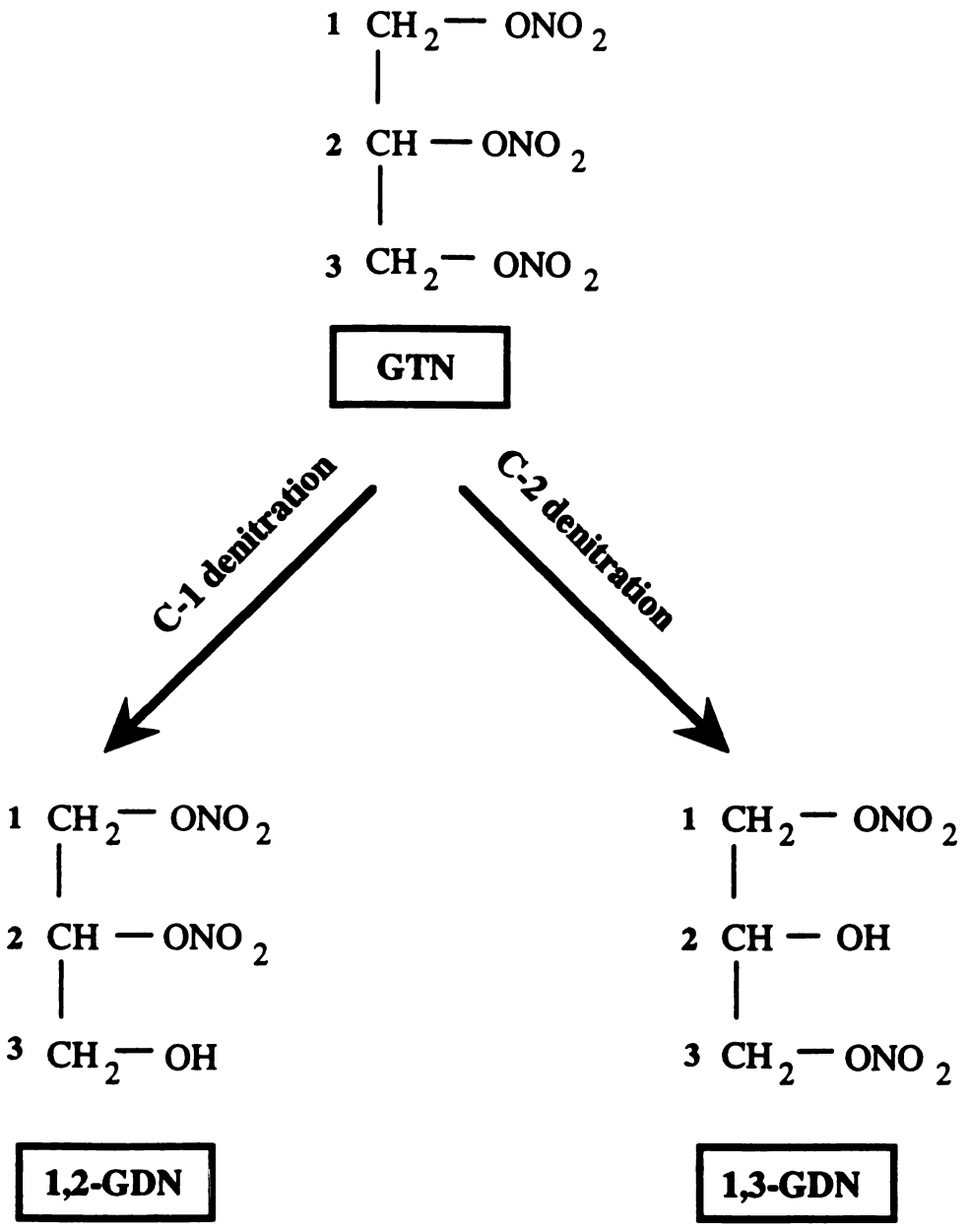


Figure 2.1 Metabolism of GTN to its dinitrate metabolites.

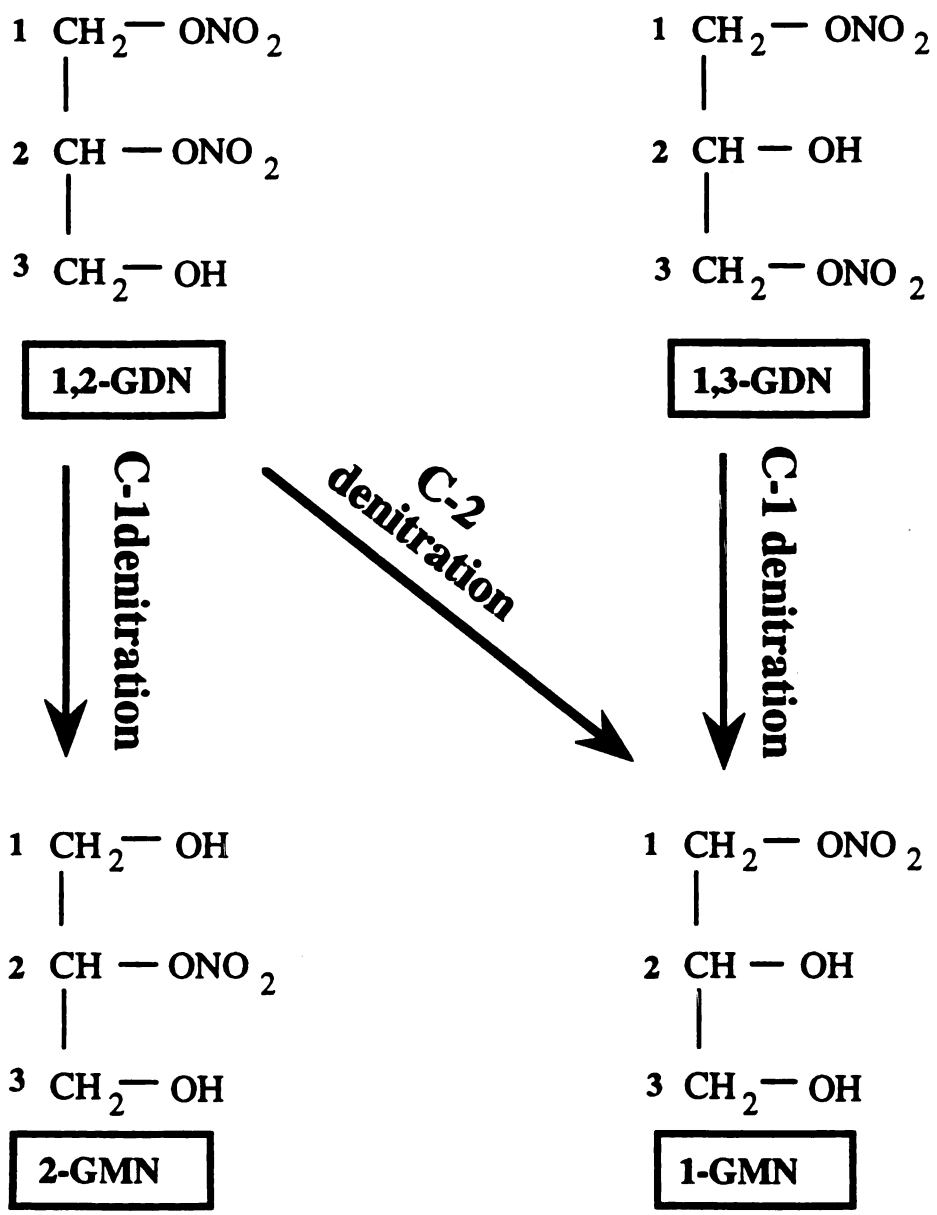


Figure 2.2 Metabolism of the dinitrates to the mononitrate metabolites.

Following ^{14}C GTN intravenous dosing the authors showed that rats and mice livers yielded ratios less than 1 while rabbits, dogs, monkeys and human livers had ratios greater than 1.

Previous studies in our laboratory have shown that the glutathione dependent enzymatic biotransformation of GTN occurs in hepatic and vascular tissues. Furthermore a distinct selectivity in the site of denitration has been observed in these cases. For example, Lau and Benet (1989) showed that the glutathione dependent metabolism of GTN via GSTs, lead to the preferential formation of 1,2-GDN in rabbit liver cytosol, while the 1,3-GDN metabolite was predominant in the microsomes. This preferential formation of 1,2-GDN has also been observed with rabbit aorta (Lau and Benet, 1990). Similarly Lau *et al.* (1992) reported the preferential formation of 1,3-GDN in bovine coronary artery subcellular fractions.

GTN is a molecule with three nitrate groups, two of which are at the terminal position. Therefore the probability of denitration at the terminal position is naturally higher and this could explain the preferential 1,2-GDN formation vs. 1,3-GDN formation in most cases. However the fact that the vasculature from different animal species and also different tissues in the same species produce different ratios of the two metabolites (sometimes yielding completely opposite denitration preferences) indicate that the differences may result not only due to structure of the molecule, but also due to the possible involvement of different isozymes with selective requirements for substrate configuration.

In order to investigate this regio selectivity of denitration further, we studied 1,2-GDN denitration. This metabolite of GTN has two nitro groups with equivalent *a priori* probabilities of denitration at the carbon-1 (C1) position or the carbon-2 (C2) position. Therefore both 1-GMN and 2-GMN can be produced. These studies lead to a better understanding of any enzyme related regioselectivities of denitration. Furthermore the further metabolism of 1,2-GDN has not been well characterized and the involvement of GSTs in this process has not been studied.

Objectives

1. To examine the profiles of 1,2-GDN metabolism and GMN formation in rabbit aortic homogenates (RAH).

2. To investigate any regio selectivity in the denitration of nitrates in rabbit aortic tissue using 1,2-GDN as a model compound.

3. To evaluate the role of GSTs in the vascular metabolism of 1,2-GDN using two GST substrate-inhibitors, ethacrynic acid (ECA) and sulfobromophthalein (SBP).

2. 2 Methods

1, 2-GDN solutions prepared by procedures described earlier (Gumbleton *et al.*, 1991) were used for the experiments. The analytical standards for GTN, 1,2-GDN, 1,3-GDN, 1-GMN and 2-GMN were purchased (>99% purity; 1 mg/ml in ethanol) from Radian (Austin, TX,

USA) and used as received. o-Iodobenzyl alcohol was available commercially at > 99% purity from Aldrich (Milwaukee, WI, USA). Methyl tert-butyl ether was purchased at the highest grade of purity (Omnisolve; EM Science, Gibbstown, NJ, USA) and before use was dried over anhydrous potassium carbonate (Fisher Scientific, Santa Clara, CA, USA). Absolute ethanol was purchased from Quantum Chemical (Tuscola, IL, USA). To prevent adsorption of the nitrates, all glassware was silanized prior to use with a 5% (v/v) solution of dichlorodimethylsilane (Aldrich, Milwaukee, WI, USA) in toluene. Nitrogen and hydrogen gases (Liquid Carbonic, Chicago, IL, USA) were of zero grade.

New Zealand white rabbits (Nitabell Rabbitry, Hayward, CA) weighing 2 to 3 kg, were used in this study. The rabbits were anesthestized using a subcutaneous injection of ketamine (40 mg/kg as 1 ml of a 100 mg/ml solution in water for injection) and decapitated 15 minutes later. The thoracic cavity was exposed and the descending aorta was surgically removed and immediately placed in a beaker containing phosphate buffer (0.13 M KH_2PO_4 -Na_2HPO_4) at pH 7.4. The aorta was cleaned, sliced and homogenized in three volumes of phosphate buffer (pH 7.4). The blood vessel homogenates were then centrifuged at 4000 x g for 10 min at 4°C and the supernatant layer was decanted and used to carry out incubations.

In each incubation, 3 mls of the homogenate (protein concentration of 2 mg/ml) was used. The incubates were placed in a water bath with the temperature maintained at 37°C. Glutathione (GSH, 2 mM) was added as a cofactor for the metabolic reaction. The mixture was then preincubated

for 5 min. For the inhibitor studies, 2 x 10^{-5}M of SBP or ECA, was added just before the 5 minute preincubation period. Following preincubation, 40 ng/ml (2.19 x 10^{-7}M) 1,2-GDN was added to the incubates. Samples (500 µl) were taken at 10, 20, 40, 60, 120 min and were frozen immediately in a mixture of dry ice and methanol. A control containing only the buffer and 2 mM GSH was also incubated with 1,2-GDN so that the amount of GMN formed by non-enzymatic degradation could be accounted for in each incubation study.

The concentrations of GDNs and GMNs were determined using the improved GC-ECD method described previously by Han *et al.* (1992) Briefly, samples were extracted by addition of 0.8 g sodium chloride. This was followed by addition of 2 ml methyl tert- butyl ether and gentle rotation and swirling. The samples were then centrifuged for 10 min at 1000 x g to separate the aqueous and organic phases and placed in a methanol-dry ice mixture, thus allowing the aqueous phase to freeze. The organic layer was transferred to another tube and the procedure was repeated. The organic phase was evaporated to 0.5 ml final volume, transferred to a 1 ml glass micro-reaction vial and then evaporated to dryness under nitrogen. The organic phase residue was reconstituted with 0.5 ml of methyl tert- butyl ether and stored at -20°C until required. A 0.5 µl aliquot of the extract was injected into the gas chromatograph. Separate standard curves were constructed for 1,2-GDN, 1-GMN and 2-GMN (Figures 2.3, 2.4, 2.5 respectively) using blank rabbit aortic homogenates, for both the GDN and GMNs (over a range of 0.4 to 15 ng/ml), with *o*- iodobenzyl alcohol as the internal standard. Linearity was

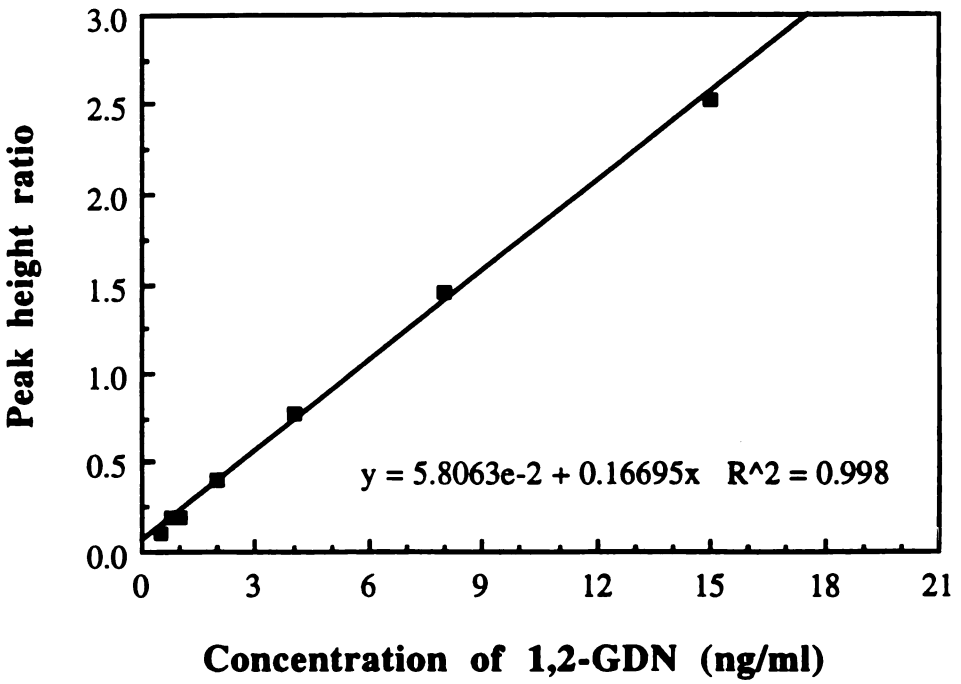


Figure 2.3 Standard curve for 1,2-GDN.

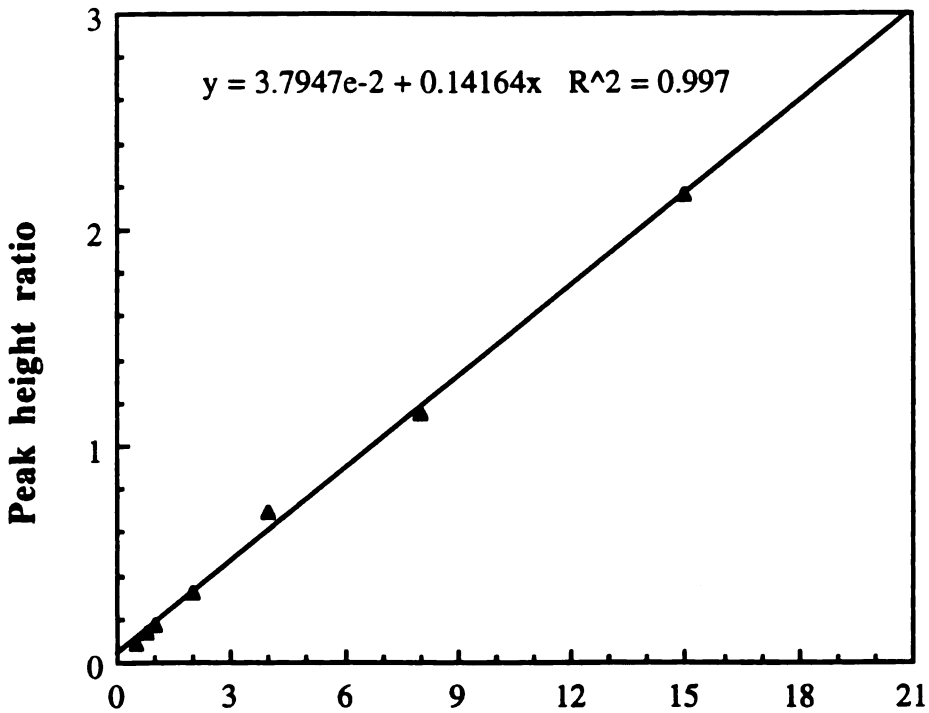


Figure 2.4 Standard curve for 1-GMN.

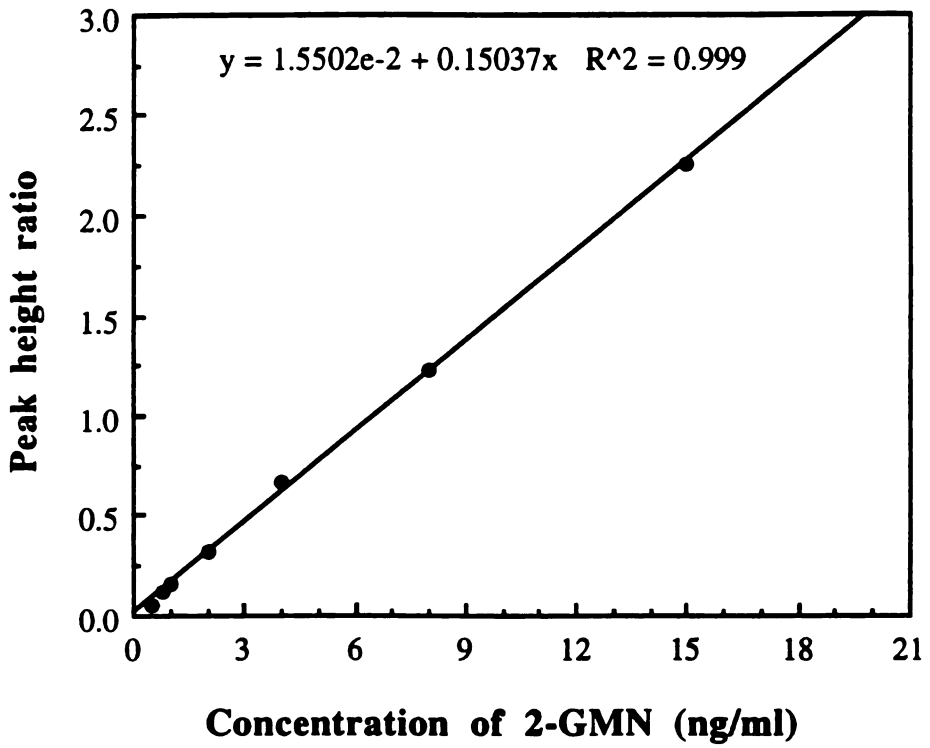


Figure 2.5 Standard curve for 2-GMN.

observed over this range, and both 1,2-GDN and the GMNs were clearly separated (Figure 2.6).

2.3 Results

2.3.1 Metabolism of 1,2-GDN in rabbit aortic homogenates

1,2-GDN was metabolized in rabbit aortic homogenates via an apparent first order degradation process. The rate constant of this degradation was calculated to be 5.25 x 10^{-3} min^{-1}. This degradation of parent compound was accompanied by formation of the two metabolites 1-GMN and 2-GMN (Figure 2.7). The concentrations of 2-GMN were much higher than the concentrations of 1-GMN. Ratios of 2-GMN : 1-GMN were calculated at each time point (see Table 2.1). The ratios ranged from 4.2 to 6.6. The area under the concentration time curves (AUC 0 to 120 mins) for both the metabolites were determined using the trapezoidal rule. AUC of 1-GMN was calculated to be 108.6 ng $min^{-1}ml^{-1}$ while the AUC of the 2-GMN metabolite was 593.8 ng $min^{-1}ml^{-1}$. The ratio of the AUC's of 2-GMN:1-GMN was 5.47. Thus about a five fold higher level of 2-GMN was formed from 1,2-GDN as compared to the level of 1-GMN formed.

2.3.2 Effect of GST inhibitors on 1,2-GDN metabolism

Upon addition of the inhibitors ECA and SBP, the metabolism of 1,2-GDN in rabbit aortic homogenates was decreased. ECA seems to inhibit this process to a much greater extent than SBP. The rate constant of 1,2-GDN degradation in the presence of ECA was decreased from 5.25

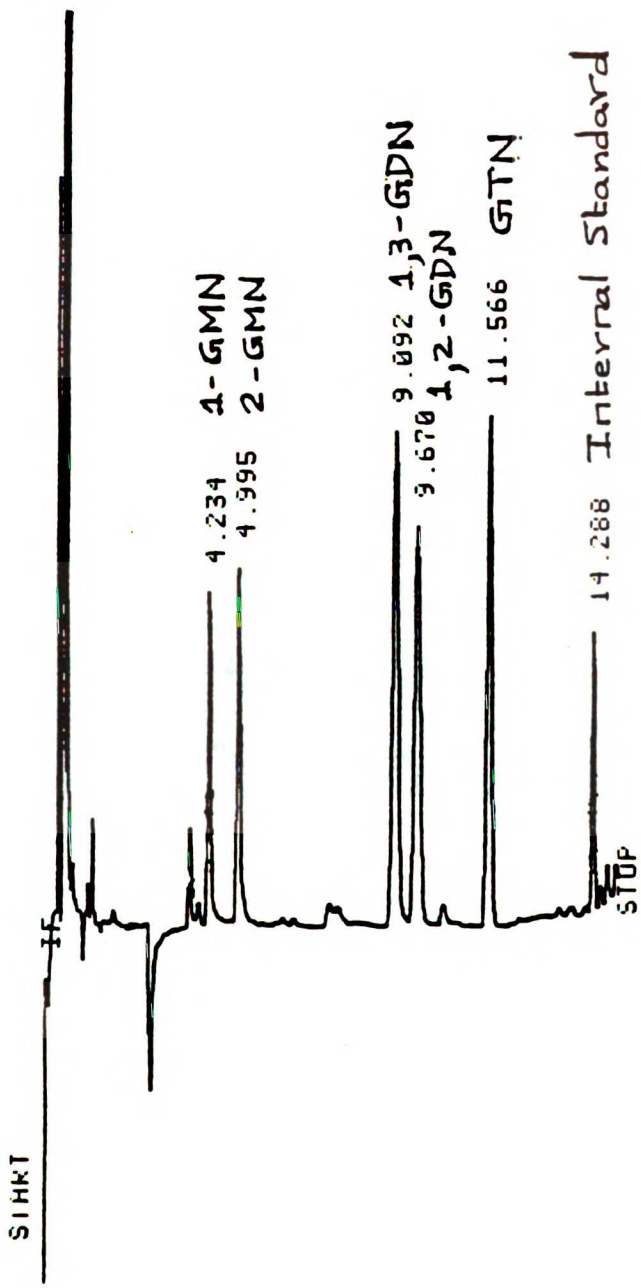


Figure 2.6 Sample chromatogram of the simultaneous detection of GTN, 1,2-GDN, 1,3-GDN, 1-GMN and 2-GMN. The internal standard was o-iodobenzyl alcohol.

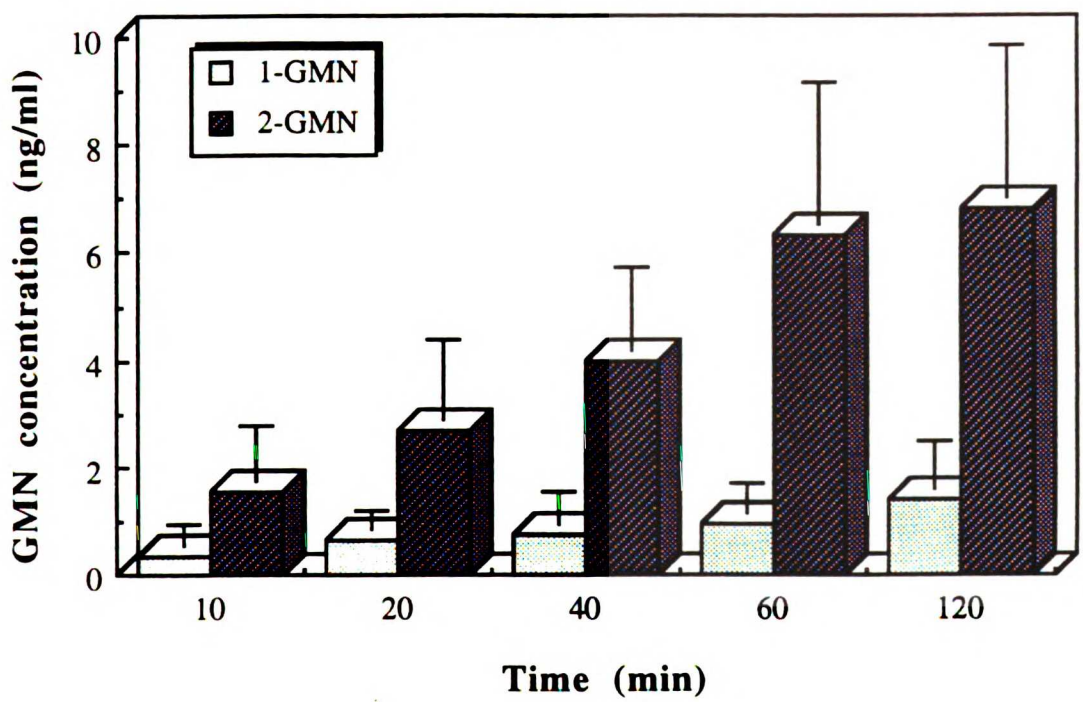


Figure 2.7 Accumulation of 2-GMN and 1-GMN from 1,2-GDN incubations (40 ng/ml) in rabbit aortic homogenates as a function of time.

TIME (MIN)	RAH	RAH + SBP	RAH + ECA
10	4.7	4.6	3.3
20	4.2	4.2	2.9
40	5.2	4.9	2.5
60	6.6	4.5	2.5
120	4.9	3.2	2.0
0 to 120 min	5.5	4.2	2.4

2-GMN : 1-GMN Concentration ratios

2-GMN : 1-GMN AUC ratios

Table 2.1 Ratio of metabolite concentrations and AUCs (2-GMN:1-GMN) in rabbit aortic homogenates (RAH) in the presence and absence of the inhibitors.

x 10^{-3} min^{-1} to 0.61 x 10^{-3} min^{-1} and to 1.24 x 10^{-3} min^{-1} upon co-incubation with SBP.

The pattern of GMN formation was also altered in the presence of these inhibitors. Table 2.1 depicts the effects of these inhibitors on the formation of 1-GMN and 2-GMN. Co-incubation with ECA produced a significant decrease in the levels of 2-GMN after 40 min incubation (Figure 2.8), while the levels of 1-GMN appear to be unaffected (Figure 2.9). As seen in Table 2.1, the ratio of 2-GMN:1-GMN was decreased in the presence of inhibitors, ECA (2.0 to 3.3) and SBP (3.2 to 4.9). The ratio of the AUCs of the two metabolites in the presence of ECA, decreased from 5.47 to 2.39, while the ratio of AUCs of the two metabolites in the presence of SBP, was affected to a lesser degree (5.47 to 4.17).

