Changes in iNOS activity, oxidative stress and melatonin levels in hypertensive patients treated with lacidipine

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Objective(s) **To study the changes in macrophage inducible nitric oxide synthase (iNOS) activity, plasma levels of nitrite, lipid peroxidation (LPO) and melatonin in human essential hypertension before and 6 months after 4 mg/day lacidipine treatment.**

Design **The study was carried out in a total of 25 subjects – 11 healthy subjects and 14 hypertensive patients. Blood pressure and peripheral blood samples were taken before and after 6 months of lacidipine treatment (4 mg/day).**

Methods **Systolic (SBP) and diastolic blood pressure (DBP), renal function, lipid and carbohydrate metabolism, renin, aldosterone and catecholamine levels were measured by routine methods. The activity of macrophage iNOS and plasma nitrite, LPO and melatonin levels were also measured.**

Conclusions **Besides reducing blood pressure, lacidipine treatment significantly decreased plasma LPO and macrophage iNOS activity, without changes in NO. Melatonin significantly increases in hypertensive patients, returning to control after lacidipine. Thus, lacidipine reduced blood pressure and free radicals, avoiding the oxidative damage to endothelium. It is suggested that administration of lacidipine plus melatonin may enhance the beneficial effects of each drug in essential hypertension.** ***J Hypertens*** **22:629–635 © 2004 Lippincott Williams & Wilkins.**

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Introduction

The endothelial enzyme nitric oxide synthase (eNOS) is constitutively expressed in endothelial cells, and plays a major role in blood pressure determination [1]. Activation of eNOS leads to nitric oxide (NO) production, which activates guanylate cyclase, causing relaxation of vascular smooth muscle [1]. Besides, NO inhibits platelet aggregation and adherence, leucocyte adherence, vascular smooth muscle cell proliferation and migration, and stimulates endothelial cell growth [2]. The inhibition of eNOS activity by N^G-monomethyl-L-arginine (L-NMMA) is followed by a sustained increase in blood pressure in humans [3]. The impaired NO-dependent vasodilatation returns to normal when blood pressure is normalized with antihypertensive drugs such as calcium-channel blockers [4]. These data suggest that alteration of NO synthesis may be related to the pathogenesis of hypertension [2,5].

Even more controversial than NO is the role that the inducible NO synthase (iNOS) plays in cardiovascular pathophysiology. Besides macrophages, iNOS is found in many other types of cells and tissues, including vascular smooth muscle and endothelium, where it may be regulated by free radicals [6]. In different types of hypertension, the expression of iNOS is upregulated and the iNOS-dependent overproduction of NO may contribute to the pathology associated with hypertension [7,8]. Free radicals may contribute to the pathogenesis of human hypertension [9], since the vascular endothelium can produce free radicals [10]. An overproduction of superoxide anion ($O_2^{\bullet-}$) and a concomitant decrease of superoxide dismutase and vitamin E have been described in human hypertension [9]. In turn, $O_2^{\bullet-}$ inactivates NO, producing the highly toxic peroxynitrite radical ($ONOO^-$). Free radicals are responsible for membrane lipid peroxidation (LPO) and nitrosation/nitrosylation of proteins, impairing endothelial cell function. An imbalance between pro-oxidant production and antioxidant defence may contribute to the high blood pressure and the endothelium impairment in spontaneously hypertensive rats [11]. Endothelium dysfunction may be improved by administration of antioxidants, suggesting that NO inactivation by free

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radicals contributes to abnormal vascular reactivity in hypertension [12].

Melatonin contributes to the mechanisms regulating arterial blood pressure [13,14], and its administration decreases blood pressure in spontaneously hypertensive rats [15,16] and in humans [17]. Melatonin is a scavenger of free radicals, which may contribute to the protection of the vascular endothelium against oxidative stress [18–20]. Besides, melatonin inhibits the expression and activity of iNOS, counteracting hypovolaemic shock during sepsis [21]. These data point to a role of melatonin in cardiovascular homeostasis.

In this study we investigate the changes in macrophage iNOS activity, oxidative stress and melatonin levels in human essential hypertension, before and after 6 months of treatment with lacidipine, a Ca^{2+}-channel antagonist. We also studied whether these changes are related to the blood pressure reduction after lacidipine treatment.

