Aortic smooth muscle cell phenotypic modulation and fibrillar collagen deposition in angiotensin II-dependent hypertension

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Abstract

\textbf{Background:} We investigated the effect of nifedipine, AT-1 and ET-1 receptor blockade on arterial smooth muscle cell phenotypes and collagen deposition in TGRen2 transgenic rat (TGR). \textbf{Methods:} Four-week-old TGR were blood pressure (BP)-matched and allocated to receive a placebo (\(n=8\)), the calcium antagonist nifedipine (\(n=6\)), the AT-1 specific receptor antagonist irbesartan (\(n=6\)), the ET\textsubscript{A}/ET\textsubscript{B} antagonist bosentan (\(n=6\)) or the ET -selective antagonist BMS-182874 (\(n=5\)). Sprague–Dawley normotensive rats served as controls (\(n=6\)). After 4 weeks of treatment animals were euthanized and the left ventricle (LV) and the structural changes in intracardiac arterioles and aorta were assessed histomorphometrically. Smooth muscle cell phenotypes and fibrillar collagen content of the aortic wall were evaluated by immunostaining, using differentiation markers—specific antibodies and Syrius red staining, respectively. The changes in ET\textsubscript{A} and ET\textsubscript{B} receptor density were also assessed with quantitative autoradiography. \textbf{Results:} Compared to placebo, only irbesartan lowered BP (\(P<0.001\)) and prevented LV and small resistance artery hypertrophy. The aorta of placebo-treated TGR showed an increase in foetal-type smooth muscle cell content and fibrillar collagen staining, compared to controls. These changes were blunted by irbesartan, which increased ET\textsubscript{A} receptors in the arterial wall, enhanced by BMS-182874 and unaffected by bosentan. Nifedipine also blunted both the VSMC and collagen changes despite having no effect on BP and ET\textsubscript{A} receptors. \textbf{Conclusions:} In TGRen2, vascular hypertrophy entails both smooth muscle cell phenotypic modulation and collagen deposition. These alterations do not follow closely the BP changes and seem to imply the dihydropyridine-sensitive calcium channels. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Increased vascular resistance is a hallmark of arterial hypertension and results from functional and structural changes in large (elastic-type) and small resistance arteries [1], which entail vascular wall hypertrophy. Molecular changes in the contractile machinery composition are involved in this process [2,3], but despite intensive research efforts the precise underlying mechanisms remain poorly understood. In a rabbit model of renovascular hypertension and in stroke-prone spontaneously hypertensive rats, the thickening of the media of large arteries implies an increase in foetal phenotype of VSMCs [4,5], i.e. a shift of VSMCs from the contractile to a synthetic phenotype and/or the expansion of a preexisting ‘immature’ medial VSMC population [3,6]. Likewise, an increased deposition of collagen and other proteins of the extracellular matrix can contribute to changing the viscoelastic properties of the vessel wall in hypertension [7]. The extracellular matrix (fibrillar collagen and fibro-
nectin matrix assembly) can play a pivotal role in the regulation of arterial VSMC differentiation and proliferation [7–9]. Thus, it would appear that the process of vascular remodeling in hypertension involves coordinated and reciprocally regulated changes in extracellular matrix proteins and VSMC phenotypes.

The TGR(mREN2)27 rat (TGR) is a monogenic model of severe hypertension created with the insertion of the mouse Ren2 gene into the rat genome [10]. The transgene is expressed in several tissues and causes increased local Ang II synthesis, which is mirrored by a twofold increase in Ang II content in the wall of the aorta [11]. The TGR is characterized by an early and prominent cardiovascular disease (CVD), and thus is a paradigm of endogenous Ang II-induced hypertension and excess CVD [10]. Accordingly, it is particularly useful to investigate the role played by Ang II and related mechanisms in hypertension-related changes in the arterial wall, including the process of VSMC phenotypic modulation and extracellular matrix deposition [12].