2.3.3 Formation of the mononitrates compared with the degradation of 1,2-GDN

The formation rates of the two metabolites were calculated up to time 60 min as depicted in Figure 2.10. For incubations where no inhibitor was present, the slope of the plot or the formation rate of 2-GMN was found to be 6.9 x 10^{-4} μM min^{-1}. The rate of formation of 1-GMN was calculated to be 1.03x10^{-4} μM min^{-1}. Upon incubating with ECA, the formation rate (initial) of 2-GMN was decreased to 0.88 x 10^{-4} μM min^{-1}; while that of 1-GMN was relatively unchanged, 1.14 x 10^{-4} μM min^{-1}(Table 2.2). Thus upon comparing the 2-GMN concentrations pre and post inhibition, there was a significant decrease in the 2-GMN formation rate while the formation rate of 1-GMN was unchanged by

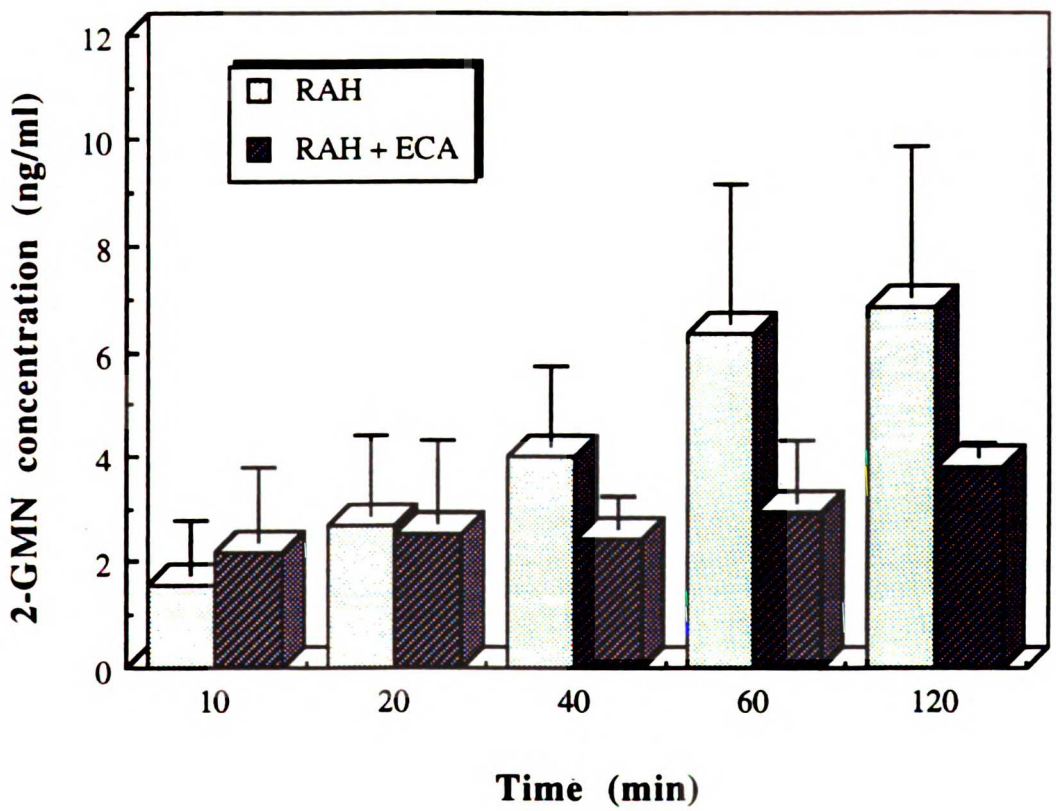


Figure 2.8 Effect of ECA treatment on the concentration of 2-GMN, formed from 1,2-GDN, in rabbit aortic homogenates (RAH).

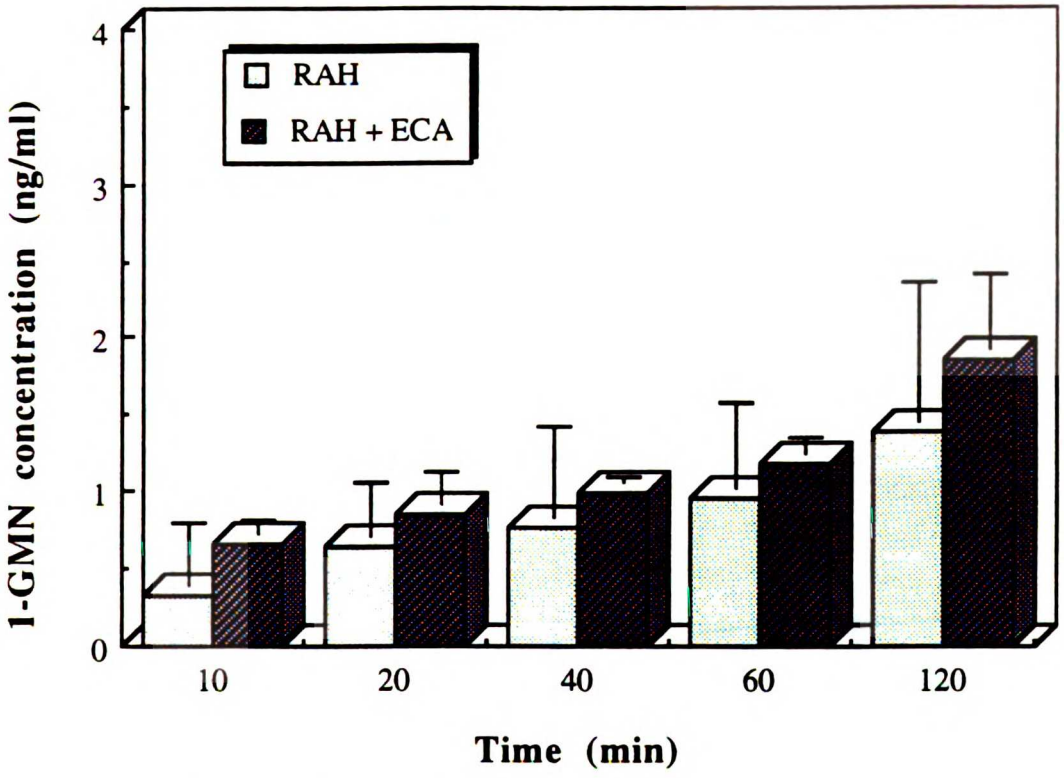


Figure 2.9 Effect of ECA treatment on the concentration of 1-GMN in rabbit aortic homogenates (RAH).

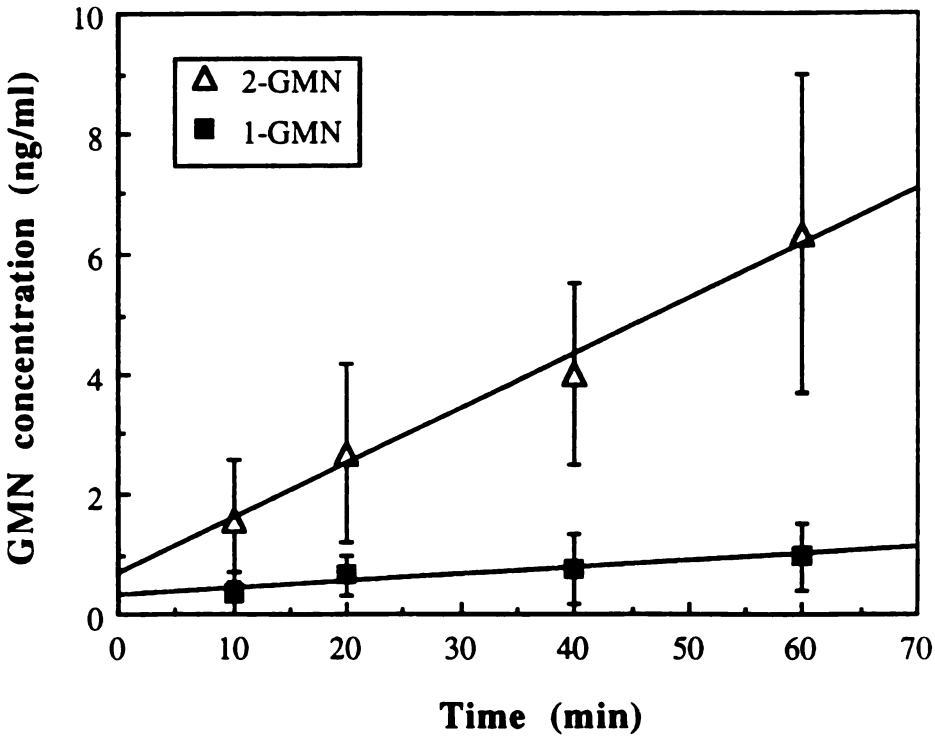


Figure 2.10 Formation rates (from 0 to 60 min) estimated from concentrations of 2-GMN and 1-GMN in RAH.

	2-GMN formation rate	1-GMN formation rate	1,2-GDN degradation rate constant
RAH	6.9×10^{-4}	1.03×10^{-4}	5.25×10^{-3}
+ ECA	0.88×10^{-4}	1.14×10^{-4}	0.61×10^{-3}
+ SBP	2.4×10^{-4}	0.42×10^{-4}	1.24×10^{-3}
Units	μM/min	μM/min	min^{-1}

Table 2.2 Formation rates of 2-GMN and 1-GMN compared to the degradation rate constants of 1,2-GDN in the presence and absence of inhibitors.

ECA treatment. This approximately 8 fold decrease in the formation of one mononitrate metabolite (2-GMN) correlated very well with the approximately 9 fold decrease in the 1,2-GDN degradation rate constant (5.25×10^{-3} min^{-1} vs. 0.61×10^{-3} min^{-1}) while the 1-GMN formation rates were essentially unaffected. In contrast, SBP produced a lesser decrease in formation rates for both mononitrate metabolites and these decreases were approximately the same for both metabolites (Table 2.2). This could indicate that SBP not only inhibits the isozyme inhibited by ECA, but that it also inhibits the isozyme that catalyzes the C-2 denitration.

2.4 Discussion

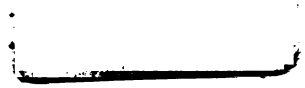
A selectivity in denitration of nitrates has been observed in *in vivo* and *in vitro* studies. These differences could be configurational i.e., a certain configurational fit to the metabolizing enzyme; leading to a specific -ONO_2 group being removed. However, in bovine coronary artery subcellular fractions a selective denitration at the C-2 position is observed. This could indicate that the differences seen in the selective denitration may be due to the presence of a specific isozyme in the tissues studied. Therefore the isozyme in bovine coronary artery which mediates denitration of a particular organic nitrate, may be different from the isozyme in rabbit aorta.

Studying the denitration selectivity of GTN is complex due to a naturally higher probability of formation of 1,2-GDN vs. 1,3-GDN (two terminal -ONO_2 groups). Although there have been no studies in the past

on the metabolism of 1,2-GDN *in vitro*, *in vivo* administration of 1,2-GDN has been carried out (Han *et al.*, 1992). These studies demonstrated a marked preference for terminal denitration in humans.

Here, using a model compound which has two -ONO_2 groups with equivalent probability of being denitrated, 1,2-GDN was incubated with rabbit aorta homogenates and an excess of glutathione. 1,2-GDN degradation rate was significantly reduced upon co-incubation with ECA and SBP indicating the involvement of GSTs in the denitration process. The formation of the 2-GMN metabolite as the major metabolite indicates a preference for C-1 denitration. Upon co-incubation with ECA, there was a significant decrease in the rate of 2-GMN formed (initial rate), while the rate of 1-GMN formation was unaffected. GMN AUC ratios were decreased significantly from 5.47 to 2.39. Thus the denitration at the C-1 position, was selectively inhibited by ECA to the same extent as 1,2-GDN, indicating that ECA acted on the selective pathway of formation of 2-GMN from 1,2-GDN. This may represent a process that is catalyzed by a specific GST isozyme. The inhibition by SBP was not specific and the inhibition of 2-GMN formation was less than that observed for ECA, indicating that SBP may inhibit both isozymes but to a lesser extent. Since the 1-GMN formation from 1,2-GDN was unaffected by ECA and to an equivalent extent as 2-GMN by SBP, 1-GMN formation probably results from a pathway which is distinct from the formation of 2-GMN and 1-GMN formation is probably catalyzed by an isozyme that is not inhibited by ECA.

These results are of special interest since ECA was a much better inhibitor of the relaxation (Lau and Benet, 1990) than was SBP.



Therefore it is possible that terminal denitration of GTN and of 1,2-GDN leads to a pharmacologically active species via a GST isozyme that is inhibited by ECA to a greater extent than by SBP. Preferential terminal denitration of GTN has been consistently observed in humans *in vivo*. However, it is unclear from these previous *in vivo* data as to which part of the metabolism is contributed by the blood vessels at the site of action, and which part comes from metabolism at other sites, possible due to different isozymes. The terminal nitrate selectivity could represent an important process for the pharmacologic effect of these drugs.

Fung and Poliszczuk (1986) have reported that the concentration of 1,2-GDN from GTN was decreased in aorta made tolerant to GTN. This may point towards a predominant role of 1,2-GDN (a product of terminal denitration), in the pharmacologic activity of GTN since a decrease in the formation of this metabolite is associated with a decrease in the effect of GTN. Our current study with rabbit aorta homogenates exhibited extensive terminal denitration of 1,2-GDN, leading to preferential formation of 2-GMN vs. 1-GMN. This preferential denitration may be important in the action of nitrates.

In conclusion, 1,2-GDN shows similar positional selectivity (i.e. preferential denitration) in its metabolism as that observed for the parent molecule GTN in rabbit aortic homogenates. Vascular tissue metabolism of 1,2-GDN is mediated in part by GST. Terminal denitration is most probably catalyzed by an isozyme of GST which is inhibited by ECA. The basis of the selectivity may well reflect the involvement of specific GST isozymes with selective requirement for substrate configuration.

CHAPTER 3

RESPONSE TO GTN : INHIBITOR STUDIES

3.1 Background

Although GTN has been a drug of choice in the treatment of angina for over a century, its mechanism of action is still not clearly elucidated. It is agreed that the site of GTN action is the vasculature, and that the drug enters vascular smooth muscle cells where it is metabolized. The final product of this metabolism is NO, which itself (Katsuki *et al.*, 1977; Feelisch and Noack, 1987; Slack *et al.*, 1988) or via the formation of an S-nitrosothiol (RSNO) acts as a first messenger and activates the enzyme GC. Activation of GC leads to the conversion of GTP to cGMP, which serves as a second messenger triggering a cascade of events leading to vasorelaxation (Ignarro *et al.*, 1981; Ignarro, 1989, Figure 3.1). Although the role of cGMP in organic nitrate induced vasorelaxation has been well established, and the necessity of a metabolic step has been agreed upon, the exact nature of the metabolic process and the particular pathway (enzymatic or nonenzymatic) responsible for the "productive" (leading to effect) metabolism of GTN have not yet been established.

Studies carried out in our laboratory (Lau and Benet, 1989; 1990; 1992 and Lau et al., 1992) have provided evidence for the possible role of glutathione-S-transferases (GSTs) in this metabolic process. On the basis of inhibitor studies in homogenates of rabbit liver (Lau and Benet, 1989 and 1990), rabbit aorta (Lau and Benet, 1992) and bovine aorta (Lau *et al.*, 1992) it has been successfully shown that GSTs catalyze the metabolic conversion of GTN to its denitrated products in these tissues. It has also been demonstrated that when rabbit aortic strips were pretreated with ethacrynic acid (ECA), an inhibitor of GST, the relaxation

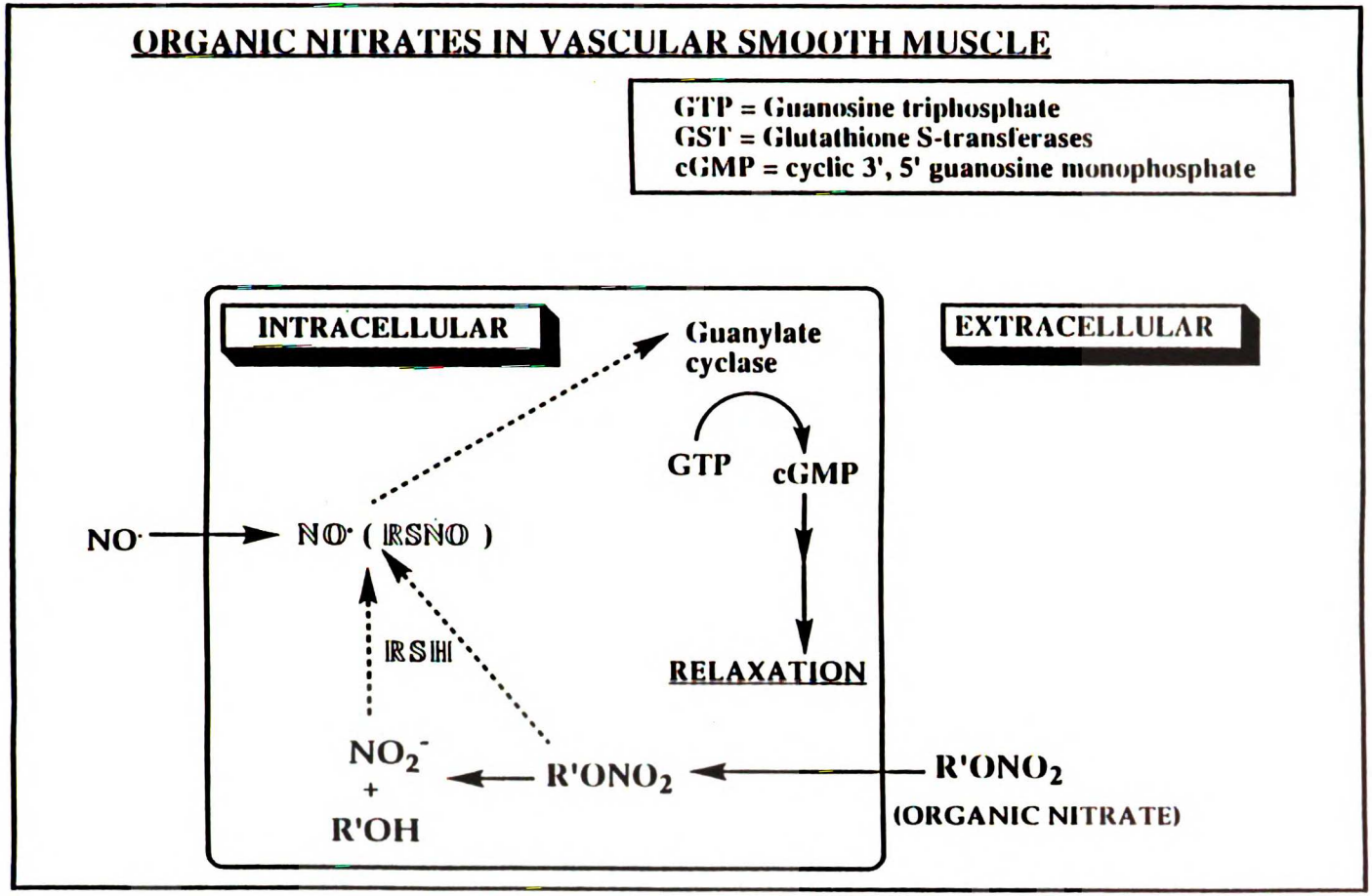


Figure 3.1 A simplified picture of the mechanism of GTN bioactivation, as proposed by Ignarro (1981).

response to GTN was reduced and this was accompanied by reduced metabolism of GTN (Lau and Benet, 1992). This has led to the conclusion that the enzyme involved in the pharmacologic activation of GTN was GST. To obtain further evidence for the hypothesis regarding the role of GST in the productive metabolism of GTN, here we are describing the investigation of the effect of ECA (GST substrate-inhibitor) on a primary biochemical response (cGMP levels). Furthermore the role of cGMP in producing relaxation is also investigated.

3.2 Methods

GTN was purchased as 10 ml vials of Tridil® (in 30 % alcohol (v/v), 30% propylene glycol and water for injection q.s) from Du Pont Pharmaceuticals (Wilmington, DE). ECA, l-phenylephrine hydrochloride (PE) and sodium nitroprusside were obtained from Sigma Chemical Co. (St. Louis, MO). Diethyl ether was purchased from Fischer Scientific Co. (Fairlawn, NJ). The tritium labeled cGMP radioimmunoassay kit was purchased from Amersham (Arlington Heights, IL). All solutions and dilutions were made in the Krebs buffer used for the vasorelaxation study.

3.2.1 Isolation of rabbit aorta for the vasorelaxation study

These techniques followed the procedures previously described (Lau and Benet, 1992). Briefly: New Zealand White rabbits (Nitabell Rabbitry, Hayward, CA; male, 2-3 kg) were anesthetized with an intramuscular injection (40 mg/kg) as 1.0 ml of a 100 mg/ml solution of

ketamine in water for injection and decapitated 15 minutes later. The thoracic cavity of the animal was opened and the descending aorta was surgically removed and immediately placed in a beaker containing Krebs buffer (NaCl, 119 mM; KCl, 4.8mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; $CaCl_2$, 2.5 mM; glucose, 11.1 mM), which was continuously gassed with carbogen (95% oxygen and 5% carbon dioxide). After isolation, excess fat and connective tissues were carefully removed; during this process the buffer was constantly replaced with fresh buffer. The endothelium was not disturbed. The helically prepared tissue was cut into four strips of dimensions 3 cm x 4 mm, which were blotted dry and weighed.

3.2.2 Vasorelaxation measurements

The aortic strips were individually suspended in Krebs buffer (composition described above) contained in 25 ml jacketed circulating water tissue baths maintained at 37°C. One of these strips was assigned for the buffer control, a second for the GTN treatment in the absence of inhibitor, and the third and the fourth as the inhibitor-treated strips. The contraction and relaxation of the strips were recorded via transducers, coupled to a Grass model 7 polygraph (Quincy, MA). The tension on the strips was maintained at 1 g. The strips were washed every 20 min during the initial equilibration period with fresh buffer. They were then allowed to equilibrate for at least 30 min to an hour before addition of any chemicals.

Upon stabilization, 100 μM PE was added to contract the strips maximally. The maximum tension produced in the strips was

recorded. The strips were then returned to baseline tension by washing with fresh Krebs buffer four to six times, approximately 3 min apart. This was followed by two longer washes for 15 min, and the strips were then allowed to stabilize. Upon stabilization the strips were treated with consecutive additions of 0.1 μM PE, until the tension in the strips reached 60-80% of the maximal contraction previously recorded. The inhibitor studies were then carried out on these strips.

3.2.3 Inhibitor studies

Upon attaining a state of submaximal contraction, the strips that were assigned for the inhibitors were incubated for 10 min with ECA at a final concentration of 0.2 mM. These inhibitor conditions were chosen based on previous results from this laboratory (Lau and Benet, 1992). The same volume of Krebs buffer solution was added to the other non inhibitor strips. At the end of the 10 min incubation, GTN solution was added to all, but the buffer control strips, at a final concentration of 0.6 μM while the same volume of buffer was added to the control strips and the relaxation was recorded for 5 min after GTN addition. The strips were then freeze clamped with tongs precooled in liquid nitrogen and immediately frozen on dry ice. Analysis of cGMP was carried out in each of these strips.

3.2.4 Analysis of cGMP levels

Each frozen strip was homogenized in 1 ml of 6% cold trichloroacetic acid (TCA) to precipitate proteins; the homogenate was then centrifuged at 4000 x g for 10 min at 2-4^{o}C to remove the protein precipitate. The supernatant was transferred into a fresh test tube and

washed with a four fold volume of water saturated diethyl ether. This procedure was repeated at least four times. Each time the ether layer was carefully removed, after the tubes were vortexed, and allowed to stand for 2-3 min. After removing the residual ether by evaporation under a stream of nitrogen, the TCA free samples were then assayed for cGMP using a ^{3}H labeled cGMP RIA kit. Each time a new kit was used standard curves (Figure 3.2) were made with six concentrations (0,0.5,1,2,4 and 8 picomoles cGMP/100 µl) prepared from standard solutions of cGMP.

The assay is based on the competition between unlabeled cyclic GMP and a fixed quantity of the tritium labelled compound for binding to an antiserum which has high specificity and affinity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample.

3.2.5 Control experiments

Sodium nitroprusside (SNP), a compound known to produce nitric oxide by a mechanism distinct from that for organic nitrates, was used as a control to test the effect of ECA on the production of cGMP in rabbit aortic strips. Concentrations of 10^{-6} M and 10^{-5} M SNP were added to the strips (PE treated) with or without ECA and cGMP levels were measured.

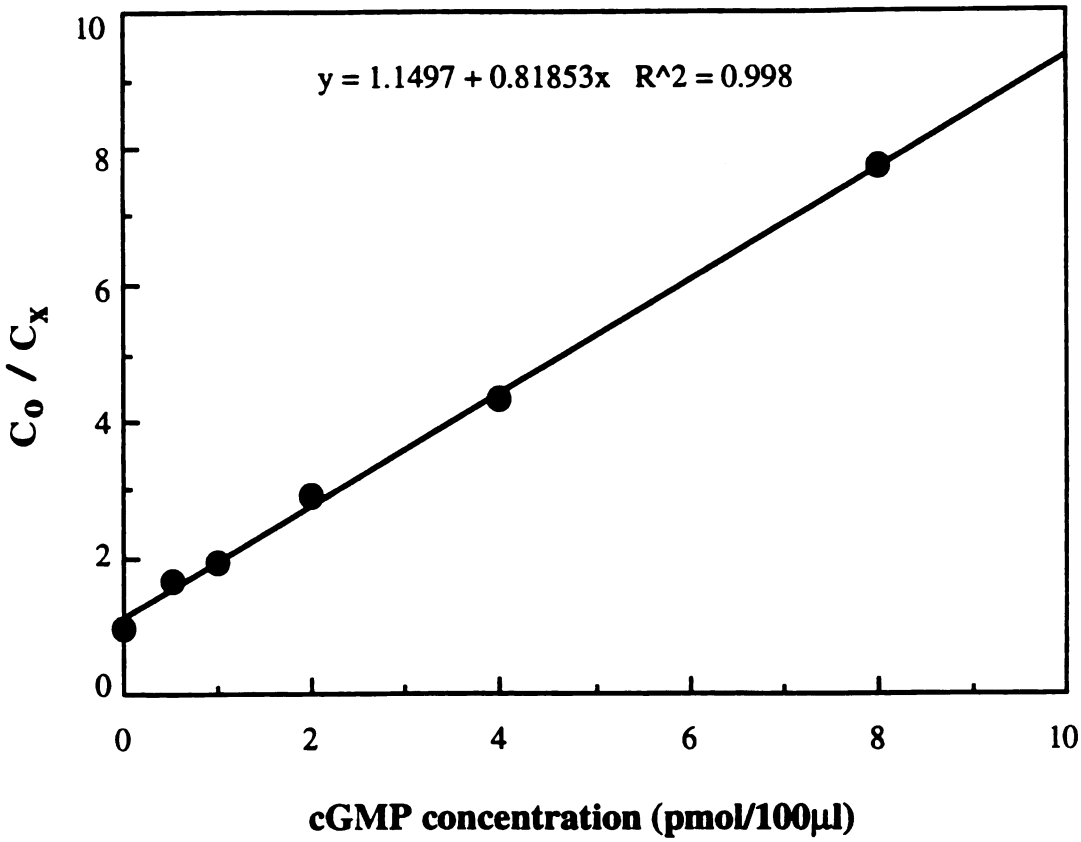


Figure 3.2 Standard curve for cGMP, (C_o / C_x = CPM bound in the absence of unlabelled cGMP / CPM bound in the presence of standard cGMP).

3.2.6 Calculations and statistical analysis of data

Relaxation was measured as the % decrease in tension below the elevated tension elicited by precontracting the aortic strips with phenylephrine. Contraction was measured as the increase in tension above the resting base line, and is expressed as the % of maximal contraction produced by phenylephrine under a given set of conditions.

The % change in relaxation due to ECA inhibition of GTN effect was calculated as [1-(% relaxation in the presence of inhibitor / % relaxation in the absence of the inhibitor)] x 100. The % change in cGMP levels was calculated as [1-(cGMP in the presence of GTN-cGMP in controls) /(cGMP in the presence of GTN plus ECA - cGMP in ECA controls)] x 100.

Statistical comparisons were made for the relaxation data and the cGMP data between groups with and without GTN and within groups with and without ECA. For this purpose a one way ANOVA was used followed by the Student Newman Keuls test. Data are expressed as means ± standard deviation (S.D). The level for statistical significance was set at $p < 0.05$.

3.3 Results

3.3.1 Relaxation measurements

On the basis of preliminary studies, a 0.6 μM GTN concentration was chosen to test the effect of ECA. This GTN

concentration produced about 80% relaxation of PE contracted rabbit aortic strips. A 59% decrease in the relaxation response to GTN was observed in the ECA pretreated strips (35.7 ± 2.9% relaxation, n=9) compared to the control strips (86.3 ± 10.0 % relaxation, n=9) that did not have a ECA pretreatment (Table 3.1). This difference was statistically significant ($p<0.05$). ECA (0.2 mM) by itself did not cause a decrease or an increase in the strip tension produced by PE. A comparison was also made between the extent of PE induced contraction in the two groups of strips tested. No significant difference was found between strips pretreated with ECA and strips not pretreated with ECA (Table 3.1, column 3) whereas both the GTN induced relaxation (g) as well as % relaxation by GTN were significantly less in the strips with ECA pretreatment (Table 3.1, columns 4 and 5).

3.3.2 cGMP analysis

The cGMP levels were measured using the RIA method described in Methods and are expressed in units of pmol cGMP produced per gram wet weight of the tissue (pmol/g wet wt). As presented in Table 3.2, 0.6 μM GTN produced a significant rise in the level of cGMP in the strips as compared to buffer controls ($p<0.05$). Strips pretreated with ECA showed much lower levels of cGMP upon GTN treatment than untreated strips with GTN treatment ($p<0.05$), although cGMP levels in the ECA pretreated strips were still significantly higher than buffer controls ($p<0.05$). The levels of cGMP in GTN treated strips were about 5-fold higher than the buffer control and upon preincubation with ECA they were decreased about 2-fold (Table 3.2). ECA pre-treatment by itself did not have any effect on the levels of cGMP. The level of cGMP

Treatment	n	PE- induced contraction (g)	GTN induced relaxation (g)	% Relaxation
A. Control	9	3.72 ± 0.31	3.22 ± 0.53	86.3 ± 10.0
B. ECA-pretreated (0.2 mM)	9	3.14 ± 0.66	1.21 ± 0.39	35.7 ± 2.9
Statistics		A = B	A > B *	A > B *

Values are mean ± S.D.
*Significant at $p<0.05$

Table 3.1 PE induced contraction and GTN (0.6 μM) induced relaxation in control and ECA pretreated groups at 5 min.

Treatment	n	cGMP levels (pmol/g wet wt)
A. Control (buffer)	4	13.2 ± 1.5
B. GTN (0.6 μM)	9	64.4 ± 8.7
C. ECA (0.2 mM) preincubation + GTN (0.6 μM)	9	29.1 ± 6.2
D. ECA treatment only	3	13.7 ± 2.3
Statistics	A < B * B > C* A < C* A = D	

Values are mean ± S.D.
*Significant at $p<0.05$

Table 3.2 cGMP levels (pmol/g wet wt) in strips incubated in buffer, upon treatment with GTN and preincubated with ECA prior to GTN treatment.

measured in ECA pretreated strips was about the same as the basal levels of cGMP, as seen in Table 3.2.

