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Enzymatic formation of nitrogen oxides from L-arginine in bovine brain cytosol

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In dialyzed bovine brain cytosol, the enzymatic formation of nitrogen oxides was directly determined. The basal formation of nitrite and nitrate was concentration-dependently enhanced by L-arginine (EC_{50} about $3 \cdot 10^{-5}$ M). Both the basal and L-arginine induced formations were inhibited by N^G-monomethyl-L-arginine (EC_{50} about $2 \cdot 10^{-4}$ M). In the presence of L-arginine, a concomitant formation of citrulline was detected. L-Arginine methyl ester also served as a substrate, but neither D-arginine, D-arginine methyl ester nor Nα-benzoyl-L-arginine ethyl ester did so. The formation of nitrite and nitrate was time-dependent, increased linearly with the protein concentration of the cytosol and was not observed when the cytosolic proteins were heat-denaturated. Exogenous NADPH (or $NADP^+$) concentration-dependently enhanced the formation of nitrite and nitrate, whereas NADH, NAD^+, FAD, Ca^{2+}, Mg^{2+} and calmodulin were ineffective. These results indicate that bovine brain contains a cytosolic enzyme which uses NADPH or $NADP^+$ as cofactors to form nitrogen oxides from both an endogenous non-dialyzable substrate and from L-arginine.

In different vertebrate cells, e.g. porcine endothelial cells [1,2], murine macrophages [3,4] and human neutrophils [5-8], the formation and release of nitric oxide or nitric oxide-containing compounds (R-NO) has been shown. R-NO activates cytosolic, soluble guanylyl cyclase and, thereby, induces an elevation of the intracellular cGMP concentration in various target cells [9,10]. R-NO formation is dependent on L-arginine, and experiments with [guanido-$^{15}N_2$]L-arginine revealed that one or both of the terminal guanidino nitrogens of L-arginine serve as physiological precursors for R-NO [11,12]. In endothelium [13], macrophages [3] and human neutrophils [8], formation and/or release of R-NO are potently inhibited by N^G-monomethyl-L-arginine [13], a structural analogue of L-arginine. The enzymatic formation of nitrogen oxides has, so far, only been demonstrated in murine macrophages [14-16]. Here, the conversion of L-arginine into R-NO and probably citrulline is catalyzed by a cytosolic enzyme(system) that depends on NADPH as a cofactor [14]. Several reports indicate that a similar biosynthetic pathway is present in the brain: L-Arginine serves as an endogenous

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activator of soluble guanylyl cyclase in cytosols of neuroblastoma cells [17], upon stimulation with N-methyl-D-aspartate, cerebellar cells release a factor with properties similar to NO [18], and in brain synaptosomes, L-arginine induces an increase in cGMP which effect is dependent on Ca^{2+} and NADPH [19]. In endothelial cells [20,21] and bovine lung [22], there is also indirect evidence (by soluble guanylyl cyclase activation) for enzymatic formation of R-NO from L-arginine. The aim of the present study was to investigate whether bovine brain cytosol contains an enzyme(system) that forms R-NO from L-arginine and, if so, to develop methods to characterize this enzyme(system) by directly measuring defined L-arginine derived nitrogen oxides, i.e. nitrite and nitrate (NO_x^-), and citrulline.

Materials and Methods

Preparation of bovine brain cytosol. Bovine brain (540 g) was washed with chilled NaCl (0.9 %, w/v). The arachnoidea, brain stem and calix were removed. The brain was then mixed with 1,620 ml of a buffer solution (pH 7.5; 3.2 mS/cm), containing (final concentrations in mM) triethanolamine/HCl (50), EDTA (2.0), reduced glutathione (GSH; 2.0), benzamidine (0.2), and were homogenized. The homogenate was centrifuged for 75 min at $100,000 \cdot g_{av}$. For the removal of low molecular weight components, e.g. Ca^{2+}, NADPH and L-arginine, the obtained cytosol was dialyzed against a buffer solution (pH 7.8; 3.2 mS/cm), containing triethanolamine/HCl (20 mM), EDTA (1.0 mM), DL-dithiothreitol (1.0 mM), benzamidine (0.26 mM), penicillin G (60 kU/l) and streptomycin (60 mg/l). The protein content was 8.3 mg/ml as estimated by the method of Bradford [23] using bovine serum albumine as standard protein. Until use, the dialyzed cytosol was stored at -80 °C for 2 months without loss of NO_x^--forming enzyme activity.

