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N-Acetylcysteine Inhibits Angiotensin Converting Enzyme *in Vivo*[1]

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ABSTRACT

Nitrate tolerance has been explained by 1) a direct loss of pharmacological effect due to reduced bioconversion and 2) an indirect effect due to activation of the renin/angiotensin system and counter-regulatory vasoconstriction. The sulfhydryl compound N-acetylcysteine (NAC) has been shown to attenuate and partly counteract tolerance to nitrates, and this effect has been attributed to a nitrate/sulfhydryl interaction and increased production of vasoactive intermediates. The effect of NAC on counter-regulatory mechanisms is, however, unknown. This study examined whether NAC modulates the function of the renin/angiotensin system in normal rats and in nitrate-tolerant healthy volunteers. Animal study: Conscious rats received NAC (5 mmol/kg/hr i.v., $n = 8$) or placebo (N-acetylserine, $n = 8$). Two hours of NAC infusion significantly reduced the pressor effect of angiotensin I (ANG I) by 39 ± 14% (mean ± SEM) and reduced angiotensin converting enzyme activity by 31% in plasma (N-acetylserine: 74 ± 9 nmol/min/mg, NAC: 51 ± 7) and 43% in kidney (N-acetylserine: 0.9 ± 0.3, NAC: 0.5 ± 0.1 nmol/min/mg protein) ($P < .05$). Clinical study: Isosorbide dinitrate (5 mg/hr) was infused into six male volunteers for 48 hr. NAC (2 g i.v. followed by 5 mg/kg/hr) was co-infused from 24 to 48 hr. Plasma angiotensin II (ANG II) increased during the first 24 hr of isosorbide dinitrate infusion and decreased from 28 ± 4 to 14 ± 2 ng/l after 2 hr of NAC infusion ($P < .05$). The results suggest that sulfhydryl supplementation modifies the function of the renin/angiotensin system *in vivo*, an effect probably mediated by inhibition of angiotensin converting enzyme activity. Thus, it is possible that sulfhydryl supplementation, in addition to its likely effect on nitrate metabolism, may attenuate nitrate-induced counter-regulatory mechanisms.

Several studies have shown that continuous exposure to organic nitrates results in tolerance development (Thadani *et al.*, 1982; Parker *et al.*, 1983; May *et al.*, 1987; Zimrin *et al.*, 1988). A "direct" loss of pharmacologic activity due to depletion of intracellular sulfhydryl compounds, with diminished nitrate bioconversion to vasoactive intermediates as a consequence, has been one explanation for the development of tolerance (Needleman and Johnson, 1973; Ignarro *et al.*, 1981; Ignarro, 1989). Recently, activation of the sympathetic nervous system and the renin-angiotensin system has also been implicated in the development of nitrate tolerance (Packer *et al.*, 1987; Dupuis *et al.*, 1990; Packer, 1990). This activation may blunt nitrate-induced hemodynamic effects and in an indirect way may be responsible for the diminished nitrate effectiveness during sustained therapy.

In support of the sulfhydryl depletion theory, the sulfhydryl donor NAC has been shown to attenuate and partly counteract tolerance to nitrates in patients with ischemic heart disease (May *et al.*, 1987; Packer *et al.*, 1987; Boesgaard *et al.*, 1992). However, controversy still exists as to whether this effect of NAC is mediated by a replenishment of depleted sulfhydryl stores or by other at present undefined sulfhydryl-dependent mechanisms (Fung *et al.*, 1988; Chong and Fung, 1991). In this respect it may be of interest that sulfhydryl compounds seem to modulate ACE-activity *in vitro* (Cushman and Cheung, 1971; Igic *et al.*, 1972; Ikemoto *et al.*, 1988).

This study examines the *in vivo* role of sulfhydryls in relation to the renin-angiotensin system. We investigated whether sulfhydryl supplementation *per se* influenced ACE activity and the blood pressure response to ANG I and ANG II in conscious rats. In addition, the effects of NAC on nitrate-induced changes in plasma renin and ANG II levels were investigated in healthy human volunteers.

MATERIALS AND METHODS

Animal Experiments

Female Wistar rats (210–250 g) were anesthetized with 1 to 3% halothane and N_2O/O_2 (2:1) for chronic catheterization. One catheter

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ABBREVIATIONS: NAC, N-acetylcysteine; ACE, angiotensin converting enzyme; ANG I, angiotensin I; ANG II, angiotensin II; NAS, N-acetylserine; ISDN, isosorbide dinitrate; MAP, mean arterial pressure; SBP, systolic blood pressure; GSH, glutathione.