Compelling evidence indicates that Ang II can turn on the synthesis of the very potent vasoconstrictor peptide Endothelin(ET)-1 in different vascular cell types, including cultured VSMC and endothelial cells (EC), via AT-1 receptors linked to protein-1/kinase C-mediated mechanisms [13–15]. ET-1 was shown to be markedly activated in TGR [16], where it could mediate the hypertrophic response to Ang II and thus play an important role in CVD and vascular remodeling [13–15] (for review, see Ref. [12]). It is also likely that BP lowering per se might elicit beneficial effects independent of blockade of specific peptide pathways [17], but this contention remains controversial because it was difficult to prove.

Thus, our aim was to investigate whether blockade of the dihydropyridine-sensitive calcium channel, the Ang II type 1 (AT-1), the ET-1 type A (ET_{A}) and B (ET_{B}) receptors, which might lower BP by different mechanisms, differentially affected VSMC phenotypic changes and collagen deposition in the arterial wall of TGR.

2. Methods

2.1. Animals and treatments

The study protocol and handling of animals followed our institutional guidelines for animal studies and were previously reported [18]. Four-week-old male heterozygous TGR were BW- and BP (tail-cuff method)-matched and randomly assigned to receive the following oral treatments: a placebo (n=8), the dihydropyridine calcium entry blocker nifedipine (30 mg/kg BW, n=6), the AT-1 receptor selective antagonist irbesartan (50 mg/kg BW, n=6), the mixed ET_{A}/ET_{B} endothelin receptor antagonist bosentan (100 mg/kg BW, n=6) or the ET_{A} selective receptor antagonist BMS-182874 (52 mg/kg BW, n=5). All these dosages were previously shown to provide maximal antihypertensive effects. At these dosages, nifedipine exerted a full-blown antihypertensive effect and normalized left ventricular (LV) mass in renovascular hypertensive rats [19]; bosentan abolished the pressor effect of exogenous ET-1 in TGR [20]; BMS-182874 exerted a maximal antihypertensive effect in rats [21] and blunted the pressor response to exogenous ET-1 [22]. The dosage of irbesartan was at least fourfold higher than that which prevented the pressor response to Ang II [23]. BMS-182874 and irbesartan were gifts of Dr James Powell of Bristol Myers Squibb (Princeton, NJ, USA) and bosentan of Dr Martine Clozel (Actelion Ltd, Allschwil, Switzerland). Nifedipine was prepared as a 0.1% stock solution in a solvent that consisted of 969 g of polyethylene-glycol 400 (Sigma), 60 g of glycerine (BDH), and 100 g of water. Since nifedipine undergoes UV destruction, the stock solution was prepared fresh every day, diluted by saline, and covered with aluminum foil. Each treatment was BW-tailored weekly and administered individually as chocolate-flavored tablets and lasted for 4 weeks; the last dosage was given 24 h before sacrifice [18]. The same chocolate-flavored tablets with no drug was given as a placebo.

2.2. Histomorphometric changes in arterioles and aorta

To assess the structural changes in the intra-myocardial arterioles, 5-μm-thick equatorial and serial sections of the LV tissue were stained with hematoxylin–eosin. All animals from each group were examined; for each rat four-to-six arterioles (i.d. 50–250 μm) cut across their transverse axis were examined at 20× magnification with a Leica DM photomicroscope equipped with QWin Standard Leica™ image software (Leica, Wetzlar, Germany). The inner diameter, calculated as the mean value of the major and minor axis, and the media thickness, estimated as the mean value of at least four values in each arteriole, were used to calculate the media cross-sectional area (MCSA). The same segment of thoracic and abdominal aorta from all rats was isolated, washed-perfused with PSS, snap frozen in dry ice-cooled isopentane and then stored in liquid nitrogen or at −80 °C until assayed. Six-μm-thick sections were then cut in the Leitz 1720 cryostat (Leitz, Wetzlar, Germany) and processed as reported [4]. Internal diameter and media thickness, estimated as specified above at magnification 5× in each section of aorta, were used to calculate the MCSA.