Methods

Chemicals

Aprotinin, leupeptin, pepstatin, phenylmethanesulphonyl fluoride (PMSF), flavin adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), inosine, bovine serum albumin (BSA), EDTA, EGTA, naphthylethylenediamine dihydrochloride, orthophosphoric acid, sulphanilamide, Tris, Hepes, nitrate reductase, dithiothreitol (DTT), H4-biopterin, L-citrulline, L-arginine and Dowex-50 W, were purchased from Sigma-Aldrich (Madrid, Spain). L-[^{3}H]arginine was purchased from Amersham Pharmacia (Madrid, Spain). Calcium chloride was purchased from Merck (Spain).

Patients

The study was carried out at Granada's University Hospital. A total of 25 subjects were included in the study. Information was given and authorization obtained from the patients and from the Hospital's Ethical Committee; the Code of Ethics of the World Medical Association was observed. Hypertensive patients included in the study were diagnosed *de novo* with essential hypertension, having a minor to moderate arterial hypertension (diastolic blood pressure between 90 and 109 mmHg in bipedestation). The following criteria were used to discard patients from the study: severe or secondary hypertension; other concomitant cardiovascular pathologies; renal insufficiency with creatinine above 1.5 mg/dl; hyperkalaemia above 5 mmol/l; proteinuria above 150 mg/24 h; obstructive respiratory diseases; hyperlipoproteinaemias; chronic alcoholism; drug abuse, and any other pathology that may impede the study, including intolerance to calcium antagonists. A complete clinical history, somatometric data and body mass index were noted.

Patients were treated with 4 mg/day oral lacidipine (Menarini Labs, Barcelona, Spain) for 6 months; control group subjects were treated with placebo. All patients in the study discontinued any antihypertensive therapy at least 15 days before lacidipine treatment. Subjects were classified in two groups: (1) a control group, comprising 11 healthy subjects (six women and five men), aged 41.5 ± 8.6 years, age- and weight-matched with patients in the hypertensive group, and (2) a hypertensive group, comprising 14 patients (seven women and seven men), aged 43.1 ± 9.4 years. Arterial blood pressure was always measured in triplicate by the same person, with intervals of 1 min between measurements, according to WHO recommendations.

Before starting the treatment, the patients came to the hospital at 0900 h and, after 10 min of rest in the supine position, blood pressure was measured with a calibrated mercury sphygmomanometer. Then peripheral blood samples were collected from the antecubital vein. This protocol was repeated 6 months after lacidipine treatment in both control and hypertensive groups. Blood samples were divided in two aliquots: one was used for macrophage extraction, and the other was centrifuged at 2500 *g* for 5 min and the plasma obtained was stored at −80°C until biochemical analyses.

Quantification of biochemical parameters

Plasma aliquots were analysed within 24 h by the biopathology laboratory of Granada University's Hospital, by routine methods. Renal function was assessed by measuring the plasma levels of creatinine, blood urea nitrogen (BUN), uric acid, sodium, potassium and chloride, and urinary levels of microalbuminuria. Lipid metabolism was assessed by measuring the plasma levels of total, high-density lipoprotein (HDL)- and low-density lipoprotein (LDL)-cholesterol, triglycerides and apolipoproteins A and B. Carbohydrate metabolism was assessed by measuring plasma glucose and insulin levels. Plasma levels of aldosterone, renin and catecholamines were also determined.

Lipoperoxidation (LPO) assay

An aliquot of plasma (200 µl) was used for assay of oxidation of plasma lipids. Malonaldehyde (MDA) and 4-hydroxyalkenal (4HDA) concentrations provide a reliable index of LPO [22]. The Bioxytech LPO-586 kit (Cayman Chemical, Ann Arbor, Michigan, USA) was used. The kit takes advantage of a chromogenic reagent that reacts with MDA and 4HDA at 45°C, yielding a stable chromophore with maximal absorbance at the 586 nm wavelength. The light wavelength and the low temperature of incubation used for these

measurements eliminate interference and undesirable artefacts.

Nitrite determination

Changes in NO levels were assessed by determination of plasma nitrite concentration [23]. Before nitrite assay, plasma nitrate was converted to nitrite as follows: 10 μl of nitrate reductase (25 mU/10 μl) plus 10 μl of NADPH in 10 mmol/l EDTA were added to 100 μl of plasma and incubated for 30 min at room temperature. Then serum nitrite concentrations were measured by the Griess reaction, by adding 140 μl of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% orthophosphoric acid) to 100 μl samples of unfiltered serum. After incubation for 20 min at room temperature, the absorbance at 550 nm (OD_{550}) was measured with the Bioteck Microplate Autoreader. Nitrite concentration was calculated by comparison with the OD_{550} of a standard solution of sodium nitrite.