(medical-grade Tygon tubing) was implanted with its tip in the ascending aorta through the left carotid artery, and two catheters were placed in the superior vena cava *via* the left jugular vein. The catheters were filled with a solution of glucose (500 g/l) and 500 IU heparin/ml and plugged with a nylon pin. Each catheter was externalized through the neck skin. After catheter implantation rats were housed individually and exposed to a 12/12-hr light-dark cycle with free access to standard rat chow and tap water. Postoperatively, rats were allowed to recover from surgery until they had regained their preoperative weight (6–8 days). Details regarding this experimental procedure with chronically catheterized conscious rats have been described previously (Boesgaard *et al.*, 1991).

Experimental Protocol

Hemodynamic response to ANG I before and after sulfhydryl administration. Conscious, unrestrained chronically catheterized healthy rats ($n = 8$) received the sulfhydryl donor NAC or placebo (NAS) intravenously for 3 hr in equimolar doses of 5 mmol/kg/hr. Each rat received NAC and NAS in random order, separated by a 48-hr interval. On both study days the blood pressure effect of an intravenous bolus injection of ANG I (0.1 μg) was determined twice: immediately before and 2 hr after the start of the infusions. The total volume of each bolus dose was 0.4 ml. Blood pressure during baseline infusion conditions (before ANG I bolus administration) and blood pressure alterations during ANG I challenge were recorded continuously by Trantec® pressure transducers (model 60-800) connected to the left carotid catheter. Tracings were displayed on a Watanabe linear recorder (Watanabe Instruments Corp., Japan).

During the infusion (5 mmol/kg/hr), plasma NAC levels reach a steady-state level of approximately 500 μM (data not shown). To investigate whether such sulfhydryl levels directly influence the biological activity of ANG I, additional hemodynamic measurements were performed in six rats receiving, in random order, ANG I (0.1 μg) and ANG I preincubated with NAC (500 μM for 1 hr).

Hemodynamic response to ANG II before and after sulfhydryl administration. In a similar setup, six rats were treated with NAC (5 mmol/kg/hr). The blood pressure effect of a bolus dose of ANG II (20 ng/kg) was determined before the start of the NAC infusion and repeated after 2 hr of infusion.

Effects of sulfhydryl administration on ACE activity in plasma and renal tissue. In another series of experiments, rats were prepared and divided into two groups (NAC treatment, $n = 7$; NAS treatment, $n = 6$). After 2 hr of infusions, arterial blood was sampled and the kidneys removed under halothane and N_2O/O_2 anesthesia for determination of ACE activity in plasma and renal tissue.

Materials. ANG I, ANG II, NAC and NAS were purchased from Sigma Chemical Co., St Louis, MO. Solutions were adjusted to pH 7.4 and prepared in 0.9% NaCl. NAS is identical to NAC except that it contains a hydroxy group in place of a sulfhydryl group and any differences in response to NAC and NAS are most likely due to this difference. ANG I (cat. no. A 9650) and ANG II were diluted in 5% glucose. Fresh solutions were prepared daily.

Clinical Study

Effects of sulfhydryl administration on plasma renin and angiotensin II levels. The study group comprised six nonmedicated healthy male volunteers ranging in age from 18 to 35 years (mean 24 years). Electrocardiograms, routine hematology and physical examinations were normal in all subjects. Informed consent was obtained from all participants and the study was approved by the Scientific Ethical Committee of Copenhagen.

Study protocol. Subjects attended the clinic at 8:00 A.M. and a cannula was inserted into a forearm vein for subsequent drug administration. After a supine rest for 1 hr, each subject received an intravenous infusion of ISDN for 48 hr. ISDN (5 mg/hr) was infused *via* polyethylene tubes in a solution of 0.9% saline (20 ml/hr). On day 2, from 24 hr and during the period from 24 to 48 hr, the ISDN infusion was supplemented by an infusion of NAC [2 g in 100 ml of 5% glucose over 15 min followed by 5 mg/kg/hr (10–12 ml/hr) for 24 hr]. [ASTRA AB, Sweden (NAC, Mucomyst) and Ercopharm A/S, Denmark (ISDN, Cardopax), generously supplied the drugs used.] Both infusions were stopped at 48 hr. Baseline values of study parameters were determined immediately before start of infusion (pretreatment baseline, 0 hr) and repeated 24 hr after termination of infusions (post-treatment baseline, 72 hr).

