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Effects of Hypotensive and Non-hypotensive Doses of Manidipine on Structure, Responses to Endothelin-1 and ICAM-1 Production in Mesenteric Small Resistance Arteries of Spontaneously Hypertensive Rats

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Objective: We have evaluated the effects of a new calcium channel blocker, manidipine, given at both high, hypotensive and low, non-hypotensive doses, on vascular morphology, response to endothelin-1 and ICAM-1 production in mesenteric small resistance arteries of spontaneously hypertensive rats (SHR). *Methods:* Ten SHR were treated with manidipine 3 mg/kg per day (high dose) and 10 with manidipine 0.3 mg/kg/ per day (low dose). The drug was administered by gavage from the 4th to 12th weeks of age. Eighteen Wistar-Kyoto (WKY) rats and 18 SHR were kept untreated as controls. Rats were killed at 13 weeks. Mesenteric small arteries were dissected and mounted on a micromyograph for determination of indexes of vascular structure (media thickness, wall thickness, media/lumen ratio). *Results:* Systolic blood pressure was significantly reduced by the high dose of the drug, while no effect was observed with low-dose manidipine. A reduction in the media/lumen ratio was observed only in SHR treated with high-dose manidipine. The response to endothelin-1 in untreated SHR was significantly lower in comparison with WKY; a significant reduction was observed in SHR treated with high-dose manidipine. ICAM-1 vascular concentrations were higher in untreated SHR than in WKY controls. Both high- and low-dose manidipine reduced ICAM-1 concentrations toward normalization. *Conclusions:* Manidipine at high, hypotensive, but not at low, non-hypotensive doses has been proven to reduce structural alterations in mesenteric small resistance arteries, and to normalize vascular responses to endothelin-1. In addition, manidipine, at both low and high doses, may reduce ICAM-1 vascular production, thus suggesting a possible anti-atherogenic effect. *Key words: adhesion molecules, calcium antagonists, calcium entry blockers, endothelin-1, endothelium, hypertrophy, ICAM-1, manidipine, vascular hypertrophy, vascular resistance.*

INTRODUCTION

Established hypertension is usually associated with the presence of structural alterations in small resistance arteries [1–3]. These alterations are characterized by an increase in the thickness of the tunica media layers and by a reduction in the internal diameter. As a consequence, the media/lumen ratio is increased. The increase in media/lumen ratio may be ascribed to hypertrophy or hyperplasia of vascular smooth muscle cells (hypertrophic remodelling) or to a re-arrangement of the same material around a narrowed lumen (eutrophic remodelling) [4]. Regardless of the mechanism involved in their onset, vascular structural alterations may play an important role in the development and/or maintenance of hypertension [1–3].

The regression of structural alterations in small resistance arteries is an appealing therapeutic goal, since, from a theoretical point of view, it would mean the correction of an hallmark of hypertension, although is not presently clear whether these alterations have a prognostic significance, independent from blood pressure values. Studies in animal models of genetic or experimental hypertension have demonstrated that it is possible to obtain a reduction of vascular structural alterations with several antihypertensive drugs. However, calcium channel blockers and ACE inhibitors seem to have some advantage in this regard [5–7]. In particular, the long-acting dihydropyridinic calcium entry blockers nitrendipine [5], isradipine [6] and lacidipine [7] (at variance with felodipine [8]) have reduced, albeit to different extents, structural alterations in mesenteric small resistance

arteries of spontaneously hypertensive rats (SHR). In these studies, a reliable *in vitro–ex vivo* micromyographic technique for the assessment of vascular morphology was used [9]. Recently, a new dihydropyridinic molecule, manidipine, was demonstrated to be able to reduce wall hypertrophy in coronary arteries when given to DOCA salt rats [10].

The development of hypertension is also often associated with the presence of endothelial dysfunction. Endothelin-1 is a 21-amino acid peptide produced by the endothelium, belonging to a family of potent vasoconstrictors, which also have the ability to induce cell hypertrophy and proliferation [11]. Endothelin-1 is five times more potent than angiotensin II in increasing peripheral vascular resistance. The activities of endothelin-2 and endothelin-3 are not well defined, although they have vasoconstrictor properties and are encoded in the human genome [11]. The role of endothelins in the pathogenesis of hypertension is still controversial [12, 13]; however, it was demonstrated that the endothelin-1 gene is expressed in small resistance arteries both in humans [14] and in spontaneously hypertensive rats (SHR) [15]. It is therefore possible that even a small amount of locally produced endothelin may act as a regulator of vascular reactivity in the circulation [16]. Very few data are presently available about the effect of antihypertensive therapy on vascular responses to endothelin-1.

Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin superfamily, is a 95-KDa cell surface glycoprotein which is constitutively expressed in some tissues, including the endothelium [17, 18]. It has been demonstrated that ICAM-1 plays a role in leukocyte extravasation through the endothelium [19]. Therefore ICAM-1 may contribute to the trapping of monocytes on local vascular walls, and therefore initiate atherogenesis. The expression of ICAM-1 seems to be increased in animal models of experimental hypertension, and it may provide a possible link between hypertension and atherogenesis [20]. It is not presently known whether antihypertensive therapy may modulate ICAM-1 production.

Therefore we considered it worthwhile to test the effects of a new calcium channel blocker, manidipine, given at both high, hypotensive and low, non-hypotensive doses, on cardiovascular morphology, response to endothelin-1 and ICAM-1 production in mesenteric small resistance arteries of SHR.

MATERIALS AND METHODS

Fifty-six rats (38 SHR and 18 Wistar-Kyoto (WKY)) were included in the study. The animals were obtained from Charles River Laboratory (Calco, Italy). All the procedures followed were in accordance with the guidelines of our institution (Medical School, University of Brescia). The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The rats were housed two to a cage in a room in which the temperature was controlled between 23°C and 25°C, and a 12-h light/dark cycle was maintained. Food and water were supplied *ad libitum*. Ten SHR were treated with manidipine 3 mg/kg per day (high dose), and 10 with manidipine 0.3 mg/kg per day (low dose). The drug was administered by gavage from the 4th to 12th weeks of age. Eighteen WKY and 18 SHR were kept untreated as controls. The drug suspension was made up freshly every day. The rats were killed at the end of the treatment period, after 3 or 4 days of therapeutic washout. Systolic blood pressure values and heart rate were measured non-invasively (tail cuff method, IITC Life Science Instruments, Woodland Hills, CA) in conscious rats every week.

On the day of death, the animals were weighed and then killed by decapitation. From each rat, mesenteric vessels corresponding to the second branch (about 140–200 μm average diameter in relaxed conditions, 2 mm long) were obtained by dissection. The vessel segments were excised free of connective and adipose tissue and two stainless steel wires of 40 μm diameter were threaded through the lumen. This ring preparation was mounted on a micromyograph, as previously described by Mulvany *et al*. [21, 22]. Total time for dissection and preparation was approximately 45 min. Vessels were then equilibrated and relaxed for at least 30 min in physiological saline solution (PSS) with the following composition (in mmol/L): NaCl 119, $NaHCO_3$ 24, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 2.5 and glucose 5.5, kept constantly at 37°C and bubbled with 5% CO_2 in O_2.

After equilibration, the micromyograph was transferred to the stage of a light microscope with immersion lens. The vessel was stretched slightly (wall tension about 0.1 N/m), and structural characteristics of the vessels were evaluated. The following parameters were measured: wall thickness, media thickness, adventitia thickness, intima thickness, internal diameter, media/lumen ratio and media cross-sectional area. Then, the normalized internal circumference L_1 was determined, as described previously by Mulvany *et al.* [21, 22], from the resting wall tension–internal circumference relation and Laplace equation (L_1 is defined as $0.9*L_{100}$, where L_{100} is an estimate of the internal circumference which the vessel would have had *in vivo* when subjected to a transmural pressure of 100 mmHg while relaxed). From L_1, the normalized internal diameter l_1 was calculated. Assuming that the cross-sectional area remains constant when the vessel is extended to L_1, the previously mentioned

Table I. *Body weight, systolic arterial pressure and morphological characteristics of the mesenteric resistance vessels*

Groups	SAP (mmHg)	Body weight (g)	MT (μm)	WT (μm)	MCSA (μm^2)	ID (μm)	M/L	RI and GI (%)
Untreated WKY (n = 18)	157 ± 14.6***	284 ± 21	20.7 ± 2.53***	35.7 ± 4.39**	16 593 ± 3896	230 ± 38 ***	0.092 ± 0.019 ***	– –
Untreated SHR (n = 18)	216 ± 24.7	269 ± 25	24.5 ± 2.43	39.1 ± 4.02	16 062 ± 3671	180 ± 33	0.140 ± 0.024	106, −0.03
SHR high-dose manidipine (n = 10)	190 ± 14.1**	288 ± 22	22.6 ± 2.01	41.4 ± 5.28	14 565 ± 2857	179 ± 25	0.128 ± 0.012*	116, −0.12
SHR low-dose manidipine (n = 10)	207 ± 12.2	287 ± 21	23.0 ± 1.14	39.1 ± 2.43	13 868 ± 1853	166 ± 26	0.142 ± 0.022	118, −0.16

SAP = systolic arterial pressure, MT = media thickness, WT = wall thickness, MCSA = media cross-sectional area, ID = normalized internal diameter; M/L = media/lumen ratio; RI = remodelling index, GI = growth index. Statistical significance of differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to untreated SHR; # $p < 0.001$.

morphological parameters were automatically calculated also in normalized conditions.

