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Diaphorase Can Metabolize Some Vasorelaxants to NO and Eliminate NO Scavenging Effect of 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO)

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Summary

Diaphorase was studied as a possible oxidoreductase participating in NO production from some vasorelaxants. In the presence of NADH or NADPH, diaphorase can convert selected NO donors, glycerol trinitrate (GTN) and formaldoxime (FAL) to nitrites and nitrates with NO as an intermediate. This activity of diaphorase was inhibited by diphenyleneiodonium (DPI) (inhibitor of some NADPH-dependent flavoprotein oxidoreductases), while it remained uninhibited by NG-nitro-L-arginine methyl ester (inhibitor of NO synthase) 7-Ethoxyresorufin (inhibitor of cytochrome P-450 1A1 and cytochrome P-450 NADPH-dependent reductase) inhibited the conversion of GTN only. Existence of NO as an intermediate of the reaction was supported by results of electron paramagnetic resonance spectroscopy. In addition to its ability to affect the above mentioned NO donors, diaphorase was able to reduce 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) and thus to eliminate its NO scavenging effect. This activity of diaphorase could also be inhibited by DPI. The reaction of diaphorase with GTN and PTIO was not affected by superoxide dismutase (SOD) or catalase. Reaction of FAL with diaphorase was lowered with SOD by 38 % indicating the partial participation of superoxide anion probably generated by the reaction of diaphorase with NADH or NADPH. Catalase had no effect. Diaphorase could apparently be one of the enzymes participating in the metabolism of studied NO donors to NO. The easy reduction and consequent elimination of PTIO by diaphorase could affect its use as an NO scavenger in biological tissues.

Key words

Formaldoxime metabolism • Glycerol trinitrate metabolism • Diaphorase • 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) reduction

Introduction

The main biosynthetic pathway discovered so far in mammals for NO formation involves NO-synthase (NOS) catalyzing oxidation of L-arginine to citrulline and NO (Moncada *et al.* 1991). However, there are substances

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with vasorelaxant effects that can produce NO either spontaneously or in some enzyme-catalyzed reactions independent of NOS (Tullett and Rees 1998, Feelish 1998). An intermediate in the conversion of L-arginine to NO, N^G-hydroxy-L-arginine (L-NOHA), can be metabolized to NO by cytochrome P-450 system in addition to NOS (Boucher *et al.* 1992, Renaud *et al.* 1993). In a similar way, some other substances bearing >C=N-OH group (e.g. oximes) can be converted to NO by the cytochrome P-450 system (Jousserandot *et al.* 1998). These substances, especially oximes, can act as efficient NOS-independent NO donors (Větrovský *et al.* 2002). Besides the cytochrome P-450 system, some NO donors are also supposed to be metabolized to NO by hitherto unspecified NAD/NADP-dependent oxido-reductases (Mohazzab *et al.* 1999, Caro *et al.* 2001, Větrovský *et al.* 2002). NAD(P)H-dependent diaphorase was examined as a representative of such oxidases. NADPH-dependent diaphorase was found to be associated with the NOS molecule histochemically in the central nervous system of some species, although this does not apply to other areas (Beesley 1995). Furthermore, NADPH-dependent diaphorase activity was found in brain nuclei following glycerol trinitrate (GTN) administration (Tassorelli and Joseph 1995). The present paper deals with the diaphorase catalyzed conversion of formaldoxime (FAL) to NO. A vasorelaxant effect of FAL has only recently been recognized (Chalupský *et al.* 2001). In this respect, FAL was compared with GTN, a well-known vasodilator. Enzymic reduction of NO scavenger, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) by diaphorase, was also investigated as this scavenger is active in its oxidized form only (Akaike *et al.* 1993).

Methods

Chemicals

Diaphorase from *Clostridium kluyveri,* NADPH, NADH, FAL, PTIO, N^G-nitro-L-arginine methyl ester (L-NAME), L-NOHA, diphenyleneiodonium chloride (DPI), diphenyliodonium chloride, sulfanilamide, N-(1-naphtyl)ethylenediamine, 7-ethoxyresorufin, catalase, superoxide dismutase (SOD), nitrate reductase, dimethylsulfoxide (DMSO), hydroxylamine hydrochloride, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) sodium salt (Na-HEPES), sodium diethyldithiocarbamate (DETC) and Trizma Base were purchased from Sigma (USA). GTN was a product of MERCK (Germany). All other chemicals were products of Lachema (Czech Republic).

