Association of increased phagocytic NADPH oxidase-dependent superoxide production with diminished nitric oxide generation in essential hypertension

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Objective **Oxidative stress has been implicated in the pathogenesis of hypertension and its complications through alterations in nitric oxide (NO) metabolism. This study was designed to investigate whether a relationship exists between phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent superoxide anion ($\bullet O_2^-$) production and NO generation in patients with essential hypertension.**

Methods **Superoxide production was assayed by chemiluminescence under baseline and stimulated conditions on mononuclear cells obtained from hypertensives ($n = 51$) and normotensives ($n = 43$). NO production was evaluated by determining serum NO metabolites, nitrate plus nitrite (NOx).**

Results **Although there were no differences in baseline $\bullet O_2^-$ production between normotensives and hypertensives, the $\bullet O_2^-$ production in phorbol myristate acetate (PMA)-stimulated mononuclear cells was increased ($P < 0.05$) in hypertensives compared with normotensives. The PMA-induced $\bullet O_2^-$ production was completely abolished by apocynin, a specific inhibitor of NADPH oxidase. Moreover, stimulation of $\bullet O_2^-$ production by angiotensin II and endothelin-1 was higher ($P < 0.05$) in cells from hypertensives than in cells from normotensives. In addition, diminished ($P < 0.001$) serum NOx was detected in hypertensives compared with normotensives. Interestingly, an inverse correlation ($r = 0.493$, $P < 0.01$) was found between $\bullet O_2^-$ production and NOx in hypertensives.**

Conclusions **Generation of $\bullet O_2^-$ mainly dependent on NADPH oxidase is abnormally enhanced in stimulated mononuclear cells from hypertensives. It is suggested that this alteration could be involved in the diminished NO production observed in these patients.** ***J Hypertens*** **22:2169–2175 © 2004 Lippincott Williams & Wilkins.**

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Introduction

A number of findings support the notion that superoxide anion ($\bullet O_2^-$) plays an important role in the pathophysiology of cardiovascular diseases, including essential hypertension [1]. An enhanced generation of $\bullet O_2^-$ may cause a loss of nitric oxide (NO) production, which leads, among other consequences, to endothelial dysfunction [2]. The nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase system is considered to be the most important source of $\bullet O_2^-$ in vascular cells [3,4]. Several studies have demonstrated the involvement of vascular NAD(P)H oxidase in experimental and clinical hypertension. In rats made hypertensive by chronic angiotensin (Ang) II infusion, vascular NAD(P)H oxidase-dependent $\bullet O_2^-$ production is dramatically increased [5]. On the other hand, the enhanced $\bullet O_2^-$ production present in the aorta of spontaneously hypertensive rats (SHR) and deoxycorticosterone acetate-salt hypertensive rats is associated with increased NAD(P)H oxidase activity [6,7]. Recently, increased NAD(P)H oxidase-dependent $\bullet O_2^-$ production has been described in smooth muscle cells from resistance arteries of hypertensives [8].

NADPH oxidase is also the major inducible source of $\bullet O_2^-$ in phagocytic cells, including lymphocytes, monocytes and neutrophils [9,10]. Although increased $\bullet O_2^-$ production in neutrophils from hypertensives has been found by several authors [11–13], no changes in this parameter were reported by others [14,15]. In addition, the enzymatic source of $\bullet O_2^-$ was not clearly demonstrated in these studies. Besides, other phagocytic cells

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may also be involved in the pathophysiology of hypertension. An association of endothelial dysfunction with subendothelial monocyte/macrophage infiltration has been reported in SHR [16]. In addition, recent data suggest that infiltration of the kidney by lymphocytes and monocytes/macrophages play a role in the development of hypertension in SHR through mechanisms involving enhanced $\bullet O_2^-$ production [17]. Moreover, available clinical data indicate that peripheral monocytes/macrophages and lymphocytes are activated in hypertensives compared with normotensives [18]. Therefore, we have hypothesized that an association may exist between $\bullet O_2^-$ overproduction by monocytes and lymphocytes and diminished NO production in patients with essential hypertension. To test this hypothesis NADPH oxidase-dependent $\bullet O_2^-$ production in peripheral mononuclear cells, monocytes and lymphocytes, as well as the serum concentration of NO metabolites (NOx), an index of systemic NO generation, were evaluated in hypertensives and normotensives.

