In Vitro Metabolism of (Nitrooxy)butyl Ester Nitric Oxide-Releasing Compounds: Comparison with Glyceryl Trinitrate

Mirco Govoni, Simona Casagrande, Raffaella Maucci, Valerio Chiroti, and Paola Tocchetti

Departments of Drug Metabolism and Pharmacokinetics (M.G., S.C., R.M., P.T.) and Medicinal Chemistry (V.C.), NicOx Research Institute, Milan, Italy

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ABSTRACT

We investigated the in vitro metabolism of two (nitrooxy)butyl ester nitric oxide (NO) donor derivatives of flurbiprofen and ferulic acid, [1,1’-biphenyl]-4-acetic acid-2-flouro-α-methyl-4-(nitrooxy)butyl ester (HCT 1026) and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid 4-(nitrooxy)butyl ester (NCX 2057), respectively, in rat blood plasma and liver subcellular fractions compared with (nitrooxy)butyl alcohol (NOBA) and glyceryl trinitrate (GTN). HCT 1026 and NCX 2057 undergo rapid ubiquitous carboxyl ester hydrolysis to their respective parent compounds and NOBA. The nitrate moiety of this latter is subsequently metabolized to inorganic nitrogen oxides (NOx), predominantly in liver cytosol by glutathione S-transferase (GST) and to a lesser extent in liver mitochondria. If, however, in liver cytosol, the carboxyl ester hydrolysis is prevented by an esterase inhibitor, the metabolism at the nitrate moiety level does not occur.

In blood plasma, HCT 1026 and NCX 2057 are not metabolized to NOx, whereas a slow but sustained NO generation in deoxygenated whole blood as detected by electron paramagnetic resonance indicates the involvement of erythrocytes in the bioactivation of these compounds. Differently from NOBA, GTN is also metabolized in blood plasma and more quickly metabolized by different GST isoforms in liver cytosol. The cytosolic GST-mediated denitration of these organic nitrates in liver limits their interaction with other intracellular compartments to possible generation of NO and/or their subsequent availability and bioactivation in the systemic circulation and extrahepatic tissues. We show the possibility of modulating the activity of hepatic cytosolic enzymes involved in the metabolism of (nitrooxy)butyl ester compounds, thus increasing the therapeutic potential of this class of compounds.

The therapeutic potential of organic nitrates has been known for more than 120 years since the use of glyceryl trinitrate (GTN) in the treatment of angina pectoris. Moreover, the pharmaceutical development of organic nitrates containing adjunct pharmacophores was reported over 40 years ago, and these were observed to manifest biological properties beyond those of the parent compound (Hodosan et al., 1969). However, there has been an explosion of activity in the area of hybrid nitrates over the past decade, stimulated by a growing realization that nitrates may represent new therapeutic agents in different areas (Keeble and Moore, 2001), the mitochondrial aldehyde dehydrogenase (Chen et al., 2002; DiFabio et al., 2003; Kollau et al., 2005) and cytochrome bc1 (Nohl et al., 2000), or the microsomal cytochrome P-450 (Servent et al., 1989; McDonald and Bennett, 1990; Schroder, 1992). Most likely this mechanism is not identical in all tissues or biological mediums, and a cooper-
ation between different enzymes and intracellular compartments is likely to occur (Kozlov et al., 2003). The first metabolite step of organic nitrates was generally thought to be the release of NO, which is consequentially oxidized to nitrite (NO$_2^-$) and nitrate (NO$_3^-$). On these bases, the main metabolic products of NO (NO$_2^-$ + NO$_3^-$) were used to quantify the NO coming from organic nitrates. However, recent findings suggest that biotransformation of organic nitrates should proceed via two steps, where NO$_2^-$ is an intermediate and precursor of NO rather than a product of NO degradation (Millar et al., 1998; Chen et al., 2002; Kozlov et al., 2003). Cyclooxygenase-inhibiting nitric oxide donors (CINODs) are a novel group of compounds with potential therapeutic applications in a variety of clinical conditions. The general structure of such molecules is a NO-releasing moiety ($\text{--ONO}_2^-$) connected via a linker to the parent compound by a carboxyl ester bond. CINODs with different linkers but the same parent compound have also been developed to modulate the extent of NO release within the same pharmacological target. Although an extensive exploration of pharmacological activities of these compounds in different pharmacological models has been carried out (Keeble and Moore, 2002), a structure-activity relationship in association with the metabolism and NO-releasing characteristics has still not been completely elucidated.

