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Effect of Ca^{2+} channel blockers on arterial hypertension and heart ischaemic lesions induced by chronic blockade of nitric oxide in the rat

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Abstract

The effects of the Ca^{2+} channel blockers diltiazem, nifedipine and amlodipine were investigated on both arterial hypertension and myocardial changes induced by chronic blockade of nitric oxide synthesis. Control male Wistar rats received N^{ω}-nitro-L-arginine methyl ester (L-NAME; 20 mg rat^{-1} day^{-1}) in the drinking water for 8 weeks; blood pressure and body weight were monitored weekly. The Ca^{2+} channel blockers were given concomitantly to L-NAME, as follows: diltiazem (13.5 mg rat^{-1} day^{-1}) and amlodipine (6.25 mg rat^{-1} day^{-1}) were administered in the drinking water whereas nifedipine (6.25 mg rat^{-1} day^{-1}) was given in the chow. N^{ω}-nitro-L-arginine methyl ester induced a time-dependent increase in blood pressure which was significantly attenuated by diltiazem (154 ± 1.6 vs. 139 ± 1.6 mm Hg, $p < 0.05$), nifedipine (166 ± 2.7 vs. 150 ± 2.1 mm Hg, $p < 0.05$) and amlodipine (208 ± 5.8 vs. 158 ± 1.8 mm Hg, $p < 0.05$) at the last week of the treatment. Rats treated with the L-NAME also developed myocardial ischaemia, as indicated by the increased percentage of fibrous tissue found in the left ventricles of these animals ($10.9 \pm 0.1\%$, $p < 0.01$) when compared to control ones ($6.3 \pm 0.1\%$). Neither diltiazem ($14.9 \pm 1.2\%$) nor nifedipine ($11.1 \pm 1.5\%$) prevented this effect whereas amlodipine ($6.9 \pm 1.1\%$, $p < 0.01$) virtually abolished the increase in fibrous tissue induced by L-NAME. The plasma concentration of the Ca^{2+} channel blockers was measured by liquid chromatography coupled to mass spectrometry at two different time points (morning and afternoon). Only amlodipine treatment was able to maintain constant levels (186 ± 46 ng ml^{-1} in the morning and 110 ± 19 ng ml^{-1} in the evening) compared to nifedipine (3003 ± 578 ng ml^{-1} in the morning and 436 ± 100 ng ml^{-1} in the evening) and diltiazem (77 ± 51 ng ml^{-1} in the morning and not detectable in the evening). In conclusion, our results indicate that amlodipine (but not diltiazem and nifedipine) can efficiently control myocardial ischaemia in nitric oxide deficient rats, probably due to its intrinsically long half-life.

Keywords: Diltiazem; Nifedipine; Amlodipine; Hypertension; Heart ischaemic lesion

1. Introduction

Continuous release of nitric oxide by the endothelium maintains vascular smooth muscle cells in a constant state of vasodilatation (Moncada and Higgs, 1993). Therefore, it has been suggested that hypertension could be the result of a diminished nitric oxide production. In fact, the relaxation induced by acetylcholine in the forearm vascular bed of hypertensive patients is attenuated in relation to normal volunteers (Calver et al., 1992; Hirooka et al., 1992; Cookcroft et al., 1994).

Chronic administration of L-arginine analogues to rats, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) provokes arterial hypertension, renal (Baylis et al., 1992; Ribeiro et al., 1992) and heart (Moreno et al., 1996) ischaemic lesions and coronary vascular remodeling (Takemoto et al., 1997). N^{ω}-nitro-L-arginine methyl ester-induced hypertension, but not myocardial lesions, is abolished by the angiotensin-converting enzyme inhibitor,

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enalapril (Moreno et al., 1995), indicating that the heart ischaemic lesions are independent of the hypertension.

The efficacy of short-acting calcium channel blockers, especially nifedipine, for treating hypertension, myocardial ischaemia and, hence, preventing mortality has been questioned (Furberg et al., 1995; Pahor et al., 1995; Psaty et al., 1995; Mackay and Sever, 1996). The major concern raised is that the rapid fluctuation of drug plasma levels could precipitate important haemodynamic changes. Thus, we have investigated the effects of Ca^{2+} channel blockers of short (diltiazem and nifedipine) and long (amlodipine) half-life on both hypertension and heart ischaemia induced by chronic nitric oxide synthesis inhibition.