Isolation of macrophages

The Dynabeads M-450 CD14 kit (Dynal A.S., Oslo, Norway) was used. These dynabeads are uniform, magnetizable polystyrene beads coated with a primary monoclonal antibody specific for the CD14 membrane antigen, which is predominantly expressed on human monocytes and macrophages. Cells were isolated directly from whole blood. During a short incubation period, the CD14-positive cells bind to Dynabeads M-450 CD14 and, subsequently, the rosetted cells can be isolated and purified using a magnet.

Assay of iNOS activity

Macrophages were sonicated (0.1 g/ml) in ice-cold buffer (25 mmol/l Tris, 0.5 mmol/l DTT, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mmol/l PMSF, pH 7.6) and stored at −20°C for total protein determination [24], or used immediately for NOS activity determination. NOS activity was measured by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline [25]. The final incubation volume was 100 μl and consisted of 10 μl crude homogenate added to prewarmed (37°C) buffer to give (final concentration) 25 mmol/l Tris, 1 mmol/l DTT, 30 μmol/l H4-biopterin, 10 μmol/l FAD, 0.5 mmol/l inosine, 0.5 mg/ml bovine serum albumin, 0.1 mmol/l $CaCl_2$, 10 μmol/l L-arginine, 40 nmol/l L-[^{3}H]arginine, pH 7.6. The reaction was started by the addition of 10 μl NADPH (0.75 mmol/l final) and continued for 30 min at 37°C. Control incubations were done by NADPH omission. The reaction was stopped by adding 400 μl of cold 0.1 mol/l HEPES, 10 mmol/l EGTA, 0.175 mg/ml L-citrulline, pH 5.5. The reaction mixture was decanted onto a 2 ml column packed with Dowex-50 W ion exchange resin (Na^+ form) and eluted with 1.2 ml of water. L-[^{3}H]citrulline was quantified by liquid scintillation spectroscopy. The retention of L-[^{3}H]arginine in this process was greater then 98%. Specific enzymatic activity is referred to as picomoles of L-[^{3}H]citrulline produced per mg protein per min.

Statistics

All values were expressed as the mean ± SD. Statistical analysis was performed using one-way ANOVA and Student's *t*-test. Correlation coefficients were used to correlate the studied variables. A value of $P < 0.05$ was considered statistically significant.

Results

Table 1 shows the systolic (SBP) and diastolic (DBP) blood pressure values before and after lacidipine treatment. Hypertensive patients have significantly higher SBP and DBP than controls ($P < 0.05$). Six months of lacidipine treatment were enough to significantly reduce both systolic and diastolic blood pressures ($P < 0.05$).

The peroxidation index, as assessed by plasma levels of MDA and 4-HDA, is shown in Figure 1b. Hypertensive patients show a significant increase in LPO ($P < 0.001$) compared to control. After lacidipine administration, the levels of LPO returned to control values. Figure 1a shows the changes in plasma melatonin before and after antihypertensive treatment. Melatonin increases significantly in hypertensive patients ($P < 0.001$), and lacidipine treatment decreases it to control values ($P < 0.001$).

Figure 2a shows the changes in iNOS activity in hypertensive patients before and after lacidipine treatment. The activity of iNOS, assessed in isolated macrophages from blood samples, was low in controls, but increased significantly in hypertensive patients ($P < 0.001$). Lacidipine treatment totally counteracted iNOS activity, returning its levels below control values ($P < 0.01$). Levels of nitrite increase significantly in hypertensive patients ($P < 0.001$), and they remained high after treatment of lacidipine (Fig. 2b).

