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Modulation of angiotensin-converting enzyme by nitric oxide

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1 The aim of the present study was to determine the effect of nitric oxide (NO) on angiotensin-converting enzyme (ACE) activity.

2 A biochemical study was performed in order to analyse the effect of the NO-donors, SIN-1 and diethylamine/NO (DEA/NO), and of an aqueous solution of nitric oxide on the ACE activity in plasma from 3-month old male Sprague-Dawley rats and on ACE purified from rabbit lung. SIN-1 significantly inhibited the activity of both enzymes in a concentration-dependent way between 1 and 100 μM. DEA/NO inhibited the activity of purified ACE from 0.1 μM to 10 μM and plasma ACE, with a lower potency, between 1 and 100 μM. An aqueous solution of NO (100 and 150 μM) also inhibited significantly the activity of both enzymes. Lineweaver-Burk plots indicated an apparent competitive inhibition of Hip-His-Leu hydrolysis by NO-donors.

3 Modulation of ACE activity by NO was also assessed in the rat carotid artery by comparing contractions elicited by angiotensin I (AI) and AII. Concentration-response curves to both peptides were performed in arteries with endothelium in the presence of the guanylyl cyclase inhibitor, ODQ (10 μM), and the inhibitor of NO formation, L-NAME (0.1 mM). NO, which is still released from endothelium in the presence of 10 μM ODQ, elicited a significant inhibition of AI contractions at low concentrations (1 and 5 nM). In the absence of endothelium, 1 μM SIN-1 plus 10 μM ODQ, as well as 10 μM DEA/NO plus 10 μM ODQ induced a significant inhibition on AI-induced contractions at 1 and 5 nM and at 1–100 nM, respectively.

4 In conclusion, we demonstrated that (i) NO and NO-releasing compounds inhibit ACE activity in a concentration-dependent and competitive way and that (ii) NO release from endothelium physiologically reduces conversion of AI to AII.

Keywords: Angiotensin-converting enzyme; nitric oxide; AI conversion; vascular smooth muscle; rat carotid artery

Introduction

Angiotensin-converting enzyme (EC 3.4.15.1, ACE) is a zinc metallopeptidase that cleaves carboxy-terminal dipeptides from several peptides. This enzyme plays a major role in the regulation of vascular tone by converting the inactive peptide angiotensin I (AI) into the vasoconstrictor and trophic angiotensin II (AII), and by inactivating the vasodilator bradykinin (BK). Vascular ACE is an ectoenzyme mainly expressed in the endothelial cells. In addition, a soluble ACE is found in plasma, which is presumably derived from the membrane-bound form by enzymatic cleavage occurring either intracellularly or at the plasma membrane (Erdös, 1990).

Nitric oxide is a highly reactive inorganic free radical. This small molecule has been shown to modulate vascular tone, platelet aggregation, inflammation and neurotransmission (Moncada *et al*., 1991; Bredt & Snyder, 1994). At the vascular level, NO induces vasodilatation and inhibits vascular smooth muscle cell proliferation (Garg & Hassid, 1989). Many of these effects are mediated by activation of soluble guanylyl cyclase and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) increases (Moncada *et al*., 1991; Bredt & Snyder, 1994). NO is also emerging as an enzyme regulator, especially of metalloenzymes. Activation of guanylyl cyclase by interaction with haeme (Arnold *et al*., 1977) or cytochrome P 450 (Wink *et al*., 1993) have been described. Other metalloproteins, non-haeme centered, such as aconitase (Hibbs *et al*., 1988) are also modulated by NO. In addition, enzymes like protein kinase C (Gopalakrishna *et al*., 1993), and glyceraldehyde 3-phosphate dehydrogenase (Molina *et al*., 1992; Dimmeler & Brune, 1992) are regulated by NO through S-nitrosylation.

Recently, several investigators have demonstrated an upregulation of cardiac and vascular ACE activity after long-term blockade of NO synthesis by the chronic administration of N-nitro-L-arginine methyl ester (L-NAME) (Michel *et al*., 1996; Takemoto *et al*., 1997). ACE expression and activity are also increased after endothelial removal in the rat carotid artery as early as 2 days after removal of the endothelium (Fernández-Alfonso *et al*., 1997).

The aim of the present study was to analyse a putative modulation of ACE activity by NO. For this purpose we have chosen both a biochemical and a functional approach. From the biochemical point of view, the objective was to study the effect on hippuril-histidine-leucine hydrolysis catalyzed by either purified rabbit lung ACE or rat plasma ACE of (i) NO-donors, SIN-1 (3-morpholinosydnonimine) and DEA/NO (diethylamine/NO adduct) or of (ii) an aqueous solution of NO. The functional approach aimed to examine both the influence of endothelial derived as well as exogenous NO on vascular ACE activity, by measuring the contractions induced by AI on rat carotid arteries.