In this article, we investigated the metabolism and associated enzymes of two NO-releasing compounds currently under development for the treatment of Alzheimer’s disease, [1,1’-biphenyl]-4-acetic acid 2-fluoro-$\alpha$-methyl-4-(nitroxy)butyl ester (HCT 1026) and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid 4-(nitroxy)butyl ester (NCX 2057), (nitroxy)butyl ester derivatives of flurbiprofen and ferulic acid, respectively (Wenk et al., 2002, 2004; Prosperi et al., 2004). These compounds bear the same aliphatic butyl linker between the parent compound and the NO-releasing group. The role of the aliphatic linker has been studied using (nitroxy)butyl alcohol (NOBA), and its metabolic properties were compared with those of the classic NO donor GTN.

Materials and Methods

Chemicals. HCT 1026, NCX 2057, and their respective dinitrated derivatives [1,1’-biphenyl]-4-acetic acid 2-fluoro-$\alpha$-methyl,4-hydroxybutyl ester (HCT 1027) and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid 4-hydroxybutyl ester (NCX 2059) (Fig. 1) were synthesized at NicOx Laboratories, Department of Medicinal Chemistry (Milan, Italy). HPLC-grade organic solvents were purchased from Carlo Erba Reagents (Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system. GTN and NOBA were kindly provided by Dipharma SpA (Milan, Italy). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Experimental Design for Liver Subcellular Fractions and Blood Plasma Incubations. Blood plasma and livers were obtained from male Sprague-Dawley rats weighing 200 to 250 g (Harlan Italy Srl, San Pietro al Natisone, Italy). Liver subcellular fractions were prepared as described by Kozlov et al. (2003). Liver fraction incubations were performed using protein concentrations reflecting the protein content of the different subcellular fractions in 1 ml of original liver homogenate (Kozlov et al., 2003) (microsomes, 1 mg·ml$^{-1}$; mitochondria, 1.3 mg·ml$^{-1}$; and cytosol, 4.1 mg·ml$^{-1}$). The following cofactor mixtures were used for each incubation: microsomes, 1 mM NADPH; mitochondria, 1 mM NADPH, 0.5 mM NAD$^+$, and 0.5 mM NADH; cytosol, 1 mM NADPH, 0.5 mM NAD$^+$, 0.5 mM NADH, and 2.5 mM reduced glutathione.

HCT 1026, NCX 2057, NOBA, and GTN as well as the inhibitors ethacrynic acid (EA), bromosulfophthalein (BSP), N-ethylmaleimide (NEM), and tetraisopropyl pyrophosphoramide (isOMPA) were dissolved in acetonitrile, dimethyl sulfoxide, or water and added to the incubation (final solvent concentration ≤1% v/v). Drugs were incubated in 0.1 M phosphate buffer, pH 7.4, with rat liver fractions or in rat blood plasma at 37°C under shaking. Inhibitors were added 15 min before drug incubation. At fixed times, 200 µl of incubation mixture was removed and deproteinized with 400 µl of acidified acetonitrile (acetonitrile + 0.5% phosphoric acid, v/v) for HPLC analysis. Another 300 µl of the incubation mixture was removed at the same time for chemiluminescence analysis.

Gas-Phase Chemiluminescence Assay. The total concentrations of inorganic nitrogen oxides (NO$_2^- +$ NO$_3^-$ + nitroxy) species = NOx) and NO$_2^-$ were determined by gas-phase chemiluminescence with a nitric oxide analyzer (NOA 280I; Sievers, Boulder, CO) after reductive cleavage and subsequent determination of the NO released into the gas phase. The apparatus was described in detail by Lundberg and Govoni (2004). NO signals were collected with NO analysis software for Windows (version 3.2; Ionic Instrument Business Group, Boulder, CO), further manipulated with Origin for Windows, version 7.0 (Microcal, Northampton, MA), and reported as area under the curve.