Blood samples were collected from the contralateral arm, after at least 1 hr of supine rest, providing conditions suitable for baseline plasma hormone determinations. For determination of renin and angiotensin II levels, blood was sampled immediately before start of infusion (0 hr, pretreatment baseline) and after 2, 24 (before NAC), 26, 48 and 72 (post-treatment baseline) hr. Analyses of hormone levels were blinded and performed by technicians unrelated to the clinical study team.

Supine blood pressure and heart rate were measured at regular intervals throughout the 72-hr study period.

Analytical Methods

Determination of rat ACE activity. Blood samples were collected directly into ice-chilled heparinized tubes, immediately centrifuged at 4°C for 15 min at 1000 × *g* and stored at −20°C until assay. The kidneys were cleaned from surrounding tissue, immediately frozen in liquid nitrogen and stored at −80°C. ACE activities in plasma and kidney tissue were measured by a spectrophotometric assay of hippurate formed from hippuryl-histidyl-leucin during incubation at 37°C for 30 min (Cushman and Cheung, 1971; Lieberman, 1975). Whole kidneys were homogenized in 9 volumes of phosphate buffer at 4°C and the 1000 × *g* supernatant was used in the assay. After lyophilization the ethyl acetate extract of hippurate was dissolved in 50% ethanol instead of water. Protein content in kidney extracts was determined by the method of Lowry *et al.* (1951). ACE activities are expressed as nanomoles of hippurate formed per minute per milliliter (for plasma) or per milligram of protein (for kidney extract).

Determination of plasma renin and plasma angiotensin II. Plasma was immediately separated and handled as described by Giese *et al.* (1981) and Kappelgaard *et al.* (1976). NAC did not influence the ANG II determinations *in vitro*.

Calculations and Statistical Analyses

MAP was estimated as diastolic pressure + (systolic pressure − diastolic pressure)/3 in millimeters of Hg. Alterations in MAP to ANG I and ANG II are presented as the ANG I-induced and ANG II-induced increase in the area under the blood pressure curve [AUC_{MAP} (0–5 min after bolus injections)].

In the clinical study, pretreatment (0 hr) and posttreatment (72 hr) baseline values were not significantly different. Thus, for all study parameters, pretreatment and post-treatment baseline values for each subject were averaged and compared with those during treatment.

Differences between baseline and treatment means were determined by Student's paired *t* test using Bonferroni's adjustment. Comparisons between treatment groups were performed using Student's *t* test for paired and unpaired observations as appropriate. All data are presented as mean ± SEM. Statistical significance was accepted for $P < .05$.

RESULTS

Animal Experiments

Hemodynamic response to ANG I before and after sulfhydryl administration. NAC infusion significantly ($P < .05$) decreased the area under the ANG I pressor response curve by 39 ± 14% (from 153 ± 20 to 83 ± 13 min × mm Hg, mean ± SEM) (fig. 1). Infusion of NAS (placebo) had no effect ($P > .05$) on the ANG I response (123 ± 20 *vs.* 118 ± 10 min × mm Hg) (fig. 1). Baseline MAP prior to ANG I injections were similar on the two study days (before NAC: 125 ± 3 mm Hg;