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Methods

Biochemical studies

ACE activity was measured by hydrolysis of Hippuril-His-Leu as described by Cushman and Cheung (1971). Briefly, 50 μl of rat plasma or 0.075 u ml^{-1} of purified rabbit lung ACE were added to 400 μl of phosphate buffered saline (NaCl 300 mM) at pH 8. The reaction was started by addition of 50 μl Hippuryl-His-Leu (10 mM). After 30 min incubation at 37°C the reaction was stopped by the addition of 1 ml 0.1 N NaOH. A 100 μl aliquot of the latter solution was incubated with 25 μl o-phthaldialdehyde for 30 min and then stopped by addition of 1 ml of 0.8 N HCl. The samples were centrifuged at 3000 *g* at 4°C for 5 min. The fluorescence was read in a Perkin-Elmer LS-3 mod fluorimeter. The excitation and emission wavelengths were 360 and 500 nm, respectively. A standard curve was performed with His-Leu between 0.1 and 10 μg 100 μl^{-1}.

Hippuryl-His-Leu (10 mM) was dissolved in 2 ml 0.1 N NaOH and diluted in distilled water to a final volume of 10 ml. A 2% disolution of o-phthaldiadehyde was prepared in dimethylsulphoxide immediately before the assay and preserved from light. His-Leu was prepared in distilled water. Blood was collected in heparin and centrifuged at 1000 *g* for 15 min to obtain plasma. Plasma aliquots were frozen and kept at −80°C.

Functional studies

Three month old Sprague-Dawley rats (250–300 g) were anaesthetized with sodium pentobarbitone and exsanguinated by cardiac puncture.

Carotid arteries were carefully isolated and placed in a physiological solution of the following composition (mM): NaCl 115, KCl 4.6, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, EDTA 0.01 and glucose 11 at 4°C. Dexamethasone (0.8 μM) was added to the physiological solution to avoid an induction of NO synthase (González *et al*., 1992). All experiments were performed in the presence of 5 μM indomethacin to avoid prostaglandin-mediated effects. The arteries were cleaned from blood and adherent tissues and divided in rings of 3 mm in length. Each vascular ring was suspended on two intraluminal parallel wires, introduced in an organ bath containing physiological solution and connected to a Piodem strain gauge for isometric tension recording. All segments were given an optimal resting tension of 2 g which was readjusted every 15 min during a 90 min equilibration period.

At the beginning of the experiment, the vessels were exposed to 75 mM KCl to check their functional integrity. Thereafter, vessels were precontracted with 0.1 μM noradrenaline and a concentration-response curve to acetylcholine (ACh) was performed (0.1 μM to 0.1 mM) in order to evaluate functionally the presence or absence of endothelium. Only vessels with more than 90% relaxation to 0.1 mM ACh were used. Drugs were preincubated with the arteries at least 30 min before the concentration-response curves to the peptides were obtained, with the exception of DEA/NO, which was incubated only for five minutes.

Nitrite measurement

Determination of NO production by NO-donors was done by nitrite measurement in the incubation medium after 30 min incubation time. Nitrite concentration was determined as described by Bennet *et al.* (1986). Briefly, aliquots of incubation medium were mixed with Griess reagent, and optical density was measured at 540 nm after 10 min. The concentration was extrapolated from a standard curve obtained with sodium nitrite.

Preparation of NO solution

NO solutions were prepared from a saturated solution (1.9 mM) of nitric oxide gas (atmospheric pressure and room temperature) in distilled water previously gassed for 45 min with 100% argon to remove the oxygen dissolved in it (Shaw & Vosper, 1977).

Reagents

Diethylamine/NO adduct (DEA/NO) was obtained from RBI (U.S.A.). SIN-1 (3-morpholinosydnonimine) was kindly provided by Cassella AG (Germany). Hip-His-Leu, His-Leu, noradrenaline, angiotensin I, angiotensin II, angiotensin converting enzyme purified from rabbit lung and N-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (U.S.A.). o-Pthalaldehyde was purchased from Fluka. 1H-[1,2,4,]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was obtained from Tocris (U.S.A.).

Drugs were dissolved in distilled water. ODQ was dissolved in dimethylsulphoxide.

Analysis of data

Contractions are expressed as the percentage of contraction produced by 75 mM KCl. For each individual concentration-response curve, the maximum and the half-maximum effective concentration (EC_{50}) were calculated by nonlinear regression.

Statistical significance was analysed by one-way ANOVA followed by Newman-Keuls' test and Student's *t* test. $P<0.05$ was considered significant.

Results

Activity of purified ACE

Linearity with respect to time of formation of His-Leu from Hip-His-Leu catalyzed by purified rabbit lung ACE was determined. Hip-His-Leu 10 mM was incubated with ACE (0.075 u ml^{-1}) for different time intervals (1, 10, 30, 50 and 60 min). A linear increase in His-Leu formation with time was observed (results not shown). Additionally, linearity between ACE concentration and formation of His-Leu from Hip-His-Leu catalyzed by ACE was determined. Incubation of 10 mmol Hip-His-Leu for 30 min with ACE (0.01, 0.05 and 0.1 u ml^{-1}) elicited a linear increase of His-Leu formation (results not shown). From these experiments, an optimal incubation time of 30 min and an optimal ACE concentration of 0.075 u ml^{-1} were chosen.