Methods

The study population consisted of two groups of subjects who attended for routine medical examination at the University Clinic of Navarra. The hypertensive group consisted of 51 patients with repeatedly documented elevated systolic blood pressure (SBP) > 139 mmHg and/or diastolic blood pressure (DBP) > 89 mmHg. Antihypertensive medication was reported by 22 patients (43%) as monotherapy or in combination. The normotensive group consisted of 43 apparently healthy subjects. All patients had appropriate clinical and laboratory evaluations to exclude hypertension secondary to other disorders [19]. Blood samples were collected after an overnight (12–14 h) fast, and plasma glucose, serum total, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, triglyceride and C-reactive protein were determined by standard laboratory protocols. All subjects gave written informed consent to participate in the study, and the Institutional Review Committee of the University Clinic approved the protocol. The study conformed to the principles of the Helsinki Declaration.

Preparation of phagocytic cells

Phagocytic cells were isolated from blood samples obtained by a Ficoll-Hypaque gradient. Briefly, blood samples (15 ml) obtained from each subject in ethylenediaminetetra-acetic acid (EDTA) K_3 tubes were diluted with physiological serum (1 : 1) and the mixture was layered on 15 ml of Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) and then centrifuged for 20 min at room temperature and 1000 *g*. The interface of phagocytic cells was carefully aspirated and washed twice in physiological serum through centrifugation for 10 min at room temperature and 300 g. The pellet was then resuspended in PBS (Gibco BRL, Paisley, Scotland, UK) ready for use. Mononuclear cells were counted in a Neubauer chamber. To obtain reproducible data soft agitation at 37°C of the final cell suspension was necessary to avoid cell aggregation. On the other hand, this agitation was soft enough to avoid endogenous stimulation.

Measurement of $\bullet O_2^-$ production in intact cells

Luminescence assays with lucigenin (10 μmol/l; Sigma, St Louis, Missouri, USA) as the electron acceptor were used to measure $\bullet O_2^-$ production in 4×10^5 mononuclear cells that were incubated at 37°C for 30 min alone or in presence of stimulus or inhibitors. The reaction was started by the addition of lucigenin to cell samples. Luminescence was measured every 11 s for 5 min in a tube luminometer (Berthold Detection System, Sirius, Pforzheim, Germany). A buffer blank was subtracted from each reading. The determinations were performed under baseline conditions and also after maximum stimulation by the protein kinase C (PKC) activator, phorbol myristate acetate (PMA, 2 mg/l, Sigma) at 37°C. A kinetic study of baseline and stimulated mononuclear cells response was carried out and the value of the area under the curve was used to quantify chemiluminescence. Data are expressed as relative light units produced per second. In some experiments, the effect of diphenylene iodonium (DPI, 5 μmol/l; Sigma), a flavoprotein inhibitor, and apocynin (2.5×10^{-3} mol/l; Calbiochem, Darmstadt, Germany), a specific intracellular inhibitor of NADPH oxidase assembly, were studied [20,21]. To verify the specificity of the lucigenin assay for $\bullet O_2^-$ production, the effect of superoxide dismutase (SOD, 10000 U/ml, Sigma), an enzymatic scavenger of $\bullet O_2^-$, was examined.

