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Effects of captopril, losartan, and nifedipine on cell hypertrophy of cultured vascular smooth muscle from hypertensive Ren-2 transgenic rats

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1 We hypothesized that tissular renin-angotensin system (RAS) induces vascular hypertrophy in hypertensive Ren-2 transgenic rats (TGR; strain name TGR(mRen2)L27). This assumption was tested in cell cultures of vascular smooth muscle (VSMC) from both hypertensive TGR and control normotensive Sprague-Dawley (SD) rats. Planar cell surface area, protein synthesis, and protein content per cell were studied, the role for locally produced angiotensin II (AII) was evaluated and the possible pharmacological interference by different drugs was analysed.

2 By use of radioimmunoassay techniques, AII could be determined in TGR cultures (10.25 ± 0.12 pg per 10^7 cells) while it could not be detected in SD ones.

3 Under serum-free conditions, VSMC from hypertensive TGR were hypertrophic when compared to SD VSMC, as they presented a higher protein content per cell (335 ± 18 and 288 ± 7 pg per cell respectively; $P < 0.05$) and increased mean planar cell surface area, as determined by image analysis ($4{,}074 \pm 238$ and $4{,}764 \pm 204$ μm^2, respectively; $P < 0.05$).

4 When exogenously added to cultured SD and TGR VSMC, AII (100 pM to 1 μM) promoted protein synthesis and protein content in a concentration-dependent manner without affecting DNA synthesis. Maximal effects were observed at 100 nM. At this concentration, AII effectively increased planar cell surface area in both SD and TGR cultures by $\sim 20\%$.

5 Treatment of TGR cultures, in the absence of exogenous AII, with the angiotensin-converting enzyme inhibitor captopril or the angiotensin AT_1 receptors antagonist losartan (100 nM to 10 μM) reduced planar cell surface area in a concentration-dependent manner. In addition, both captopril and losartan (10 μM), decreased protein synthesis by $\sim 15\%$.

6 Treatment of SD VSMC, in the absence of exogenous AII, with both captopril and losartan had no effect either on planar cell surface area or protein synthesis.

7 Treatment with the Ca^{2+} antagonist nifedipine (100 nM to 10 μM) reduced cell size in both SD and TGR cultures. Maximal cell reduction reached by nifedipine averaged 906 ± 58 and $1{,}292 \pm 57$ μm^2, in SD and TGR, respectively ($P < 0.05$). In addition, nifedipine, nitrendipine and nisoldipine (all at 10 μM) decreased protein synthesis in both cell types by 15–25%.

8 We concluded that cultured VSMC from TGR are hypertrophic in comparison with those from SD. This cell hypertrophy can be the consequence of the expression of the transgene Ren-2 that activates a tissular RAS and locally produces AII, which acts in a paracrine, autocrine, or intracrine manner. Cell hypertrophy in TGR cultures could be selectively reduced by RAS blockade, while nifedipine decreased cell size and protein synthesis in both hypertrophic and non hypertrophic cells.

Keywords: Hypertensive transgenic rats; cultured vascular smooth muscle; cell hypertrophy; angiotensin II; captopril; losartan; nifedipine

Introduction

Medial thickening of blood vessels is closely related to arterial hypertension. In addition to remodeling processes (Mulvany, 1993), the enhanced thickness is achieved by increased smooth muscle mass that can be due to cellular hyperplasia and/or hypertrophy (Owens, 1989). These structural changes may represent an adaptative mechanism of the vessel to high blood pressure (Folkow, 1982). However, some studies indicate that the increase in wall thickness can precede the establishment of hypertension (Lee, 1985; Loeb *et al*., 1986; Eccleston-Joyner & Gray, 1988), thus suggesting that structural alterations may be responsible, at least in part, for the increase in blood pressure.

Among the factors that can directly promote growth of vascular smooth muscle cells (VSMC), many studies have focussed on the role played by the renin-angiotensin system (RAS) through the production of angiotensin II (AII). In this way, AII has been shown to induce VSMC hypertrophy either when infused *in vivo* (Holycross *et al*., 1993) or when exogenously added to cell cultures (Geisterfer *et al*., 1988; Berk *et al*., 1989; Millet *et al*., 1992). In addition to the circulating RAS, increasing evidence indicates that AII may be locally generated in many tissues, including blood vessels (Okunishi *et al*., 1987; Naftilan *et al*., 1991; Gohlke *et al*., 1992; Stock *et al*., 1995). It has been proposed that vascular AII plays an important role in the maintenance of the vessel structure and repair processes (Unger *et al*., 1991; Johnston, 1994). In this way, vascular angiotensin converting enzyme (ACE) activity has been shown to be increased in many forms of vascular remodeling (Okamura *et al*., 1986; Rakugi *et al*., 1991), while the administration of ACE inhibitors leads to regression of cardiovascular hypertrophy associated with hypertension (Owens, 1987; Levy *et al*., 1993).

