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Reversible inactivation of endothelial nitric oxide synthase by N^G-nitro-L-arginine

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N^G-Methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA) inhibited NO-induced cGMP accumulation in porcine aortic endothelial cells with half-maximally effective concentrations of 15 and 3.4 μM, respectively. The effects of both compounds were reversible, but the L-NNA-induced inhibition was only reversed by wash-out in the presence of 1 mM L-arginine. In short-term incubations (45 s) of membrane fractions, L-NMA and L-NNA exhibited similar potencies to inhibit endothelial NO synthase, but L-NNA was markedly more potent than L-NMA after prolonged incubation periods (≥ 3 min) due to induction of a pronounced, reversible enzyme inactivation.

Endothelial cell; Nitric oxide synthase; Inhibitor; Inactivation; L-Arginine

1. INTRODUCTION

Endothelium-derived nitric oxide (NO) is a potent endogenous vasodilator and inhibitor of platelet activation [1]. NO release is probably mediated by intracellular free Ca^{2+} required for activation of a constitutively expressed, calmodulin-dependent NO synthase (NOS) [2,3]. Whereas cytokine-inducible and neuronal isoforms of NOS are predominantly cytosolic [4,5], the endothelial enzyme is associated with membranes [6] due to posttranslational myristilation at its N-terminus [7,8]. Recently it has been shown that phosphorylation of endothelial NOS results in its translocation from membranes to the cytosol [9].

Albeit regulated in a quite different manner, all NOS isoforms described so far appear to be functionally and structurally similar (for reviews see: [10–12]). They are homodimers with subunit molecular masses ranging from 130 to 160 kDa and catalyze an oxidation of L-arginine to stoichiometrical amounts of L-citrulline and NO through reductive activation of molecular oxygen at their heme-containing catalytic domain. The electrons required for oxygen reduction are shuttled from the essential cofactor NADPH to the heme by the reduced flavins FAD and FMN, suggesting that NOS isozymes exhibit combined cytochrome P_{450} oxygenase and reductase activities. In fact, neuronal NOS exhibits Ca^{2+}/calmodulin-dependent cytochrome reductase activity which seems to be independent from the catalytic site because it is neither affected by the substrate analogs L-NMA and L-NNA nor by drugs interfering with heme-catalyzed reactions [13,14].

NOS-catalyzed reduction of molecular oxygen uncouples from L-arginine oxidation at low substrate concentrations, leading to the generation of hydrogen peroxide [15,16]. L-NMA has no effect on this L-arginine-independent reaction, but surprisingly L-NNA turned out as a powerful inhibitor of H_2O_2 formation. Thus, L-NNA seems to have effects unrelated to substrate competition, and recently we found that it induced a rapid inactivation of brain NOS [14,17]. Previous reports indicate that L-NMA inhibits constitutive and inducible NOS with similar potencies, whereas L-NNA seems to exhibit selectivity for the endothelial and neuronal enzymes [18–20]. These results suggest that L-NNA-induced enzyme inactivation may be specific for constitutive NOS. Since selective inhibition of NOS isozymes is of considerable therapeutical relevance, we have addressed this issue and studied the effects of L-NMA and L-NNA on endothelial NOS. We found that the endothelial enzyme is indeed reversibly inactivated by L-NNA, indicating that the catalytic domains of the two constitutive isozymes are closely similar but differ significantly from that of inducible NOS.

2. MATERIALS AND METHODS

2.1. *Materials*

L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was from MedPro (Amersham), Vienna, Austria. All other chemicals were obtained from Sigma Deisenhofen, Germany or from sources described previously [13,15,21].

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Abbreviations: PAEC, porcine aortic endothelial cells; NOS, NO synthase; L-NMA, N^G-methyl-L-arginine; L-NNA, N^G-nitro-L-arginine; IC_{50}, concentration producing 50% inhibition.