The velocity of ACE reaction was determined at different Hip-His-Leu concentrations ranging from 0.1 to 6 mM. K_m and V_{max}, estimated from Lineweaver-Burk plots, were of 1.18 mM and of 30.3 nM, respectively (Figure 1). A concentration of 0.5 mM Hip-His-Leu was selected for further assays.

In order to determine whether NO influences ACE activity, the enzyme was incubated with the NO donors, SIN-1 and DEA/NO, which have a different pattern of NO release. SIN-1 induced a significant concentration-dependent decrease in ACE activity from 1 to 100 μM (Figure 2a). Incubation of ACE with DEA/NO (10 nM to 10 μM) induced a substantial

decrease in ACE activity (Figure 2b). A non-specific effect of diethylamine on ACE inactivation was excluded, since diethylamine did not affect ACE activity (ACE activity as % of control: DEA 1 μM: 94.5$\pm$4.7; DEA 10 μM: 90$\pm$9.8; DEA 100 μM; 98.1$\pm$4.7). Since ACE activity is very sensitive to small pH changes a possible reduction of pH induced by NO donors was analysed. pH values did not change significantly with NO-donors used (control: pH = 7.7; SIN 10 μM: pH = 7.7; DEA/NO 10 μM: pH = 7.7).

The effect of NO chelators, oxyhaemoglobin and carboxy-PTIO, on ACE activity was analysed in order to test if ACE inhibition by NO-donors was due only to NO release and not to non-specific mechanisms. These studies failed because these compounds interfered with the emission of fluorescence (results not shown). Therefore, the effect of an aqueous NO solution on ACE activity was analysed. NO solution induced a concentration-dependent inhibition of ACE activity at 0.1 and 0.15 mM (Figure 2c). Additionally, nitrites were determined in the incubation medium. NO-donors, SIN-1 and DEA/NO, exhibited a concentration-dependent nitrite release (Table 1).

The velocity of ACE reaction at varying Hip-His-Leu concentrations in the presence of 1 and 10 μM SIN-1 was determined. Lineweaver-Burk plots indicated an apparent competitive inhibition (Figure 3).

Plasma ACE activity

Plasma ACE from 3 month old Sprague-Dawley rats was analysed. Linearity of His-Leu release with increasing plasma concentrations (10 to 100 μl) was demonstrated (results not shown). For further experiments, a volume of 50 μl plasma and 30 min incubation time were chosen as optimal parameters.

The effect of substrate concentration on reaction velocity is shown in Figure 4; 0.5 mM Hip-His-Leu was selected for further assays. The K_m determined from the Lineweaver-Burk plot was 2.16 mM and V_{max} 68.5 nM.

Both, SIN-1 and DEA/NO induced a significant concentration-dependent decrease in ACE activity from 1 μM to 0.1 mM (Figure 5a and 5b). In order to exclude the interference of other plasmatic proteases on activity determination, the inhibitory effect of NO-donors was compared with the effect of 0.1 μM captopril as a control (Figure 5). The velocity of plasma ACE reaction at varying Hip-His-Leu concentrations in the presence of 10 μM SIN-1 was determined and compared to the inhibition elicited by captopril. From Lineweaver-Burk plots an apparent competitive inhibition was observed (Figure 6).

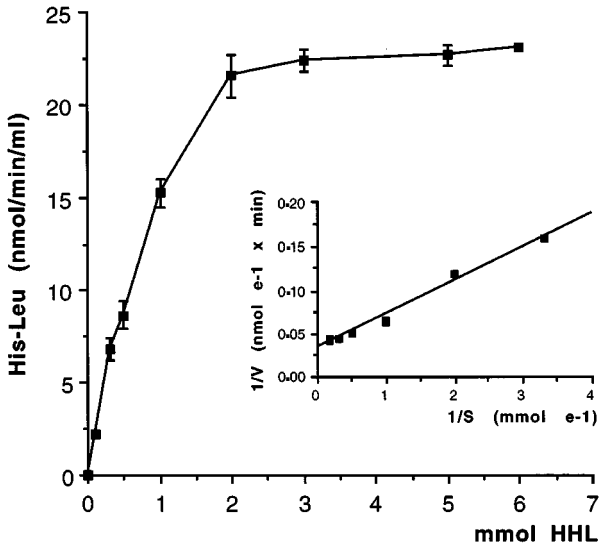

Figure 1 Velocity of formation of L-histidyl-L-leucine catalyzed by purified rabbit lung ACE as a function of the concentration of Hip-His-Leu (HHL). Data show the mean $n = 12$. Insert: Lineweaver-Burk plot of Hip-His-Leu hydrolysis by purified rabbit lung ACE. Vertical lines indicate s.e.mean.

