Effect of *cis*-Unsaturated Fatty Acids, Prostaglandins, and Free Radicals on Angiotensin-Converting Enzyme Activity *in Vitro* (44106)

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Abstract. **Angiotensin-converting enzyme (ACE) is known to play an important role in the pathobiology of human essential hypertension. Similarly, *cis*-unsaturated fatty acids, prostaglandins, and free radicals are believed to play a role in the control of blood pressure. It was observed that all the *cis*-unsaturated fatty acids tested can inhibit ACE activity to a significant degree. On the other hand, prostaglandins and free radicals, superoxide anion, hydrogen peroxide, and hydroxyl radical did not show significant inhibitory effects on ACE activity *in vitro*. But, the nitric oxide donor sodium nitroprusside showed a potent inhibitory action on ACE activity, suggesting that one of the possible mechanism(s) by which nitric oxide can bring about its anti-hypertensive action might be by modulating ACE activity in addition to its direct vasodilator action. These results indicate that there is a close interaction among ACE activity, *cis*-unsaturated fatty acids, and nitric oxide, which may have relevance to the pathobiology of human essential hypertension.** [P.S.E.B.M. 1997, Vol 214]

Angiotensin-coverting enzyme (ACE, EC 3.4.15.1) is a dipeptidyl carboxypeptidase of 15 kDa, which is widely distributed in many tissues (1, 2). ACE converts the inactive prohormone angiotensin I to the potent hormone angiotensin II. Angiotensin II performs two important functions, one involving the control of blood pressure by constricting arterial vessels; and the other being the control of body fluid volume by increasing renal retention of salt and water and modulating renin-angiotensin-aldosterone system.

ACE is present in many different cell types, such as leukocytes, alveolar macrophages, peripheral monocytes, neuronal cells, renal proximal tubular cells, and epididymal cells (3). The principle location of ACE is in the endothelial cells, and, thus, the major site of angiotensin II production in the body is the blood vessels.

ACE inactivates vasodilatory substances like bradykinin and other peptides. It has been postulated that angiotensin produced *in situ* may interact with other humoral regulators to exert specific action on the growth and metabolism of vascular smooth muscle cells (4). ACE is known to be involved in the pathophysiology of hypertension. Inhibition of ACE activity by drugs such as captopril and enalapril can lower blood pressure. These ACE inhibitors can also inhibit the degradation of bradykinin. It has been shown that oxidation induces an increase in the activity of ACE in the rat kidney (5).

Epidemiological studies suggested that people who subsist on vegetarian diet have lower blood pressure than the general population (6, 7). Vegetarians eat more polyunsaturated fatty acids (PUFAs) and significantly less saturated fat and cholesterol. It has been demonstrated that saturated fats can induce hypertension and that PUFAs have hypotensive action. Mills and Ward (8) have shown that γ-linolenic acid (GLA) can block stress-induced hypertension. Hassal and Kirtland (9) have demonstrated that dietary supplementation of dihomo-GLA (DGLA) enhanced the synthesis of prosta-

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Table I. Details of the Controls and Patients with Hypertension

Group	No. of cases	Mean age ± SD	Sex (M/F)	Mean blood pressure (mm Hg sys/dia)	Duration of hypertension
Normal control	18	42.5 ± 8.0	10/8	110–130/70–90	—
Untreated hypertensives	18	45.8 ± 10.0	11/7	160–190/100–130	1 week to 2 years

glandin E_1 (PGE_1) and reduced hypertension. Increasing GLA intake had no effect on the plasma thromboxane level, but significantly increased the production of the aortic vasodilator PGI_2 in the hamster. Dietary n-6 and n-3 fatty acids have been demonstrated to reduce systemic blood pressure in both animals and humans. Singer *et al.* (10) showed that there is a defect in the desaturation and elongation of both n-6 and n-3 fatty acids in patients with hypertension. In the SHR (spontaneously hypertensive rat) there is an abrupt fall in the plasma levels of arachidonic acid (AA) and eicosapentaenoic acid (EPA) in the phospotidylcholine fraction (11). Administration of large doses of linoleic acid (LA) and α-linolenic acid (ALA) failed to raise the percentage of GLA and EPA, respectively, in serum lipids (10), indicating that humans with hypertension seem to desaturate and elongate the precursor essential fatty acids slowly. In an earlier study, we showed that in patients with essential hypertension the plasma concentrations of unsaturated fatty acids such as AA, which forms the precursor to prostacyclin, and nitric oxide (NO) are decreased (12, 13), but no information is available as to the interaction among unsaturated fatty acids, prostaglandins, and ACE activity. Hence, the effect of various *cis*-unsaturated fatty acids and prostaglandins on ACE activity in PMNLs (polymorpho-nuclear leukocytes) *in vitro* and on purified ACE enzyme was studied, and the results are reported here.