2.2. *Cell culture and determination of cGMP levels*

PAEC were cultured in 6-well plastic plates as described [21]. Prior to experiments, the cells were washed twice with isotonic HEPES buffer, pH 7.1, containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$, and preincubated for 15 min at 37°C in 1.4 ml of the same buffer in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM indomethacin and NOS inhibitors as indicated. For wash-out of inhibitors, cells were incubated for further 15 min with or without 1 mM L-arginine and washed twice with buffer prior to starting the incubations by addition of A 23,187 (1 μM final concentration). Reactions were terminated 4 min later by removal of the buffer and addition of 1 ml of 0.01 M HCl. Cyclic GMP was measured in the supernatants by radioimmunoassay.

2.3. *Preparation of crude subcellular fractions from PAEC*

PAEC were suspended in a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA (buffer A), and subsequent to addition of phenylmethylsulfonyl fluoride (0.5 mM final concentration) the cells were sonicated (3 × 15 s) and centrifuged at 100,000 × *g* for 40 min. The pellets were washed once with buffer A containing 1 M KCl and then twice with buffer A without salt. Finally, pellets were suspended in buffer A, containing 10% (v/v) glycerol and stored at −70°C until use. Similarly, supernatants were adjusted to 10% glycerol prior to storage at −70°C. All procedures were carried out at 4°C. Protein was determined by the method of Bradford with bovine serum albumin as a standard [22].

To test for reversibility of NOS inhibition, cells were pretreated for 5 min at 4°C in the absence and presence of 0.1 mM L-NMA or L-NNA (0.1 mM each) prior to homogenization and centrifugation at 100,000 × *g* for 20 min. The obtained pellets were washed three times with buffer A and finally resuspended directly in the reaction mixture used for the determination of L-citrulline formation (see below).

2.4. *Determination of L-citrulline formation*

Unless otherwise indicated, subcellular fractions of PAEC (0.2–0.7 mg of protein) were incubated for 10 min at 37°C in a total volume of 0.1 ml of a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 3 μM free Ca^{2+}, 10 μg/ml calmodulin, 0.5 mM NADPH, 5 μM FMN, 5 μM FAD, 10 μM (6*R*)-5,6,7,8-tetrahydrobiopterin, and 10 μM L-[2,3,4,5-^{3}H]arginine (~ 90 nCi/nmol). Isolation of [^{3}H]citrulline was performed by column chromatography on Dowex cation exchange resin AG 50W-X8 as previously described [23].

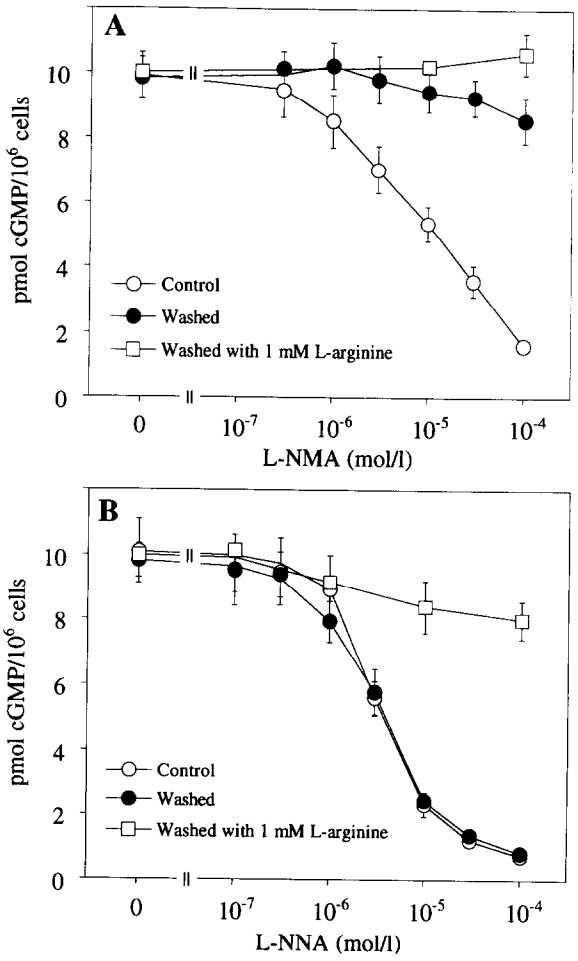

Fig. 1. Effects of L-NMA (A) and L-NNA (B) on A 23,187-stimulated accumulation of cGMP in PAEC. Cultured PAEC were preincubated for 15 min at 37°C with increasing concentrations of L-NMA (A) or L-NNA (B), incubated for 4 min in the presence of 1 μM A 23,187, and assayed for intracellular cGMP by radioimmunoassay as described in section 2. Where indicated, inhibitors were washed-out with buffer in the absence and presence of 1 mM L-arginine prior to incubation. Data are means ± S.E. of three separate experiments assayed in duplicate.

