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ARE FREE RADICALS INVOLVED IN THE PATHOBIOLOGY OF HUMAN ESSENTIAL HYPERTENSION?

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Possible involvement of reactive oxygen species and nitric oxide in the pathogenesis of human essential hypertension was investigated. It was observed that both superoxide anion and hydrogen peroxide production by polymorphonuclear leukocytes and the plasma levels of lipid peroxides are higher in uncontrolled essential hypertension compared with normal controls. Nitric oxide levels measured as its stable metabolite nitrite, as an index of nitric oxide synthesis, revealed its levels to be low in hypertensive patients. Superoxide anion, hydrogen peroxide, lipid peroxides and nitric oxide levels reverted to normal values after the control of hypertension by drugs. The concentrations of anti-oxidants such as vitamin E and superoxide dismutase were found to be decreased in patients with uncontrolled hypertension. Several anti-hypertensive drugs inhibited lipid peroxidation *in vitro*. Angiotensin-II, a potent vasoconstrictor, stimulated free radical generation in normal leukocytes which could be blocked by calmodulin antagonists. These results suggest that an increase in free radical generation and a simultaneous decrease in the production of nitric oxide and anti-oxidants such as SOD and vitamin E occurs in essential hypertension. This increase in free radical generation can inactivate prostacyclin and nitric oxide and decrease their half life which can lead to an increase in peripheral vascular resistance and hypertension.

KEY WORDS: Free radicals; hypertension; angiotensin-II; lipid peroxidation; nitric oxide; anti-oxidants; anti-hypertensive drugs

INTRODUCTION

Endothelial damage can be linked to vascular diseases, such as atherosclerosis and hypertension. Endothelial cells produce neurohumoral mediators such as prostacyclin (PGI_2), endothelium derived vascular relaxing factor (EDRF), and endothelin and thus, contribute to the control of local vascular diameter and tone[1]. A number of studies have confirmed that endothelial cells are a prerequisite to evoke relaxation with acetylcholine and other neurohumoral substances[1,2]. These evidences suggest that dysfunction of endothelial cells contribute to inappropriate vasoconstriction and platelet aggregation which are early signs of vascular diseases such as atherosclerosis, hypertension and coronary or cerebral vasospasm or occlusion[1].

In addition, it is now believed that reactive oxygen species play critical roles in the pathogenesis of various diseases, such as collagen vascular diseases, reperfusion injury, septicemia, ARDS (adult respiratory distress syndrome), and cerebrovascular and cardiovascular injury[3,4]. Superoxide radicals can inactivate EDRF (which

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is now identified as nitric oxide, NO), which is a potent vasodilator and platelet anti-aggregator. Hence, it has been suggested that superoxide radicals may affect vascular resistance by inactivating EDRF[5]. To verify this possibility, we measured superoxide and hydrogen peroxide generation in unstimulated and stimulated polymorphonuclear leukocytes of hypertensive patients, lipid peroxides and nitrite, as an index of nitric oxide synthesis, in their plasma and the results are reported here. We also studied the effect of various anti-hypertensive agents on lipid peroxidation process *in vitro* and the levels of vitamin E, superoxide dismutase, catalase and glutathione peroxidase in the RBCs of patients with essential hypertension.

MATERIALS AND METHODS

Selection of Patients

Newly detected patients of essential hypertension were selected for the study. None of these patients were on any drugs at the time of collection of blood samples. Volunteers matched for age, sex and social status formed the control group (table 1). To control hypertension, these patients were given propranolol, metoprolol, nifedipine, verapamil and atenolol either alone or in combination. After the control of hypertension, heparinised blood was collected again for various studies.

Estimation of Free Radicals

Both superoxide anion and hydrogen peroxide were estimated in leukocytes separated from the heparinised blood samples collected from the controls and patients with hypertension by dextran sedimentation technique as described earlier[6,7].