Materials and Methods

Selection of Normal Controls and Patients with Hypertension. Healthy voluntary blood donors whose blood pressure was within normal limits were selected as controls for this study. Blood samples were collected in our blood bank, after blood pressure was checked. These normal volunteers matched for age, sex, and social status compared with the hypertensive group formed the control group (Table I).

Newly detected patients diagnosed to have essential hypertension were also selected for the study (Table I). The diagnosis of hypertension was made by an independent group of physicians and cardiologists not connected with the present study. These patients were subjected to various investigations such as electrocardiogram (ECG), 2-D echo test, stress test, and determination of blood sugar, urea, creatinine, serum lipid profile, and serum sodium and potassium levels. The following tests were also performed to rule out the existence of secondary hypertension in these patients: ultrasound examination of the abdomen for renal sizes, 24-hr urinary albumin and VMA (vanillylmandelic acid), creatinine clearance, and, where necessary, isotope renogram and renal angiogram examinations. Only those patients who were negative for all these investigations and who were determined to have only essential hypertension were included in the study.

Separation of PMNLs. Blood samples were collected from randomly selected normal healthy control persons. Fifteen milliliters of venous blood was drawn and collected in a heparinised tube. PMNLs were separated from the heparinised blood by dextran sedimentation technique as described earlier (14, 15). The viability of the separated PMNLs was checked by Trypan blue dye exclusion method. More than 90% of the cells were found to be viable.

Isolation of PMNL Membrane. PMNLs were suspended in 50 m*M* Tris-buffer (pH 7.4) and lysed by sonication (with five bursts of 5 sec each). The lysed cells were centrifuged at high speed for 1 hr at 4°C. The membranes were washed with Tris-buffer twice and finally suspended in Tris-buffer. This leukocyte lysate was used for estimating the ACE activity, and the values obtained were referred to as leukocyte ACE activity.

Estimation of Angiotensin-Converting Enzyme Activity. The ACE converts the substrate 2-furanacryloyl-*L*-phenylalanyl-glycylglycine (FAPGG) to 2-furanacryloyl-*L*-phenylalanine (FA-phe) and glycylglycine (Gly-Gly), which results in the change in absorbance that can be determined at 345 nm. The ACE activity in serum and in the leukocyte lysate was estimated by the method of Ronca-Testoni (16). The reaction mixture contained 50 μl Tris-buffer (pH 8.2), 0.3 m*M* NaCL, 0.8 μ*M* FAPGG, 50 μl of serum or lysate (leukocyte lysate). The initial reading was taken after incubating the mixture for 3 min at 37°C and the final reading after 20 min of incubation. ACE activity was calculated by the following formula:

$$\text{U/l} + (\text{A/min}) \times V_1 \times 1000/0.5 \times V_2,$$

where V_1 is the assay volume, and V_2 is the sample volume. One unit (1 U) of ACE is the amount of enzyme that converts 1 μmol of FAPGG to FA-phe and Gly-Gly per minute at 37°C. For studies pertaining to

Table II. Serum Angiotensin-Converting Enzyme Levels in Untreated Hypertensives

Control (n = 18)	58 ± 22 U/l
Untreated hypertensives (n = 18)	102 ± 50 U/l[a]

[a] $P < 0.05$, compared with control.

the effect of fatty acids and prostaglandins on purified enzyme, purified ACE was obtained from Sigma Chemical Co. (St. Louis, MO).

Estimation of Protein. The concentration of protein present in the sample was determined by the method of Lowry *et al.* (17).

Source of Chemicals Used. All the chemicals in the study including fatty acids and prostaglandins were obtained from Sigma. Purified ACE was also obtained from Sigma and was used to study the effect of various fatty acids and prostaglandins.

Statistical Methods. The data obtained was analyzed by using Student's t test. All values are expressed as Mean ± SD.