The 70% decrease in the cGMP response that was observed in the strips pretreated with ECA corresponded well with the 59% decrease in relaxation response to GTN upon pretreatment with ECA (Figure 3.3).

3.3.3 Control experiments with SNP

At the two concentrations of SNP tested here no significant difference was observed in cGMP levels measured in the strips pretreated with ECA as compared to those not pretreated with the inhibitor. At the 10^{-6}M concentration of SNP, the levels of cGMP were 56.0 ± 0.2 pmol/g wet wt in control strips vs. 50.6 ± 5.6 pmol/g wet wt in the ECA pretreated strips (n=3) and at the10^{-5}M SNP concentration, the levels of cGMP were 72.3 ± 13.0 pmol/g wet wt in control strips vs. 70.7 ± 3.6 pmol/g wet wt in the ECA pretreated strips (n=3).

3.4 Discussion

The currently accepted hypothesis for the mechanism of action of GTN and all other organic nitrates emphasizes the necessity of a biotransformation process for GTN to produce its effect (Ignarro, 1989, Figure 3.1). Brien *et al.* (1986 and 1988) previously demonstrated a relationship between nitrate metabolism and cGMP elevation in rabbit aortic strips, where the increase in cGMP levels was found to correlate with the extent of GTN metabolism, as measured by the formation of GDN metabolites. Although the exact nature of the enzyme responsible

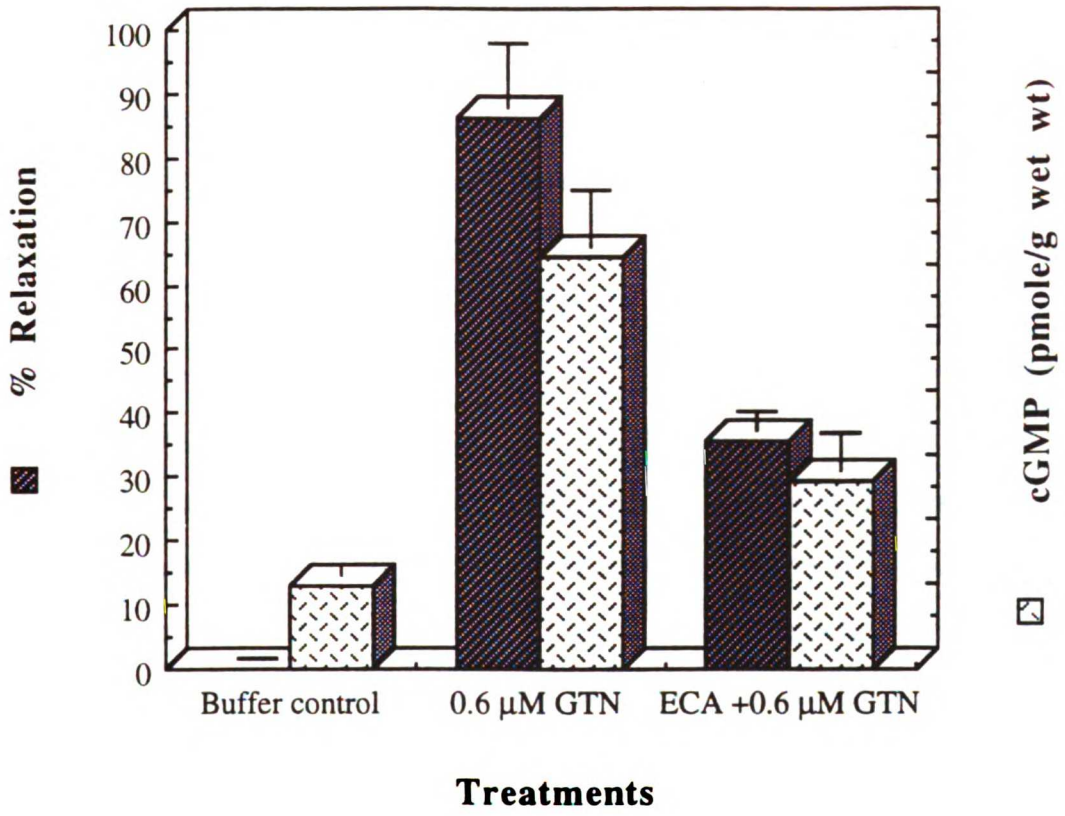


Figure 3.3 Responses to 0.6 µM GTN (cGMP response and % relaxation) in the presence and absence of ECA. Buffer control depicts the basal levels of cGMP.

for the activation of GTN has as yet not been identified, various theories have been suggested. Feelisch and Noack (1987) proposed that the non enzymatic decomposition of organic nitrates in the presence of cysteine is of importance for the pharmacologic effect. Servent *et al.* (1989), McDonald and Bennett (1990), Schröder and Schror (1990) and Schröder (1992) have suggested that cytochrome P-450's may be involved in the formation of NO from GTN. Brien *et al.* (1986) have proposed that the biotransformation of GTN in rabbit aorta occurs due to direct interaction of one of the nitrate groups of GTN with the iron (Fe^{++}) of guanylate cyclase bound heme. Chung and Fung (1990) and Fung *et al.* (1992) proposed that the enzyme responsible for the activation of GTN to nitric oxide is located in the plasma membrane and is thiol dependent. Most recently, Fung *et al.* (1992) reported that this enzyme is a 160KD protein that is distinct from GSTs. We and others have suggested that GST plays an important role in the activation process (Kukovetz and Holzmann, 1985; Lau and Benet, 1989,1990 and 1992; Lau *et al.*, 1992).

In our laboratory, Lau *et al.* (1992) found in bovine coronary artery cytosol that metabolism of GTN was a cytosolic thiol dependent enzymatic process and that ECA and SBP significantly reduced the rate of this process. In an earlier publication, Yeates *et al.* (1989) reported that GTN induced relaxation could be antagonized by SBP in rabbit aortic strips in muscle bath studies. However, Lau *et al.* (1992) were unable to reproduce this inhibition of GTN induced relaxation by SBP, whereas ECA produced a significant inhibition (Lau *et al.*, 1992). Needleman *et al.* (1973) previously reported a decrease in the relaxation due to GTN in rabbit aortic strips upon preincubation with ECA. Similar observations

were also made by Moffat *et al.* (1985) in canine dorsal pedal artery rings. Here, in this study we chose to measure not only the relaxation response to GTN but also a primary biochemical response which leads to the relaxation process, cGMP levels in the tissue. We hoped that since the two responses were measurable in the same strips we would have stronger evidence for the role of GST in the GTN bioactivation process by studying the effect of a GST inhibitor on this process.

In this study, the addition of ECA led to a decrease in the GTN induced vasorelaxation in all strips tested. This was accompanied by a parallel decrease in the levels of cGMP in the strips pretreated with ECA compared to those without pretreatment. In fact we found a correlation between the decrease in the relaxation response and the biochemical response to GTN (Figure 3.3). The results here can be used to address two important points. First, the results highlight the potential role of GST in the GTN activation process. If ECA specifically inhibits the activity of a GST isozyme, or a group of GST isozymes, then we would expect that by preincubating strips with this compound, the response to GTN would be affected only if the isozyme (or group of isozymes) is responsible for the metabolic activation of GTN. Secondly, the correlation between the decrease in the two responses provides evidence against a nonspecific effect of ECA on a point in the process (Figure 3.1) after cGMP production. However, these results do not rule out the possibility that ECA may have produced its effect on the guanylate cyclase enzyme itself or on other putative enzymes involved in the NO-activation process. To test this possibility we investigated the effect of ECA on cGMP levels in rabbit aortic strips exposed to sodium nitroprusside, a compound for

which it has previously been believed there was no requirement for an enzymatic conversion to activate GC but which more recently has been suggested to yield nitric oxide by a metabolic mechanism distinct from that for organic nitrates (Bates *et al.*, 1990; Marks *et al.*, 1991; Kowaluk *et al.*, 1992). At the two concentrations of SNP tested, no significant differences were observed in the cGMP levels induced by SNP in the presence and absence of ECA. This is consistent with the previous findings of Rapaport and Murad (1988) who reported that 0.1 mM ECA did not affect either cGMP levels or GC activation in such tissues. We also studied the effect of ECA on the tension and cGMP levels in the PE treated strips. ECA did not change the basal level of cGMP nor did it cause any changes in the tension in the tissue (Table 3.1).

Cytosolic GSTs consist of a family of isozymes broadly classified into three groups described as alpha, mu and pi on the basis of their subunit compositions, kinetic properties and immunoreactivities. These isozymes are expressed to different extents in different organs in the body, as well as in different species (Vos and van Bladeren, 1990). Since it is clear that the vasculature is the site of action of GTN, the expression of the GST isozymes in these tissues is of interest to us. The limitation of our study is that we cannot rule out possible cross reactivities of ECA with different GST isozymes. However, Ploemen *et al.* (1990) demonstrated that ECA most strongly inhibits the mu class GST. Furthermore, Tsuchida *et al.* (1990) successfully purified GSTs with activities towards GTN from the human heart and aorta. Two of these seven purified forms belonged to the GST pi family while the other five were immunologically related to the GST mu family. Two of the five

isozymes (mu) were found to exhibit high activities towards ECA and one of these two (pI=8.3) also possessed high activity to GTN. Furthermore, recent studies, reported in abstract form (Chern *et al.*, 1991), found that the metabolism of GTN correlated with the activity of the mu class of GST. Affinity chromatography, immunoprecipitation and immunoblots indicated that in rabbit aorta cytosol the mu class GST mediated the metabolism of GTN (Lanzo *et al.*, 1992).

In conclusion, we observed a correlation between the decrease in GTN induced cGMP levels and the decrease in GTN induced vasorelaxation. These results, together with previous metabolism studies (Lau and Benet, 1990; Lau *et al.*, 1992) indicate that GSTs may be involved in the productive metabolism of GTN. Furthermore the apparent preferential mu specificity of ECA inhibition (Ploemen *et al.*, 1990; Tsuchida *et al.*, 1990) and the results of Chern *et al.* (1991) and Lanzo *et al.* (1992) point to a possible involvement of the mu isozyme of GST. Further testing of this hypothesis will be examined in subsequent chapters.

CHAPTER 4

CORRELATION BETWEEN GST ACTIVITIES IN DIFFERENT TISSUES AND PHARMACOLOGIC RESPONSE TO GTN

4.1 Background

Although the critically important vasoactive species of GTN has not yet been identified, cGMP has been shown to be the second messenger of GTN induced vasorelaxation. The nature of the process involved in the production of this second messenger is also not clear. It is our working hypothesis that glutathione S-transferases (GSTs) are involved in the bioactivation of GTN. On the basis of inhibitor studies in homogenates of rabbit liver (Lau and Benet, 1989 and 1990), rabbit aorta, (Lau and Benet, 1992) and bovine coronary arteries (Lau *et al.*, 1992), it has been demonstrated that GST catalyzes the metabolic conversion of GTN to its products. When the effects of a GST inhibitor were studied in an aortic strip model, the relaxation response to GTN was decreased in parallel with reduced metabolism of GTN (Lau and Benet, 1992) and also with decreased cGMP concentrations (Kenkare and Benet, 1993, Chapter 3) suggesting that the biotransformation of GTN may be productive in terms of the pharmacologic effects.

It is known that GSTs are expressed in the body as multiple isoforms. In rabbit aorta the distribution of the different GST isozymes has been studied. Chern *et al.* (1991) and Lanzo *et al.*(1992) have found that the majority of GSTs occur as the pi isozyme (85%) while the mu isozyme accounts for approximately 15% of total GSTs. The alpha isozyme could not be detected in rabbit aorta. Using affinity chromatography, immunoprecipitation and immunoblotting techniques, the GST mu isozyme has been shown to mediate GTN metabolism in rabbit aortic cytosol. In immunoprecipitation studies on rabbit aortic

cytosol, antiserum against the pi isozyme did not affect GTN metabolism (Lanzo *et al.*, 1992) while antiserum against mu isozyme decreased the GTN metabolism significantly (Chern *et al.*, 1991, Table 4.1). Furthermore, Tsuchida *et al.* (1990) have successfully purified GST mu isozymes with activities towards GTN from the human heart and aorta. In the human aorta these authors found the same distribution of GST isozymes as in rabbit aorta with the pi being abundant, mu isozyme was a smaller subset while the alpha was not detectable. Tsuchida et al. (1990) also showed that the pi isozyme had no activity to GTN (Table 4.2), while the mu isozymes exhibited high activities towards GTN. In the previous chapter a good correspondence was observed in aortic strips pretreated with ECA, a GST mu preferential inhibitor between decreases in relaxation and cGMP levels induced by GTN (Kenkare and Benet, 1993). Thus, the mu isozyme of GST, an efficient metabolizer of GTN, appears to be important in the bioactivation of GTN (i.e., the production of pharmacologic effects).

The activity of the enzyme/isozyme of interest can be measured using specific substrates. Seidegard *et al.* (1984 and 1987) have found that trans stilbene oxide (TSO) is a specific substrate for the GST mu isozyme isolated from human liver, exhibiting a 2000 fold higher activity to the GST mu isozyme over other GST isozymes. 1-chloro-2,4 dinitrobenzene (CDNB) is a well known general substrate of GST (Habig *et al.*, 1974). Therefore the object of the present work is to investigate the relationship in rabbit aorta, a site of action of GTN, between the activity of GSTs, especially the mu isozyme, and the generation of the responses to GTN.

Anti-serum against	Percent decrease		
	Total GST activity	GST mu activity	GTN metabolism
Pi isozyme	87%	3.1 %	6.0 %
Mu isozyme	<20 %	> 75 %	> 75 %

Table 4.1 Percent decrease in total GST activity, GST mu isozyme activity and GTN metabolism upon immunoprecipitation with antiserum against the pi isozyme (Chern *et al.*,1991) and the mu isozyme (Lanzo *et al.*, 1992).

GST forms		Activity to GTN (µmol/min/mg protein)
pI 8.3	**Mu**	**0.78**
pI 6.6	**Mu**	**0.43**
pI 4.8	**Pi**	**< 0.01**
GST -I	(Liver alpha)	0.85
GST-pi	(Placenta)	<0.01
pI 6.0	(Liver mu)	1.08

Table 4.2 GSTs forms purified by Tsuchida *et al.* (1990) from human aortas (top section) compared to previously purified GST isozyme forms (bottom section).

However, activity in the aorta cannot be detected in intact animals or in humans for the purpose of clinical monitoring. Quite a few studies have been published (Seidegard *et al.*,1985; 1986 and1987; Wiencke *et al.*, 1990) describing GST mu isozyme activity in human mononuclear leukocytes. Furthermore, approximately 50% of the population lacks GST mu isozyme activity in mononuclear leukocytes measured using *trans*-stilbene oxide. Preliminary studies in our laboratory indicate that GST mu isozyme activity in rabbits varies substantially. We plan to test if the GST mu isozyme activity in mononuclear leukocytes correlates with that in aorta. Where GST mu isozyme is important for GTN bioactivation, the activity of this isozyme in mononuclear leukocytes could be a predictor of GTN action.

There is no evidence that patients lacking GST mu isozyme activity in mononuclear leukocytes respond differently to GTN than those patients which do exhibit mu isozyme activity. It can therefore be either argued that the GST mu isozyme is not the enzyme mediating GTN bioactivation, or that GST mu isozyme activity in mononuclear leukocytes does not correlate with that in aorta. To investigate these possibilities, GST and its mu isozyme activities in rabbit aortas, livers and mononuclear leukocytes (RMLs) were measured and the relationship between them was also investigated. The results of these two studies are presented in this chapter.

4.2 Methods

The techniques utilized here followed procedures described previously by Lau and Benet (1992) and Kenkare and Benet (1993). Briefly: eight male New Zealand white rabbits (Nitabell Rabbitry, Hayward CA) weighing 2 to 3 kg, were used in this study. The rabbits were anesthestized using a subcutaneous injection of ketamine (40 mg/kg as 1.0 ml of a 100 mg/ml solution in water for injection) and decapitated 15 minutes later. The thoracic cavity was exposed and the descending aorta was surgically removed and immediately placed in a beaker containing Krebs buffer (NaCl, 119 mM; KCl, 4.8 mM;KH_2PO_4, 1.2mM; $NaHCO_3$, 25mM; $CaCl_2$, 2.5 mM; glucose, 11.1 mM), which was continuously gassed with carbogen (95% oxygen and 5% carbon dioxide). After isolation of the aorta, excess fat and connective tissues were carefully removed; during this process the buffer was constantly replaced with fresh buffer. The endothelium was not disturbed. The helically prepared tissue was cut into four strips of dimensions 3 cm x 4 mm. Two of the four strips were frozen immediately for enzyme assays to be performed later, while the remaining strips were used for cGMP and relaxation measurements.

The aortic strips frozen for enzyme assays were homogenized with three volumes of phosphate buffered saline (PBS). The homogenate was centrifuged using an Eppendorf centrifuge at maximum speed, and the clear upper layer was frozen and stored at -80°C. In a second set of rabbits blood was collected as described above and mononuclear lymphocytes were separated as described in section 4.2.3 below. A piece

of liver was surgically removed and homogenized in three volumes of PBS. This homogenate was centrifuged at 4000 xg for 10 min, and the supernatant frozen and stored at -80°C.

4.2.1 Response measurements

Aortic strips were individually suspended in Krebs buffer (composition described above) contained in 25 ml jacketed circulating water tissue baths maintained at 37°C . One strip from each tissue was assigned as buffer control while the others as GTN treated. The contraction and relaxation of the strips were recorded via transducers, coupled to a Grass model 7 polygraph (Quincy, MA). The precontraction of the strips was performed as described in section 3.2.2 of Chapter 3. The relaxation produced by GTN was recorded at the end of 5 minutes, which is about the time for maximal relaxation. Immediately after the 5 minute measurement, the strips were freeze clamped using tongs precooled in liquid nitrogen and frozen on dry ice. Analysis of cGMP was then carried out in each of these strips, usually the next day.

4.2.2 Analysis of cGMP levels

The frozen strips were homogenized in 1 ml of 6% cold trichloroacetic acid (TCA) to precipitate proteins; the homogenate was then centrifuged at 4000 rpm for 10 minutes at 2-4°C to remove the protein precipitate. The supernatant was transferred into a fresh test tube and washed with a four-fold volume of diethyl ether saturated with water. This procedure was repeated at least four times. Each time the ether layer was carefully removed, after the tubes were vortexed, and allowed to stand for some time. After removing the residual ether by evaporation

under a stream of nitrogen, the TCA free samples were then assayed for cGMP using a ^{3}H labeled cGMP radioimmunoassay kit (Amersham, Arlington Heights, IL). Each time a new kit was used, standard curves were made with six concentrations (0, 0.5, 1, 2, 4 and 8 picomoles cGMP/100 µl) prepared from the standard solutions of cGMP.

4.2.3 Rabbit mononuclear leukocytes (RML) separation

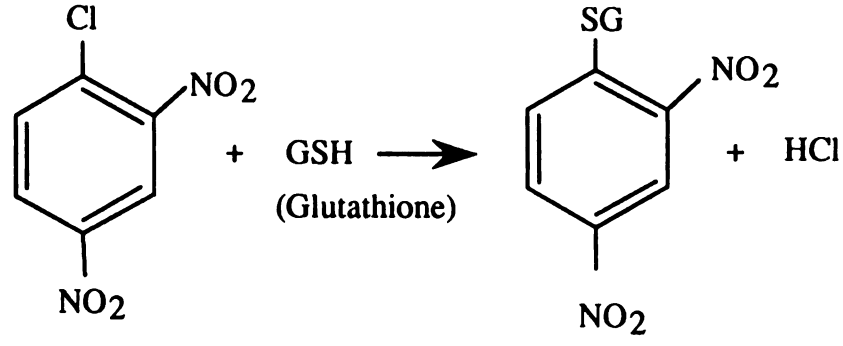
In a clear conical centrifuge tube, 3 ml of separation medium (Histopec, Sigma, St. Louis, MO) was added to the bottom of the tube and 3 ml of heparinized rabbit blood was carefully added on top of the medium. The tube was then centrifuged for 30 min at 400 x g. The middle cloudy band in the tube, which is RML, was aspirated into another conical tube. The RMLs were washed twice by suspension in 10 ml of PBS and pelleted by centrifugation at 250 x g for 10 min. The final pellet was mixed with 0.5 ml of PBS and stored at -80°C. Prior to enzyme analysis, the RMLs were thawed and sonicated for 2 min to release the cytosol.

4.2.4 Enzyme assays

The strips frozen for enzymatic analysis were homogenized using three times the volume of phosphate buffer saline (pH 7.4) and spun at 4000 x g for 5 minutes. The supernatants were then used for enzyme assays.

Total GST activity (1-chloro 2,4-dinitrobenzene activity)

Total GST activity was measured using the procedure (Figure 4.1) of Habig *et al.* (1974). Briefly the assay was carried out in a 3 ml plastic cuvette that contained 1 mM CDNB, 5 mM glutathione, 0.1mM



1-chloro-2,4-dinitrobenzene (CDNB)

Conjugate λ = 340 nm

Figure 4.1 Spectrophotometric assay of total GST activity (Habig *et al.*,1974).

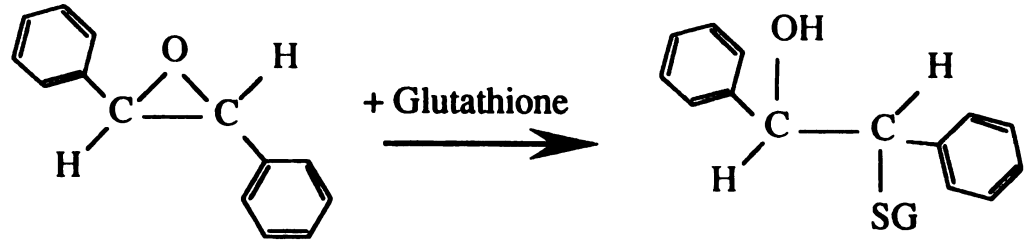
potassium phosphate buffer pH 6.65 and 10 µl of the aortic supernatant preparation (total assay volume = 3 ml). The increase in absorbance was measured at 340 nm at room temperature. Each liver sample used was diluted 100 fold. The extinction coefficient is 9.6 mM and one unit of activity was defined as one µmol/min. CDNB activities were expressed in µmole CDNB /min/mg protein.

GST mu isozyme activity (trans stilbene oxide activity)

GST mu isozyme activity was measured using a radiometric assay (Figure 4.2) similar to that described by Seidegard *et al.* (1984). Briefly, the samples were incubated with 4 mM glutathione and 250 µM [^{3}H] TSO (specific activity = 15 Ci/mmol) in PBS, pH=7.4, at 37°C for 10 min (total assay volume = 100 µl). The reaction was terminated by extraction with 200 µl of hexanol. The % radioactivity in the aqueous phase (product of TSO and GSH) was calculated from the radioactivity measurements in the organic phase and aqueous phase using liquid scintillation counting. The unit of activity was expressed as nmol TSO/min/mg protein or normalized by total GST activity as nmol TSO/min/CDNB unit.

4.2.5 Protein assays

The analysis of proteins were conducted using the procedure of Lowry and Freedman (1951). Protein concentrations were expressed in mg/ml. A representative standard curve is shown in Figure 4.3.



Trans-stilbene oxide (TSO)
Soluble in hexanol

Glutathione conjugate
Soluble in water

Figure 4.2 Radiometric assay of GST mu activity (Seidegard *et al.*, 1984).

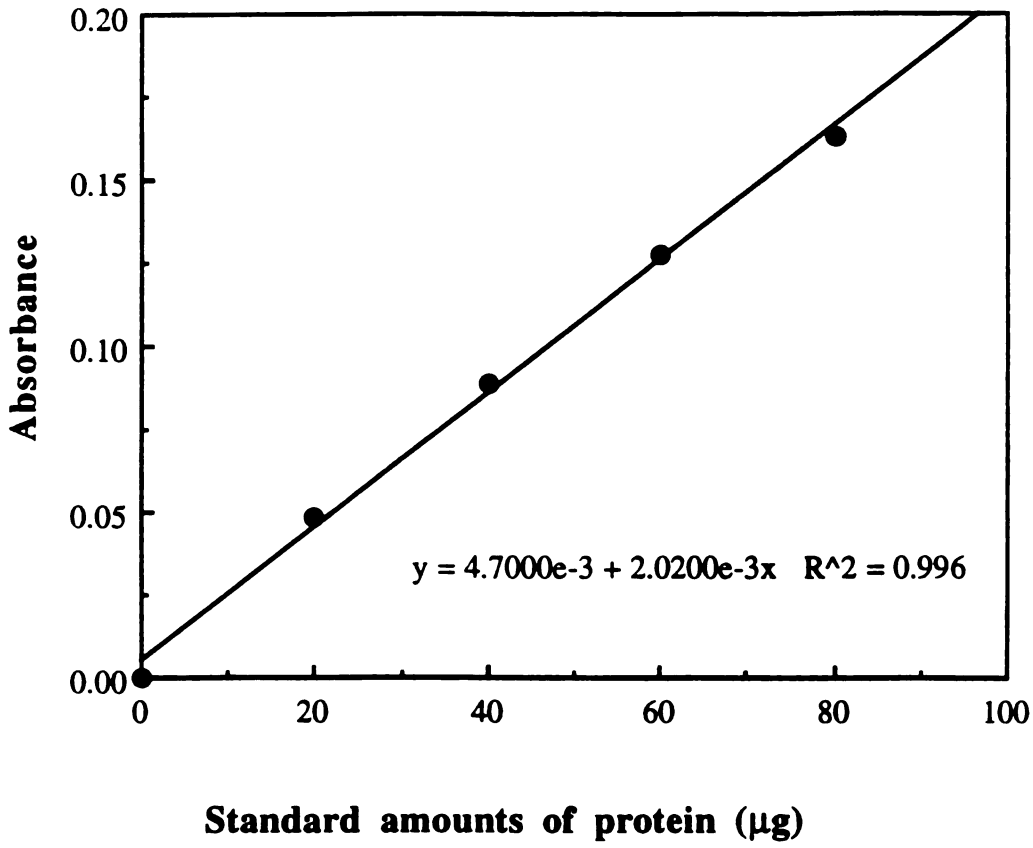


Figure 4.3 A standard curve for the estimation of proteins by the method of Lowry and Freedman (1951).

4.2.6 Data analysis

GTN induced relaxation was measured as % decrease in tension below the elevated tension elicited by precontracting the strips with PE. Fold increases in cGMP were calculated as cGMP in GTN treated strips divided by cGMP in buffer control. Linear regressions for all the correlations were carried out using the Cricket Graph software.

4.3 Results

4.3.1 GST mu activity compared to the responses to GTN in aorta

Basal cGMP levels in the aortas of the 8 rabbits were found to average 14.5 ± 2.1 pmol/g wet wt. The rise in cGMP on GTN treatment in an individual rabbit aorta was compared to its own basal cGMP level. Upon treatment with 0.5 μM GTN, the cGMP levels in the aortic strips increased from 2 to 4.2 fold relative to basal levels. A good correlation (r^2=0.789) was observed between the fold increase in cGMP and the % relaxation in the 8 rabbit aortas. GST mu isozyme activity was measured in nmol TSO/min/CDNB unit. Six of the eight rabbit aortas had TSO activities ranging from 1.42 to 2.27 nmol/min/CDNB unit. These rabbit aortas also showed a 2.2 to 3.5 fold increase in cGMP levels and a corresponding 56 to 100% relaxation. One rabbit aorta, with a GST mu activity of 0.99 nmol/min/CDNB unit, exhibited a much lower increase in cGMP and a correspondingly lower % relaxation in response to GTN. The eighth rabbit, with a very high GST mu activity of 3.58

nmol/min/CDNB unit, yielded the greatest increase in cGMP in response to the same concentration of GTN. When the GST mu activity was compared to the increase in cGMP levels an excellent correlation was found (r^2=0.948, Figure 4.4). A lower but obviously significant correlation (r^2=0.715) was observed upon comparison of GST mu activity with % relaxation. When the enzyme activity was normalized to protein content (nmol TSO/min/mg protein) rather than total GST content, the correlations were lower than those previously obtained. That is, comparison of the TSO activity normalized to protein content with the increase in cGMP yielded an r^2 =0.776 (Figure 4.5) and r^2=0.539 with % relaxation (data not shown).

4.3.2 Total GST activity compared to the responses of GTN in aorta

Total GST activity was measured in terms of μmol CDNB/min/mg protein. The total GST activities of the different rabbit aortas were compared and were found to range from 0.18 to 0.6 μmol/min/mg protein. When total GST activities were compared with responses to GTN in aortas of individual rabbits, no correlation was found with the increase in cGMP levels (r^2=0.099, Figure 4.6) or the % relaxation (r^2=0.067, data not shown) in the 8 rabbits studied.