2. Materials and methods

2.1. Experimental design

Male Wistar rats (150–200 g) were provided by the Central Animal House (CEMIB-UNICAMP). The animals were maintained under light and temperature-controlled conditions (12 h day/12 h night, 25°C) and were fed with a standard chow (Nuvilab CR-1®, Nuvital Nutrientes, Curitiba, Brazil). All experiments were in accordance with the guidelines of State University of Campinas (UNICAMP) for animal care.

2.1.1. Diltiazem protocol

1. Control ($n = 15$), rats that received tap water alone;
2. L-NAME ($n = 16$), rats that received L-NAME alone (20 mg rat^{-1} day^{-1});
3. L-NAME + diltiazem ($n = 17$), rats that received both L-NAME and diltiazem (20 and 13.5 mg rat^{-1} day^{-1}, respectively); and
4. diltiazem ($n = 19$), rats that received diltiazem alone (13.5 mg rat^{-1} day^{-1}).

Both L-NAME and diltiazem were dissolved in the drinking water. Because diltiazem is photosensitive, the drinking bottles were painted black. The experiment was carried out for 8 weeks. The dose of diltiazem was chosen according a previous study (Natsume et al., 1985).

2.1.2. Nifedipine study

1. Control ($n = 11$), rats that received standard chow;
2. L-NAME ($n = 13$), rats that received L-NAME alone (20 mg rat^{-1} day^{-1});
3. L-NAME + nifedipine ($n = 12$), rats that received both L-NAME and nifedipine (20 and 6.25 mg rat^{-1} day^{-1}, respectively); and
4. nifedipine ($n = 13$), rats that received nifedipine alone (6.25 mg rat^{-1} day^{-1}).

Nifedipine were daily added to the chow. The animals had free access to tap water. The experiment was carried out for 8 weeks. The dose of nifedipine was chosen according our previous study (Ribeiro et al., 1995).

2.1.3. Amlodipine study

1. Control ($n = 11$), rats that received tap water alone;
2. L-NAME ($n = 13$), rats that received L-NAME alone (20 mg rat^{-1} day^{-1});
3. L-NAME + amlodipine ($n = 12$), rats that received both L-NAME and amlodipine (20 and 6.25 mg rat^{-1} day^{-1}, respectively); and
4. amlodipine ($n = 13$), rats that received amlodipine alone (6.25 mg rat^{-1} day^{-1}).

Both L-NAME and amlodipine were dissolved in the drinking water. The experiment was carried out for 8 weeks. Regarding the dose used of amlodipine, we decided to employ the same dose as that of nifedipine.

2.2. Measurement of plasma concentration of diltiazem, nifedipine and amlodipine by liquid chromatography coupled to mass spectrometry (LC-MS)

A separate group of animals received diltiazem (13.5 mg rat^{-1} day^{-1}; $n = 10$), nifedipine (6.25 mg rat^{-1} day^{-1}; $n = 10$) or amlodipine (6.25 mg rat^{-1} day^{-1}; $n = 10$). Both diltiazem and amlodipine were dissolved in the drinking water whereas nifedipine was added to the chow. After 2 weeks of treatment, blood samples from each animal were taken in the morning (8:00–10:00 a.m.) and evening (6:00–8:00 p.m.). On each occasion, the animals were slightly anaesthetised with ether and one 1-ml sample was taken via the tail vein. The blood samples were centrifuged at $2000 \times g$ for 10 min and plasma removed and stored at −20°C until assay.

A liquid–liquid extraction was performed before drug analysis. Briefly, to 0.2 ml of rat plasma, nicardipine (1 μg ml^{-1}) was added. Diethyl-ether/hexane (80/20; 3 ml) was added; the samples were then vortex-mixed and centrifuged (3000 rpm, 10 min) and the supernatant collected and dried under nitrogen at 37°C. The residue was resuspended with mobile phase (200 μl) and an aliquot (20 μl) were injected into the liquid chromatograph.

The mobile phase (67% CH_3CN, 33% H_2O, 5 mM acetate) was infused through a isocratic system at a flow rate of 1 ml min^{-1}. The chromatography was performed on a Genesis column 4 μm C_{18} 150 × 4.6 mm fitted with a guard column of the same material. The calcium channel blockers were analysed by tandem mass spectrometry with positive ion electrospray using selected daughter ion monitoring (MRM). The daughter ions selected were as follows (m/z): diltiazem (415.0 > 177.7), nifedipine (347.0 > 193.8), amlodipine (409.0 > 237.8) and nicardipine (480.0 > 314.9). The study was performed in a Hewlett-Packard 100 liquid chromatograph coupled to a Micromass Quattro II mass spectrometer.

