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Nitric oxide donors and angiotensin-converting enzyme inhibitors act in concert to inhibit human angiotensin-converting enzyme activity and platelet aggregation in vitro

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Abstract

This study investigates the effects of exogenous and endogenous nitric oxide (NO) on human circulating and endothelial angiotensin-converting enzyme activity and platelet aggregation. The NO donor *S*-nitroso-*N*-acetylpenicillamine (10^{-8}–10^{-6} M) significantly and dose-dependently inhibited serum angiotensin-converting enzyme activity. The concomitant addition of *S*-nitroso-*N*-acetylpenicillamine to angiotensin-converting enzyme inhibitor-treated (captopril or enalaprilat) serum, further reduced angiotensin-converting enzyme activity. In cultured endothelial cells from human umbilical veins (HUVECs), both *S*-nitroso-*N*-acetylpenicillamine and 3-morpholinosydnonimine (SIN-1) significantly reduced angiotensin-converting enzyme activity. An additative effect was seen with a combined treatment of captopril and *S*-nitroso-*N*-acetylpenicillamine. Treatment with the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) did not affect angiotensin-converting enzyme activity. Thrombin inhibited endothelial angiotensin-converting enzyme activity, an effect that was abolished when cells were pretreated with L-NMMA. Adenosine 5′-diphosphate (ADP)-induced platelet aggregation was inhibited with *S*-nitroso-*N*-acetylpenicillamine, SIN-1 and nitroglycerine. Captopril did not affect aggregation, while a high concentration of enalaprilat (10^{-4} M) reduced it. The concomitant addition of 10^{-5} M angiotensin-converting enzyme inhibitor to NO donor-treated platelets resulted in a further reduction of platelet aggregation. This effect was most evident with SIN-1 and enalaprilat. In conclusion, both exogenous and endogenous NO inhibit human angiotensin-converting enzyme activity. NO donors and angiotensin-converting enzyme inhibitors act in concert to inhibit angiotensin-converting enzyme and platelet aggregation.

Keywords: Angiotensin-converting enzyme; Angiotensin-converting enzyme inhibitor; Endothelial cells, cultured; Umbilical veins, human; Nitric oxide (NO); Platelet aggregation

1. Introduction

Angiotensin-converting enzyme (EC 3.4.15.1) is a key enzyme in the renin–angiotensin system regulating blood pressure, and water and electrolyte balance in the body. Angiotensin-converting enzyme is an ecto-enzyme anchored in the vascular endothelial cell membrane by its hydrophobic C-terminal and the active sites protruding out into the lumen (Hooper et al., 1987; Ryan et al., 1975). It may, by proteolytic cleavage, lose its C-terminal anchor and become soluble in the blood (Baudin et al., 1997; Hooper et al., 1987). Angiotensin-converting enzyme contains two homologous (Bernstein et al., 1989; Soubrier et al., 1988) and independently active sites (Wei et al., 1991), each containing a Zn^{2+} ion (Ehlers and Riordan, 1991; Wei et al., 1991). Angiotensin-converting enzyme has a broad substrate specificity, it not only converts angiotensin I to the active angiotensin II (Skeggs et al., 1956), but also acts on a variety of other peptides (Erdös and Skidgel, 1986). One of these is bradykinin which angiotensin-converting enzyme degrades to inactive peptide fragments (Yang and Erdös, 1967). Bradykinin is an inflammatory mediator capable of inducing endothelium-dependent relaxation of the vascular smooth muscle cells (Hall, 1992). Bradykinin-induced relaxation is most often mediated by the bradykinin B_2 receptor (Hall, 1992) and the subsequent production of nitric oxide (NO), prostacyclin and/or en-

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dothelium-derived hyperpolarizing factor (EDHF) (Busse and Fleming, 1996; D'Orléans-Juste et al., 1989; De Nucci et al., 1988; Mombouli and Vanhoutte, 1995). Angiotensin-converting enzyme may thus augment blood pressure and reduce local blood flow by increasing angiotensin II generation and bradykinin degradation. Inhibitors of angiotensin-converting enzyme (e.g. captopril and enalapril) are first-line drugs in the treatment of hypertension and heart failure (Brown and Vaughan, 1998).

NO is a free radical synthesised and released by the vascular endothelium in response to several stimuli (Lüscher and Barton, 1997). NO synthase in healthy vessels (eNOS) is constitutive, and responsible for both the basal and receptor-mediated release of NO. Acetylcholine, bradykinin and thrombin are examples of endogenous agonists capable of activating eNOS (Lüscher and Barton, 1997). NO-mediated effects in the cardiovascular system are vasodilation (Furchgott and Zawadski, 1980), inhibition of platelet aggregation (Radomski et al., 1990), and anti-proliferation (Garg and Hassid, 1989). It has previously been shown that NO donors inhibit angiotensin-converting enzyme from rats (Ackermann et al., 1998) and pigs (Persson and Andersson, 1999). In humans, the precursor of NO, L-arginine, reduces angiotensin-converting enzyme activity (Higashi et al., 1995).