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The transgenic rats harbouring the mouse Ren-2 gene (TGR; strain name TGR(mRen2)L27) have been developed from the Sprague-Dawley rat (SD) as a new model of genetic hypertension (Mullins *et al*., 1990). The insertion of the mouse Ren-2 gene into the rat genome produces severe hypertension that can be reverted by treatment with the ACE inhibitor, captopril (Mullins *et al*., 1990), or the AII receptor antagonist, losartan (Bader *et al*., 1992), thus indicating that the RAS, and particularly AII, is involved in the elevation of blood pressure. In addition, TGR express the Ren-2 gene at high levels in different tissues (Ganten *et al*., 1991), including blood vessels (Hilgers *et al*., 1992). Furthermore, enhanced release of angiotensin I (AI) and AII has been found at the vascular level (Hilgers *et al*., 1992; Campbell *et al*., 1995). These data suggest that tissular rather than circulating RAS is responsible for high blood pressure in TGR. In addition, previous data from our laboratory indicate that AII synthesized at the vascular level enhances the contractile response to different agonists in these rats (Arribas *et al*., 1994). Therefore, we hypothesize that tissular RAS could also participate in the development of vascular hypertrophy, which has been demonstrated in several arteries from TGR (Bachmann *et al*., 1992; Gross *et al*., 1995; Struijker-Boudier *et al*., 1996). To study the possible VSMC alterations leading to such structural changes, cell cultures from both hypertensive TGR and normotensive Sprague-Dawley (SD) rats were obtained, some growth parameters were studied, the role for locally produced AII was evaluated and the possible pharmacological interference by different drugs was analysed.

Methods

Cell culture

Primary cultures of VSMC were obtained as previously described (Peiró *et al*., 1995) by enzymatic dissociation of femoral arteries from 20 week old heterozygous Ren-2 transgenic rats (strain name TGR(mRen2)L27) and control Sprague-Dawley rats (SD); these control animals were derived from the Hannover Sprague-Dawley strain used to create the transgenic line (which was obtained from the Zentralinstitut für Versuchstierkunde, Hannover, Germany). Animals were obtained at 4 weeks of age from the University of Heidelberg (Germany) and then bred at the facilities of the Facultad de Medicina Autónoma (Madrid, Spain).

Rats were anaesthetized with sodium pentobarbitone (70 mg kg^{-1}, i.p.) and blood pressure was determined by cannulating the carotid artery. The cannula was connected to a transducer (Letica, Barcelona, Spain) and pressure was registered on a polygraph (2006, Letica). Pressure values, obtained from five animals of each strain, were (mean arterial blood pressure) 180.2 ± 5.0 and 97.2 ± 4.5 mmHg in TGR and SD rats, respectively. Afterwards, animals were exsanguinated and femoral arteries were carefully dissected, cleaned free of fat and connective tissue, cut into small pieces and placed in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, New York) containing 0.1% bovine serum albumin (BSA) (Sigma) and 4 mg ml^{-1} collagenase (type II, Sigma) for 90 min incubation at 37°C in a humidified atmosphere of CO_2 (5%) and air (95%). After washing three times by centrifugation, cells were resuspended in DMEM supplemented with 10% foetal calf serum (Gibco), 100 μml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} Amphotericin B (Sigma), and seeded into 25-cm^2 culture flasks (Nunc, Roskilde, Denmark). Cells were characterized as vascular smooth muscle by two different criteria: (1) morphologically, confluent cultures exhibited a 'hill and valley' pattern typical of smooth muscle; (2) immunohistochemical staining with smooth muscle-specific monoclonal antibody to α-actin (Dakopatts, Glostrup, Denmark), using the avidin-biotin peroxidase complex method (Hsu *et al*., 1981), with 98% of the cells showing positive stain. At confluence, cells were passaged by trypsinization with 0.05% trypsin-0.02% ethylenediamine-tetraacetic acid (EDTA; Gibco). Experiments were performed in VSMC pooled from five animals of each rat strain. Cells between passages 5 and 10 were used.