4.3.3 Comparison of GST mu isozyme in rabbit aorta and in RML

In eight rabbits, TSO activities in aortas ranged from 0.99 to 3.58 nmol/min/CDNB while those in RMLs from the same rabbits ranged from negligible to 5.1 nmol/min/CDNB. A poor correlation (r^2=0.234) of

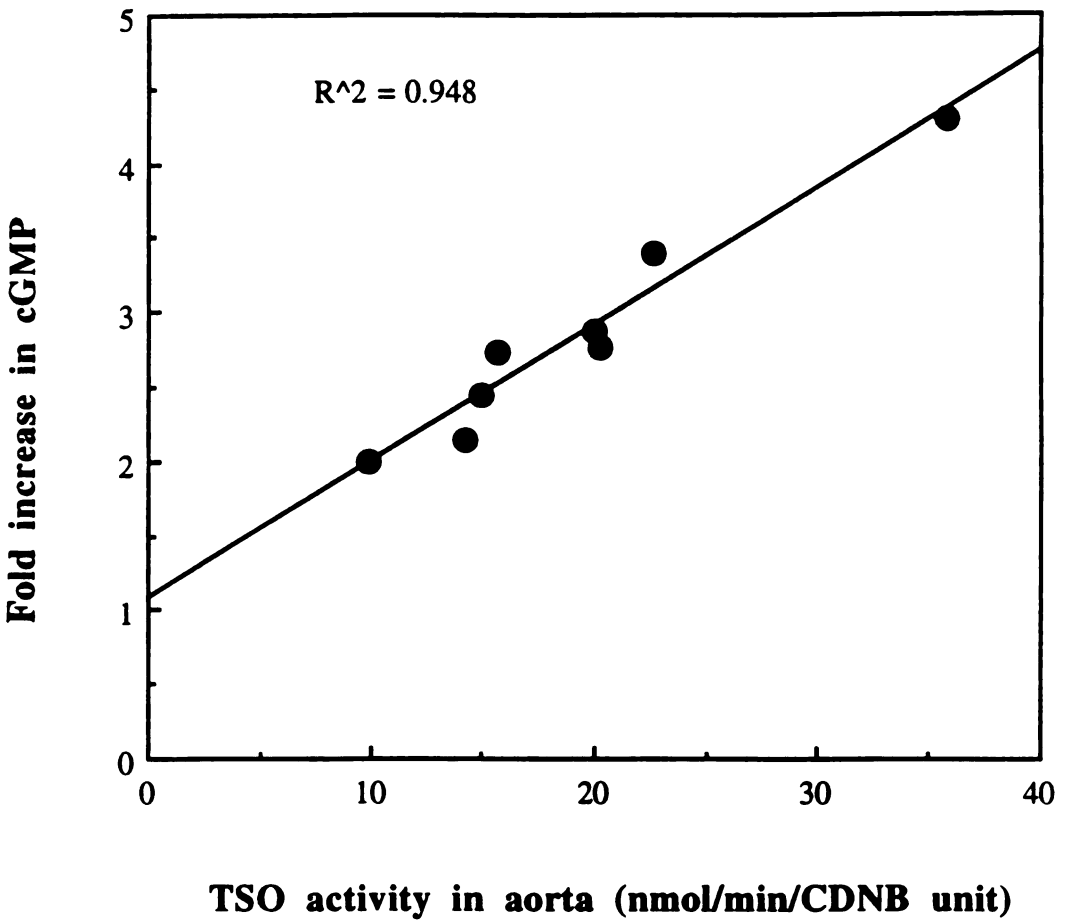


Figure 4.4 TSO activity in aorta correlated with fold increases in cGMP.

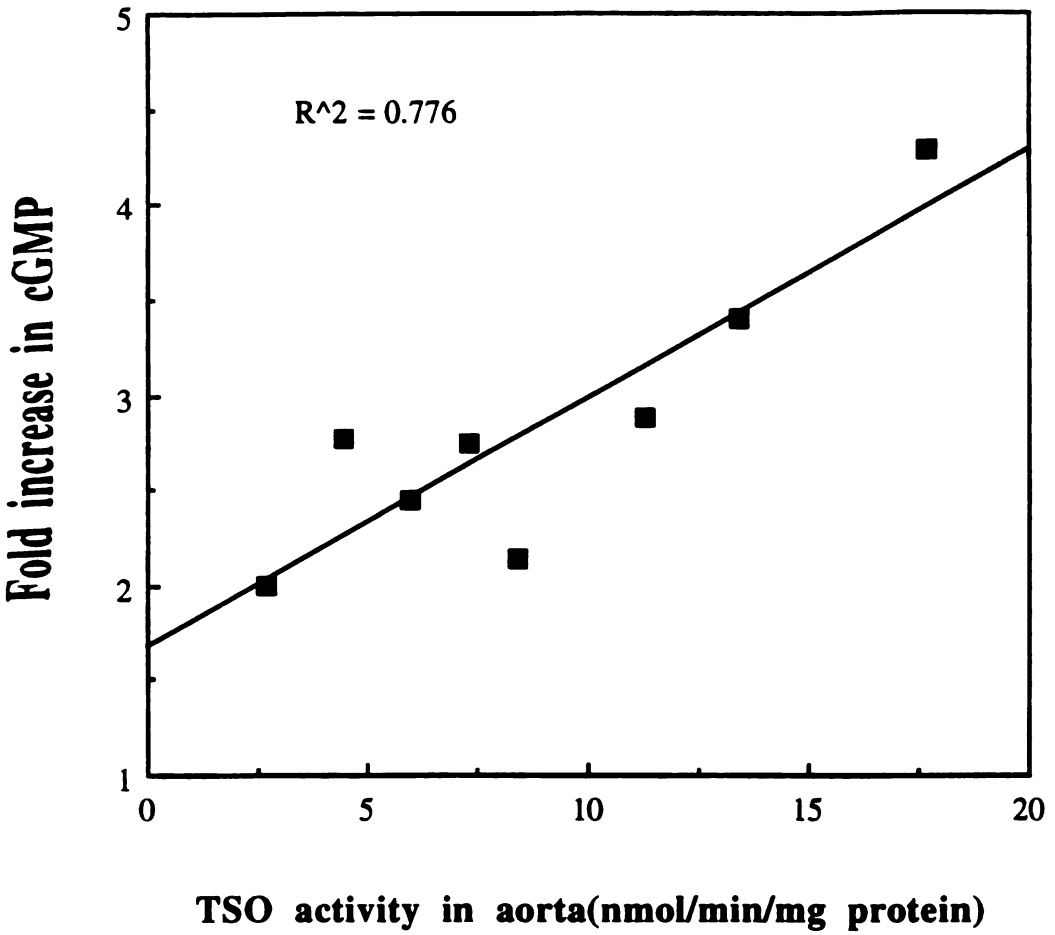


Figure 4.5 TSO activity in the aorta (normalized to protein content) correlated with the increases in cGMP.

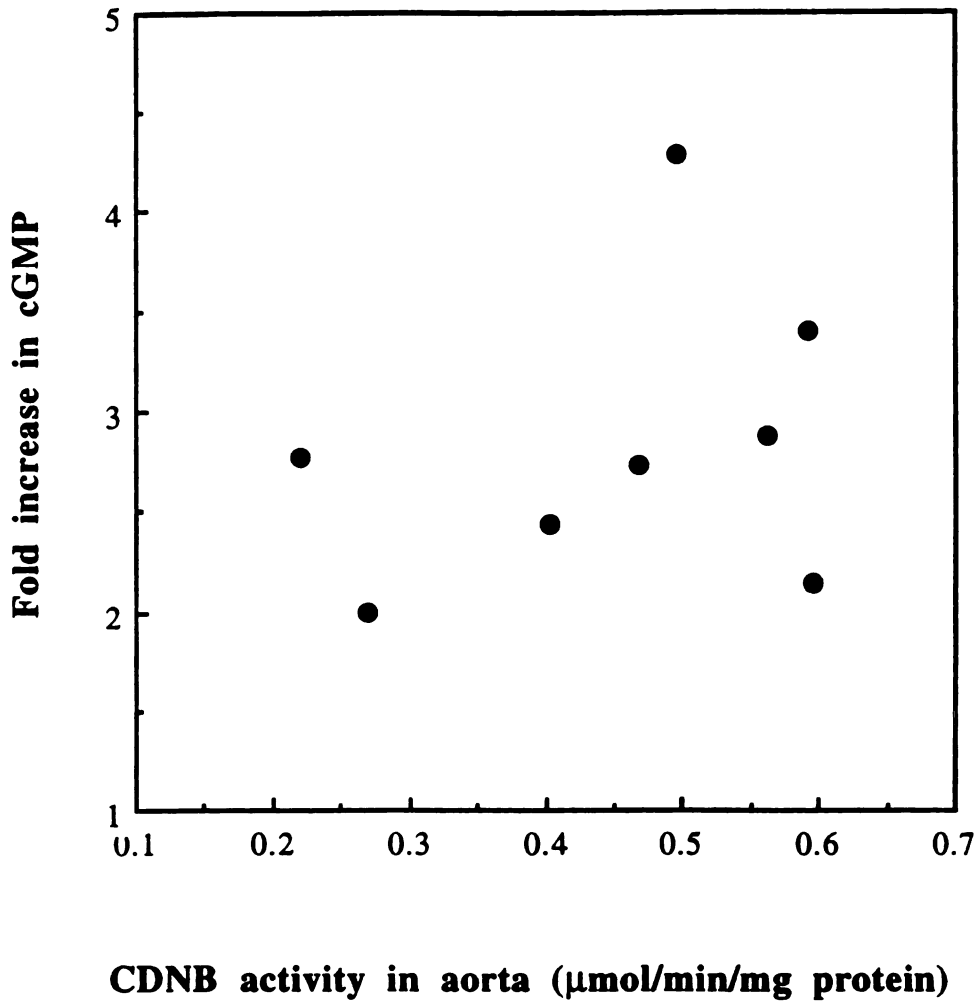


Figure 4.6 Lack of correlation between the CDNB activity in the aorta and the increase in cGMP.

activities between these two tissues in terms of TSO (mu isozyme) activity was found as shown in Figure 4.7. A much better correlation for measures of CDNB (total GST) activities in aortas with those in RMLs was observed in the same rabbits (r^2=0.607, Figure 4.8).

4.3.4 Comparison of GST mu isozyme activities in liver and in RML

In rabbit liver, TSO activities ranged from 0.08 to 0.42 nmol/min/CDNB unit, (n=9). When these activities were compared to those in RMLs from the same rabbits, a good correlation was found (r^2=0.814, Figure 4.9). A comparable correlation was also observed (r^2=0.775) if TSO activities were normalized to protein concentration rather than CDNB activities (data not shown).

4.3.5 Relative enzyme activities in the different rabbit tissues studied

The CDNB and TSO activities are summarized in Table 4.3. Total GST activity is markedly higher in rabbit liver (26.7 μmol CDNB/min/mg protein) than in the aorta (0.45 μmol CDNB/min/mg protein). TSO activity in the liver (6.08 nmol TSO/min/mg protein) is about 8 times higher than that in RML (0.77 and 0.81 nmol TSO/min/mg protein in two different groups of rabbits), while CDNB activity in livers is about 100 times higher than that in RML. The CDNB and TSO activities in the aorta were comparable to the CDNB and TSO activities respectively in the RMLs (Table 4.3).

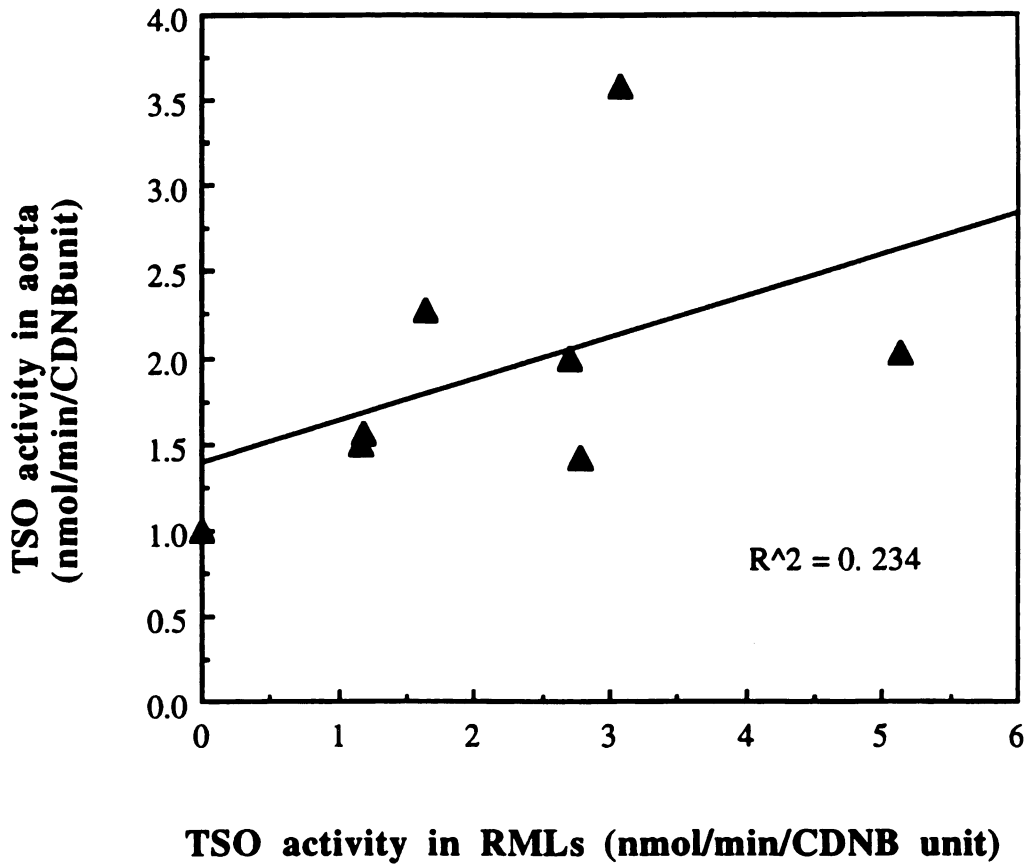


Figure 4.7 Poor correlation between the TSO activity in the RMLs and in aorta.

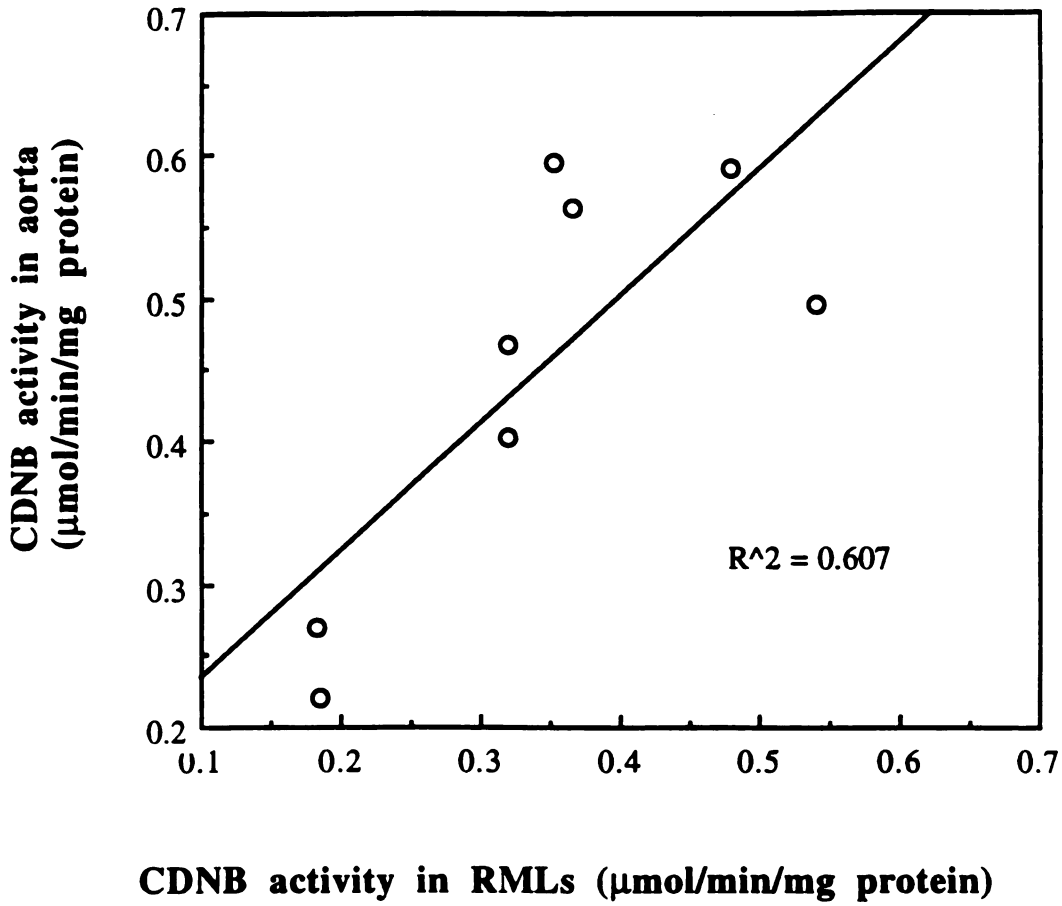


Figure 4.8 Correlation between the CDNB activity in the RMLs and in the aorta.

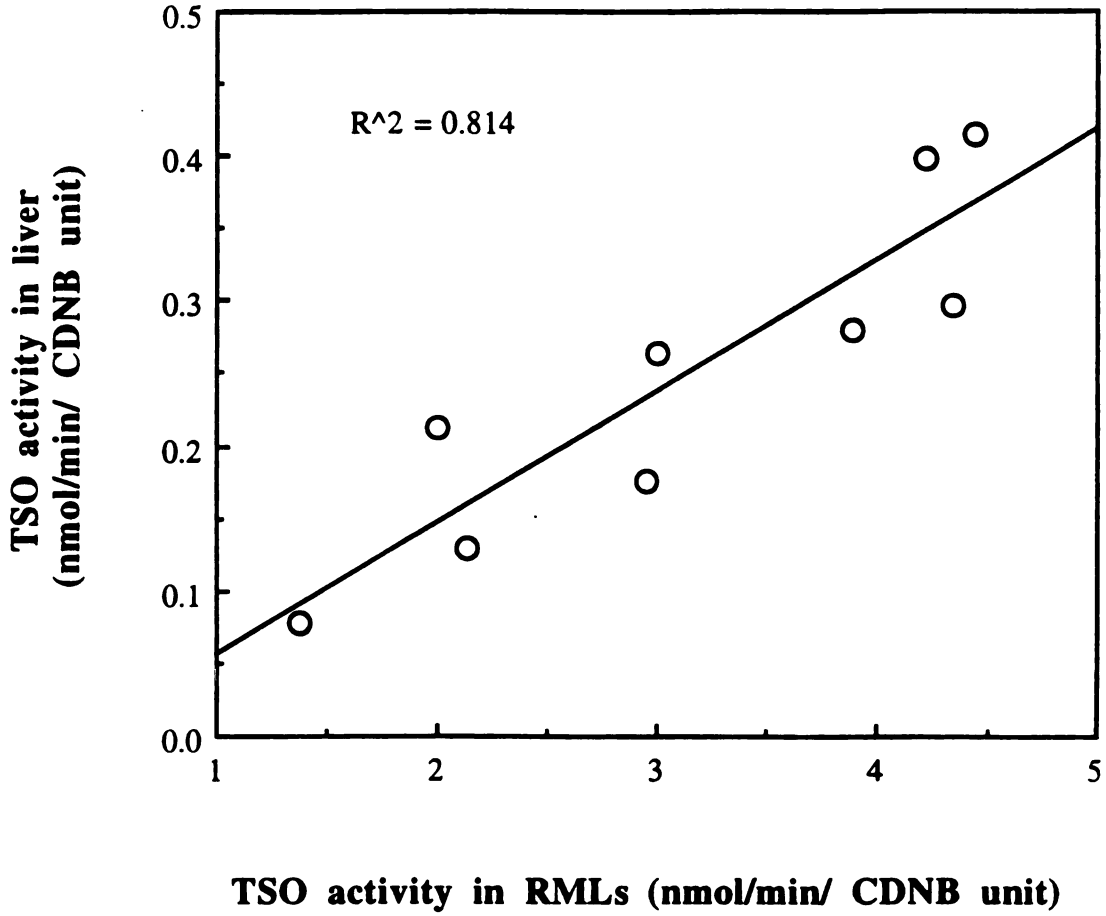


Figure 4.9 Correlation between the TSO activity in the RMLs and in the liver.

A	n = 8	Aorta	RML
Total GST	**Mean**	0.45	0.34
(μmol CDNB/min/mg pro.)	**± SD**	0.13	0.12
	%CV	29.8	34.1
GST mu isozyme	**Mean**	0.89	0.77
(nmol TSO/min/mg pro.)	**± SD**	0.47	0.48
	%CV	52.3	62.5

B	n = 9	Liver	RML
Total GST	**Mean**	26.7	0.26
(μmol CDNB/min/mg pro.)	**± SD**	4.11	0.05
	%CV	15.4	18.1
GST mu isozyme	**Mean**	6.08	0.81
(nmol TSO/min/mg pro.)	**± SD**	2.65	0.29
	%CV	43.5	35.8

Table 4.3 Summary of the GST activities in aortas and mononuclear leukocytes of 8 rabbits (set A) and in livers and mononuclear leukocytes of 9 rabbits (set B).

4.4 Discussion

Metabolism of GTN is known to be important for the production of pharmacologic effects of this drug in vascular tissue. Different theories have been proposed to explain the metabolism of GTN in vascular and nonvascular tissues. Feelisch and Noack (1987) proposed the non enzymatic thiol dependent decomposition of GTN, while more recently a number of investigators (Servent *et al.*, 1989; McDonald and Bennett, 1990; Schröder and Schror, 1990; Schröder, 1992; Delaforge *et al.*, 1993) have suggested that cytochromes P-450 may be involved in the formation of nitric oxide from GTN. Brien *et al.* (1986) have proposed that the biotransformation of GTN in rabbit aorta occurs due to a direct interaction of one of the nitrate groups of GTN with the iron (Fe^{++}) of guanylatecyclase bound heme. Chung and Fung (1990) proposed that the enzyme responsible for the activation of GTN to nitric oxide is located in the plasma membrane and is thiol dependent. Most recently, Fung *et al.* (1992) reported that this enzyme is a 160 KD protein that is distinct from GSTs. It has been suggested (Needleman *et al.*, 1969; Lau and Benet, 1989; Lau and Benet, 1990; Hill *et al.*, 1992; Lau and Benet, 1992; Lau *et al.*, 1992) that GSTs are important in the metabolic activation of GTN and that the majority of the metabolism is associated with the cytosolic fraction.

Three different classes of GST isozymes, designated alpha, pi and mu, have been identified. These isozymes are expressed to different extents in different organs of the body and in different species (Mannervik, 1985). As reported in the Background to this chapter, the results of Tsuchida *et al.* (1990), Chern *et al.* (1991), Lanzo *et al.* (1992)

indicate that in human and in rabbit vasculature, the GST mu isozyme metabolizes GTN while pi isozyme, the abundant isozyme is not active in metabolizing GTN. The alpha isozyme was not detectable. We have recently shown that GTN responses in rabbit aortic strips (measured as % relaxation and elevation of cGMP levels) were modulated when the strips were pretreated with a GST mu selective inhibitor (Chapter 3 and Kenkare and Benet, 1993).

In the present study the activity of total GST enzymes and the activity of the mu isozyme were measured together with the response to a specific concentration of GTN in the same rabbit aorta. Since the basal cGMP levels were also measured in the same animal the cGMP increases represent specific rises in the level of the second messenger in the aorta of each rabbit. As the enzyme activity measurements and the response measurements were done on each individual rabbit aorta, it was possible to correlate individual tissue responses with the enzyme activities in those rabbits.

The results of this part of the study showed that total GST activity, measured as μmole CDNB/mg protein did not correlate well with the GTN induced increase in cGMP levels of each rabbit aorta (r^2=0.099). However on comparing the GST mu isozyme activity, measured as nmol TSO/CDNB unit, a very good correlation (r^2=0.948) was observed between these activities and the increase in cGMP levels. This indicates that GST mu activity and not the total GST activity is a good marker of the biochemical response to GTN in rabbit aorta. The increase in cGMP levels and the % relaxation in the different rabbits correlated well (r^2=0.789) as seen in our previous study (Kenkare and Benet, 1993),

which is in agreement with the hypothesis that cGMP is the second messenger of GTN induced relaxation in vascular smooth muscle. A marked but somewhat lower correlation (r^2=0.715) was observed between the mu isozyme activity and the % relaxation in aortas. This decreased correlation is predictable since relaxation of aortic strips is a secondary response to GTN, preceded by the cGMP increase, a more primary biochemical response.

We also observed a decrease in the r^2 values when cGMP increases and % relaxation were correlated with GST mu isozyme activity/ mg of protein rather than when GST mu isozyme activity was normalized to CDNB measurements. This is probably due to the fact that the protein level accounts for all the proteins in the tissue (GSTs and others), which we would expect to vary more from animal to animal as opposed to the relative fraction of the GSTs, a single enzyme system. It could be argued that the observed relationship between the TSO activity and the response to GTN may be coincidental and an increased GST mu isozyme activity was due to a higher level of expression of all proteins in individual rabbits. However, this explanation is probably unlikely, since rabbit aortas with high GST mu activities did not have high total GST activities (data not shown).

The results of this study provide evidence for a relationship between mu isozyme activity (TSO activity) in vascular tissue and the pharmacologic response to GTN, in the rabbit model. This study does not exclude the possibility of other enzymes being involved in the metabolism and/or bioactivation of GTN, however together with evidence obtained previously, it does indicate that the mu isozyme of GST may play a role in

the activation of GTN in the rabbit aorta in the *in vitro* system used in this study.

In examining the correlation between the activity of the GST mu isozyme in aortas, mononuclear leukocytes and livers we have shown that mu isozyme activity in RMLs do not correlate well (r^2=0.234) with that in rabbit aortas. That is, the mu isozyme activity in rabbit aorta is not predicted by the mu isozyme activity in RMLs.

If our observation of the lack of correlation between GST mu isozyme activity in the RMLs and in the rabbit aorta holds true in humans, this may explain why although 50% of the population lacks the TSO activity in mononuclear leukocytes there is no evidence that this population does not respond to GTN. Previously, it was reported that the difference in expression of human GST mu (TSO activity) is due to a gene deletion (Seidegard et al., 1988). Recently, Haefeli *et al.* (1993) compared GTN response using the hand vein compliance technique in subjects who were found, using the PCR technique on mononuclear leukocytes, to be genetically with and without expression of GST mu isozyme. No differences were found in the effects of GTN between the two groups. Haefeli et al. (1993) concluded that the GST mu isozyme may not be important in GTN bioactivation.

However, there is evidence that more than one form of GST mu exists in the human body and in the vasculature. At least five GST sub forms have been purified from human aorta and from the heart (two and three respectively). All five forms are immunologically related to GST-μ (mu form from liver) (Tsuchida *et al.*, 1990). These five heart and aorta

mu isoforms were found to exhibit different pI values and each possess different amino acid sequences which are also different from that of GST-μ. Except for one of the heart mu forms with a pI 5.3, all of the other four have activity to GTN. Suzuki *et al.* (1987) also reported two types of human GST(μ) and one of GST-5, which cross react with GST(μ) antibody. The human GST mu class isozymes are encoded by a dispersed gene family with a minimum of six genes located on at least three different chromosomes. DeJong *et al.* (1991) also suggested that the GST-μ gene(s), which account(s) for the isozyme expression in liver and other tissues located on chromosome 13, is/are only member(s) of a dispersed gene family. Furthermore, gene deletion for the null phenotype may not be the best explanation. The primers used in the Haefeli study were synthesized on the basis of the sequence of the human liver cDNA GST-μ clone. These primers may or may not share marked homology with others mu genes. It is thus possible that the mu isozymes in the vasculature, may be products of a different gene, which may or may not be polymorphic.

The results of this study also indicated that total GST activity in different rabbits is less variable than GST mu isozyme activity. The highest GST activity was found in hepatic tissue. Although the mean value of TSO activities in livers was about 8 times higher than that in RMLs, the mean value of CDNB activities was almost 100 times higher than that in RMLs. Similar results were obtained when livers were compared with aorta. Thus, the fraction of GSTs which have activity to TSO is larger in RML and rabbit aorta than that found in the liver.

According to Seidegard *et al.* (1984), TSO is a specific substrate of GST mu isozyme. However, these earlier studies of mu isozyme activity were based on the GST-μ, which was isolated from human liver GST. The specificity of TSO activity toward GST mu sub forms and the specificity in terms of genes requires further elucidation. Further studies are also required to determine which sub form(s) is responsible primarily for the metabolism and the activation of GTN at its site of action.

CHAPTER 5

INVESTIGATING TOLERANCE TO GTN

5.1 Background

Tolerance to organic nitrate esters is defined as a condition in which increasing doses of the drug are required to induce a given hemodynamic or anti-anginal effect. Stewart (1888) was the first to report a description of tolerance to GTN in humans. Later, several publications described the existence of tolerance in humans and in experimental animals *in vivo* (Ebright, 1914; Myers and Austin, 1929; Crandall *et al.*, 1931; Bogaert, 1968; Bogaert *et al.*, 1968; Clark, 1969). These findings prompted the investigation of the possible existence of tolerance in isolated vascular smooth muscle taken from animals made tolerant to organic nitrate esters *in vivo* (Needleman, 1970; Herman and Bogaert, 1971). Although *in vivo* tolerance to organic nitrates could be a complex combination of pharmacokinetic alterations, physiologic alterations (such as neurohormonal compensatory responses) and biochemical alterations (see Chapter 1); *in vitro* tolerance, in isolated vascular tissues is probably the direct result of one or many biochemical alterations in the vascular smooth muscle cells by organic nitrates.

In the past, various hypotheses have been proposed to describe tolerance to GTN at a cellular level. These have included a reduction in thiol groups or thiol depletion (Needleman and Johnson, 1973; Torresi *et al.*, 1985; May *et al.*, 1987; Noack, 1990), reduced guanylate cyclase activity due to a desensitization of this enzyme (Axelsson and Karlsson, 1984; Kukovetz and Holzmann, 1987; Ahlner *et al.*, 1986; Schroder *et al.*, 1990) and reduced vascular biotransformation of the drug (Fung and Poliszczuk, 1986; Slack *et al.*, 1989; Bennett *et al.*, 1989;

Mulsch *et al.*, 1989; Brien *et al.*, 1986; Forster *et al.*, 1991; Schror *et al.*, 1991).