This study was performed to investigate whether exogenous and endogenous NO are able to inhibit human angiotensin-converting enzyme. We also studied if there are any differences in NO-mediated inhibition of soluble serum or endothelial (endothelial cells from human umbilical vein (HUVEC)) angiotensin-converting enzyme. To investigate if a combined action of NO and angiotensin-converting enzyme inhibitors elicit functional effects in humans, we studied platelet aggregation.

2. Methods

2.1. Serum angiotensin-converting enzyme activity

Venous blood was collected in silicone-coated vacutainer tubes, allowed to sediment for 2 h, and then centrifuged ($1000 \times g$ for 20 min, 4°C). The serum was removed and stored at −20°C until analysed.

Serum was incubated with 10^{-9}–10^{-7} M angiotensin-converting enzyme inhibitor (captopril or enalaprilat) and/or 10^{-8}–10^{-6} M *S*-nitroso-*N*-acetylpenicillamine for 5 min in 37°C water bath. Drugs were dissolved in 0.1 M potassium phosphate buffer pH 7.8, and corresponding volumes of solvent was added to controls. Then, angiotensin-converting enzyme activity was analysed with a commercial radioenzymatic assay (ACE-direct REA, Bühlmann Laboratories, Allschwil, Switzerland). In short, the synthetic substrate [^{3}H]hippuryl-glycyl-glycine was added and cleaved by angiotensin-converting enzyme to [^{3}H]hippuric acid. After 1 h incubation in 37°C water bath, the reaction was stopped by adding 50 μl 1 M HCl, scintillation liquid was added and each sample was counted in a scintillation counter. The experiments were performed in darkness due to the instability of *S*-nitroso-*N*-acetylpenicillamine when exposed to light.

2.2. Cultured endothelial cells from HUVEC

Human umbilical cords were obtained after normal vaginal delivery (after informed consent from the mothers), and kept in sterile bottles containing phosphate-buffered saline (PBS), penicillin, streptomycin and gentamicin. Endothelial cells were then isolated by treatment with 0.5 mg/ml collagenase for 15 min at 37°C as described previously (Nyhlén et al., 2000). In short, the collagenase + cell perfusate was washed twice, and then resuspended in culture medium (Dulbecco's modified Eagle's medium (DMEM)) supplemented with nonessential amino acids (1:100), oxalacetic acid (1.2 mM), insulin (0.24 IE/ml), penicillin (5 U/ml), streptomycin (0.5 μg/ml), HEPES (10 mM), endothelial cell growth factor (ECGF, 30 μg/ml), heparin (20 U/ml) and 17% inactivated fetal calf serum. Resuspended endothelial cells were seeded in 25 cm^2 tissue culture flasks coated with 0.2% gelatin, and kept in an incubation chamber. Medium was replaced every 48–62 h. At confluence, cells were harvested with trypsin-EDTA for 5–10 min, and then reseeded 1:2. Second passage was seeded in a 96-well microtiter plate, and angiotensin-converting enzyme activity was analysed as described below.

2.2.1. Angiotensin-converting enzyme activity in cultured endothelial cells from HUVEC

When the cells had reached confluence in the 96-well microtiter plate, medium was removed from each well and replaced with medium without fetal calf serum. All drugs were dissolved in medium without fetal calf serum. Medium without fetal calf serum was used to avoid discrepancies in results due to angiotensin-converting enzyme present in the serum (Bramucci et al., 1999). Cells were pretreated for 15 min with N^G-monomethyl-L-arginine (L-NMMA) and for 5 min with captopril, *S*-nitroso-*N*-acetylpenicillamine, *S*-morpholinosydnonimine (SIN-1), or

Table 1
Angiotensin-converting enzyme activity (U) in human serum

Concentration (M)	Captopril	Enalaprilat	*S*-nitroso-*N*-acetylpenicillamine
Control	39.4 ± 4.3	32.0 ± 5.1	39.4 ± 4.3
10^{-9}	28.4 ± 3.8[a]	15.3 ± 2.6[b]	n.d.
10^{-8}	19.2 ± 4.0[b]	3.3 ± 0.7[b]	31.0 ± 4.7
10^{-7}	7.4 ± 2.0[b]	2.3 ± 0.5[b]	27.1 ± 5.7[a]
10^{-6}	n.d.	n.d.	25.7 ± 6.3[b]

$n = 5$–6, n.d. = not done.
[a] One-way ANOVA, $P < 0.05$.
[b] One-way ANOVA, $P < 0.01$.

thrombin, before analysis. Then, angiotensin-converting enzyme activity was analysed with the radioenzymatic assay described above, with the following modifications. Blank and standard serum were added to wells with corresponding volumes of medium without fetal calf serum. The substrate was then added directly to all the wells of the microtiter plate. Cells were incubated with substrate for 2 h (in the incubation chamber), then 150 μl medium + substrate was transferred from each well into scintillation vials containing 50 μl 1 M HCl (to stop the enzymatic reaction). Scintillation liquid was added and the samples were counted in a scintillation counter.