2.3. Immunohistochemistry and VSMC immunophenotyping

Immunophenotyping of VSMC from normal and hypertensive rats was accomplished with the use of the monoclonal antibodies to VSMC lineage (SM α-actin) and SMC differentiation (SM and MyHC-Apla2 myosin;
Table 1
Monoclonal antibodies used for the immunophenotypic characterization of VSMC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>VSMC</th>
<th>Ref./source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF-48</td>
<td>SM1-, SM2-type myosin heavy chain (MyHC)</td>
<td>Fetal- and adult-type VSMC</td>
<td>[24]</td>
</tr>
<tr>
<td>CloneA4</td>
<td>SM-type α-actin</td>
<td>Fetal- and adult-type VSMC</td>
<td>[3,6]</td>
</tr>
<tr>
<td>NM-G2</td>
<td>Platelet-type MyHC isoforms MyHC-Apla2</td>
<td>Fetal-type VSMC</td>
<td>[3,6]</td>
</tr>
<tr>
<td>IST-9</td>
<td>EIIIA-Fibronectin (Fn) isoform</td>
<td>Fetal-type VSMC</td>
<td>[5,25]</td>
</tr>
<tr>
<td>ET_A</td>
<td>Endothelin Receptor Subtype A</td>
<td>VSMC</td>
<td>Alomone Labs, Jerusalem, Israel</td>
</tr>
</tbody>
</table>

1 Cross-reacting with striated-type MyHC.
2 Purchased from Sigma, St Louis, MO.
3 IST-9 was a generous gift from Dr L. Zardi, Genoa, Italy.

EIIIA-Fn markers [3,6,24,25] which are shown in Table 1. In the abdominal rat aorta, coexpression of SM-MyHC, MyHC-Apla2 and EIIIA-Fn identifies the foetal-type VSMC, whereas down-regulation of the latter two markers in the presence of high SM immunoreactivity pertains to a well differentiated adult-type VSMC [5,6]. A commercially available polyclonal antibody to ET_A receptors was also used. The antibodies were applied to freshly cut unfixed cryosections as described [4]; the secondary antibody was the swine IgG anti-mouse IgG, or goat IgG anti-rabbit IgG, coupled with horseradish peroxidase (Dako, Dakopatts a/s, Glostrup, Denmark). Non-immune rat or rabbit serum was added to the secondary antibody to saturate non-specific binding-sites. Bound IgG was revealed by incubation in amino-ethyl-carbazole solution. Counterstaining was carried out with Harris’s hematoxylin. Non-immune IgG instead of the primary antibody were used in control experiments. Quantification of staining was carried out blindly by a single examiner, using the aforementioned photomicroscope and software. To minimise operator-dependent variability, a specific routine automatically detecting the red-stained areas corresponding to binding to the specific antigen was developed. All animals from each group were examined. The entire surface of aorta cryosections was measured at 40× magnification after careful exclusion of spoiled portions. At least five views were captured and analysed for each section. Results are expressed as percentage of tunica media area stained with chosen antibodies.

2.4. Endothelin receptor subtype density

Autoradiography was used to assess ET receptor density on iliac artery sections processed as reported [26]. All animals from each group were examined. In brief, ET-1 binding sites were labelled by incubation at room temperature for 120 min with 100 pmol/l [125I]ET-1 (Amersham Laboratories, Aylesbury, UK, specific activity 2000 Ci/mmol). Non-specific binding and selective displacement of [125I]ET-1 were studied by adding 1 μmol/l of unlabeled ET-1, or 100 nmol/l BQ-123 or BQ-788 (all from Neosystem Laboratories, Strasbourg, France), respectively. Washing thrice the samples in cold 50 nmol/l Tris–HCl buffer terminated the reaction. After rinsing in distilled water, the sections were rapidly dried, fixed in paraformaldehyde vapours at 80°C for 120 min, and then coated with NTB2 Kodak Nuclear emulsion (Eastman Kodak, Rochester, NY). Exposure lasted for 2 weeks at 4°C; undiluted D19 Kodak was used for development. ET_A and ET_B receptor density was quantified with computer-assisted image analysis software (Casti Imaging, Venice) coupled to a Leitz Laborlux™ microscope.