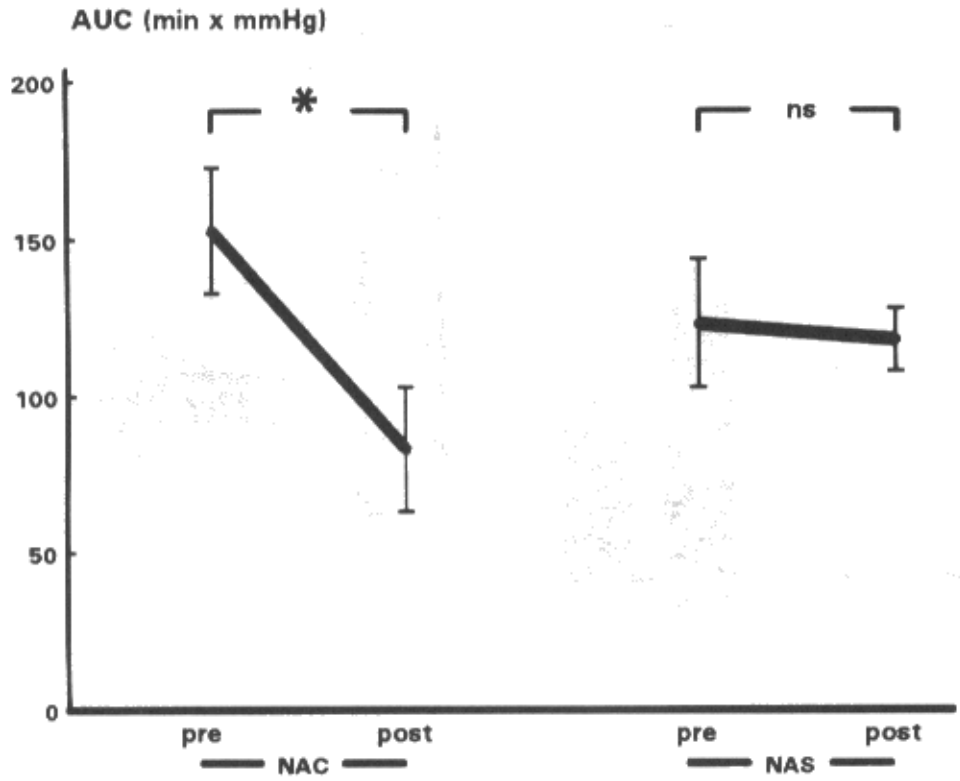

Fig. 1. Blood pressure response (mean ± SEM) to angiotensin I before and after infusion of NAC and NAS, 5 mmol/kg/hr i.v. for 2 hr in conscious rats ($n = 8$). AUC, ANG I-induced increase in the area under the mean arterial pressure curve (0–5 min). *$P < .05$; ns, nonsignificant.

before NAS: 121 ± 3 mm Hg, $P > .05$). Infusion of NAC or NAS did not affect the MAP.

Preincubation of ANG I in 0.5 mM NAC for 1 hr did not change the pressor response to ANG I (ANG I + NAC: 131 ± 12 min × mm Hg; ANG I: 116 ± 12 min × mm Hg, $P > .05$).

Hemodynamic response to ANG II before and after sulfhydryl administration. Infusion of NAC did not affect the ANG II-induced changes in MAP (before NAC: 85 ± 9 min × mm Hg; after NAC 93 ± 7 min × mm Hg, $P > .05$).

Effects of sulfhydryl administration on ACE activity in plasma and renal tissue. Figure 2 shows the effect of NAC infusion on ACE activities in plasma and kidney extracts. The plasma and kidney ACE activities in animals treated with NAC were 31% and 43% lower than in placebo-treated animals (NAS), respectively (plasma: 51 ± 7 *vs.* 74 ± 9 nmol/min/ml; kidney: 0.5 ± 0.1 *vs.* 0.9 ± 0.3 nmol/min/mg protein, $P < .05$).

Clinical Study

Heart rate and blood pressure responses. Pretreatment (0 hr) and post-treatment baseline (72 hr) blood pressure levels were similar (mean difference ± SEM; 3 ± 2 mm Hg, $P > .05$), and infusion-induced changes therefore seem related to treatment.

Infusion of ISDN was associated with a significant decrease in SBP after 2 hr of infusion (table 1). Compatible with the development of tolerance to ISDN, the effect of ISDN was significantly attenuated at 24 hr as compared with its acute effect (table 1). Addition of NAC partially restored the SBP response to ISDN, suggesting some reversal of tolerance on SBP. However, this effect was short lasting and not detectable for more than 60 min despite continued NAC infusion.

There was a trend toward increased heart rate within the first 24 hr, but heart rate and diastolic blood pressure were not significantly altered during the study period (table 1). No rebound hemodynamic changes were observed within the 2-hr follow-up period (48–50 hr).

Plasma renin and ANG II levels. Plasma renin increased in five of six subjects during the first 24 hr of ISDN infusion. A further increase was seen in four of six during the ISDN + NAC infusion. These changes, however, did not reach statistical significance ($P > .05$) (fig. 3).

