J. Pharm. Pharmacol. 1993, 45: 430–433
Received May 19, 1992

In-vivo Binding of (+)-[^{3}H]PN 200-110 to Peripheral Tissues and Brain of Spontaneously Hypertensive Rats: Effect of Lacidipine

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Abstract—The time-course of dihydropyridine receptor occupancy by lacidipine and its relationship with pharmacological activity has been studied in spontaneously hypertensive rat (SHR), as measured by the inhibition of specific (+)-[^{3}H]PN 200-110 binding in-vivo. After oral administration of doses active in reducing blood pressure, lacidipine did not show tissue target differences in respect to binding sites labelled by (+)-[^{3}H]PN 200-110 in cerebral cortex, heart, ileum, bladder and thoracic aorta. The relative occupancy of receptors in heart 60 min after oral administration of 1 mg kg^{-1} lacidipine was 75%. After 12 h, when lacidipine was still effective in reducing blood pressure in SHR, a low (15%) but detectable proportion of receptors was still occupied by the drug. The percentage decrease of blood pressure was linear with the percentage of receptor occupancy obtained by different doses of lacidipine; that is, there was a close correspondence between ED25 for decrease in blood pressure (0·33 mg kg^{-1}) and ED25 for inhibition of (+)-[^{3}H]PN 200-110 specific binding in the heart (0·36 mg kg^{-1}). The long-lasting effect of lacidipine on blood pressure might be explained by its selective interaction with dihydropyridine binding sites labelled in-vivo by (+)-[^{3}H]PN 200-110.

Lacidipine, a new 1,4-dihydropyridine calcium entry blocker, has been pharmacologically characterized as a potent and long-lasting antihypertensive drug after both oral and intravenous administration (Micheli et al 1990). Like other calcium antagonists, its blood pressure lowering action is specifically linked to the inhibition of calcium-induced contraction in vascular smooth muscle, by interaction with the dihydropyridine receptors (Salomone et al 1992). The stereospecific, saturable, high-affinity binding site for the dihydropyridine calcium-channel antagonist (+)-[^{3}H]PN 200-110 has been demonstrated in cell membranes from different tissue homogenates (Lee et al 1984; Supavilai & Karobath 1984; Ichida et al 1989), intact tissue strips (Morel & Godfraind 1987; Dacquet et al 1989) and solubilized receptor preparations (Schneider & Hofmann 1988; Tuana et al 1988; Krizanova et al 1989). Despite the importance of in-vitro binding studies, in-vivo experiments offer unique advantages of cell integrity and intact local environmental conditions. Moreover, pharmacokinetic factors (absorption, plasma protein binding, distribution, metabolism and excretion) would contribute to determine the drug concentration available for interaction with the receptors.

We have, therefore, planned a study in which lacidipine was orally administered to spontaneously hypertensive rats (SHRs) at the same dosages used for the determination of the antihypertensive ED25 (dosage reducing blood pressure by 25%), before intravenous injection of the radioactive dihydropyridine (+)-[^{3}H]PN 200-110. The inhibition of (+)-[^{3}H]PN 200-110 specific binding by lacidipine in cerebral cortex, heart, thoracic aorta, ileum and urinary bladder, was evaluated after 60 min, and related to the antihypertensive effect.

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Materials and Methods

Antihypertensive activity in spontaneously hypertensive rats (SHRs)

Male SHRs (Charles River), weighing about 250 g, were used. Systolic blood pressure and heart rate were measured indirectly from the tail of conscious trained animals by means of a blood pressure recorder (W+W 8005), before treatment and at intervals after the oral administration of drug. Lacidipine was administered by gastric gavage (0·25, 0·5 and 1 mg kg^{-1}).

In-vivo (+)-[^{3}H]PN 200-110 binding measurement

Male SHRs (Charles River, Italy), weighing about 250 g, were pretreated orally with lacidipine (0·25, 0·5 or 1 mg kg^{-1}) 60 min before (+)-[^{3}H]PN 200-110 treatment, or with 1 mg kg^{-1} of lacidipine 720 min before (+)-[^{3}H]PN 200-110 treatment.

