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Renin Inhibits the Vasorelaxation Induced by Nitroso Albumin

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Nitrosated proteins exhibit actions characteristic of free NO. As the vasorelaxation effect of nitrosated albumin is rapidly inactivated in plasma, we postulated that a protease could remove or modify the NO attached to albumin. We found that the ability of plasma to inactivate the vasorelaxing action of NO-bovine serum albumin (NO-BSA) is restricted to a plasma fraction containing macromolecules. We also found that a crude preparation of renal renin also inactivated the vasorelaxation action of NO-BSA and UV-spectrophotometric analysis showed that the 335-nm signal of NO-BSA was significantly decreased by renin. This decrease could be prevented by a renin inhibitor or by immunodepleting the renin preparation with a monoclonal antibody to renin. The data suggest that renin accelerates the uncoupling of NO to albumin. Such a function may be important in the control of vascular tone and blood pressure.

Nitric oxide (NO)[1] has, among its many biological actions, a critical role in the control of vascular smooth muscle tone and blood pressure (1). NO is a potent endogenous vasodilator and is tonically activated since blood pressure increases upon inhibition of NO synthesis (2, 3). Further, NO synthesis increases during extracellular volume expansion (4, 5), and inability to do so leads to hypertension (4, 6). NO relaxes vascular smooth muscle in part by activating guanylate cyclase (1) and in part, by its covalent attachment to potassium channels, thereby favoring hyperpolarization (7).

Covalent linkage of NO to proteins [nitrosation reaction (8)] is of additional interest because it may provide a mechanism for NO delivery. Because NO is an extremely reactive free radical which can be inhibited by many substances (9, 10) it is unclear how after its synthesis in endothelial cells, free NO diffuses to its targets. In this regard, NO generated in the presence of low molecular weight thiols such as cysteine yields nitrosothiols (11) and these compounds are more stable than NO and have biological effects that more closely resemble endogenously generated endothelium-derived relaxing factor [EDRF (11, 12)]. Further, Stamler *et al.* (13, 14) and Keaney *et al.* (15) found that nitrosoproteins have EDRF-like effects, including vasodilation (13–15) and that NO circulates in plasma primarily as an adduct of albumin [NO-albumin (13)].

When NO-albumin is in physiological solutions, its vasodilatory action has a half life of ~24 h but in plasma, the half life is only ~40 min (14) suggesting that a factor in plasma removes or modifies the nitroso group on albumin. Acid catalyzed and covalent catalyzed hydrolysis of nitrosated amino acids have been described (15). Since proteases, esterases and acyl transferases employ these mechanisms in the transformation of preferred substrates, we speculated that a plasma enzyme from one of these classes might be found to hydrolyze NO-albumin. Because NO regulation of vascular tone is modulated by the state of the extracellular fluid volume (4, 5) and renin has a central role on the control of both vascular tone and extracellular fluid volume, we postulated that this protease could modify NO-albumin.

METHODS

Synthesis of nitroso-bovine serum albumin (BSA). Nitrosation of BSA (NO-BSA) was carried out by incubating equimolar amounts of BSA (3 g, fraction V, fatty acid free; Sigma, St. Louis, MO) and $NaNO_2$ (1 mg; Fisher Scientific, Springfield, NJ) in 50 ml of 0.5 N HCl for 30 min at room temperature, as described (14). The solution was next neutralized to pH 7.4 by addition of 1 N NaOH and small amounts of 150 mM NaCl with 10 mM Tris, pH 7.4.

Spectrophotometric analysis of nitroso-BSA. UV-visible spectroscopy of NO-BSA was done with a Beckman spectrometer (Beckman Instruments, Inc., Fullerton, CA) at a concentration of protein of 30 mg/ml by diluting the stock solution of NO-BSA 1:1 with distilled water. Background signal of non-nitrosated BSA was subtracted from reported values.

Blood vessel bioassay. Rings from descending aortas of Sprague–Dawley rats were mounted in glass chambers containing Krebs-

[1] Abbreviations used: BSA, bovine serum albumin; EDRF, endothelium-derived relaxing factor; NO, nitric oxide; NO-BSA, nitrosated bovine serum albumin.