Although lucigenin concentration was low enough to avoid autoxidation, the measurements were validated against an independent measurement of $\bullet O_2^-$ production using SOD-inhibitable ferrycytochrome *c* reduction. Mononuclear cells (4×10^5) were incubated in 500 μl of buffer containing ferrycytochrome *C* (80 μmol/l; Sigma) at 37°C for 60 min in the presence or absence of SOD (10000 U/ml, Sigma) and finally the absorbance was measured at 550 nm. The PMA-stimulated production measured by lucigenin-enhanced chemiluminescence was closely related with measurements determined in parallel by ferricytochrome *c* reduction.

In some experiments $\bullet O_2^-$ production in mononuclear cells was analysed in response to physiological agonist, such as Ang II (0.1 μmol/l; Sigma) and endothelin (ET)–1 (0.01 μmol/l, Sigma). Furthermore, the upstream signalling pathways involved in NADPH oxidase-mediated $\bullet O_2^-$ production were also investigated with specific inhibitors of PKC (bisindolinmalenide

(BIS) I, 10 μmol/l, Calbiochem), phosphatidylinositol-3-kinase (wortmannin, 1 μmol/l, Sigma), MEK (PD 98059, 50 μmol/l; Calbiochem), p38 MAPK (SB 203580, 1 μmol/l, Sigma) and tyrosine kinase (tyrphostin, 1 μmol/l, Sigma).

Determination of serum NOx

Serum samples collected after an overnight (12–14 h) fast were prepared after centrifugation blood samples. Nitrate and nitrite (NO_2^-/NO_3^-) are stable NO metabolites and their serum concentration reflects systemic NO production [22]. After serum ultrafiltration, by means of centrifugation at 14 000 *g* for 20 min and filters with a cut-off of 30 kDa, NOx concentration was evaluated directly from the supernatant using a colorimetric assay based on Griess reaction (Cayman Chemical Company, Ann Arbor, Michigan, USA). The absorbance of the samples was determined at 540-nm wavelength and compared with those of known concentrations of sodium nitrite.

Statistical analysis

Data are reported as means ± standard error (SEM). Statistical differences between mean values were tested by Student's *t* test for unpaired data once normality was demonstrated (Shapiro–Wilk test); otherwise a Mann–Whitney U test was used. When more than two groups were analysed Kruskal-Wallis followed by a Mann-Whitney U test was employed. Correlation between continuously distributed variables was analysed by calculation of the linear regression and correlation coefficients. The significant level for all tests was taken as $P < 0.05$.

Results

The clinical characteristics of the studied subjects are summarized in Table 1. Hypertensives were older than normotensives. No differences were found between normotensives and hypertensives with regard to gender, frequency of smoking, body mass index and prevalence of diabetes mellitus or hypercholesterolemia. The values of blood pressure were higher in the hypertensives than in the normotensives. As expected, and since the antihypertensive treatment was ineffective in treated patients, no differences were found in blood pressure values between treated and untreated hypertensive patients.

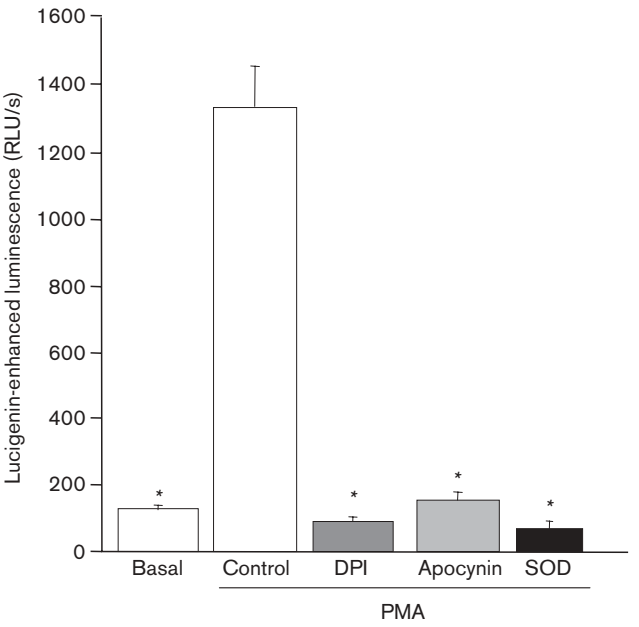
Table 1 Clinical, demographic and biochemical characteristics of normotensive subjects and hypertensive patients