2.3. Platelet aggregation

Venous blood was collected in silicone-coated vacutainer tubes and anticoagulated with 3.8% trisodium citrate (blood/anticoagulant 9:1). All samples were drawn after informed consent from healthy volunteers who refrained from antiplatelet drugs such as aspirin for 2 weeks prior to the study. Platelet-rich plasma was obtained by centrifugation at $220 \times g$ for 20 min; platelet poor plasma was prepared by further centrifugation at $1500 \times g$ for 10 min. In vitro platelet aggregation was measured with modifications of a recently described microplate-based technique (Fratantoni and Poindexter, 1990; Hoylaerts et al., 1996). Platelet-rich plasma (150 μl) was incubated with drugs or solvent (saline) at room temperature (22°C) for 5 min before platelet aggregation was induced by adenosine 5′-diphosphate (ADP) at treshold concentrations of 1–5 μM. The microplate (Maxi-Sorp, Nunc, Roskilde, Denmark) was incubated with continuous agitation at 37°C in a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The decrease of optical density (OD) at 632 nm within 10 min was determined and expressed as the percentage of the difference between platelet-rich plasma and platelet-poor plasma. In platelet-rich plasma with solvent, the aggregation was set at 0% and in platelet poor plasma, it was set at 100%. Experiments were performed in triplicate.

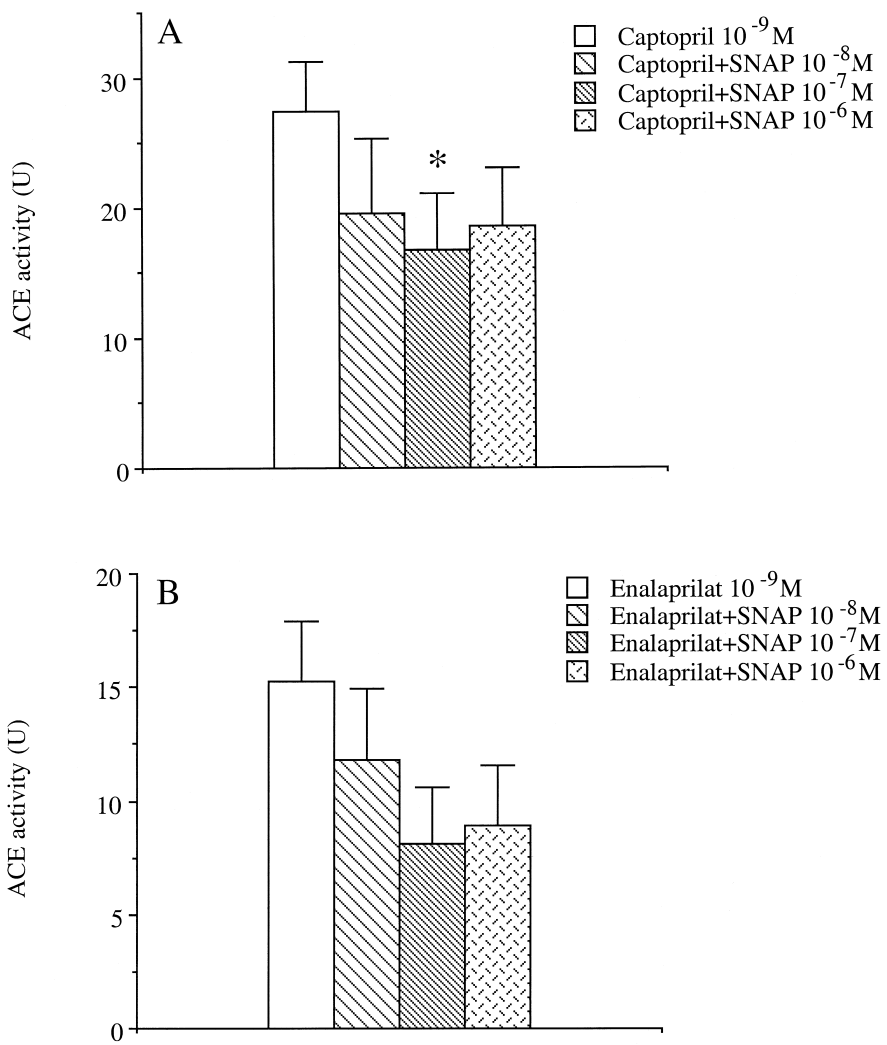

Fig. 1. Angiotensin-converting enzyme activity in human serum concomitantly treated with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP), and 10^{-9} M captopril (A; $n = 5$) or enalaprilat (B; $n = 5$) for 5 min. Significance is calculated with one-way ANOVA, and denoted as $^{*}P < 0.05$.