2.3. Blood pressure measurements

The arterial blood pressure and body weight gain were evaluated weekly. For each animal, mean blood pressure

was measured at least in triplicate by a tail-cuff method (Zatz, 1990).

2.4. Stereological procedures

Stereological analysis was performed according to the method described by Aherne (1970). For this procedure, formalin-fixed left ventricle and septum were cut into five equidistant rings perpendicular to the long axis of the ventricle. The rings were then embedded in paraffin, and 5 μm sections were stained with Masson's trichrome. Analysis of the slides were blinded performed on a light microscope (Zeiss, Germany) and the relative volume occupied by each element of the ventricle (myocardial fibers, fibrous tissue or vessels) was measured with an special ocular containing a 25-point reticulum (five parallel lines with five points each, kpl 8×, Zeiss, Germany). For counting, 50 microscopic fields were evaluated and the relative volume (Ppi) occupied by each component was calculated as follows: $Ppi = p/P - R$, where p is the number of reticular points hitting each cardiac element, P is the total number of reticular points and R is the number of points hitting artefactual retraction areas.

2.5. Drugs

N^{ω}-Nitro-L-arginine methyl ester was purchased from Sigma (USA) and sodium pentobarbital from Rhône-Merieux (France). Diltiazem was provided by Boehringer de Angeli (Brazil). Nifedipine and amlodipine were provided by Laboratorios Biosintetica (Brazil).

2.6. Data and statistical analysis

Results are expressed as the mean ± S.E.M. Analysis of variance followed by Bonferroni's test was applied in order to assess the differences in body weight and tail-cuff pressure. For stereological procedures, analysis of variance was followed by Tukey's test. A p-value < 0.05 was considered to be significant.

3. Results

3.1. Body weight

After 8 weeks of treatment, body weight did not differ significantly between experimental groups in diltiazem study (339 ± 8, 314 ± 8, 330 ± 11, 348 ± 7, for control, L-NAME, L-NAME + diltiazem and diltiazem animals, respectively). In the nifedipine study, no significant difference in body weight was observed between control, L-NAME and L-NAME + nifedipine (308 ± 7, 294 ± 7 and 295 ± 5 g, respectively). A significant increase in body weight was observed in the animals treated with nifedipine alone (337 ± 10 g, $p < 0.05$). In the amlodipine study, a slight (but significant) decrease in body weight was observed in animals treated with either L-NAME (261 ± 6 g, $p < 0.05$) or L-NAME + amlodipine (254 ± 5 g; $p < 0.05$) compared to control (298 ± 6 g) and amlodipine alone (289 ± 7 g).

3.2. Tail-cuff pressure

Chronic administration of L-NAME (20 mg rat^{-1} day^{-1}) induced a time-dependent increase in blood pressure (Fig. 1). At the second week of treatment, blood pressure was significantly higher ($p < 0.01$) in L-NAME-treated ani-