Enzyme incubation. Aliquots of the dialyzed cytosol (0.33 mg protein/ml) were incubated (37 °C; 18 h), if not otherwise stated, in the presence of EDTA (0.08 mM), triethanolamine/HCl (50 mM; pH 7.8), free Mg^{2+} (2.0 mM), free Ca^{2+} (0.01 mM), L-arginine (0.3 mM), NADPH (1.0 mM). The reaction was stopped by heat-denaturating the samples (70 °C, 20 min). Incubates were then assayed for NO_x^- and citrulline.

Calcium buffers. The free Ca^{2+} and Mg^{2+} concentrations were adjusted by adding $CaCl_2$ and $MgCl_2$ as calculated from the respective stability constants given by Holloway and Reilley [24]. The final EDTA concentration (which was used for the calculations) was $8 \cdot 10^{-4}$ M.

Determination of nitrite and nitrate. The concentration of nitrite and nitrate in the incubates was determined by a chemiluminescence method [1] after reduction of nitrate to nitrite with nitrate reductase. A 100 µl-aliquot of the incubate was combined with nitrate reductase (20 mU) and (final concentrations in mM) phosphate buffer (50; pH 7.5), FAD (5.0) and NADPH (0.6) to a final volume of 120 µl and incubated (1 h; 37 °C). Under these conditions, nitrate was reduced to quantitatively yield nitrite. When nitrate reduction had completed, the 120 µl assay mixture was injected into a solution of KI (1 %, w/v, in glacial acetic acid). Under these conditions, nitrite is reduced to NO. NO was extracted from this reaction mixture by a constant flow of nitrogen gas into an NO gas analyser. There was a linear correlation between the NO chemiluminescence signal and the amount of nitrite injected (detection limit 30 pmol; linear range 30-3000

pmol). Equimolar standard solutions of nitrite and nitrate incubated with nitrate reductase gave equal NO chemiluminescence signals.

Determination of citrulline. Citrulline was determined according to a modification of the method described by Rothnagel and Rogers [25]. A 200 μl aliquot of the incubate was pretreated with urease (100 mU; 1 h; 37 °C) in a final volume of 210 μl and thereafter combined with 400 μl of acid solution (conc. H_2SO_4:conc. H_3PO_4:H_2O; 25:20:55 v/v; supplemented with 41.7 mg/500 ml $FeCl_3 \cdot 6H_2O$) and 200 μl of reagent solution (thiosemicarbazide, 0.01 % w/v; diacetylmonoxime, 0.5 %, w/v, in H_2O). The mixture was heated (100 °C; 15 min), cooled at room temperature for 15 min, centrifuged ($10,000 \cdot g_{av}$), and the absorption at 540 nm was measured spectrophotometrically.

Data evaluation. Each experiment was performed 3-4 times, and each point was estimated in triplicates expressed as mean ± S.E.M.

Materials. All compounds were of analytical grade and were obtained from Sigma (Deisenhofen, F.R.G.), except for buffer salts (Merck, Darmstadt, F.R.G) and N^G-monomethyl-L-arginine acetate (Calbiochem, Frankfurt, F.R.G). D-Arginine methyl ester was a kind gift from Dr. P. Del Soldato (Italfarmaco Research Center, Milano, Italy).

Results

When dialyzed bovine brain cytosol was incubated at 37 °C, a basal formation of nitrite and nitrate (NO_x^-) was detected (190 ± 39 pmol NO_x^-/mg protein; Fig. 1), which was concentration-dependently increased by L-arginine (EC_{50} about $3 \cdot 10^{-5}$ M) up to 2.5 fold (473 ± 38 pmol NO_x^-/mg protein). In the presence of L-arginine, the formation of NO_x^- was time-dependent and increased linearly with the protein concentration. When heat-denaturated cytosolic proteins were incubated under standard conditions, no NO_x^- formation was observed (not shown). In the presence of L-arginine, concomitantly to the formation of NO_x^- the formation of citrulline was observed (11.3 pmol/mg protein). An effect similar to that of L-arginine was observed with L-arginine methyl ester but not with the respective D-enantiomers or with Nα-benzoyl-L-arginine ethyl ester. The latter compound apparently inhibited the basal NO_x^- formation. N^G-Monomethyl-L-arginine concentration-dependently inhibited both the basal, i.e. in the absence of L-arginine, and the L-arginine induced NO_x^- formation. The NO_x^- formation in the presence of L-arginine was not dependent on exogenous NADPH or NADP, but was enhanced by these cofactors (Fig. 2). Exogenous NADH, NAD and FAD (up to 1 mM) did not serve as cofactors. The NO_x^- forming enzyme activity was not affected by the concentrations of free Ca^{2+} and Mg^{2+} (in the range of 10^{-5}-10^{-2} M; not shown) or by Ca^{2+}/calmodulin (in the range of 0.3-10 U/ml; not shown).