Results

Human Leukocyte Angiotensin-Converting Enzyme Activity in Patients with Essential Hypertension. Angiotensin-converting enzyme activity was estimated in normal serum controls and in patients with essential hypertension, and the results are given Table II. These results showed a significant increase in the activity of serum ACE in patients with untreated hypertension.

Effect of Fatty Acids on ACE Activity *in Vitro*. Studies were performed to look at the possible effect of various c-UFAs on ACE activity in normal human leukocytes (1×10^4) *in vitro*. The concentration of fatty acids used in these studies was 20 μg/ml. The results, given in Figure 1, indicate that all the fatty acids tested can inhibit ACE activity to a significant degree. Of all the fatty acids tested, n-3 fatty acids EPA and DHA were the most effective.

Similar studies were also performed with purified ACE (Sigma), and these results are given in Figure 2. All the n-6 and n-3 fatty acids inhibited ACE activity to a significant extent when tested at 10 μg/ml concentration. ALA, EPA, and DHA of the n-3 series were the most effective in inhibiting ACE activity *in vitro*. A dose-dependent study with arachidonic acid (Fig. 3) showed that AA inhibits ACE activity in a dose-dependent fashion.

Effect of Prostaglandins on ACE Activity. The effect of various prostaglandins (PGE_1, PGE_2, PGI_2, and $PGF_{2\alpha}$) on the activity of purified ACE was studied, and the results are given in Figure 4. These results indicate that prostaglandins when used at a concentration of 10 μg/ml have a small but not a statistically significant action on the activity of the purified enzyme (ACE).

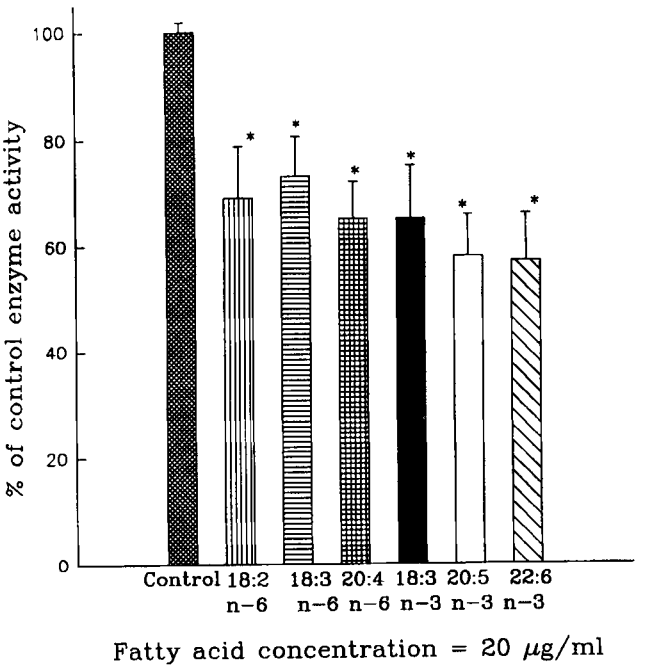

Figure 1. Effect of *cis*-unsaturated fatty acids on leukocyte ACE activity. All values are expressed as mean ± SD of 10 experiments, and each experiment was done in duplicate. The enzyme activity in control is 773 ± 55 U/mg protein was taken as 100%. *$P \leq 0.05$, compared with control.

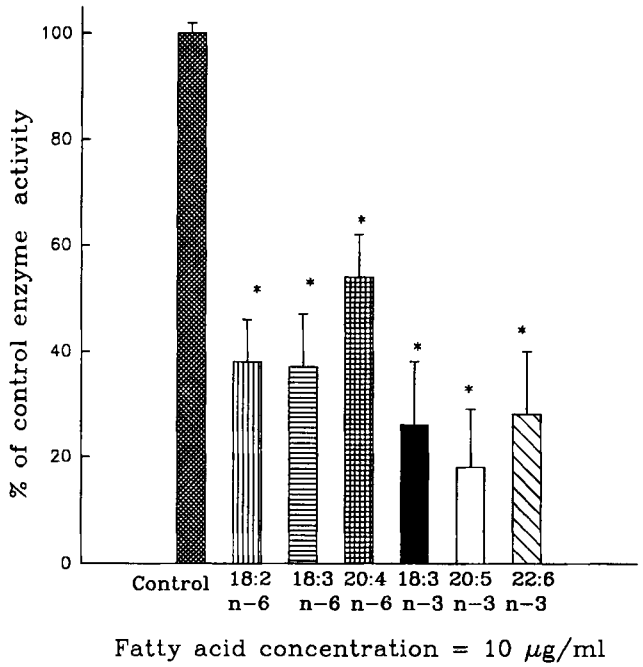

Figure 2. Effect of *cis*-unsaturated fatty acids on ACE (purified) activity. All values are expressed as mean ± SD of 10 experiments, and each experiment was done in duplicate. The enzyme activity in control is 1195 ± 224 U/l was taken as 100%. *$P \leq 0.05$, compared with control.