2.5. Effects of additional antihypertensive agents

The effect of BP-lowering on VSMC phenotypes in the abdominal aorta by different treatments was investigated in 5-week-old TGRen2 rats, which received a placebo (n=6), the dual ACE and neutral vasopeptidase inhibitor MDL-100,240 (MDL, 40 mg/kg BW, n=8), or ramipril (RAM, 5 mg/kg BW, n=8), as reported in detail [27]. In brief, treatment lasted for 4 weeks at the end of which the abdominal aorta was collected and processed as described above for the immunohistochemistry experiments. We carried out the immunostaining for SM α-actin and EIIIA-Fn [3,5,6].

2.6. Statistical analysis

Results are expressed as mean±S.D., or S.E.M. One-way ANOVA with Bonferroni’s post-hoc test and unpaired t-test was used for comparisons. Natural log-transformed values were used whenever Levene’s test showed a non-gaussian distribution. Statistical significance was set at α<0.05.

3. Results

3.1. Body weight, blood pressure and cardiac weight

Body weight was higher in the nifedipine than in the bosentan group but there were no other differences between groups (Table 2). BP was markedly lower in the irbesartan than in the other groups starting from the first week of treatment; by the end of the study period, it was
3.3. VSMC phenotypes

In the aortas from normotensive Sprague-Dawley rats (BP = 126 ± 26 mmHg at 8 weeks of age) the immunostaining for SM myosin and SM α-actin did not change appreciably during the developmental stages (19 days foetus, 7 days, 4 and 8 weeks) that were examined. In contrast, the proportion of VSMCs stained for non-muscle myosin and EIIIA-Fn decreased progressively from foetal, to neonatal and to adult stage (Fig. 1). Thus, in adult Sprague-Dawley rats, VSMCs expressing these antigens were barely detectable and mainly confined to the subendothelial region [3]. Compared to age-matched Sprague-Dawley rats, TGR (Fig. 1G,H) exhibited increased percentages of non-muscle myosin- and EIIIA-Fn-stained VSMCs in the media, despite a similar proportion of SM α-actin positive cells (Fig. 2). A significant correlation (r = 0.835, P < 0.0001) between EIIIA-Fn and MyHC-Apla2 immunostaining was found in these rats.

The different antihypertensive agents elicited quite different effects on VSMC phenotypes. The proportion of SM myosin positive VSMCs was similar in all groups, including placebo, but that of SM α-actin was variable and lower in nifedipine (33.3 ± 20.8%, P < 0.05) and BMS-182874 (40.2 ± 6.9%, P < 0.01) than in placebo (69.7 ± 14.2%), bosentan (67.2 ± 10.6%) and irbesartan groups (75.5 ± 12.7%).

3.4. Fibrillar collagen

The results of staining for fibrillar collagen of abdominal aorta sections from adult TGR receiving the different treatments are shown in Fig. 7. The proportion of stained vessel wall did not differ between placebo and BMS-182874 groups, but was markedly lower in the nifedipine group. The decrease in the bosentan and irbesartan groups did not achieve statistical significance.

3.5. Endothelin receptor subtypes density

 Autoradiography demonstrated specific [125I]ET-1 binding sites in the iliac artery wall; displacement with BQ-123 and BQ-788 showed the coexistence of both receptor...
subtypes. Table 2 shows the changes in $ET_\alpha$ and $ET_\beta$ receptor subtype density in the five experimental groups. Irbesartan increased significantly $ET_\alpha$ receptor density, compared to both placebo and BMS-182874; no differences between the other groups were seen. The $ET_\beta$ receptor density was similar in all treatment groups.

### 3.6. Effects of additional antihypertensive agents

The effects of MDL-100,240 and ramipril, compared to placebo, on BP and cardiac and vascular structure were recently reported in detail and therefore will be mentioned only briefly [26,27]. Both treatments proved to be similarly

