Volume 294, number 3, 221–224 FEBS 10491 December 1991

On the substrate specificity of nitric oxide synthase

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Received 9 October 1991

Nitric oxide (·NO) synthase (NOS) activity in subcellular fractions from cultured endothelial cells (EC) and lipopolysaccharide-activated J774.2 monocyte/macrophages was investigated by monitoring the ·NO-mediated increase in intracellular cyclic GMP in LLC-PK$_1$ pig kidney epithelial cells. The constitutive NOS in EC (NOS$_c$) was largely membrane-bound, whereas the inducible NOS in J774.2 cells (NOS$_i$) was equally distributed among cytosol and membrane(s). Both the cytosolic NOS$_c$ in EC and the membrane-bound NOS$_i$ in J774.2 cells were strictly Ca^{2+}-dependent, whereas the membrane-bound NOS$_c$ in EC and the cytosolic NOS$_i$ in J774.2 cells were not. L-Homoarginine and L-arginine-containing small peptides, such as L-arginyl-L-phenylalanine, replaced L-arginine as a substrate for the NOS$_c$ in EC and the Ca^{2+}-independent NOS$_i$ in J774.2 cells, but not the Ca^{2+}-dependent NOS$_c$. Thus, irrespective of their intracellular localisation, at least three isoforms of NOS exist, which can be differentiated by their substrate specificity and Ca^{2+}-dependency.

Nitric oxide synthase; Calcium; Structure-activity relationship, L-Arginine analogue; Endothelial cell; Monocyte/macrophage

1. INTRODUCTION

Nitric oxide (·NO) is a potent vasodilator and antithrombotic agent, and plays an important role in the cytotoxicity of activated macrophages and as a neurotransmitter (for a recent review see [1]). Its formation from L-arginine (L-Arg) is catalyzed by an NADPH-dependent dioxygenase, referred to as ·NO synthase (NOS), which can exist in at least two distinct forms [2]. One enzyme, a constitutive agonist-triggered and Ca^{2+}/calmodulin-dependent NOS, is mainly present in neuronal cells [3] and endothelial cells [4], whereas the other enzyme, a Ca^{2+}-independent inducible NOS, is found predominantly in macrophages [5] and smooth muscle cells [6] after activation by cytokines or bacterial lipopolysaccharide (LPS). The reaction catalysed by both types of NOS is likely to be identical, as N^G-hydroxy-L-arginine (L-HOArg) is a substrate for both the inducible [7] and constitutive NOS [8]. An important way of elucidating the chemical nature of this mechanism is to characterise the relative substrate specificity of these enzymes. Recently, we have proposed that L-arginine-containing dipeptides serve as additional or alternative substrates for the biosynthesis of ·NO by cultured endothelial cells (EC), and that this could also be important for the flow-induced release of ·NO from the endothelium in vivo [9]. Consequently, we have investigated whether these dipeptides are converted to ·NO by NOS preparations derived from cultured EC and monocyte/macrophages.

Abbreviations: EC, endothelial cell(s); EGTA, ethylene glycol-bis(β-amino ethyl ether) N,N,N′-N′-tetraacetic acid; L-MeArg, N^G-monomethyl-L-arginine; ·NO, nitric oxide; L-NO$_2$Arg, N^G-nitro-L-arginine; L-HOArg, N^G-hydroxy-L-arginine; D-HOArg, N^G-hydroxy-D-arginine; SOD, superoxide dismutase.

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2. MATERIALS AND METHODS

2.1. *Materials*

D-Arg, L-Arg, bestatin, calmodulin (from bovine brain), L-homoarginine, and superoxide dismutase (from bovine erythrocytes; SOD) were obtained from Sigma Chemical Co; L-MeArg acetate salt from Calbiochem, L-NO$_2$Arg acetate salt and the various peptides from Bachem Feinchemikalien AG; and (6*R*,*S*)-5,6,7,8-tetrahydro-L-biopterin (THB) from Dr. B. Schircks Laboratories. L-HOArg and D-HOArg (purity ≥98%) were synthesized by Dr. Paul L. Feldman [10]. All other reagents and solvents were of the highest commercially available quality from either Sigma Chemical Co. or Merck Ltd.

