Possible Metabolic Pathways of Conversion of Formaldoxime and Glyceryl Trinitrate to NO

K. Chalupský, P. Bartík, S. Eklová and G. Entlicher

Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic

Abstract. The oxidation of N-hydroxylated compounds may result in production of nitrogen oxides, including nitric oxide (NO). Oxidation may be independent on NO-synthase. Production of nitrites and nitrates *via* NO from formaldoxime and glyceryl trinitrate was studied and compared. Superoxide ion, ions Fe^{2+} and Fe^{3+}, methemoglobin and methemoglobin + NADPH + methylene blue, oxyhemoglobin and oxyhemoglobin + NADPH + methylene blue in the presence of atmospheric oxygen were used as oxidoreductive agents. Formaldoxime (triformoxime) was chosen as a newly recognized atypical cyclic oxime which can be converted to NO and glyceryl trinitrate as a well-known NO donor of quite different structure. From the oxidoreductive agents used, glyceryl trinitrate was not converted to nitrites or nitrates by Fe^{2+}or Fe^{3+} and by methemoglobin alone. Formaldoxime was resistant to the action of superoxide ion and methemoglobin alone. Importance of these possible metabolic pathways for production of NO from examined vasodilators is discussed.

Key words: Formaldoxime — Glyceryl trinitrate — Oxyhemoglobin — Methemoglobin — Superoxide — Fe^{2+} — Tolerance

Abbreviations: CYP, cytochrome P-450; GTN, glyceryl trinitrate; FAL, formaldoxime; Hb, hemoglobin; metHb, methemoglobin; oxyHb, oxyhemoglobin; MB, methylene blue; Tris, tris-(hydroxymethyl)-aminomethane.

Introduction

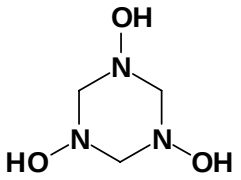
Nitric oxide (NO) is now considered a main endogenous messenger, which plays a key role in diverse biological effects. It has since been implicated in e.g. vasodilation, inhibition of adhesion and aggregation of platelets, bronchodilation, modulation of intestinal mobility, contractions of heart and skeletal muscle, erectile function, neurotransmission and nonspecific immunity (Stamler and Feelisch 1996). Many of these biological effects are mediated by NO stimulation of soluble

Correspondence to: Prof. Gustav Entlicher, Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic
E-mail: entg@seznam.cz

guanylate cyclase (Moncada et al. 1991). Nitric oxide is rapidly oxidized in biological systems by way of reactions with oxygen, superoxide and some metals. Usually, nitrite is the major product of NO metabolism in oxygenated systems (Stamler and Feelisch 1996). Nitric oxide is mainly produced by oxidation of L-arginine by a group of enzymes called the NO-synthases. Of course, there is an increased interest in compounds, from which NO can be produced in cells. These compounds can be classified into several groups that include: 1. organic nitrates (e.g. glyceryl trinitrate (GTN)); 2. organic nitrites (e.g. isoamyl nitrite); 3. inorganic nitroso compounds (e.g. sodium nitroprusside); 4. sydnonimines (e.g. molsidomine (SIN-1)), and S-nitrosothiols (e.g. S-nitrosoglutathione). All these compounds differ in their need of specific necessary metabolic agents to release NO (Tullett and Rees 1998).

All the above mentioned groups of substances should act as potent vasodilators. However, there are many other substances, which can exert a vasodilatory effect in different systems and which can be hardly classified into the above mentioned groups. The list of known vasodilating substances is presented for example in The Merck Index (1996).

It seems that, among others, one relatively simple substance that can generate NO has not been given attention till recently. Formaldoxime (triformoxime) (FAL) has a formula, which does not signalize the possibility of NO production at first sight. The name FAL is used for the unstable monomer as well as for the stable trimer of this substance. From this point of view, triformoxime should be the proper name for the trimer of FAL. However, the name FAL for the trimer is generally accepted.



FAL

Only recently, vasodilatory effects of FAL have been investigated (Chalupsky et al. 2001). It was found that FAL is a relatively potent, endothelium independent vasodilator ($EC_{50} \sim 3$ μmol/l). Its conversion to NO was not found to be mediated by NO-synthase. The present paper deals with the basic properties of this substance from the metabolic point of view. In these respects, FAL was compared with the well-known vasodilator, GTN. As for the reactivity, the effect of superoxide ion, Fe^{2+}, Fe^{3+}, methemoglobin (metHb) or oxyhemoglobin (oxyHb) with added NADPH and methylene blue (MB) was studied. We wanted to study the effect of hemoglobin (Hb) due to known enzymic acitivity of this protein (Giardina et al. 1995). This chromoprotein may exhibit, among other enzymic activities, the activity characteristic of the monooxygenase function of the liver microsomal cytochrome P-450 (CYP). NADPH stimulates the monooxygenase-like activity of oxyHb in the presence of electron carrier, such as MB (Blisard and Mieyal 1981).