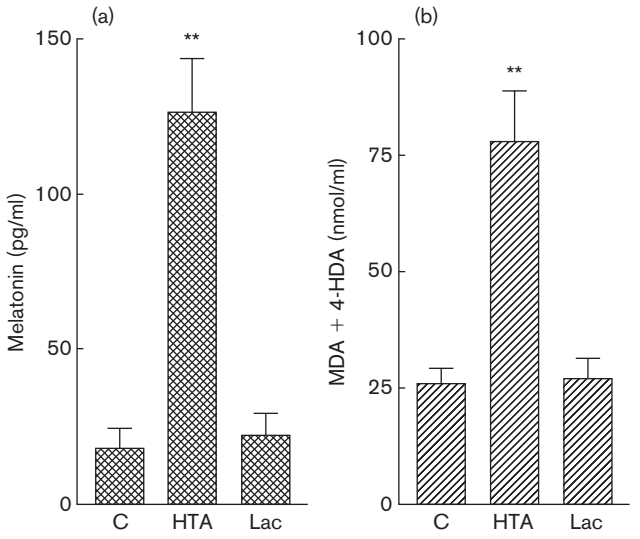
Table 2 shows the results of the correlation analysis. Before lacidipine treatment, a positive correlation was

Table 1 Systolic (SBP) and diastolic (DBP) blood pressure values in the studied groups

Group	SBP (mmHg)	DBP (mm/Hg)
Control	125.8 ± 5.6	82.5 ± 4.3
HTA	152.55 ± 8.4*	97.1 ± 6.7*
HTA + lacidipine	137.1 ± 4.6	86.7 ± 8.2

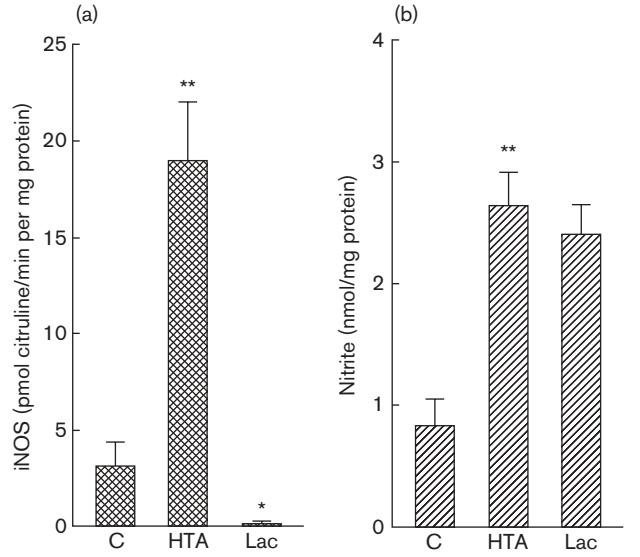
Data are the mean ± SD. Lacidipine was administered at doses of 4 mg/day for 6 months. HTA, hypertensive group; HTA + lacidipine, hypertensive group treated with lacidipine (4 mg/day per 6 months). *$P < 0.05$ versus control and lacidipine.

Fig. 1



Effects of lacidipine on (a) plasma levels of melatonin and (b) lipid peroxidation (LPO) in hypertensive patients before (HTA) and after lacidipine (Lac) treatment, compared with normotensive subjects (control, C). (a) Plasma melatonin, $^{**}P < 0.001$ versus control and lacidipine. (b) LPO index, $^{**}P < 0.001$ versus control and lacidipine. MDA, malonaldehyde; 4HDA, 4-hydroxyalkenal

Fig. 2



Effects of lacidipine on macrophage inducible nitric oxide synthase (iNOS) activity (a) and plasma nitrite levels (b) in hypertensive patients before (HTA) and after lacidipine (Lac) treatment, compared with normotensive subjects (control, C). (a) Macrophage iNOS activity, $^{*}P < 0.01$ versus control; $^{**}P < 0.001$ versus control and lacidipine. (b) Plasma nitrite, $^{**}P < 0.001$ versus control.

found between most of the studied variables. These correlations disappeared after lacidipine administration, coinciding with the normalization of most of these variables.

Table 2 Regression analysis between the studied variables in the hypertensive patients before and after lacidipine treatment

	HTA group		HTA + lacidipine group	
	R	*P*	*R*	*P*
melatonin/NO	+0.5604	$P < 0.05$	+0.0107	NS
melatonin/LPO	+0.8121	$P < 0.01$	−0.0327	NS
melatonin/iNOS	+0.6540	$P < 0.05$	–	
NO/LPO	+0.7771	$P < 0.01$	−0.2663	NS
iNOS/NO	+0.8014	$P < 0.01$		
iNOS/LPO	+0.7121	$P < 0.01$	–	

HTA, hypertensive group; HTA + lacidipine, hypertensive group treated with lacidipine (4 mg/day per 6 months). NO, nitric oxide; LPO, lipid peroxidation; iNOS, inducible nitric oxide synthase.