3. RESULTS AND DISCUSSION

Formation of nitric oxide by cultured PAEC was assayed as Ca-ionophore A 23,187-induced accumulation of intracellular cGMP [21]. As shown in Fig. 1, cGMP formation was inhibited by L-NMA (A) and L-NNA (B) with IC_{50} values of 15.0 and 3.4 μM, respectively. To find out whether inhibition was reversible, PAEC were pretreated with increasing concentrations of L-NMA or L-NNA and washed in the absence and presence of 1 mM L-arginine prior to incubation with A 23,187. Fig. 1 shows that the inhibitory effects of L-NMA were almost completely reversed after wash-out with buffer, whereas reversal of L-NNA-induced effects was only achieved with L-arginine additionally present. Thus, different molecular mechanisms seem to account for the inhibitory effects of L-NMA and L-NNA, but both compounds turned out as reversible inhibitors of endothelial NOS. The pronounced effect of added L-arginine shown in Fig. 1B should be due to competition with L-NNA at the substrate site of NOS, suggesting that intracellular L-arginine levels were considerably enhanced by the exogenously added amino acid. Alternatively, it is also conceivable that the substrate may have direct access from the extracellular space to the catalytic domain of membrane-bound NOS.

The data shown in Table I confirm the previous characterization of endothelial NOS as a membrane-associated enzyme [6]. We found that KCl-insoluble fractions of PAEC contained 80–95% of total NOS with a specific activity ranging from 50 to 100 pmol L-citrulline × mg^{-1} × min^{-1}, whereas NOS activity was below 5 pmol × mg^{-1} × min^{-1} in the supernatants. These results may explain the previously observed poor L-arginine-induced stimulation of purified soluble guanylyl cyclase co-incubated with PAEC cytosols [2].

Table I

Distribution of NOS in subcellular fractions of cultured PAEC

Fraction	Insoluble	Soluble
Protein (mg)	7.0	19.2
Specific NOS activity (pmol L-citrulline × mg^{-1} × min^{-1})	59.0	3.4
Total NOS activity (pmol L-citrulline × min^{-1})	413	65.3
Distribution of NOS activity (%)	86	14

Membranes and supernatants were prepared and assayed for NOS activity and protein as described in section 2. Data are representative for 4 similar preparations.

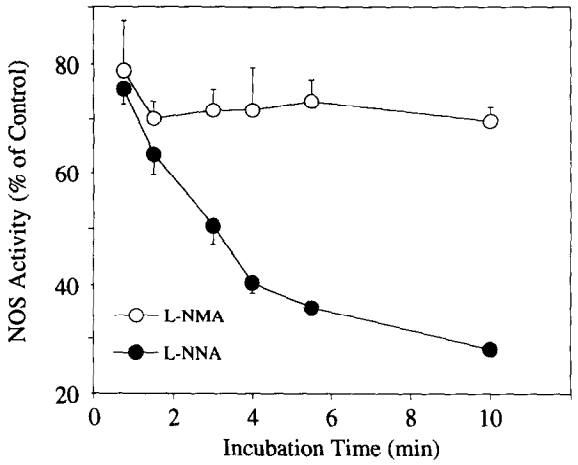

Fig. 2. Time course of endothelial NOS inhibition by L-NMA and L-NNA. Membrane fractions (0.7 mg of protein) obtained from cultured PAEC were incubated at 37°C in a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 3 μM free Ca^{2+}, 10 μg/ml calmodulin, 0.5 mM NADPH, 5 μM FMN, 5 μM FAD, 10 μM (6*R*)-5,6,7,8-tetrahydrobiopterin, and 10 μM L-[2,3,4,5-^{3}H]arginine (~ 90 nCi/nmol) in the absence and presence of L-NMA and L-NNA (1 μM each). At the indicated time points, 0.1 ml aliquots were removed and assayed for [^{3}H]citrulline. Data are means ± S.E. of three separate experiments assayed in duplicate.