AII radioimmunoassay

AII was measured by radioimmunoassay in cell extracts and supernatants from both SD and TGR cultures. Confluent cultures were growth-arrested by replacing FCS-containing medium with vehicle medium, i.e., DMEM supplemented with 0.1% BSA and the aboved mentioned antibiotics, for 24 h. Cultures were then incubated for further 24 h with fresh basal medium. Supernatants were collected and supplemented with 1 mM captopril, 2 mM phenylmethylsulphonyl fluoride (PMSF; Sigma) and 5 μg ml^{-1} aprotinin (Sigma), whereas VSMC were dettached with trypsin-EDTA and washed twice by centrifugation in phosphate-buffered saline. The resulting pellets were resuspended in the following mixture: 140 mM NaCl, 20 nM Tris buffer pH 8.0, 0.1% Triton X-100 (Sigma), 1% Nonidet P-40 (Sigma), 2 mM EDTA, 1 mM captopril, 2 mM PMSF and 5 μg ml^{-1} aprotinin, lysed by three cycles of freezing and thawing and centrifuged at 800 *g* for 15 min to eliminate insoluble pellet fractions. Afterwards, both supernatants and cell soluble fractions were concentrated in a phenyl-silica column (Amersham, Buckinghamshire, U.K.) and submitted to radioimmunoassay, as described by the supplier (Angiotensin II radioimmunoassay kit, Diagnostics Pasteur, Marnes la Coquette, France).

Planar cell surface area

VSMC were sparsely seeded into 75-cm^2 culture flasks in medium containing 10% FCS. After cell attachment, culture medium was switched to vehicle medium either alone or containing the different drugs tested. After 24 h, the medium was renewed, cells cultured for additional 24 h and then fixed with 1% glutaraldehyde. Planar cell surface area was quantified by computer-assisted morphometry. Randomly selected images were transmitted to an Apple Macintosh Power 7100 computer by a video camera (Sony Corporation, Tokyo, Japan) connected to the microscope (Nikon, Tokyo, Japan) and therefore submitted to analysis with an appropriate software (NIH Image). Measurements were performed in a blind manner. At least 100 cells were counted for each treatment.

Determination of protein content per cell

To determine protein content per cell, VSMC were plated at different densities into 24-well plates, grown for 48–72 h in the presence of 10% FCS and then 24 h growth-arrested. Afterwards, wells were washed twice with a cold phosphate-buffered saline solution and cell layers solubilized with 0.2 N NaOH to determine protein content per well by the method of Bradford (1976), with bovine serum albumin (BSA) as standard. Cell number per well was determined in replicated plates according to the method described by Gillies *et al*. (1986). For each cell density, protein content per cell was determined in triplicate.

Protein and DNA synthesis

To determine protein synthesis, VSMC were plated into 24-well culture plates at a density of 5×10^4 cells/well. When confluent, VSMC were 24 h growth-arrested and then incubated with fresh vehicle medium containing [^{14}C]-leucine (0.5 μCi ml^{-1}; 50–60 mCi $mmol^{-1}$, Amersham), either alone or with the different drugs tested, for an additional 24 h. The medium was then aspirated, cells washed rapidly three times with cold phosphate-buffered saline solution and incubated at 4°C for 30 min in 10% trichloroacetic acid. The acid-insoluble material was solubilized by incubation with 0.2 N NaOH at 4°C overnight. Radioactivity was then measured in a Beckman LS8100 (Beckman Instruments, Inc., Fullerton, CA) liquid

scintillation counter while protein content per well was determined by the method of Bradford (1976). To determine DNA synthesis, the same procedure was followed except that [^{14}C]-leucine was replaced by [^{3}H]-thymidine (0.5 μCi ml^{-1}; 50–60 mCi mmol^{-1}, Amersham) during the labelling period.

Materials and statistical analysis

Captopril was from Squibb (Princeton, New Jersey) and nifedipine from Bayer AG (Wuppertal, Germany). Losartan was a generous gift from DuPont Merck (Brussels, Belgium). Unless otherwise stated, all other drugs or reagents were purchased from Sigma.

Values are given as mean ± s.e.mean. The statistical analysis was evaluated by unpaired Student's t test for single data points or by two-way analysis of variance (ANOVA) for curves, with the level of significance chosen at $P<0.05$.

Results

AII radioimmunoassay

In cell extracts from TGR VSMC, AII content determined by radioimmunoassay was 10.25 ± 0.12 pg/10^7 cells (results from three independent experiments). In cell extracts from SD-derived cultures, AII was undetectable. No AII could be detected in the supernatant from both types of cultures.