2.4. Calculations

Results are expressed as mean ± S.E.M. One unit (U) of angiotensin-converting enzyme activity is defined as the amount of enzyme required to release 1 μmol of hippuric acid per minute and liter. Platelet aggregation is given as percent change in ADP-induced aggregation (i.e. negative values means inhibition of aggregation). Statistical calculations were done with Student's one sample *t*-test (platelet aggregation experiments) or one-way analysis of variance (ANOVA) repeated measures for matched data followed by Dunnett's post-hoc test. Statistical significance is denoted with $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ or, in the tables, with $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$.

2.5. Drugs

ADP, collagenase, oxalacetic acid, *S*-nitroso-*N*-acetylpenicillamine, and thrombin were obtained from Sigma (St. Louis, MO, USA). N^{G}-monomethyl-L-arginine (L-NMMA) and 3-morpholinosydnonimine (SIN-1) were bought from Alexis, San Diego, CA, USA. Nitroglycerine was obtained at Tika Läkemedel, Lund, Sweden. Captopril was a gift from Bristol-Myers Squibb, Princeton, NJ, USA, and enalaprilat was obtained at Merck Sharp and Dohme, Haarlem, Netherlands. DMEM, nonessential amino acids, penicillin, streptomycin, HEPES, trypsin-EDTA (10×), and fetal calf serum were all bought from Life Technologies, Scotland, UK. Insulin (Velosulin®) was from Novo Nordisk, Denmark, ECGF from Boehringer-Mannheim, Germany, and heparin from Kabi Pharmacia, Sweden.

3. Results

3.1. Serum angiotensin-converting enzyme activity

As expected, the angiotensin-converting enzyme inhibitors captopril and enalaprilat decreased serum angiotensin-converting enzyme activity in a dose-dependent manner (Table 1). Serum angiotensin-converting enzyme was also dose-dependently and significantly inhibited by the NO donor *S*-nitroso-*N*-acetylpenicillamine (10^{-8}–10^{-6} M) (Table 1). Combined treatment with 10^{-9} M captopril or enalaprilat and *S*-nitroso-*N*-acetylpenicillamine resulted in a trend towards an additative inhibitory effect on serum angiotensin-converting enzyme (Fig. 1).

3.2. Angiotensin-converting enzyme activity in cultured endothelial cells from HUVEC

Both captopril, *S*-nitroso-*N*-acetylpenicillamine and SIN-1 dose-dependently and significantly inhibited angiotensin-converting enzyme activity in the cultured endothelial cells (Fig. 2). The concomitant addition of 10^{-9} M captopril and *S*-nitroso-*N*-acetylpenicillamine showed