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**Fig. 1.** Immunohistochemistry of aortic cryosections from 19-day-old fetus (A, B), day 2 (C, D) and 4 weeks (E,F) control Sprague-Dawley rats exposed to antibodies to EIIIA-Fn (A,C,E) and MyHC-Apla2 (B,D,F). Note that immunostaining of medial VSMC with both antibodies decreases during development. Asterisks indicate localization of antigens in the subendothelial portion of the media (m). Bars: 100 $\mu$m (panels A–D); 80 $\mu$m (panels E,F). Panels G and H show the exemplary results obtained with EIIIA-Fn (G) and MyHC-Apla2 (H) antibodies in an adult (8-week-old) male heterozygous TGR rat. Note the marked increase in VSMCs expressing the two antigens compared to an age- and sex-matched normotensive Sprague-Dawley rat (panels E and F). Bar: 100 $\mu$m (panels G,H); a, adventitia; m, media; vv, vasa vasorum.
the ‘contractile’ protein content [29], whereas VSMC hyperplasia would be mirrored by a shift of VSMC from a contractile to a synthetic and/or proliferating phenotype [2,3]. Aortic rings from TGR were found to exhibit increased maximal developed tension responses to receptor and non-receptor mediated vasoconstricting stimuli, which indicated an enhanced efficiency of the contractile machinery via qualitative or quantitative changes in VSMC [18]. Thus, the present and previous findings collectively indicate that vascular hypertrophy occurs early in the life span of TGR and involves both elastic and resistance type arteries.

We also found that the increased MCSA of the abdominal aorta occurring with the development of severe hypertension implied two consequences: (1) significant alterations of the proportion of VSMC expressing antigens that are normally found during foetal development, but vanish significantly (P<0.05) lower percentages of EIIIA-Fn and MyHC-Apla2 of foetal-type VSMC in the aortic media of normotensive animals compared to TGR rats.

effective in lowering BP and in preventing the development of cardiac hypertrophy and vascular structural changes. At the end of the fourth week of treatment, the immunostaining of the abdominal aorta for EIIIA-Fn [3,5], expressed as percent of immunostained area [6], was significantly lowered by both MDL-100,240 (0.46±0.13%, P=0.001) and ramipril (0.22±0.04%, P<0.001), compared to placebo (3.9±1.0%). Similar statistically significant differences between active treatments and placebo were seen when results were normalised for differences in SM α-actin immunostaining.

4. Discussion

One of the most relevant results of this study is the demonstration that the aorta, which has a twofold increase in Ang II content in this transgenic rat model [28], and the intramyocardial resistance artery wall developed marked hypertrophy, as indicated by increased media cross-sectional area. These changes were already evident at 8 weeks of age and are consistent with previous results in small mesenteric resistance arteries [18].

4.1. Mechanisms of vascular hypertrophy

Ang II-dependent hypertension has been linked with eutrophic remodelling of small resistance arteries and hypertrophy of elastic arteries in rats [1], but the underlying mechanisms remained to be clarified. According to one theory, VSMC hypertrophy would involve an increase in...
4.3. Effects of AT-1 receptor blockade

Up-regulation of non-muscle myosin and/or Fn in VSMC might be related to hypertension-induced mechanical stretch [29] and/or to paracrine effects of Ang II, which may occur via ET-1 [35,36]. To investigate the relative importance of the two peptides, we used an AT-1 antagonist and drugs interfering with ET-1 pressor mechanisms (see later). Irbesartan hindered the development of both hypertension and cardiac hypertrophy, a finding that accords with data with telmisartan [37] and with results with MDL-100,240 and ramipril [27]. However, although cultured VSMC from arteries of TGR showed hyperplastic changes and increased Ang II content [38], neither ACE nor AT-1 receptor blockade prevented DNA synthesis and cell proliferation in vitro. Furthermore, mesangial cells from TGR exhibited blunted \([Ca^{2+}]\) responses to Ang II [39]. Thus, it would appear that the VSMC from TGR are resistant to Ang II effects and thereby to AT-1 blockade. Accordingly, the favourable effects of irbesartan on VSMC phenotypic modulation and collagen deposition might be predominantly due to the BP lowering rather than to blockade of the autocrine–paracrine effects of Ang II. The fact that MDL-100,240 and ramipril, which lowered BP, accomplished a prevention of VSMC phenotypic modulation similar to that seen with irbesartan does not clarify this issue.