The in-vivo binding of (+)-[^{3}H]PN 200-110 has not yet been characterized; therefore we have used the method described by Supavilai & Karobath (1984) for (+)-[^{3}H]PY 108-068. Both compounds are benzoxadiazole 1,4-dihydropyridine derivatives, carrying respectively an ethyl-ester (PY 108-068) or a methyl-ester (PN 200-110) substituent to the dihydropyridine ring. In-vitro studies have demonstrated that the two compounds interact in a similar way with the same apparent binding sites (Supavilai & Karobath 1984). Animals received (+)-[^{3}H]PN 200-110 injection into the tail vein (28 μCi). Parallel groups of animals were co-injected with 0·5, 1·0 or 2·5 mg kg^{-1} nifedipine to determine non-specific binding. Rats were decapitated 5 min after the administration of (+)-[^{3}H]PN 200-110, a time at which the in-vivo binding of the analogue (+)-[^{3}H]PY 108-068 is maximal and corresponds to non-metabolized radioactivity (Supavilai & Karobath 1984). Cerebral cortex and peripheral

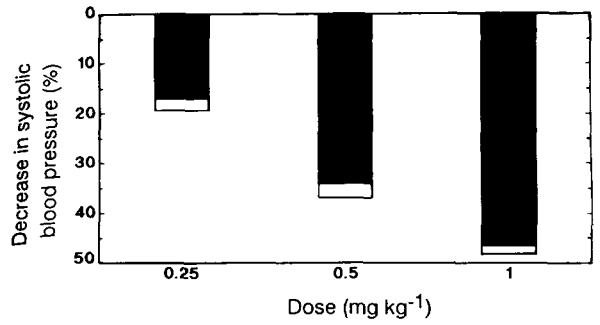

FIG. 1. Maximum percentage decreases in systolic blood pressure induced by different oral doses of lacidipine in conscious spontaneously hypertensive rats (tail-cuff method). ED25 (dose that reduces systolic blood pressure by 25% of basal values) was 0·33 (0·30–0·41) mg kg^{-1}. Data are means ± s.e. of eight animals.

tissues (heart, ileum, thoracic aorta and bladder) were rapidly dissected and homogenized in 50 vol of 50 mM Tris-HCl, pH 7·7, containing 10 mM $CaCl_2$, with an Ultra-Turrax TP 1810 for 20 s at maximal velocity setting. Two samples (0·5 mL) were placed directly into two scintillation vials (total radioactivity). Total radioactivity bound to particulate material (total bound radioactivity) was determined immediately by filtration of two 0·5 mL homogenates over Whatman GF/B glass fibre filters which were immediately washed twice with 5 mL ice-cold buffer.

Protein content in the homogenates was determined by Peterson's method (1977) using bovine serum albumin as standard.

Drugs

(+)-[^{3}H]PN 200-110 (sp. act. 84 Ci $mmol^{-1}$) was obtained from Amersham, UK, and was diluted with 0·9% NaCl in order to inject 28 μCi/200 μL per rat. Nifedipine was obtained from Sigma Chemical Co. and was co-injected with (+)-[^{3}H]PN 200-110.

Lacidipine was synthesized by Glaxo (Verona, Italy), and was administered orally as a suspension in 0·5% carboxymethylcellulose with 0·01% Tween 80 (5 mL kg^{-1}). Control rats were pretreated with vehicle alone.

Data calculations and statistics

For the evaluation of antihypertensive activity eight animals per group were used. Systolic blood pressure values were expressed as mean ± s.e. Comparisons were carried out by a paired Student's *t*-test. ED25 (dose which reduces systolic blood pressure by 25%) was calculated by linear regression analysis of dose-response curves.

For (+)-[^{3}H]PN 200-110 binding, because of differences in animals' total radioactivity, direct comparison of binding between rats was facilitated by calculating the percentage of particulate-bound radioactivity for each tissue of each animal as particulate bound radioactivity divided by total radioactivity.

Data are presented as mean ± s.d. of four animals per group. Statistical analysis was done by analysis of variance and Tukey's test.