	Normotensives	Hypertensives	*P*
Age (years)	54.5 ± 1.9	61.2 ± 1.3	0.016
Gender (M/F)	29/14	38/13	0.445
BMI (kg/m^2)	28.5 ± 0.4	29.8 ± 0.6	0.213
Smoking (yes/no)	17/26	15/36	0.164
Diabetes mellitus (yes/no)	10/33	15/36	0.180
Hypercholesterolemia (yes/no)	22/21	26/25	0.777
SBP (mmHg)	122.1 ± 2.1	150.1 ± 1.9	0.000
DBP (mmHg)	78.9 ± 1.3	91.5 ± 1.2	0.000
MAP (mmHg)	94.2 ± 1.2	110.5 ± 1.0	0.000
PP (mmHg)	45.1 ± 1.4	59.5 ± 2.0	0.000
C-reactive protein (mg/dl)	0.29 ± 0.03	0.32 ± 0.02	0.635
Glucose (mg/dl)	98 ± 2	103 ± 2	0.075
Total cholesterol (mg/dl)	229 ± 6	234 ± 5	0.680
HDL-cholesterol (mg/dl)	47 ± 2	51 ± 2	0.110
LDL-cholesterol (mg/dl)	158 ± 5	161 ± 4	0.885
Triglyceride (mg/dl)	111 ± 8	112 ± 7	0.775

Values are mean ± SEM or number of subjects. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; HDL, high-density lipoproteins; LDL, low-density lipoproteins.

Preliminary experiments showed that PMA (2 mg/l) achieved the maximum $\bullet O_2^-$ production after 10 min of incubation. Concentration and time of incubation was determined after dose– and time–response curves had been constructed (data not shown). The PMA-stimulated $\bullet O_2^-$ production was inhibited by DPI (5 μmol/l) and apocynin (2.5×10^{-3} mol/l). SOD (10000 U/ml) also abolished the cellular-induced increase in chemiluminescence PMA-stimulated conditions (Fig. 1). No differences with basal levels of chemiluminescence derived from unstimulated cells were observed. Thus, these results suggest that the enzymatic complex of NADPH oxidase may be the enzymatic source of $\bullet O_2^-$ from mononuclear cells in the conditions of the present study.

Fig. 1



Effects of diphenylene iodonium (DPI; 5 μmol/l), apocynin (2.5×10^{-3} mol/l) and superoxide dismutase (SOD; 10 000 U/ml) on phorbol myristate acetate (PMA)-stimulated $\bullet O_2^-$ generation assayed in control conditions. Mononuclear cells were stimulated for 10 min with PMA (2 mg/l) before lucigenin (10 μmol/l) was added. Data are expressed as mean + standard error (SE). RLU/s, relative light units per second. *$P < 0.01$ versus control.