Discussion

Bovine brain cytosol contains an enzyme(system) that forms R-NO and citrulline from L-arginine. R-NO was determined as nitrite and nitrate

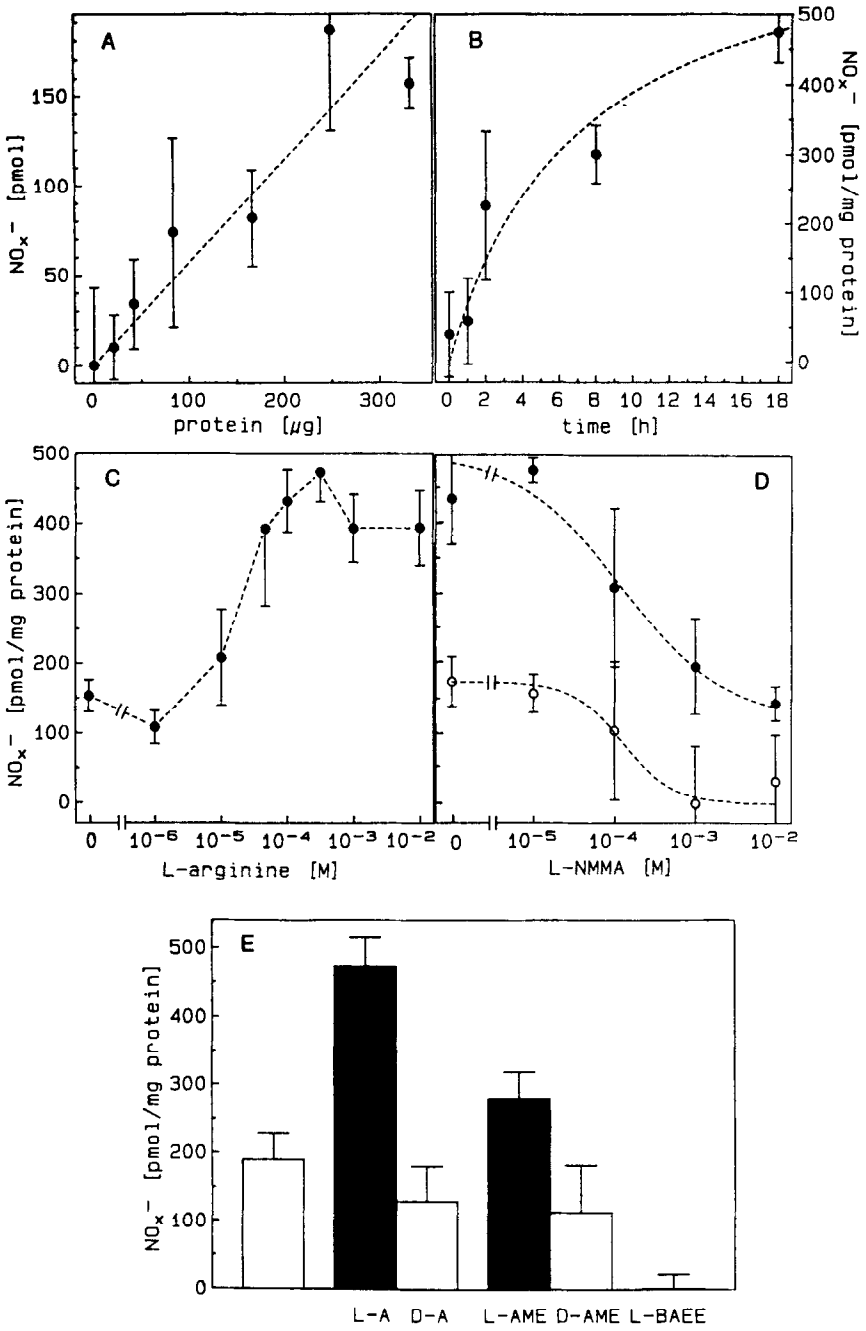

Fig. 1. Effects of protein concentration, incubation time and arginine analogues on the formation of nitrite and nitrate (NO_x^-) in dialyzed bovine brain cytosol. Standard incubation conditions were: 0.33 mg of protein, 1 mM NADPH, 50 mM triethanolamine/HCl, 0.01 mM free Ca^{2+}, 2 mM free Mg^{2+}, 0.8 mM EDTA, no arginine (o) or 0.3 mM L-arginine (●), 18 h, 37 °C. Panel A: Effects of protein concentration. Panel B: Effect of incubation time. Panel C: Effect of the L-arginine concentration. Panel D: Effects of N^G-monomethyl-L-arginine on the NO_x^- formation in the absence (o) and in the presence of 0.3 mM (exogenous) L-arginine (●). Panel