Planar cell surface area

After 48 h incubation in serum-free vehicle medium, mean planar cell surface area, determined by image analysis, was significantly higher in TGR cultures when compared with SD ones (Table 1). The addition to the medium of 100 nM AII enhanced cell size in both types of culture, this effect being abolished by the simultaneous administration of 10 μM losartan, antagonist of AT_1 receptors (Table 1).

In the absence of exogenous AII, the addition of the ACE inhibitor captopril (100 nM to 10 μM) or losartan (100 nM to 10 μM) to vehicle medium for 48 h reduced concentration-dependently the cell planar surface area in TGR while no effects were observed in SD cultures (Figure 1). Maximal cell reduction produced by both drugs in TGR cells were similar and reached 773 ± 62 and 621 ± 29 μm^2, for captopril and losartan, respectively.

Analogous experiments in the presence of the calcium channel antagonist nifedipine (100 nM to 10 μM) induced significant concentration-dependent reductions of cell size either in SD and TGR cultures (Figure 2). Maximal cell reduction reached by nifedipine averaged 906 ± 58 and 1,292 ± 57 μm^2, in SD and TGR, respectively ($P<0.05$). In TGR cultures, the maximal effect achieved by nifedipine was higher than that obtained with captopril or losartan ($P<0.05$).

Protein content determinations and protein synthesis experiments

Under serum-free conditions, VSMC derived from TGR rats showed a higher protein content than those derived from SD rats (335 ± 18 versus 288 ± 7 pg per cell, $P<0.05$, results from three independent experiments). For each strain, no differences in protein content per cell was found depending on culture cell density.

After 24 h incubation in serum-free vehicle medium, TGR VSMC showed enhanced basal [^{14}C]-leucine uptake in comparison with SD VSMC (3,168 ± 490 and 5,515 ± 480 d.p.m./well for SD and TGR cultures, respectively; $P<0.05$). The addition of AII (100 pM to 1 μM) to the medium during the labelling period promoted protein synthesis in both SD and TGR cultures; thus, there was a concentration-dependent increase of [^{14}C]-leucine uptake, which was maximal at 100 nM for both cell strains (Figure 3). However, the threshold concentration of AII was 100 pM and 1 nM for TGR and SD cultures, respectively. The effect of AII on protein synthesis was receptor-dependent as it was abolished by the addition of 10 μM losartan (data not shown). This enhancement of protein synthesis was accompanied by a parallel increase in protein content per well, which was maximal at 100 nM AII and reached 18.7 ± 3.5% and 22.1 ± 3.1% in SD and TGR cultures, respectively. The increase in protein synthesis and protein

Table 1 Comparative mean planar cell surface area of SD and TGR cultures: effect of 48 h treatment with AII (100 nM) and losartan (10 μM)

	Mean planar cell surface area (μm^2)	
	SD	*TGR*
Basal	4,074 ± 238	4,764 ± 204†
AII	4,844 ± 291*	5,745 ± 350*,†
AII + losartan	3,992 ± 215	3,969 ± 217*

Results are expressed as mean ± s.e.mean of three independent experiments. In each experiment, at least 100 cells were counted per treatment. *$P<0.05$ compared to basal. †$P<0.05$ compared to SD.