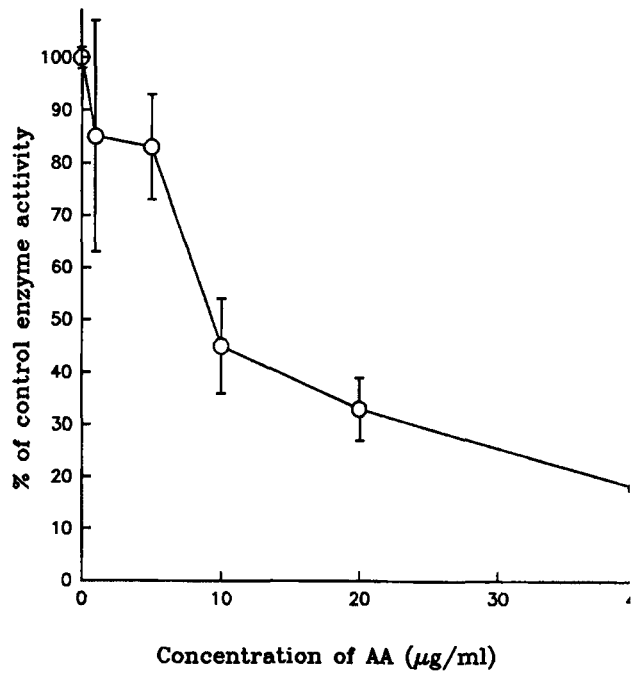

Figure 3. Effect of arachidonic acid (AA) on angiotensin-converting enzyme (purified) activity *in vitro*. Each point represents a mean ± SD of three experiments, and each experiment was done in duplicate. The enzyme activity in control is 1175 ± 224 U/l was taken as 100%.

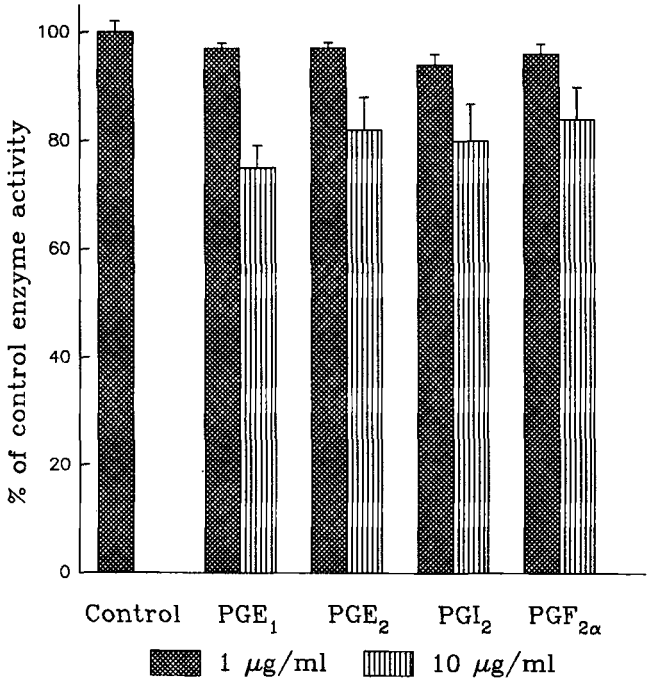

Figure 4. Effect of prostaglandins (PGs) on ACE (purified) activity. All values are expressed as mean ± SD of four experiments, and each experiment was done in duplicate. The enzyme activity in control is 1205 ± 226 U/l was taken as 100%.

Effect of Superoxide Anion, Hydroxyl Radical, and Hydrogen Peroxide on the Activity of ACE. The effect of superoxide anion, generated by xanthine and xanthine oxidase; hydroxyl radical, generated by Fe, *L*-ascorbic acid; and H_2O_2 on purified ACE activity was studied. The results (Fig. 5) revealed that superoxide anion, hydroxyl radical, and hydrogen peroxide show insignificant inhibitory effect on ACE activity *in vitro*.