E: Effect of L- and D-arginine (L- and D-A), L- and D-arginine methyl ester (L- and D-AME) and Nα-benzoyl-L-arginine ethyl ester (L-BAEE; 0.3 mM each). Points and bars represent means, vertical lines represent S.E.M. (n = 9-12).

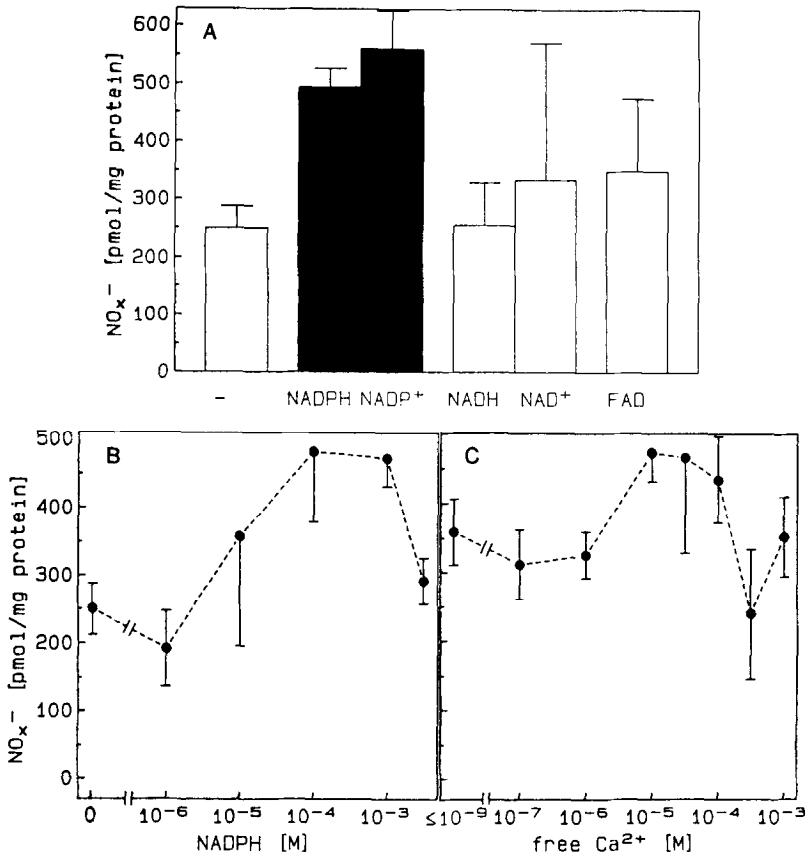

Fig. 2. Effects of cofactors and Ca^{2+} on the formation of nitrite and nitrate (NO_x^-) in dialyzed bovine brain cytosol. Standard incubation conditions were: 0.33 mg of protein, 1 mM NADPH, 50 mM triethanolamine/HCl, 0.01 mM free Ca^{2+}, 2 mM free Mg^{2+}, 0.8 mM EDTA, 0.3 mM L-arginine (●), 18 h, 37 °C. Panel A: Effects of NADPH, $NADP^+$, NADH, NAD^+ and FAD (1 mM each). Panel B: Effects of increasing NADPH concentrations. Panel C: Effects of increasing free Ca^{2+} concentrations. Points and bars represent means, vertical lines represent S.E.M. (n = 9).