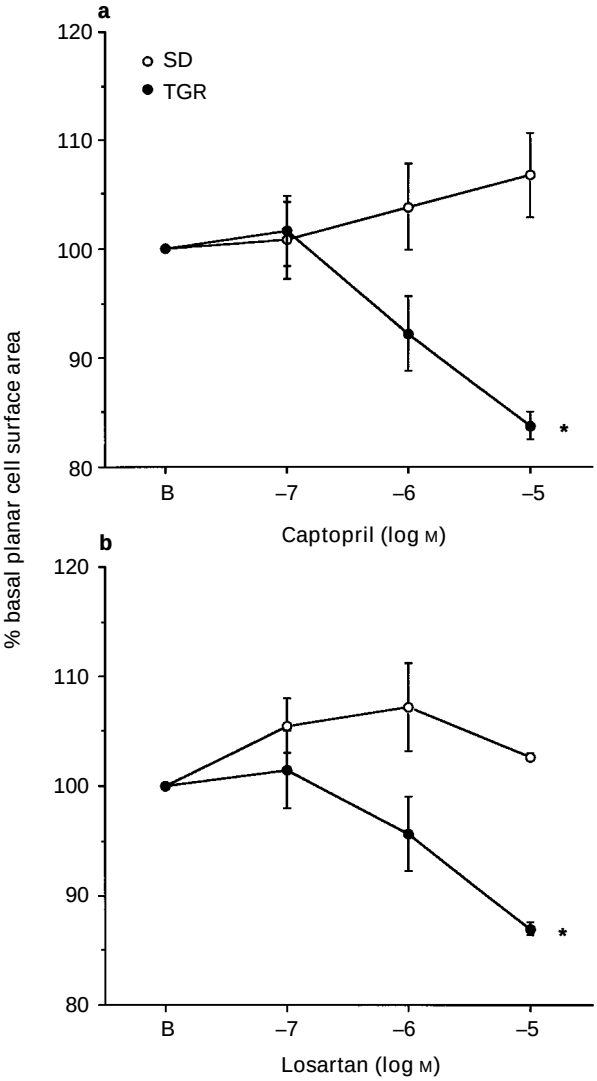

Figure 1 Planar cell surface area of SD and TGR cultured VSMC after 48 h treatment with captopril (a) or losartan (b). Data points represent mean of three independent experiments; vertical lines show s.e.mean. Results are expressed as the percentage of planar cell surface area of VSMC treated with vehicle medium for the same period. Analysis of the data was carried out by two-way ANOVA. *$P<0.05$ compared to SD.

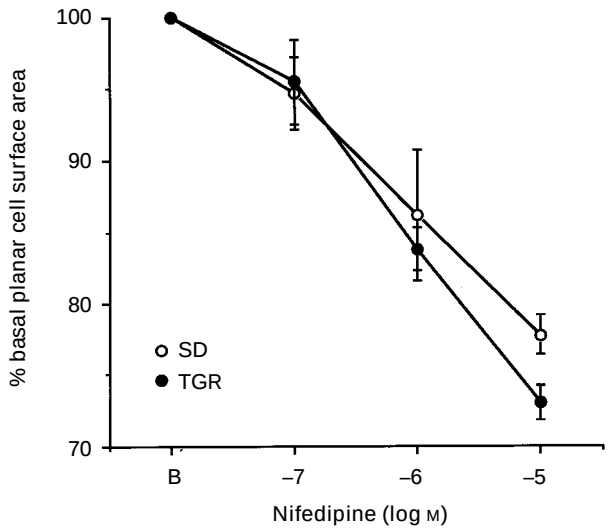

Figure 2 Effect of 48 h treatment with nifedipine on planar cell surface area of SD and TGR cultured VSMC. Data points represent mean of three independent experiments; vertical lines show s.e.mean. Results are expressed as the percentage of planar cell surface area of VSMC treated with vehicle medium alone for the same period.

content elicited by AII was not due to a proliferative effect, as AII failed to modify [^{3}H]-thymidine uptake at any of the concentrations used (data not shown).

Analogous experiments in both cell types were performed, in the absence of exogenous AII but in the presence of the RAS blockers captopril (10 μM) or losartan (10 μM). In TGR cultures, a significant reduction of [^{14}C]-leucine uptake was observed with either captopril or losartan (Figure 3). The degree of the reduction in basal protein synthesis achieved by both compounds was around 15%. These agents failed to modify protein synthesis in SD VSMC (Figure 3). On the other hand, the administration of 10 μM nifedipine in the same experimental conditions reduced [^{14}C]-leucine uptake in either SD or TGR cells to a similar degree (Table 2). Analogous results were observed with other dihydropiridinic calcium channel antagonists (Table 2). The reduction of protein synthesis induced by dihydropyridines in TGR VSMC was significantly higher than that produced by captopril or losartan ($P<0.05$).

Discussion

Enhanced wall thickness is a key feature of arterial hypertension. Whether it is a cause or result of high blood pressure, the enlargement of the vessel wall together with the reduction of the lumen diameter markedly contributes to the maintenance or even the amplification of hypertension (Folkow, 1982). As observed in other animal models of chronic hypertension (Owens & Schwartz, 1982; 1983), typical vascular structural alterations related to hypertension have been found in hypertensive TGR rats; thus, either in aorta, coronary, renal, and mesenteric vasculature from TGR, a marked enlargement of tunica media has been observed (Bachmann *et al*., 1992; Gross *et al*., 1995; Struijker-Boudier *et al*., 1996). Consistent with these observations, in the present work we observed that planar cell surface area and protein content per cell under basal conditions were significantly higher in TGR cultures in comparison with SD cells, indicating that VSMC from the hypertensive animals were hypertrophic.

