Metabolism and Pathways for Denitration of Organic Nitrates in the Human Liver

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Received January 18, 2013; accepted April 11, 2013

ABSTRACT

Liver first-pass metabolism differs considerably among organic nitrates, but little information exists on the mechanism of denitration of these compounds in hepatic tissue. The metabolism of nitrooxybutyl-esters of flurbiprofen and ferulic-acid, a class of organic nitrates with potential therapeutic implication in variety of different conditions, was investigated in comparison with glyceryl trinitrate (GTN) in human liver by a multiple approach, using a spontaneous metabolism-independent nitric oxide (NO) donor [3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5)] as a reference tool. Nitrooxybutyl-esters were rapidly and quantitatively metabolized to their respective parent compounds and the organic nitrate moiety nitrooxybutyl-alcohol (NOBA). Differently from GTN, which was rapidly and completely metabolized to nitrite, NOBA was slowly metabolized to nitrate. In contrast to the spontaneous NO donor NOC-5, NOBA and GTN did not generate detectable NO and failed to suppress the activity of cytochrome P450, an enzyme known to be inhibited by NO. The direct identification of NOBA after liver metabolism targets this compound as the functional organic nitrate metabolite of nitrooxybutyl-esters. Moreover, the investigation of the pathways for denitration of NOBA and GTN suggests that organic nitrates are not primarily metabolized to NO in the liver but to different extents of nitrate or nitrite depending in their different chemical structure. Therefore, cytochrome P450-dependent metabolism of concomitant drugs is not likely to be affected by oral coadministration of organic nitrates. However, the first pass may differently affect the pharmacological profile of organic nitrates in connection with the different extent of denitration and the distinct bioactive species generated and exported from the liver (nitrate or nitrite).

Introduction

Organic nitrates have been harnessed therapeutically since 1879, when glyceryl trinitrate (GTN) was first described as a remedy for angina pectoris (Murrell, 1879). GTN, acting through the liberation of nitric oxide (NO) in vascular tissue, is still widely used in the treatment of coronary artery disease and congestive heart failure (Parker and Parker, 1998). This compound is rapidly and efficiently absorbed from the mouth, leading to a rapid onset of action that is suitable for the treatment of acute angina attacks. After absorption from the mouth or the skin, GTN is rapidly denitrated by hepatic metabolism (systemic t1/2, ∼1 minute) (Needleman, 1976) to its dinitrate form (GDN), which has a higher systemic half-life (t1/2, ∼2 hours) (Needleman, 1976) but a lower potency than does GTN (Münzel et al., 2005). When GTN is given by oral route, it undergoes extensive liver first-pass metabolism (Needleman et al., 1976), leaving its dinitrate form to circulate systemically with little or no parent compound appearing in blood (Needleman, 1976; Yu et al., 1988). For this reason, GTN is administered orally only for prophylaxis purposes because of its slower onset of action and higher doses required to reach therapeutic efficacy in comparison with sublingual or transdermal administration.

Efforts to increase its duration of action and prophylaxis have led to the synthesis of other organic nitrates, of which the most important is isosorbide mononitrate (ISMN). ISMN has similar pharmacological actions as GTN, but it is longer acting and it undergoes low hepatic metabolism after oral administration (Abshagen, 1992). Furthermore, the increasing realization that nitrates may represent new therapeutic agents in different areas led to the development of organic nitrates containing adjunct pharmacophores to introduce biologic properties beyond those of the parent compound (Hodosan et al., 1969; Keeble and Moore, 2002). In particular, nitrooxybutyl-ester derivatives is a novel class of hybrid organic nitrates consisting of a (-ONO2) structural unit connected via a butyl linker to the parent compound by a carboxyl-ester bond.

Although the capacity of organic nitrates to liberate NO in vascular tissue is quite well established (Mülach et al., 1995;
Kleschyov et al., 2003; Münzel et al., 2005), the exact mechanism of denitration is still a matter of debate. Moreover, whether organic nitrates are at all capable of generating NO in extravascular tissues, as for example, in the liver, is still controversial. Some studies have provided evidence for NO generation from GTN incubated in subcellular liver fractions (Servent et al., 1989; Kozlov et al., 2003), but a generalized concept (although not supported by any data) is that the liver cannot metabolize GTN to NO. Indeed, it is well established that GTN undergoes extensive hepatic metabolism, causing loss of vascular bioactivity (Needleman et al., 1972), but this does not necessarily imply that the liver is not capable of acute NO generation from GTN. A better understanding of how organic nitrates are metabolized in the liver is important when characterizing novel organic nitrates that are under development. As an example, NO has been reported to be directly and indirectly involved in the inactivation of cytochrome P450 (P450) (Khatsenko et al., 1993; Wink et al., 1993; Stadler et al., 1994), suggesting that liver P450-dependent drug metabolism may be drastically affected by oral coadministration of organic nitrates undergoing first-pass metabolism.