NOx were reduced to NO with a solution of vanadium(III) chloride in 1 M hydrochloric acid (saturated solution) at 95°C. In this condition, organic nitrates eventually present in the sample do not convert to NO or, at worst, they slightly convert, increasing the baseline but not affecting the shape and the recovery of NOx. Despite this, samples were extracted before analysis with chloroform to remove organic nitrates potentially present and stabilize the baseline and deproteinized with ice-cold ethanol.

Nitrates were reduced to NO with a solution consisting of 45 mM potassium iodide and 10 mM iodine in glacial acetic acid at 80°C. Samples were directly injected into the reducing solution without pretreatment. The methods for NOx and NO$_2^-$ determination have been described previously by Lundberg and Govoni (2004).

HPLC Analysis. Liquid chromatography analyses were carried out on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA).

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Fig. 1. Chemical structures of HCT 1026 and NCX 2057 and their hypothetical metabolites arising from biotransformation pathways involving hydrolytic cleavage of the carboxylic and nitric ester functions in the molecule.
sequentially, after 6 h of incubation (Fig. 2, G and H). The direct denitrate metabolites of HCT 1026 and NCX 2057 (HCT 1027 and NCX 2059, respectively) were never observed over the incubation time.

A parallel set of incubations with NOBA, focused on the metabolism of the aliphatic nitrate moiety, showed qualitatively the same results (Fig. 3A) as those for HCT 1026 and NCX 2057 (Fig. 2) in terms of NOx generation. In fact, the NOx formation in blood plasma and liver microsomes after incubation of NOBA (250 μM) was negligible over the incubation time, whereas liver mitochondria showed a slightly higher metabolic activity with NOx formation reaching 28% of the incubated after 6 h. Again, maximum metabolic activity was observed in liver cytosol with sustained NOx generation (V_{max} = 0.25 ± 0.01 nmol · mg^{-1} · min^{-1}) and complete conversion of the nitrate moiety to NOx species (>99% after 6 h of incubation).

Incubations with the vehicle in the same experimental conditions did not produce any NOx concentration increase. Moreover, as indexes of enzymatic activity, HCT 1026, NCX 2057, and NOBA were found to be stable in deactivated boiled liver cytosol (data not shown). Enzymatic Michaelis-Menten parameters measured in cytosol incubations for the disappearance of HCT 1026 or NCX 2057 reflected their fast metabolism at the carboxyl ester level, whereas parameters related to the subsequent formation of NOx species were not significantly different (Table 1). Moreover K_m and V_{max} constants related to the cytosolic NOx formation from NOBA were similar to those of HCT 1026 or NCX 2057 (Table 1). The kinetics of NOx generation at the different NOBA concentrations used for K_m and V_{max} calculations are depicted in Fig. 3B. The analysis of nitrite (NO_2^-) in all cytosolic incubations showed a very low formation of these species over the incubation time, accounting for less than 2% of the incubated over 6 h (data not shown).

**Metabolism of GTN in Rat Blood Plasma and Liver Subcellular Fractions.** GTN incubated in blood plasma at a concentration of 250 μM showed a very rapid NOx formation (V_{o} = 12.8 ± 2.5 nmol · ml^{-1} · min^{-1}), achieving an almost complete conversion to NOx species in 2 h of incubation (Fig. 3C). In liver microsomes and in liver mitochondria, GTN showed an increase of the NOx levels just in the first minutes of incubation (~16% in relation to one nitrate moiety of GTN) and remained stable afterward. In contrast, in liver cytosol GTN was very quickly and completely metabolized to NOx species (80% within 15 min of incubation, V_{o} = 4.5 ± 0.15 nmol · mg^{-1} · min^{-1}). NOx levels reached a complete conversion to NOx species after 1 h of incubation and did not change significantly up to 6 h of incubation (Fig. 3C).