A "remodelling index" and a "growth index" were calculated in untreated and treated SHR, according to Heagerty *et al*. [4], who expanded a previous observation of Baumbach & Heistad [23]. The remodelling index quantifies how much of the vascular structural alteration may be explained by a rearrangement of the same otherwise normal material around a narrowed lumen, without cell growth. By contrast, the growth index quantifies the contribution net of cell growth (hypertrophy or hyperplasia) to the structural change.

Each vessel was then stimulated as follows:

- three stimulations (2 minutes for each) with PSS in which NaCl was substituted with KCl on an equimolar basis (K-PSS), and two stimulations with K-PSS containing 10 μmol/L norepinephrine;
- a cumulative dose-response curve to endothelin-1 at the following concentrations: 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} μmol/L, 3 min per concentration.

(For further details about the methods used, see [24] and [25].)

Then the vessel's content in ICAM-1 was quantitated by an immunoenzymatic method (Soluble ICAM-1 immunoassay, R&D systems, Minneapolis, MN, USA). Each mesenteric small artery was homogenated, and then ICAM-1 local concentrations were analysed as described in the immunoassay kit. The assay involves the simultaneous reaction of ICAM-1 present in the sample to two antibodies directed against different epitopes on the ICAM-1 molecule. One antibody coats the walls of the microtiter wells and the other is conjugated to the enzyme horseradish peroxidase. Any ICAM-1 present forms a bridge between the two antibodies. After removal of

Fig. 1. Time course of blood pressure from the 6th to 13th weeks in untreated SHR (n = 18), untreated WKY (n = 18) and in SHR treated with high-dose manidipine (n = 10) and low-dose manidipine (n = 10). Data at "13 weeks" correspond to the time of death. See text for statistical significance of differences between curves. Data are expressed as mean ± SEM.

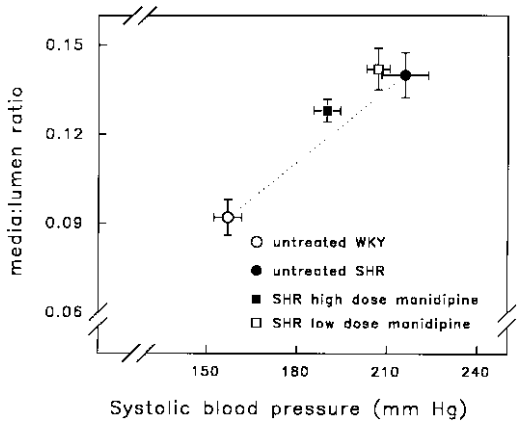

Fig. 2. Media/lumen ratio in mesenteric small arteries plotted against systolic blood pressure in untreated SHR (*filled circles*), untreated WKY (*open circles*), SHR treated with high-dose manidipine (*filled squares*) and SHR treated with low-dose manidipine (*open squares*). The value of the media/lumen ratio observed in treated SHR is similar to that expected from the regression between systolic blood pressure and media/lumen ratio in untreated SHR and WKY. Data are expressed as mean ± SEM.

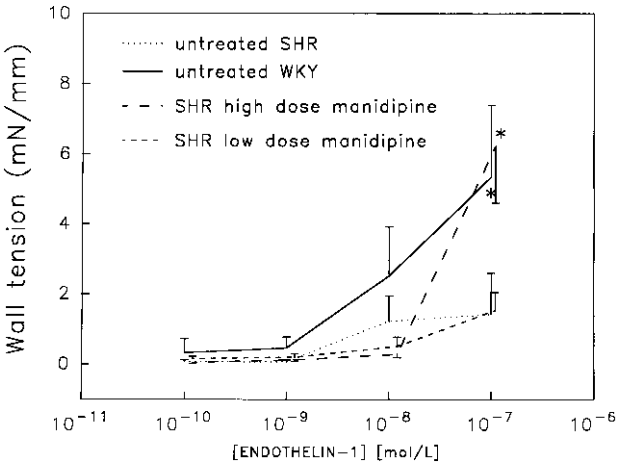

Fig. 3. Dose-response curve to endothelin-1 in treated and untreated SHR as well as in WKY. The vascular response is expressed as active media stress. Data are expressed as mean ± SEM. The response to endothelin-1 in untreated SHR is significantly lower than that of WKY (ANOVA $p < 0.05$). A significant reduction toward normalization was observed in SHR treated with high-dose manidipine (ANOVA $p < 0.05$). Statistical significance of differences in comparison with untreated SHR (Student's *t*-test): * $p < 0.05$.