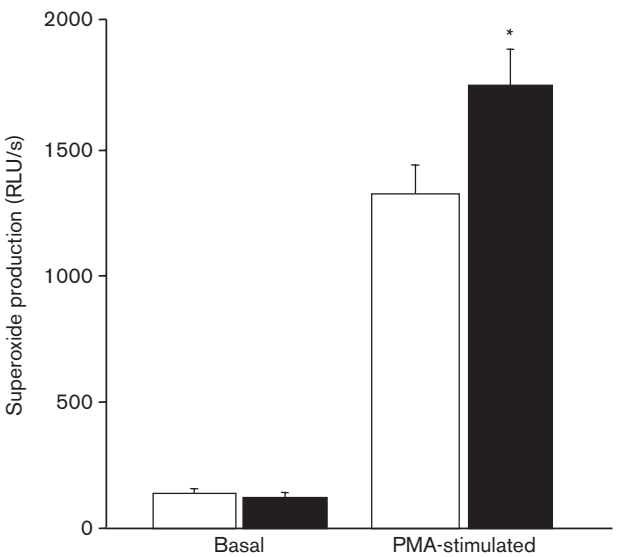
The values of baseline and PMA-stimulated $\bullet O_2^-$ production in mononuclear cells from hypertensives and normotensives are shown in Figure 2. The mean levels of $\bullet O_2^-$ production from non-stimulated mononuclear cells were similar in the two groups of subjects. The addition of PMA stimulated $\bullet O_2^-$ production both in normotensives (134.9 ± 18.9 versus 1320.7 ± 148.1 relative light units (RLU)/s; $P < 0.001$) and hypertensives (131.7 ± 16.5 versus 1748.7 ± 147.4 RLU/s; $P < 0.001$). The magnitude of this stimulation was higher ($P < 0.05$) in cells from hypertensives than in cells from normotensives. Since hypertensives were older than normotensives, and in order to discard an effect of age on PMA-stimulated $\bullet O_2^-$ production, this parameter was evaluated across quartiles of age, in the whole population and in the hypertensive group and no significant tendency was found. On the other hand, there were no differences in the values of PMA-stimulated NADPH oxidase-dependent $\bullet O_2^-$ production between treated and untreated ($n = 22$, 1562 ± 196 RLU/s versus $n = 29$, 1910 ± 215 RLU/s, respectively, $P = 0.261$) hypertensives. As the study was not randomized, nothing about the prophylaxis of the antihypertensive therapy (doses, time of treatment.) was controlled. Interestingly, we possessed information concerning the ability of the antihypertensive agents to interfere with the renin–angiotensin system. No differences were found in PMA-stimulated $\bullet O_2^-$ production depending on the antihypertensive therapy.

Finally, and in order to check if the mononuclear cells from hypertensives showed a higher response than normotensives to physiological agonist, we evaluated the $\bullet O_2^-$ production induced by Ang II (0.1 μmol/l) and ET-1 (0.01 μmol/l). $\bullet O_2^-$ production was significantly higher in hypertensives than in normotensives in response to both Ang II (226.8 ± 7.9 versus 130.1 ± 14.9 RLU/s; $P < 0.05$) and ET-1 (211.4 ± 6.1 versus 133.8 ± 15.1 RLU/s; $P < 0.05$) (Fig. 3).

Furthermore, we investigated the upstream signalling pathways involved in the PMA-stimulated $\bullet O_2^-$ production in a subgroup of individuals. BIS I (10 μmol/l), a protein kinase C (PKC) inhibitor, completely inhibited $\bullet O_2^-$ production. Wortmannin 1 μmol/l, inhibited $\bullet O_2^-$ production by 80% and both, PD 98059 50 μmol/l and SB 203580 1 μmol/l inhibited by 70% the effect measured in the present study. Finally, tyrphostin 1 μmol/l inhibited by 50%. No differences were found in the effect of the tested inhibitors on $\bullet O_2^-$ production between normotensives ($n = 10$) and hypertensives ($n = 10$).

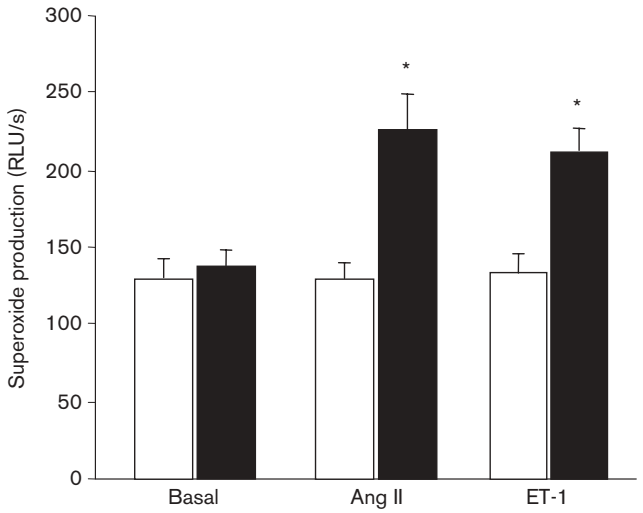
The NOx levels were lower ($P < 0.001$) in hypertensives than in normotensives (Fig. 4). Again, there were no differences in the values of NOx between