Plasma ANG II increased significantly during the first 24 hr of ISDN infusion (baseline: 15 ± 2; 24 hr: 28 ± 4 ng/l) (fig. 3). This rise in ANG II levels was counteracted by NAC, which significantly decreased ANG II levels by approximately 50% (from 28 ± 4 to 14 ± 2 ng/l) within 2 hr and for the rest of the infusion period (fig. 3) (Five of six subjects showed a small increase in ANG II between 26 and 48 hr, $P > .05$.)

DISCUSSION

Depletion of sulfhydryl compounds necessary for the metabolism of organic nitrates to vasoactive S-nitrosothiols and/or nitric oxide has been proposed as a "direct" mechanism for development of nitrate tolerance (Needleman and Johnson, 1973; Ignarro *et al.*, 1981; Ignarro, 1989). However, in several studies, a close concordance between activation of neurohormonal systems (especially the renin-angiotensin system) and tolerance development also suggests a role for counter-regulatory vasoconstriction as an "indirect" additional mechanism for tolerance development (Packer *et al.*, 1987; Dupuis *et al.*, 1990; Packer, 1990). NAC has been shown to delay or partly counteract tolerance (Torresi *et al.*, 1985; May *et al.*, 1987; Packer *et al.*, 1987; Boesgaard *et al.*, 1992), but controversy remains as to the exact mechanisms involved. At present, it is believed that the effect of NAC is mediated either by repletion of depleted sulfhydryl groups, and/or by other nitrate/thiol

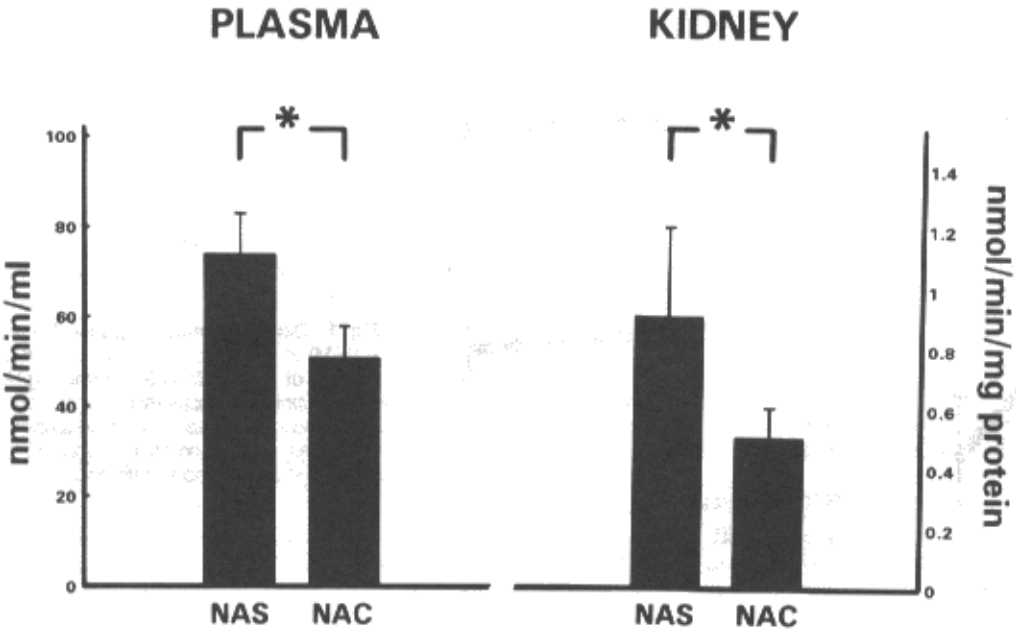

Fig. 2. Rat plasma and kidney ACE activity (mean ± SEM) after treatment with NAC (5 mmol/kg/hr i.v., $n = 7$) or equimolar doses of NAS (placebo, $n = 6$) for 2 hr. *P < .05.

TABLE 1

Systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) (mean ± SEM) at baseline and during infusion with ISDN and ISDN + NAC in six healthy volunteers

			Nac			
			ISDN			
Hours	Baseline[a]	2	24	25	48	50
HR supine (beats/min)	68 ± 5	64 ± 5	75 ± 5	67 ± 6	71 ± 6	67 ± 4
SBP supine (mm Hg)	124 ± 4	110 ± 3[b,c]	120 ± 4	116 ± 3[b,c]	121 ± 6	120 ± 5
DBP supine (mm Hg)	83 ± 2	80 ± 4	80 ± 3	81 ± 4	80 ± 4	79 ± 4

[a] Baseline = mean of pretreatment baseline (0 hr) and post-treatment baseline (72 hr).
[b] $P < 0.05$ compared with baseline.
[c] $P < 0.05$ compared with 24 hr.

interactions, increasing nitrate bioconversion and production of vasoactive intermediates (nitrosothiols and/or nitric oxide).