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Henseleit buffer (17) at 37°C and equilibrated with 95% O_2/5% CO_2. The rings were connected to tension transducers (Harvard Apparatus, Dover, MA) and tension was measured with a transducer amplifier (CB Sciences, Milford, MA) and a MacLab (AD Instruments, Milford, MA). Sustained contractions were induced with 50 μM phenylephrine (Sigma, St. Louis, MO). To test the effect of NO-BSA on the contracted rings, 5 ml of the NO-BSA stock solution were added to the bathing media for a final concentration of 10 μM.

Incubation of NO-BSA with plasma. Five milliliters of 60 mg/ml of NO-BSA prepared as described were incubated with 5 ml of human plasma (blood collected in Vacutainer tubes containing sodium citrate; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) for 3 h at 37°C. The mixture was then added to the contracted vessels.

To obtain a protein-free plasma fraction, 10 ml of human plasma were placed in a Centricon-10 concentrator with a nominal molecular weight cut-off membrane of 10 kDa (Amicon, Beverly, MA) and ultrafiltration was induced by centrifugation at 2500 rpm at 4°C. Centrifugation was stopped when 5 ml of plasma ultra filtrate were obtained. The 5 ml sample of plasma containing proteins and the 5 ml sample of protein-free ultrafiltrate each was incubated with 5 ml of NO-BSA for 3h at 37°C. The two samples were finally added to contracted vessels.

Incubation of NO-BSA with renin and other proteases. Five milliliters of NO-BSA (60 mg/ml) were supplemented with 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4$, 137 mM NaCl, 0.8 mM Na_2HPO_4, pH 7.4 and 5.6 mM glucose and mixed with 2 units of a crude preparation of hog kidney renin (Sigma, St. Louis, MO) dissolved in 0.5 ml of 0.9 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4 (PBSC/M); control NO-BSA was mixed with 0.5 ml of PBSC/M. The mixtures were incubated for 3 h at 37°C and added to contracted rings. UV-visible spectrophotometric analysis of the samples was done before and after the three hours of incubation at 37°C.

Five milliliters of 60 mg/ml of NO-BSA were incubated with 2 units of renin. Inactivation of renin was done by boiling for 5 min. 5 ml of 60 mg/ml of NO-BSA were also incubated with cathepsin B (12 units), cathepsin D (100 units) and thrombin (65 NIH units), all from Sigma (St. Louis, MO).

Incubation of NO-BSA with renin and renin inhibitor. Fifteen milliliters of NO-BSA (60 mg/ml) were supplemented with 1.3 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4$, 137 mM NaCl, 0.8 mM $Na_2 HPO_4$, pH 7.4 and 5.6 mM glucose. This sample was divided in three 5 ml samples, one of which was mixed with 0.5 ml PBSC/M alone, another with 0.5 ml PBSC/M containing 2 units of hog kidney renin and the third sample with 0.5 ml PBSC/M containing 2 units of hog kidney renin and 10 mg of the renin inhibitor (18) Nα-CBZ-Arg-Arg-Pro-Phe-His-Sta-Ile-His-N$_\epsilon$-BOC-Lys (Sigma, St. Louis, MO). UV-visible spectrophotometric analysis of the three samples was done before and after 3h of incubation at 37°C.

Immunodepletion of renin. Hybridoma F32 VIIIC4 [anti-hog kidney renin (19)] was purchased from ATCC and hybridoma 1C-1, which produces an IgG_1 that binds cocaine, has been described (20). Both hybridomas were grown as described (20). 2 L of hybridoma supernatant were harvested, concentrated with a Centricon-10 (Amicon, Beverly, MA) and the concentration of IgG measured. 50 μg of anti-hog renin antibody (10-fold molar excess of IgG over renin) and 50 μg of 1C1 antibody were bound to 0.75 ml of Protein G Plus-agarose (Oncogene Research Products, Cambridge, MA) by incubation at 4°C for 30 min on a shaker. After pelleting by centrifugation, the agarose beads were washed three times with PBSC/M. Afterwards, the beads with IgG were incubated with 2 units of renin in 0.5 ml of PBSC/M for 1 h at room temperature. After pelleting the beads by centrifugation, the supernatants were incubated with NO-BSA as described above. UV-visible spectroscopy of the samples was done before and after 3h of incubation at 37°C.