Fig. 2



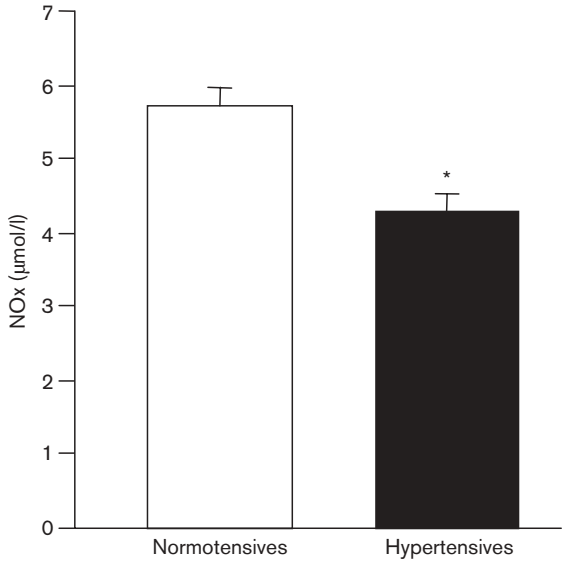
Baseline and phorbol myristate acetate (PMA)-stimulated $\bullet O_2^-$ generation in mononuclear cells obtained from normotensives (open bars) and hypertensives (closed bars). Intact mononuclear cells were incubated for 10 min with PMA (2 mg/l) and the rate of $\bullet O_2^-$ production was evaluated by lucigenin (10 μmol/l) chemiluminescence. Data are expressed as mean + standard error (SE). RLU/s, relative light units per second. *$P < 0.05$ versus normotensive controls.

Fig. 3



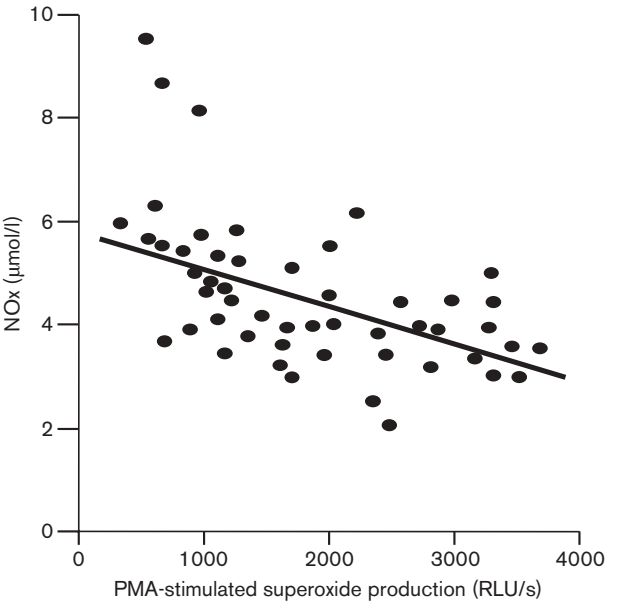
Baseline and angiotensin (Ang) II (0.1 μmol/l) and endothelin (ET)–1 (0.01 μmol/l)-stimulated $\bullet O_2^-$ generation in mononuclear cells obtained from normotensives (open bars, $n = 10$) and hypertensives (closed bars, $n = 10$). The rate of $\bullet O_2^-$ production was evaluated by lucigenin (10 μmol/l) chemiluminescence. Data are expressed as mean + standard error (SE). RLU/s, relative light units per second. *$P < 0.05$ versus normotensive controls.