Nitroxybutyl-ester derivatives of anti-inflammatory and/or antioxidant agents, such as flurbiprofen and ferulic acid, is a novel class of organic nitrates with potential therapeutic implication in a variety of different conditions (Keeble and Moore, 2002; Scatena, 2004), such as neurodegenerative diseases (Wenk et al., 2002, 2004; Prosperi et al., 2004; Gasparini et al., 2005), inflammation and nociception (Ronchetti et al., 2009), learning and memory (Boultadakis et al., 2010), airway inflammation and relaxation (Larsson et al., 2007, 2009), osteoporosis, and bone resorption (Idris et al., 2004). Despite extensive pharmacological characterization of these compounds, their metabolism and pathways of denitration remain unknown. In particular, being targeted mainly for chronic diseases, these molecules are generally designed to be administered by oral route, and an evaluation of their liver metabolism is therefore critical.

In the present study, we investigated the metabolism of nitroxybutyl-ester compounds in comparison with GTN and ISMN in the human liver by a multiple approach that includes (1) a chemiluminescent nitrite/nitrate/S-nitrosothiols (RSNO) metabolic profile assessment, (2) a test of NO-mediated inhibition of liver CYP1A2 activity, (3) a direct electrochemical detection of NO, and (4) the use of a spontaneous metabolism-independent NO donor 5-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) as a reference tool.

### Materials and Methods

#### Biologic Material and Chemicals

Human liver microsomes, cytosol, and mitochondria were purchased from Tebu-bio, Magenta, Italy, as pools of different mixed-sex donors. High-performance liquid chromatography (HPLC)-grade organic solvents were purchased from Carlo Erba reagents (Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system. [1,1'-Biphenyl]-4-acetic acid, 2-fluoro-a-methyl, 4-nitroxybutyl ester (HCT 1026), [1,1'-biphenyl]-4-acetic acid, 2-fluoro-α-methyl, 4-hydroxybutyl ester (HCT 1027), 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid 4-nitroxybutyl ester (NCX 2057), and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid 4-hydroxybutyl ester (NCX 2059) were provided by Dipharma S.p.A. (Milan, Italy). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

GTN and (nitroxy)butyl alcohol (NOBA) were kindly provided by Dipharma S.p.A. (Milan, Italy). The optimization of the conditions for evaluation of the inhibitory potential of GTN and microsomal CYP1A2 was conducted according to the FDA Guidance for Industry, Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations (2012; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf). In brief, the kinetics of acetaminophen formation from phenacetin incubated in human liver microsomes was tested at three different protein concentrations (chosen to avoid enzymatic saturation) for five different substrate concentrations. The experimental Michaelis-Menten constant values were calculated by Lineweaver-Burk equation for each protein concentration tested.

Inhibition experiments were further performed (1) at the lowest microsomal protein concentration tested (0.5 mg ml⁻¹), (2) incubating the substrate at the experimental calculated Km value (50 µM), and (3) incubating the substrate for a period during which the formation of acetaminophen is linear and does not exceed the 30% of substrate depletion (30 minutes).

#### Liver Subcellular Fractions Incubations

In vitro incubations were performed in reconstituted human liver homogenate (microsomes, 0.5 mg ml⁻¹, mitochondria, 0.65 mg ml⁻¹, cytosol, 2.05 mg ml⁻¹) (Kozlov et al., 2003) or in the single subcellular fraction at the same individual protein concentration. The following cofactor mixtures were used for all incubations: 2 mM NADPH, 0.5 mM NAD⁺, 0.5 mM NADH, and 2.5 mM reduced glutathione (GSH). Reconstituted human liver homogenate (microsomes, mitochondria, and cytosol) or individual subcellular fractions and cofactors were incubated at the final working concentration in Na-phosphate buffer (pH 7.4) and kept under slight shaking at 37°C during the experiments. Drugs (HCT 1026, NCX 2057, NOC-5, GTN, ISMN, and NOBA) were dissolved in acetonitrile or water and added to human liver fractions at a final solvent concentration <0.5% (v/v).

