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Nifedipine, losartan and captopril effects on hyperplasia of vascular smooth muscle from Ren-2 transgenic rats

Concepción Peiró [a], Javier Angulo [a], Javier Regadera [b], José L. Llergo [a], Alberto Sánchez-Ferrer [c], Leocadio Rodríguez-Mañas [c], Carlos F. Sánchez-Ferrer [a,*]

[a] *Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, c / Arzobispo Morcillo 4, 28029-Madrid, Spain*

[b] *Departamento de Morfología, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain*

[c] *Unidad de Investigación y Servicio de Geriatría, Hospital Universitario de Getafe, Madrid, Spain*

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Abstract

Vascular smooth muscle cells from hypertensive transgenic rats for the mouse Ren-2 gene exhibited radioimmunoassayable angiotensin II and hyperplasia in comparison with cells from Sprague-Dawley rats. However, neither captopril, losartan, saralasin, nor PD123319 (all at 10 μM) modified DNA synthesis or cell number observed in 4-day growth curves with 10% fetal calf serum. Nifedipine reduced DNA synthesis in both cell types, the concentration required being significantly higher in Sprague-Dawley- (1 μM) than in transgenic-derived cultures (100 nM). The EC_{50} values were of 2.43 ± 0.32 and 1.0 ± 0.17 μM, respectively ($P < 0.05$). In both cell types, only 10 μM nifedipine reduced serum-induced cell proliferation, but inhibition percentage was higher in transgenic-derived cultures. In conclusion, hyperplasia of transgenic-derived vascular smooth muscle cells is not blocked by angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists, but these cells are more sensitive to the antiproliferative effects of nifedipine.

Keywords: Hyperplasia; Smooth muscle, vascular; Cell culture; Nifedipine; Captopril; Losartan; Transgenic rat

1. Introduction

Hypertrophy and/or hyperplasia of the vascular smooth muscle cells are events closely related to arterial hypertension (Schwartz et al., 1990; Heagerty et al., 1993). This is not merely the result of adaptative changes to increased blood pressure but other genetically determined mechanisms may be involved. Indeed, it is well known that cell cultures obtained from vascular smooth muscle of spontaneously hypertensive rats exhibit higher rates of proliferation than those from normotensive Wistar-Kyoto controls, when exposed to serum or other growth factors (Yamori et al., 1981; Clegg et al., 1986; Scott-Burden et al., 1991), indicating that these cells retain in vitro some abnormal proliferative characteristics. Although the mechanisms responsible for excessive vascular smooth muscle growth in hypertension are not well understood, different growth factors, vasoactive peptides, and intracellular mediators, like Ca^{2+}-dependent signaling mechanisms, have been implicated (Hamet et al., 1991; Bohr et al., 1991; Scott-Burden et al., 1992).

The transgenic rats harbouring the mouse Ren-2 gene have been developed from the Sprague-Dawley rat as a model of experimental 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 inhibitor of the angiotensin-converting enzyme captopril (Mullins et al., 1990) or the antagonist of angiotensin II receptors losartan (Bader et al., 1992), thus indicating that the renin-angiotensin system, and particularly angiotensin II, may be involved in the elevation of blood pressure. In addition, hypertensive transgenic rats express the Ren-2 gene at high levels in

* Corresponding author. Tel.: (34-1) 397-5470; Fax: (34-1) 3397-5353; e-mail: carlosf@mvax.fmed.uam.es

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different tissues (Ganten et al., 1991), including blood vessels (Hilgers et al., 1992). Indeed, enhanced release of both angiotensin I and angiotensin II at the vascular level (Hilgers et al., 1992, 1994; Campbell et al., 1995) has been described in these rats. These data suggest that tissular rather than circulating renin-angiotensin system is responsible for high blood pressure in this hypertensive model. In addition, previous data from our laboratory indicate that angiotensin II synthesized at the vascular level is enhancing the contractile response to different agonists in these rats (Arribas et al., 1994).