FAL and GTN decomposition to nitrites and nitrates by diaphorase

Diaphorase (0.3 mg/ml, 5.2 U/mg of solid) and NADPH or NADH (1 mmol/l) in 0.05 mol/l Tris-HCl pH 7.6 was incubated with 0.2 mmol/l FAL or with 0.2 mmol/l GTN at 37 °C for 2 h. Alternatively, DPI (0.03 mmol/l), diphenyliodonium chloride (0.03 mmol/l), L-NAME (up to 2 mmol/l), 7-ethoxyresorufin (0.01 mmol/l), SOD (100 U/ml) or catalase (100 U/ml) were added. All concentrations given are final concentrations in the reaction mixture. DPI, 7-ethoxyresorufin as well as diphenyliodonium chloride were first dissolved in dimethylsulfoxide and then added to the reaction mixture (final DMSO concentration sulfoxide was 1 %). In the reaction mixture, bacterial growth was prevented with toluene atmosphere. After incubation, nitrites were determined with Griess reagent (Green *et al.* 1982) and nitrates were determined as nitrites with Griess reagent after reduction with nitrate reductase (Granger *et al.* 1996).

Diaphorase catalyzed NO formation from FAL [electron paramagnetic resonance (EPR) spectroscopy]

For EPR spectroscopy the method of Mordvintcev *et al.* (1991) was used. Fe^{2+}-DETC complex $[Fe(DETC)_2]$ for NO radical detection by EPR was used. As this complex is not well soluble in water, denaturated yeast was employed as a solvent. Yeast (0.3 g) was denaturated by boiling in 1 ml of Krebs solution: NaCl 99.01, KCl 4.69, $CaCl_2$ 2.50, $MgSO_4$ 1.20, K_2HPO_4 1.03, glucose 11.1, Na-HEPES 10.0 (mmol/l, final concentrations), pH 7.4. The pellet after centrifugation was suspended in 2.5 ml of freshly prepared colloid $[Fe(DETC)_2]$ ($FeSO_4$ .7 H_2O in distilled deoxygenated water and DETC in deoxygenated Krebs solution). Diaphorase, 200 µl, (final activity 1.54 U/ml), 25 µl of NADPH (final concentration 1 mmol/l) and 25 µl of FAL (final concentrations 1 mmol/l) were added to 250 µl of $Fe(DETC)_2$ (final concentration 0.25 mmol/l) in a yeast suspension. The sample was incubated at 37 °C for 1 h, after that frozen in liquid nitrogen and used for measurement with EPR spectrometer Miniscope (Magnettech, Germany). Control samples contained all substances in the same concentrations except diaphorase. Also representative EPR spectrum of NO-$[Fe(DETC)_2]$ was measured (2-(N,N-diethylamino)-diazenolate-2-

oxide diethylammonium salt, DEA-NONOate, final concentration 30 μmol/l was used as NO donor). The instrument settings were: microwave power 10 mW, amplitude modulation 1 mT, modulation frequency 100 kH, sweep time 100 s and number of scans 10.

Reduction of PTIO in the presence of diaphorase and NADPH

Diaphorase (3 μg/ml, 16 mU/ml) and NADPH (1 mmol/l) in 0.05 mol/l Tris-HCl buffer pH 7.6 was incubated with PTIO (0.3 mmol/l) at 37 °C in the dark. Alternatively DPI (0.03 mmol/l) or SOD (200 U/ml) were added. The reduction of PTIO was determined spectrophotometrically at 550 nm. All concentrations given are final concentrations in the reaction mixture.

Expression of results and statistical analysis

Results were expressed as mean ± S.E.M. of *n* experiments. Student's test for paired or unpaired data (as appropriate) was used for other statistical comparisons, with $p<0.05$ considered to be statistically significant.