GTN was stable in boiled deactivated cytosol (data not shown). Enzymatic Michaelis-Menten parameters measured in cytosol incubations for the formation of NOx reflected the fast metabolism at the nitrate moiety level of GTN in comparison to NOBA (V_{max} GTN >> V_{max} NOBA) (Table 1). The kinetics of NOx generation at the different GTN concentra-
Fig. 2. Metabolic profile of HCT 1026 and NCX 2057 and their metabolites in blood plasma (A and B), liver microsomes (C and D), liver mitochondria (E and F), and liver cytosol (G and H). HCT 1026 and NCX 2057 were incubated at a final concentration of 250 μM, and their metabolic profile was followed over the incubation time (\(\text{t} \)). \(\Delta\), kinetics of flurbiprofen and ferulic acid formation from HCT 1026 and NCX 2057, respectively, whereas the direct denitrated metabolites HCT 1029 and NCX 2059 were not observed over the incubation time either in blood plasma or in liver subcellular fractions. \(\bigcirc\), time course of NOx species generated over the incubation time. NOx values are subtracted from basal (time 0). Data are presented as means ± S.D., \(n \geq 3\).
Effect of isoOMPA on the Metabolism of HCT 1026 and NCX 2057 in Liver Cytosol. A selective inhibitor of butyrylcholinesterase, isoOMPA, incubated at the concentration of 2.5 mM in liver cytosol inhibited 1) the metabolism of HCT 1026 and NCX 2057 (incubated at a final concentration of 250 µM) to flurbiprofen and ferulic acid, respectively, and 2) the metabolism of NOBA and GTN (incubated at a final concentration of 250 µM) to NOx species. The kinetics of NOx generation at different NOBA and GTN concentrations (50, 100, 250, and 500 µM) in liver cytosol were studied. Values are subtracted from basal (time 0). Data are presented as means ± S.D., n ≥ 3.

TABLE 1
Michaelis-Menten kinetic constants in pooled rat liver cytosol

<table>
<thead>
<tr>
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<th>Carboxyl Ester Metabolism: [P] = Flurbiprofen/Ferulic Acid</th>
<th>Nitric Ester Metabolism: [P] = NOx</th>
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<tr>
<td></td>
<td>$K_m^a$ (µM)</td>
<td>$V_{max}^b$ (nmol·mg⁻¹·min⁻¹)</td>
</tr>
<tr>
<td>HCT 1026</td>
<td>324.58 ± 28.02</td>
<td>28.51 ± 4.20</td>
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<tr>
<td>NCX 2057</td>
<td>281.55 ± 4.58</td>
<td>96.01 ± 1.47</td>
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<tr>
<td>NOBA</td>
<td>248.93 ± 3.69</td>
<td>0.47 ± 0.01</td>
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<tr>
<td>GTN</td>
<td>171.43 ± 40.03</td>
<td>7.32 ± 0.67</td>
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[P], product of the enzymatic reaction; [S], substrate of the enzymatic reaction.

a Calculations were performed based on the disappearance of HCT 1026 and NCX 2057.
b Calculations were performed based on the formation of NOx species.
2) the metabolism of the nitrate moiety to NOx species. Moreover, HCT 1027 and NCX 2059 were never observed during the incubation time. In particular, as shown in Fig. 4A, the concentration of HCT 1026 was less than 20% reduced after 6 h of incubation with the inhibitor, whereas the metabolites flurbiprofen and NOx did not exceed 16% of formation.

Differently from HCT 1026 and NCX 2057, the metabolism of NOBA incubated in the same condition was not affected by isoOMPA. The NOx generation was, in fact, exactly the same in the presence or in the absence of the inhibitor (Fig. 4B).

Nitric Oxide Generation from HCT 1026 and NCX 2057 in Whole Deoxygenated Blood. HCT 1026 and NCX 2057 incubated in deoxygenated whole blood at a concentration of 100 µM produced a slow and sustained HbFe(II)NO accumulation (Fig. 5A). HbFe(II)NO formation from HCT 1026 and NCX 2057 was not significantly different, ranging from 0.2 ± 0.1 to 29.8 ± 5.6 µM for HCT 1026 and from 0.7 ± 0.5 to 28.0 ± 6.7 µM for NCX 2057 after 15 min and 4 h of incubation, respectively (Fig. 5B). Analogously, the initial rate (Vp) of HbFe(II)NO formation accounted for 29.2 nmol · min⁻¹ and 27.6 nmol · min⁻¹ for HCT 1026 and NCX 2057, respectively. The effects could not be attributed to endogenous NO production, because the vehicle was devoid of any effect under these experimental conditions.