In addition to haemodynamic forces related to hypertension, several substances have been shown to possess the ability to promote VSMC growth directly. Thus, the trophic action of AII, initially identified as a vasoactive compound, has been clearly demonstrated (Schelling *et al*., 1991). The TGR provide a genetic model of hypertension that differs from their normotensive SD control by a single gene defect, i.e., the insertion of the Ren-2 gene into the rat genome (Mullins *et al*., 1990). Among other tissues, the expression of the Ren-2 transgene has been demonstrated in the vascular wall (Bader *et al*., 1992;

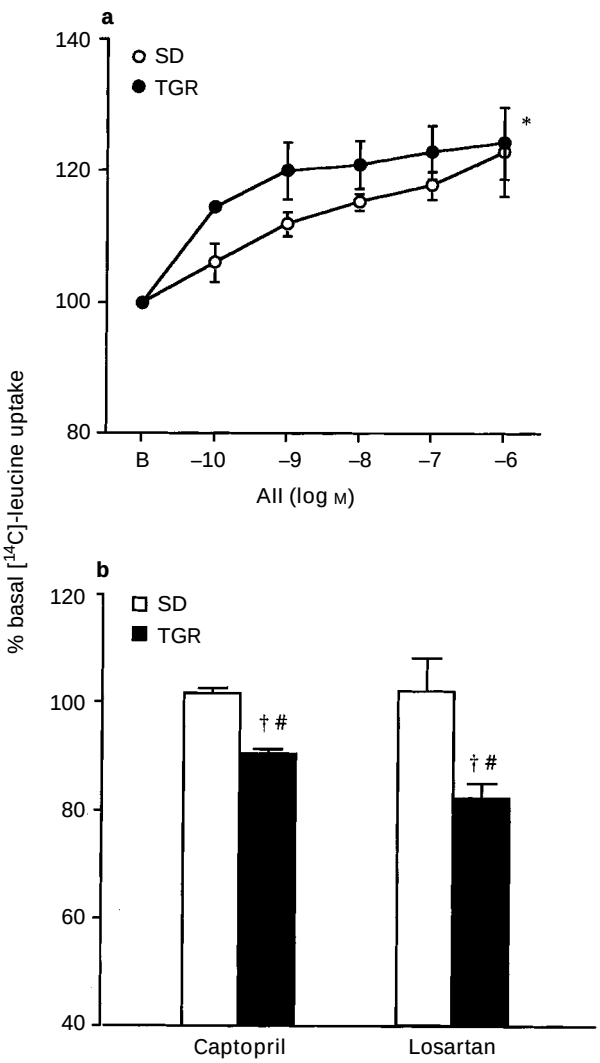

Figure 3 (a) Dose-dependent effect of AII on protein synthesis of SD and TGR cultured VSMC. Data points represent mean of three independent experiments each performed in triplicate; vertical lines show s.e.mean. Results are expressed as the percentage of [^{14}C]-leucine uptake in VSMC treated with vehicle medium. Analysis of the data was carried out by two-way ANOVA. *$P<0.05$ compared to SD. (b) Effect of 24 h treatment with captopril and losartan (both at 10 μM) on protein synthesis of SD and TGR VSMC. Columns represent means ± s.e.mean of three independent experiments each performed in triplicate. Analysis of the data was carried out by Student's *t* test. †$P<0.05$ compared to basal. #$P<0.05$ compared to SD.

Table 2 Effect of nifedipine (10 μM), nisoldipine (10 μM) and nitrendipine (10 μM) on basal protein synthesis of SD and TGR VSMC

	[^{14}C]-leucine uptake (% of basal)	
	SD	*TGR*
Nifedipine	73.2 ± 2.7*	76.9 ± 3.4*
Nisoldipine	75.5 ± 1.6*	81.6 ± 2.9*
Nitrendipine	80.5 ± 2.5*	86.3 ± 1.2*

Results are expressed as mean ± s.e.mean of three independent experiments performed in triplicate. Values of basal [^{14}C]-leucine uptake are in the text. *$P<0.05$ compared to basal.

Hilgers *et al*., 1992). Consistent with these results, in the present culture conditions, significant amounts of AII were detected in the cell extracts from TGR cells by radioimmunoassay, while no traces of AII were observed in SD ones. However, AII could not be detected by radioimmunoassay in the supernatant from both types of culture. It seems reasonable to propose that the differences in cellular AII content may be due to the expression of the Ren-2 transgene in cultures from TGR, which is activating an *in situ* RAS and synthesizing AII. Studies *in vivo* and *in vitro* suggest increased RAS activity in the vessels of Ren-2 transgenic rats (Hilgers *et al*., 1992; Arribas *et al*., 1994), which is consistent with a paracrine, autocrine or even intracrine regulation of the vascular tone by tissular RAS through the local production of AII (Arribas *et al*., 1994).