2.2. *Cell culture*

Bovine aortic endothelial cells were harvested and grown to confluence on Cytodex-3 microcarrier beads (Pharmacia-LKB Ltd.) in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 4 mM L-glutamine and 10% (v/v) fetal calf serum (FCS; Gibco). J774.2 cells (murine monocyte/macrophage cell line, ECACC 85011428) and LLC-PK$_1$ pig kidney epithelial cells (ATCC CL 101) were obtained from the European Collection of Animal Cell Cultures. The J774.2 cells were grown to a density of 1–2×10^6 cells/ml in stirrer bottles in 100–200 ml DMEM supplemented with 4 mM L-glutamine and 10% FCS, and the LLC-PK$_1$ cells were seeded into 96-well plates and grown to confluence in medium 199 (Flow Laboratories) supplemented with 10% FCS.

2.3. *Preparation of subcellular fractions*

All of the following procedures were carried out at 0–4°C. Approximately 10^9 bovine aortic EC on beads or 0.5×10^9 J774.2 cells, induced by mouse interferon (500 U/ml; Sigma) for 6 h followed by LPS

(serotype 0127:B8; 1 μg/ml; Sigma) for 18 h or non-induced, were suspended in 50 mM Tris·HCl buffer, pH 7.4, containing 10 mM EDTA, 5 mM glucose, 1.15% (w/v) KCl, 0.1 mM DL-dithiothreitol (DTT), 200 U/ml SOD, 2 mg/l leupeptin, 2 mg/l pepstatin A, 10 mg/l trypsin inhibitor and 44 mg/l phenylmethylsulfonyl fluoride (PMSF). The cell suspensions were bubbled with helium for 15 min, sonicated (Sonic & Materials Inc., model GE 375, 5 cycles of 10 s duration with intermittent 50 s cooling periods), and the homogenate centrifuged in three subsequent steps at 1000 × *g* for 10 min, 10 000 × *g* for 20 min and 200 000 × *g* for 30 min. The 200 000 × *g*-supernatant (cytosol) was concentrated by using disposable Centricon-10 filters (MW cut-off 10 000; from Amicon), and the 200 000 × *g*-pellet (microsomes) was resuspended in 50 mM Tris·HCl buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM DTT, 2 mg/l leupeptin, 2 mg/l pepstatin A, 10 mg/l trypsin inhibitor, 44 mg/l PMSF and 10% (v/v) glycerol. Protein concentrations were determined by using Peterson's modification of the micro-Lowry method with bovine serum albumin as a standard [11].

2.4. *Determination of NOS activity*

Aliquots of the cytosol or microsomes (25 μl corresponding to ca. 50 μg of protein) were added to monolayers of LLC-PK$_1$ cells in 96-well plates (6.25 × 10^4 cells/well) pretreated for 10 min with 1 mM isobutylmethylxanthine and SOD (200 U/ml) in 50 μl of Dulbecco's phosphate buffered saline (DPBS, with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}, pH 7.4). The various substrates (400 μM diluted in 25 μl of DPBS buffer containing 400 μM NADPH, 400 μM DTT, 4 μM THB and 0.4 μM calmodulin) or 25 μl DPBS buffer were added (total volume of 100 μl) and the incubation continued for 10 min at 37°C. In some experiments, co-incubations with L-NO_2Arg or L-MeArg (final concentration 100–300 μM), EGTA (1–3 mM) or bestatin (50 μg/ml) were carried out. The incubations (performed in triplicate for each NOS preparation), were terminated by aspirating the supernatant, addition of 100 μl 50 mM sodium acetate (pH 4.0) and rapid freezing with liquid nitrogen. After thawing, centrifugation at 10 000 × *g* for 10 min and acetylation of the supernatant, cyclic GMP levels were determined (in duplicate) by radioimmunoassay [8].

2.5. *HPLC analysis*

The proteolytic metabolism of the various L-Arg-containing peptides by subcellular fractions from either EC or J774.2 cells was determined by reversed-phase HPLC/fluorescence detection analysis as described [12].