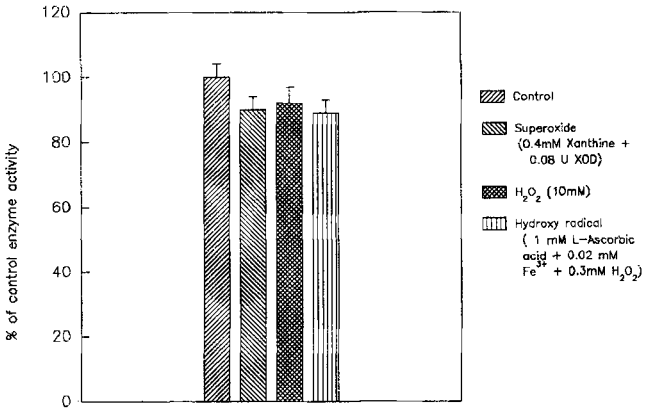

Figure 5. Effect of free radicals on ACE (purified) activity *in vitro*. All values are expressed as mean ± S.D. of four experiments and each experiment was done in duplicate. The enzyme activity in control is 1160 ± 236 U/L was taken as 100%.

Effect of Sodium Nitroprusside on the Activity of Purified ACE. Sodium nitroprusside, a potent vasodilatory compound and a donor of nitric oxide, which is responsible for its vasodilatory action, was tested on purified ACE activity *in vitro*. The concentrations of sodium nitroprusside used were 0.2 and 5 m*M*. These results showed that 5 m*M* concentration of sodium nitroprusside can completely inhibit the activity of ACE (Fig. 6). On the other hand, Sodium nitroprusside at 0.2 m*M* did show a small but not a statistically significant inhibitory action of ACE activity. When compared with the inhibitory action of captopril, a known inhibitor of ACE activity, these results indicate that 5 m*M* of sodium nitroprusside is equivalent to 25 μ*M* of captopril in terms of its inhibitory action on ACE activity.

Discussion

Endothelial cells produce many biologically active substances, including nitric oxide and PGI_2, which are potent vasodilators and platelet anti-aggregators (18, 19). Free radicals, especially superoxide anion, are known to inactivate NO and PGI_2 (20–22). Since free radical generation is high in patients with uncontrolled hypertension (12), both NO and PGI_2 may be readily inactivated, and this may lead to an increase in peripheral vascular resistance and hypertension (13). Thus, free radicals can modulate the tone of vascular smooth muscles directly by acting on the smooth muscle cells and also indirectly by altering the half-life of biologically active vasoactive mediators. Katuse and Vanhoutte (23) suggested that superoxide anion itself could be an endothelial-derived vasoconstrictor. This suggests that increase in free radical generation observed in untreated

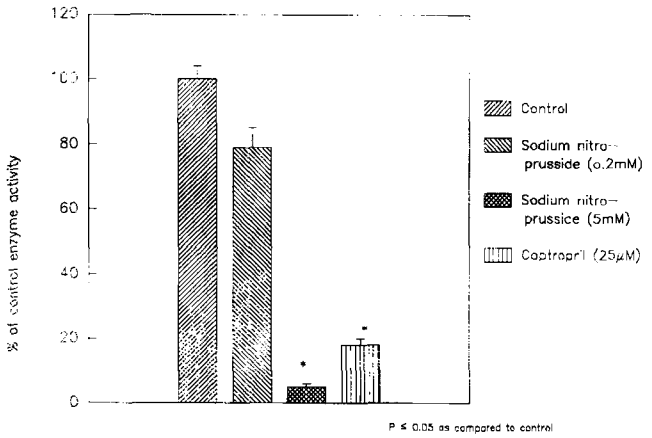

Figure 6. Effect of sodium nitroprusside on ACE (purified) activity *in vitro.* All values are expressed as mean ± S.D. of four experiments and each experiment was done in duplicate. The enzyme activity in control is 1160 ± 236 U/L was taken as 100%.

hypertensives (12) could be one of the factors responsible for the heightened peripheral vascular resistance seen in these patients. Further support for this concept is derived from the studies performed by Nakazono *et al.* (24), who showed that a fusion protein (HB-SOD) consisting of human Cu-Zn type SOD and a C-terminal basic peptide with a high affinity for heparin sulfate on endothelial cells not only can localize to the vascular walls but also can effectively prevent the development of hypertension in the spontaneously hypertensive rats (SHR). This lends direct evidence for the involvement of superoxide anion in the pathogenesis of hypertension in SHR animals and, possibly, in humans.

