Metabolism and Cytotoxic Mechanisms of Nitroglycerin in Isolated Rat Hepatocytes

Hossein Niknahad,*, Peter J. O’Brien

*Pharmaceutical Sciences Research Center, and School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

Abstract
It has been proposed that organic nitrates such as glyceryl trinitrate (GTN), used in the treatment of cardiovascular diseases, act by producing nitric oxide (NO). However, the biochemical pathway for NO formation from GTN is not well understood. In the present study, we showed that nitrate formation from GTN, by isolated rat hepatocytes, was inhibited about 50% when cellular glutathione was depleted and about 40% when cytochrome P-450 was inactivated by SKF525A. This suggests that GTN is metabolized and/or NO is formed by three pathways in rat hepatocytes: 1) denitrification of GTN by GSH/GSH transferase system; 2) reduction of GTN by reduced cytochrome P-450; and 3) GTN can directly react with protein thiol groups of cellular macromolecules (transnitrosation). At much higher concentrations, GTN was toxic towards hepatocytes (LC50= 2 mM for 2 h of incubation) and cytotoxicity was accompanied by GSH and ATP depletion. Depleting GSH and/or inactivating cytochrome P-450 beforehand markedly increased GTN cytotoxicity. The permeable thiol reductant dithioteritol unlike antioxidants was found to be an effective antidote, even if added to the cells an hour after GTN. The results suggest that GTN-induced cytotoxicity is mediated by transnitrosylation of mitochondrial, structural and vital protein thiols.

Key words: Cytochrome P-450 inhibition; Glyceryl trinitrate; GSH depletion; Isolated rat hepatocyte; Mitochondria.

Received: November 10, 2011; Accepted: February 1, 2012.

1. Introduction
The ability of mammalian cells to convert the organic nitrate nitroglycerin (GTN), to vasoactive nitric oxide (NO) or S-nitrosothiol (SNO) played a significant part in the discovery that NO functions as an endogenous physiological mediator [1]. GTN has long served as a principal therapeutic agent for acute angina and congestive heart disease [2-5]. It is thought that GTN induces vasorelaxation by generating NO or a related SNO. However, some observations have thrown this idea into doubt, with many studies demonstrating that NO is present only when there are high
concentrations of GTN [6].

Both enzymatic and nonenzymatic mechanisms of GTN metabolism and biotransformation have been proposed. The list of candidate enzymes includes glutathione S-transferases [7], the cytochrome P-450 system [8], xanthine oxidoreductase [9], and mitochondrial aldehyde dehydrogenase (ALDH-2) [10-13]. Direct interactions of GTN with low molecular weight thiols may also produce vasodilator SNOs [14]. NO and SNOs activate the target enzyme soluble guanylyl cyclase (sGC), increasing tissue levels of the second messenger cGMP. A cGMP-dependent protein kinase I (cGK-I) mediates vasorelaxation by phosphorylating proteins that regulate intracellular Ca^{2+} levels [15]. Nitroglycerin can also dilate blood vessels through a cGMP-independent pathway [10].

Although multiple cellular activities mediating GTN metabolism have been characterized, the mechanisms that specifically subserve GTN bioactivation have remained elusive. However, several studies have demonstrated that GTN in vivo is rapidly metabolized by the liver to nitrite and glycerol dinitrate [16]. Because glutathione (GSH) was depleted and the oxidized glutathione (GSSG) formed was effluxed into the bile, the initial step is believed to be mediated by glutathione S-transferase to form glutathione sulfonyl nitrite, which reacts directly with GSH to yield nitrite and GSSG [17]. NO is believed to be formed from the released nitrite; however, the mechanism of formation of NO from nitrite is not known yet. Figure 1 shows proposed mechanism of action of GTN which involve glutathione S-transferase and subsequent steps.