Effect of NEM on the Metabolism of HCT 1026, NCX 2057, NOBA, and GTN in Liver Cytosol. The metabolism at the carboxyl ester function of HCT 1026 and NCX 2057 incubated at the concentration of 250 µM in liver cytosol was not inhibited by the presence of NEM, a –SH alkylating compound (incubated at 5 mM). Already after 30 min of incubation, HCT 1026 and NCX 2057 completely disappeared, whereas concomitant and stable formation of flurbiprofen and ferulic acid was observed (Fig. 6, A and B). The metabolism of both compounds was not significantly different, ranging from 63% at an EA concentration of 250 µM up to 93% at a BSP concentration of 2.5 mM (Fig. 8A). According to the fast metabolism of GTN, its level of inhibition was calculated on the basis of the NOx concentration after 6 h of incubation, ranged from 11% at a BSP concentration of 250 µM up to 93% at a BSP concentration of 2.5 mM (Fig. 8C). An analog series of incubations was conducted in the presence of another widely used inhibitor of GST, EA (a diuretic agent currently on the market). Interestingly, EA strongly inhibited the metabolism of GTN in a concentration-dependent manner (from 63% at an EA concentration of 250 µM up to 83% at an EA concentration of 2.5 mM) (Fig. 8D) but did not affect the metabolism of NOBA even at the highest concentration of 2.5 mM (Fig. 8B).

Discussion

The metabolism of (nitroxy)butyl ester compounds such as HCT 1026 and NCX 2057 is summarized in Fig. 9. The ubiquitous fast carboxyl ester hydrolysis of HCT 1026 or NCX 2057 yields the formation of two distinct entities that might exert dual properties, those of the parent compound and those related to an organic nitrate (NOBA).

It is reported that the in vitro activities of CINODs and hybrids in vitro are influenced by addition of esterases (Paul...
Fig. 6. Effects of NEM (5 mM) on the metabolism of HCT 1026 (A), NCX 2057 (B), NOBA (C), and GTN (D) incubated at a final concentration of 250 μM in liver cytosol. A, metabolic profile of HCT 1026 (▼) and its metabolite flurbiprofen (▲) and NOx generation (○) over the incubation time. B, metabolic profile of NCX 2057 (▼), its metabolite ferulic acid (▲), and NOx generation (○) over the incubation time. C and D, time course of NOx species generated over the incubation time after metabolism of NOBA and GTN in the presence (●) or absence (▲) of NEM. NOx values are subtracted from basal (time 0). Data are presented as means ± S.D., n = 3.
Clark et al., 2000; Keeble and Moore, 2002). Moreover, previous observations demonstrated that the fast biotransformation of HCT 1026 in S9 liver fraction incubations was not influenced by the presence or absence of cofactors (S. C. Casagrande, M. G. Govini, R. M. Maucci, and P. T. Tocchetti, unpublished data). This finding indicated that the initial carboxyl ester hydrolysis of the compound is not P450-mediated and might result from the action of carboxyl esterases.

Our results related to the metabolism of HCT 1026, NCX 2057, and NOBA in the presence of a selective inhibitor of butyrylcholinesterase (Fig. 4) allow us to conclude that 1) the first fast and extensive carboxyl ester hydrolysis to the respective parent compound and NOBA is esterase-dependent, 2) the metabolism at the nitrate moiety level is not esterase-mediated, and 3) this latter result does not occur unless carboxyl ester hydrolysis takes place. Justifying these results are the assertions that 1) the first carboxyl ester metabolic step leading to the formation of the parent compound and NOBA is essential for the metabolism of the NO-releasing moiety and 2) NOBA is the active metabolite retaining NO bioactivity. As also supported by similar metabolism at the nitrate moiety level of NOBA (Fig. 3, A and B) in comparison with that of HCT 1026 and NCX 2057 (Fig. 2) and by an analysis of the Michaelis-Menten parameters in the cytosolic fraction (Table 1), NOBA is thus the compound to note when searching for NO-releasing properties and associated enzyme(s) involved with (nitrooxy)butyl ester NO-releasing compounds such as HCT 1026 and NCX 2057.