The superoxide anion can reduce nitroblue tetrazolium (NBT) to the insoluble formazan. It is generally accepted as a reliable and simple method of assaying superoxide anion and possibly other free radicals[8]. The ability to reduce NBT was assayed by incubating polymorphonuclear leukocytes (PMNs) with 0.1% NBT with or without phorbolmyristate acetate (PMA) as detailed earlier[9].

The amount of hydrogen peroxide released by PMNs with and without PMA was estimated by horse-radish peroxidase method[10] and as described earlier[11].

For both superoxide anion and hydrogen peroxide assays, the total number of PMN cells taken were 1×10^6 cells/assay.

TABLE I
Details of the hypertensive patients and controls

Group ±S.E	Mean age ±S.E	Sex	Mean Blood pressure		Duration of illness
			Pretreatment	Post treatment	
		M/F	Sys/Dias in mm of Hg	Sys/Dias	
Control	42.5 ± 8.2	10/8	110–30/ 70–90	–	–
Hypertensives	44.8 ± 10.9	14/16	160–190/ 100–130	120–150/ 80–90	One week to 23 years

Estimation of Nitric Oxide as Nitrite in the Plasma

Nitric oxide (NO) is the effector molecule of the L-arginine pathway and it decomposes rapidly in aerated solutions to form stable nitrite/nitrate products. In our study, nitrite concentrations were determined and used an index of NO synthesis. Nitrite was quantified colorimetrically after its reaction with the Griess reagent[12].

Estimation of SOD, Catalase and Vitamin E in RBCs

Preparation of RBC lysate:
Two ml of blood from normal individuals and from patients with essential hypertension was centrifuged for 10 min at 3000 g and the serum was removed by suction. The sedimented erythrocytes were suspended in 1 ml of distilled water and lysed by keeping at 4°C for 2 hrs (volume of lysate: 2ml). To 1.88 ml of this lysate, 0.8 ml of a mixture of chloroform: ethanol (3:1, v/v) and 0.3 ml of water was added to precipitate the hemolysate, which was centrifuged at 3000 g for 10 min. The activities of SOD, catalase and vitamin E was estimated as described earlier[13-15].

Hemoglobin was estimated by the method of Drabkin and Austin[16] in the erythrocyte lysate using 20 ul for 5 ml of Drabkin's solution.

Lipid Peroxidation Products

The total amount of lipid peroxidation products present in the plasma samples of the controls and the hypertensives was estimated using the thiobarbituric acid (TBA) method[17,18] which measures the malondialdehyde (MDA) reaction products and hence is referred to as MDA-eq ((MDA-equivalents) substances. The TBA reaction was done using 0.5 ml of plasma as described earlier[17,18]. 1,1,3,3-tetraethoxy propane (TEP) was used as the standard.

The data obtained from these studies was analysed using students "t"-test.

RESULTS

The results of the NBT-reduction as a measure of superoxide anion generation by PMN cells in normals and patients with hypertension with and without PMA stimulation are given in Fig. 1. It is clear from these results that superoxide is produced in significantly large amounts in uncontrolled hypertensive patients compared with controls both by unstimulated and stimulated PMNs. Similarly, hydrogen peroxide is also produced in increased amounts by PMNs in uncontrolled hypertension (fig. 1).

The results of MDA-eq estimated in the controls and uncontrolled hypertensives are given in table II, which shows that there is increase in the levels of lipid peroxides in the hypertensives. This indicates that there is indeed an increase in free radical generation in uncontrolled hypertension. It was also observed that the levels of free radicals (superoxide and hydrogen peroxide) and lipid peroxides (MDA-eq) returned to control values after the control of hypertension by drugs. (the various drugs used in these patients included: nifedipine, a calcium antagonist, metoprolol, a beta-blocker., enalopril, an ACE inhibitor) (fig. 1 and table II).

In table 3, the levels of SOD, catalase and vitamin E in the RBC of normal controls and in patients with essential hypertension are given. It is evident from these results that there is a significant decrease in SOD and vitamin E levels in the RBC

of uncontrolled hypertensives. The concentrations of both catalase and glutathione peroxidase were also found to be low in uncontrolled hypertensives but did not reach statistically significant levels. Similar to free radicals and lipid peroxides, the concentration of SOD returned to control values after the control of hypertension by anti-hypertensive drugs (table III).