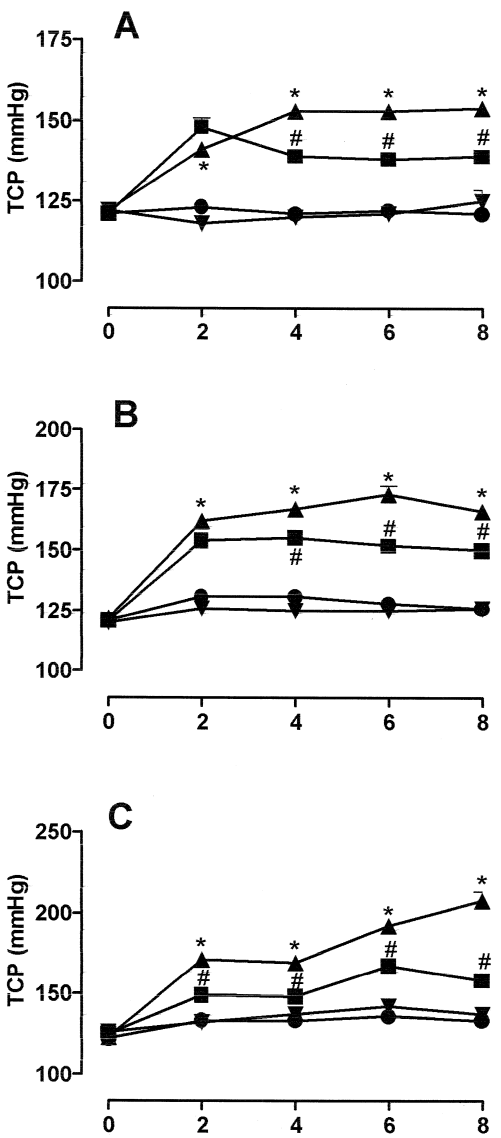

Fig. 1. Effect of diltiazem (Panel A), nifedipine (Panel B) and amlodipine (Panel C) on N^{ω}-nitro-L-arginine methyl ester (L-NAME)-induced hypertension. Tail-cuff pressure (TCP; mm Hg) was measured weekly in control (●), L-NAME (▲), L-NAME + calcium channel blockers (■) and calcium channel blockers alone (▼) animals. Results are expressed as mean ± S.E.M. $^{*}p < 0.05$ compared to control group, $^{\#}p < 0.05$ compared to L-NAME group.

mals compared to control animals in the three studies. In rats in which L-NAME was co-administered with diltiazem, nifedipine or amlodipine, blood pressure was also significantly attenuated but still elevated compared to their respective control group (Fig. 1). At the end of the study, blood pressure was similarly reduced by diltiazem and nifedipine (approximately 10% reduction; $p < 0.01$) whereas the reduction by amlodipine was approximately 25% ($p < 0.01$; Fig. 1). Administration of diltiazem, nifedipine or amlodipine alone had no significant effect on blood pressure.

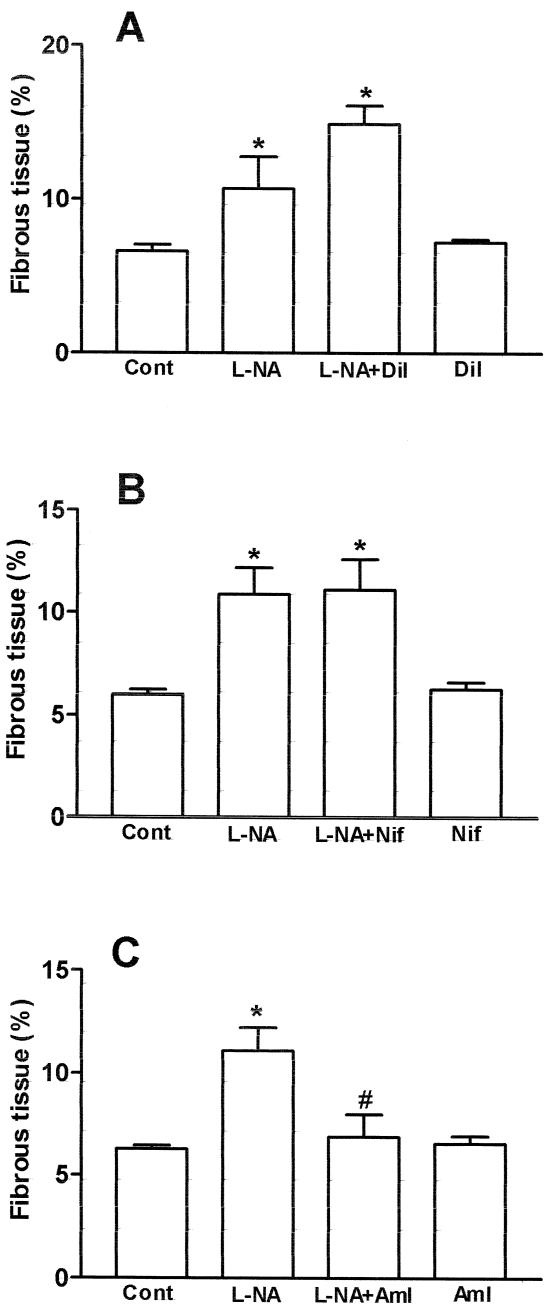

Fig. 2. Effect of diltiazem (Panel A), nifedipine (Panel B) and amlodipine (Panel C) on N^{ω}-nitro-L-arginine methyl ester (L-NAME)-induced myocardial ischaemia. Fibrous tissue represents the sum of postnecrotic fibrous scars, perivascular and interstitial fibrosis found in the left ventricle. Results are expressed as mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control and diltiazem/nifedipine. Dilt, diltiazem; Nif, nifedipine; Aml, amlodipine; L-NA, L-NAME.

3.3. Mortality

In the diltiazem study, 20% of the animals treated with L-NAME and 15% of those treated with L-NAME + diltiazem died. All the animals from control and diltiazem groups survived. In the nifedipine study, no death was observed during the experimental period. In the amlodipine study, 23% of the animals treated with L-NAME died whereas all the animals from control, L-NAME + diltiazem and diltiazem groups survived.