Cytochrome P-450 also is thought to mediate biotransformation of GTN. Servent et al. have reported that GTN was denitrated by rat liver microsomes in the presence of NADPH with formation of complexes of glyceryl dinitrates and mononitrites [18]. They have also recorded formation of P-450-Fe-NO complex during the reaction. McDonald and Bennet also have reported microsomal biotransformation of GTN to 1,3-GDN and 1, 2-GDN which has been shown to be NADPH dependent, inhibited by cytochrome P-450 inhibitors, such as carbon monoxide and SKF525A [19].

In the following, the effect of GTN on isolated rat hepatocytes and mitochondria has been investigated. Nitrite formation correlated with GSH depletion and was prevented by depleting GSH and/or inactivating cytochrome P-450 dependent mixed function oxidase. At higher concentrations protein S-nitrosylation occurred, respiration was inhibited and cytotoxicity ensued. This could be averted by addition of dithiothreitol.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats (300-350 g), fed a standard chow diet and given water ad libitum, were used in all experiments.
Nitroglycerin metabolism and toxicity

2.2. Chemicals
Nitroglycerin was obtained from Du Pont Pharmaceuticals as injectable USP from (Tridil®). Collagenase (from Clostridium histolicum) and HEPES were purchased from Bohringer-Mannheim. Trypan blue, thiobarbituric acid, fluoro-2,4-dinitrobenzene, and iodoacetic acid were obtained from Sigma. Sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride were purchased from Aldrich. Other chemicals were of the highest commericial grade available.

2.3. Preparation of isolated rat hepatocytes
Hepatocytes were isolated from male Sprague-Dawley rats by a two-step collagenase perfusion, as described previously. The cells were suspended (10⁶ cells/ml) in Krebs-Henseleit buffer containing 12.5 mM HEPES and incubated under a stream of 95% O₂ and 5% CO₂ in continuously rotating round-bottomed 50 ml flasks at 37 °C [20-22].

2.4. Glutation determination
The total GSH and GSSG content of hepatocytes was measured by the HPLC analysis of deproteinized samples (5% metaphosphoric acid), after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene, by high performance liquid chromatography, using a C18 µBondapak NH2 column (Waters Associates, Milford, MA) [23]. GSH and GSSG were used as external standards. A Waters 6000A solvent delivery system equipped with a model 600 solvent programmer, a Wisp 710 automatic injector and a Data Module were used for analysis.

2.5. Determination of ATP
ATP in hepatocytes was extracted using an alkaline extraction procedure quantified by HPLC, using C18 µBondapak reverse phase column (Waters Associates Milford, MA) as explained by Stocchi et al. [24].

2.6. Isolation of mitochondria
The liver was removed with small scissor and minced in a cold manitol solution containing 0.225 M D-manitol, 75 mM

---

Table 1. Cytotoxicity of GTN in freshly isolated rat hepatocytes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cytotoxicity (%) at Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22±2</td>
</tr>
<tr>
<td>GTN 0.3 mM</td>
<td>23±3</td>
</tr>
<tr>
<td>GTN 3 mM</td>
<td>30±4</td>
</tr>
<tr>
<td>GTN 3 mM+DTT 5 mM</td>
<td>33±4a</td>
</tr>
<tr>
<td>GTN 0.3 mM+GSH Depleted</td>
<td>34±3a</td>
</tr>
<tr>
<td>GTN 0.3 mM+P-450 inhibited</td>
<td>36±3b</td>
</tr>
<tr>
<td>GTN 0.3 mM+GSH Depleted+P-450 inhibited</td>
<td>38±3b</td>
</tr>
</tbody>
</table>

All data are given as mean±SD (n=6). Cells were incubated at a concentration of 1×10⁶ cells/ml. *Significantly different from control p<0.05. **Significantly different from GTN (3 mM) treated hepatocytes (p<0.05). ***Significantly different from GTN (0.3 mM) treated cells (p<0.05).