As observed in other models of hypertension, morphological vascular changes have been described in Ren-2 transgenic rats such as an enhanced thickness of tunica media in aorta, coronary, and renal arteries (Bachmann et al., 1992; Gross et al., 1995). The activation of the renin-angiotensin system has been proposed as an important trophic factor for the vascular wall by the production of angiotensin II (Daemen et al., 1991; Lever et al., 1992; Van Kleef et al., 1992). However, conflictive results have been obtained with respect to the mitogenic ability of this peptide in cultured vascular smooth muscle cells, while its ability to increase protein synthesis is well documented (Geisterfer et al., 1988; Berk et al., 1989; Harris et al., 1990; Scott-Burden et al., 1991; Morton et al., 1995). In a previous work from our laboratory, we reported a higher rate of proliferation in cultured vascular smooth muscle cells from Ren-2 transgenic rat arteries, in comparison with their respective Sprague-Dawley controls (Peiró et al., 1992). In the present work, we further analyzed the growth characteristics of these cultures, as well as its possible interference with the renin-angiotensin system and Ca^{2+} channel blockers.

2. Materials and methods

2.1. Cell culture

Primary cultures of vascular smooth muscle cells 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; these control animals were derived from the Hannover Sprague-Dawley strain used to create the transgenic line (which were 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 anesthetized with 70 mg/kg i.p. sodium pentobarbital 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) of 180.2 ± 5.0 and 97.2 ± 4.5 mmHg in transgenic and Sprague-Dawley 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, NY, USA) containing 0.1% bovine serum albumin (Sigma, St. Louis, MO, USA) and 4 mg/ml 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% fetal calf serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml 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. No staining was observed when cultures were treated with an antibody raised against *Ulex europaeus* agglutinin (Vector, Burlingame, CA, USA), which binds endothelial cells. At confluence, cells were passaged by trypsinization with 0.05% trypsin-0.02% EDTA (Gibco). Experiments were performed in vascular smooth muscle cells pooled from five animals of each rat strain. Cells between passages 5 and 10 were used.

2.2. Angiotensin II radioimmunoassay

Angiotensin II was measured by radioimmunoassay in cell extracts from both Sprague-Dawley- and transgenic-derived cultures. Confluent cultures were growth-arrested by replacing fetal calf serum-containing medium with vehicle medium, i.e., DMEM supplemented with 0.1% bovine serum albumin and the above mentioned antibiotics, for 24 h. Cultures were then incubated for a further 24 h with fresh vehicle medium. Cells were then detached 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 phenylmethylsulfonyl fluoride (Sigma) and 5 μg/ml aprotinin (Sigma), lysed by three cycles of freezing and thawing and centrifuged at $800 \times g$ for 15 min to eliminate insoluble pellet fractions. Afterwards, the soluble fractions were concentrated in a phenyl-silica column (Amersham, Buckinghamshire, UK) and subjected to radioimmunoassay, as described by the supplier (Angiotensin II radioimmunoassay kit, Diagnostics Pasteur, Marnes la Coquette, France).

2.3. Growth curves

Cell number was determined by the method of Gillies et al. (1986). Briefly, vascular smooth muscle cells were trypsinized, resuspended in culture medium containing 10% fetal calf serum and plated into 24-well culture plates (Nunc). After different time intervals, cells were fixed with 1% glutaraldehyde, washed twice with phosphate-buffered saline, stained with 1% crystal violet (Fluka, Buchs, Switzerland) for 30 min and extensively washed with deionized water. Plates were allowed to dry overnight. Crystal violet was extracted from cells by adding 2 ml of 10% acetic acid per well and the resulting color was measured by absorbance at 595 nm in an EL-340 automated microplate reader (Bio-Tek Instruments, Winooski, VT, USA). To validate this method for vascular smooth muscle cells, a standard curve was carried out to establish the relationship between optic density and cell number counted by hemocytometer ($r = 0.978$).

2.4. DNA synthesis

After trypsinization, vascular smooth muscle cells were plated into 24-well culture plates at a density of 5×10^4 cells/well and grown to confluence. Cells were then 24 h growth-arrested and then incubated for an additional 24 h with fresh vehicle medium containing [^{3}H]thymidine (0.5 μCi/ml, 50–60 mCi/mmol, Amersham). Afterwards the supernatant was aspirated, cells washed rapidly three times with cold phosphate-buffered saline and incubated at 4°C for 30 min in 10% trichloroacetic acid. The acid-insoluble material was solubilized by incubation with 0.2 M NaOH at 4°C overnight and radioactivity measured in a Beckman LS8100 (Beckman Instruments, Fullerton, CA, USA) liquid scintillation counter. The different compounds to be tested were added during the labeling period. Analogous experiments were performed to determine protein synthesis, with an identical design except that [^{3}H]thymidine was replaced by [^{14}C]leucine (0.5 μCi/ml, 50–60 mCi/mmol, Amersham) during the labeling period.