Because diazeniumdiolates, such as NOC-5, release 2 moles of NO per mole of parent compound ( Fitzhugh and Keever, 2000), for comparison purposes, the compounds were incubated under equinormal conditions, meaning a concentration of GTN, ISMN, or NOBA double that of NOC-5 (GTN) = ISMN = NOBA = 2 [NOC-5].

#### Incubation for HCT 1026 and NCX 2057 Metabolic Assessment

The experiment was started by adding the compound (HCT 1026 or NCX 2057 at a final concentration of 250 µM) to reconstituted homogenate. At fixed time points (0, 1, 5, 7, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 minutes), 50 µl of the incubation mixture was removed and deproteinized with 100 µl of methanol for HPLC-ENO20 analysis. The remaining 200 µl of incubation mixture was removed at the same time, deproteinized with 400 µl of acetonitrile plus phosphoric acid 1% (v/v), and processed for HPLC/UV HCT 1026 or NCX 2057 metabolite profiling.

#### Incubations for NO₂⁻ and NO₃⁻ Assessment

The experiment was started by adding the compound (NOC-5 GTN, ISMN, or NOBA at a final concentration of 250–500 µM) to reconstituted homogenate or single fractions. At fixed time points, 50 µl of incubation mixture was removed, deproteinized with 100 µl of methanol, and submitted for HPLC-VIS NO₂⁻ and NO₃⁻ analysis.

#### Incubations for CYP1A2 Inhibition and RSNO Assessment

For each incubation, furafylline was used as standard control (reference CYP1A2 inhibitor). After 30 minutes of incubation in reconstituted homogenate with the drug (NOC-5, GTN, ISMN, or
Assay for HCT 1026 and NCX 2057 Metabolite Profiling

The metabolic profile of HCT 1026 and NCX 2057 and their respective metabolites was performed by liquid chromatography analysis on an Agilent 1100 system (Agilent Technologies, Santa Clara, CA) equipped with a UV/Visible diode array programmable detector. Separations were achieved by reverse phase elution with a Synergy Hydro-RP 80 Å column (150 × 4.6 mm i.d.; particle size, 4 μm) (Phenomenex, Torrance, CA) equipped with an AQ-C18 precolumn (4 × 3 mm i.d.) (Phenomenex) maintained at 25°C. The mobile phase; acetone (solvent A) and 25 mM sodium phosphate buffer (pH 2; solvent B) was delivered at a flow rate of 1 ml min⁻¹. HCT 1026 and its metabolites were detected at λ = 246 nm with the following gradient composition: 0 minute A, 20%; 1–8 minutes A, 80%; and 9–11 minutes A, 20%. Under these conditions, the retention times for HCT 1026, its direct denitrated derivative HCT 1027, and flurbiprofen were 6.8, 4.7, and 4.2 minutes, respectively.

NCX 2057 and its metabolites were detected at λ = 325 nm with the following gradient composition: 0 minute A, 20%; 4–8 minutes A, 80%; and 10–12 minutes A, 20%. Under these conditions, the retention times for NCX 2057, its direct denitrated derivative NCX 2059, and ferulic acid were 6.1, 4.6, and 4.1 minutes, respectively.

NCX 2057, HCT 1026, and their metabolites were chromatographically separated and characterized on the basis of the typical UV fingerprint of the pure compounds. Chromatographic peaks were processed for spectral purity characterization by statistical analysis for automated comparison of spectra (Agilent Chemstation software; Agilent Technologies). Only peaks showing spectral matching factors higher than 99% were considered to be acceptable. Chromatograms were integrated with Agilent Chemstation software, and the area under the curve was converted into concentration with use of reference compounds.

Assays for NO²⁻, NO₃⁻, and NOBA analysis

NO²⁻, NO₃⁻, and NOBA concentrations in liver fractions were measured after methanol precipitation of proteins (1:2 v/v) by using a dedicated HPLC system (ENO-20; EiCom, Kyoto, Japan) coupled with and Agilent 1100 autosampler. The method (Yamada and Nabeshima, 1997) is based on the separation of NO²⁻, NO₃⁻, and NOBA by reverse-phase/ion exchange chromatography, followed by online reduction of NO₃⁻ and NOBA to NO⁻ with cadmium and reduced copper. NO₂⁻ is then derivatized with the Griess reagent, and the level of diazo compounds is measured by a visible detector at 540 nm. The retention times for NO₂⁻, NO₃⁻, and NOBA were 5.2, 8.6, and 29.5 minutes, respectively.