---

Figure 2. Effect of treatment with SKF 525A (50 µM) and 1-bromoheptane (200 µM) on GTN (150 µM) induced changes in nitrite release after 1 h. All data are given as mean±SD (n=6). Cells were incubated at a concentration of 1×106 cells/ml. All curves are significantly different from control (p<0.001).
sucrose and 0.2 mM ethylenediaminetetraacetic acid (EDTA). Approximately 30 g of minced liver was gently homogenized in a glass homogenizer with a Teflon pestle and then centrifuged at 700×g for 10 min. at 4 °C to remove nuclei, unbroken cells and other non-subcellular tissues. The supernatants were centrifuged at 7000×g for 20 min. These second supernatant were pooled as the crude microsomal fraction and the pale loose upper layer, which was rich in swollen or broken mitochondria, lysosomes and some microsomes, of sediments was washed away. The dark packed lower layer (heavy mitochondrial fraction) was resuspended in the mannitol solution and recentrifuged twice at 7000×g for 20 min. The heavy mitochondrial sediments were suspended in Tris solution containing 0.05 M Tris-HCl buffer (pH 7.4) 0.25 M sucrose, 20 mM KCl, 2.0 mM MgCl$_2$ and 1.0 mM Na$_2$HPO$_4$ at 4 °C before assay.

2.7. Protein determination

Protein concentration was determined using the method developed by Bradford [25].

2.8. Depletion of mitochondrial GSH

GSH-depleted hepatocytes were obtained by preincubating hepatocytes with 0.2 mM 1-bromoheptane for 20 min. [26].

2.9. Mitochondrial calcium release assay

Calcium release was monitored in freshly isolated mitochondria by measuring the absorbance change of the chromatophoric dye Arzenazo III (40 μM), using the wavelength pair 654-685 nm. Mitochondria (2.0 mg/ml) were loaded with Ca$_{2+}$ before addition of GTN.

2.10. Oxygen consumption assay

Oxygen uptake was monitored at 37 °C using a Clark-type oxygen electrode (Yellow Spring, OH) in a 2 ml chamber with 2 mg/ml protein.

2.11. Measurement of nitrite concentration

A colorimetric determination of nitrite concentration by diazo coupling of N-(1-Naphthyl) ethylene diamine dihydrochloride and sulfanilamide was used. Aliquots were taken in specific time points and added to 1.1 ml of 13.2 mM sulfanilamide in 0.91 M HCl. After addition of 0.1 ml of 11.2 mM N-(1-Naphthyl) ethylenediamine dihydrochloride the sample were allowed to stand for at least 1 h at room temperature before the absorbance was measured at 540 nm.

2.12. Statistical analysis

All values were expressed as mean±SD of 6 samples. Analysis of variance (ANOVA) followed by student Newmans-Keuls test was used to evaluate the significance of the results obtained. All computations were analysis by computer using SPSS software.

3. Results

3.1. Effect of GSH depletion and/or P-450 inactivation on biotransformation of GTN by hepatocytes

Incubation of GTN (150 μM) with isolated rat hepatocytes resulted in a time-dependent formation of nitrite (about 110 μM at 60 min) (Figure 2). Preincubation of hepatocytes with 200 μM bromoheptane, a GSH depletory, inhibited metabolism of GTN to nitrite by
Nitroglycerin metabolism and toxicity

about 50% after 1 h of incubation (Figure 2). Inactivation of cytochrome P-450 by SKF 525A beforehand also had a similar effect to GSH depletion, i.e., it inhibited formation of nitrite from GTN, but its inhibitory effect was less than GSH depletion (Figure 2). Inactivation of cytochrome P-450 and depletion of GSH at the same time inhibited the metabolism of GTN to nitrite by hepatocytes by a large extent (about 90%) (Figure 2).