2.5. Materials and statistical analysis

Captopril was from Squibb (Princeton, NJ, USA), and nifedipine from Bayer (Wuppertal, Germany). Losartan was a generous gift from DuPont Merck (Brussels, Belgium). PD123319 was a generous gift from Parker-Davis (Ann Arbor, MI, USA). Nitrendipine and nisoldipine were a generous gift from Laboratorios Alter (Madrid, Spain). All other drugs or reagents were purchased from Sigma.

Values are given as mean ± S.E.M. Results were obtained in separate experiments performed in different days, at least three or four, each one performed in triplicate. 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 $P < 0.05$. Student's *t*-tests were also employed in the statistical comparison of the EC_{50} values, the latter defined as the concentration required to produce half the maximum effect.

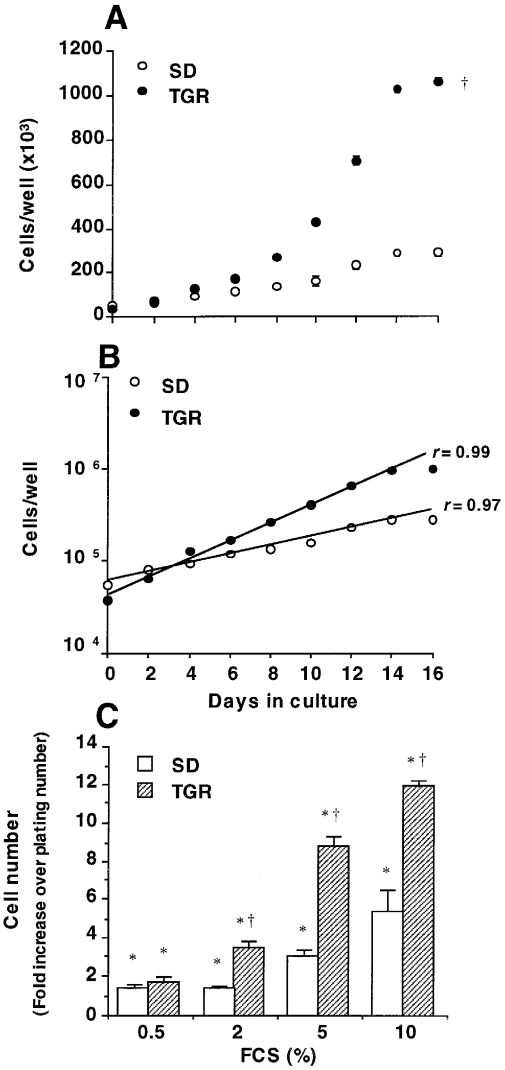

Fig. 1. (A) Growth kinetics of vascular smooth muscle cells from Sprague-Dawley (SD) and transgenic (mRen-2)L27 (TGR) rats. Cells were plated into 24-well plates and grown in culture medium containing 10% fetal calf serum (FCS), which was renewed every 2 days. Cell number per well was determined at 48-h intervals as described in Section 2. Data points represent mean ± S.E.M. of four separate experiments performed in triplicate. † $P < 0.05$ between SD and TGR cultures (ANOVA). (B) Semi-logarithmic representation of panel A. The slope of the linear portion, which corresponds to the exponential phase of cell growth, permits to calculate the population doubling time according to the following equation: doubling time = log 2/slope. The values of the doubling time are given in the text. *r*, linear regression coefficient. (C) Effect of growing concentrations of fetal calf serum on vascular smooth muscle cell proliferation. Cells were plated at a density of 5×10^4 cells/well in culture medium containing 10% fetal calf serum to permit cell attachment. After 3 h, medium was replaced by fresh medium containing 0.5, 2, 5 or 10% fetal calf serum. Cell number per well was determined after 6 days of culture. Data are expressed as mean ± S.E.M. of four separate experiments performed in triplicate. * $P < 0.05$ versus plating number, † $P < 0.05$ versus SD.