Plasma cholesterol significantly increased in hypertensive patients compared to control ($P < 0.05$, Table 3). The increase in total plasma cholesterol during hypertension depended on the increase in LDL-cholesterol ($P < 0.05$), since HDL-cholesterol remained unchanged. After lacidipine treatment, both total and LDL-cholesterol returned to control values ($P < 0.05$). Triglycerides and apolipoproteins A and B were also unchanged (data not shown). Plasma electrolytes, urea and urinary excretion of microalbumin were similar in all groups of patients, with no significant differences between them (data not shown). Plasma uric acid was not affected by hypertension, but lacidipine treatment significantly increased it ($P < 0.001$). Plasma creatinine was significantly higher in hypertensive patients than in controls ($P < 0.05$), returning to control values after treatment with lacidipine. The other biochemical parameters measured did not show significant changes between the studied groups (data not shown).

In all cases, we did not find statistical differences between the variables measured in the control group at the beginning of the experimental protocol and 6 months later. Thus, we only showed the data of the control group corresponding to the first determination.

Discussion

Increasing experimental evidence suggests a role for free radicals in the pathogenesis of essential hypertension [9–12]. Free radicals cause extensive cellular

Table 3 Plasma levels of some biochemical variables in the studied groups

Variable (mg/dl)	Control	HTA	HTA + lacidipine
Total cholesterol	185.01 ± 25.3	210.28 ± 29.5*	194.21 ± 21.9
LDL-cholesterol	117.27 ± 32.39	133.17 ± 25.94*	119.93 ± 20.63
HDL-cholesterol	56.82 ± 19.98	53.07 ± 12.03	53.93 ± 12.46
Creatinine	0.89 ± 0.14	0.95 ± 0.13*	0.85 ± 0.14
Uric acid	5.05 ± 1.91	5.08 ± 1.42	8.26 ± 3.49**

Data are the means ± SD. HTA, hypertensive group; HTA + lacidipine, hypertensive group treated with lacidipine (4 mg/day per 6 months); LDL, low-density lipoprotein; HDL, high-density lipoprotein. $^{*}P < 0.05$ versus control and lacidipine; $^{**}P < 0.001$ versus control and HTA.

damage, facilities LPO, and increase intracellular calcium [10], hence increasing peripheral resistance and blood pressure. An increase in $O_2^{\bullet-}$-producing enzymes has been demonstrated in hypertension; this may damage the endothelium and initiate oxidation of LDL [26]. Our results show that lacidipine treatment counteracted hypertension-dependent LPO increase and reduced blood pressure. These findings suggest a relationship between oxidative stress and hypertension. The results also support previous data showing that the therapeutic benefit of antihypertensive drugs, such as calcium antagonists, could be, in part, due to an inhibition of free-radical production [27]. Lacidipine treatment also induced a significant increase in uric acid, which may be a side-effect of the drug.

We found a significant increase of macrophage iNOS activity in hypertensive patients, which agrees with previous data reporting an increase in iNOS in different types of hypertension [28,29]. The induction of iNOS causes an increase of NO, which contributes to the pathogenesis of hypertension [7,30]. Besides, the endothelium dysfunction during essential hypertension leads to a decrease in eNOS activity [31,32]. The decline of eNOS may contribute to the development of hypertension, whereas the increase of iNOS may be a consequence of the pathological state of vessels associated with hypertension. Both isoenzymes, i.e. eNOS and iNOS, produce considerable amounts of $O_2^{\bullet-}$. Both $O_2^{\bullet-}$ and NO react very rapidly to form peroxynitrite. This reaction is approximately three times faster than the dismutation of $O_2^{\bullet-}$ by superoxide dismutase (SOD), implying that an increased production of $O_2^{\bullet-}$ in the vascular wall may very well inhibit the physiological functions of NO [33]. In addition, peroxynitrite can form peroxynitrous acid, which, under homolytic cleavage, can yield HO• [33]. Among others, hypercholesterolaemia and hypertension have been now proposed as consequences of the production of these radicals [33]. This explains the hypercholesterolaemia found in the hypertensive patients in our study. Since lacidipine does not directly affect plasma levels of cholesterol [34], the decrease in iNOS-dependent free radicals may explain, at least in part, the reduction in both total and LDL-cholesterol after lacidipine treatment. Besides, both the antioxidant plus the increase of endogenous cholesterol clearance produced by melatonin could also participate in the hypocholesterolaemic effect here reported [21,35].