3.2. Effect of GTN on GSH

Incubation of hepatocytes with different concentrations of GTN decreased GSH content of hepatocytes. Addition of 300 µM GTN depleted the GSH content of hepatocyte to about 55% and 20% at 15 min and 1 h, respectively (Figure 3A). Toxic doses of GTN (3 mM) depleted all of the GSH content of the cells only in 15 min. of incubation (Figure 3A) and did not recover during the rest of the incubation time. The concentration of GSSG did not increase parallel to decrease of GSH (Figure 3B). Addition of dithiothreitol (DTT), a thiol reductant, at 15 min. or even later immediately restored the GSH content of the cells to levels even more than the original level (Figure 3A), but GSSG was almost disappeared (Figure 3B).

3.3. Effect of GTN on ATP content of hepatocytes

Figure 4 shows the effect of GTN on ATP level in isolated rat hepatocytes. At the beginning, decrease of ATP was fast with high concentrations of GTN (1-3 mM), and after 30 and 60 min of incubation of hepatocytes with 3 mM GTN more than 60% and 90% of ATP was depleted, respectively. However, 150 or 300 µM of GTN did not effectively deplete ATP content of the cells (data not shown). The decrease in ATP content of hepatocytes is in the same direction as GSH, but the rate of GSH depletion is much faster.

3.4. Cytotoxicity of GTN in isolated hepatocytes

GTN in low concentrations (up to 300 µM) did not show significant toxic effects towards isolated rat hepatocyte, but with higher concentrations it was toxic and its LC₅₀ for 2 h was about 2 mM (Table 1). Its toxicity was dose and time-dependent.

3.5. Effect of dithiothreitol on GTN cytotoxicity

Hepatocytes were protected against cytotoxicity of GTN by DTT (Table 1). Addition of DTT even 30 min. after GTN prevented cytotoxicity of GTN.

3.6. Effect of GSH depletion and cytochrome P-450 inhibition on cytotoxicity of GTN

GSH depletion and/or cytochrome P-450 inactivation increased the cytotoxicity of GTN towards hepatocytes. Even 300 µM GTN was toxic in GSH depleted and/or P-450 inactivated hepatocytes (Table 1). Inactivation of cytochrome P-450 increased the toxicity more than GSH depletion. If both pathways of metabolism of GTN to nitrite were inactivated, cytotoxicity was increased very sharply and 300 µM GTN resulted in about 70% cell death at 3 hr (Table 1).

Figure 4. Effect of GTN on ATP content of hepatocytes after 2 h of incubation. All data are given as mean±SD (n=6). Cells were incubated at a concentration of 1×10⁶ cells/ml. All curve are significantly different from control (p<0.001).
3.7. Effect of GTN on mitochondrial respiration

GTN inhibited mitochondrial respiration. As shown in Figure 5, 1 or 3 mM GTN inhibited oxygen uptake by isolated mitochondria by 36% and 46%, respectively. GSH depletion did not affect mitochondrial respiration.

3.8. Effect of GTN on mitochondrial calcium release

GTN increased the release of calcium from mitochondria even with low concentrations (100 μM). Figure 6 shows the effect of GTN on calcium release from normal and GSH depleted mitochondria. GSH depletion potentiated the effect of GTN on calcium release.

4. Discussion

Various hypotheses on the molecular mechanism of organic nitrate-induced vasodilation considered the biotransformation of organic nitrates, such as nitroglycerin in the smooth muscle cells as an initial step for vasodilation [15, 27, 28].

Metabolism of GTN occurs in the liver as well [13], but probably it is not important in the vasodilatory effect of GTN because creation of NO in the smooth muscle cells of the vessels is critical for its vasodilatory effect and because of its short half life, it is unlikely to be able to travel from liver to vascular smooth muscle. Therefore, metabolism of GTN by the liver seems to be a clearance pathway [8, 29].