3. Results

3.1. Angiotensin II radioimunoassay

In vascular smooth muscle cells derived from transgenic rats, angiotensin II content determined by radioimmunoassay was of 13.3 ± 0.97 pg/10^7 cells (results from three independent experiments). In Sprague-Dawley-derived cultures no angiotensin II could be detected.

3.2. Growth curves

The proliferative response of vascular smooth muscle cells from both transgenic and Sprague-Dawley rats to fetal calf serum was compared by determining cell number per well at 48-h intervals. As shown in Fig. 1A, vascular smooth muscle cells derived from hypertensive transgenic rats showed a much higher proliferation rate than those derived from normotensive control rats when cultured with 10% fetal calf serum ($P < 0.05$). In these specific experimental conditions, in which culture medium was renewed every 2 days, population doubling time, calculated during the logarithmic phase of growth, was significantly shorter for transgenic cultures (70 ± 5 vs. 145 ± 4.5 h, $P < 0.05$) (Fig. 1B). When serum concentration was switched to 0.5% no differences of proliferation were observed between both cell strains after 6 days of culture (Fig. 1C), thus suggesting that the hyperplastic response of transgenic cells was dependent on fetal calf serum. Indeed, the addition of growing concentrations (2%, 5% and 10%) of fetal calf serum to culture medium resulted in concentration-dependent differences between the cell number achieved in Sprague-Dawley- and transgenic-derived cultures (Fig. 1C).

To assess whether the hyperresponsiveness of vascular smooth muscle cells from Ren-2 transgenic rats to fetal calf serum was related to the production of angiotensin II, growth curves were performed in the presence of the angiotensin-converting enzyme inhibitor captopril (10 μM). As shown in Fig. 2A, the addition of captopril to culture medium did not modify the proliferation rate induced by 10% fetal calf serum either in Sprague-Dawley- or transgenic-derived cultures. Furthermore, no changes in growth curves were observed when cultures were treated with the angiotensin AT_1 receptor antagonist losartan (10 μM) (Fig. 2B). The non-specific angiotensin receptor antagonist saralasin (10 μM) also failed to modify the mitogenic effect of fetal calf serum (Fig. 2C). Similar results were obtained with the angiotensin AT_2 receptor antagonist PD123319 (10 μM) (Fig. 2D).

In the presence of the Ca^{2+} channel blocker nifedipine (10 μM), cell proliferation was reduced in both Sprague-Dawley- and transgenic-derived cultures from 2 days of treatment (Fig. 3A). However, the inhibitory effects of nifedipine were significantly higher in cultures from trans-