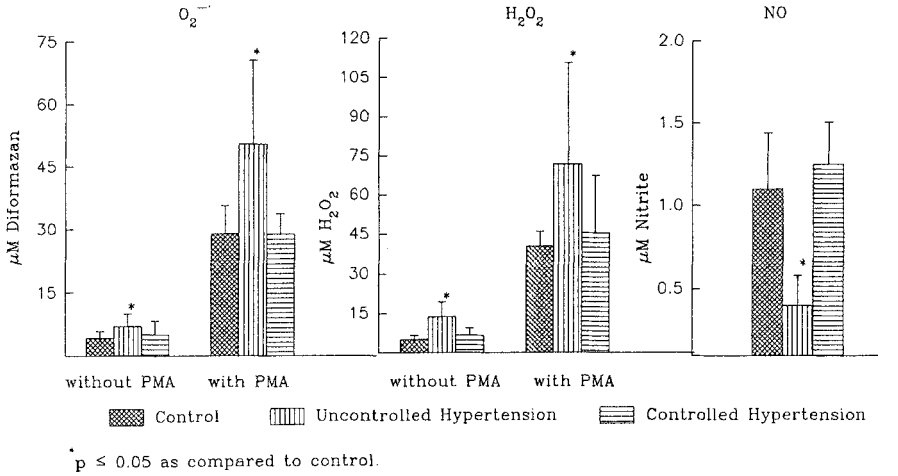

FIGURE 1 Free radical generation by PMNs and levels of Nitric oxide in plasma of normals and patients with hypertension.

TABLE II
Lipid peroxide levels in plasma of normals and hypertensives

	Nanomoles of MDA-eq
1. Control (n = 18)	2.4 ± 0.4
2. Hypertensives (n = 25)	3.64 ± 0.4*
3. Post-treated Hypertensives (n = 18)	2.25 ± 0.7

*p < 0.05 compared to control.

TABLE III
Levels of SOD, catalase, glutathione peroxidase and vitamin E in RBCs in control and hypertensives

	Controls	Hypertensives	Post-treated Hypertensives
1. SOD (U/g of Hb)	1148 ± 360	580 ± 208*	1140 ± 212
2. Catalase (KU/g of Hb)	1146 ± 304	1128 ± 220	1266 ± 300
3. Glutathione peroxidase (U/g of Hb)	17.0 ± 1.4	14.9 ± 1.6	18.7 ± 3.5
4. Vitamin E (ug/g of Hb)	1.54 ± 0.6	0.63 ± 0.27*	ND

*p < 0.05 compared to control (n = 12), ND = Not done.

In order to know whether the return to normal values of free radicals and lipid peroxides in hypertensives is due to the control of hypertension and/or the effect of the drugs used, we studied the effect of propranolol, metoprolol and atenolol, known beta-blockers, and verapamil, a known calcium antagonist, on free radical-induced lipid peroxidation in leukocyte membranes using iron (Fe^{3+}), ascorbic acid and ADP system[19]. The results of this study given in fig. 2 shows that all the drugs tested, propranolol, metoprolol and atenolol, all betablockers., and verapamil, a calcium antagonist, can inhibit lipid peroxidation *in vitro*. Hence, some of the beneficial effect of anti-hypertensive drugs can be attributed to their anti-oxidant action.