As indicated above, AII content in the culture medium of TGR VSMC was below the detection limit of the assay, so that the amount of AII appears to be very low. The fact that AII was not detected in the supernatant but was present inside VSMC suggests that AII can be rapidly internalized. Supporting this hypothesis, we have immunocytochemical data showing that AII is present within the cytoplasm of TGR VSMC, presumably in granule-like structures (unpublished results). The presence of AII in granules after an internalization process has been shown by other authors (Anderson *et al*., 1993). It is possible that, once inside the cell, AII may exert its action in an intracrine manner. This possibility has been reinforced by a recent study (Haller *et al*., 1996), which shows that microinjection of AII into cultured VSMC initiates a number of signalling mechanisms mediated by intracellular angiotensin receptors. These events may lead to the translocation of protein kinase C towards the nucleus, maybe affecting cell growth (Haller *et al*., 1996). In the present experiments, an intracrine action of AII is also consistent with the fact that the increases in protein synthesis or content and planar cell surface area in TGR VSMC are of the same magnitude independently of the culture density, indicating they are not related to the levels of AII in the culture medium.

There is controversy about the trophic role of AII when exogenously added to VSMC in culture, but most studies suggest this peptide acts only as a promotor of cell hypertrophy (Geisterfer *et al*., 1988; Berk *et al*., 1989; Millet *et al*., 1992). Previous data from our laboratory indicate that AII is not proliferative either in SD or TGR VSMC (Peiró *et al*., 1992). In the present work, we further confirmed that AII was unable to enhance DNA synthesis. Nevertheless, this peptide increased planar cell surface area in both types of cultures, which was abolished by losartan, indicating its dependence on AT_1 receptors. Furthermore, AII enhanced protein synthesis in both cell types in a concentration-dependent manner, although TGR cultures were more sensitive. The enhanced sensitivity to the effects of AII has also been observed in contractility experiments performed in our laboratory with TGR (Arribas *et al*., 1994). In this work, the isolated aortic rings from TGR required lower concentrations of AII than those from SD to exhibit vasoactive responses. Although at present we cannot provide a complete explanation, the higher sensitivity of VSMC from TGR may be due to: (1) an increased number of receptors on the cell surface; (2) an enhanced receptor affinity for the ligand; and (3) a higher amplification of intracellular signal transduction. At present, no studies exist, to our knowledge, to support any of these possibilities. However, an interesting finding was that the increased AII sensitivity was observed not only in isolated vascular segments but also in cell cultures, suggesting some involvement of genetic alterations that may be related to the inserted transgene Ren-2.

Our results suggest that AII is essentially hypertrophic for VSMC, which is consistent with most data concerning growth actions of this peptide in vascular smooth muscle (Schelling *et al*., 1991). Based on this, we tested whether the cell hypertrophy observed in VSMC from TGR was mediated by the endogenous production of AII, as this peptide could be detected by radioimmunoassay only in these cultures. In addition, recent work suggests that vascular hypertrophy in mesenteric arteries from TGR is mediated, at least partially, by enhanced tissular RAS activity (Struijker-Boudier *et al*., 1996). Therefore, we used different RAS blockers, like captopril and losartan, which have the ability to reduce high blood pressure in TGR *in vivo* (Mullins *et al*., 1990; Gardiner *et al*., 1995). Confirming our hypothesis, in the present experimental conditions, either the inhibition of ACE with captopril or the antagonism of AT_1 receptors with losartan decreased planar cell surface area in a concentration-dependent manner only in cells from TGR, while SD VSMC were unaffected. Both mechanisms of blocking the RAS activity in TGR cultures lead to a similar reduction of cell size. Additionally, protein synthesis was reduced by these drugs in TGR-derived but not in SD cells. The percentage of protein synthesis inhibition reached by both blockers of RAS was rather approximate. It is worthy of note that high concentrations of losartan were needed to inhibit the growth effects of either endogenous or exogenously added AII. In fact, we do not have a full explanation for such a result, although similar findings regarding AII-induced growth have been obtained by others (Ullian *et al*., 1993; Natarajan *et al*., 1994). There is evidence of a slightly lower affinity of angiotensin AT_1 receptors for losartan than for AII in rat cultured VSMC (Bunkenburg *et al*., 1992; Corriu *et al*., 1994). Another hypothetical explanation is that the internalization of receptors together with AII, which occurs very rapidly in VSMC (Anderson *et al*., 1993), may protect them from the antagonism by losartan. If so, this would reinforce an intracrine role for AII in promoting VSMC growth. However, at present this hypothesis requires further experimental support.