2.6. *Statistical analysis*

Unless otherwise stated, all values in the figures and text are expressed as mean ± SEM of *n* observations. A one-way analysis of variance (ANOVA) was used to assess the statistical significance of results with $P<0.05$ considered significant.

3. RESULTS

Incubations of L-Arg, L-HOArg, L-homoarginine, the various peptides, L-MeArg, L-NO_2Arg or EGTA with LLC-PK$_1$ cells alone had no effect on their cyclic GMP level, and D-Arg or D-HOArg, when incubated with either EC, induced J774.2 cytosol or microsomes also did not cause any significant increase in cyclic GMP ($n=3$ for each compound and NOS preparation). The contamination by free L-Arg of the various peptides was checked by HPLC analysis and found to be ≤0.1%.

3.1. *Characterisation of the NOS activity present in cultured EC*

The NOS activity from cultured EC was largely recovered in the membrane fraction (84–92%, $n=4$, see

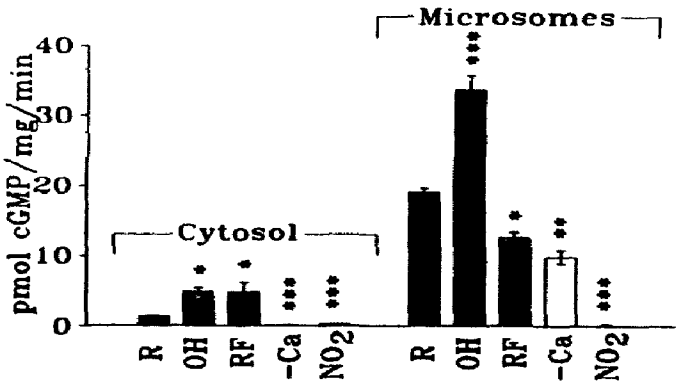

Fig. 1. Distribution and characterisation of the NOS activity in cultured EC. The figure (representative of $n=5$ individual preparations of EC cytosol and $n=12$ individual preparations of EC microsomes) shows the net increase in LLC-PK$_1$ cell cyclic GMP (mean ± SEM, expressed as pmol cyclic GMP/mg of protein added/min) of triplicate incubations of L-Arg (R), L-HOArg (OH) or L-Arg-L-Phe (RF, all at 100 μM) with the cytosolic or microsomal protein from one batch of cultured EC (filled columns). The average increase in LLC-PK$_1$ cell cyclic GMP with L-Arg was 0.99 ± 0.2 pmol cGMP/mg/min for the cytosol (3.64 ± 0.55 pmol cGMP/mg/min for L-HOArg) and 10.82 ± 1.82 cGMP/mg/min for the microsomes (18.75 ± 3.96 pmol cGMP/mg/min for L-HOArg), rspectively. The figure also shows the strong inhibitory effect of 1 mM EGTA (–Ca, empty columns) and 300 μM L-NO_2Arg (NO_2) on the increase in LLC-PK$_1$ cell cyclic GMP with L-Arg (*$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared to L-Arg).

[4]). When compared to L-Arg, incubations with L-HOArg consistently produced a stronger increase in cyclic GMP with both the cytosolic (3.7 ± 0.6-fold, $n=4$, $P<0.05$ by one sample two-tailed Student's *t*-test) and microsomal fraction (3.1 ± 0.6-fold, $n=11$, $P<0.01$), suggesting a more rapid conversion of L-HOArg to ·NO than L-Arg. The formation of ·NO from L-Arg by both cytosol and microsomes was virtually abolished by co-incubations with 100–300 μM L-NO_2Arg (Fig. 1). Similarly, the conversion of L-HOArg to ·NO was substantially inhibited by 100 μM L-NO_2Arg (80%, $n=3$) or L-MeArg (97%, $n=3$). Interestingly, removal of Ca^{2+} by co incubations with 1 mM EGTA abolished the formation of ·NO from L-Arg by the cytosol (Fig. 1), but not the microsomes (46 and 67% inhibition for L-Arg and L-HOArg, respectively, $n=3$). Increasing the EGTA concentration to 3 mM still did not abolish (85% inhibition, $n=3$) the conversion of L-Arg to ·NO by the microsomal enzyme, suggesting that EC membranes may contain both a Ca^{2+}-dependent and Ca^{2+}-independent NOS.