Statistical analysis. All data are reported as mean ± SEM and were analyzed by unpaired t test. Differences were termed significant if the t value exceeded the critical value for the 5% level.

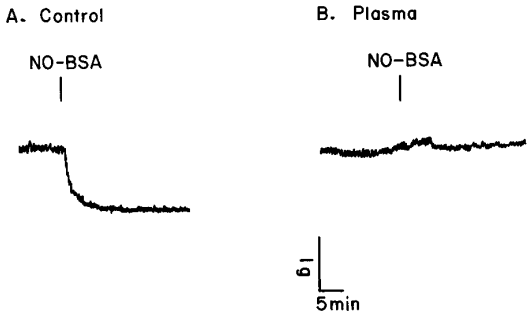

FIG. 1. Vasorelaxation of constricted aortic rings induced by NO-BSA (A) and by NO-BSA incubated with plasma (B).

RESULTS

NO-BSA vasorelaxation is inhibited by a plasma macromolecule. As previously shown (14), NO-BSA relaxed contracted aortic rings (Fig. 1A) 10 μM of NO-BSA induced a 77 ± 10% decrease in tension (n = 4). As predicted from the previous observation that the vasodilatory action of NO-BSA has a half life of 40 min in plasma (14) and as illustrated in Fig. 1B, incubation of the NO-BSA with plasma for 3 h at 37°C completely inhibited the effect of NO-BSA (0% decrease; n = 4; $p < 0.01$). To determine whether the loss of NO-BSA vasorelaxation induced by plasma was due to a macromolecule, we fractionated plasma by ultrafiltration. The plasma ultrafiltrate and the plasma fraction containing macromolecules were incubated with NO-BSA for 3 h at 37°C. Whereas the ultrafiltrate of plasma had no effect on the vasorelaxation induced by NO-BSA (86 ± 3% decrease in tension; n = 3) the plasma fraction containing macromolecules markedly inhibited the NO-BSA effect (17 ± 4% decrease in tension; n = 3; $p < 0.01$). This suggests that a plasma macromolecule(s), likely a protein, is responsible for the rapid inactivation of NO-BSA in plasma.

NO-BSA vasorelaxation is inhibited by renin. Renin had a marked inhibitory effect on the vasorelaxation induced by NO-BSA (Fig. 2). In this group of experiments in which NO-BSA was incubated at 37°C for 3 h, NO-BSA decreased tension by 53 ± 7% (n = 4) but when NO-BSA was incubated in the presence of renin, it only decreased tension by 16 ± 5% (n = 5; $p < 0.01$). In contrast, renin had no effect on the vasorelaxing effect of 10 μM sodium nitroprusside (not shown; Abbott Labs, North Chicago, IL).

Spectroscopic analysis of NO-BSA incubated with renin. To test whether the inhibition of NO-BSA vasorelaxing action by renin was due to loss of NO from the protein, we examined the effect of renin on the UV-visible spectroscopic signal of NO-BSA. Absorption maxima of NO-BSA are detected at 335 and 545 nm (14, 21, 22) with the former wavelength giving, by far,