In this study the pressure response to ANG I in normal rats was decreased by administration of NAC but unaffected by NAS, suggesting a sulfhydryl-mediated effect on the renin-angiotensin system. Preincubation of NAC and ANG I did not alter the ANG I response, and the effect of NAC is therefore not related to a direct interaction with ANG I. Similarly, the pressor effect of ANG II was preserved during NAC infusion, suggesting that the reduced pressor response to ANG I is not due to alterations in the vasoconstrictor effects of ANG II.

Glucocorticoids are known to increase ACE activity, but physiological mechanisms involved in the regulation of ACE activity are not well understood. *In vitro*, using similar assays as in the present study, plasma ACE activity is reduced by 50% (IC_{50}) after incubation with the reduced sulfhydryl compound GSH at concentrations in the range of 10 to 100 μmol/l (Igic *et al.*, 1972). A similar degree of enzyme inhibition is also found in kidney and lung homogenates incubated with low micromolar concentrations of GSH (Igic *et al.*, 1972; Ikemoto *et al.*, 1988). Other reduced sulfhydryl compounds like cysteine and NAC also show weak ACE inhibitory properties *in vitro* with an IC_{50} of around 500 μmol/l (lung) and 1.5 mmol/l (kidney), respectively (Cushman and Cheung, 1971; Ikemoto *et al.*, 1988).

In the present *in vivo* study, rat plasma and kidney ACE activities were significantly decreased after NAC treatment as compared with placebo (NAS). This finding suggests that the reduced blood pressure response to ANG I after NAC treatment is related to a sulfhydryl-induced inhibition of ACE activity. GSH (synthesized from cysteine, glutamate and glycine) constitutes the major cellular sulfhydryl pool and is present in high concentrations (0.5–10 mM) in various tissues (Meister, 1984). NAC provides a supply of cysteine for GSH synthesis (Meister *et al.*, 1986), and NAC, in the same amount as in the present study, significantly raises plasma and vascular cysteine and GSH levels (Boesgaard *et al.*, 1993). When the concentrations of NAC, cysteine and GSH measured in that study are cumulated, plasma sulfhydryls (cysteine, GSH and NAC) are increased by a factor of 55 (to 993 μmol/l) and sulfhydryl levels in aorta are increased by 84% (to 1.4 mmol/l) during infusion of NAC (5 mmol/kg/hr). Thus, sulfhydryl concentrations known to inhibit ACE activity *in vitro* are readily available during the infusion of NAC *in vivo*.