Gas-Phase Chemiluminescence Assay for RSNO

RSNOs were determined by gas-phase chemiluminescence with use of an NO analyzer (Aerocline AB, Solna, Sweden) after reductive cleavage and subsequent determination of the NO released into the gas phase. The method has been described in detail elsewhere (Lundberg and Govoni, 2004). NO signals were collected and reported as area under the curve with Azur (version 3.0) chromatographic software (Datalys, Saint-Martin d’Hères, France). RSNOs were reduced to NO with a solution consisting of 45 mM potassium iodide and 10 mM iodine in glacial acetic acid at 60°C, and measurement was performed using direct sampling injection (300 μl).

Because the reducing solution is able to convert both NO²⁻ and nitroso/nitrosyl compounds to NO, RSNOs were quantified by simple subtraction of the peak area of sample aliquots pretreated with sulfanilamide and mercuric chloride at room temperature for 30 minutes [10% (v/v) of a solution 5% sulfanilamide and 0.2% HgCl₂ in 1 N HCl] from that of sample aliquots treated only with sulfanilamide at room temperature for 15 minutes [10% (v/v) of a solution 5% sulfanilamide in 1 N HCl]. Under these conditions, NO₂⁻ reacts with sulfanilamide to form a stable diazonium ion that is not converted to any appreciable extent to NO, and HgCl₂ selectively cleaves the S-NO bond (Feilisch et al., 2002) without affecting peak shape or recovery of other detectable NO species. The calibration curve was obtained with freshly prepared S-nitrosothiolane.

HPLC-MS/MS Analysis for CYP1A2 Inhibition Evaluation

The phenacetin O-deethylation activity of CYP1A2 was evaluated using liquid chromatography. Analyses were performed on an Agilent 1100 system coupled with a triple quadrupole mass spectrometer (API 2000 LC-MS/MS system; Applied Biosystems/MDS Sciex, Toronto, ON, Canada). Separations were achieved by reverse phase elution with a Phenomenex Synergy Hydro-RP 80 Å (50 × 4 mm i.d., particle size 4 μm) equipped with a Phenomenex AQ-C18 precolumn (4 × 3 mm i.d.) maintained at 25°C. During the mobile phase, water containing 0.1% formic acid (solvent A) and acetone (solvent B) was delivered at a flow rate of 0.35 ml min⁻¹ at the following gradient composition: 0–1 minute A, 100%; 1.10–24 minutes A, 0%; and 4.40–15 minutes A, 100%. Under these conditions, the retention times for acetalaminophen, phenacetin, and ketoprophin (internal standard) were 3.87, 4.12, and 4.4 minutes, respectively.

The following mass spectrometer settings were used: turbo ionspray interface maintained at 300°C; detection (positive ion mode) of the analytes using the multiple reaction monitoring scan mode was performed using collision-activated dissociation gas at 5 psi, curtain gas at 20 psi, turbo ion spray voltage at +5500 V, channel electron multiplier at 2400 V, source gas (GS1 and GS2) pressure at 40 and 50 psi, respectively; declustering potential, focusing potential, and entrance potential were set at 31, 370, and 10.05 V, respectively; and experiments were performed using a dwell time of 50 milli-seconds. The spectra were acquired at unit resolution. The mass transitions (multiple reaction monitoring) of acetaminophen, phenacetin, and ketoprofen were m/z 152 → 110, m/z 180 → 138, and m/z 255 → 105, respectively. Chromatograms were integrated using Analyst software (version 1.2; Applied Biosystems, Foster City, CA), and area under the curve was converted to concentration with use of reference compounds.

Assay for Electrochemical Detection of NO

The measurement of NO release was performed using the inNO nitric oxide measurement system with use of the amiNO-700 sensor electrode (Innovative Instruments Inc., Indian Trail, NC). The output current was monitored and recorded on a Windows 98 compatible system using analysis software provided with the inNO system. The sensor was calibrated by adding known volumes of standard NO₂⁻ solution to an acidified solution in the presence of iodide ion according to the manufacturer’s specifications and settled for temperature compensation before the experiment.