In an effort to identify the possible stimuli for the increased free radical generation observed in patients with essential hypertension, we studied the effect of various hormones on free radical generation by human leukocytes *in vitro*. Of all the chemicals tested including adrenaline, nor-adrenaline, histamine, dopamine, angiotensin-I, angiotensin-II, a potent vasoconstrictor, alone could induce free

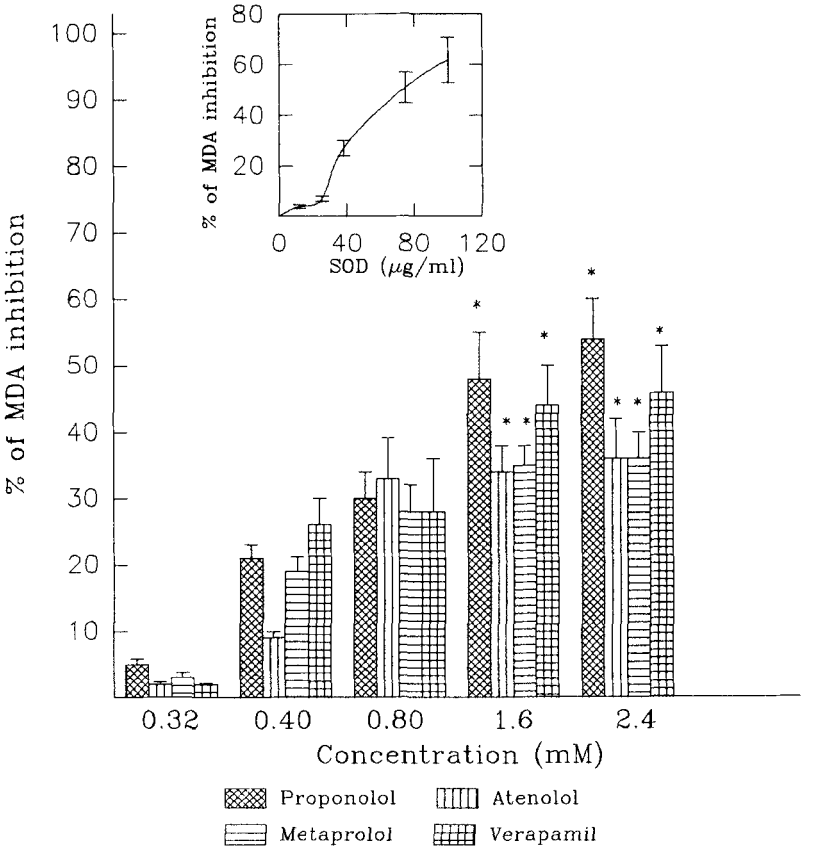

FIGURE 2 Inhibition of lipid peroxidation by anti-hypertensive drugs. Membranes (200 μg) were pre incubated with drugs in 20 mM HEPES, 0.29 M Kcl, pH.7.4 for 1/2 hour at 37°C and the peroxidation reaction was initiated with 1 mM ascorbic acid, ADP/Fe^{3+} (250 μM/50 μM) and 50 μM H_2O_2. MDA-eq. were estimated after 1/2 hour by TBA method. The MDA levels produced in controls were 90 ± 10 nM (n = 4). *$p \leq 0.05$ as compared to control.

radical generation in human leukocytes *in vitro* (fig. 3). Angiotensin-II induced free radical generation was found to be a calmodulin dependent process since it could be inhibited by calmodulin antagonists such as trifluoperazine (TFP) and chlorpromazine (CPZ) (data not shown).

As nitric oxide (NO) is the physiological antagonist of angiotensin-II and is a powerful vasodilator and platelet anti-aggregator, we have also measured its levels (as its stable metabolite nitrite/nitrate) in patients with hypertension. The results of this study given in fig. 1, clearly suggest that the NO levels are low in uncontrolled hypertensives and it returned to normal values after the control of hypertension by drugs.