Fig. 2 shows the time courses of endothelial NOS inhibition by L-NMA and L-NNA (1 μM each), assayed as formation of L-citrulline from 10 μM L-arginine by PAEC membrane fractions. L-NMA inhibited the reaction in a time-independent manner to ~70% of controls, whereas L-NNA induced a pronounced enzyme inactivation down to ~ 30% of controls during the 10 min of incubation. Both inhibitors were equally potent after short-term incubations (45 s), indicating that L-NMA and L-NNA have similar affinities to endothelial NOS and that the apparent high potency of L-NNA results from inactivation of the enzyme. Although the inhibitory effect of L-NMA was independent of time, our findings do not rule out the possibility that the endothelial enzyme metabolizes this compound to an as yet unidentified inactivating species as described for macrophage and brain NOS [17,24,25]. Metabolism of L-NMA is rather slow and enzyme inactivation could become substantial after prolonged periods of incubation only.

Reversibility of enzyme inactivation was studied by determination of NOS activities in membrane fractions obtained from PAEC pretreated with L-NNA or L-NMA (0.1 mM each) and comparing the data with the respective enzyme activities of untreated preparations. The data shown in Table II demonstrate that the effects of both compounds were reversed when the membranes were washed with buffer, albeit reversibility of L-NNA-induced inhibition was not complete even when 1 mM L-arginine had been included during wash-out of the inhibitor (n = 2; data not shown). This latter finding is not necessarily in conflict with the results obtained with intact cells (Fig. 1B), since experimental conditions are hardly comparable. Due to instability of endothelial NOS under cell-free conditions, we had to rapidly equilibrate and wash-out the membranes at 4°C, while removal of the inhibitors from intact cells was performed at 37°C for more extended periods of time. Recently we found that dissociation of L-NNA from purified brain NOS followed first order kinetics with a remarkably slow rate constant of 7.4×10^{-2} min^{-1} [17]. Thus, dissociation of L-NNA from both constitutive isozymes seems to be slower than that of L-NMA, and this may be the reason why L-NNA has been described as irreversible inhibitor of constitutive NOS [26].

Together with previous results [17], the present findings indicate that similar mechanisms may account for inhibition of endothelial and neuronal NOS by L-NNA. Both enzymes undergo substantial inactivation in the presence of this drug, and the effect is clearly reversible, even though rates of dissociation are rather slow. However, NOS present in cytosolic fractions of γ-interferon/LPS-activated RAW-264.7 macrophages was not inhibited by L-NNA in the presence of equimolar amounts of L-arginine (B. Mayer, unpublished observation), confirming that the inducible enzyme is considerably less sensitive to inhibition by this compound than constitu-

Table II

Reversibility of endothelial NOS inhibition by L-NMA and L-NNA

Incubation	NOS activity (% of untreated controls)	
	L-NMA-pretreated	L-NNA-pretreated
Buffer	106.9 ± 7.66	58.4 ± 6.05
Inhibitor (10 μM)	26.2 ± 2.73	4.4 ± 1.04

PAEC were pretreated with L-NMA or L-NNA (0.1 mM each) for 10 min at 4°C. Membrane fractions, prepared and washed as described in section 2, were assayed for NOS activity in the absence and presence of 10 μM of the respective inhibitors. Controls (= 100%) refer to NOS activities of membranes obtained from untreated cells determined in the absence of inhibitors. Data are means ± S.E. of three preparations assayed in duplicate.

tive NOS [19,20]. The pronounced L-NNA-induced inactivation we have seen appears to be specific, therefore, for the endothelial and neuronal enzymes, suggesting that the catalytic sites of the constitutive isozymes structurally differ from the substrate binding domain of inducible NOS.

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