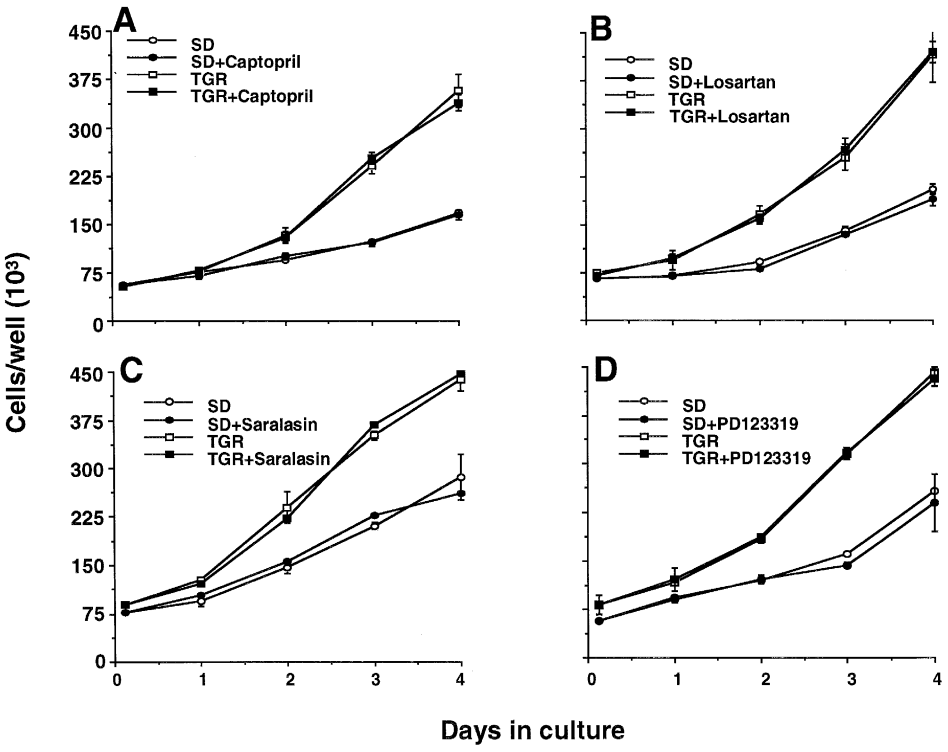

Fig. 2. Effect of 10 μM captopril (A), 10 μM losartan (B), 10 μM saralasin (C) or 10 μM PD123319 (D) on 10% fetal calf serum-induced proliferation. Vascular smooth muscle cells from Sprague-Dawley (SD) and transgenic (mRen-2)L27 (TGR) rats were seeded into 24-well plates in medium containing 10% fetal calf serum that was replaced after 3 h by the same medium either alone or containing captopril or losartan. Fresh medium was added every day. Data points represent mean ± S.E.M. of three separate experiments performed in triplicate.

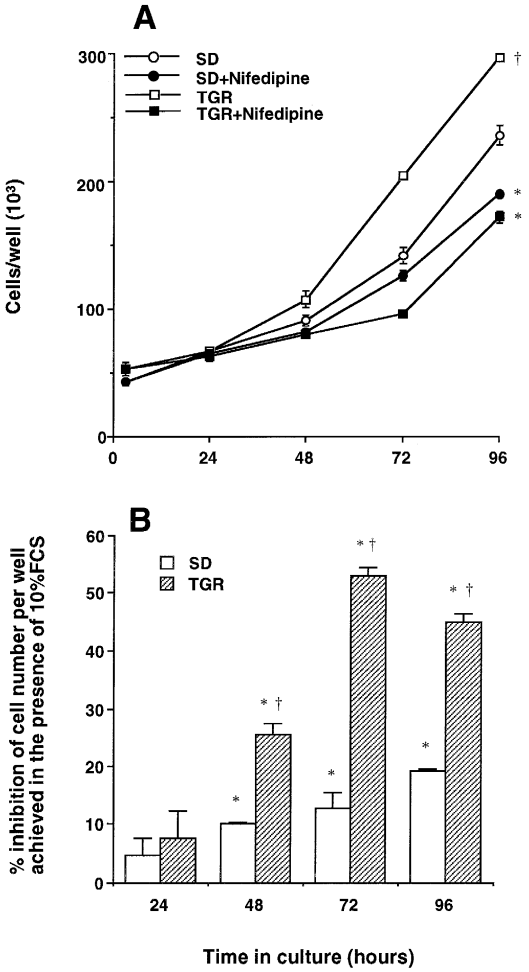

Fig. 3. (A) Effect of 10 μM nifedipine on 10% fetal calf serum (FCS)-induced proliferation. Vascular smooth muscle cells from Sprague-Dawley (SD) and transgenic (mRen-2)L27 (TGR) rats were seeded into 24-well plates in medium containing 10% fetal calf serum that was replaced after 3 h by the same medium either alone or containing nifedipine. Fresh medium was added every day. Data points represent mean ± S.E.M. of three separate experiments performed in triplicate. * $P < 0.05$ versus cultures of the same strain treated with 10% fetal calf serum alone (ANOVA), † $P < 0.05$ versus SD cultures treated with 10% fetal calf serum (ANOVA). In panel B, the effect of nifedipine is expressed as the percentage of inhibition of cell number per well obtained in the presence of 10% fetal calf serum alone. * $P < 0.05$ versus untreated cultures, † $P < 0.05$ versus SD.

genic compared to Sprague-Dawley rats (Fig. 3B). Indeed, in these specific experimental conditions, in which medium was renewed every day, nifedipine increased population doubling time by 6.6 ± 1 and 28 ± 4 h (i.e., from 40.6 ± 1.2 to 47.2 ± 1.4 h and from 31.3 ± 3.3 to 54.6 ± 3 h) in Sprague-Dawley- and transgenic-derived cultures, respectively. Treatment with 1 μM nifedipine failed to significantly reduce 10% fetal calf serum-induced proliferation in both types of cultures (data not shown). To discard toxic effects of nifedipine, the cultures were checked every day along growth curves, as well as before and after nifedipine treatment in [^{3}H]thymidine uptake experiments. When observed under microscope, cell cultures did not present a damaged aspect nor enhanced cell loss was observed. At the concentrations used in this study, nifedipine had no toxic effects on cultured vascular smooth muscle cells, as reported by different authors (see Jackson and Schwartz, 1992). In our experimental conditions, microscopical cell damage was observed only at very high concentrations of nifedipine (1 mM or higher).