The fact that analogous effects were obtained with two different RAS blockers is consistent with the presence of a complete and functional RAS in TGR-derived cultures, which promotes cell hypertrophy. In agreement with our assumptions, previous data support the existence of a complete RAS in VSMC cultures. Functional studies performed in rat aortic VSMC in culture have shown the presence of captopril-sensitive ACE activity (Andre *et al*., 1990). In addition, other studies have confirmed the presence of ACE activity in cultured VSMC, which shows high sensitivity to ACE inhibitors (Millet *et al*., 1992; Ideishi *et al*., 1993), being dynamically regulated by glucocorticoids or FGF (Fishel *et al*., 1995). In addition, not only ACE activity but also ACE mRNA has been detected in cultured VSMC, further confirming that ACE can be synthesized by this cell type (Fishel *et al*., 1995). Although the present experiments were performed after 24–48 h of serum deprivation, we cannot completely discard the possibility that angiotensinogen may have been trapped into VSMC from serum-containing medium used before the growth arrest. However, this possibility is not unique; indeed, in the medial smooth muscle layer of rat aorta, angiotensinogen mRNA has been detected at low levels (Naftilan *et al*., 1991), thus indicating the ability of VSMC to synthesize angiotensinogen that may be the precursor of locally synthesized AII.

It is interesting to note that the hypertrophic cellular mechanisms seem to be constitutively activated in TGR cultures, without requiring the administration of any additional trophic factor. Furthermore, these cells are hypertrophic in the absence of high blood pressure conditions. These facts, in agreement with data previously obtained (Struijker-Boudier *et al*., 1996), support a role for local RAS in enhancing wall thickness *in vivo*, suggesting also that cell hypertrophy rather than hyperplasia accounts for the structural alterations in the TGR vasculature (Bachmann *et al*., 1992). Additionally, it is possible to speculate about the chronological relationship between the development of hypertension and vascular wall enlargement in this hypertensive model. As the Ren-2 transgene is expressed in many organs at the time of birth (Zhao *et al*., 1993), it is reasonable to assume that locally synthesized AII could induce VSMC growth before the establishment of the hypertensive phenotype.

In contrast to the antihypertrophic effects of the RAS blockers, which were selective for the TGR cultures, planar cell

surface area and protein synthesis of either SD or TGR VSMC were reduced by the administration of dihydropyridine calcium channel antagonists. While many studies have investigated the effectiveness of these drugs in reducing the replication of VSMC (see review by Jackson & Schwartz, 1992), none, to our knowledge, have focused on the elucidation of their influence on VSMC hypertrophy. However, some studies in isolated cardiac myocytes have suggested that the hypertrophy induced by AII, endothelin-1 or noradrenaline is sensitive to dihydropyridines (Grohé *et al*., 1994; Lubic *et al*., 1994). In the present experiments, rather high concentrations of these agents were required, which were in the same range as those needed to reduce cell proliferation (Jackson & Schwartz, 1992). These elevated concentrations could mean that unspecific mechanisms of the dihydropyridines are involved in reducing cell size. Furthermore, although the effects of nifedipine were slightly higher in TGR cells, the cultures from SD also showed marked reductions in planar cell surface area and protein synthesis, indicating that the effects of the dihydropyridines were not selective for hypertrophic TGR cells, in contrast to the effects of the RAS blockers captopril and losartan.

In spontaneously hypertensive rats, hypertension-induced vascular hypertrophy *in vivo* may be reduced by Ca^{2+} channel blockers to a similar extent as with an ACE inhibitor (Morishita *et al*., 1992). The mechanism for such an effect is not well defined but it is not solely related to the antihypertensive action of dihydropyridines (Morishita *et al*., 1992). In conscious TGR, the use of a dihydropyridine Ca^{2+} antagonist is effective in lowering elevated blood pressure (Hirth-Dietrich *et al*., 1994), although no studies have been published concerning the possible *in vivo* antihypertrophic cardiovascular effects of these agents in this specific model of hypertension.

We conclude that cultured VSMC from hypertensive Ren-2 transgenic rats are hypertrophic in comparison with cells from normotensive SD. This cell hypertrophy could be the consequence of the expression of the renin gene that activates a tissular RAS and produces AII locally, which then acts in a paracrine, autocrine or intracrine manner. Cell hypertrophy in TGR cultures could be selectively reduced by RAS blockade, whereas dihydropyridines decreased cell size and protein synthesis in both hypertrophic and non hypertrophic VSMC.

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