(NO_x^-). The formation of NO_x^- was dependent on the time and protein concentration. In the present study, an incubation time of 18 h at 37 °C had to be chosen. In studies on the formation of L-arginine-derived NO_x^- in murine macrophage cytosol, the required incubation times for the accumulation of NO_x^- were also long, i.e. 19 h at 25 °C [16] and 3 h at 37 °C [15], respectively. A non-dialyzable factor of bovine brain cytosol, exogenous L-arginine and L-arginine methyl ester were substrates for NO_x^- formation. The effect of arginine analogues on NO_x^- formation was stereospecific since D-arginine and D-arginine ethyl ester were ineffective. N^G-Monomethyl-L-arginine concentration-dependently inhibited both the basal (i.e. in the absence of exogenous L-arginine) and the L-arginine-induced NO_x^- formation.

Several findings indicate, that besides free L-arginine peptides that contain L-arginine are also substrates for the NO_x^- forming enzyme(system): In neuroblastoma cells, an endogenous activator of the cytosolic guanylyl cyclase is formed from L-arginine but also from peptides containing L-arginine [17]. In the cytosol of lipopolysaccharide- and interferon-γ-activated murine macrophages, a constitutive high-molecular weight factor greatly enhanced NO_x^- formation [15]. In endothelial cells, Nα-benzoyl-L-arginine ethyl ester, a substrate for various peptidyl-L-arginine-utilizing enzymes, has been suggested to be a substrate for R-NO formation [26]. When bovine brain cytosolic proteins were separated on a Mono Q anion exchange column, preliminary findings suggest that a factor that did not bind to Mono Q anion exchange resin when recombined with dialyzed bovine brain cytosol was utilized by the NO_x^- forming enzyme(system) to generate NO_x^- (not shown). This factor is likely to account for the basal NO_x^- formation observed in dialyzed bovine brain cytosol, i.e. in the absence of added L-arginine. However, in bovine brain cytosol, Nα-benzoyl-L-arginine ethyl ester was not a substrate for NO_x^- formation.

NADPH serves as a cofactor for the formation of NO_x^- from L-arginine. However, the fact that the formation of NO_x^- was also observed in the absence of potential (exogenous) cofactors may indicate that cofactors are utilized that are tightly bound to protein components of the dialysate.

It is currently a matter of debate, whether the R-NO forming enzyme is regulated by the free Ca^{2+} concentration. In neuronal tissues, a stimulatory effect of L-arginine on guanylyl cyclase activity has been observed both in de-ionized cytosol of neuroblastoma cells [17] and in a completely Ca^{2+} dependent manner in brain synaptosomes [19]. Similarly, in endothelial cell cytosol, positive [20] and negative [21] findings on regulatory effects of Ca^{2+} have been reported. In the present study, neither free Ca^{2+}, Mg^{2+} nor Ca^{2+}/calmodulin significantly affected the formation of NO_x^- in the presence of exogenous L-arginine. However, due to the obligatory long incubation time, regulatory effects of Ca^{2+} or Ca^{2+}-dependent mechanisms may have been masked and may only be detectable by methods which require shorter incubation times (in the minute range), e.g. the use of soluble guanylyl cyclase stimulated by L-arginine-derived R-NO [20,22].

Citrulline has been suggested to be the co-product of NO_x^- formation from L-arginine [3,4,12,14-17,19]. L-arginine-dependent citrulline formation was detected in dialyzed bovine cytosol. However, the molar ratio of the citrulline vs. the NO_x^- formation was 24:1, suggesting that, in bovine brain cytosol, either citrulline is not the co-product of NO_x^- formation from L-arginine or that bovine brain cytosol contains an L-arginine deiminase like activity, forming citrulline but not NO_x^- from L-arginine, or that NO_x^- is not the major breakdown product of the L-arginine-derived R-NO. With

respect to the latter possibilty, it has to be taken into account that R-NO may be identical with hydroxylamine (H_2NOH) formed by hydrolysis of N^G-hydroxy-L-arginine. H_2NOH degradates only partially to NO_x^-, with N_2 and NH_3 being the major breakdown products [27].

In conclusion, bovine brain is the second vertebrate tissue where enzymatic formation of nitrogen oxides from L-arginine has been demonstrated. The nitrate reductase/chemiluminescence assay for NO_x^- formation appears to be a useful tool to further characterize and purify the enzyme(system), cofactors, endogenous non-dialyzable substrate(s) and to elucidate whether H_2NOH or other R-NO are released from L-arginine.

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