It has been known for a long time that CYP can substitute NO-synthase in the decomposition of hydroxy-L-arginine into NO and citrulline (Boucher et al. 1992a). Among different CYP isoforms, CYP 3A subfamily with a very close homology with NO-synthase in the heme-binding domain was found to be the most potent (Renaud et al. 1993). Also other hemeproteins (horseradish peroxidase, catalase or Hb) were found to catalyze the oxidation of hydroxy-L-arginine to citrulline and NO_2^- by H_2O_2 or cumylhydroperoxide (Boucher et al. 1992b). In a similar way N-hydroxyguanidines, amidoximes and ketoximes are oxidized to nitrogen oxides by microsomal CYP (Jousserandot et al. 1998). Biotransformation of GTN with isolated Hb has also been reported (Bennett et al. 1984; Marks et al. 1989). NO formation from nitrovasodilators was also found to be independent of Hb or non-heme iron (Feelisch and Noack 1987). However, to the best of our knowledge CYP-like activity of oxyHb (with the addition of NADPH and MB to the reaction mixture) has not been considered so far with respect to possible metabolism of NO donors and the production of NO.

Materials and Methods

FAL hydrochloride (triformoxime hydrochloride) was bought from Fluka (Buchs, Switzerland), ferric chloride (standard solution; 1,000 g in 1 l) from Merck (Darmstadt, Germany) and glyceryl trinitrate (nitroglycerin, GTN, Lénitral) from Vidal (Paris, France), sulfanilamide, pig metHb, MB, NADPH, N-(1-naphthyl)ethylenediamine, nitrate reductase from *Aspergillus niger*, hypoxanthine, potassium superoxide, N^G-hydroxy-L-arginine, catalase from bovine liver, xanthine oxidase from buttermilk and all other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

Determination of nitrites and nitrates

Nitrite concentrations were determined with Griess reagent: 1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine in 2.5% orthophosphoric acid (Green at al. 1982). Griess reagent was mixed with an equal volume of a sample at room temperature. Absorbance was determined at 540 nm against the blank 10 min later (sample buffer with Griess reagent). For calibration curve sodium nitrite was used as standard. Nitrates were converted to nitrites by NADPH-dependent nitrate reductase (150 mU/ml) and NADPH (40 μmol/l) (final concentrations) (Granger et al. 1996), and the latter were determined with Griess reagent as described above.

Effect of superoxide ion

Potassium superoxide, as a source of superoxide ion (Forman and Fridowich 1973), was dissolved in anhydrous dimethyl sulfoxide in a convenient concentration. The reaction was started simply by the addition of 0.1 ml of the above described potassium superoxide solution to 0.9 ml of an aqueous solution of tested compounds in 0.1 mol/l phosphate buffer, pH 7.4. Final concentration of potassium superoxide was 0.1 mmol/l. Nitrites and nitrates were determined 5 min later. Superoxide

anion was also generated by the hypoxanthine-xanthine oxidase system (Horecker and Heppel 1955).

Reaction with Fe^{2+} and Fe^{3+} ions

Ferrous sulphate or ferric chloride 10 μmol/l, 100 μmol/l, 1000 μmol/l (final concentrations) in 0.05 mol/l Tris-HCl, pH 7.6 (fresh solutions) was mixed with 200 μmol/l FAL (final concentration) or with 200 μmol/l GTN (final concentration), and incubated at 37℃ for various time intervals. After incubation nitrates and nitrites were determined.

Preparation of oxyHb

OxyHb was prepared either by oxidation and oxygenation of metHb, or from fresh rabbit erythrocytes.

MetHb (2.5 mg) was dissolved in 5 ml of 0.05 mol/l Tris-HCl buffer, pH 7.4 and sodium dithionite (35 mg) was added. The mixture was applied to Sephadex G-25 column (15.0 × 1.2 cm) equilibrated with distilled water. Hb was eluted from the column with distilled water and Hb solution was converted to oxyHb by bubbling with oxygen.