Data Analysis

NOx (NO₃⁻ + NO₂⁻), NO₂⁻, and NO₃⁻ values in the incubations were subtracted from the basal (time 0). One-way analysis of variance
was used to evaluate differences between groups, and it was followed by a Tukey post hoc test for comparisons among multiple groups. Differences were considered to be significant at $P < 0.05$. IC50 values for enzyme inhibition were obtained by fitting to the sigmoid equation: $Y = Y_0 + [\alpha / 1 + (X/IC50)^b]$, where $Y =$ inhibition and $X =$ inhibitor concentration. Data were modeled using nonweighted nonlinear regression analysis with use of SigmaPlot, version 8.02 (Systat Software, Inc., Point Richmond, CA).

**Results**

**Liver Metabolism of Nitrooxybutyl-Ester Compounds.**

The chemical structures of HCT 1026, NCX 2057, and their metabolites that arise from biotransformation pathways involving hydrolytic cleavage of the carboxylic and nitric ester functions in reconstituted human liver homogenate (cytosol + microsomes + mitochondria) with cofactors. HCT 1026 (A) and NCX 2057 (B) were incubated at a final concentration of 250 μM, and their metabolic profile was observed over the incubation time. Filled triangles (▲) show the kinetic of flurbiprofen and ferulic acid formation from HCT 1026 and NCX 2057 (○), respectively, whereas the direct denitrated metabolites HCT 1029 or NCX 2059 were not observed over the incubation time. Open squares (□) represent the kinetics of NOBA generation from HCT 1026 (A) and NCX 2057 (B), and filled diamonds (●) show the time course of NOx species generated over the incubation time. NOx values are subtracted from the basal (time 0). Data are presented as mean ± S.D., $n \geq 3$.

HCT 1026 was converted to flurbiprofen with $V_0 = -19.32 ± 5.79 \mu$M/min, consistent with $V_0$ of flurbiprofen and NOBA formation of $18.63 ± 1.54 \mu$M/min and $18.39 ± 3.05 \mu$M/min, respectively, ($-V_0 \text{HCT 1026} \approx V_0 \text{Flurbiprofen} \approx V_0 \text{NOBA}$) (Fig. 1A).

NCX 2057 was converted to ferulic acid with $V_0 = -13.87 ± 2.51 \mu$M/min, consistent with $V_0$ of ferulic acid and NOBA formation of $12.93 ± 0.15 \mu$M/min and $11.30 ± 1.23 \mu$M/min, respectively ($-V_0 \text{NCX 2057} \approx V_0 \text{Ferulic Acid} \approx V_0 \text{NOBA}$) (Fig. 1B).

**Fig. 1.** Chemical structures and metabolic profile of HCT 1026 (A), NCX 2057 (B), and their metabolites arising from biotransformation pathways involving hydrolytic cleavage of the carboxylic and nitric ester functions in reconstituted human liver homogenate (cytosol + microsomes + mitochondria) with cofactors. HCT 1026 (A) and NCX 2057 (B) were incubated at a final concentration of 250 μM, and their metabolic profile was observed over the incubation time. Filled triangles (▲) show the kinetic of flurbiprofen and ferulic acid formation from HCT 1026 and NCX 2057 (○), respectively, whereas the direct denitrated metabolites HCT 1029 or NCX 2059 were not observed over the incubation time. Open squares (□) represent the kinetics of NOBA generation from HCT 1026 (A) and NCX 2057 (B), and filled diamonds (●) show the time course of NOx species generated over the incubation time. NOx values are subtracted from the basal (time 0). Data are presented as mean ± S.D., $n \geq 3$.

**Fig. 2.** Extent of denitration of GTN and NOC-5 in reconstituted human liver homogenate (microsomes + mitochondria + cytosol) with cofactors. GTN (□) and NOC-5 (☓) were incubated at equinormal conditions (500 μM and 250 μM for GTN and NOC-5, respectively), and the profile of NOx species generated was observed over the incubation time. Values are subtracted from the basal (time 0). Data are presented as mean ± S.D., $n \geq 3$. 

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HCT 1027 and NCX 2059 were not observed over the incubation time.

Incubations with the vehicle in liver homogenate or with the compounds alone in buffer did not produce any increase in NOx concentrations.