It might be proposed that the changes elicited by irbesartan on BP and VSMC could be ascribed to a different mechanism. As irbesartan, at the dosage used in this study, was shown to cross the blood–brain barrier in an acute study of spontaneously hypertensive rats [40], it might accomplish blockade of angiotensin receptors located in the central nervous system [41,42] and thereby reduce peripheral efferent sympathetic nerve activity [42]. However, this contention needs further investigation on the central effects of oral chronic irbesartan administration in TGR since the sympathetic inhibitory effect of irbesartan was not confirmed in all studies [43].

4.4. Effects of blockade of the endothelin receptor subtypes

ET-1 is a likely mediator of the pressor and growth-
promoting effects of Ang II [13–15] and was shown to be markedly activated in TGR [16], where it could mediate the hypertrophic response to Ang II and thus play an important role in CVD and vascular remodeling (for review, see Ref. [12]). To investigate the role of ET-1 and of its receptor subtypes we used the mixed ET$_{\alpha}$/ET$_{\beta}$ antagonist bosentan and an ET$_{\alpha}$ antagonist. This strategy was preferred to a head-to-head comparison of an ET$_{\alpha}$- and an ET$_{\beta}$-selective agent, because experience with AT-1 and AT-2 antagonist indicated that blockade of a single receptor subtype was associated with enhanced activation of the other, thus making enigmatic the interpretation of results. The information on ET-1 antagonist in experimental hypertension is conflicting (for review, see Refs. [12,18]). In rats treated with either infusion of exogenous Ang II [44] or administration of mineralocorticoid and/or salt [45], ET$_{\alpha}$ antagonists lowered BP and protected from CVD [46]. In contrast, neither a mixed nor an ET$_{\alpha}$-selective receptor antagonist [20–22,47] elicited such effects in this and previous studies in TGR [20]. Furthermore, ET$_{\alpha}$ blockade not only failed to prevent hypertension and cardiac hypertrophy, but worsened aortic hypertrophy, VSMC phenotypic modulation and fibrillar collagen deposition. In this regard the most striking of our findings was the increase in foetal-type VSMC population in BMS-182874 treated animals (Figs. 3G,H and 4), which was accompanied by a high (Figs. 6E and 7) level of fibrillar collagen deposition in the abdominal aorta tunica media. These nefarious effects accord with findings of impaired renal function in TGR [18], and of increased mortality following administration of an ET$_{\alpha}$ antagonist in spontaneously hypertensive rats [48]. Unlike BMS-182874, bosentan did not adversely affect VSMC phenotypic modulation (Figs. 2 and 3C,D) and collagen deposition (Figs. 6C and 7), possibly because the detrimental effect of ET$_{\alpha}$ blockade was counterbalanced by a favourable outcome of ET$_{\beta}$ blockade.

The density of ET$_{\alpha}$ and ET$_{\beta}$ receptors in the artery wall was similar in all groups with the exception of irbesartan that significantly increased the density of ET$_{\alpha}$ receptor, compared to placebo (Table 2), possibly because it prevented the down-regulation of ET$_{\alpha}$ receptor seen in placebo-treated TGR. The increase in foetal-type VSMC population in the latter TGR was associated with a patchy expression of ET$_{\alpha}$ immunostaining (Fig. 5C). Thus, collectively these results indicate that severe Ang II-dependent
hypertension implies a decreased and heterogeneous expression of ET$_A$ receptors within the arterial tunica media. The lack of BP lowering effect and the detrimental outcome on CVD of ET$_A$ blockade in TGR might be explained on this ground.

5. Conclusions

The development of severe hypertension in TGR is associated with early and prominent vascular structural changes that involve both elastic-type and small resistance arteries. In the aortic tunica media, these changes imply both an increase in foetal VSMC phenotypes and an increased fibrillar collagen deposition. AT$_1$ receptor blockade prevented hypertension and the vascular structural changes. Blockade of the dihydropyridine-sensitive calcium channels also protected TGR from collagen deposition even despite the lack of any BP lowering effect. In contrast, both VSMC phenotypic modulation and fibrillar
collagen deposition were enhanced by an ET₆ receptor antagonist, possibly because of the shift of endogenous ET-1 on the ET₆ receptors. Thus, in this model of Ang II-dependent hypertension, ET-1 intervenes in the autocrine–paracrine processes leading to vascular changes.

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