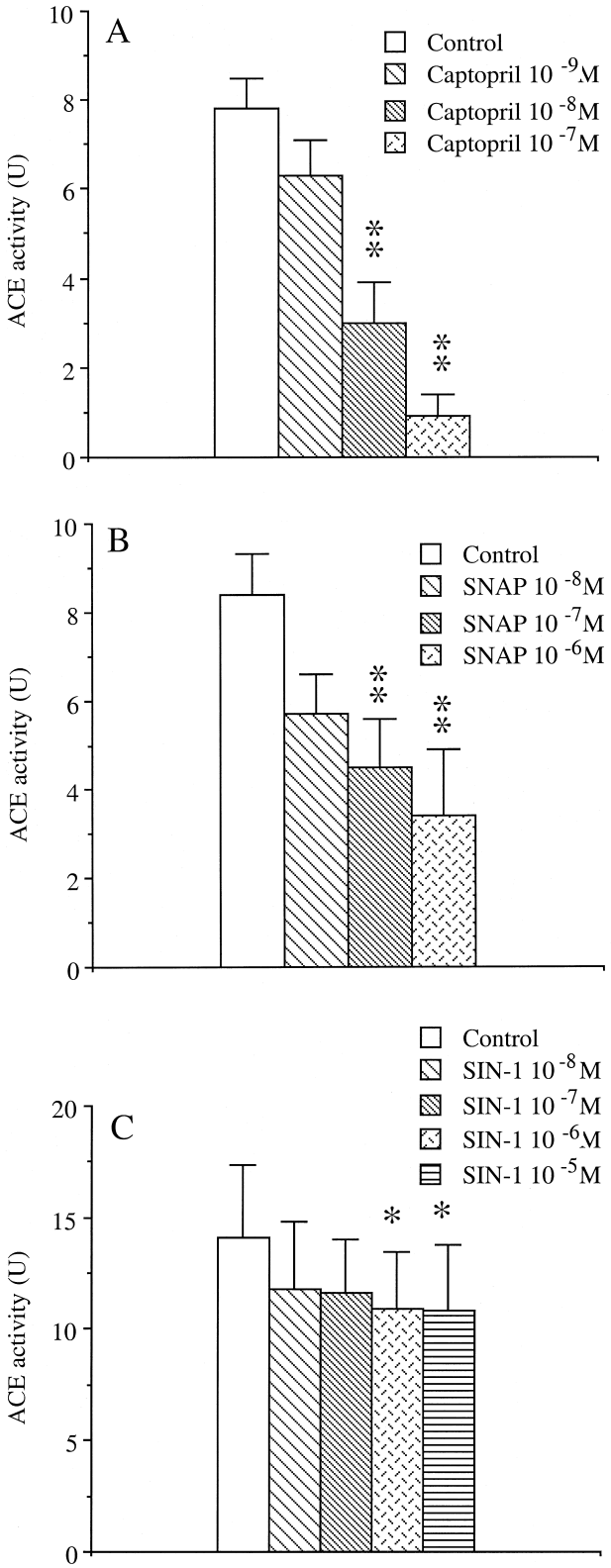

Fig. 2. Angiotensin-converting enzyme activity in cultured endothelial cells from human umbilical veins treated with captopril (A; $n = 5$), *S*-nitroso-*N*-acetylpenicillamine (SNAP; B; $n = 6$), or SIN-1 (C; $n = 5$) for 5 min. Significance is calculated with one-way ANOVA, and denoted as $^{*}P < 0.05$ and $^{**}P < 0.01$.

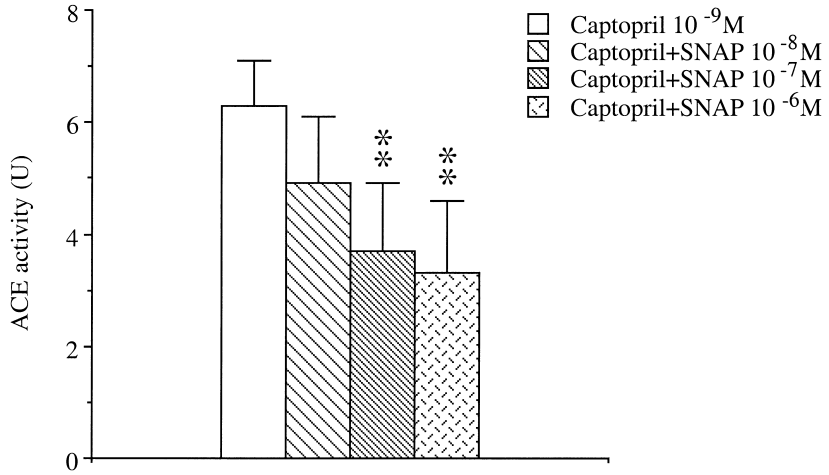

Fig. 3. Angiotensin-converting enzyme activity in cultured endothelial cells from human umbilical veins (HUVEC) concomicantly treated with *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 10^{-9} M captopril, $n = 5$. Significance is calculated with one-way ANOVA and denoted as $^{**}P < 0.01$.

an additative inhibitory effect (Fig. 3). The NOS inhibitor L-NMMA did not affect angiotensin-converting enzyme activity in the endothelial cells, 17.5 ± 1.6 U as compared to control 16.2 ± 1.2 U, $n = 8$. When the cells were stimulated with 0.1 or 1 U/ml thrombin, no effect on angiotensin-converting enzyme activity was seen. Angiotensin-converting enzyme activity was significantly reduced by 10 U/ml thrombin (13.0 ± 1.6 U) compared to controls (15.7 ± 1.7 U), $P < 0.01$, $n = 4$. In cells pretreated with L-NMMA, 10 U/ml thrombin did not significantly affect angiotensin-converting enzyme activity (14.8 ± 1.8 U), $n = 4$.

3.3. Platelet aggregation

The NO donors SIN-1, *S*-nitroso-*N*-acetylpenicillamine, and nitroglycerine dose-dependently inhibited platelet aggregation (Table 2). Captopril (10^{-8}–10^{-4} M) did not significantly affect platelet aggregation, while a high dose (10^{-4} M) enalaprilat showed an inhibitory effect (-13.6 ± 1.8% change in aggregation, $P < 0.001$, $n = 6$). When 10^{-5} M captopril or enalaprilat was added to the platelets together with a threshold dose of NO donor, an additional reduction was seen with SIN-1 (Fig. 4). A trend towards a similar effect was seen with nitroglycerine (data not shown). With *S*-nitroso-*N*-acetylpenicillamine, only enalaprilat could further inhibit platelet aggregation (Fig. 5). When higher concentrations of the NO donors were used, no additional effect with the angiotensin-converting enzyme inhibitors were seen (data not shown).