Fig. 4



Serum levels of nitric oxide metabolites (NOx) in normotensive subjects and hypertensive patients. Data are expressed as mean + standard error (SE). *$P < 0.001$ versus normotensive controls.

Fig. 5



Inverse correlation between phorbol myristate acetate (PMA)-stimulated $\bullet O_2^-$ production and nitric oxide metabolites (NOx) in hypertensive patients; $y = 6.61 - 0.00067x$; $r = 0.493$, $P < 0.01$. RLU/s, relative light units per second.

treated and untreated hypertensives (4.5 ± 0.2 µmol/l versus 4.3 ± 0.2 µmol/l, respectively, $P = 0.261$). An inverse correlation was found between NOx concentration and PMA-stimulated $\bullet O_2^-$ generation in the hypertensive population (Fig. 5).

Discussion

The major finding of this study is that NADPH oxidase-dependent $\bullet O_2^-$ production is abnormally enhanced in mononuclear cells from hypertensive patients. The NADPH oxidase is a multicomponent enzyme that has a membrane portion collectively known as cytochrome b_{558}, that is inactive until it is associated with the cytosolic components (see [9,10] for review). Apocynin is a potent intracellular inhibitor of the assembly of the NADPH oxidase upon stimulation, since it impedes the assembly of the $p47^{phox}$ and $p67^{phox}$ subunits within the membrane NADPH oxidase complex [20,21]. This agent has no known inhibitory effects upon the other potential sources of reactive oxygen species [20]. Thus, our findings showing complete inhibition of PMA-stimulated $\bullet O_2^-$ production observed in the presence of apocynin strongly suggests that the enzymatic complex of NADPH oxidase is the source of $\bullet O_2^-$.

The exaggerated response to PMA of hypertensive cells might be the result of a state of preactivation. In fact, preactivated monocytes exhibit increased adherence to endothelial cells and increased release of cytokines upon stimulation in hypertensives [23–25]. Several potential stimulating factors of the NAD(P)H oxidase enzymatic system deserve to be considered in the setting of arterial hypertension. The role of humoral factors in the upregulation of NAD(P)H oxidase activity has been extensively documented [26]. Ang II- and ET-1-enhanced NAD(P)H oxidase activity has been described in several kinds of cells, including smooth muscle cells [27,28] and monocytes [23,29]. Our findings showing a higher $\bullet O_2^-$ production in response to Ang II and ET-1 in hypertensives than in normotensives support a potential role of these factors in the activation of mononuclear cells on the hypertensive state. In addition, Ungvari *et al.* [30] have recently demonstrated that mechanical stress associated with high pressure induces $\bullet O_2^-$ production in isolated arteries via stimulation of NADPH oxidase. The question as to whether mechanical forces may also play a role on phagocytic enzymatic system merits investigation. Nevertheless, our finding that PMA-stimulated $\bullet O_2^-$ production is increased in those treated hypertensives in whom blood pressure remains raised suggest that mechanical forces may play an important regulatory role in the activation of phagocytic NADPH oxidase. Additionally, previous works support that a genetic background may also contribute to the activation of NAD(P)H oxidase [31–33].

Underlying mechanisms for this increased $\bullet O_2^-$ production are unclear, although data from several studies suggest a potential role for p38 and ERK MAPKs, tyrosin kinases and PKC [34–36]. Data here presented support the involvement of all these pathways in the regulation of NADPH oxidase-mediated $\bullet O_2^-$ production in phagocytes. Moreover, PMA is thought to stimulate NADPH oxidase to produce $\bullet O_2^-$ by activation of PKC. In accordance with this view, increased PMA-stimulated $\bullet O_2^-$ production was blocked by BIS, an inhibitor of PKC. The potential relevance of these data is remarked by a number of findings showing that hemodynamic and humoral factors, including Ang II and ET-1, activate NADPH oxidase through PKC-dependent mechanisms [30,37,38].