Results

Diaphorase + NAD(P)H converted GTN and FAL to nitrites/nitrates (Fig. 1). The effect on GTN was much more pronounced than that on FAL. Either diaphorase alone or NADPH or NADH showed no activity. When NADH was used instead of NADPH, no significant change in the activity was observed. Nitrites were the major final product of the reaction. Only a small amount of nitrates was formed. The same diaphorase system was not able to oxidize L-NOHA to nitrites/nitrates but hydroxylamine conversion was between those of GTN and FAL (data not shown). The enzyme kinetic data of FAL and GTN decomposition by diaphorase are summarized in Table 1. An inhibitor of some NADPH-dependent flavoprotein oxidoreductases, DPI (Cross and Jones 1986, Stuehr *et al.* 1991) decreased the diaphorase activity with FAL to about 25 % and with GTN to about 3 % of the original value. Resulting 1 % DMSO concentration (dimethylsulfoxide solution of DPI was used) did not affect the diaphorase activity. Diphenyliodonium chloride had a much lower inhibitory effect than DPI. An inhibitor of NOS, L-NAME (Rees *et al.* 1990) had no effect on the diaphorase catalyzed reactions. 7-ethoxyresorufin (an inhibitor of cytochrome P-450 $1A_1$ and cytochrome P-450 NADPH-dependent reductase) (Dutton *et al.* 1989, Tassaneeyakul *et al.* 1993) inhibited the reaction of diaphorase with GTN while the reaction with FAL remained unaffected. The addition of catalase also had no influence on the diaphorase activity. SOD did not affect the diaphorase catalyzed conversion of GTN, whereas conversion of FAL was decreased by 38±8 % (n=9). Diaphorase activity was fully destroyed by heating to 100 °C for 5 min.

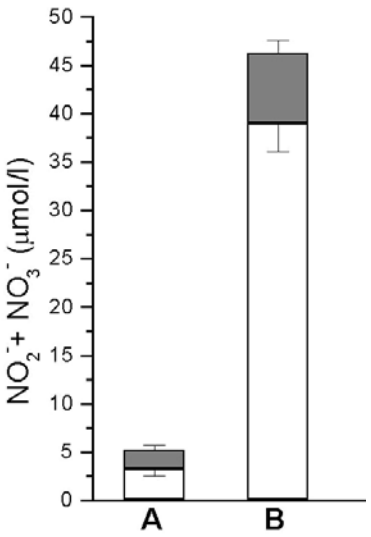

Fig. 1. FAL and GTN decomposition to nitrites and nitrates by diaphorase. A – FAL + diaphorase + NADPH; B – GTN + diaphorase + NADPH; Columns represent nitrite + nitrate concentrations; the white parts - the concentrations of nitrites, the gray parts - the concentrations of nitrates. Results are expressed as mean ± S.E.M. (n=3). Reactions were performed under non-saturating conditions.

Table 1. Enzyme kinetic data of FAL and GTN decomposition by diaphorase.

	V_{max} (nmol.l^{-1}.s^{-1})	K_m (μmol.l^{-1})	Specific activity (pmol.s^{-1}.mg^{-1}of protein) (5.6 U/mg of protein)
A	1.1 ± 0.2	278 ± 6	4.1
B	26.0 ± 0.3	985 ± 7	97.0

The data were calculated with the use of program Hyper 1.1 (http://www.liv.ac.uk/~jse/software.html). Results are expressed as mean ±S.E.M. (n=5). **A** – FAL + diaphorase + NADPH; **B** – GTN + diaphorase + NADPH;

EPR spectroscopy was used to support the assumption that NO radical was formed during the studied reactions catalyzed by diaphorase. Results with FAL are presented in Figure 2. A significant signal of NO was observed. Similar results were obtained with GTN

(data not shown).

Reduction of PTIO in the presence of diaphorase and NADPH or NADH was studied spectrophotometrically as the oxidized form of PTIO has a distinct absorption maximum at about 550 nm (Fig. 3). Reduction of this substrate by diaphorase was inhibited by DPI to about 30 % of the original one, but was completely unaffected by even higher concentration of SOD (200 U/ml).

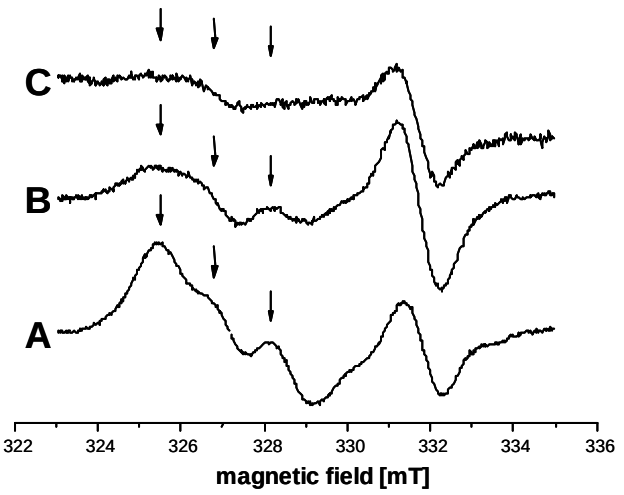

Fig. 2. Diaphorase catalyzed NO formation from FAL (EPR spectroscopy). Black arrows indicate typical triplet hyperfine structure of NO-[Fe(DETC)$_2$]. **A** - standard spectrum of NO-[Fe(DETC)$_2$], **B** – FAL + diaphorase + NADPH, **C** - control (FAL + NADPH)