Although much conflicting data exist regarding thiol depletion and desensitization of guanylate cyclase as possible mechanisms for the development of tolerance to GTN, the extent of involvement of these mechanisms in the tolerance development process has not been equivocally confirmed. In contrast, most of the data regarding reduced vascular biotransformation as an important cause of GTN tolerance are quite consistent. However no mechanistic explanations have been forwarded so far. As the mechanism of action of GTN is quite complex and involves a cascade of various reactions occurring in parallel and in series, for which intermediates are still not clear, a direct cause and effect demonstration is difficult.

The vasodilating action of GTN is intimately linked to its metabolism to the dinitrate metabolites. It has been shown that the time course of dinitrate formation from GTN in vascular segments closely parallels that of vasodilation and that *in vitro* tolerance to GTN results in a significantly reduced level of dinitrates in the aortic wall, suggesting reduced biotransformation of GTN in the vascular wall (Brien *et al.*, 1986). Reduced levels of metabolites of both GTN and isosorbide dinitrate were found in tolerant rabbit aortic strips and a significant cross tolerance between GTN and ISDN mediated effects on metabolite concentrations within the vascular wall has been reported (Slack *et al.*, 1989). Such an effect has also been demonstrated in cell cultures of vascular and non vascular origin (Bennett *et al.*, 1989). It has therefore been suggested that the decreased biotransformation could be due to a

change or a decrease in the activity of the enzyme systems involved in metabolizing these organic nitrates, or alternatively, due to co-factor depletion.

On the basis of our previous studies (Chapters 3, 4) and the results of Chern et al. (1990) and Lanzo *et al.* (1992), a role for GST mu in the bioactivation of GTN has been postulated. Therefore, it seems reasonable to hypothesize that the reduced metabolism of GTN, observed in tolerance development could be, at least in part due to an alteration in the activity of the GST mu isozyme. The overall aim of this chapter is to evaluate this hypothesis.

The objectives of the studies described in this chapter are:

1. To demonstrate tolerance development to GTN in our *in vitro* rabbit aortic strip model.

2. To study the changes (if any) in GST (mu) activity during tolerance development.

3. To compare the changes in GST (mu) activity to the decreased response to GTN during the development of tolerance.

4. To determine if thiol levels are affected during tolerance to GTN.

5. To test the possibility that a desensitization of guanylate cyclase may be responsible for tolerance development to GTN.

5.2 Methods

Most of the techniques utilized here followed procedures described in previous chapters as reported by Lau *et al.* (1992) and Kenkare and Benet (1993). Briefly: male New Zealand white rabbits (Nitabell Rabbitry, Hayward, CA) weighing 2 to 3 kg, were used in these studies (four rabbits for the studies described in sections 5.2.1 and 5.2.4 each, seven rabbits for the studies described in section 5.2.2 and three for the studies in section 5.2.3). The rabbits were anesthestized using a subcutaneous injection of ketamine (40 mg/kg as 1.0 ml of a 100 mg/ml solution in Water for Injection) and decapitated 15 minutes later. The thoracic cavity was exposed and the descending aorta was surgically removed and immediately placed in a beaker containing Krebs buffer (NaCl, 119 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25mM; $CaCl_2$, 2.5 mM; glucose, 11.1mM), which was continuously gassed with carbogen (95% oxygen and 5% carbon dioxide). After isolation of the aorta, excess fat and connective tissues were carefully removed; during this process the buffer was constantly replaced with fresh buffer. The endothelium was not disturbed. The aorta was then helically cut and divided into two equal segments. The experiments then performed are described in the subsections 5.2.1, 5.2.2, 5.2.4.

5.2.1 Tolerance to GTN: Dose response curves

Two strips from each aorta were utilized for studying the effect of GTN tolerance on the relaxation response to various doses of GTN. The protocol is demonstrated in Figure 5.1. Each strip was suspended in Krebs buffer contained in 25 ml jacketed circulating water

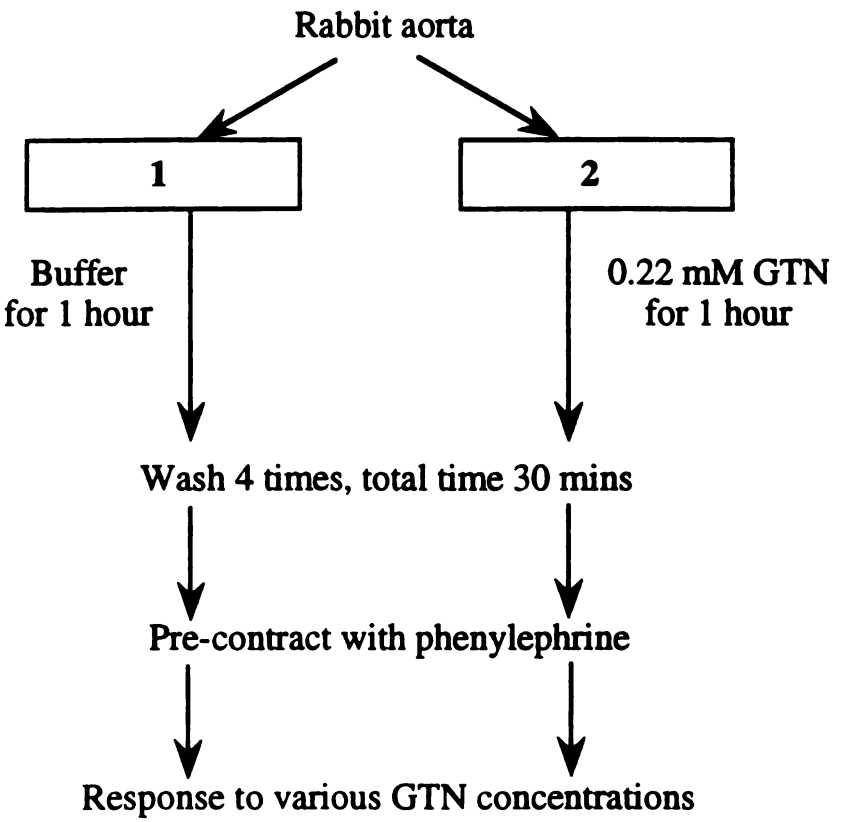


Figure 5.1 Protocol for GTN dose-response studies.

tissue baths maintained at 37°C. One of the two strips was assigned as control and the other for tolerance development (strip 2). GTN (0.22 mM) was added to strip 2, while the same volume of buffer was added to strip 1. The two segments were allowed to stand for an hour. At the end of the hour, each strip was washed for a total time of 30 min (buffer was changed approximately 4 times during this period). The strips were then precontracted with submaximal doses of phenylephrine (PE) as described in chapters 3 and 4 and increasing concentrations of GTN were added to the strips. A dose-response curve was then constructed, using the cumulative percent relaxation from PE induced contraction of strips as the response measurement.

A sigmoidal Emax model (Hill equation) was used to fit the dose-response data. This model is described as follows:

$$\text{Effect} = \frac{E_{max} \times [\text{Drug}]^{\gamma}}{EC_{50}^{\gamma} + [\text{Drug}]^{\gamma}}$$

where:

E_{max} = the maximal effect (% relaxation) produced by the drug

[Drug] = concentration of drug

EC_{50} = concentration of drug which produces 50 % of maximal relaxation.

γ = Hill coeffecient

5.2.2 Enzyme activity and GTN response studies

Each of the two segments was suspended in Krebs buffer contained in 25 ml jacketed circulating water tissue baths maintained at 37°C. One of the two segments was assigned as tolerant (segment A, Figure 5.2) while the other as pre-tolerant (segment B, Figure 5.2) .

GTN (0.22 mM) was added to segment A while the same volume of buffer was added to segment B. The two segments were allowed to stand for an hour . At the end of the hour, each segment was divided into two strips (A1, A2, B1, B2 in Figure 5.2). One strip of each segment was immediately frozen between blocks of dry ice, for enzyme assays to be performed later. The remaining strip was washed for a total time of 30 min (4 times) and then effect measures were performed on these strips.

5.2.2.1 Effect measurements

After being washed for 30 min, each strip (pre-tolerant and tolerant) was treated with 0.5 μM GTN. At the end of 5 min, the strips were frozen using tongs precooled in liquid nitrogen and immediately frozen on dry ice. Analysis of cGMP was then carried out in each of these strips. In four of the seven rabbit aortas, the relaxation effect of GTN was also measured along with the cGMP levels. The procedures used for measuring vasorelaxation were the same as described in previous chapters and by Kenkare and Benet (1993). Briefly the strips were precontracted using phenylephrine (approximately 0.3 to 0.6 μM) and relaxation effect

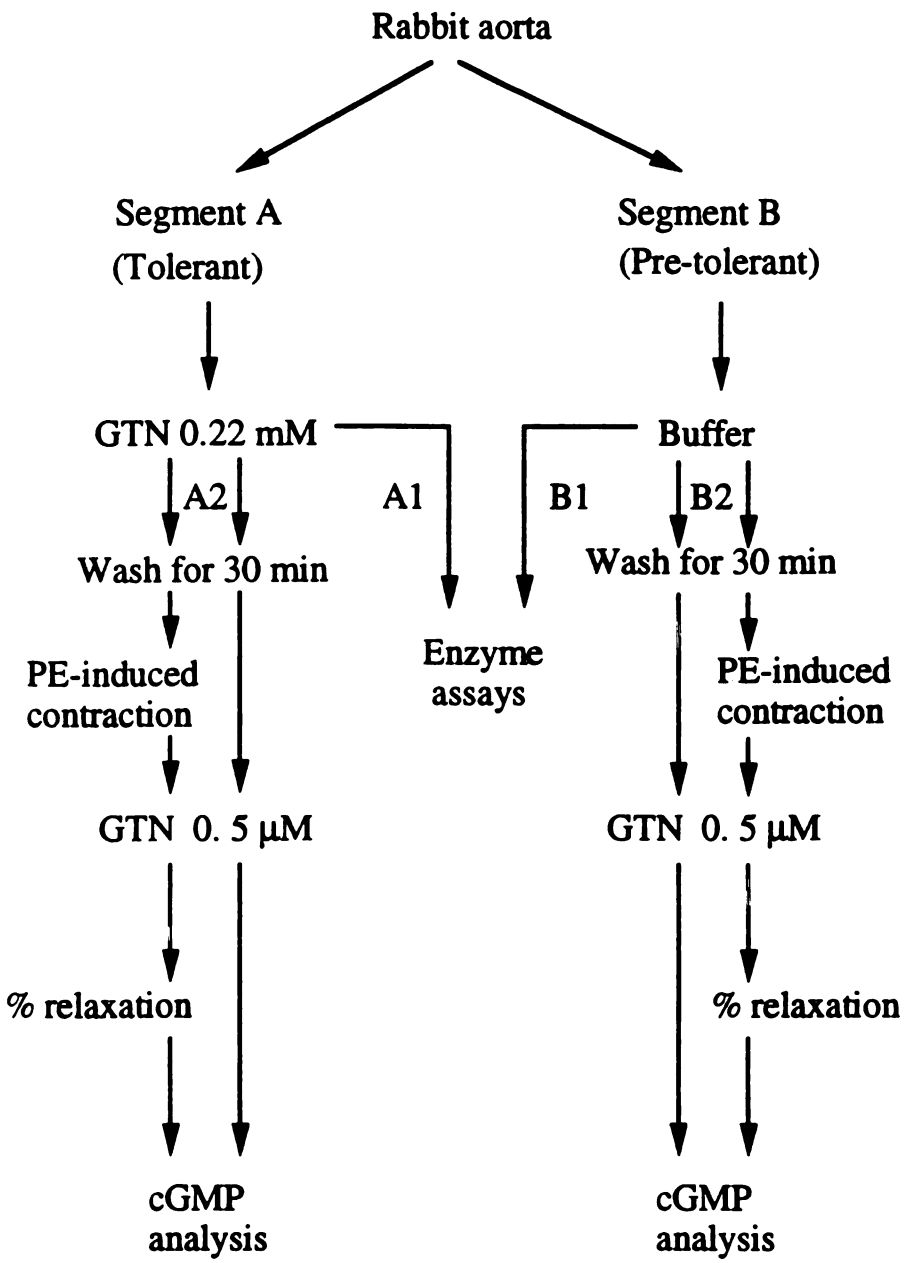


Figure 5.2 Protocol for the enzyme activity and response studies.

of GTN was recorded via transducers coupled to a Grass model 7 polygraph (Quincy, MA). These strips were then frozen for cGMP analysis together with the other strips described above for this protocol.

5.2.2.2 Analysis of cGMP levels

cGMP levels were analyzed using the procedure previously described by Kenkare and Benet (1993). Briefly, the frozen strips were homogenized in 1 ml of 6% trichloroacetic acid (TCA) to precipitate proteins; the homogenate was then centrifuged at 4000 rpm for 10 min at 2-4^{o}C to remove the protein precipitate. The supernatant was transferred into a fresh tube and washed with a four-fold volume of diethyl ether saturated with water. This procedure was repeated at least four times. Each time the ether layer was carefully removed, after the tubes were vortexed, and allowed to stand for some time. After removing the residual ether by evaporation under a stream of nitrogen, the TCA free samples were then assayed for cGMP using a ^{3}H labeled cGMP radioimmunoassay kit (Amersham, Arlington Heights, IL).

5.2.2.3 Enzyme activity measurements

The strips frozen for enzymatic analysis were homogenized using three times the volume of phosphate buffer saline (pH 7.4) and spin at 4000 rpm for 5 min. The supernatants were then used for the enzyme assays.

Total GST activity (1-chloro 2.4 -dinitrobenzene activity)

Total GST activity was measured using the procedure of Habig *et al.* (1974). Briefly the assay was carried out in a 3 ml plastic

cuvette that contained 1 mM CDNB, 5 mM glutathione, 0.1 mM potassium phosphate buffer pH 6.65 and 10 µl of the aortic supernatant preparation. The increase in absorbance was measured at 340 nm at room temperature. The extinction coefficient is 9.6 mM and one unit of activity was defined as one µmole/min. CDNB activities are expressed in µmole CDNB/min/mg protein.

GST mu isozyme activity (trans stilbene oxide activity)

GST mu activity was measured using a radiometric assay similar to that described by Seidegard *et al.* (1984). Briefly the aortic supernatant fraction (10 µl) was incubated in a final volume of 100 µl containing phosphate buffered saline, pH 7.4, 4 mM glutathione and 250 µM [^{3}H] TSO (specific activity= 15 Ci/mmol). The reaction mixture was incubated at 37^{o}C for 10 min and the reaction terminated by extraction with 2 volumes of hexanol. The % radioactivity in the aqueous phase was calculated from the radioactivity measurements in the organic and aqueous phases using liquid scintillation counting. The non enzymatic control rates of reaction, determined from inactivated boiled tissue control was subtracted from the experimentally determined rates to yield the reported activities as (nmol/min)/CDNB unit and (nmol/min)/mg protein.

Protein assays

Protein was estimated using the procedure of Lowry and Freedman (1951). Protein concentrations were expressed in mg/ml.

5.2.3 Measurement of thiol levels in tolerant vs. non-tolerant aorta

The levels of thiols (glutathione and cysteine) in rabbit aortic tissues were determined using an HPLC analysis technique with fluorimetric derivatization and detection (Haj-Yehia and Benet, 1993). Briefly 100 mg of aortic tissue, either made tolerant by treatment with 0.22 mM GTN (N=3) or by treatment with buffer control (N=3), were homogenized using 20 mM EDTA in 40% acetonitrile. Pre-column derivatization was performed using 2-(4-maleimidophenyl)-6-methylbenzthiazole yielding fluorescent derivatives with excitation λ = 310 nm and emission λ = 405 nm. The following HPLC conditions were used:

Column: ODS 25 cm Beckmann column

Mobile phase: 10 mM KH_2PO_4 ,

0.1% hexane sulphonic acid,

35% acetonitrile and pH was adjusted to 4.5.

Standard curves (Figures 5.3, 5.4) were made for standard solutions of glutathione and cysteine (0-10 nmol/ml) using N-acetyl cysteine as an internal standard. A typical chromatogram is depicted in Figure 5.5.

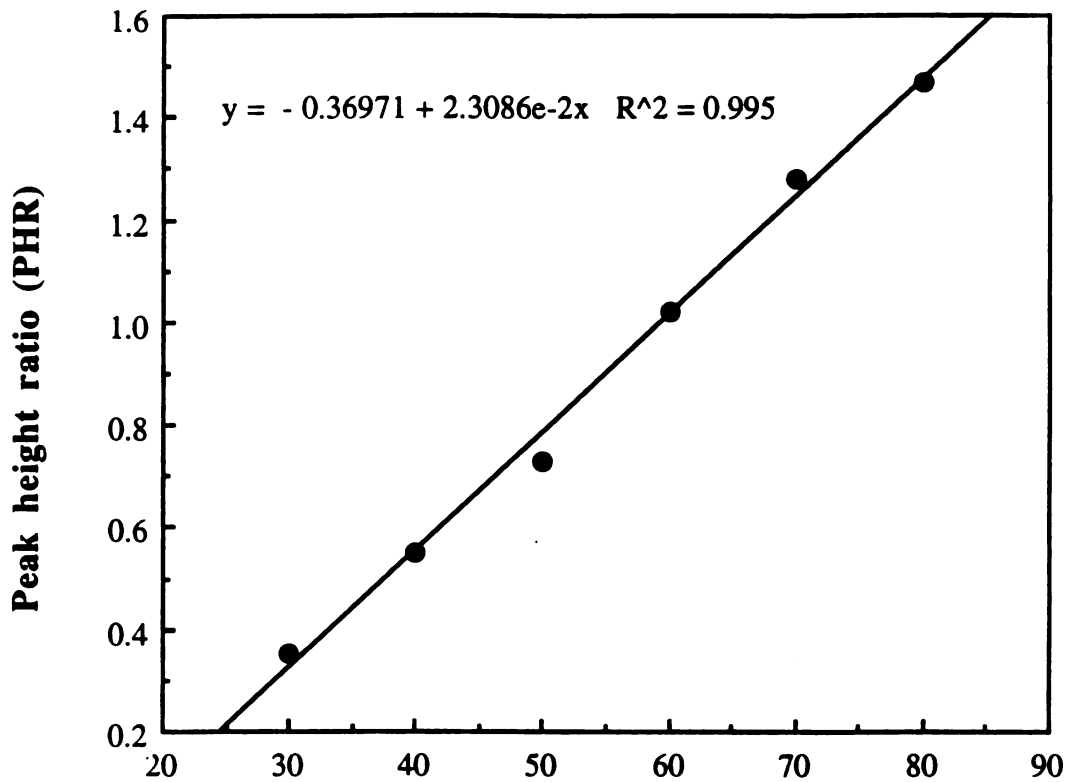


Figure 5.3 Standard curve for glutathione.

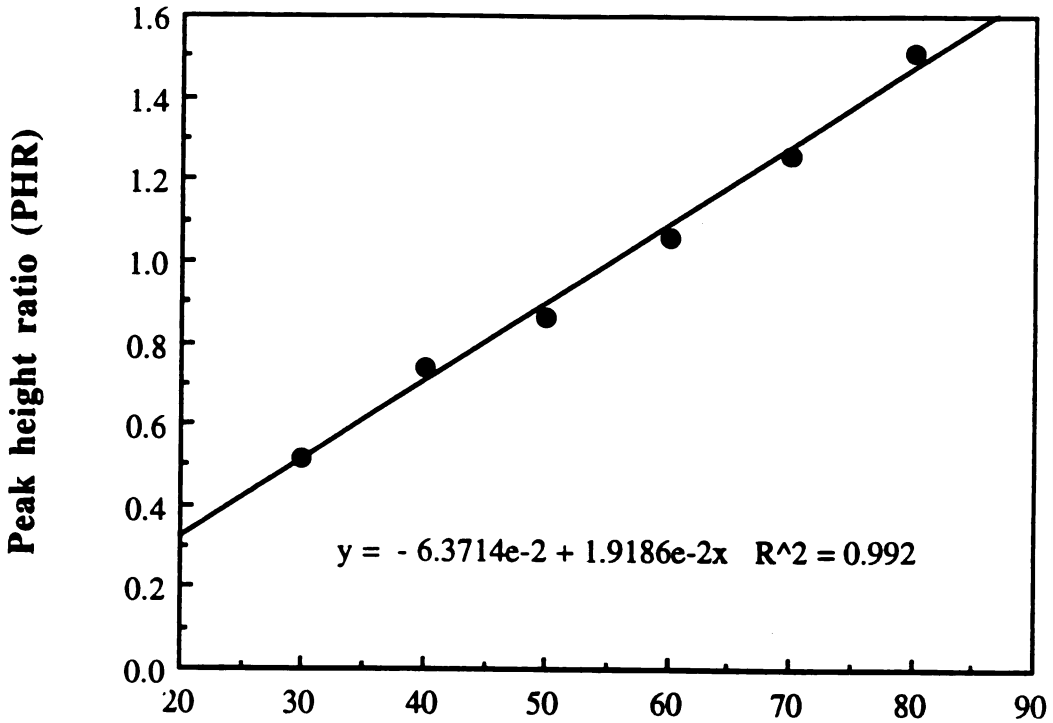
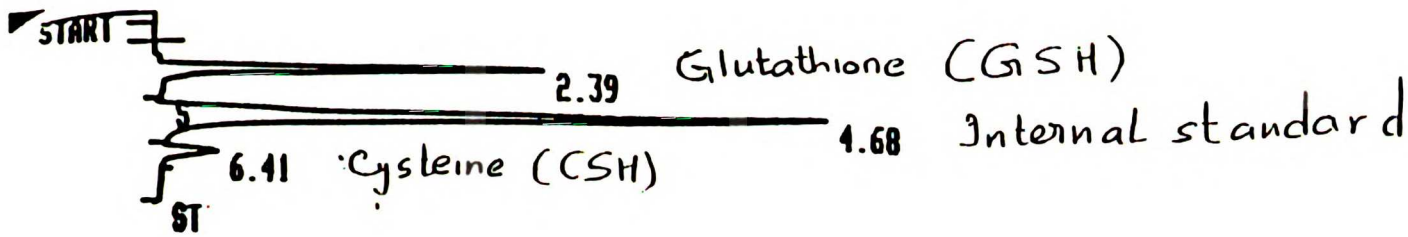


Figure 5.4 Standard curve for cysteine.



RUN # 242
WORKFILE ID: C
WORKFILE NAME:

HEIGHT%

RT	HEIGHT	TYPE	AR/HT	HEIGHT%	
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4.68	1848058	SPB	0.476	60.192	IS
6.41	146508	DTBB	0.402	4.772	Cysteine

Figure 5.5 A sample chromatograph of the analysis of thiols, glutathione and cysteine. Internal standard (I.S.) was N-acetyl cysteine.

5.2.4 S-nitroso-acetyl penicillamine response in GTN-pretreated aorta.

S-nitroso-penicillamine is a nitrosothiol compound that can directly activate guanylate cyclase. A protocol is described in Figure 5.6. Each aorta was divided into four equal strips, with each strip suspended in a separate tissue bath. Two strips were assigned as buffer pretreated and two as GTN pretreated strips. Strips 2 and 4 were treated with 0.22 mM GTN for an hour while strips 1 and 3 were pretreated with equal volume of buffer. After an hour the strips were washed about four times for a total period of 30 min and then contracted with a submaximal dose of PE. Strips 1 and 2 were then exposed to various concentrations of GTN while strips 3 and 4 were exposed to various concentrations of S-nitroso acetyl penicillamine (SNAP). A sigmoidal Emax model was used to fit the data, similar to that used for GTN.

5.3 Data analysis

Data are expressed as mean ± S.D. Statistical comparisons between the pre- and post tolerant enzyme activities, cGMP levels and % relaxation in the different rabbit aortas was made using paired t-tests. Statistical significance was accepted as $P< 0.05$. For every pair of aortic segments the following three ratios were calculated to describe the extent of tolerance development:

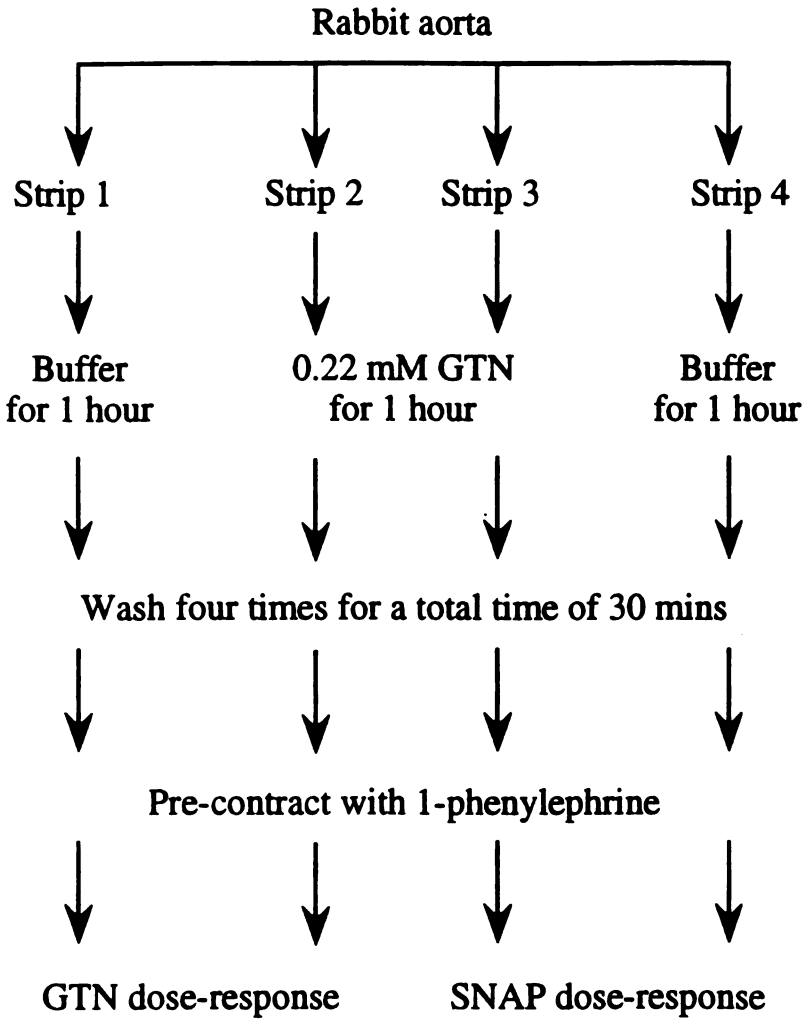

Figure 5.6 Protocol for SNAP cross-tolerance studies.

cGMP measurements:

$$\frac{\text{Tolerant}}{\text{Pre-tolerant}} = \frac{\text{Fold increase in cGMP to 0.5 μM GTN in tolerant aorta}}{\text{Fold increase in cGMP to 0.5 μM GTN in pre-tolerant control}}$$

Since both fold increase measurements are compared to the same baseline values this ratio may be calculated from cGMP measurements:

$$\frac{\text{Tolerant}}{\text{Pre-tolerant}} = \frac{\text{cGMP levels in response to 0.5 μM GTN in tolerant aorta}}{\text{cGMP levels in response to 0.5 μM GTN in pre-tolerant control}}$$

Relaxation measurements:

$$\frac{\text{Tolerant}}{\text{Pre-tolerant}} = \frac{\text{Relaxation by 0.5 μM GTN in tolerant aorta}}{\text{Relaxation by 0.5 μM GTN in pre-tolerant control}}$$

Enzyme activity measurements:

$$\frac{\text{Tolerant}}{\text{Pre-tolerant}} = \frac{\text{Enzyme activity in tolerant strip}}{\text{Enzyme activity in pre-tolerant control}}$$

All dose-response curves were prepared from the cumulative % relaxation following PE induced contraction in aortic strips. The individual data were fit to a sigmoidal Emax model and the parameters of the Hill equation were determined. The parameters obtained from the individual fits were averaged. The statistical comparisons for the Hill parameters for SNAP effects pre- and post-tolerance were done using the student Newman Keuls test. Statistical significance was accepted as $P < 0.05$.

5. 4 Results

5.4.1 Effect of pre-treatment with GTN on the response to additional GTN

The % relaxation in response to GTN can be described by a sigmoidal E_{max} type of relationship. The average value of E_{max} estimated from the model fitting was 116.8% while the average EC_{50} estimate was 1.07×10^{-7} M. The average Hill coefficient estimate was 0.866. Upon pretreatment with GTN (0.22 mM) for an hour, there was a marked decrease in the % relaxation. The curves for GTN dose-response in the GTN pre-treated strips were shifted to the right considerably as compared to the dose-response curves for the untreated tissues. In the case of the pretreated strips the effect did not reach a maximum at the highest concentration used, however, the effect at this concentration was significantly lower than the effect in the untreated strips which had already reached the maximum. The EC_{50} for the tolerant tissues could not be estimated. However, as seen in Figure 5.7, the % relaxations at the largest concentrations of GTN were much lower than the effects at those concentrations in the untreated strips. The dose-response curves are shown in Figure 5.7 and the Hill equation parameters for the pre-tolerant GTN dose response are shown in Table 5.1 (middle column). The significant rightward shift in the dose response curve for GTN with pretreatment (Figure 5.7) demonstrates the development of tolerance to GTN in our *in vitro* rabbit aortic strip model.