Table 1 Nitrite concentration in the incubation medium of the enzyme assay after addition of NO-donors

	Nitrite concentration (μM)
SIN-1 1 μM	0.307$\pm$0.09
SIN-1 10 μM	2.76$\pm$0.3
SIN-1 100 μM	24.4$\pm$0.37
DEA/NO 0.1 μM	0.39$\pm$0.09
DEA/NO 1 μM	7.64$\pm$0.01

Data are presented as means$\pm$s.e.; $n = 5-8$.

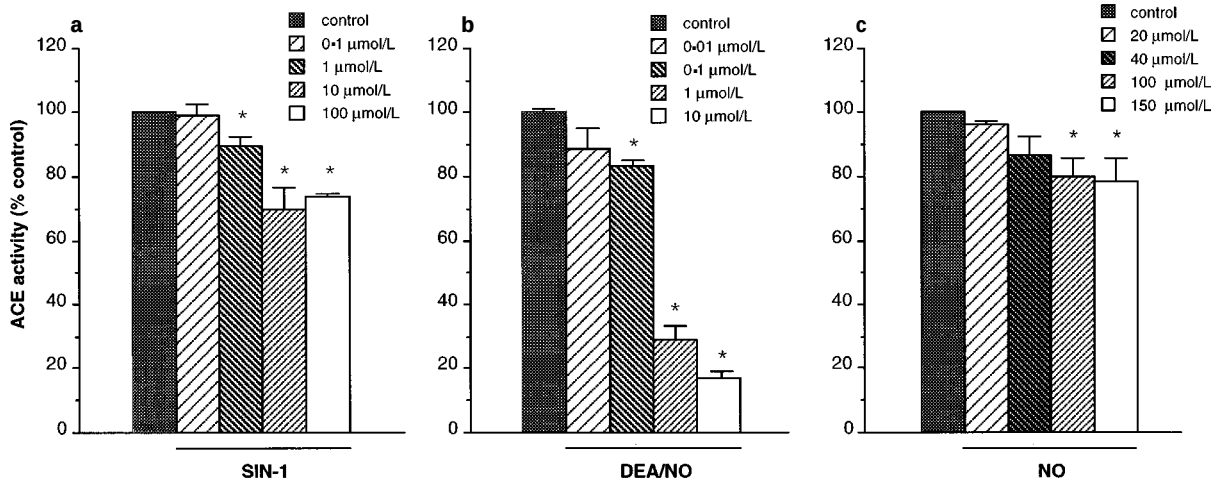

Figure 2 Effect of different concentrations of (a) SIN-1, (b) DEA/NO and (c) an aqueous NO solution on Hip-His-Leu hydrolysis by purified rabbit lung ACE. Activity is expressed as % of control (without NO-donor) $\pm$s.e.mean; $n = 7-15$. *$P < 0.05$.

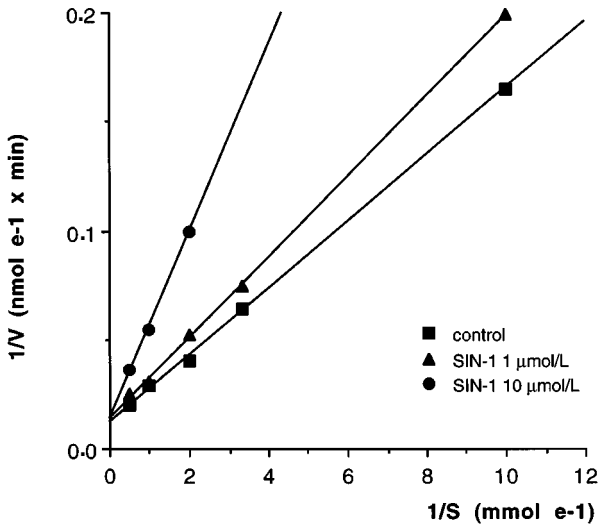

Figure 3 Lineweaver-Burk plot of Hip-His-Leu hydrolysis by purified rabbit lung ACE in presence of SIN-1; $n=6-7$.

Functional studies

In order to assess the modulation of vascular ACE activity by endogenous NO, we compared contractions elicited by AI and AII in rat carotid artery with intact endothelium.

Concentration-response curves for AI and AII were started at 1 nM in order to avoid desensitization at higher concentrations of the peptide. AI elicited a contraction from 5 nM (Figure 7a). Higher concentrations than 100 nM induced a loss of tone. AI contractions were completely abolished by 10 μM captopril and reduced by 1 μM losartan, demonstrating conversion of AI to AII (results not shown). AII elicited a contraction, which started at 1 nM. Lower concentrations, 0.1 nM AII elicited no response ($0\pm0\%$, $n=8$) and 0.5 nM $0.4\pm0.4\%$ of control ($n=8$). The concentration-response curve reached a maximum at 50 nM (Figure 7b). Tone was lost at higher concentrations.

Preincubation with 0.1 mM L-NAME, an inhibitor of NO-synthase, induced a significant increase of both AI- and AII-induced contractions at all concentrations used (Figure 7). EC_{50} values indicated a shift to the left of the concentration-response curve (Table 2).