4. Discussion

Both exogenous (NO donor) and endogenous NO inhibited angiotensin-converting enzyme from human serum and cultured endothelial cells. *S*-nitroso-*N*-acetylpenicillamine inhibited angiotensin-converting enzyme at much lower concentrations (10^{-8}–10^{-6} M) than has previously been described with *S*-nitroso-*N*-acetylpenicillamine and SIN-1 (Ackermann et al., 1998; Persson and Andersson, 1999). The inhibition of angiotensin-converting enzyme by NO is not altogether surprising, as NO is able to bind to Fe^{2+} in guanylyl cyclase and hemoglobin. Angiotensin-

Table 2
Change in ADP-induced human platelet aggregation (%)

Concentration (M)	SIN-1	*S*-nitroso-*N*-acetylpenicillamine	Nitroglycerine
10^{-7}	1.8 ± 0.9	n.d.	-0.6 ± 2.8
10^{-6}	-2.9 ± 1.3	-16.8 ± 4.3[a]	-10.3 ± 4.5
3×10^{-6}	n.d.	-35.4 ± 3.8[b]	n.d.
10^{-5}	-19.3 ± 2.2[b]	-35.6 ± 3.2[b]	n.d.
10^{-4}	-33.3 ± 2.4[b]	n.d.	n.d.

$n = 6$–10, n.d. = not done.
[a] Student's one sample *t*-test, $P < 0.01$.
[b] Student's one sample *t*-test, $P < 0.001$.

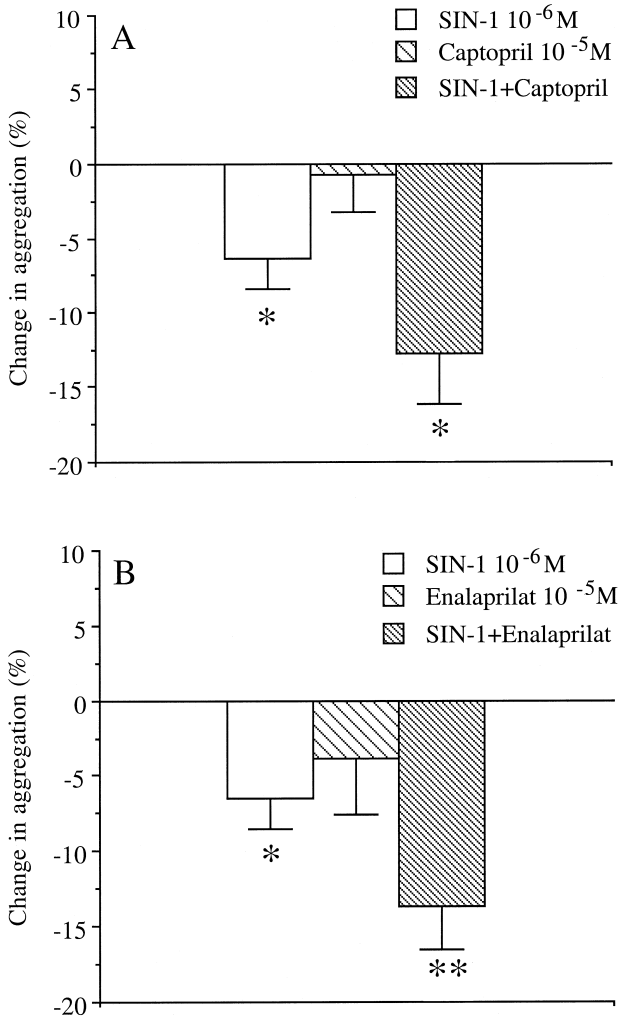

Fig. 4. Change in ADP-induced human platelet aggregation, when treated in vitro with the NO donor SIN-1, and the angiotensin-converting enzyme inhibitors captopril (A; $n = 7$) or enalaprilat (B; $n = 6$), alone or in combination. Signficance is calculated with Student's one sample *t*-test, and denoted as $^{*}P < 0.05$ and $^{**}P < 0.01$.

converting enzyme contains Zn^{2+} (Ehlers and Riordan, 1991; Wei et al., 1991), and angiotensin-converting enzyme inhibitors inhibit angiotensin-converting enzyme by binding to Zn^{2+} (Guthrie, 1993).

The additional effect of captopril and *S*-nitroso-*N*-acetylpenicillamine was less evident in serum than in cultured endothelial cells from HUVEC. This is probably due to the fact that the low dose captopril used (10^{-9} M) did not significantly inhibit angiotensin-converting enzyme in the endothelial cells. However, since captopril was less, and *S*-nitroso-*N*-acetylpenicillamine was more potent in inhibiting angiotensin-converting enzyme in the cells, a difference between circulating and endothelial angiotensin-converting enzyme cannot be excluded.