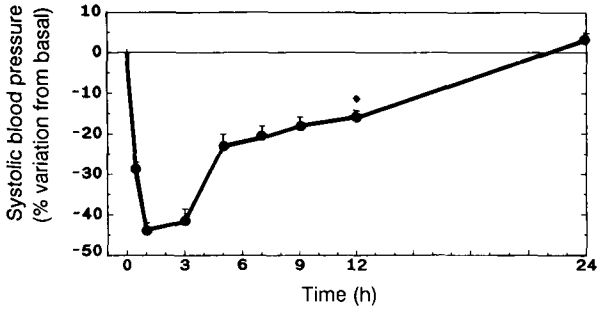

FIG. 2. Effects of lacidipine (1 mg kg^{-1}) on systolic blood pressure at various time intervals after administration. Data are means ± s.e. of eight animals per group. *Last significant effect $P < 0.05$ (Student's paired *t*-test).

Results

Antihypertensive activity in SHRs

Lacidipine, administered orally at doses ranging from 0·25 to 1 mg kg^{-1}, caused a dose related fall in systolic blood pressure with an ED25 value of 0·33 mg kg^{-1} (Fig. 1). The antihypertensive effect induced by all tested doses reached peak effect 1 h after administration, as indicated in Fig. 2 for the dose of 1 mg kg^{-1}. The systolic blood pressure reduction induced by 1 mg kg^{-1} was significant 12 h after lacidipine administration (Fig. 2).

In-vivo (+)-[^{3}H]PN 200-110 binding

After intravenous administration of (+)-[^{3}H]PN 200-110 to control rats 5 min before they were killed, total radioactivity was found in comparable amounts in cerebral cortex, heart and ileum (about 13 ± 1 fmol (mg protein)$^{-1}$), lower in the bladder (9 ± 1·2 fmol (mg protein)$^{-1}$) and higher in thoracic aorta (33 ± 7 fmol (mg protein)$^{-1}$). Total radioactivity was not significantly influenced by co-injection with three different doses of nifedipine (0·5, 1·0, 2·5 mg kg^{-1}) used to determine the nonspecific binding (data not shown).

Total bound radioactivity, expressed as percentage of total radioactivity, was highest in cerebral cortex, intermediate in heart and in ileum, and lowest in thoracic aorta and urinary bladder (Table 1). Total bound radioactivity was significantly reduced by co-injection of nifedipine in all tissues tested. The inhibition obtained with 0·5 mg kg^{-1} of nifedipine was maximal, since no further inhibition could be obtained with 1·0 or 2·5 mg kg^{-1} (data not shown). The mean of the bound values obtained with the three different doses of nifedipine was therefore taken as the measure of nonspecific bound radioactivity (Table 1). Specific bound radioactivity

Table 1. In-vivo (+)-[^{3}H]PN 200-110 binding in SHR tissues.

	Total	Nonspecific	Specific
Cerebral cortex	81 ± 5	48 ± 4**	33 ± 5
Heart	66 ± 7	49 ± 3**	17 ± 5
Ileum	57 ± 4	45 ± 3**	13 ± 3
Bladder	41 ± 8	28 ± 4**	12 ± 5
Thoracic aorta	47 ± 2	42 ± 2*	5 ± 2

Data are presented as % radioactivity bound and are the means ± s.d. of four animals per group. Rats were injected intravenously with 28 μCi of (+)-[^{3}H]PN 200-110 alone (total) or together with excess of nifedipine (nonspecific) 5 min before killing. Mean specific binding was calculated as difference between the mean values for total and nonspecific binding. *$P < 0.05$, **$P < 0.01$ compared with (+)-[^{3}H]PN 200-110 alone.