Fig. 7. Effects of allopurinol (2.5 mM) on the metabolism of NOBA (A) and GTN (B) incubated at a final concentration of 250 μM in liver cytosol. Figures represent the time course of NOx species generated over the incubation time after metabolism of NOBA and GTN in the presence (●) or in the absence (■) of allopurinol. Values are subtracted from basal (time 0). Data are presented as means ± S.D., n ≥ 3.

Fig. 8. Effects of BSP and EA on the metabolism of NOBA (A and B) and GTN (C and D) incubated at a final concentration of 250 μM in liver cytosol. A, NOx generation after metabolism of NOBA after 6 h of incubation in the presence of BSP at a final concentration ranging from 0 to 2.5 mM. B, time course of NOx species generated over the incubation time after metabolism of NOBA in the presence (●) or absence (■) of 2.5 mM EA. C and D, generation of NOx after metabolism of GTN after 2 h of incubation in the presence of BSP (C) or EA (D) at a final concentration ranging from 0 to 2.5 mM. Values are subtracted from basal (time 0). Data are presented as means ± S.D., n ≥ 3. *, p < 0.05 versus control in the absence of inhibitor (0 μM).
Direct evidence of NO generation from HCT 1026 and NCX 2057 has been obtained by using EPR. Both HCT 1026 and NCX 2057 exhibited the same profile of HbFe(II):NO formation, suggesting that the final transformation to bioactive NO comes from the same chemical entity. In effect, the fast carboxyl ester hydrolysis metabolism of HCT 1026 and NCX 2057 with subsequent formation of NOBA and the stability of the latter in blood plasma might allow NOBA to reach erythrocytes and be transformed into bioactive NO within this compartment. This is a further confirmation of the key role played by NOBA in the delivery and biotransformation of this class of compounds. Moreover, this finding demonstrates that an active role is played by erythrocytes in the bioactivation of (nitroxy)butyl ester compounds and suggests that hemoglobin is the possible mediator of this biotransformation (Bennett et al., 1986; Cosby et al., 2003).

Although the chemical structures of the NO-releasing moieties of GTN and NOBA are identical (–ONO2), it is clear that the NO-donating characteristics are different. As also demonstrated by our results, a comparison between the in vitro metabolism of NOBA and GTN showed significant differences both in terms of specificity to the incubation matrices and extent of metabolic products (NOx) formed over the incubation time (Fig. 3, A and C). In particular and differently from NOBA, the rapid metabolism at the nitrate moiety level of GTN in blood plasma might be related to the rapid fall of systemic blood pressure associated to this NO-donor drug.

Recently, the hypothesis that the first metabolic step of organic nitrates is a direct enzymatic bioactivation to NO with a consequent rapid oxidation of this latter to NOx has been replaced by evidence of a direct 1e− reduction to NO2− (Chen et al., 2002; Kozlov et al., 2003). NO2− can then be nonenzymatically or enzymatically converted into bioactive NO (Lundberg and Weitzberg, 2005) and/or can be oxidized to NO3−. In view of this latter mechanism of NO generation, the measurement of NOx species gives information regarding the extent of the first metabolic conversion to species (NO2−) retaining potential NO bioactivity.

In liver, NOBA is metabolized to NOx mainly in the cytosolic fraction and to a minor extent in the mitochondrial fraction. A negligible metabolism to NOx species occurs in microsomes, and this leads to exclusion of the superfamily of P450 as species involved in a direct catalytic activity of the nitrate moiety of (nitroxy)butyl ester compounds. Instead, a role in the metabolism of NOBA is played by mitochondria as recently reported also for GTN (Chen et al., 2002; DiFabio et al., 2003; Kollau et al., 2005).

Considering the cytosolic enzymes, XO has recently been reported to catalyze the anaerobic reduction of GTN to NO2− and then to NO (Millar et al., 1998; Doel et al., 2001). However, incubations of NOBA or GTN in liver cytosol in the presence of an excess of a selective inhibitor of XO did not change the metabolism of these compounds (Fig. 7). Thus, XO, in oxygenated conditions, seems not to be directly involved in the metabolism of the nitrate moiety of both (nitroxy)butyl ester compounds and GTN.