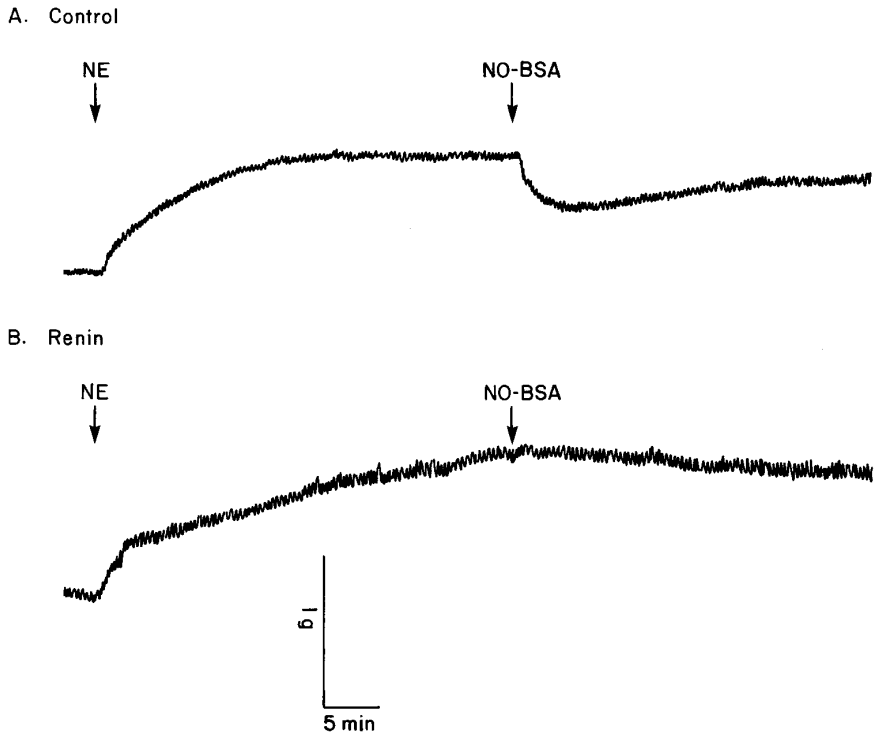

FIG. 2. Vasorelaxation induced by NO-BSA (A) and by NO-BSA incubated with renin (B). PE, phenylephrine.

the greatest signal (14). Further, polynitrosylated proteins (in which NO, in addition of being attached to protein sulfhydryl groups, is also attached to other nucleophilic side chains) are most easily detected at ~340 nm (21). Thus, to examine the effect of renin on the spectra of NO-BSA, we used the signal at 335 nm as an index of protein nitrosation. As shown in Fig. 3, at 335 nm NO-BSA gave an easily detectable signal above that of BSA and the signal decreased by about 50% after 24 h of incubation at 22°C, concomitant with the half life of ~24 h of NO-BSA in these conditions (14).

As illustrated in Fig. 4A, NO-BSA incubation at 37°C for 3 h resulted in a modest but significant decrease in signal at 335 nm (13 ± 2%; $n = 6$). Of interest, however, is the finding that when NO-BSA was incubated under identical conditions in the presence of renin (see Fig. 4B) the decrease in signal at 335 nm was (40 ± 8%; $n = 4$; $p < 0.02$). When NO-BSA was incubated with a renin preparation which had been inactivated by boiling, there was no significant effect on the 335 nm signal (not shown).

The hog kidney renin used for these studies is a crude preparation of the enzyme. To further establish that the effect of the renin preparation on NO-BSA was indeed

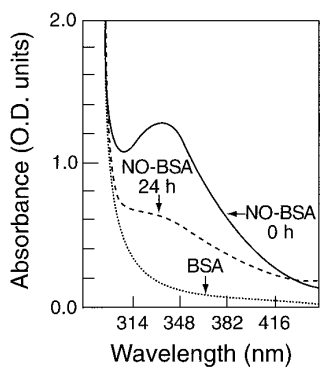

FIG. 3. UV-visible absorption spectra of BSA, NO-BSA immediately after synthesis (0 min) and of NO-BSA after 24 h at room temperature.

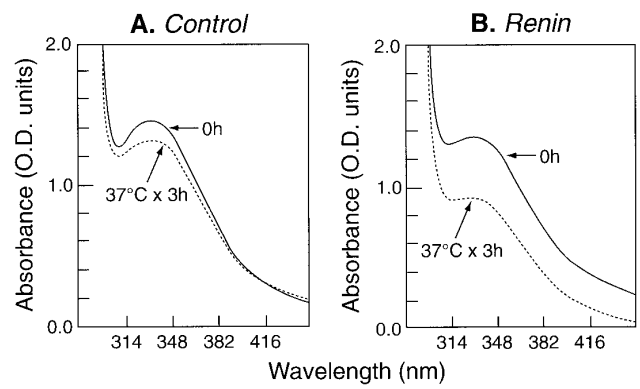

FIG. 4. UV-visible absorption spectra of NO-BSA incubated at 37°C for 3 h in the absence (B) or presence (B) of renin.