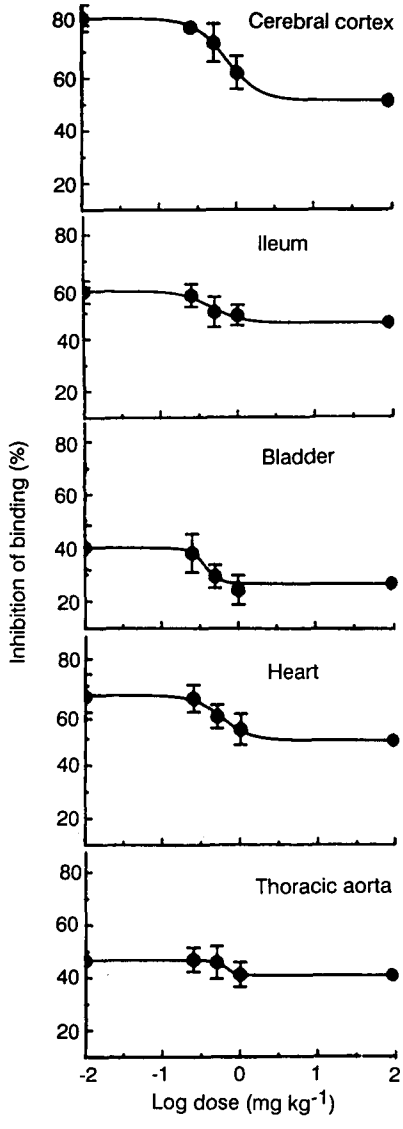

FIG. 3. In-vivo inhibition of (+)-[^{3}H]PN 200-110 binding by lacidipine. Male SHRs were injected intravenously with 28 μCi of (+)-[^{3}H]PN 200-110 dissolved in 0·2 mL of 0·9% NaCl, 60 min after oral administration of different doses of lacidipine and were killed 5 min later. The highest point in each curve corresponds to the % of bound radioactivity in control rats (total binding); the lowest corresponds to the % of bound radioactivity obtained in animals co-injected with excess of nifedipine (nonspecific binding). Each point represents the mean value ± s.d. of four animals per group.

was highest in cerebral cortex (33%), intermediate in heart, ileum and urinary bladder (12–17%) and a very low amount of specific (+)-[^{3}H]PN 200-110 was found in thoracic aorta (5%) (Table 1).

Lacidipine administered orally 60 min before the radioligand, for comparison with pharmacological experiments, dose-dependently decreased (+)-[^{3}H]PN 200-110 bound (Fig. 3), without affecting the total radioactivity present in tissues (data not shown).

From the data presented in Fig. 3 it was possible to calculate the doses of lacidipine giving half-maximal inhibition of specific (+)-[^{3}H]PN 200-110 binding in-vivo in all the tissues studied. For each curve we have determined the maximum value of (+)-[^{3}H]PN 200-110 bound, corresponding to total bound radioactivity; the minimum value of (+)-[^{3}H]PN 200-110 bound, corresponding to nonspecific bound radioactivity, and three intermediate bound values obtained with the different doses of lacidipine. Such experimental design of families of sigmoidal curves allows the use of the logistic function (De Lean et al 1978), which calculates the dose of inhibitor giving half-maximal effect and the slope of the inhibition curve. These slopes were greater than 1 and not significantly different amongst the different tissues, but their meaning in in-vivo studies is less clear than in-vitro, where they represent the Hill coefficient.

Table 2. In-vivo potencies (mg kg^{-1}) of lacidipine for inhibition of (+)-[^{3}H]PN 200-110 in different tissues.

	ED50 ± s.d.	ED25
Cerebral cortex	0·74 ± 0·16	0·43
Thoracic aorta	0·65 ± 0·09	0·55
Heart	0·57 ± 0·08	0·36
Ileum	0·45 ± 0·10	0·27
Bladder	0·37 ± 0·12	0·29

Lacidipine was orally administered 60 min before intravenous injection of (+)-[^{3}H]PN 200-110 (28 μCi). ED50 ± s.d. values were calculated from the data presented in Fig. 3 using the ALLFIT program (De Lean et al 1978); ED25 values were obtained by extrapolation of the curve fitting.

The ED50 values also were not statistically different amongst tissues: the lowest ED50 values (Table 2) were found in bladder and in ileum: 0·37 ± 0·12 and 0·45 ± 0·10 mg kg^{-1}, respectively; intermediate in heart: 0·57 ± 0·08 mg kg^{-1}; and highest in thoracic aorta and cerebral cortex 0·65 ± 0·09 and 0·74 ± 0·16 mg kg^{-1}, respectively.