unbound material by aspiration and washing, the amount of conjugated antibody bound to the well is detected by reaction with a substrate specific for the enzyme, which yields a coloured product proportional to the amount of conjugated antibody (and thus ICAM-1 in the sample). The coloured product was quantified photometrically. Typical sensitivity of these assays was less than 1 ng/mL. The ICAM-1 concentrations were expressed as ng/g of vascular tissues.

All data are expressed as mean ±SD, unless otherwise stated. One-way ANOVA and Bonferroni's correction for multiple comparisons were used to evaluate differences among groups. Two-way ANOVA for repeated measures was used for blood pressure (group time) (BMDP Statistical Software programs 7D, 1V and 2V, BMDP Statistical Software Inc., Los Angeles, CA, USA).

RESULTS

No difference in body weight was observed among groups (Table I).

Systolic blood pressure and heart rate

Systolic blood pressure (SBP) values in untreated and treated SHR and WKY from the 4th to 13th weeks are reported in Fig. 1, while SBP values at the time of death are reported in Table I. At 4 weeks of age, no statistically significant difference in SBP was observed between untreated SHR and WKY. During the treatment period, SBP was significantly higher in untreated SHR than in WKY controls (ANOVA $p < 0.001$). At 12 weeks of age, the SHR treated with high-dose manidipine showed a significant reduction in SBP (ANOVA $p < 0.05$ vs untreated SHR during the treatment period), while no significant change was observed with low-dose manidipine. Heart rate was higher in untreated SHR (420 ± 30.0 beats/min) than in WKY controls (354 ± 26.4 beats/min, $p < 0.001$). The antihypertensive treatment did not affect heart rate (high-dose manidipine: 428 ± 30.5 beats/min, low-dose manidipine: 433 ± 39.8 beats/min).

Vascular morphology

Values of media thickness, wall thickness, media cross-sectional area, internal diameter and media/lumen ratio in mesenteric small resistance arteries of SHR and WKY are reported in Table I. Untreated SHR clearly showed the presence of vascular structural alterations. Treatment with high-dose manidipine induced a significant reduction of the media/lumen ratio in the SHR. No effect was observed with low-dose manidipine. The value of the media/lumen ratio observed in the SHR treated with low- and high-dose manidipine is similar to that expected from the regression between SBP and the media/lumen ratio in untreated SHR and WKY (Fig. 2). The remodelling index in untreated and treated SHR was greater than 100%. Therefore, almost all of the increase in the media/lumen ratio could be explained by a eutrophic remodelling process, while cell growth played a minor role, if any, as demonstrated by the negative values of the growth index. The differences in media cross-sectional area among groups were not statistically significant, thus suggesting that a

Table II. *Vascular content of ICAM-1*

Groups	ICAM-1 (ng/g of tissue)
Untreated WKY ($n = 14$)	135 ± 69**
Untreated SHR ($n = 14$)	366 ± 233
SHR high-dose manidipine ($n = 8$)	178 ± 43*
SHR low-dose manidipine ($n = 10$)	186 ± 62*

Statistical significance of differences in comparison to untreated SHR: * $p < 0.05$, ** $p < 0.01$.

correction of eutrophic remodelling occurred in the SHR treated with high-dose manidipine.

Vascular function

No significant difference among the groups (untreated WKY and treated and untreated SHR) in response to KPSS was observed (data not shown) in mesenteric small resistance arteries. The response to KPSS + norepinephrine was modestly increased in untreated SHR (about 25%), but no significant changes were observed during the different treatments (data not shown). The response to endothelin-1 in untreated SHR was significantly lower compared with WKY (ANOVA $p < 0.05$) (Fig. 3); a significant trend toward normalization was observed in the SHR treated with high-dose manidipine (ANOVA $p < 0.05$) (Fig. 3).