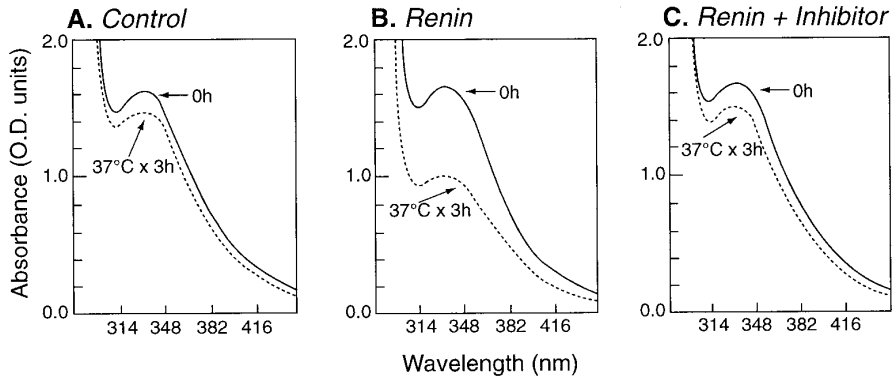

FIG. 5. UV-visible absorption spectra of NO-BSA incubated at 37°C for 3 h in the absence (A) or presence (B) of renin and in the presence of renin plus renin inhibitor (C).

due to renin, two experiments were performed. First, we examined whether an inhibitor of renin modified the effect of this enzyme on the 335 nm signal of NO-BSA. The renin inhibitor Nα-CBZ-Arg-Arg-Pro-Phe-His-Sta-Ile-His-N$_\epsilon$-BOC-Lys is an analogue of the amino acid sequence at the cleavage site of angiotensinogen (18) and, as shown in Fig. 5, it inhibited the ability of renin to remove NO from BSA. In this experiment, after 3h of incubation at 37°C, the 335 nm signal of NO-BSA decreased by 12% in the absence of renin (Fig. 5A), by 49% in the presence of renin (Fig. 5B) and by 12% when renin was present together with its inhibitor (Fig. 5C). Second, we examined whether the effect of the renin preparation on the 335 nm signal of NO-BSA could be inhibited by immunodepleting renin from the renin preparation. As detailed in Methods, a monoclonal antibody to hog kidney renin and a monoclonal antibody to cocaine were coupled to agarose beads linked to protein G. The beads were then incubated with the renin preparation and after pelleting the agarose, both supernatants were incubated with NO-BSA. Although the presence of the antibody supernatant resulted in a lower 335 nm absorption signal of NO-BSA, as shown in Fig. 6, the renin preparation incubated with the irrelevant antibody decreased this signal by 61%, whereas the preparation depleted of renin by the anti renin antibody decreased the signal by only 13%. Thus, the decrease in absorbance of NO-BSA at 335 nm caused by the renin preparation was prevented by an inhibitor of renin and by immunoprecipitating the renin with an anti renin monoclonal antibody, strongly suggesting that the effect of the renin preparation was indeed due to this enzyme.

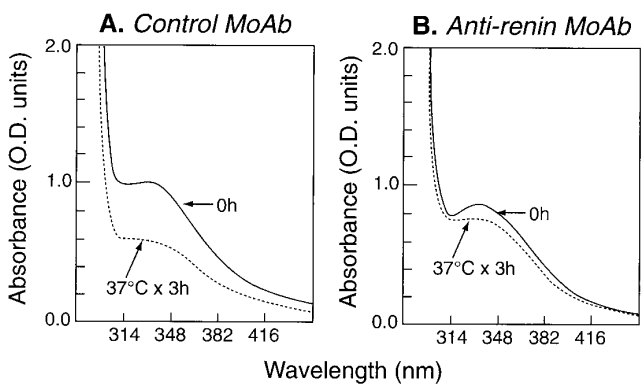

FIG. 6. UV-visible absorption spectra of NO-BSA incubated with a renin preparation previously immunodepleted with an antibody to hog renal renin (A) or a control antibody (B).