It is possible that ACE activity is inhibited by NAC and

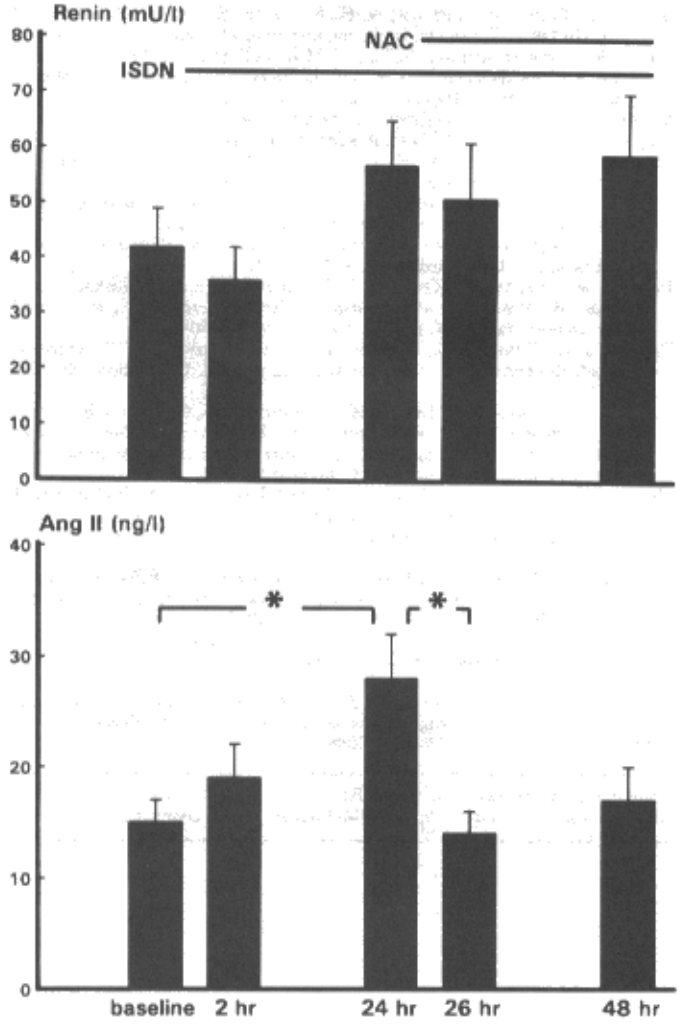

Fig. 3. Changes (mean ± SEM) in plasma renin and plasma angiotensin II levels in six healthy male volunteers during a 48-hr infusion of ISDN. NAC was co-infused from 24 to 48 hr. Baseline = mean ± SEM of pretreatment baseline (0 hr) and post-treatment baseline (72 hr). *P < .05.

other reduced sulfhydryl compounds because these compounds bind a metal cofactor of the enzyme (Cushman and Cheung, 1971). In contrast, exposure to O_2 or oxidation of sulfhydryl compounds (which decreases the number of free —SH groups) in rat and human kidney extracts significantly increases the activity of ACE (Tominaga *et al.*, 1988; Ikemoto *et al.*, 1989). Put together, these observations suggest that ACE activity may depend on the cellular redox status.

GSH protects the cell membrane against oxidative and other types of damage, and GSH export also involves the reduction of compounds located in the immediate environment on both sides of the cell membrane (Meister, 1989). In addition, the cellular redox status depends on levels of cysteine and reduced GSH, and changes in redox state may be associated with alterations in the biological activity and regulation of a variety of enzymes (Gilber, 1989). ACE is located at the cell membrane, and one may speculate that the effect of NAC reflects an increase in cellular reductive capacity and subsequent inhibition of ACE activity in the vascular system.

ACE inhibition interferes with bradykinin metabolism, and some of the physiological effects produced by converting enzyme inhibition may be related to changes in bradykinins and enhanced release of nitric oxide. In general, however, the main effect of ACE inhibition is thought to be mediated through inhibition of the renin-angiotensin system and reduced production of ANG II. This conception has recently been supported by results showing that specific ANG II receptor blockade reduces the pressor response to ANG I (Christen *et al.*, 1991) and produces hemodynamic changes similar to ACE inhibition in conditions with an activated renin angiotensin system (Raya *et al.*, 1991).

Activation of the renin angiotensin system may play a role in the development of nitrate tolerance. The finding of a reduced hypotensive effect of ISDN over 24 hr paralleled by an increase in plasma ANG II levels and a clear upward trend in plasma renin levels is in accordance with this neurohormonal activation hypothesis. Absolute changes were minor, but ANG II levels were significantly decreased after NAC administration, suggesting that, in addition to possible effects on nitrate metabolism, high doses of NAC may exert an inhibitory effect on the renin angiotensin system in the clinical setting.

Despite an effect on ANG II levels during NAC infusion, NAC supplementation, as previously described (Packer *et al.*, 1987), had no prolonged effect on blood pressure. However, measurements of systemic blood pressure do not necessarily correlate with sulfhydryl-induced improvements in antianginal and venodilatatory effects of nitrates (Levy *et al.*, 1991; Boesgaard *et al.*, 1992). Thus, the present results do not rule out the possibility that NAC may have useful effects in relation to nitrate tolerance. In fact, the use of conventional ACE inhibitors has recently been shown to help maintain nitrate-induced venodilatory effects (Katz *et al.*, 1991).

In conclusion, the results suggest that sulfhydryl supplementation modifies the function of the renin angiotensin system *in vivo*, an effect probably mediated by inhibition of ACE activity and reduced conversion of ANG I to ANG II. Thus, it may be that sulfhydryl compounds facilitate the metabolism of nitrates to vasoactive intermediates and, at the same time, attenuate nitrate-induced counter-regulatory mechanisms.

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