The liver metabolism of other compounds belonging to the class of nitroxybutyl-ester derivatives was investigated and showed very similar metabolic properties to HCT 1026 and NCX 2057 (unpublished data).

**NOC-5, GTN, ISMN, and NOBA Liver Metabolism.** NOC-5 and GTN were rapidly and extensively metabolized to NOx species. The kinetics of NOx generation from the two compounds overlapped over 90 minutes of incubation with an almost identical V0 (13.0 ± 0.9 µM/min and 12.9 ± 0.4 µM/min, for NOC-5 and GTN, respectively) (Fig. 2). However, the generation of NO2− and NO3− as individual species was different. Although GTN generated NO2− as the main metabolite (V0 NO2− = 13.1 ± 0.1 µM/min), V0 NOx > V0 NO3− = 0.4 ± 0.1 µM/min) (Fig. 3A), NOC-5 generated both NO2− and NO3− with similar V0 (V0 NO2− = 7.3 ± 0.2 µM/min, V0 NO3− = 5.7 ± 0.2 µM/min) (Fig. 3B).

NOBA incubated under the same experimental conditions revealed a slower rate of metabolism to form NOx than GTN, although NOx formation from ISMN was very low (Fig. 4). An analysis of the individual species generated from NOBA also showed differences in terms of metabolic products. In fact, NOBA led exclusively to the generation of NO3− with a V0 NO3−/V0 NOx = 1.2 ± 0.1 µM/min and a generation of NO3− of 70 and 460 µM after 60 and 240 minutes of incubation, respectively (Fig. 3C). Incubations with the vehicle in liver homogenate or with the compounds alone in buffer did not produce any increase in NOx concentrations.

**RSNO Formation.** Figure 5A shows the generation of RSNO measured 1 hour after incubation of NOC-5 and GTN at different concentrations. Although the amount of RSNO formed was much lower in comparison with the initial concentration, NOC-5 showed a higher nitrosating capacity than did GTN, in particular at the highest concentrations tested, where RSNO levels reached 9.8 ± 2.1 µM for NOC-5 and 3.5 ± 0.3 µM for GTN (Fig. 5A). Incubations with NOBA or ISMN under the same experimental conditions did not produce any RSNOs, nor did incubations with vehicle in liver homogenate or with the compounds alone in buffer.

**Inhibition of CYP1A2.** The effects of GTN, ISMN, NOBA, and NOC-5 on CYP1A2 activity are shown in Fig. 5B. The estimated IC50 values obtained showed that organic nitrates, such as ISMN, NOBA, and GTN, did not significantly inhibit the phenacetin O-deethylase activity of CYP1A2. In contrast, NOC-5 completely inhibited CYP1A2 activity at a concentration of 500 µM with an IC50 of 114.2 µM. Incubations were...
performed in homogenate (microsomes, mitochondria, and cytosol) to guarantee the presence of a complete pool of liver enzymes and complete liver metabolic activity.

**Direct Measurement of NO Formation.** Generation of NO was assessed using an electrochemical sensor, and large differences were noted between NOC-5 and the organic nitrates GTN, ISMN, and NOBA. The spontaneous metabolism-independent NO donor NOC-5 showed a rapid generation of NO peaking ($t_{\text{max}}$) at 6 minutes and reaching a maximum concentration ($C_{\text{max}}$) of 4.15 μM (Fig. 5C). In contrast, GTN, NOBA, and ISMN did not show any NO increase over the study period (Fig. 5C). Lower concentrations of organic nitrates were also tested, but again, no formation of NO was detected (unpublished data).

Incubations with the vehicle in reconstituted homogenate or with compounds alone in buffer did not produce any NO.

**Metabolism of GTN in Different Liver Compartments.** Conversion of GTN to NOx differed between the various liver compartments studied. Among the fractions, the cytosol was able to metabolize GTN, with the highest rate $V_0_{\text{cytosol}} = 7.8 \pm 0.5$ μM/min being more than 4 times higher than the metabolism in the other fractions ($V_0_{\text{mitochondria}} = 1.0 \pm 0.1$ μM/min, $V_0_{\text{microsomes}} = 1.7 \pm 0.1$ μM/min) (Fig. 6A). No significant differences were observed when GTN was incubated in homogenate (microsomes, mitochondria, and cytosol), compared with incubations in the cytosolic fraction ($V_0_{\text{cytosol}} \approx V_0_{\text{microsomes} + \text{mitochondria} + \text{cytosol}} = 7.9 \pm 0.6$ μM/min) (Fig. 6A). Moreover, the generation of NO$_2$ during the first part of the pharmacokinetic profile (0–10 minute) in homogenate and cytosol was similar (Fig. 6B).