A second finding of this study is that the serum nitrate plus nitrite concentration was lower in hypertensives than in normotensives. This finding confirms previous data reported by others in the hypertensive population [39,40]. NO is rapidly oxidized to nitrite and then to nitrate by oxygenated hemoglobin, molecular oxygen and $\bullet O_2^-$, so the major oxidative metabolites are nitrite and nitrate. Although NO is able to react with $\bullet O_2^-$ to form peroxynitrate, this compound may lead to nitration of tyrosine or may isomerase to nitrates [41]. Nevertheless, serum nitrotyrosine levels were undetectable in our population (data not shown). Thus, NOx measurements obtained in this study clearly reflect real changes in NO generation. Impaired expression/activity of endothelial NO synthase (eNOS) could cause the diminished production of NO observed in hypertensives (Reviewed in [42]). In addition, eNOS-mediated NO production may be reduced when the enzyme is deprived of its critical cofactor tetrahydrobiopterin or its substrate L-arginine [43]. This phenomenon has been referred to as eNOS uncoupling. Oxidation of tetrahydrobiopterin by peroxynitrite may also lead to uncoupling of eNOS [44]. Landmesser *et al.* [45] have recently demonstrated that NAD(P)H oxidase is critically important in producing reactive oxygen species that ultimately oxidize tetrahydrobiopterin in blood vessels of hypertensive animals. Our finding of an inverse correlation between NADPH oxidase-dependent $\bullet O_2^-$ production and NO metabolites in hypertensives supports this possibility. In agreement with this, blockage of the β-receptors inhibits the phagocytic NADPH oxidase activity and prevents eNOS uncoupling in experimental hyperlipidemia [46].

The potential role of phagocytic NADPH oxidase in the pathogenesis of hypertension is unclear. Several groups have demonstrated an increased $\bullet O_2^-$ generation in neutrophils from hypertensives [11–13]. It has also been reported that ROS production is increased in immortalized lymphocytes from hypertensives [47]. Augmented infiltration of monocytes and lymphocytes in the vascular wall of SHR, associated with enhanced $\bullet O_2^-$ production that impair, among others, the endothelial function have been demonstrated [16,17]. Thus, the findings here reported showing a higher NADPH oxidase-dependent $\bullet O_2^-$ production in stimulated mononuclear cells from hypertensives suggest that infiltrated mononuclear cells may play a relevant role in the development of oxidative stress in the vascular wall, favouring endothelial dysfunction, among other mechanisms, by an impaired NO production. Further studies are required to demonstrate the molecular evidence linking an increased $\bullet O_2^-$ generation with a diminished NO production.

Some limitations of the present study should be acknowledged. First, half of the patients were under antihypertensive treatment but it was inadequate in terms of blood pressure control and apparently it did not influence $\bullet O_2^-$ or NO production. Second, oral intake of NO_2 and NO_3 was not restricted in the overall population although some authors have indicated that dietary nitrates are eliminated from the blood by urinary excretion after approximately 12 to 16 h, and therefore measurement of NOx in blood collected after an overnight fast could reliably reflect endogenous NO production [48]. Third, since the study was performed in a small population, further studies in larger samples are required to confirm the current results.

In conclusion, this study demonstrates for the first time that the production of $\bullet O_2^-$ by NADPH oxidase is increased in PMA-stimulated mononuclear cells from hypertensive patients. Furthermore, our results show an association between exaggerated phagocytic NADPH oxidase-dependent $\bullet O_2^-$ production and diminished NO generation in these patients. Thus, it is tempting to speculate that phagocytic NADPH oxidase might be involved in diminished NO availability observed in essential hypertension.

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