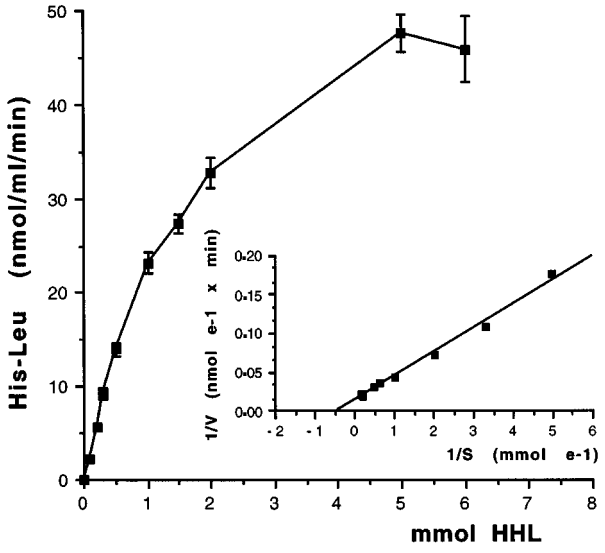

Figure 4 Velocity of formation of His-Leu catalyzed by rat plasma ACE as a function of the concentration of Hip-His-Leu (HHL). Insert: Lineweaver-Burk plot of Hip-His-Leu hydrolysis by plasma ACE. Data show the mean, $n=21$; vertical lines indicate s.e.mean.

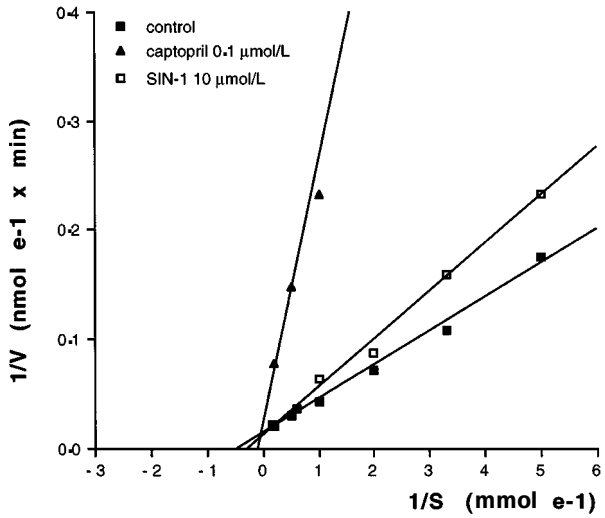

Figure 6 Lineweaver-Burk plot of Hip-His-Leu hydrolysis by rat plasma ACE in presence of SIN-1 and captopril. Data show the mean; $n=5-16$.

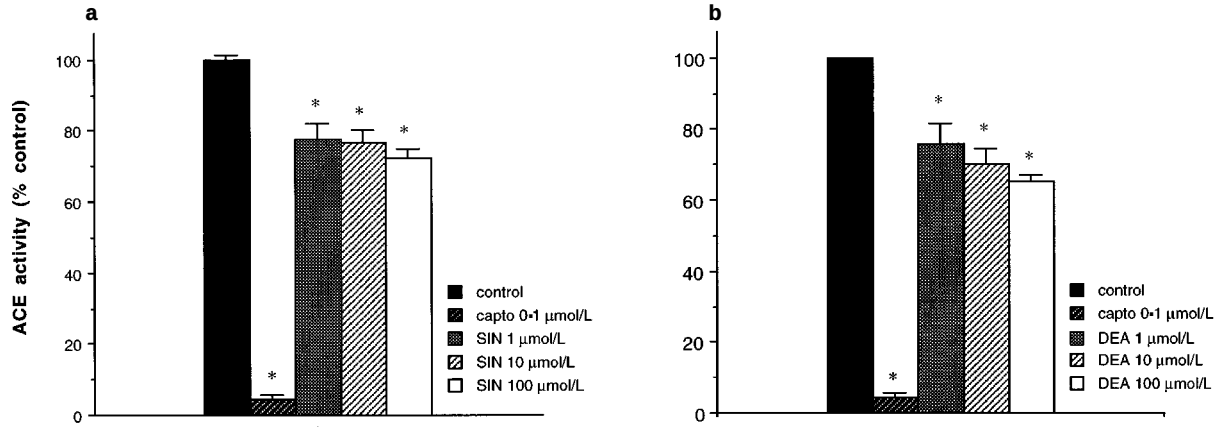

Figure 5 Effect of different concentrations of (a) SIN-1 and (b) DEA/NO on Hip-His-Leu hydrolysis by rat plasmatic ACE compared to the inhibitory effect of captopril (capto). Activity is expressed as % of control (without NO-donor) $\pm$s.e.mean; $n=6-22$. $^*P<0.05$.