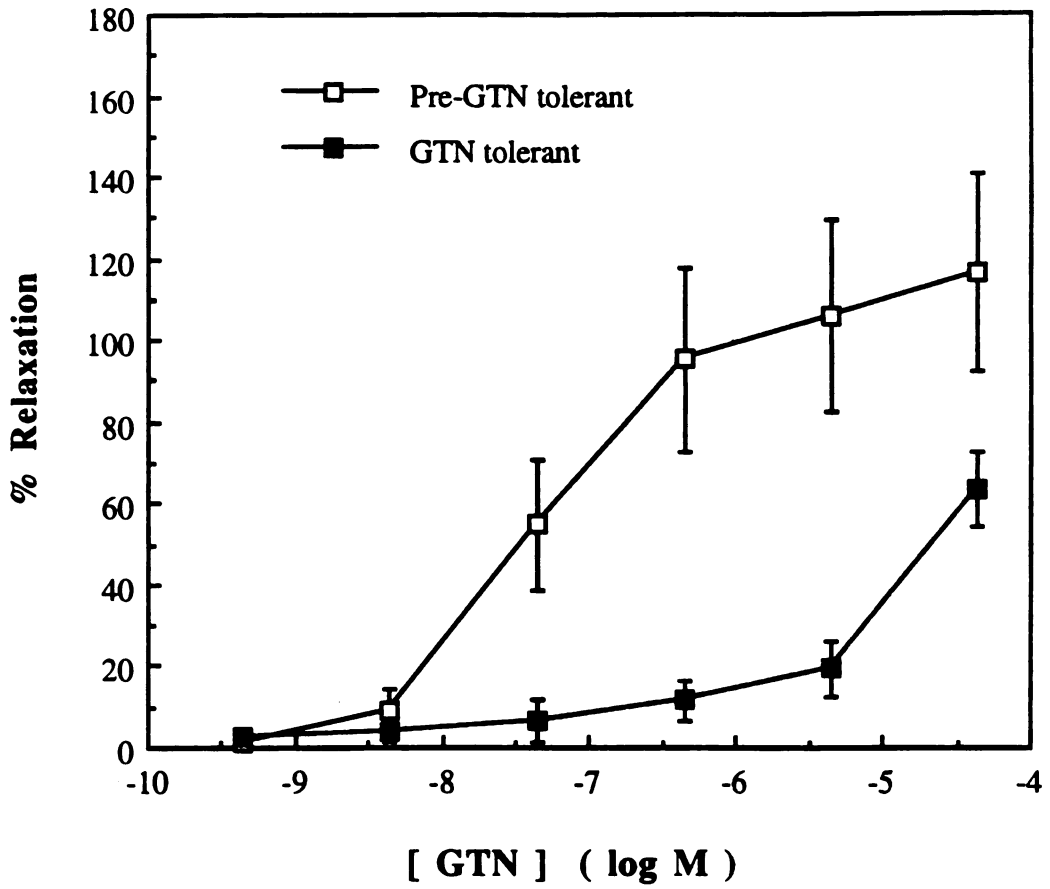

Figure 5.7 Dose response curve for GTN (pre and post tolerance development).

	GTN	SNAP
E_{max} (%)	116.8 ± 21.6	94.9 ± 5.6
EC_{50} (M)	$1.07 \times 10^{-7} \pm 1.29 \times 10^{-7}$	$2.14 \times 10^{-7} \pm 0.39 \times 10^{-7}$
γ	0.87 ± 0.33	0.97 ± 0.46

Table 5.1 Parameters of the sigmoidal E_{max} model fitted to GTN and SNAP dose-response data.

5.4.2 GTN responses correlate to the GST activities in tolerant tissues

cGMP levels were measured in response to GTN (0.5 μM) in rabbit aortae pre- and post tolerance development *in vitro*. In our previous studies with rabbit aortic strips we observed that this concentration of GTN could produce significant increases in cGMP levels. In segments that were pretreated with buffer control for an hour, cGMP levels were found to be 50.2 ± 11.0 pmol/g wwt (N=7), while the segments pretreated with a high concentration of GTN (0.22 mM) for an hour had lower cGMP levels of 26.4 ± 12.2 pmol/g wwt (statistically significant, paired t-test, $P<0.05$, N=7), indicating tolerance development (Figure 5.8, right hand side).

Two of these seven rabbit aortae showed resistance to the development of tolerance to GTN (tolerant/pre-tolerant ratio for cGMP response was > 0.9) , with mean cGMP levels of 44.2 pmol/ g wwt in pre-tolerant segments vs. 40.3 pmol/g wwt in tolerant segments (Figure 5.9 B). In the remaining five rabbit aortae, a significant difference was observed in the cGMP levels (Figure 5.9 A) upon inducing tolerance (52.6 ± 11.9 pmol/g wwt in pre tolerant vs. 20.9 ± 8.83 pmol/g wwt in tolerant segments, N=5).

There was a large variability in the extent of tolerance development in the various rabbit aortae. That is, the tolerant/pre-tolerant ratio for cGMP response varied from 0.18 to 0.93. No statistically significant difference was found in the cGMP levels between the pre-tolerant rabbit aortae that were precontracted with phenylephrine (N=4,

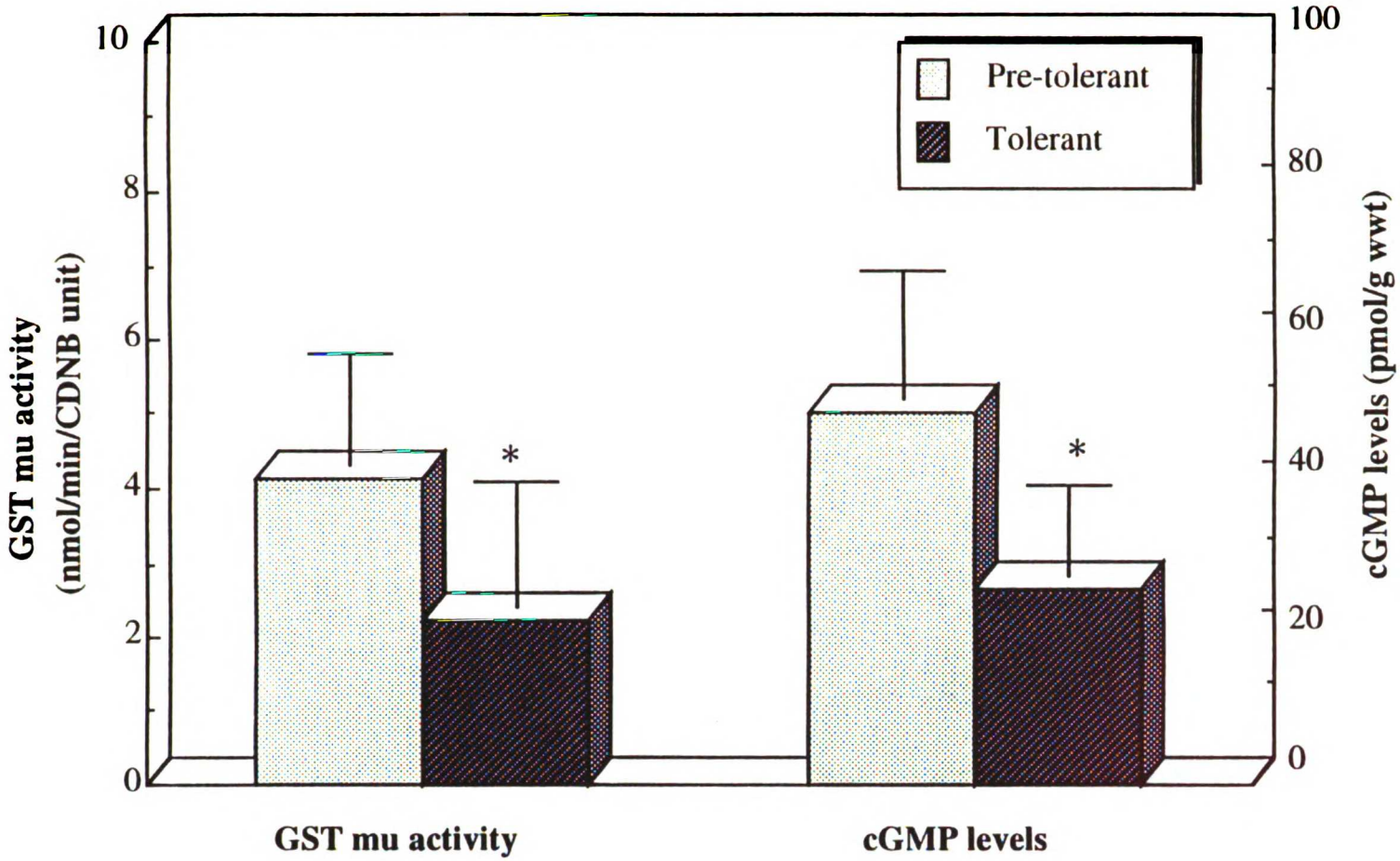

Figure 5.8 GST mu isozyme activity and cGMP levels were decreased by pre-treatment with GTN (0.22 mM). * Statistically significant difference, Paired t-test, $P < 0.05$.

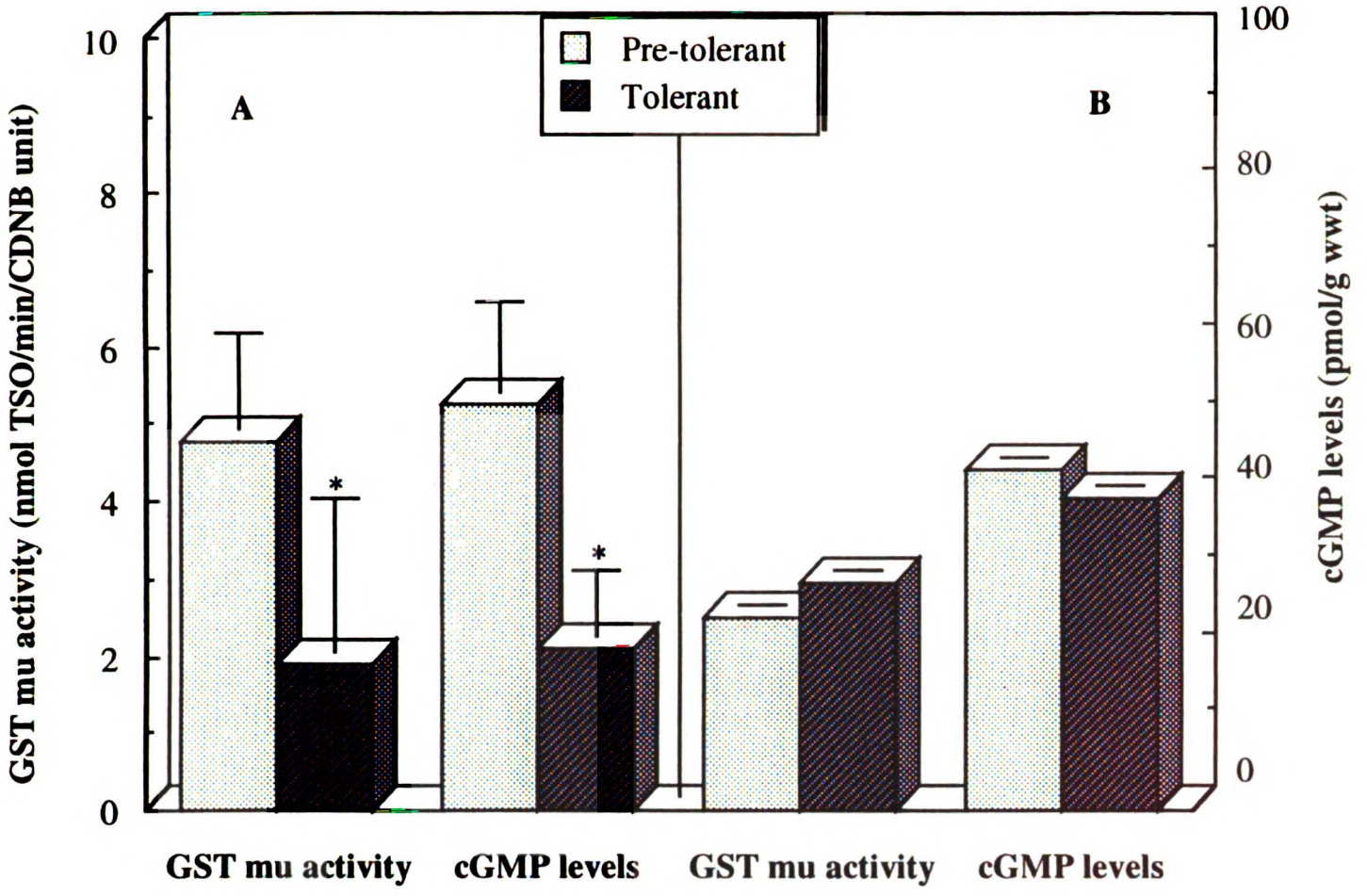

Figure 5.9 Comparison of the GST mu activity and cGMP levels in aortas that exhibited tolerance to GTN (n = 5, **A**) and the GST mu activity and cGMP levels in aortas that did not exhibit tolerance (n = 2, **B**). * Statistically significant differences (Paired t-test, $P < 0.05$, n=5, **A**).

53.8 ± 13.3 pmol/g wwt) for relaxation measurements and those that were directly treated with GTN (N=3, 45.3 ± 5.91 pmol/g wwt).

5.4.2.1 Percent relaxation in tolerant vs. pre-tolerant segments

The % relaxation in response to 0.5 μM GTN was measured in 4 of the 7 rabbit aortae studied. In the segments that were pretreated with buffer control for one hour, a 89.9 ± 7.6% relaxation to GTN was observed while a 47.0 ± 25.8% relaxation was observed in the segments pretreated with GTN (0.22 mM) for an hour (Figure 5.10). As observed with the cGMP levels, a considerable variability, ranging from 0.22 to 0.78 was observed in the tolerant/pre-tolerant ratios for the relaxation responses to GTN. The GTN tolerant strips that exhibited a low relaxation response to 0.5 μM GTN compared to their paired controls, also showed low cGMP levels compared to their pre-tolerant controls (Table 5.2). There was a good correlation (r^2=0.778) between relaxation and cGMP in the tolerant and pre-tolerant rabbit aortas (Figure 5.11).

5.4.2.2 Total GST activity in rabbit aorta during tolerance to GTN

Total GST activity in the segments pretreated with GTN (0.22 mM), 0.32 ± 0.06 μmol CDNB/min/mg protein was not significantly different from the total GST activity in the segments that were pretreated with same volume of buffer, 0.34 ± 0.10 μmol CDNB/min/mg protein. Additionally, protein concentrations were not significantly different in the tolerant segments (3.23 ± 1.09 mg/ml of aortic homogenate) vs. the pre-tolerant segments (3.55 ± 0.83 mg/ml of aortic homogenate).

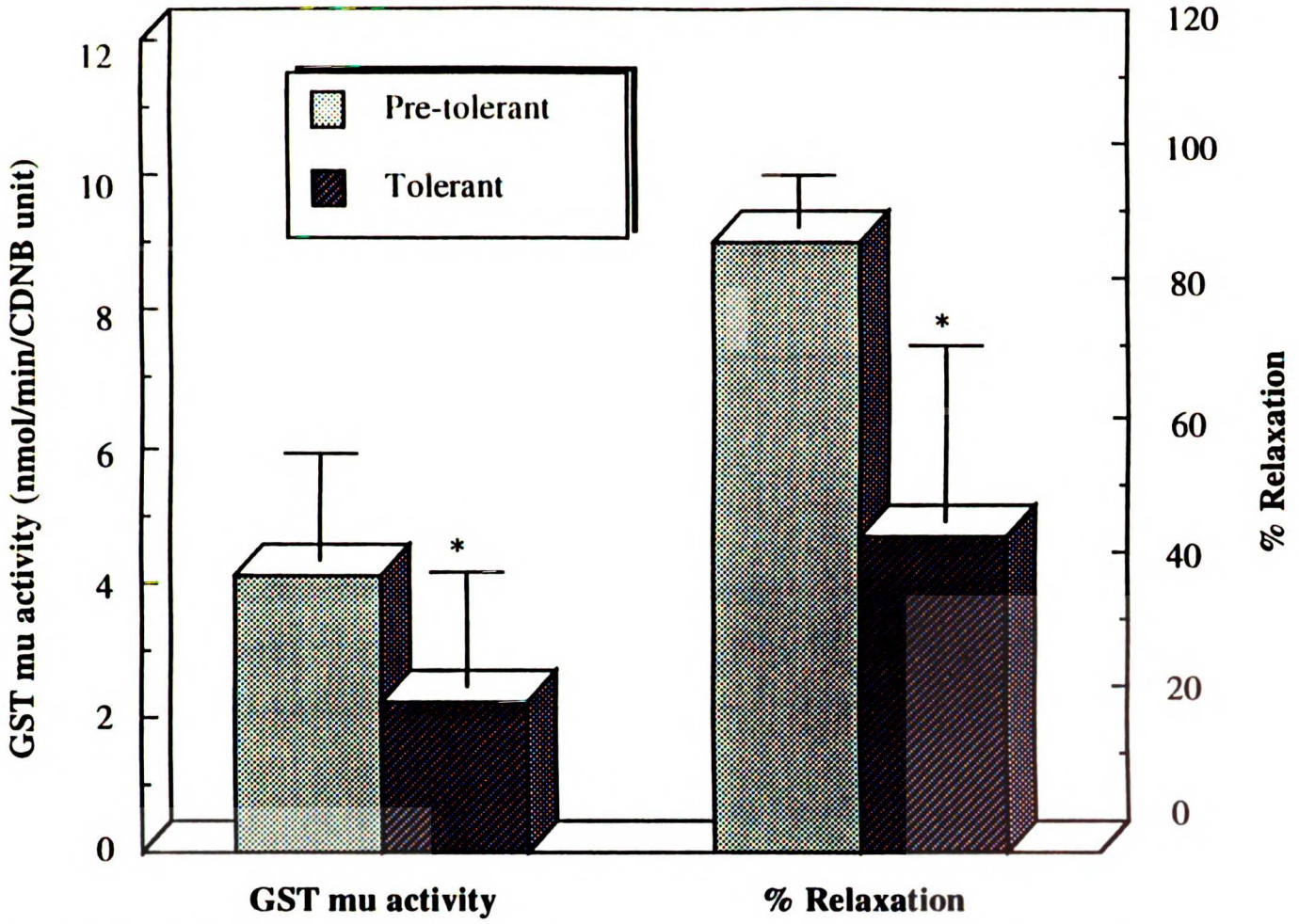

Figure 5.10 GST mu activities and relaxation responses in rabbit aortas pre- and post-tolerance to GTN. * Statistically significant difference, Paired t-test, $P < 0.05$.

Rabbit #	cGMP Tolerant/Pre-tolerant	Relaxation Tolerant/Pre-tolerant
1	0.901	0.785
2	0.443	0.328
3	0.296	0.221
4	0.926	0.785

Table 5.2 The tolerant/pre-tolerant ratios of the cGMP measurements and relaxation measures, compared in the four rabbit aortas.

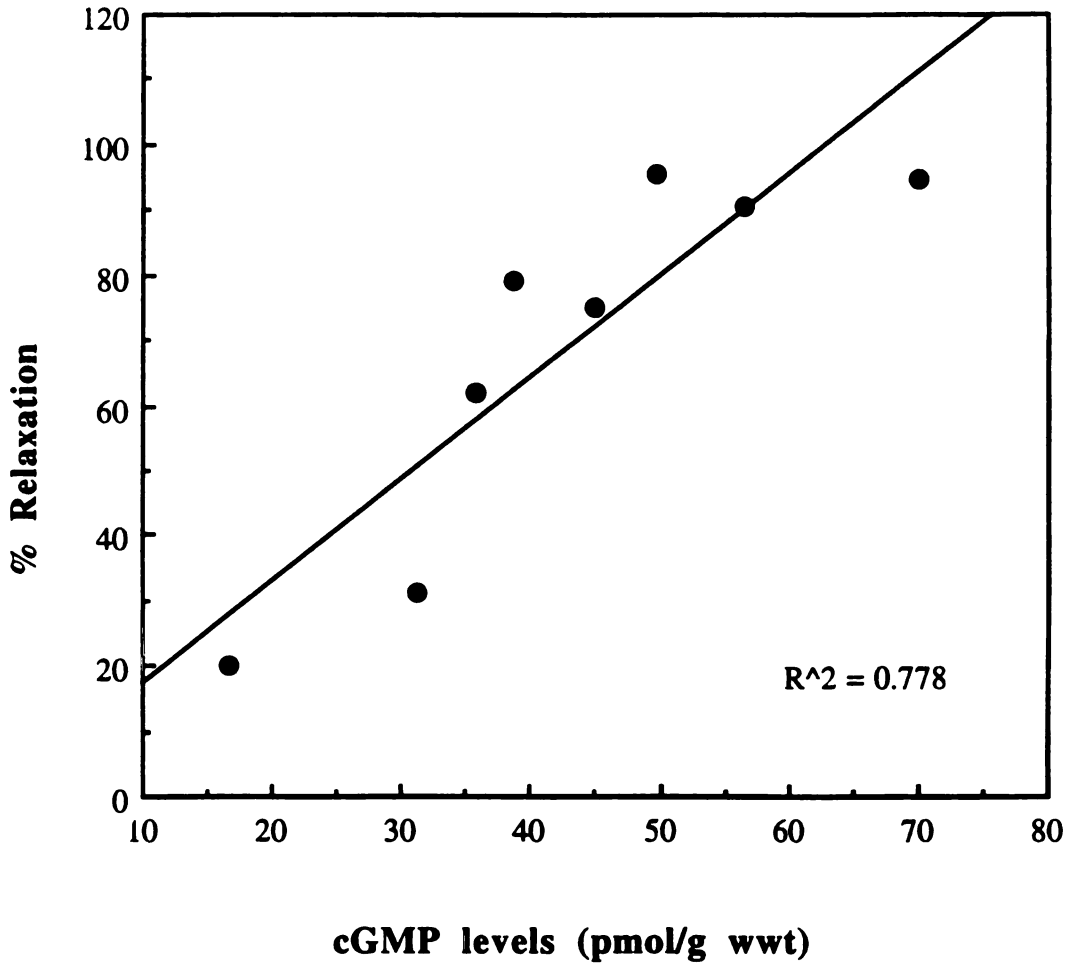

Figure 5.11 Plot of relaxation and cGMP levels produced in response to GTN (0.5 μM) in tolerant and nontolerant strips.

Glutathione S-transferase mu isozyme activity in GTN tolerant aortae

Activity of the mu isozyme of GST (normalized to total GST) was significantly lower in the segments that were pretreated with GTN (0.22 mM), 2.22 ± 1.71 nmol TSO/min/CDNB unit as compared to those that were not pretreated, 4.12 ± 1.53 nmol TSO/min/ CDNB unit, Figure 5.8. GST mu activity in two of the seven rabbit aortae studied remained unchanged (average activity of 2.96 TSO/min/CDNB unit in pre-tolerant vs. 2.51 nmol TSO/min/ CDNB unit in tolerant segments, Figure 5.9 B). In the remaining five aortae the mu isozyme activity in the tolerant segments was significantly lower than the pre-tolerant segments (4.76 ± 1.28 nmol TSO/min/CDNB unit in pre-tolerant vs. 1.92 ± 1.97 nmol TSO/min/CDNB unit in tolerant, N=5, Figure 5.9 A). On an equiprotein basis, GST mu activity in tolerant aortic strips was 61% of the GST mu activity in pre-tolerant strips (1.25 nmol TSO/min/mg protein in pre-tolerant vs. 0.76 nmol TSO/min/mg protein in tolerant segments of aortae).

Correlation of effect measures to the activity measures

A 40% decrease in the mean GST mu activity was observed in the 7 rabbit aortas, i.e., the mean tolerant/pre-tolerant ratio for the GST mu isozyme activity was 0.6. This correlated very well with the tolerant/pre-tolerant ratio of 0.55 for the cGMP response to 0.5 μM GTN (Figure 5.8, right hand side). Furthermore the tolerant/pre-tolerant ratio for relaxation response to 0.5 μM GTN was 0.53 which also correlated well with the tolerant/pre-tolerant ratio for GST mu activity, i.e. 0.6 (Figure 5.10). Furthermore upon closer inspection, it was seen that of the

7 rabbit aortas, 2 seemed to be resistant to becoming tolerant and the GST mu activities in those 2 rabbit aortas also remained unchanged (Figure 5.9 B). On the other hand the remaining five rabbits became tolerant (as demonstrated by the decrease in cGMP levels) and a corresponding decrease was also observed in the GST mu activity (Figure 5.9 A). When the individual tolerant/pre-tolerant ratios of the cGMP responses was compared to the tolerant/pre-tolerant ratios of the GST mu isozyme activities, a very good correlation, r^2=0.8 was also observed (individual data not shown).

5.4.3 Does thiol depletion explain this decrease in isozyme activity ?

The levels of glutathione and cysteine were measured in aortic tissues made tolerant to GTN and compared to those treated with a buffer control. Glutathione concentration in the tolerant strips was 0.82 ± 0.18 µmole/g wwt compared to 0.70 ± 0.07 µmole/g wwt in the pre-tolerant strips. Cysteine concentration in the tolerant tissue was 0.12 ± 0.03 µmole/g wwt compared to 0.11 ± 0.01 µmole/g wwt. There was no significant difference (paired t-test) between the levels of the two thiols in tolerant vs. pretreated aortic strips (Figure 5.12).

5. 4. 4 Cross-tolerance to SNAP in the strips made tolerant to GTN

The potency of SNAP and GTN in the rabbit aortic strips was comparable. Strips of rabbit aorta which were pretreated with a high dose of GTN did not show a decreased response to SNAP (Figure 5.13), while aortic strips from each of these rabbits showed significant tolerance to

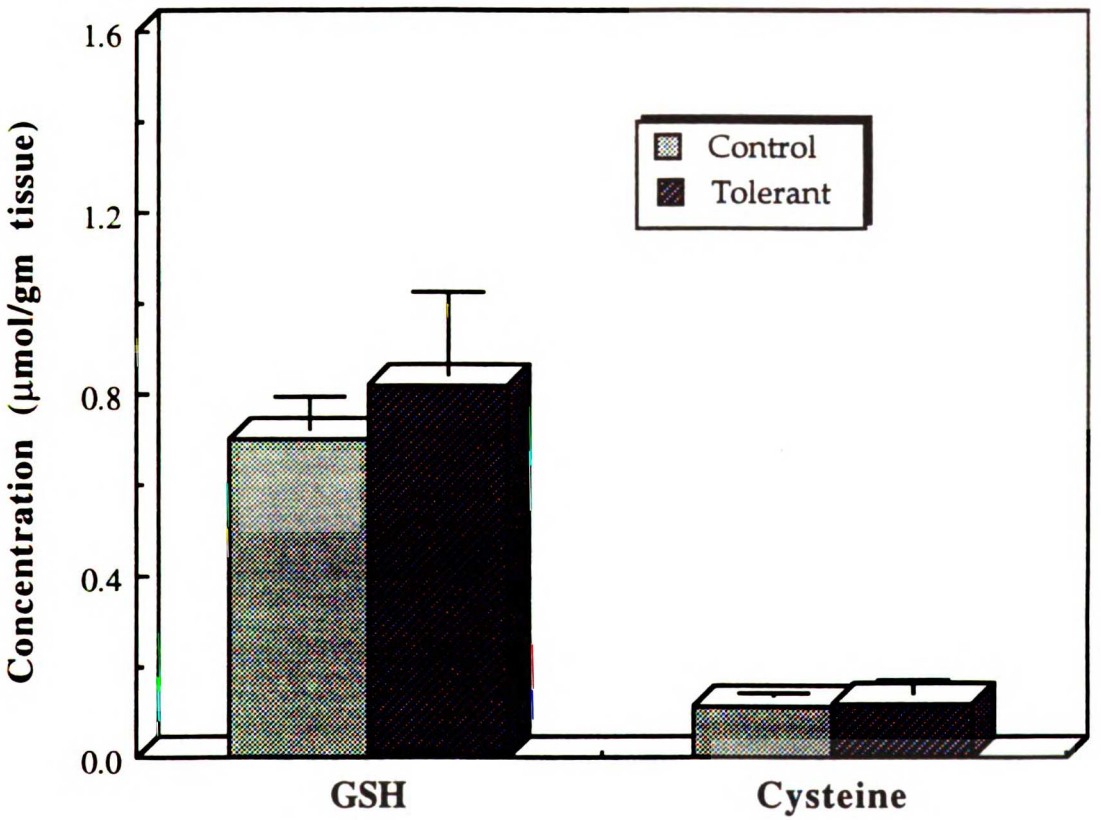

Figure 5.12 Concentrations of glutathione (GSH) and cysteine in control and GTN pre-treated strips.

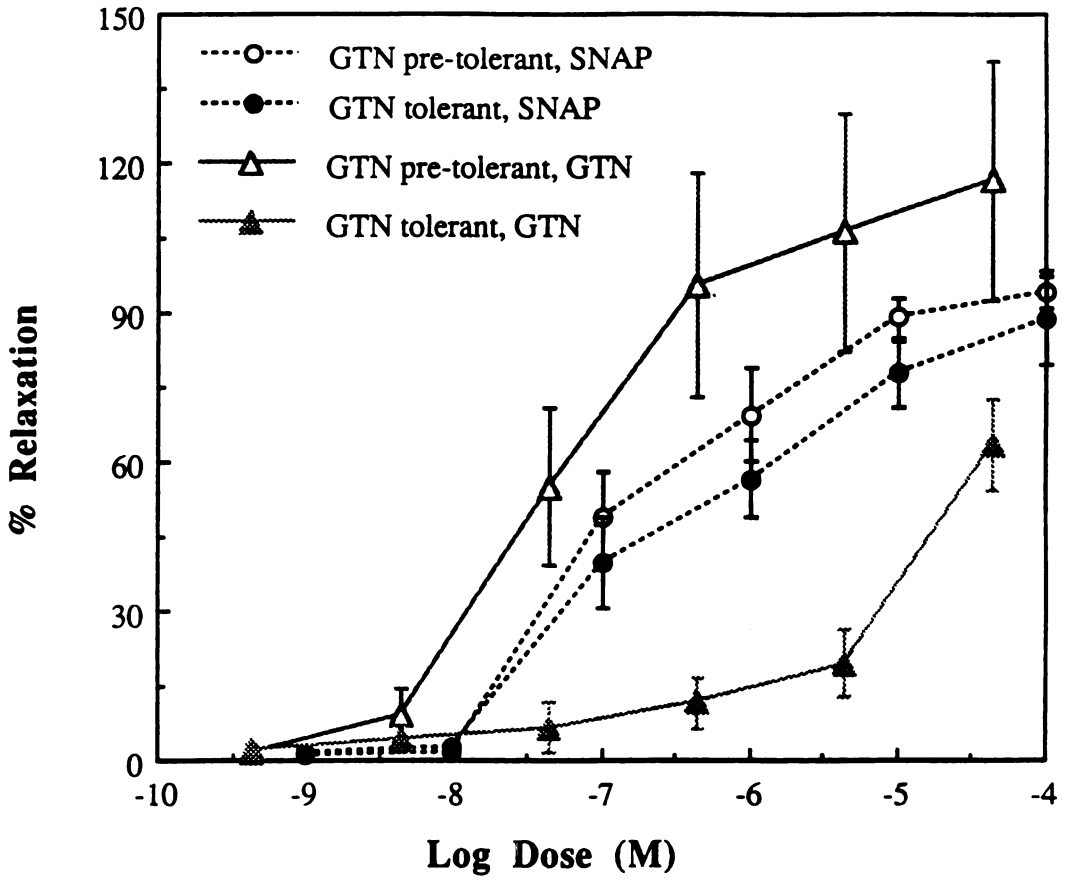

Figure 5.13 Dose response to GTN (solid lines) and SNAP (dotted lines) in control and GTN pre-treated strips.