Melatonin efficiently scavenges the hydroxyl radical [18,20] and peroxynitrite [36], counteracting peroxynitrite-induced toxicity [36,37], and reduces the expression and activity of iNOS [21]. Melatonin significantly improved nitrate tolerance of coronary vascular reactivity in hypertension [37], a finding that may be applicable in our study. Thus, the increase of melatonin in hypertensive patients may reflect a protective mechanism against free radical-induced oxidative damage [38]. When hypertensive status disappears, e.g. after lacidipine treatment, melatonin also decreases. Under normal conditions, melatonin levels are not affected by calcium antagonists [39], suggesting that its reduction was not due to lacidipine itself. Probably, the reduction of the oxidative stress, that stimulates melatonin production, together with its metabolization after scavenging free radicals [20], may explain the decrease in plasma levels of melatonin in hypertensive patients treated with lacidipine.

The antihypertensive effects of melatonin are well documented. Although in specific experimental conditions melatonin may antagonize the efficacy of calcium-channel blockers [40], most of the experimental evidence suggests that the indoleamine reduces both normal and increased arterial blood pressure. Melatonin affects cardiovascular function by acting directly on blood vessels and systemically reducing sympathetic activity [38,41]. Through its antioxidant activity, melatonin administration reduces blood pressure in spontaneously hypertensive rats, which are suspected to have a lower cellular content of antioxidants [13,42]. The hypotensive effect of melatonin has also been linked to a direct effect on the endothelium, improving the vascular NOS pathway [41,43]. Taken together, the antihypertensive actions of melatonin support its participation in the regulation of cardiovascular functions as an endogenous hypotensive factor [13].

Since oxidative stress impairs endothelium-dependent vasodilatation in hypertension, endothelial dysfunction should improve on administration of antioxidants [6,11,12]. In several models of hypertension, including spontaneously hypertensive rats, antioxidants such as ascorbic acid and glutathione significantly produced dose-dependent aortic relaxation. Supplementation with α-tocopherol enhanced the total antioxidant status, including SOD activity, with an accompanying reduction of LPO and an increase in NOS activity in blood vessels, thus preventing the development of increased blood pressure [12,44]. The levels of $O_2^{\bullet-}$ and oxidized LDL were also decreased by administering antioxidants such as vitamins E and C [45]. But high doses of vitamin E impair acetylcholine-induced aortic relaxation, due to its pro-oxidant activity at these doses [46]. Vitamin C seems to improve the synthesis of eNOS-derived NO and/or protect NO by scavenging $O_2^{\bullet-}$ and peroxynitrite [47], at doses above 500 mg/day *in vitro*, but not *in vivo*. High doses of this vitamin are also pro-oxidant and genotoxic [48]. So, the use of these vitamins in high doses as antioxidants against hypertension is not recommended.

A last point of interest is the recently reported partici-

pation of mitochondria in the pathophysiology of hypertension. The mitochondrial NOS isoform (mtNOS) is overexpressed in inflammatory states, leading to an increase in NO [49]. Hypertension causes important changes in mtNOS activity and NO production, and the inhibition of mtNOS prevents both calcium- and phosphate-induced mitochondrial permeability transition pore opening and apoptosis [50]. Of great interest is that melatonin, but not vitamins C and E, maintains glutathione homeostasis in mitochondria under strong oxidative stress [51,52]. Melatonin counteracts the expression and activity of the mtNOS and prevents NO production in these pathological situations [49,53]. The possibility that the increase in melatonin levels during hypertension was related to a reduction in mtNOS, counteracting the toxicity of intramitochondrial NO, should be explored.

In conclusion, the oxidative damage induced by $O_2^{\bullet-}$ and the broad activation of NO synthesis coexist in human essential hypertension. Besides its calcium antagonism, two recently described lacidipine actions may be related to its therapeutic effects: reducing adhesion molecules and oxidase expression [54], and scavenging peroxynitrite [55]. These actions may participate in the protection of the endothelium against the attack of free radicals during hypertension. Taken together, lacidipine and melatonin can improve endothelial dysfunction by restoring vascular NOS pathway activity and NO availability, through a mechanism probably related to an antioxidant effect [43,56]. Thus, the use of melatonin as a therapeutic agent in combination with lacidipine may be of interest in the treatment of hypertension.

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