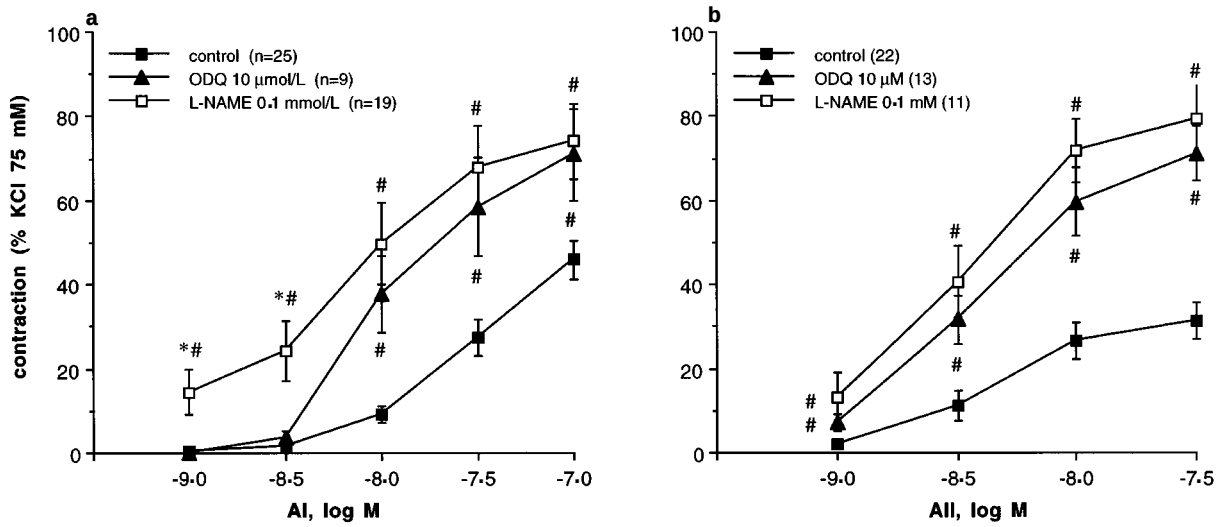

Figure 7 Comparison of the effect of 0.1 mM L-NAME and 10 μM ODQ on (a) AI-concentration-response curves and (b) AII-concentration-response curves. Contraction is expressed as % of a previous response elicited by 75 mM KCl and vertical lines show s.e.mean; n = number in parentheses. *$P<0.05$ compared AI- or AII-concentration-response curve with both, in presence of L-NAME and in presence of ODQ. #$P<0.05$ with respect to control.

Table 2 EC_{50} values for AI and AII-induced concentration-response curves in the rat carotid artery in the absence and presence of ODQ and L-NAME

	AI (log M)	*AII* (log M)
Control	−7.46 (−7.7, −7.23)	−7.84 (−8.16, −7.52)
+10 μM ODQ	−7.43 (−7.81, −7.1)	−8.56 (−8.93, −8.18)*
+0.1 mM L-NAME	−8.21 (−8.52, −7.91)*	−8.61 (−8.88, −8.36)*
E-	−8.17 (−8.45, −7.89)*	
E- + 10 μM ODQ plus 1 μM SIN-1	−7.47 (−7.75, −7.15)#	
E- + 10 μM ODQ pus 10 μM DEA/NO	−6.56 (−7.02, −6.11)#	

Data are presented as geometric means and confidence intervals. *$P<0.05$ with respect to control. #$P<0.05$ with respect to arteries without endothelium (E-).

A concentration of the guanylyl cyclase inhibitor ODQ of 10 μM was chosen because it abolished acetylcholine-induced relaxation. ODQ (10 μM) induced a shift to the left of the concentration-response curve for AII (Figure 7b) and of the EC_{50} value (Table 2). Contractions elicited by AI were only increased at high concentrations of the peptide, but not at 1 and 5 nM (Figure 7a). The EC_{50} value was equal to control (Table 2). Comparison of the contractions elicited by AI in presence of L-NAME with those elicited in presence of ODQ, showed a significant difference at 1 and 5 nM (Figure 7a). A putative inhibitory effect of ODQ on ACE activity was excluded (results not shown).

In vessels without endothelium AI elicited a concentration-response curve which was shifted to the left as compared to the control (Figure 8). The EC_{50} value was significantly different from control (Table 2). After preincubation of arteries without endothelium with 10 μM ODQ plus 1 μM SIN-1, contractions elicited by AI showed a significant decrease at 1 and 5 nM (Figure 8), whereas the contractions elicited by AII were unaffected (results not shown). ODQ 10 μM plus DEA/NO 10 μM induced a significant shift to the right of the concentration-response curve to AI (Table 2) with no effect on AII-induced responses (results not shown). ODQ 10 μM was chosen because it abolished the relaxant effect elicited by both SIN-1 1 μM and DEA/NO 10 μM in arteries precontracted with 0.1 μM noradrenaline.

Discussion

We analysed the effect of NO on ACE activity. For this purpose we performed a biochemical study by analysing the hydrolysis of Hip-His-Leu by ACE in presence of NO-donors, SIN-1 and DEA/NO, as well as in presence of an aqueous solution of NO. From a functional point of view, we examined whether endothelial NO modulates AI-induced contractions in rat carotid artery. This study demonstrates for the first time that NO inhibits ACE activity *in vitro*. This inhibition seems to occur also for vascular ACE by NO of endothelial origin.