Vascular content of ICAM-1

ICAM-1 vascular concentrations were higher in untreated SHR than in WKY controls ($p = 0.0015$) (Table II). Both high-dose manidipine ($p = 0.023$) and low-dose manidipine ($p = 0.03$) reduced ICAM-1 concentrations toward normalization (Table II). No significant correlation between ICAM-1 concentrations and indexes of vascular structure or of functional response to endothelin-1 was observed.

DISCUSSION

In the present study we observed a significant reduction of structural alterations in mesenteric small resistance arteries of SHR after treatment with high-dose manidipine. No effect was observed with the lower dose of the drug. Calculation of the remodelling and growth indices suggests that a correction of eutrophic remodelling of mesenteric small resistance arteries occurred in SHR treated with high-dose manidipine. Similar data have been observed in a recent study in which enalapril and losartan were evaluated [26]. The potential mechanism involved in this vascular protective effect of manidipine is probably to be ascribed to the ability of the drug to reduce intracellular calcium. Calcium ions act as an intracellular messenger mediating a large variety of responses, including cell growth processes [27].

Alterations in small artery structure are often, but not always, appropriate to the actual blood pressure [28]. Therefore the hemodynamic effect of an antihypertensive drug is an important factor, although modulation of vascular growth may also be obtained independently from blood pressure reduction [25]. In our study, the beneficial effect of antihypertensive therapy on vascular structure was that predicted on the basis of the regression line between systolic blood pressure and structural parameters in untreated SHR and WKY. However, the hypotensive dose of manidipine (3 mg/kg per day) used in the present study was much lower than that used in similar studies with other calcium entry blockers (e.g. nitrendipine, isradipine, lacidipine, 10–30 mg/kg/day) [6–8]. Consequently, the extent of blood pressure reduction obtained was only moderate, compared with almost complete normalization observed in other studies. However, our purpose was to induce a reduction, but not necessarily a normalization of blood pressure; the dose of manidipine was chosen accordingly.

In the present study we observed a depressed responsiveness to endothelin-1 in SHR, compared with WKY, thus confirming previous reports [29–33]. The results could be explained, at least in part, by a downregulation of the endothelin receptors on vascular smooth muscle, due to increased vascular production of the peptide or as a consequence of high blood pressure itself [12]. Antihypertensive therapy with high-dose manidipine completely normalized this altered response. In a previous study, Deng & Schriffin [31] observed a normalization of vascular responses to endothelin-1 in mesenteric small resistance arteries of two kidney-one clip rats after chronic treatment with cilazapril, while the vasoconstrictor responses in metoprolol-treated and hydralazine-treated rats remained significantly blunted. Therefore, it appears that calcium channel blockers and ACE inhibitors may have some advantage in this regard, compared with other antihypertensive drugs. However, it should be taken into account that in the previously mentioned study [31], metoprolol and hydralazine were devoid of effects on vascular structure, while cilazapril nearly normalized the media/lumen ratio.

An increase in soluble ICAM-1 levels has been associated with atherosclerosis, and therefore may be a possible marker of atherogenic risk. The possible link between hypertension and soluble or tissue ICAM-1 may be an increased cellular expression in response to hypertension-related changes in endothelial structure and function. In our study ICAM-1 vascular content was greater in untreated SHR, compared with WKY normotensive controls, and was reduced by both manidipine treatments, thus suggesting a direct interaction of the

drug with the local production of the molecule. It has previously been demonstrated that mechanical strain can stimulate the expression of ICAM-1 by endothelial cells [20]. Therefore it would be expected that only a hypotensive dose of a drug should modulate ICAM-1 production through a reduction in strain. However, it is also possible that calcium entry blockers may have direct effects on endothelial cells, not related to their hemodynamic properties. It has been demonstrated that some calcium channel blockers may be capable of antioxidant action [34]. Therefore it is possible that additional properties, beyond blood pressure reduction, may explain the beneficial effect of manidipine on ICAM-1 expression even at low, non-hypotensive doses. Our data may also suggest that manidipine could be effective in preventing the adhesion of inflammatory cells. It is therefore possible that manidipine may possess anti-atherogenic properties; however, further studies are needed to elucidate these aspects. In fact, small arteries are not the site of atheroma, and different levels of the vasculature may be differently affected by environmental insults and by drugs.

In conclusion, manidipine at high, hypotensive, but not at low, non-hypotensive doses has been proven to reduce structural alterations in mesenteric small resistance arteries, and to normalize vascular responses to endothelin-1. In addition, manidipine, at both low and high doses, may reduce ICAM-1 vascular production.

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