Alternatively oxyHb was prepared from fresh rabbit erythrocytes following the described procedure (Pluta et al. 1997). Concentrations of oxyHb or metHb were determined spectrophotometrically using molar absorption coefficients 0.58×10^5l/mol/cm, 0.98×10^5 l/mol/cm at maxima at 430 nm and 415 nm, respectively (Nelson and Cox 2000).

Effect of metHb or oxyHb

MetHb or oxyHb (0.5 mg/ml) in 0.05 mol/l Tris-HCl, pH 7.6 was incubated with 200 μmol/l FAL (final concentration) or with 200 μmol/l GTN (final concentration) at 37℃ for 24 h. In the reaction mixture the bacterial growth was prevented with toluene atmosphere. After incubation nitrates and nitrites were determined. Alternatively NADPH (1 mmol/l) and MB (1 μmol/l) (final concentrations) were added to the reaction mixtures. In this case the reaction mixtures were incubated in dark.

Results

Effect of superoxide ion

Superoxide ion produced nitrites from GTN whereas FAL remained resistant to its action. Concentration dependence of the formation of nitrites from GTN is shown in Fig. 1. The similar results were obtained when superoxide ion was generated from KO_2 or by hypoxanthine-xanthine oxidase system.

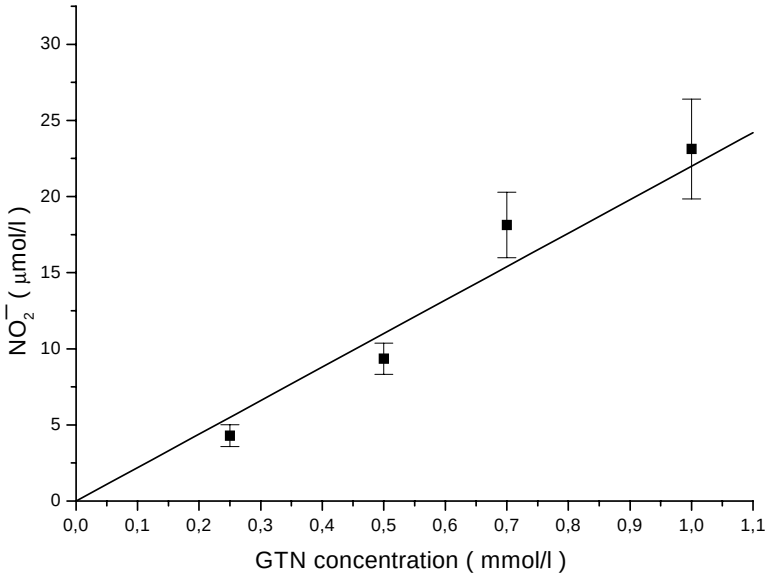


Figure 1. Effect of superoxide ion (100 μmol/l) on GTN conversion to nitrites ($n = 3$).

Effect of Fe^{2+} and Fe^{3+}

The Fe^{2+} and Fe^{3+} ions showed negligible effect on GTN, whereas especially Fe^{2+} ions caused decomposition of FAL and formation of nitrites. Fe^{3+} ions elicited lesser effect than Fe^{2+} ions (results not shown). Concentration (Fe^{2+}) and time dependence of the formation of nitrites from FAL by Fe^{2+} ions are presented in Fig. 2.

No nitrates were found besides nitrites in the reaction mixture.

Effect of metHb or oxyHb + NADPH + MB

Formation of nitrites/nitrates from FAL and GTN by metHb or oxyHb + NADPH + MB is compared in Fig. 3. The effect of NADPH + MB or metHb alone on the conversion of both FAL and GTN to nitrites/nitrates was negligible. The total conversion of FAL and GTN to nitrites/nitrates was about the same. The effect of metHb + NADPH + MB was similar to that of oxy Hb + NADPH + MB. Absorption spectra measurements showed that oxyHb (prepared from rabbit erythrocytes) incubated alone can persist in the oxygenated form for at least 24 hours. All observed reactions with metHb or oxyHb were prevented by heating reaction mixture above 80 ℃. Kinetic data of the reaction of GTN and FAL with oxyHb + NADPH + MB are presented in Table 1.