The biochemical study was performed by using Hip-His-Leu as ACE substrate. Purified rabbit lung ACE and rat plasma ACE were used and kinetic parameters were compared. K_m values were 1.18 mM and 2.16 mM, respectively. These values are very similar to those previously described in the literature for Hip-His-Leu hydrolysis. K_m for human ACE from serum is 1.33 mM (Friedland & Silverstein, 1976) and for rabbit lung enzyme is 2.6 mM. Recombinant human ACE and human kidney ACE exhibit a K_m of 1.54 mM and 1.61 mM, respectively (Wei *et al.*, 1991).

Both SIN-1, a long-lasting NO-donor (Noack & Feelisch, 1989), and DEA/NO, a short-lived NO-donor (Maragos *et al.*, 1991), elicited a concentration-dependent inhibition of both purified and plasma ACE activity. This inhibitory effect was less pronounced in plasma ACE than in purified ACE probably due to the protein content of plasma, where the NO released might react with SH groups of several proteins (Stamler *et al.*, 1992). Nitrite levels, which are an estimate of NO release into the incubation medium, were also concentration-dependent. Under our experimental conditions, DEA/NO was more potent than SIN-1 in inhibiting ACE activity. Such an effect was not due to diethylamine, which is also released by DEA/NO during the incubation period. The higher potency of DEA/NO might be related to its high rate of NO release.

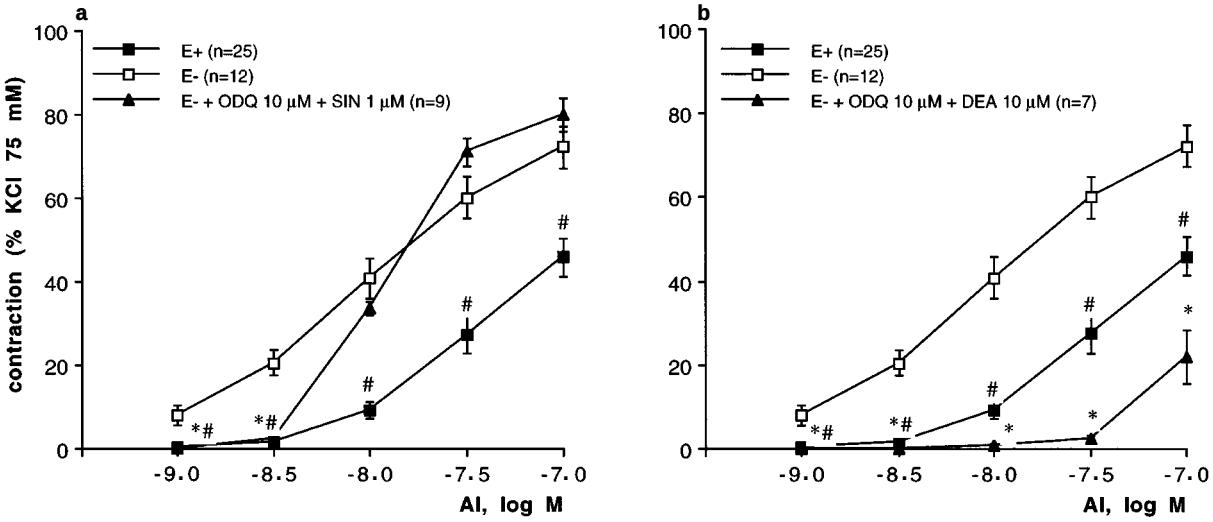

Figure 8 Effect of (a) 1 μM SIN-1 plus 10 μM ODQ and (b) 10 μM DEA/NO plus 10 μM ODQ on angiotensin I (AI)-induced concentration-response curves in absence of endothelium. Contraction is expressed as % of a previous response elicited by 75 mM KCl of n number of responses; vertical lines show s.e.mean. $^*P<0.05$.

DEA/NO led to significantly higher nitrite concentrations than SIN-1. A direct relationship between rate of NO release by NO-donors and parameters such as inhibiton of DNA synthesis (Nakaki *et al*., 1990) or cell viability (Mooradian *et al*., 1995) has been shown.

These results suggest that inhibition of ACE activity induced by NO-donors might be a specific effect for NO. This assumption is further supported by the inhibitory effect elicited by an aqueous NO solution on ACE activity. The fact that the inhibition of ACE by NO is less potent than the one induced by SIN-1 and DEA/NO is probably due to the short half-life (5 s) of NO (Moncada *et al*., 1991). Whereas these NO-donors release NO more or less constantly during the incubation period (30 min), NO is rapidly oxidized after addition to the incubation medium.

A loss of ACE activity due to pH reduction, as described by Ehlers and Riordan (1990), elicited by NO or NO-donors was excluded.