GTN . The dose-response relationship was a typical sigmoidal E_{max} type of relationship and therefore the data was fitted to a model, for which the parameters E_{max}, EC_{50} and Hills coeffecient were determined. No significant changes were observed in the parameters for SNAP (Table 5.3).

5. 5 Discussion

While tolerance to the hemodynamic effects of GTN is well known, the mechanism of tolerance development remains controversial. A reduced intracellular metabolic activation of GTN, either due to a decrease in the cofactors that are essential for the reactions or a decrease in the enzyme activity (or alteration of the crucial enzyme) have been proposed as a mechanisms for tolerance development to GTN. Such a supposition is supported by the findings of Fung and Poliszczuk, (1986), Slack *et al.* (1989), Bennett *et al.* (1989), Mulsch *et al.* (1989), Brien *et al.* (1986), Forster *et al.* (1991) and Schror *et al.* (1991). Other hypotheses have been proposed to explain the development of tolerance to GTN such as a decrease in the guanylate cyclase activity (Axelsson and Karlsson, 1984; Ahlner *et al.*, 1986; Kukovetz and Holzmann, 1987; Schroder *et al.*, 1990) or reduced intracellular thiol levels in the vascular smooth muscle cells (Needleman and Johnson, 1973; Torresi *et al.*, 1985; May *et al.*, 1987; Noack, 1990). All these mechanisms are depicted in Figure 5.14.

Studies were carried out as described in this chapter to investigate tolerance development and more specifically to evaluate the role of GST mu isozyme in this process. We have been able to

	Pre-tolerant	GTN-tolerant
E_{max} (%)	94.9 ± 5.6	104.3 ± 30.6
EC_{50} (M)	$2.14 \times 10^{-7} \pm 0.39 \times 10^{-7}$	$1.49 \times 10^{-6} \pm 1.69 \times 10^{-6}$
γ	0.97 ± 0.46	0.71 ± 0.51

Table 5.3 Parameters of the sigmoidal E_{max} model fitted to SNAP pre- and post-tolerance dose-response data. No statistically significant differences were observed between the parameters, pre- and post-tolerance, Student Newman Keuls test.

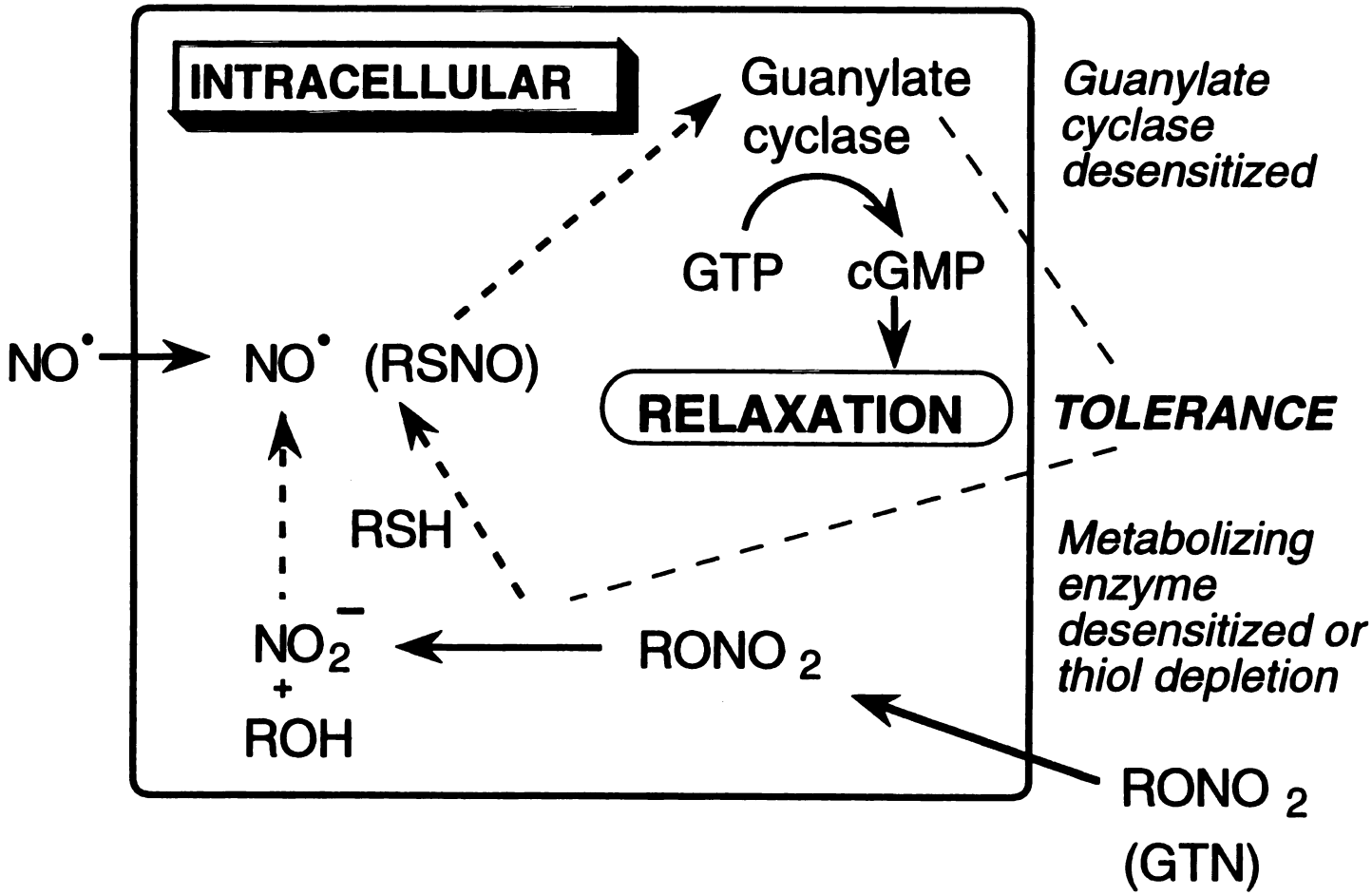

Figure 5.14 Possible mechanism of tolerance to GTN.

demonstrate the development of tolerance (reduced response to GTN) by pretreatment of the isolated aortic strip with a high concentration of GTN. This method of inducing tolerance has been previously demonstrated by Rosen *et al.* (1987), Marks *et al.* (1989), Slack *et al.* (1989) and Kowaluk and Fung (1990).

To evaluate the cause/s of GTN tolerance, the three different hypothesis of GTN tolerance were investigated. It is our hypothesis that the mu isozyme of GST is a major catalyst in the metabolism and bioactivation of GTN. Therefore, we measured the activity of this isozyme in individual rabbit aortas, pre and post GTN pretreatment. We found that the GST mu isozyme activity was significantly reduced in tolerant tissues vs. the pre-tolerant tissues. This is consistent with the observations of Chern et al. (1991) who found that there was about a 50% decrease in GST mu activity upon induction of GTN tolerance *in vivo*. The total GST activity did not change upon pretreatment with GTN for one hour. This indicated a specific alteration of the mu isozyme activity only. Although no direct cause/effect can be established, we compared the reduced enzyme activities to the reduced response to GTN in each individual rabbit aorta. The ratios of the tolerant to pre-tolerant cGMP levels seen in the rabbits compared well with those of % relaxation upon GTN pre-treatment. Two of the seven rabbits exhibited less than a 10% decrease in response to GTN after the GTN pretreatment. We arbitrarally set greater than 10% reduction in effect as a cut-off for defining that a aortic tissue became "tolerant". Therefore two of the seven rabbit aortas appeared resistant to the development of tolerance. Interestingly the GST mu isozyme activity in those two rabbits also did not decrease.

The good correlation we observed could indicate that the decreased response to GTN could be a result of the decreased enzyme activity/level. However it could also be a secondary effect of some other biochemical change in the vascular smooth muscle which then produces a decrease in the enzyme activity. At this point our results do not differentiate between these two possibilities and it is very difficult to determine if the second mechanism is occurring. However it is reasonable to say that a decrease in GST mu isozyme may be a possible reason for the tolerance to GTN, based upon the good correlation observed between the response to GTN and the isozyme activity.

The positive aspects of this study are that for the first time such a correlation between the isozyme activity and the response to GTN has been demonstrated. Furthermore each rabbit aorta served as its own control and this problem of GTN tolerance could be examined on an individual basis at the site of action, using a biochemical marker of response such as cGMP formation.

To determine if desensitization of guanylate cyclase could play a role in the development of tolerance to GTN, we studied the effect of GTN pretreatment on the dose response of SNAP, a S-nitrosothiol which either directly or via the spontaneous liberation of nitric oxide activates guanylate cyclase and therefore does not require a metabolism step. The dose-response of SNAP was practically unchanged pre and post-GTN treatment. The Hill equation parameters were unchanged, indicating that in our *in vitro* model of tolerance, the decreased response to GTN is not due to a desensitization of guanylate cyclase. In these studies we looked at SNAP dose response in tolerant aorta in which a decreased

response to GTN was observed suggesting that tolerance to GTN is specific and is probably due to a step(s) involved in the formation of the vasoactive species and not in the step(s) related to the further actions of the vasoactive species. Our experiments, therefore, indicate that desensitization of guanylate cyclase is probably not be a major cause of tolerance in our *in vitro* model of tolerance.

Similar conclusions were also made by Berkenboom *et al.* (1988) and Holtz *et al.* (1989) for their studies where the responses to SIN-1 a compound that liberates nitric oxide spontaneously were not affected by GTN tolerance *in vitro* or *in vivo*. Boesgaard *et al.* (1994) in plasma and vessels, Mulsch *et al.* (1989) in cultured aortic smooth muscle cells, Fung *et al.* (1989b) and Henry *et al.* (1989a) both in isolated blood vessels have also made the same conclusion.

To examine thiol depletion as a possible mechanism for tolerance development, concentrations of thiols, glutathione and cysteine were measured in aortic tissues made tolerant to GTN and compared to their non tolerant controls. There was no difference between the levels of thiols pre- or post tolerance. This suggests that in our *in vitro* model of tolerance, thiol depletion was not important. It also indicates that the decrease in isozyme activity that we observed in tolerant tissues was not due to a decrease in thiol cofactors, but is probably a direct result of inactivation of the isozyme. These results indicate that depletion of intracellular thiols could not be an explanation for intracellular tolerance, either directly or via a decrease in the activity of the isozyme/enzyme.

To summarize, our studies have shown that GST mu isozyme activity is reduced in GTN tolerant tissues and that this decrease correlates well with the decrease in the effect of GTN, as measured by cGMP levels. Overall our studies indicate that in our *in vitro* model, GTN tolerance in rabbit aortic strips is accompanied by an inactivation of GST mu isozyme. These results point towards a possible role of GST mu in the bioactivation and tolerance development to GTN.

The enzyme inactivation observed could be via a suicidal mechanism where the enzyme is inactivated by a metabolite whose formation it catalyzes. Evidence for a metabolite induced inhibition of the enzyme systems involved, has been presented by Cossum and Roberts (1985) and Cossum *et al.* (1986). Such a metabolite inhibition may also be operative in degradation/elimination reactions and could possibly explain the reduced plasma clearance and accumulation of metabolite after infusion of organic nitrates *in vivo*, as shown for GTN and ISDN by Sutton and Fung (1984), Noonan *et al.* (1985) and Chong and Fung (1989). This mechanism also requires further probing.

The phenomenon of resistance to GTN tolerance, as was demonstrated in two of the rabbits examined has been reported in clinical cases where some individuals require much higher doses/time to develop tolerance than others. In the rabbits that did not become tolerant we did not increase the dose of GTN. It would be interesting to see if those higher doses could eventually render these tissues tolerant. Finally the process of tolerance to GTN can be and has been reversed, in the clinic and also in animals following a nitrate free interval. This process will be investigated

CHAPTER 6

CAN TOLERANCE TO GTN BE REVERSED?

6.1 Background

GTN tolerance is a significant clinical problem. Many reports have suggested that intermittent GTN treatment, incorporating a daily nitrate-free interval, can avoid the tolerance associated with continuous GTN therapy. This has lead to treatment regimens such as 12 hour GTN patches followed by a GTN free 12 hour period or administration of smaller doses of GTN over 14 day periods. In *in vitro* preparations of isolated coronary arteries (Henry *et al.*, 1989), rat abdominal aortic rings (Mehta *et al.*, 1991; Kowaluk *et al.*, 1989b), and in cell cultures (Schröder *et al.*, 1988), such a reversal of effects has been demonstrated by washing the tissues free of nitrates. However this process has not been well characterized.

Schröder *et al.* (1988) showed that rat lung fibroblast cells made tolerant to GTN could be rendered effective or responsive by overnight incubation in nitrate free media without addition of thiols. Furthermore these authors also suggested that incubation with cycloheximide, a protein synthesis inhibitor decreased the recovery. This led Schröder et al. (1988) to hypothesize that reversal is a function of synthesis of a protein essential for the mechanism of action of GTN.

Henry *et al.* (1989) showed that the reversal of tolerance was dependent on the GTN pre-exposure concentration, duration of GTN exposure, as well as the GTN free-period. Kowaluk *et al.* (1989) showed that GTN tolerance could be avoided by use of an intermittent GTN exposure vs. a continuous exposure. Furthermore a sufficiently long wash time was useful in reversing tolerance. Mehta *et al.* (1991) showed that in

rat abdominal aortic rings a second exposure of GTN decreased the effect if done 30 or 60 min later but not 120 min following the first exposure.

In Chapter 5 we successfully demonstrated the development of tolerance (decreased effect of GTN) in our *in vitro* rabbit aortic strip model. We also observed a decrease in the GST mu isozyme activity in tissues made tolerant to GTN. We therefore decided to investigate if this tolerance to GTN could be reversed by washing (similar to a nitrate free interval in clinical situations), to characterize the optimum times and frequencies of washing that would lead to a significant reversal of tolerance and also to study if the GST mu isozyme activity is changed from its low activity in tolerant tissues to a higher activity in reversed tissues.

Therefore, the objectives of this work are:

1. To demonstrate the *in vitro* reversal of tolerance in rabbit aortic strips.

2. To understand whether this reversal is due to metabolite removal or due to the synthesis of new metabolizing enzyme.

6.2 Methods

Most of the techniques utilized here follow procedures described in previous chapters, as well as by Lau and Benet (1992) and Kenkare and Benet (1993). Briefly: seven male New Zealand white rabbits (Nitabell Rabbitry, Hayward, CA), weighing 2 to 3 kg, were used in this study. The rabbits were anesthestized using a subcutaneous

injection of ketamine (40 mg/kg as 1.0 ml of a 100 mg/ml solution in Water for Injection) and decapitated 15 minutes later. The thoracic cavity was exposed and the descending aorta was surgically removed and immediately placed in a beaker containing Krebs buffer (NaCl, 119 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25mM; $CaCl_2$, 2.5 mM; glucose, 11.1mM), which was continuously gassed with carbogen (95% oxygen and 5% carbon dioxide). After isolation of the aorta, excess fat and connective tissues were carefully removed; during this process the buffer was constantly replaced with fresh buffer. The endothelium was not disturbed. The aorta was then helically cut and divided into four equal strips. The experiments then performed are described below. A general protocol is shown in Figure 6.1.

6.2.1 Effect of different washing times on the recovery of GTN tolerance

The four aortic strips were treated as follows. One of the four strips was contracted with phenylephrine (PE), as described in Chapters 3, 4 and 5 and the relaxation response to GTN (0.5 μM) was determined. This strip was then exposed to 0.22 mM GTN for an hour, followed by either a 30 min washing period (buffer was changed once every 10 min during this period) or a 60 min washing period (buffer was changed once every 10 min during this period) or a 120 min washing period (buffer was changed once every 10 min during this period). Following the washings, the strip was precontracted with PE and the response to GTN (0.5 μM) was determined. The remaining three strips

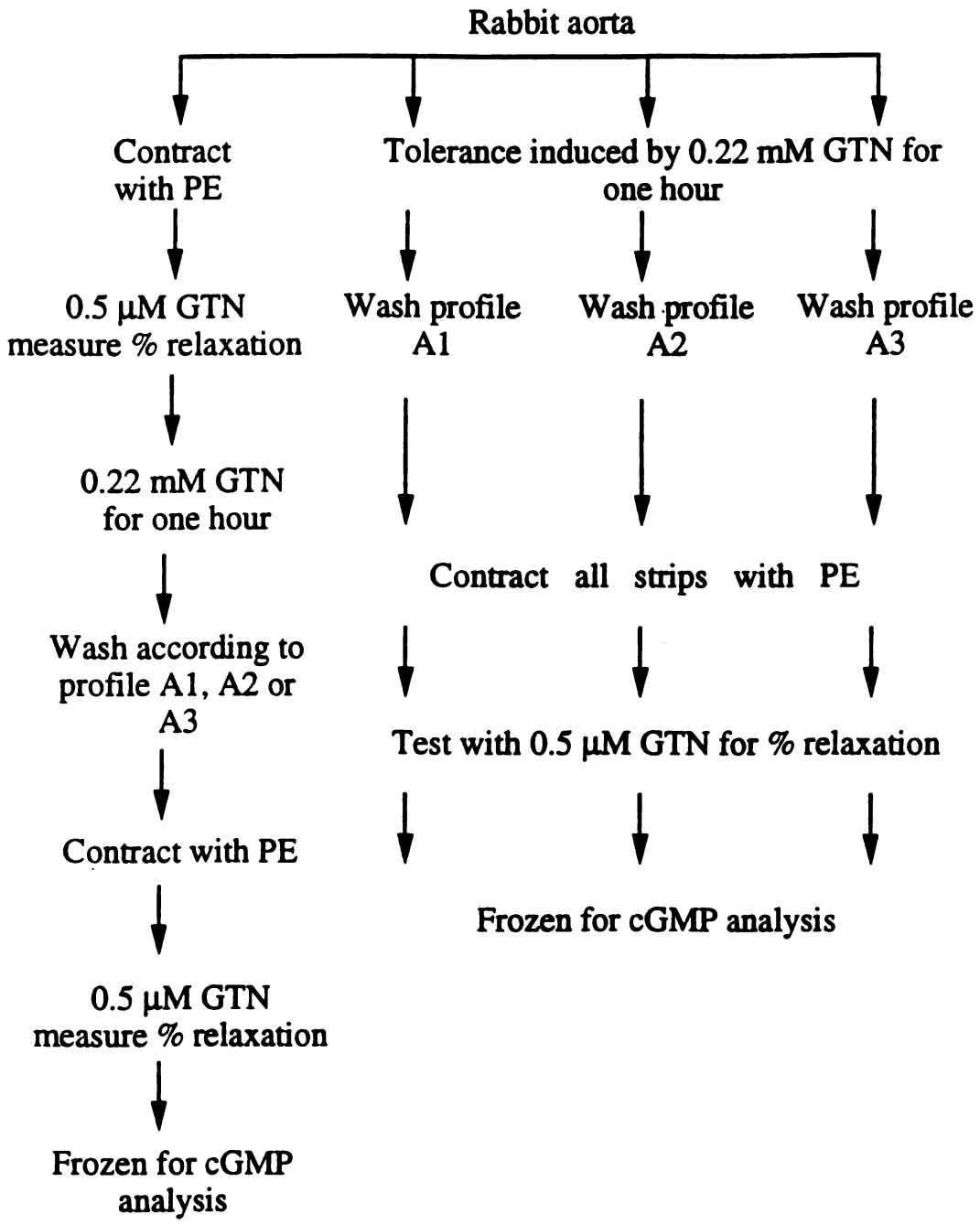

Figure 6.1 General protocol for the reversal of tolerance studies.

were first treated with 0.22 mM GTN for an hour, after which they were washed in the following fashion:

Strip 2: Change buffer every ten minutes for 30 min (A1).

Strip 3: Change buffer every ten minutes for 60 min (A2).

Strip 4: Change buffer every ten minutes for 120 min (A3).

The washed strips were contracted with PE and relaxation was measured using 0.5 μM GTN. All samples were then frozen quickly on dry ice for cGMP analysis. cGMP analysis was carried out as previously described in Chapter 5.

6.2.2 Effect of frequency of washings on tolerance reversal

Four aortic strips were treated as follows. One of the four strips was contracted with phenylephrine (PE) and the relaxation response to GTN (0.5 μM) was determined. This strip was then exposed to 0.22 mM GTN for an hour, followed by either a 30 min washing period (buffer was changed once every 5 min during this period) or a 30 min washing period (buffer was changed once every 10 min during this period) or a 30 min washing period (buffer was changed once every 15 min during this period). Following the washings, the strip was then precontracted with PE and then the response to GTN (0.5 μM) was determined. The remaining three strips were first treated with 0.22 mM GTN for an hour, after which they were washed in the following fashion:

Strip 2: Changed buffer every five minutes for 30 min (A1).

Strip 3: Changed buffer every ten minutes for 30 min (A2).

Strip 4: Changed buffer every fifteen minutes for 30 min (A3).

The washed strips were contracted with PE and relaxation was measured using 0.5 μM GTN. All samples were then frozen quickly on dry ice for cGMP analysis.

6.2.3 Effect of time between washings and total washing time on tolerance reversal

Four aortic strips were treated as follows. One of the four strips was contracted with phenylephrine (PE) and the relaxation response to GTN (0.5 μM) was determined. This strip was then exposed to 0.22 mM GTN for an hour, followed by either a 30 min washing period (buffer was changed once every 5 min during this period) or a 60 min washing period (buffer was changed once every 20 min during this period) or a 120 min washing period (buffer was changed once every 40 min during this period). Following the washings, the strip was precontracted with PE and the response to GTN (0.5 μM) was determined. The remaining three strips were first treated with 0.22 mM GTN for an hour, after which they were washed in the following fashion:

Strip 2: Buffer changed every five minutes for 30 min (A1).

Strip 3: Buffer changed every twenty minutes for 60 min (A2).

Strip 4: Buffer changed every forty minutes for 120 min (A3).

The washed strips were contracted with PE and relaxation was measured using 0.5 μM GTN. All samples were then frozen quickly on dry ice for cGMP analysis.

6.2.4 Control experiment

In a separate set of experiments, the effect of washing a non-tolerant tissue was determined to prove that the reversal observed is not due to an improved effect due to washing which may occur even in non-tolerant tissues.

For this purpose, each of four strips was suspended in a jacketed water bath chamber and allowed to stand for either 0 min, 30 min (buffer changed every 10 min in this period), 60 min (buffer changed every 10 min during this period) and 120 min (buffer changed every 10 min during this period). At the end of the wash periods the strips were contracted with PE and the response to 0.5 μM GTN was determined.

6.2.5 Enzyme activity

In a separate set of experiments, the aorta from each of 6 rabbits were divided into two segments. Each of these segments was suspended into individual muscle bath chambers and treated with 0.22 mM GTN for an hour. After one hour incubation, one of the segments was washed for a short period of time (0 or 30 min) while the other was washed for a longer period of time (60 or 120 min). The buffer was changed once every 10 min over the entire 30, 60 and 120 min periods. At the end of the washing period the strips were frozen on dry ice and prepared for enzyme analyses. For a pair of segments from each rabbit aorta, one was therefore washed for a shorter time while the other for a longer time. Thus a pairwise comparison could be made of the enzyme activities in these rabbit aortas. The GST mu isozyme activity, total GST

activity and protein levels were measured using methods previously described in Chapters 4 and 5.

6.3 Data analysis

The % relaxation and cGMP levels obtained from each treatment were averaged. All data is expressed as mean ± SD for % relaxation and cGMP levels, GST mu, total GST activity and protein levels. All statistical analysis for the % relaxation and cGMP levels were carried using ANOVA and Student Newman Keuls test while the analyses for enzyme activities were done using paired t-test. $P<0.05$ was denoted as a statistically significant difference.

6.4 Results

6.4.1 Effect of washing times on recovery of GTN tolerance

As shown in Figure 6.2, aortic strips had 94.7 ± 5.0 % relaxation in response to 0.5 μM GTN. Upon pre-incubation with GTN for an hour and washing for 30 min the relaxation was decreased to 29.2 ± 11.4 % while after 60 min washing the relaxation was about 47.0 ± 14.1%. Upon washing for 120 min the relaxation was measured to be 81.3 ± 7.1 %. Thus a significant increase in relaxation response was observed upon washing the tissue for longer times. The frequency of washing was kept constant in this set of experiments.

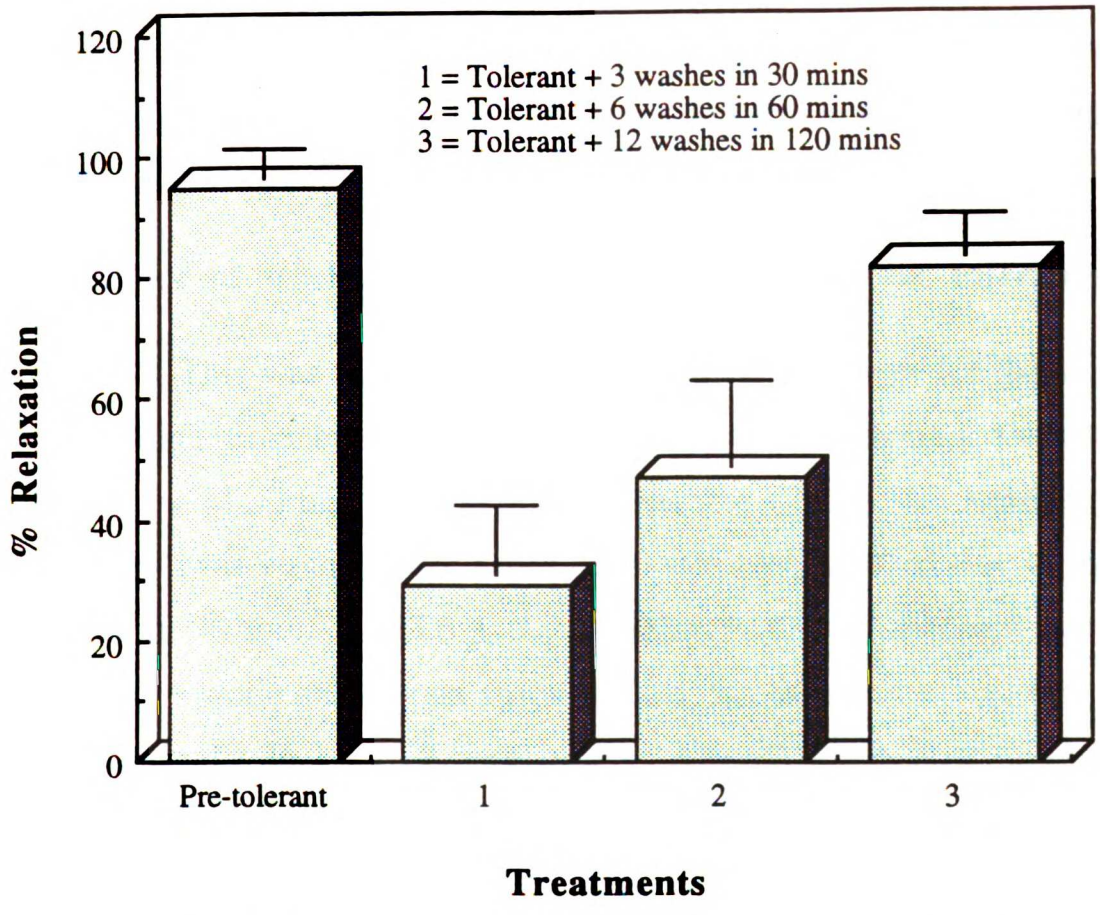

Figure 6.2 Effect of different washing times (same frequency of washing) on relaxation response to GTN (0.5 μM).

cGMP levels also increased from 37.9 ± 9.3 pmol/g wwt after 30 min of washing tolerant tissues to 47.3 ± 12.4 pmol/g wwt after 60 min washing. After washing for 120 min the cGMP levels were increased to 66.2 ± 5.8 pmol/g wwt (Figure 6.3). Due to the variability in tolerance development, statistically significance was not observed at $P < 0.05$ for 60 min vs. 30 min washings, although the trend was towards an increase in cGMP levels. At 120 min the cGMP levels were significantly higher than after 30 min washing.

6.4.2 Effect of washing frequency on recovery from tolerance

This was tested by washing the different strips for the same total washing time but at different frequencies of washing, i.e. total washing time of 30 min but one of the strips was washed two times in 30 min, second was washed three times in 30 min and the third was washed six times in 30 min. A 88.6 ± 4.9 % relaxation was observed in the control strips while the tolerant strips washed two times for a total time of 30 min showed a 26.2 ± 12.9 % relaxation. Upon washing three times for a total period of 30 min the % relaxation in response to 0.5 μM GTN was 36.8 ± 22.2 % and upon washing six times over a period of 30 min, they showed a 35.7 ± 12.5 % relaxation. There was no statistically significant difference between the responses of the three groups of strips, washed at different frequencies for a period of 30 min (Figure 6.4).