Although GTN was found to be stable in phosphate buffer (unpublished data), it was slightly unstable in the presence of cofactors. Incubations performed in presence of each cofactor alone (NAD$^+$, NADH, NADPH, or GSH) showed that this instability is attributable to GSH slowly reacting with GTN and generating NOx species (unpublished data).

**Discussion**

In a previous study (Govoni et al., 2006), we showed that nitrooxybutyl-ester derivatives undergo ubiquitous and fast esterase-mediated carboxyl-ester hydrolysis in rat liver and plasma, yielding to the formation of the parent compound and an organic nitrate moiety speculatively identified as the NOBA. The direct and unequivocal identification of NOBA after metabolism of nitrooxybutyl-ester (Fig. 1) has been made possible only in the current study with the development of a novel chromatographic method based on the online conversion of organic nitrates to nitrite and subsequent derivatization with the Griess reagent. Therefore, NOBA is in fact the active organic nitrate metabolite of nitrooxybutyl-esters, and for these reasons, it has been used on its own to investigate the mechanism of denitration of this class of compounds in human liver in comparison with GTN and generating NOx species (unpublished data).
NO donor NOC-5. The various organic nitrates displayed considerably different rates of liver metabolism to NOx, with GTN being rapidly metabolized, NOBA showing an intermediate rate, and ISMN being negligibly metabolized (Fig. 4). Of note, NOC-5 and GTN generated identical quantities of NOx (Fig. 2). Assuming that the first metabolic step of organic nitrates in the liver is associated with the release of NO, which is consequently oxidized to nitrite and nitrate, these data might have been taken as evidence for GTN and NOC-5 having the same NO releasing kinetics in the liver. However, when looking at the generation of NO2− and NO3− separately (Fig. 3, A and B), it became evident that the mechanism of denitration is different from these two molecules. Although NOC-5 denitration is consistent with a direct generation of NO, followed by oxidation to both NO2− and NO3−, GTN denitration led almost exclusively to the generation of NO2− (Fig. 7). Of note, in addition, the metabolic profile of NOBA was not consistent with a direct generation of NO leading exclusively to the generation of NO3− species (Fig. 3C). In addition to the different NO2− NO3− metabolic profile, GTN, NOBA, and NOC-5 also showed different nitrosating capacity measured as S-nitrosothiol generation. In fact, consistent with the direct generation of NO in turn forming the potent nitrosating agent N2O3 (Fig. 7), S-nitrosothiols formation was higher for NOC-5 (Fig. 5A). A further indirect assessment of GTN and NOBA metabolism comes from the experiments looking at the activity of CYP1A2, an enzyme potently inhibited by NO (Stadler et al., 1994). NOC-5 was found to extensively inhibit CYP1A2 activity, whereas the different organic nitrates did not (Fig. 5B).

These results clearly suggest that organic nitrates share a common pathway of denitration in the liver not primarily being metabolized to NO. Instead, depending on their chemical structure, they are metabolized to nitrate or nitrite. This was further demonstrated by measuring NO formation, as shown in Fig. 5C. Although NOC-5 directly released NO, GTN and NOBA did not show any detectable NO formation.

Of importance, the denitration of GTN in liver homogenate is exclusively performed by the cytosolic fraction with a negligible contribution from the mitochondrial and the microsomal compartments. This is clearly evidenced by the fact that the denitration of GTN in the presence of a complete pool of liver enzymes (microsomes, mitochondria, and cytosol) was the same as when using only cytosolic enzymes (Fig. 6). Of interest, it has been shown previously (Kozlov et al., 2003) that the cytosolic fraction of the liver is not capable of significant GTN

![Fig. 6.](A) Extent of denitration of GTN in the different liver compartments. GTN was incubated at a final concentration of 250 μM, and the profile of NOx species generated was observed over the incubation time in reconstituted human liver homogenate (microsomes + mitochondria + cytosol) with cofactors (x), human liver cytosol with cofactors (o), human liver microsomes with cofactors (v), human liver mitochondria with cofactors (♦), and buffer + cofactors (□). (B) Nitrite formation in liver homogenate (microsomes + mitochondria + cytosol) and cytosol during the first 10 minutes of incubation. Values are subtracted from the basal (time 0). Data are presented as mean ± S.D., n = 3.