NO readily reacts with SH-containing compounds forming nitrosothiols (Girard and Potier, 1993), which have been proposed as intermediates in NO-induced effects (Ignarro et al., 1981). Captopril is a sulfhydryl-containing angiotensin-converting enzyme inhibitor, and may as such act as a free radical scavenger (Chopra et al., 1989; Mak et al., 1990). It can thus combine with NO to form nitroso-captopril, and one could speculate that this compound is more effective than captopril or NO alone in inhibiting angiotensin-converting enzyme and platelet aggregation. We cannot exclude the possibility that nitroso-captopril is formed in our experiments and contribute to the results. However, when using the non-sulfhydryl-containing enalaprilat, the same effects were seen, and enalaprilat was even more potent than captopril in inhibiting both serum angiotensin-converting enzyme and platelet aggregation. Enalaprilat is previously known as a more potent an-

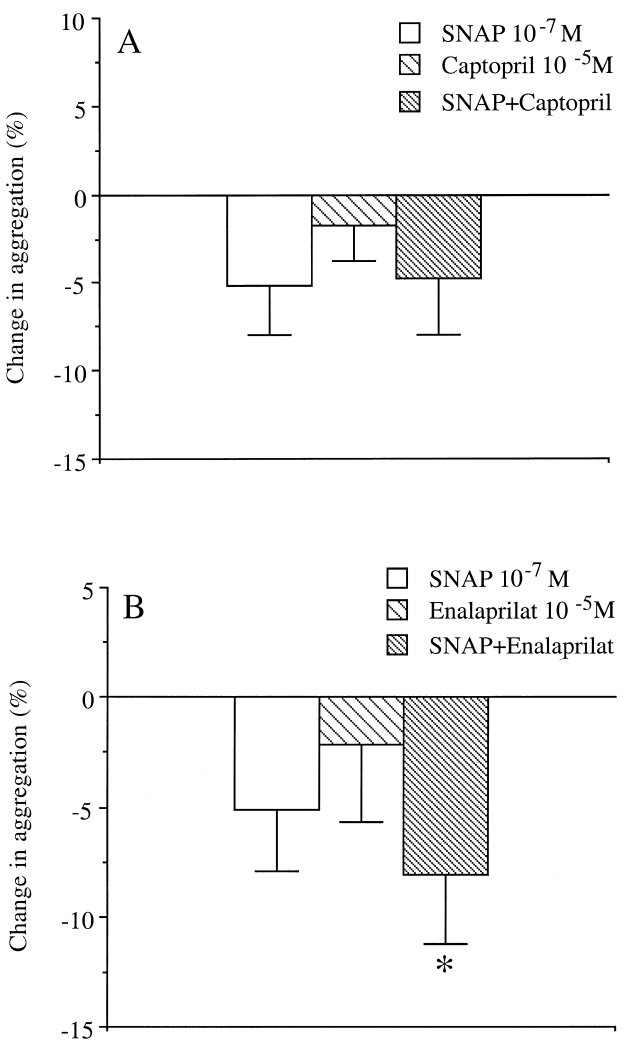

Fig. 5. Change in ADP-induced human platelet aggregation, when treated in vitro with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP), and the angiotensin-converting enzyme inhibitors captopril (A; $n = 6$) or enalaprilat (B; $n = 6$), alone or in combination. Signficance is calculated with Student's one sample *t*-test, and denoted as $^{*}P < 0.05$.

giotensin-converting enzyme inhibitor than captopril (Johnston et al., 1988).

To study the effect of endogenous NO on angiotensin-converting enzyme activity, we used cultured endothelial cells from HUVEC. These cells are shown both to express angiotensin-converting enzyme (Graf et al., 1992; Johnson and Erdös, 1977) and to release NO (Sorrentino et al., 1990; Tsukahara et al., 1993). Basal NO release does not seem to be enough to inhibit angiotensin-converting enzyme activity, as the NO synthase inhibitor L-NMMA had no significant effect in our study. It has been shown that shear stress reduce angiotensin-converting enzyme expression and activity (Rieder et al., 1997), and also induce eNOS expression and thus increase basal NO production (Lüscher and Barton, 1997). Whether shear stress may reduce angiotensin-converting enzyme activity by increasing NO-mediated angiotensin-converting enzyme inhibition is unknown.

Thrombin increases receptor-mediated NO release from cultured endothelial cells from HUVEC (Tsukahara et al., 1993). The present results show a significant reduction in angiotensin-converting enzyme activity in human endothelial cells stimulated with thrombin. This effect was abolished with L-NMMA, suggesting that thrombin indeed inhibits angiotensin-converting enzyme via increased NO production. Thus, receptor-mediated NO release may induce vasodilation and increased local blood flow, not only by a direct effect on the vascular smooth muscle cells, but also indirectly by decreased angiotensin-converting enzyme-mediated angiotensin II generation and/or bradykinin degradation.