Kinetic parameters of plasma ACE in the presence of either NO or NO-donors were calculated from Lineweaver-Burk plots. The values obtained indicated that NO acts as a competitive inhibitor of ACE. This suggests that NO could interact with the active centre/s of the enzyme. ACE has two active centres, which contain the sequence H-His-Glu-Met-Gly-His-OH (Soubrier *et al*., 1988). Zn is bound by two histidine residues, both essential for the corresponding function of the active site (Wei *et al*., 1992). From our results we cannot elucidate the nature of the interaction between NO and the active centre/s.

In order to analyse the possibility that vascular ACE is modulated physiologically by endothelial NO, we estimated ACE activity in rat carotid artery rings by quantifying the contractions elicited by AI. As AI has no vasoconstrictor activity by itself, contractions elicited by this peptide reflect in an indirect way conversion to AII, i.e. ACE activity. This is demonstrated by the reduction of AI-induced contractions observed in presence of the ACE inhibitor captopril and the AII AT_1-receptor antagonist losartan (results not shown). The concentration-response curve to AI was always compared to the contractions elicited by AII, in order to discriminate the effect of NO on ACE activity from its effects on AII-induced contractions. To ascertain that the endothelial function was intact we only used arteries with more than 90% relaxation to 0.1 mM acetycholine.

L-NAME, an inhibitor of NO synthase, induced a similar increase of AI and AII responses. L-NAME inhibits basal endothelial NO release and induces a potentiation of contractile effects elicited by several vasoconstrictors, as described previously (Conrad & Whittemore, 1992).

As NO-induced effects are mediated by stimulation of vascular smooth muscle guanylyl cyclase and cyclic GMP increases (Moncada *et al*., 1991), we chose an inhibitor of guanylyl cyclase with the aim of discriminating the effect of NO on vascular tone from its possible effect on ACE activity. A concentration-response curve to AI and AII was performed in the presence of the guanylyl cyclase inhibitor, ODQ (Moro *et al*., 1996). Under these conditions, NO synthesis is still maintained, whereas vascular effects of NO are inhibited. AI-induced contractions, but not those elicited by AII, were equal to control at 1 and 5 mM.

Based on these results, we postulate that basal endothelial release of NO inhibits ACE. The fact that this inhibition is observed at low AI concentrations is in good agreement with the competitive inhibition elicted by NO on ACE activity. For AII the increased contraction observed both in presence of L-NAME and ODQ is due to the abolishment of the vascular effects of NO. However, for AI, the significant difference between L-NAME and ODQ at low concentrations suggest a difference between the inhibition of NO synthesis and the inhibition of its vascular effect. We suggest that NO synthesis, which is still maintained in the presence of ODQ, affects the conversion of AI to AII. An inhibitory effect of ODQ on ACE can be excluded, because guanylyl cyclase inhibition by ODQ is due to oxidation of Fe^{2+} to Fe^{3+} on the haeme group (Garthwaite *et al*., 1995). This oxidation is not possible on the zinc atom of the active centre of ACE. The lack of effect of ODQ on ACE activity under our experimental conditions was, nevertheless, excluded.

The hypothesis that basal endothelial release of NO inhibits ACE was further confirmed by removing the endothelium and mimicking endothelial NO release by the addition of an NO-donor. After endothelial removal, AI-elicited contractions were similar to those observed in presence of L-NAME in arteries with endothelium. This effect has already been

described by Arnal *et al*. (1994), who observed an increased ACE activity in arteries without endothelium as compared with arteries with endothelium. This is probably due to the presence of ACE in the adventitial and in the vascular smooth muscle layer (Arnal *et al*., 1994; Fernández-Alfonso *et al*., 1997). The addition of NO donors, SIN-1 1 μM and DEA/NO 10 μM, in the presence of 10 μM ODQ in order to avoid relaxation, mimicked the endothelial effect and AI-induced contractions were inhibited. The inhibitory effect was more pronounced for DEA/NO, according to the results previously observed on ACE activity.

Our finding, ACE inhibition by NO, is supported by results published recently by two groups (Michel *et al*., 1996; Takemoto *et al*., 1997). Chronic treatment of normotensive rats with L-NAME induces an increase in blood pressure which is associated with an upregulation of ACE activity specifically in blood vessels and heart, but not in serum and other organs (Takemoto *et al*., 1997). After a similar treatment, Michel *et al*. (1996) also demonstrated an increase in vascular ACE activity, restricted to the media and adventitia, without an effect on plasma ACE activity. This finding is in good agreement with the results observed previously by our group, where endothelium removal resulted in a quick upregulation of ACE in the media even after 2 days (Fernández-Alfonso *et al*., 1997).

In conclusion, we demonstrated that the hydrolysis of Hip-His-Leu by ACE is inhibited by NO-donors, SIN-1 and DEA/NO, and by an aqueous solution of NO in a concentration-dependent and competitive way. In addition, basal endothelial NO release inhibits the conversion of AI to AII in rat carotid artery. This effect was observed at low concentrations of AI, supporting the competitive inhibition elicited by NO. Taking into account that circulating concentrations of AI and AII are in the nanomolar range, we suggest that NO might be a physiological modulator of both endothelial and plasma ACE.

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