Since ACE is also known to be involved in the pathophysiology of essential hypertension, and as ACE inhibitors are the drugs of choice in the treatment of essential hypertension, it is interesting to know about the possible interaction between free radicals, including NO, and ACE activity. This is particularly compelling in light of our earlier observation that in patients with essential hypertension the concentrations of NO and those of some antioxidants are low compared with normal controls (13).

The interaction between dietary nutrients and blood pressure is complex yet highly relevant to contemporary attempts to reduce the risk of cardiovascular disease. Both n-3 and n-6 fatty acids can lower blood pressure. Sanders *et al.* (25) were the first to describe a hypotensive effect of cod liver oil in healthy volunteers. Goodfriend and Ball (26) showed that the angiotensin II receptors can be inhibited by unsaturated fatty acids. Dietary supplementation of fish oil lowers blood pressure in animals and humans. Hui *et al.* (27) demonstrated that the antihypertensive action of LA and fish oil is independent of PG synthesis. On the other hand, others have shown that fish oil supplementation increases PGI_3 synthesis (28, 29).

Based on these observations, it can be suggested that there is no clear consensus as to the mechanism(s) by which fatty acids lower blood pressure. It is possible that the blood pressure lowering by polyunsaturated fatty acids may be due to changes in the lipid composition and fluidity of the cell membranes at the receptor sites of vasoactive hormones and/or neural transmitters. Further, the plasma levels of several unsaturated fatty acids were found to be low in untreated hypertensives (12). The mechanism by which these fatty acids lower blood pressure may involve an increase in 8 GI_3 synthesis (29) and/or by enhancing NO generation by the endothelial cells (30).

The results of the present investigation indicate that ACE activity is significantly higher in patients with hypertension than in normal controls. The increase in ACE activity sustains the hypertension *via* an enhanced generation of angiotensin II. This is supported by the fact that in SHR the development of hypertension coincides with an increase in aortic ACE activity (31). This enhanced ACE activity could be due to an overexpression of ACE gene in these animals (32). This may explain the effective antihypertensive action of ACE inhibitors such as captopril and enalapril in almost all hypertensives.

The present observation that both n-3 and n-6 fatty acids, but not prostaglandins, inhibited the activity of ACE to a significant degree suggests that these fatty acids themselves can serve as antihypertensive agents. It may be added here that plasma concentrations of n-3 and n-6 fatty acids vary anywhere between 26 and 723 μg/ml in normal individuals, as reported by Fujioka *et al.* and others (33–35). Hence, the interaction between n-3 and n-6 fatty acids and ACE activity as studied here has clinical significance.

In addition, it was also noted in the present study that free radicals do not have any significant action on ACE activity. These results indicate that the increase in the generation of free radicals observed in patients with hypertension may not be responsible for the increase in blood pressure due to their action on ACE activity but may be due to some other property such as inactivating NO and/or PGI_2. This is supported by the observation that sodium nitroprusside, which can donate NO, is a powerful inhibitor of ACE activity. Similar results were reported by Park and Means (36). It is possible that this inhibitory action of nitroprusside on ACE activity could be due to NO generated by it. Hence, it can be suggested that one of the mechanisms by which sodium nitroprusside/NO act as antihypertensive agents is by blocking ACE activity. Thus, NO may have a more significant role in the pathobiology of human essential hypertension in comparison to other free radicals such as superoxide and hydroxyl radicals since it is sodium nitroprusside/NO that showed inhibitory action on ACE activity. Though the amount of sodium

nitroprusside used in the present study at which inhibitory action on ACE activity was observed is high (5 m*M*), it is likely that even at lower concentrations this inhibitory action may be present, since it is seen from the results shown in Figure 5B that sodium nitroprusside, when used at 0.2 m*M*, can inhibit ACE activity. However, this needs to be verified. It is nevertheless clear from the present study that sodium nitroprusside/NO can inhibit the ACE activity, and this may have relevance for the role of NO in understanding and controlling hypertension.

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