DISCUSSION

Endothelial cells produce biologically active substances such as EDRF (nitric oxide, NO), EDCF (endothelium-derived vascular contracting factor), and prostacyclin (PGI_2)[20]. These 3 substances can contribute to changes in arterial calibre in response to various stimuli. Since NO and PGI_2 are vasodilators and EDCF is a vasoconstrictor, it can be suggested that any alterations in their production and/or action can lead to changes in peripheral vascular resistance and facilitate the

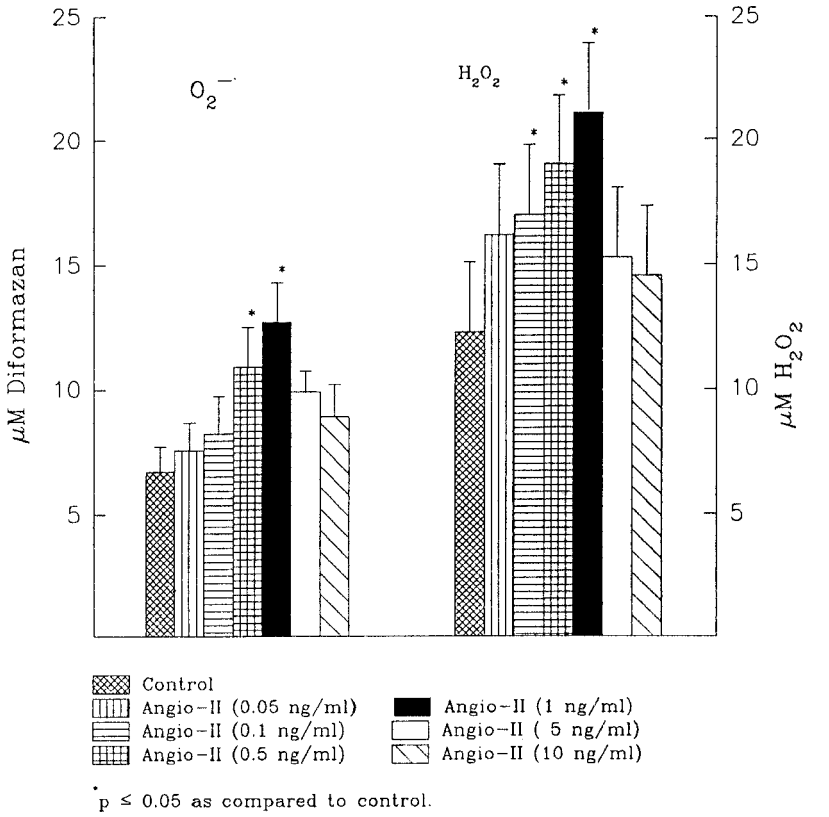

FIGURE 3 Effect of Angiotensin-II on Free radical generation in PMNs. in vitro.

development of hypertension. Free radicals, especially superoxide anion ($O_2^{-\cdot}$) is known to inactivate both NO and PGI_2[1,2,21]. PMNs which can generate free radicals, are in close proximity to endothelial cells. Hence, it is likely that if PMNs generate an excess of free radicals close to the proximity of endothelial cells, inactivation of NO and PGI_2 can occur, and this may lead to hypertension.

The results presented here suggest that in uncontrolled hypertension there is an increase in free radical generation and a simultaneous decrease in anti-oxidants such as SOD and vitamin E; and also of NO (fig. 1 and table III). This can lead to decrease in the half life of NO and PGI_2 and thus, predispose to the development of hypertension. One of stimuli for this increase in free radical generation appears to be angiotensin-II, a physiological antagonist of NO. In this context, the study by Aisaka *et al.*[22] is interesting wherein they reported that N^G-monomethyl-L-arginine (NMMA), an inhibitor of NO synthase increased blood pressure of both normal rats and SHR (spontaneously hypertensive rats). Endothelial cells produce superoxide radicals[23] and are known to oxidize lipoproteins extracellularly. Thus, in situations wherein superoxide radicals are produced in excess, either by endothelial cells and/or PMNs, they may react with NO and PGI_2, inactivate them and lead to increase in peripheral vascular resistance and hypertension. This hypothesis is further supported by the studies of 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 high affinity for heparan sulfate on endothelial cells can localise within vascular walls and decrease the blood pressure of spontaneously hypertensive rats (SHR). Thus, hypertension in SHR and in humans may be suggested as a "free radical-dependent disease". If so, use of SOD and its analogues, and L-arginine to increase NO levels may form new therapeutic strategies in hypertension.

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