![Fig. 7.](Metabolism of NOC-5, GTN, and NOBA in human liver. Although NOC-5 is spontaneously converted to NO, which in turn, is metabolized in the liver to nitrite, nitrate, and nitrosothiols, the organic nitrates GTN and NOBA are directly metabolized to nitrite and nitrate, respectively.)
bioactivation to NO. Taken together, these findings suggest that the absence of intrahepatic NO generation is explained by the much higher rate of denitration performed by the cytosolic enzymes, thereby consuming GTN before it reaches the other compartments.

Recently, it has been reported that the enzymatic metabolism of organic nitrates might be concentration dependent (Daiber et al., 2009), and therefore, a careful evaluation of the test concentrations used is needed when investigating the metabolism of these compounds. GTN, ISMN, and nitro-oxybutylester compounds can be taken by the oral route in doses of 46 μmole, 628 μmole, and 2.2 mmole, respectively (Parker and Parker, 1998; Fagerholm and Björnsson, 2005). These molecules are lipophilic and very rapidly absorbed to reach the liver. GTN is almost completely metabolized during first passage, leaving the dinitrate metabolite NOBA to circulate systemically (Needleman, 1976; Yu et al., 1988). The \( t_{\text{max}} \) of intact detectable GTN in vivo in plasma after oral intake is 5 minutes after administration (Yu et al., 1988). This suggests that the entire dose taken is rapidly concentrated in the liver during first pass. Nitrooxybutylester derivatives are rapidly absorbed from the gastrointestinal tract, and their parent compound appears in the circulation with almost complete bioavailability (Fagerholm and Björnsson, 2005). This suggests formation of NOBA at relevant concentration in the liver. According to these considerations, the micromolar concentrations tested in the present work seem to be appropriate for analyzing the metabolic pathway during first passage. These concentrations might, however, not be suitable to assess the functional metabolism of organic nitrates after the first passage, because the concentrations of intact drug reaching the target tissues is orders of magnitude lower.

NO donor compounds are known to inhibit P450 by forming nitrosyl-heme P450 reversibly in competition with oxygen (Ignaoro et al., 2002). Such decreased P450-mediated activity might have implications for drug interactions when NO donors are given in association with other drugs metabolized by P450s. In this in vitro study, organic nitrates were not found to inhibit CYP1A2 (Fig. 5B), most likely because of lack of NO release (Fig. 5C). Translated in vivo, these findings suggest that P450-dependent drug metabolism of concomitant drugs would not be affected by coadministration of organic nitrates.

Another implication of the results of current study is that, although the systemic bioavailability of organic nitrates might be decreased by its liver metabolism to nitrate or nitrite, this specific metabolic route may support another recently described NO pathway. Nitrate can be converted in vivo to nitrite that is emerging as a stable circulating NO pool, and a variety of pathways have been described for the reduction of nitrite to NO in blood and tissues (Lundberg et al., 2009, 2009). Such NO formation is expected to be slower and more prolonged than direct release of NO from most organic nitrates and can significantly contribute to the final therapeutic effect of these molecules in connection to the extent and identity of species generated (nitrate or nitrite) and exported from the liver.

In conclusion, our data suggest that organic nitrates are not primarily metabolized to NO in the liver and their different chemical structures not only dramatically change the extent of denitration in this organ but also the entity of the metabolic species generated. ISMN undergoes negligible liver metabolism, and GTN is rapidly and extensively metabolized primarily to nitrite. Nitrooxybutyl-ester compounds, on the other hand, are first rapidly metabolized to the organic nitrate moiety NOBA, which is eventually slowly metabolized to inorganic nitrate. If the lower extent of denitration of NOBA in comparison with GTN was also reflected in vivo during first passage, NOBA might have the potential to overcome the liver and reach (at least in part) the systemic circulation, allowing for bioactivation to NO in the vascular compartment elsewhere, where a different enzymatic pool than in the liver exists. In summary, the first pass may differently affect the pharmacological profile of organic nitrates in connection with the different extent of denitration and the distinct species generated and exported from the liver (nitrate or nitrite).

References


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