Strips washed two times over a total period of 30 min had cGMP levels of 33.1 ± 2.03 pmol/g wwt while those washed three times had levels of 30.5 ± 4.2 pmol/g wwt. Strips washed six times in 30 min

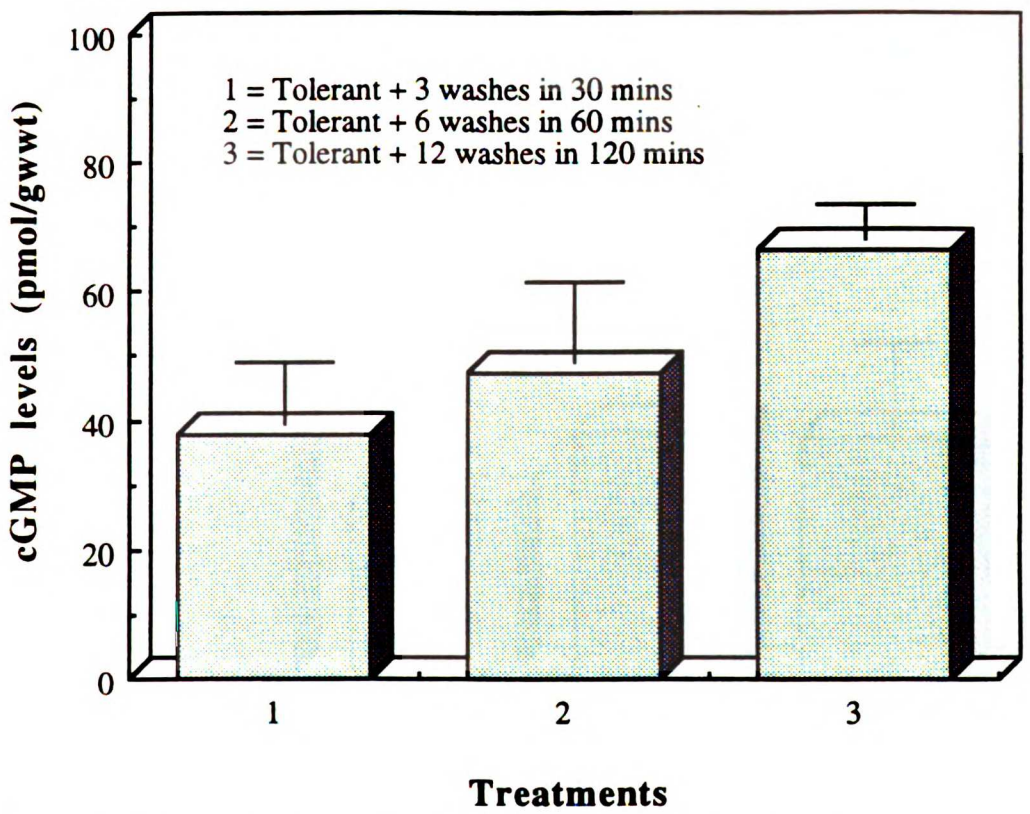

Figure 6.3 Effect of different washing times (same washing frequency) on the cGMP levels produced in response to 0.5 μM GTN.

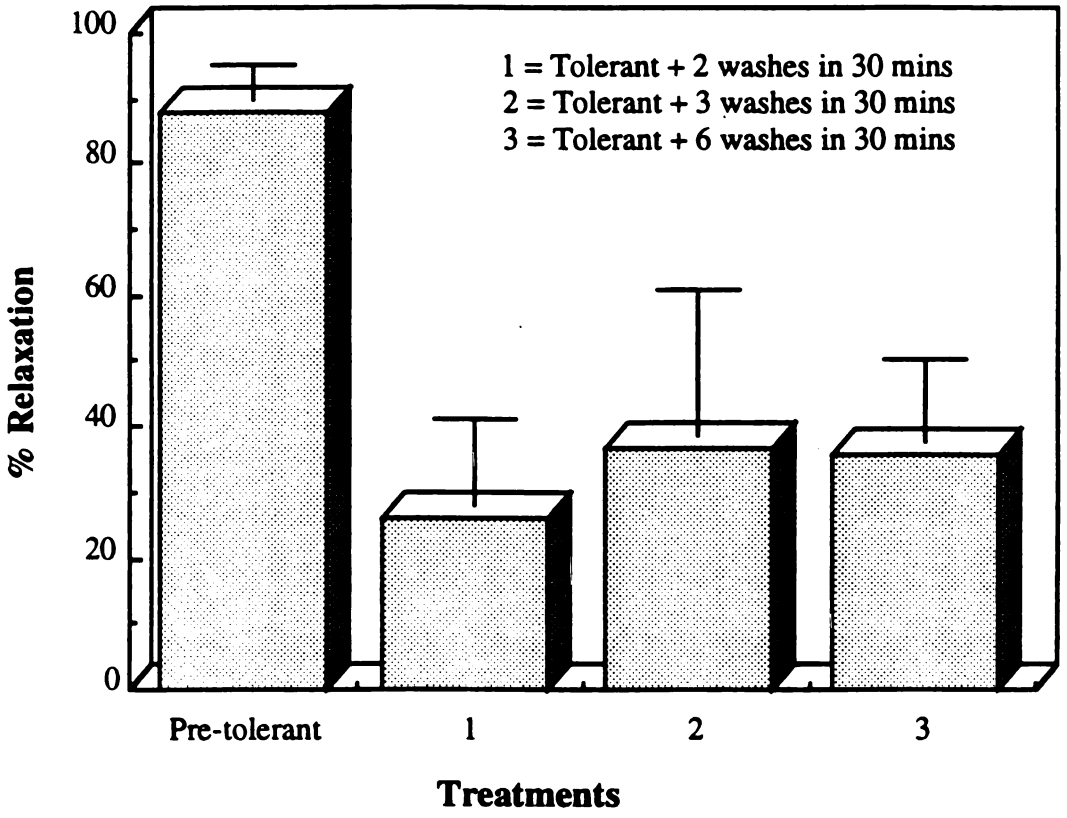

Figure 6.4 Effect of different washing frequencies on the relaxation response to 0.5 μM GTN.

had cGMP levels of 39.2 ± 10.3 pmol/g wwt. There was no statistically significant difference between the levels in the three groups (Figure 6.5).

6.4.3 Effect of washing times and frequency on recovery from tolerance

As demonstrated in Figure 6.6, a 95.0 ± 8.7 % relaxation was observed in the pre-tolerant aortic strips which was then decreased to 22.8 ± 13.9 % relaxation post-tolerance when buffer was changed every 10 minutes over a period of 30 min. In the strips where the buffer was changed every 20 min over a period of 60 min, the response was 52.0 ± 7.2 % while in the strips whose buffer was changed every 40 min over a period of 120 min, the response was 64.0 ± 12.8 %.

cGMP levels in the 30 min wash were 40.2 ± 7.9 pmol/g wwt while the levels in the 60 min wash averaged 50.7 ± 9.9 pmol/g wwt. Upon washing for 120 min the levels of cGMP were found to be 69.8 ± 4.7 pmol/g wwt (Figure 6.7). This trend was very similar to that of the experiments in section 6.3.1, where the total times were the same as in these experiments although the frequency of washing was higher.

In the control experiment, no difference in the % relaxation was observed in response to GTN in the strips washed for 0, 30, 60 and 120 min without any GTN pretreatment (data not shown).

6.4.4 Measurement of GST activity

In this set of experiments, for every pair of segments made from a single aorta and pretreated with the high concentration of GTN, one was washed every 10 min over a shorter period of time (0 or 30 min)

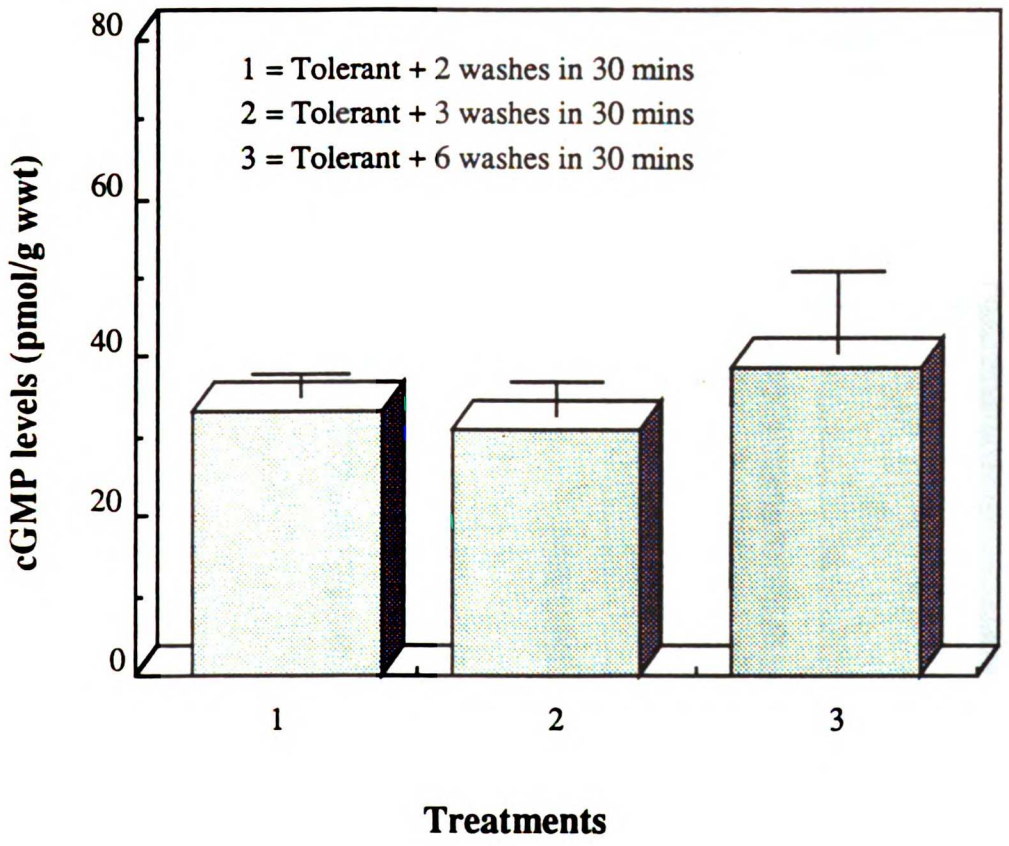

Figure 6.5 Effect of different washing frequencies on the cGMP levels produced in response to 0.5 μM GTN.

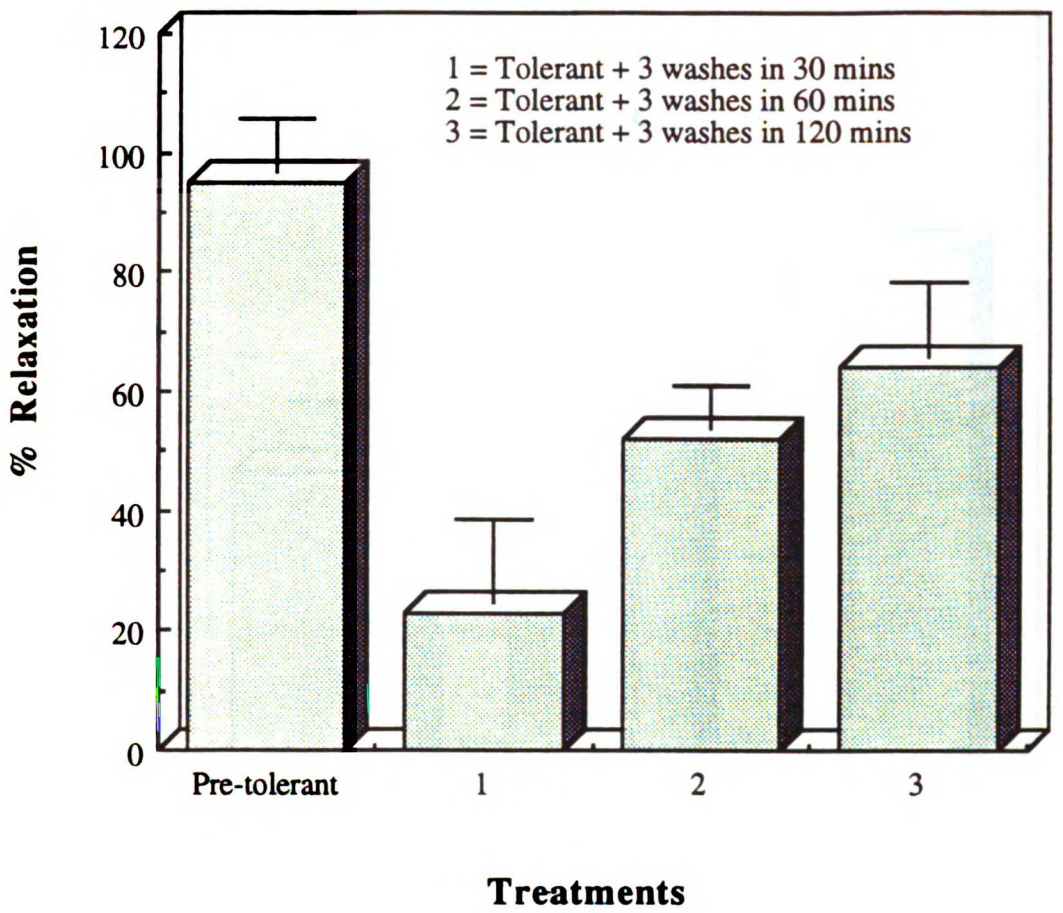

Figure 6.6 Effect of different washing times and frequencies on the relaxation response to 0.5 μM GTN.

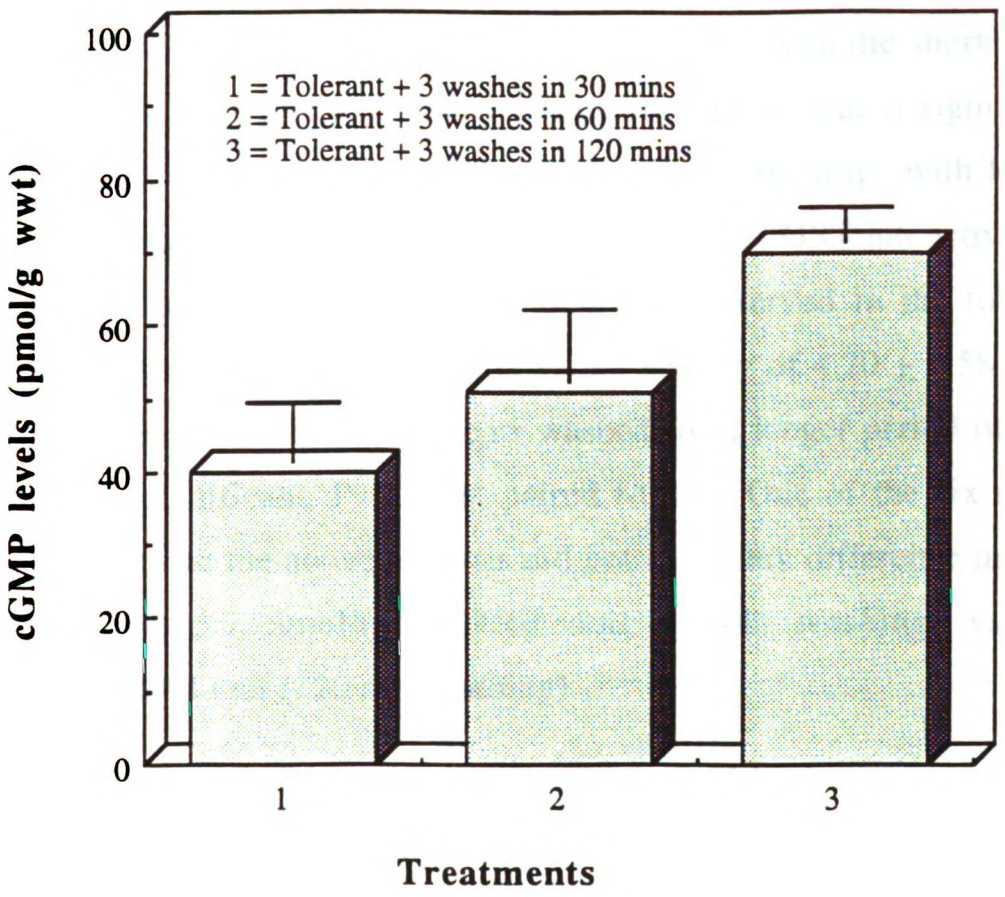

Figure 6.7 Effect of different washing times and frequencies on the cGMP levels produced in response to 0.5 μM GTN.

while the other was washed every 10 min over a longer period of time (60 or 120 min). At the end of the total periods the strips were frozen and the enzyme activities were then determined.

The GST mu activities in the six rabbit aortas with the shorter and longer washes post tolerance were compared. There was a significant increase in the activity of the GST mu isozyme in the strips with longer washes as compared to those with shorter washes. A GST mu activity of 2.15 ± 0.98 nmol TSO/min/ CDNB unit was observed in the tolerant segments washed for a shorter period vs. an activity of 4.20 ± 0.58 nmol TSO/min/CDNB unit in the segments washed for a longer period of time (statistically significant, P= 0.006, paired t-test). One of the six rabbit aortas included in the above analysis did not show any difference in GST mu activities, 3.7 nmol/min/CDNB unit (0 min washing) vs. 3.8 nmol/min/CDNB unit (120 min washing).

Total GST activity was also compared in the six rabbits and no significant difference was observed (Paired t-test, P=0.256). The total GST activity in the rabbit aorta segments washed over shorter time periods was 0.43 ± 0.11 μmol CDNB/min/mg protein as compared to 0.39 ± 0.11 μmol CDNB/min/mg protein in the segments washed over longer time periods.

6.5 Discussion

Reversal of tolerance to GTN has been reported clinically as well as in animals and in *in vitro* preparations of tissues and cell cultures.

In this chapter we were able to demonstrate a reversal of the tolerance to GTN *in vitro* in our rabbit aortic strip model. In rat abdominal aorta, Mehta *et al.* (1991) showed that a second exposure to GTN 30 or 60 min following the first exposure resulted in tolerance vs. a second exposure at 120 min which did not result in tolerance. Therefore it was of interest to determine in our rabbit aortic strip model the relationship between time of washing and recovery of tissue from the tolerant state after pretreatment with a high concentration of GTN. Furthermore it was not clear if the effect of washing on the reversal of tolerance is due to the removal of GTN/metabolite or due to a synthesis of new enzyme to make up for an inactivated metabolic enzyme. Our studies were undertaken to better elucidate this process.

Our previous studies have shown that the tolerance to GTN is accompanied by a decrease in GST mu isozyme activity in aortic tissue with no changes in total GST activity. Therefore it was also our objective to determine the isozyme activity in tissues washed after tolerance development to study any possible changes in activity from the reduced activity in tolerant tissues.

In the first set of washing experiments, we observed that the recovery of the strips from the tolerant state towards its pre-tolerant state was dependent on the total washing time. Thus the strips washed for a total period of 120 min showed close to the pre-tolerant response, although the strips washed for 30 min exhibited a very low response to the same concentration of GTN. We found that a test dose given 2 hours after tolerance development (by GTN pretreatment) produced a response close to that observed in pre-tolerant state. These results are consistent with the

observations of Mehta *et al.* (1991) who found that the second dose of GTN would produce the same response as the first only after a washing period of about 2 hours. In our experiments all the strips were washed at the same frequency. Therefore it was difficult to conclude if the longer washing time or the more number of washes was responsible for the better recovery in the 120 min washed tissue vs. the 30 min washed tissue.

In order to determine the true effect of efficient washing on the recovery process, we carried out the second set of experiments where all the strips were washed over the same time period, but the frequency of washing was varied. Although the strips were all washed over the same time period, some were washed only twice while others were washed three or six times. The relaxation and cGMP response to GTN (0.5 μM) were not significantly different in the different treatments. These results indicate that no matter how fast or slowly the tissues are washed, recovery is determined by the time before which the next exposure is made.

We then repeated the experiments in the first set, where we increased the time of washing from 30, 60 to 120 min. However we also washed the strips the same number of times i.e. three times. Thus the efficiency of washing was the same but the total length of washing time was different. Although the % relaxation at 120 min in this experiment was slightly lower (but not significantly different) than that in the previous experiment (section 6.2.1), we observed a very similar trend in the response to 0.5 μM GTN in this experiment even though the strips were not washed as efficiently. These results are in agreement with our results from section 6.4.2, which also indicate that the reversal process

seems to be a function of the total period before the next exposure to GTN, rather than the efficiency of GTN removal.

Since it has been suggested that tolerance to GTN is due to a decrease in the activity of the metabolizing enzyme, the recovery of tolerance could be due to an increase in enzyme activity to pretolerance levels. Although there is evidence that tolerance could also be due to a desensitization of guanylate cyclase (Axelsson and Karlsson, 1984; Kukovetz and Holzmann, 1987; Ahlner *et al.*, 1986; Schröder *et al.*, 1990) our experiments with SNAP in Chapter 5 have already shown that this probably does not occur in our model. As we observed a decrease in GST mu isozyme activity in tolerant tissues in Chapter 5, we were interested in investigating the possibility of the GST mu isozyme being reactivated during the washing process.

We observed an increase in GST mu isozyme activity in 5 of the 6 rabbit aortas upon washing for 1 or 2 hours vs. their matched pairs which were either not washed or washed for only half an hour. The one rabbit aorta that did not show any change could be an example of a resistant rabbit as shown in Chapter 5. Furthermore in this rabbit aorta, since the tissue was washed up to 120 min with no effect on activity, it is unlikely that it is not yet recovered since in most of the aortas that we studied, the response to GTN was close to pre-tolerant response after 120 min of washing. These observations of increased activity of the isozyme indicate that the isozyme is being revived. This could be either due to removal by washing of metabolite(s) that inactivates the isozyme, or due to protein synthesis returning the level of destroyed protein to its pre-tolerant level. Because Schröder *et al.* (1988) found that in rat lung

fibroblast cells, incubation with a protein synthesis inhibitor such as cycloheximide decreased the recovery, the latter explanation is possible. However no direct conclusion in this regard can be made at this time.

Thus our results indicate that the reversal of *in vitro* tolerance to GTN can be demonstrated. These results also show that the reversal of tolerance by washing is probably a time dependent phenomenon which may or may not be assisted by the washing process. Furthermore washing the aortic strips for longer time periods causes an increase in GST mu isozyme activities from their low tolerant values. The mechanism of this increase in isozyme activities requires further investigation.

CHAPTER 7

SUMMARY OF FINDINGS

7.1 Selective denitration of glyceryl nitrates

A selectivity in denitration of GTN via GSTs has been observed in different tissues and different species of animals. In order to investigate this selectivity in denitration, 1,2-GDN, a metabolite of GTN, with equivalent probability of denitration from either of two positions was used as a model compound and its metabolism in rabbit aortic homogenates was studied. Furthermore, the effects of ethacrynic acid (ECA) and sulfobromophthalein (SBP), two GST inhibitors on the formation rates of the two metabolites, 1-GMN and 2-GMN were investigated.

The 1,2-GDN degradation rates were significantly reduced upon co-incubation with ECA and SBP indicating an involvement of GSTs in the denitration process. 2-GMN was the predominant product of 1,2-GDN metabolism. This preferential formation of 2-GMN indicates a preference for C-1 denitration. Pretreatment with ECA produced a specific decrease in the formation of 2-GMN while the formation of 1-GMN was unaffected. The decrease in 2-GMN formation rate correlated well with the decrease in 1,2-GDN degradation rate. SBP however, produced a non specific decrease in the formation of both metabolites. Furthermore the inhibition by SBP of 2-GMN formation was less than that observed for ECA, indicating that SBP may inhibit both isozymes but to a lesser extent. 1-GMN probably results from a pathway which is distinct from the formation of 2-GMN.

7.2 Responses to GTN: inhibitor studies

cGMP (formed by activation of the enzyme guanylate cyclase) has been proposed to be the second messenger for GTN induced vasodilation. The concentration of this second messenger and the relaxation response to GTN (0.6 μM) were measured in intact rabbit aortic strips. The effect of ECA (0.2 mM) pretreatment on these responses to GTN was studied. The ECA pretreatment led to a decrease in the GTN induced vasorelaxation in all the strips tested. This was accompanied by a parallel decrease in the levels of cGMP in the strips pretreated with ECA compared to those without ECA pretreatment. A correlation was observed between the decrease in the relaxation response and the cGMP increase in response to GTN. These results strengthen the hypothesis that the enzyme involved in the vascular metabolism of GTN possesses activity to ECA and belongs to the GST family.

The possible nonspecific effects of ECA on the cascade of events in GTN bioactivation was studied. There was no decrease in the cGMP levels produced in response to sodium nitroprusside (10^{-5} and 10^{-6} M), a compound that produces nitric oxide by a mechanism distinct from that of GTN. The basal levels of cGMP in the strips pretreated with ECA were also not different from the basal levels of cGMP in the strips that were not pretreated with inhibitor. These results suggest that the decrease in GTN responses observed after ECA pretreatment are probably due to a specific effect on the metabolizing enzyme and not due to a non-specific effect on guanylate cyclase or some other step in the production of effects to GTN.

7.3 Correlation between the GST activities in different tissues and pharmacologic response to GTN

The activity of the mu isozyme of GST can be measured using a specific substrate trans-stilbene oxide (TSO), while total GST can be quantitated using 1-chloro 2,4-dinitrobenzene (CDNB). These isozyme activities and the responses to GTN were measured in aortic tissues from different rabbits and the relationship between them was evaluated.

A very good correlation was observed between the increase in cGMP levels and the TSO activity across rabbits (r^2=0.948) while total GST activity did not correlate very well with these responses (r^2=0.099). This indicated that GST mu activity (TSO activity) and not the total GST activity (CDNB activity) is a good marker of the biochemical response to GTN in rabbit aorta. A marked but somewhat lower correlation was observed between the TSO activity and relaxation in response to GTN (r^2=0.715). The TSO activity normalized to CDNB units correlated with the GTN responses better than the TSO activity normalized to amount of protein.

In examining the correlation between the activity of the GST mu isozyme (TSO activity) in aortas, mononuclear leukocytes and livers, we have shown that this activity in the RMLs does not correlate well (r^2=0.234) with that in rabbit aortas. A poor correlation was also observed between the TSO activities in the aorta and in RMLs while the CDNB activities in the aortas and mononuclear leukocytes correlated well. In another set of rabbits, TSO activities in the RMLs correlated very well with the TSO activities in the livers. In comparing the activities of GSTs

and the mu isozyme in these tissues, we observed that the fraction of GSTs which have activity to TSO in the RMLs and in rabbit aorta is much larger than the fraction found in the liver. These results suggest that the mu isozyme with activity to TSO in the aorta may be different from the isozyme in liver and RMLs and that the GST mu isozyme activity in the aorta correlates with the vascular response to GTN in the rabbit.

7.4 Investigating tolerance to GTN

Tolerance to GTN was studied in our rabbit aortic strip model. Aortic strips were pretreated with a high concentration of GTN (0.22 mM) and the development of tolerance was demonstrated by measuring the relaxation response at different concentrations of GTN (0.5 μM). In seven rabbits responses to GTN and the enzyme activities were measured in aortas in which each aorta was divided for tolerant and pre-tolerant treatments.

During tolerance there was a significant decrease in TSO activity in the aorta while CDNB activity was not affected. This indicates a specific alteration of the mu isozyme activity only. The reduced enzyme activities were compared to the reduced responses to GTN. The decrease in cGMP levels correlated well with the decrease in TSO activity in all the rabbits studied. Two of the seven rabbit aortas exhibited less than a 10 % decrease in response to GTN. These two rabbit aortas appeared resistant to the development of tolerance. The TSO activity in these two rabbits also did not decrease.

To examine thiol depletion as a possible mechanism for tolerance development, concentrations of thiols, cysteine and glutathione in GTN tolerant and pre-tolerant rabbit aortas were measured. No significant decreases in the levels of each of the thiols was observed. This suggests that in our *in vitro* model of tolerance, thiol depletion is not a possible cause of tolerance nor is it responsible for the decreased activity of the mu isozyme observed in tolerant tissues.

To determine if desensitization of guanylate cyclase could play a role in the development of tolerance to GTN, we studied the effect of GTN pretreatment on the dose response to SNAP, a S-nitrosothiol compound which either directly or via the spontaneous liberation of nitric oxide activates guanylate cyclase and thereby does not require a metabolic step. In aortic strips that showed significant tolerance to GTN, there was no change in the dose response of SNAP compared to non-tolerant strips. These results indicate that the tolerance to GTN is probably due to a step(s) involved in the formation of the vasoactive species and not in the step(s) related to further actions of the vasoactive species.

7.5 Can tolerance to GTN be reversed ?

Tolerance to GTN has been reversed *in vitro* and in the clinics in humans. Reversal of GTN tolerance was demonstrated in our rabbit aortic strip model by the process of washing. The cGMP and relaxation responses reverted to the pre-tolerant state by washing for 2 hours post tolerance. Aortic strips were made tolerant to GTN and then were washed for either 30, 60 or 120 min. The strips washed for 2 hours

post tolerance development produced close to pre-tolerant effects in response to GTN. The recovery of the aortas seems to be time dependent. The efficiency of the washing process did not seem to affect the pattern of tolerance reversal. These results indicated that the reversal process seemed to be a function of the total period before the next exposure to GTN, rather than the efficiency of GTN removal.

The TSO and CDNB activities were compared in tolerant aortic strips which were washed for a short period of time (0 or 30 min) and the tolerant aortic strips washed for a longer time period (60 or 120 min). The TSO activities in the strips washed for longer time periods were higher than the TSO activities in the strips washed for shorter time periods (close to the level in tolerant strips). This result suggests that the isozyme which was altered in the tolerant tissues is revived. Whether this is a result of new protein synthesis or removal of an inactivating metabolite requires further investigation.

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