3.2. *Characterisation of the NOS activity present in J774.2 cells*

No NOS activity was detected in the cytosol prepared from non-induced J774.2 cells, whereas traces of activity were present in the membrane fraction (2–6% of the NOS activity found in microsomes from induced J774.2

cells (see below), $n=3$), suggesting that a small portion of the NOS activity in these cells is constitutively active. After treatment with interferon and LPS for 24 h, NOS activity increased substantially in both fractions, and was equally distributed among them with 51–60% of the total activity present in the cytosol ($n=4$). Again L-HOArg was converted to ·NO faster than L-Arg (cytosol: 2.0 ± 0.3-fold, $n=7$, $P<0.05$ by one sample two-tailed Student's t-test; microsomes: 1.9 ± 0.3-fold, $n=4$, $P<0.06$) and the metabolism of both compounds was either abolished (microsomes) or substantially impaired (cytosol) by co-incubations with L-MeArg (100–300 μM, Fig. 2, 75–100% inhibition, $n=3$) or L-NO_2Arg (100–300 μM, 40–83% inhibition, $n=3$). Importantly, removal of Ca^{2+} did not affect the NOS activity in the cytosol, but almost completely blocked that in the microsomes (Fig. 2), suggesting that interferon/LPS induces a membrane-bound NOS in J774.2 cells which is strictly Ca^{2+}-dependent.

3.3. *Substrate specificity of the NOS isoenzymes present in cultured EC*

L-Homoarginine (Table I) and L-Arg-L-Phe were both good substitutes for L-Arg when incubated with the cytosolic NOS. The substrate specificity of the membrane-bound enzyme from EC is depicted in Fig. 3. L-Homoarginine and L-Arg-L-Arg-L-Arg were relatively poor substrates with approximately 25% of the activity of L-Arg, whereas L-Arg-L-Phe, L-Ala-L-Arg or L-Arg-L-Arg replaced L-Arg without a major loss in activity (Table I). As with L-Arg, the formation of ·NO from L-Arg-L-Phe was abolished by co-incubations with 300 μM L-NO_2Arg (from 12.41 ± 0.86 to 0.07 ± 0.01 pmol cGMP/mg/min, $n=3$, $P<0.001$).

Cultured EC rapidly cleave L-Arg-containing dipeptides to L-Arg [9,12] which may subsequently serve as a substrate for NOS. To study this possibility, we quantitated the metabolism by both cytosol and microsomes of L-Ala-L-Arg, the dipeptide most extensively metabolized by EC [9]. HPLC analysis revealed that the cytosol produced 33% (i.e. 33 μM) L-Arg from L-Ala-L-Arg over 10 min, whereas the microsomes were less active

Table I

Comparison of the substrate specificity of the various isoforms of NOS

NOS source	Relative activity (%)						
	R	OH	CH_2	RF	AR	RR	RRR
EC cytosol	100 (5)	370 (4)	80 (1)	186 (2)	ND	ND	ND
EC microsomes	100 (12)	310 (11)	25 (2)	77 (4)	108(41*) (2)	165 (2)	26 (2)
J774.2 cytosol (induced)	100 (12)	200 (8)	28 (1)	60 (3)	108 (2)	122(74**) (2)	ND
J774.2 microsomes (induced)	100 (8)	190 (4)	5 (1)	3 (2)	3 (3)	252 (3)	ND

(R, L-Arg; OH, L-HOArg; CH_2, L-homoarginine; RF, L-Arg-L-Phe; AR, L-Ala-L-Arg; RR, L-Arg-L-Arg; RRR, L-Arg-L-Arg-L-Arg; ND, not determined; numbers in parentheses indicate the number of individual NOS preparations tested from either source.

*Corrected for a 62% inhibition ($P<0.05$) in the presence of bestatin.
**Corrected for a 39% inhibition ($P<0.05$) in the presence of bestatin.