Spectroscopic analysis of NO-BSA incubated with other proteases. The 335-nm absorbance of NO-BSA as not significantly affected by cathepsin B_1 (12% decline after 3 h of incubation), cathepsin D (8% decline) and thrombin (10% decline) relative to control (13% decline).

DISCUSSION

In physiological solutions, the half-life of the vasorelaxing activity of NO-BSA is ~24 h (20) whereas it is only ~40 min in plasma (14). In agreement with these observations, we found complete inhibition of the vasorelaxing action for NO-BSA when incubated with plasma for 3 h at 37°C. We also found that the ability of plasma to inactivate NO-BSA was restricted to the plasma fraction containing macromolecules, suggesting that a protein may release NO from albumin. On theoretical grounds (16), plasma proteins with the capacity for acyl hydrolysis or transfer were potential candidates in accelerating the cleavage of NO from albumin. Thus, we originally examined transglutaminases but in preliminary experiments found that transglutaminase I had no effect on the vasorelaxing action of NO-BSA. Proteases comprise a larger class of candidates. Of these, renin was deemed an attractive candidate on biological grounds, as regulation of vasoactive mechanisms shows a high degree of interaction, and on mechanistic grounds, as renin plays a central role

in blood pressure control. We found that a crude preparation of hog kidney renin markedly inhibited the vasorelaxing effect of NO-BSA. Evidence that this was likely due to an accelerated loss of NO from BSA was obtained by spectrophotometric analysis of NO-BSA as renin significantly decreased its characteristic 335 nm absorbance.

Because the renin used in this study was a crude preparation of the enzyme, we demonstrated the specificity of the effect by two methods. As shown in Fig. 5, an inhibitor of renin prevented the decrease in the 335 nm signal of NO-BSA caused by renin and, as shown in Fig. 6, the renin preparation lost its ability to significantly affect the 335 nm signal of NO-BSA when immunodepleted with an antibody to renin. All these data suggest that renin accelerates the rate of loss of the NO attached to albumin, thereby inhibiting the vasorelaxing action of NO-BSA.

A comparison of the effect of plasma to that of renin in inhibiting the vasorelaxing action of NO-BSA makes clear that the former has a greater inhibitory action than the latter. This raises the possibility that other macromolecules may be involved in the uncoupling of NO and albumin. In this regard, it is of interest that although nitrosation occurs preferentially at reduced thiol groups, it may also occur at other nucleophilic centers (21, 22) and while BSA has 0.4 ± 0.1 mol of SH/mol of protein (22), estimation of the degree of nitrosation of our NO-BSA preparation suggests an NO to protein ratio of ~0.86, indicating that other residues, like tryptophan (22), may have also been nitrosated.[2] It is thus possible that NO coupled to different nucleophilic centers may be targets for different enzymes.

The finding that renin is able to accelerate the uncoupling of NO from albumin indicates that activation of this enzyme results in two actions (angiotensin generation and inactivation of NO-albumin) important in determining vascular smooth muscle tone with both actions promoting vasoconstriction and increasing blood pressure. A similar example of two mechanisms participating in the same over-all function has been found for hemoglobin where two different active sites, one for O_2 and one for NO, participate in the regulation of oxygen delivery to tissues (23). However, in contrast to hemoglobin, our results with the renin inhibitor suggest that the catalytic site for angiotensinogen in renin is also involved in the removal of NO from albumin.

[2] At 335 nm, the absorbance of our NO-BSA preparation was ~1.5 O.D. units and the absorptivity of NO-BSA is 3869 M^{-1} cm^{-1} (14). Thus, the estimated concentration of NO-BSA would be ~3.88×10^{-4} M, slightly lower (~0.86) than the concentration of BSA in the reaction mixture (4.5×10^{-4} M).

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