Within the vascular smooth muscle and liver cells there may be more than one biotransformation pathway. Needleman et al. [30] were the first to suggest that cellular sulphhydril groups are involved in the biotransformation of GTN. GSH is the major non-protein thiol present in cells and is required for the metabolism of GTN by glutathione S-transferase [14, 15, 27]. Other thiol groups also have been shown to react with GTN and produce nitrite in smooth muscle cells [27]. Our results also approved that GSH is involved in the metabolism of GTN by hepatocytes, as depletion of GSH resulted in about 50% decrease in nitrite formation (Figure 2). Also, we found that depletion of GSH and inactivation of cytochrome P-450, another pathway for metabolism of GTN, at the same time did not completely block formation of nitrite from GTN. This may be due to involvement of other cellular thiol groups in the metabolism of GTN.

We found that inhibition of cytochrome P-450 by SKF 525A also decreases the formation of nitrite from GTN by isolated...
Nitroglycerin metabolism and toxicity

rat hepatocytes, but its role in nitrite formation was less than GSH. Previously, also metabolism of GTN by rat liver microsomes has been reported [15, 16]. McDonald and Bennett [19] have reported that the involvement of cytochrome P-450 in the metabolism of GTN in rat liver microsomes is region selective and produces preferentially 1,3-dinitroglycerin, while GSH transferase produces preferentially 1,2-dinitroglycerin in rabbit liver cytosol [7].

At higher concentrations, GTN was toxic towards isolated rat hepatocytes. Its cytotoxicity was dose-dependent and started from about 300 μM and its LC_{50} for 2 h was about 2 mM. Dithiothreitol (DTT) was able to protect hepatocytes against GTN cytotoxicity, which implies that oxidative stress and thiol oxidation is involved in the mechanism of GTN cytotoxicity.

GTN with toxic doses depleted GSH very fast, but did not increase the GSSG concentration parallel to GSH depletion (Figure 3A and B). This means that a part of GSH is in the form of conjugate with nitrite possibly by transnitrosylation. Depleting GSH and/or inactivating cytochrome P-450 beforehand sharply increased the cytotoxic effect of GTN. Even 300 μM GTN was highly toxic when both pathways were blocked.

GTN with toxic and non toxic doses depleted ATP content of the cells. It also inhibited mitochondrial respiration and increased calcium release from mitochondria even with non toxic doses (150 μM). GSH depletion potentiated the effect of GTN on mitochondrial calcium release. Respiratory inhibitors, i.e. rotenon and cyanide, were found to increase cytotoxicity of GTN (data not shown). Inhibition of respiration and ATP depletion could probably be due to oxidation of thiol groups of mitochondrial proteins by GTN.

Putting all above data together, we propose the following mechanisms for GTN metabolism and cytotoxicity which is also depicted in Figure 1. There are three pathways for metabolism of GTN: 1) GTN can be metabolized by GSH/GSH transferase system to form glutathione sulfonylnitrite (GS-NO2) and complexes of glyceryl dinitrites (GDN), preferentially 1,2-GDN. GS-NO2 later reacts with GSH to yield nitrite and GSSG. Nitrite is believed to be converted, by an unknown mechanism, to nitrite oxide which by activating guanylate cyclase and liberating cGMP causes vascular smooth muscle relaxation and vasodilation. 2) Cytochrome P-450 metabolizes GTN to glyceryl dinitrites, preferentially 1,3-GDN, and releases nitrite. 3) GTN can directly react with protein thiol groups of cellular macromolecules (transnitrosation). DTT can reduce these thiol groups and protect cells from GTN induced cytotoxicity. Transnitrosation of mitochondrial thiol groups causes deformation of mitochondrial membrane or inactivation of enzymes which results in decrease in ATP production by mitochondria. ATP depletion leads to inactivation of ATP dependent calcium pumps which results in elevation of calcium level in the cell and finally by activation of proteases and phospholipases results in cell death. Denaturation of structural and other vital proteins by transnitrosylation are possibly involved in cytotoxic mechanism of GTN.

References