Inhibition of endogenous NO production increases angiotensin I-induced vasoconstriction in porcine iliac arteries (Persson and Andersson, 1999), showing the physiological importance of NO-mediated angiotensin-converting enzyme inhibition in local blood flow. NO acts as an autocrine inhibitor of vascular smooth muscle cell contraction (Yeh et al., 1996). The NO-mediated angiotensin-converting enzyme inhibition previously shown in rats (Ackermann et al., 1998) and pigs (Persson and Andersson, 1999), and in this study confirmed in humans, may be yet another way of NO to affect local vascular tone.

Increased vascular angiotensin-converting enzyme activity has been seen in hypertension (Shiota et al., 1992) and atherosclerosis (Diet et al., 1996; Persson, unpublished results) and is proposed as a causal factor in these diseases. Hypertension and atherosclerosis are also associated with endothelial dysfunction and reduced NO bioavailability (Lüscher and Noll, 1995). According to the results of this study, the increased angiotensin-converting enzyme activity may partly be due to the removal of NO, an endogenous angiotensin-converting enzyme inhibitor.

To investigate if a combined action of NO and angiotensin-converting enzyme inhibitors elicits any functional effects in humans, we chose to study platelet aggregation. The organic nitrate nitroglycerine (Schafer et al., 1980), the *S*-nitrosothiol *S*-nitroso-*N*-acetylpenicillamine (Salas et al., 1994; Whiss and Larsson, 1998) and the sydnonimine SIN-1 (Nishikawa et al., 1982), are all described as inhibitors of platelet aggregation. In this study, the angiotensin-converting enzyme inhibitor captopril did not affect platelet aggregation, but a high concentration of enalaprilat (10^{-4} M) reduced aggregation. Since this concentration is higher than the plasma concentrations seen when treating patients with enalaprilat, an inhibitory effect on platelet aggregation in vivo with this drug is unlikely. But considering an eventual higher tissue level of this drug, a reduction of platelet aggregation at local sites cannot be ruled out.

When concomitantly adding an angiotensin-converting enzyme inhibitor to NO donor-treated platelets, a further reduction in aggregation was achieved. There was some discrepancies in results, since adding enalaprilat, but not captopril, to *S*-nitroso-*N*-acetylpenicillamine-treated platelets affected the aggregation. Furthermore, the experiments with nitroglycerine did not reach significance. This may be due to differences in action between the NO donors. For instance, the various classes of NO donors differ in their need of specific cofactors to release NO, and the pathways of bioactivation are clearly different (Feelisch, 1991). Nitroglycerine requires metabolic conversion to release NO, *S*-nitroso-*N*-acetylpenicillamine probably needs metabolism partially, and SIN-1 appears to not require any metabolism. Nitroglycerine has been shown to be metabolised to NO by blood platelets, although to a lesser extent than by vascular smooth muscle cells (Weber et al., 1996). *S*-nitroso-*N*-acetylpenicillamine is reported to release NO spontaneously (Kankaanranta et al., 1996) but it has also been suggested that the release of NO from *S*-nitroso-*N*-acetylpenicillamine occurs inside the platelet (Salas et al., 1994), and that *S*-nitroso-*N*-acetylpenicillamine requires activation of glyceraldehyde-3-phosphate dehydrogenase for the transport of NO in platelets (McDonald et al., 1993). SIN-1 spontaneously release both NO and superoxide (O_2^-) leading to the formation of peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH°) (Feelisch, 1991). The effect of SIN-1 appears not to depend on interactions with cofactors. Differences in requirement of cofactors of the NO donors may have an impact on the inhibiting effect of platelets in various disease states (Whiss and Larsson, 1998).

Since the results on serum and cultured endothelial cells show a more pronounced inhibition of the angiotensin-converting enzyme when an NO donor is added together with captopril or enalaprilat, it is concluded that in order to affect platelet aggregation enzyme activity has to become very low.

Of all the NO donors used in this study, only nitroglycerine is used clinically as an anti-anginal drug. Whether the additional decrease in platelet aggregation, seen when simultaneously adding nitroglycerine and an angiotensin-converting enzyme inhibitor, is present also in patients

with combination therapy is yet unknown. But one may speculate that such a combination should be of great value in patients at risk of myocardial infarction.

In conclusion, NO donors are able to inhibit circulating and endothelial angiotensin-converting enzyme from humans. This reduction in angiotensin-converting enzyme activity is additative with captopril and enalaprilat. Endogenous basal NO release is not enough to inhibit angiotensin-converting enzyme, but thrombin-stimulated NO release is. Captopril and enalaprilat do not, in reasonable concentrations, affect platelet aggregation. But adding any of these angiotensin-converting enzyme inhibitors to NO donor-treated platelets, an obvious further inhibition of aggregation is observed, showing that the additative reduction in angiotensin-converting enzyme activity may have impact on functional responses in humans. This NO-mediated angiotensin-converting enzyme inhibition may account for yet another way of NO to affect vascular tone and cardiovascular diseases.

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