3.4. Stereological analysis

In the stereological analysis, we assumed fibrous tissue as the sum of postnecrotic fibrous scars and interstitial and perivascular fibrosis. In both studies, left ventricles of L-NAME-treated animals presented more fibrous tissue than control ones ($p < 0.05$, Fig. 2A,B). Administration of either diltiazem or nifedipine did not reverse the increase in fibrous tissue induced by L-NAME ($p < 0.05$ vs. control, Fig. 2). The values obtained in diltiazem or nifedipine-treated animals did not differ significantly from those of control group. However, administration of amlodipine virtually prevented the increase in fibrous tissue induced by L-NAME ($p < 0.05$ vs. L-NAME; Fig. 2).

3.5. Calcium channel blockers plasma concentration

The measurement of diltiazem, nifedipine and amlodipine plasma concentrations revealed higher concentrations in the morning (77 ± 51, 3003 ± 578 and 186 ± 46 ng ml^{-1}, respectively; $n = 10$), as compared to evening concentrations (< 5 ng ml^{-1}, 436 ± 100 and 110 ± 19 ng ml^{-1}, respectively; $n = 10$).

4. Discussion

Our results clearly demonstrated that amlodipine, but not nifedipine nor diltiazem, prevented the ischaemic lesions induced by chronic nitric oxide blockade. Interestingly, the three calcium channel blockers only attenuated the hypertension, indicating that the heart ischaemic lesions and the increase in blood pressure are dissociated in this particular model (Moreno et al., 1996). Indeed, enalapril treatment prevents the development of L-NAME induced-hypertension and kidney lesions (Baylis et al., 1992), but failed to prevent the ischaemic lesions developed in the heart (Moreno et al., 1995). Inhibitors of nitric oxide synthesis cause coronary vasoconstriction (Humph-

ries et al., 1991; Huckstorf et al., 1994) and reduction of coronary blood flow (Amrani et al., 1992; Moreno et al., 1997) which can induce the myocardial infarcts observed in this experimental model. Thus, the prevention of hypertension would not compensate for the lack of nitric oxide in the coronary circulation.

The failure of diltiazem and nifedipine to prevent the development of the heart lesions may be related to its intrinsically short half-lives. For instance, short half-life calcium antagonists have been implicated in increase in mortality in patients with coronary heart disease (Furberg et al., 1995; Pahor et al., 1995; Psaty et al., 1995), the possible explanations being the established proischaemic effect, negative inotropic effects, marked hypotension and possibly proarrhythmic effects, all caused by the large fluctuations in the plasma concentrations. Indeed, as demonstrated in this study, diltiazem did not maintain levels during the whole day (being undetectable in the evening samples). Similarly, nifedipine levels were also largely reduced in the evening samples (9 times less). Although amlodipine levels were also significantly reduced in the evening samples, the degree of fluctuation (less than twice) was reasonably smaller.

Diltiazem, nifedipine and amlodipine attenuated, but not abolished, the hypertension induced by chronic blockade of nitric oxide synthesis. Similar results were observed with verapamil (Takase et al., 1996) and nifedipine (Ribeiro et al., 1995), which partially reduced L-NAME-induced hypertension, although the latter prevented the renal fibrosis. The reason for the failure of the calcium channel blockers here employed to avoid the raise in blood pressure in this particular model is yet to be understood. L-NAME-induced hypertension can be prevented by inhibition of the renin–angiotensin system, suggesting an important role for angiotensin II in mediating this effect (Baylis et al., 1992; Ribeiro et al., 1992; Moreno et al., 1995; Takemoto et al., 1997). Angiotensin II acts on angiotensin AT_1 receptor subtype to activate phospholipase C with subsequent formation of inositol 1,3,4-triphosphate (IP3) and diacylglycerol (DAG). The former mobilises calcium from intracellular stores causing the opening of voltage-gated calcium channel. Therefore, in conditions in which vasoconstrictors are markedly activated, such as in L-NAME-induced hypertension, calcium channel blockers may not be expected to be as effective as the receptor antagonist, since they would not antagonise the effect of the intracellular released calcium. Accordingly, the impaired response to acetylcholine was normalised in hypertensive patients given captopril, but not nifedipine (Hirooka et al., 1992).

The finding that only amlodipine prevented the heart ischaemic lesions observed in this model possibly reflects its intrinsic long half-life. The results here presented clearly emphasise the importance of monitoring drug plasma concentrations in animal experiments in order to assess treatment efficacy.

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