Because GTN metabolism is mediated by a cytosolic glutathione-dependent organic nitrate reductase (Needleman, 1976), our evidence that an (–SH)-alkylating agent such as NEM in liver cytosol inhibited the metabolism to NOx species of NOBA and GTN (Fig. 6, C and D) and that the metabolism of both compounds was inhibited in a concentration-dependent manner by BSP (Fig. 8, A and C) is a clear demonstration of the involvement of GST in the direct metabolism of the nitrate moiety. Interestingly though, another widely used inhibitor of GST, EA, did not affect the metabolism of NOBA but still inhibited the metabolism of GTN (Fig. 8, B and D).

Rat liver is a very complex tissue containing at least 14 GST isoenzymes belonging to the alpha, pi, mu, and theta classes. However, the alpha and mu GST classes are widely represented (56 and 45%, respectively) (Turella et al., 2003). BSP and EA exhibit different inhibition values (Ki) on the alpha and mu GST isoforms of rat liver (Singhal et al., 1996). Differently from that of GTN, the negligible inhibition of EA on liver cytosolic NOBA metabolism suggests a prevalent metabolic activity of one of these two isoforms rather than a wider involvement of different GST isoforms.

The major rat hepatic cytosolic GST isoforms are not normally present in blood plasma and are released in blood only after liver damage (Igarashi et al., 1988). Differently from NOBA, the evidence of an extensive metabolism at the nitrate moiety level of GTN in blood plasma suggests the involvement of other enzymes (in addition to GST) in the direct denitrification of this drug.

Kozlov et al. (2003) demonstrated that in liver GTN is directly metabolized to NO2− in the cytoplasm, and NO2− can be subsequently metabolized to NO in the mitochondria or endoplasmic reticulum by different enzymes. The lack of
physiological response of GTN in liver could be explained by the difficulty of $\text{NO}_2^-$ to reach other subcellular compartments also because of its rapid oxidation to $\text{NO}_3^-$ (Ignarro et al., 1993) as, in fact, is evidenced by the analysis of the ratio $\text{NO}_2^-/\text{NO}_3^-$ in our cytosolic incubations. We identified the cytosolic enzyme involved in the transformation of GTN and NOBA to NOx species as GST. GST had already been shown to be involved in the metabolism of GTN in vascular tissue. However, this enzyme was demonstrated to be capable of reduction to $\text{NO}_2^-$ but not to NO (Kurz et al., 1993). Thus, the cytosolic GST-mediated denitration of these organic nitrates in liver might partially prevent the compounds from reaching other subcellular compartments (such as mitochondria) or tissues intact and being transformed into bioactive NO. For these reasons, GST might be seen as an enzyme responsible for the deactivation of organic nitrates in cytosol, acting, at least in liver, as a scavenger of NO bioavailability.

We demonstrated that, differently from GTN, NOBA seems to be more specifically metabolized by GST (alpha or mu isofrom). Moreover, although GTN is completely and rapidly converted to NOx species by cytosolic GST, NOBA is consistently more slowly metabolized in a linearly dependent manner. The slower liver cytosolic metabolism of NOBA, compared with that of GTN, might allow this compound to reach (at least in part) the subcellular compartment (such as mitochondria) capable of bioactivation to NO or diffuse back into blood, being slowly converted to nitrosyl hemoglobin within erythrocytes and/or reach the vascular system and exert the NO action. Moreover, selective inhibition of GST might decrease the scavenging effect on these organic nitrates in liver, suggesting the possibility of increasing the bioavailability of NO in other tissues.

In conclusion, it is likely that the mechanism of NO generation from organic nitrates depends on the location in which the metabolites occurs, from pathological conditions and varies in relation to the chemical nature of the organic nitrate. Although these aspects complicate the search for the mechanisms of NO generation, the therapeutic potential of these drugs will not be unlocked until a clear identification of the metabolic steps by which they finally provide NO is established.

References