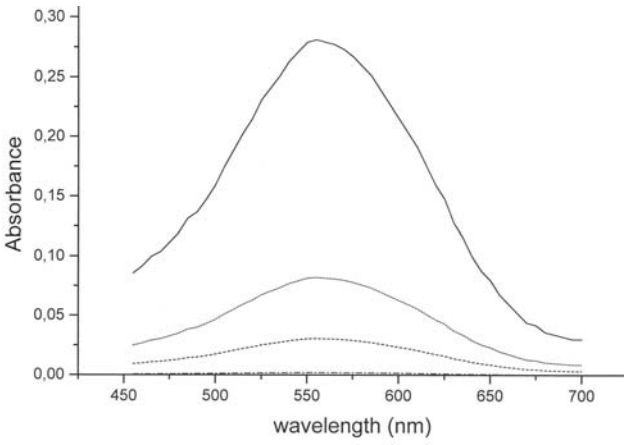

Fig. 3. Reduction of PTIO in the presence of diaphorase and NADPH _____ t = 0 (without diaphorase); **.....** t =10 min; **--------** t = 30 min; _._._._. t = 60 min.

Discussion

Diaphorase could apparently be one of the enzymes participating in the metabolism of studied NO donors to NO. This activity of diaphorase has not been described so far. However, this ability of diaphorase is in accordance with the supposed activity of unidentified NAD(P)H-dependent oxidases in the metabolic transformation of some NO donors to NO (Mohazzab *et al.* 1999, Caro *et al.* 2001, Větrovský *et al.* 2002). Inhibition of diaphorase with DPI is also in accordance with the findings that DPI can antagonize vascular relaxations caused by GTN (Bennett *et al.* 1994) as well as those caused by 4-chlorobenzamidoxime (Větrovský *et al.* 2002). Recently, mitochondrial aldehyde dehydrogenase was found to be responsible for metabolic transformation of GTN to NO (Chen *et al.* 2002, Ignarro 2002). It seems that several metabolic pathways may exist simultaneously. Apparently, this activity of diaphorase could be physiologically very important as this enzyme is present in cells of all aerobic organisms, but diaphorase used in our experiments was a commercial product of microbial origin. The observed effect of diaphorase on PTIO was completely unexpected. According to Akaike *et al.* (1993), PTIO should act as a NO scavenger by transformation of NO to an equimolar mixture of nitrites and nitrates. In our experiments, PTIO served as a very good substrate for diaphorase. This easy reduction and consequent elimination of PTIO by diaphorase and possibly by other NAD(P)H-dependent oxidases could affect its use as an NO scavenger in biological tissues.

In the case of the two examined NO donors and PTIO, the used diaphorase in fact catalyzed two types of reactions. With GTN and PTIO it is a simple reduction, while with FAL it is oxidation in the presence of O_2 and NAD(P)H (NAD or NADP were found to be ineffective as cofactors). This oxidation is, perhaps, similar to that of oximes with cytochrome P450 and NADPH (Jousserandot *et al.* 1998). Reactions with GTN and PTIO were completely unaffected by SOD while that with FAL was decreased by 38 %. This indicates that conversion of FAL by diaphorase is potentiated by the superoxide ion generated probably by the diaphorase reaction with NADH or NADPH.

Conversion of GTN to NO by aldehyde dehydrogenase isolated from mitochondria and activities of diaphorase (commercial preparation of microbial origin) described above are interesting from the point of view of general biochemistry. Hypothesis can be presented that both enzymes could form a part of the enzyme complex (containing dihydrolipoyl transacetylase, pyruvate or oxoglutarate dehydrogenase

and dihydrolipoyl dehydrogenase) accomplishing oxidative decarboxylation of α-oxoacids (pyruvate, α-oxoglutarate).

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