To compare the inhibition of specific (+)-[^{3}H]PN 200-110 binding in-vivo with the antihypertensive effect of lacidipine, expressed as ED25, the ED25 for the inhibition of in-vivo (+)-[^{3}H]PN 200-110 binding has been extrapolated from the curves shown in Fig. 3. The results, also reported in Table 2, show that in all the tissues studied, the dose of lacidipine that induces 25% inhibition of (+)-[^{3}H]PN 200-110 binding, is very close to the dose which causes 25% of maximum decrease in blood pressure (0·33 mg kg^{-1}).

When lacidipine was injected at the dose of 1·0 mg kg^{-1} 720 min before the radioligand, a small but still detectable decrease of specific (+)-[^{3}H]PN 200-110 binding was measurable in cerebral cortex (21%), heart (14%), ileum (34%) and bladder (12%).

Discussion

In the present study, (+)-[^{3}H]PN 200-110 was used to label dihydropyridine receptors in-vivo because of its low degree of nonspecific binding, as compared with [^{3}H]nitrendipine at physiological temperatures (Schoemaker et al 1983; Lee et al 1984). The tissue distribution of (+)-[^{3}H]PN 200-110 binding in SHRs is similar to that reported for its analogue (+)-[^{3}H]PY 108-068 by Supavilai & Karobath (1984) in normal rats. These authors also reported that the in-vivo binding of (+)-[^{3}H]PY 108-068 is maximal 5 min after intravenous administration. Therefore, we applied this condition to

measure the in-vivo binding of (+)-[^{3}H]PN 200-110 to avoid metabolism of the labelled material. We found the maximum amount of specific binding to be in the cerebral cortex of SHRs as already reported in normal rats for [^{3}H]nitrendipine (Schoemaker et al 1983; Roeske et al 1985) and for (+)-[^{3}H]PY 108-068 binding in-vivo (Supavilai & Karobath 1984).

Although the dihydropyridines such as PN 200-210 have high affinity for vascular smooth muscle, in the brain (+)-[^{3}H]PN 200-110 binds predominantly to synaptic regions and not to blood vessels (Cortes et al 1984). It is, however, difficult to give anatomical definition of brain binding of (+)-[^{3}H]PN 200-110 in our study, since we studied binding to homogenates without cellular resolution.

It is surprising that there is a very low amount of specific (+)-[^{3}H]PN 200-110 in thoracic aorta, in spite of the high content of total radioactivity found in the homogenate. From in-vitro binding studies it has been reported that the large-sized arteries, such as aorta, have higher B_{max} values for (+)-[^{3}H]PN 200-110 binding than the medium to low sized arteries (Pinquier et al 1988). Moreover, [^{3}H]nitrendipine binding in-vivo in thoracic aorta of normal rats has been found to be comparable or higher than that in ileum (Schoemaker et al 1983; Roeske et al 1985). One possibility for such a discrepancy is that in our study we have used SHRs, in order to compare the pharmacological activity of lacidipine with its degree of occupancy of dihydropyridine receptors.

The data obtained indicate that lacidipine does not show tissue target differences in its interaction with calcium antagonist binding sites labelled by (+)-[^{3}H]PN 200-110, having a similar ED50 in all the tissues tested.

The relative occupancy of receptors in heart, 60 and 720 min after oral administration of 1 mg kg^{-1} lacidipine, was 75 and 15%, respectively, paralleling the effect on blood pressure.

Moreover, the percentage decrease of blood pressure was linear with the inhibition of specific (+)-[^{3}H]PN 200-110 binding in-vivo obtained by different doses of lacidipine, so that there was a close correspondence between the ED25 for decrease in blood pressure (0·33 mg kg^{-1}) and the ED25 for inhibition of (+)-[^{3}H]PN 200-110 specific binding (0·36 mg kg^{-1}).

The conclusion of the present study is that the long-lasting antihypertensive effect of lacidipine might be explained by the degree of occupancy of dihydropyridine binding sites labelled in-vivo by (+)-[^{3}H]PN 200-110.

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