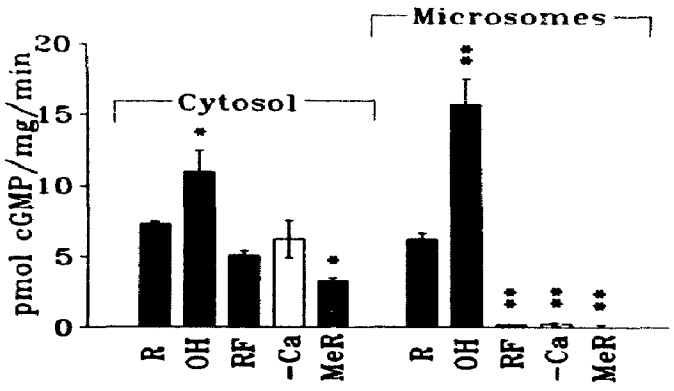

Fig. 2. Distribution and characterisation of the NOS activity in activated J774.2 cells. Details and abbreviations are as in Fig. 1. The figure is representative of $n=12$ individual preparations of J774.2 cell cytosol and $n=7$ individual preparations of J774.2 cell microsomes with average increases in LLC-PK$_1$ cell cyclic GMP with L-Arg of 11.2 ± 2.1 pmol cGMP/mg/min for the cytosol (19.6 ± 5.4 pmol cGMP/mg/min for L-HOArg) and 7.32 ± 0.78 cGMP/mg/min for the microsomes (14.43 ± 3.28 pmol cGMP/mg/min for L-HOArg), respectively. Unlike Fig. 1, the figure shows the effects of 300 μM L-MeArg (MeR, hatched columns) instead of L-NO_2Arg (*$P<0.05$, **$P<0.01$ when compared to L-Arg).

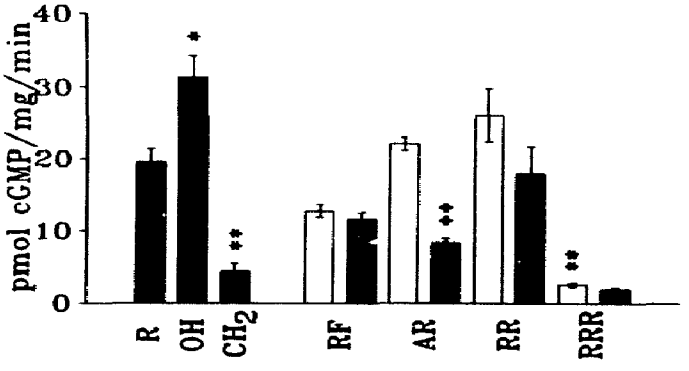

Fig. 3. Substrate specificity of the membrane-bound NOS$_c$ from cultured EC. Details are as in Fig. 1. The figure is representative of at least $n=2$ individual preparations of EC microsomes and is different from the preparation depicted in Fig. 1. It shows the increase in LLC-PK$_1$ cell cyclic GMP after incubations of the microsomal protein with L-Arg (R), L-HOArg (OH) or L-homoarginine (CH_2, filled columns) or L-Arg-L-Phe (RF), L-Ala-L-Arg (AR), L-Arg-L-Arg (RR) or L-Arg-L-Arg-L-Arg (RRR, all at 100 μM) in the presence (hatched columns) or absence (empty columns) of 50 μg/ml bestatin (*$P<0.05$, **$P<0.01$ when compared to L-Arg, **$P<0.01$ when compared to L-Ala-L-Arg without bestatin).

with 2% L-Arg from L-Ala-L-Arg, and 1 and 7% from L-Arg-L-Phe and L-Arg-L-Arg, respectively (n=3). Interestingly, the aminopeptidase inhibitor bestatin (50 μg/ml corresponding to ca. 100 μM) strongly inhibited (83%, n=3) the cleavage of L-Ala-L-Arg by the cytosol and was, therefore, included in the NOS assay. Bestatin had no significant effect on the conversion of L-Arg-L-Phe to ·NO by the cytosolic NOS (n=3). The formation by the membrane-bound enzyme of ·NO from L-Arg-L-Phe, L-Arg-L-Arg or L-Arg-L-Arg-L-Arg was also not affected by bestatin (Fig. 3), whereas that from L-Ala-L-Arg decreased by 62%, indicating that this peptide only owed a major part of its activity to the liberation of L-Arg (Table I).