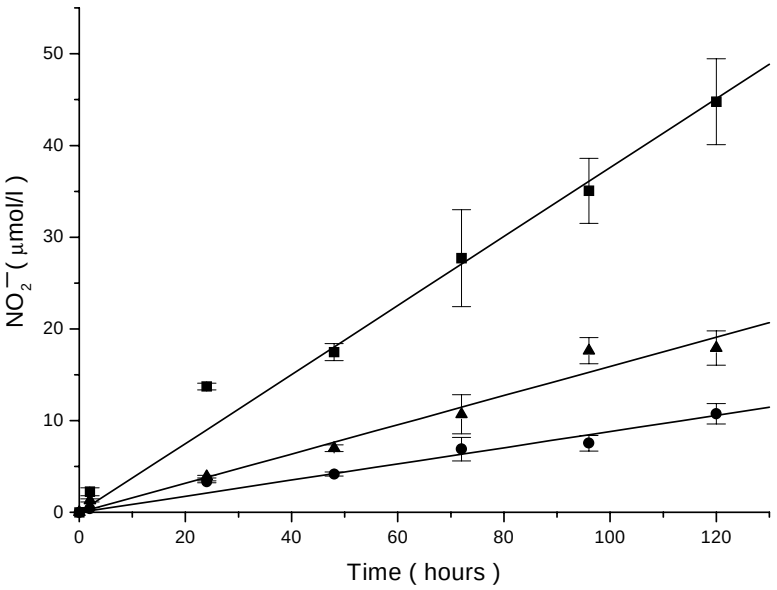


Figure 2. Effect of Fe^{2+} on FAL (200 μmol/l) decomposition to nitrites. ●, Fe^{2+} (10 μmol/l); ▲, Fe^{2+} (100 μmol/l); ■, Fe^{2+} (1000 μmol/l).

Table 1. Enzyme kinetic data[a] of FAL and GTN decomposition

	V_{max} (nmol·l^{-1}·s^{-1})	K_m (μmol·l^{-1})	Specific activity (nmol·s^{-1}·μg^{-1} of protein)
A	1.02 ± 0.02	488 ± 17	2.04
B	2.59 ± 0.48	1195 ± 331	5.18

A, FAL + oxyHb + NADPH + MB; B, GTN + oxyHb + NADPH + MB; [a] Substrate concentrations 200, 400, 600 and 1000 μmol·l^{-1} and other conditions as described in Materials and Methods were used for K_m and V_{max} determination; $n = 5$. The data were calculated with the use of program Hyper 1.1 (`http://www.liv.ac.uk/~jse/software.html`).

Production of nitrates

In all reaction mixtures obtained during the incubation of GTN and FAL with various substances, nitrates were determined in addition to nitrites. Under the experimental conditions used, the found amount of nitrates was different (Fig. 3) but always much lower than that of nitrites. Only with oxyHb the amount of nitrates reached more than half of the total amount of nitrites/nitrates (Fig. 3).

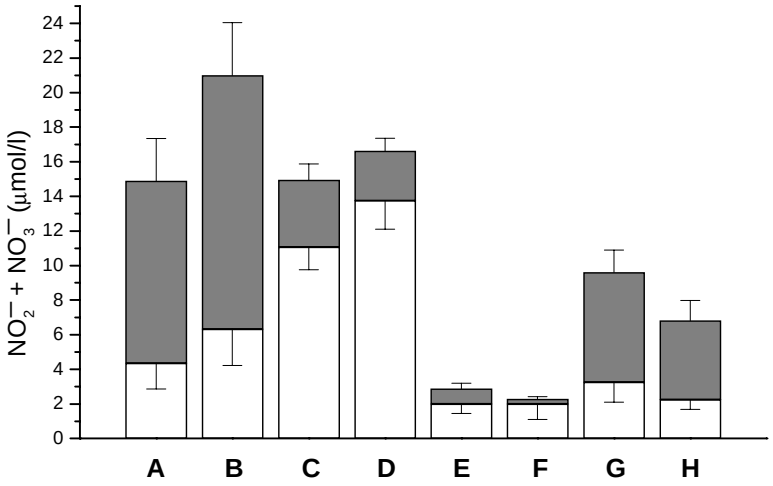


Figure 3. FAL and GTN decomposition to nitrites and nitrates by metHb and oxyHb. **A**, FAL + oxyHb + NADPH + MB; **B**, GTN + oxyHb + NADPH + MB; **C**, FAL + metHb + NADPH + MB; **D**, GTN + metHb + NADPH + MB; **E**, FAL + metHb; **F**, GTN + metHb; **G**, FAL + oxyHb; **H**, GTN + oxyHb. Columns represent nitrite + nitrate concentrations; the white parts – the concentrations of nitrites, the gray parts – the concentrations of nitrates.