3.4. *Substrate specificity of the NOS isoenzymes present in J774.2 cells*

The substrate specificity of the cytosolic and membrane-bound NOS from induced J774.2 cells is summarised in Table I. As with the microsomal EC enzyme, L-homoarginine was a weak substrate for the cytosolic NOS from these cells, whereas all three L-Arg-containing dipeptides were good substitutes for L-Arg. Bestatin did not affect the formation of ·NO from L-Arg-L-Phe, but significantly (P<0.05) decreased that of L-Arg-L-Arg by 39% (n=3), suggesting that part of its activity was due to the liberation of L-Arg. Interestingly, L-homoarginine, L-Arg-L-Phe or L-Ala-L-Arg were not substrates for the microsomal NOS from J774.2 cells, whereas L-Arg-L-Arg was an excellent substitute for L-Arg (Table I) and was not affected by bestatin. As with L-Arg, co-incubations of L-Arg-L-Arg with 1 mM EGTA substantially impaired the formation of ·NO (77% inhibition, n=3).

4. DISCUSSION

This study demonstrates that L-Arg-containing dipeptides are excellent substrates for the various isoforms of NOS, and confirms earlier reports showing that L-homoarginine and an L-Arg-containing dipeptide, L-Arg-L-Asp, can replace L-Arg as a substrate for the biosynthesis of ·NO by cytokine-activated RAW 264.7 monocyte/macrophages [13]. Thus, the active site of NOS seems to be flexible enough to accommodate molecules different from L-Arg in size, charge and hydrophobicity. This should be considered when proposing a chemical mechanism for the biosynthesis of ·NO from L-Arg or when designing more selective inhibitors of NOS. Moreover, these findings substantiate our earlier suggestion that the availability of L-Arg-containing small peptides may represent an important regulatory factor for the biosynthesis of ·NO by the endothelium in vivo [8].

The small NOS_c activity in non-induced J774.2 cells, like the NOS_c in cultured EC, is largely membrane-bound (see [4]), whereas the NOS_i in J774.2 cells is equally distributed among membrane(s) and cytosol. Interestingly, the capacity of these isoforms of NOS to produce ·NO is very similar on the basis of the amount of protein included in the assay, suggesting that the burst-like biosynthesis of ·NO by EC, in contrast to the continuous production of ·NO by cytokine-activated macrophages, is tightly controlled by intracellular signals. The cytosolic NOS_c and the membrane-bound NOS_i are both strictly Ca^{2+}-dependent, whereas the cytosolic NOS_i is not. The partial resistance to removal of Ca^{2+} of the membrane-bound NOS activity in EC may indicate the presence of another Ca^{2+}-independent NOS. This activity is likely to be constitutive although it possibly could be induced by traces of endotoxin in the culture medium. Interestingly, the membrane-bound NOS_i in J774.2 cells is strictly Ca^{2+}-dependent and has a different substrate specificity from the Ca^{2+}-independent NOS_i in the cytosol. A similar membrane-bound and Ca^{2+}-independent NOS_i activity was also reported in rat peritoneal macrophages activated by *Bacillus Calmette-Guérin* [14]. Thus, irrespective of their intracellular localisation, at least three isoforms of NOS exist, all of which have a higher apparent v_{max} for L-HOArg when compared to L-Arg consistent with the hypothesis that L-HOArg is an intermediate in the biosynthesis of ·NO.

Acknowledgements: The authors are indebted to Dr. Paul L. Feldman for the generous supply of L-HOArg and D-HOArg, Dr. Artur Zembowicz for his discussions and help with the LLC-PK$_1$ cell assay, Elizabeth G. Wood for the steady cell supply, and Ivana Vojnovic for expert technical assistance. This work was supported by a grant from Glaxo Group Research Ltd.

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