Discussion

FAL is able to induce endothelium independent relaxation of isolated rat aorta (Chalupsky et al. 2001). According to the effects of NO-synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), (Zembowicz et al. 1993), cell permeable scavenger of the NO free radical, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), (Akaike et al. 1993) and inhibitor of NO induced guanylate cyclase activation, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), (Hwang et al. 1998), this relaxation was apparently caused by the conversion of the FAL to NO (Chalupsky et al. 2001). The conversion was not mediated by NO-synthase (Chalupsky et al. 2001). This presumption is supported by presented results of the study of FAL reactivity. The differences found in reactivity between GTN and FAL are not surprising. At least, these substances have different chemical structures, GTN being organic ester of nitric acid and FAL an atypical cyclic oxime. On the other hand, the effect of some substances, e.g. oxyHb, is surprisingly similar. It is interesting that the effect of Fe^{2+} on FAL is more pronounced than that of Fe^{3+} and that the effect of Fe^{2+} bound in oxyHb is roughly 30 times larger than the effect of free Fe^{2+} ions. The effect of metHb + NADPH + MB is unexpected. Only Hb in its oxygenated form should exert CYP-like activity and metHb should be inactive according to the literature data (Giardina et al. 1995). The results obtained with metHb indicate that either metHb could have also some CYP-like activity or metHb exerts some

new enzyme-like activity. All reactions studied showed concentration dependence with respect to both the "substrate" and the agent (in addition to those presented in Figs. 1 and 2). An access of air oxygen was necessary for the reaction to proceed. The presence of oxygen for the conversion of hydroxy-L-arginine to NO by CYP is also necessary (Boucher et al. 1992a). However, in our experiments hydroxy-L-arginine was neither converted to nitrites/nitrates by metHb + NADPH + MB, nor by oxyHb + NADPH + MB (results not shown).

We determined nitrites and nitrates as possible final reaction products of NO in our experiments. Of course, nitrite formation is merely indirect evidence for NO intermediate. In accordance to the published results (Stamler and Feelisch 1996; Bonner and Stedman 1996) we found that nitrites were the main final product also of FAL or GTN decomposition (Fig. 3). Only with oxyHb the share of nitrates was much larger. This can be explained by the known reaction of NO with oxyHb which leads to nitrates: oxyHb + NO $\rightarrow$ metHb + NO_3^- (Mateo and De Artinano 2000). The effects of NADPH + MB addition is more pronounced with metHb than with oxyHb. Discussion of these findings with respect to mechanism of NO release would be very difficult as the metabolic transformation of GTN to NO should be reductive in principle while that of FAL should be oxidative. OxyHb acts as an NO scavenger (Mateo and De Artinano 2000) and thus the CYP-like activity of oxyHb could have only limited significance for NO production from NO donors. Nevertheless, it could be important in elimination of NO donors without effective NO formation and thus participate in tolerance induction. Also found reaction of superoxide ion with GTN (and consequent reaction of superoxide ion with NO) is in accordance with findings that increased superoxide production could be related to *in vivo* GTN tolerance induction (Munzel et al. 1999). In this respect the fact that FAL is not affected by superoxide ion is interesting. We used two preparations of oxyHb. All the presented experiments were carried out with lysates of erythrocytes which contain in addition of oxyHb also some oxidoreductases. However, similar results were obtained with oxyHb prepared by reduction of metHb and oxygenation. But, oxyHb prepared in this way was relatively quickly (during 4 h) converted back to metHb. In spite of the fact that CYP-like activity of oxyHb was not considered with respect to oxidation of NO donors, this activity of oxyHb is not surprising. Indeed, the similar activity of metHb was unexpected. Unfortunately, this possible activity of metHb is irrelevant from the physiological point of view. Only very low percentage of Hb ($\sim$1%) is in the form of metHb under physiological conditions in the erythrocyte (Giardina et al. 1995). Physiological significance of measured relatively low enzymic activities is disputable. However, the relative concentrations of the reacting components were given by the detection limits of the method used. Under physiological conditions the concentrations would be shifted to a much higher agent/"substrate" ratio. Moreover, *in vivo* systems might be much more effective.

It is evident that NO donors can be metabolized to NO by several pathways simultaneously. Among them the superoxide pathway may be important for some donors (GTN) under certain circumstances (Munzel et al. 1999). OxyHb CYP-like activity could also be important in metabolism of studied NO donors. However,

both pathways lead to degradation of NO donors without effective NO production and thus they could be important mainly in connection with tolerance to some NO donors.

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