3.3. DNA synthesis

To determine the rate of DNA synthesis, vascular smooth muscle cells were plated at a density of 5×10^4 cells/well and grown in the presence of 10% fetal calf serum untill confluence. After 24 h of growth arrest, the cultures were incubated with [^{3}H]thymidine for an addi-

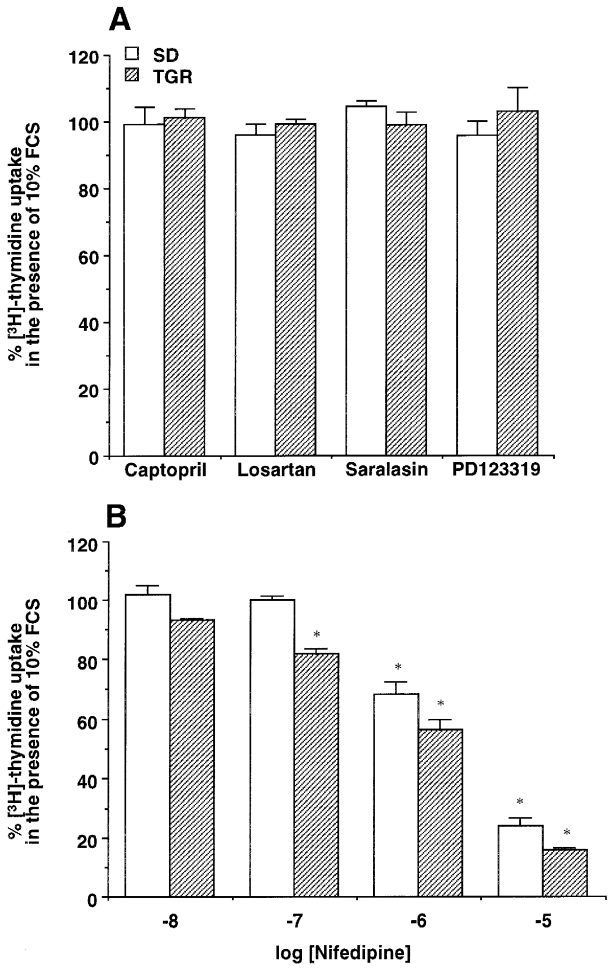

Fig. 4. (A) Effect of captopril, losartan, saralasin and PD123319 (all at 10 μM) and (B) nifedipine (10 nM to 10 μM) on [^{3}H]thymidine uptake induced by 10% fetal calf serum (FCS) in vascular smooth muscle cells from Sprague-Dawley (SD) and transgenic (mRen-2)L27 (TGR) rats. Results are expressed as percentage of the uptake induced by fetal calf serum, which absolute values are given in the text. Bars represent mean ± S.E.M. of four separate experiments performed in triplicate. * $P < 0.05$ versus fetal calf serum-induced uptake. The response of SD and TGR vascular smooth muscle cells to nifedipine was statistically different, as evaluated by ANOVA ($P < 0.05$).

tional 24 h in serum-free vehicle medium. In these conditions, [^{3}H]thymidine uptake was of 4986 ± 188 dpm/well and 19 305 ± 1286 dpm/well for Sprague-Dawley- and transgenic-derived cultures, respectively ($P < 0.05$; results from six experiments performed in triplicate). When 10% fetal calf serum was added to the culture medium during the labeling period, DNA synthesis was markedly increased in both cell types reaching 60 685 ± 2149 and 156 848 ± 3560 dpm/well in Sprague-Dawley- and transgenic-derived cultures, respectively ($P < 0.05$; results from six experiments performed in triplicate).

Fetal calf serum-induced [^{3}H]thymidine uptake was not altered when cultures from Sprague-Dawley or transgenic rats were treated during the labeling period either with captopril, losartan, saralasin, or PD123319 (all at 10 μM) (Fig. 4A). However, nifedipine (10 nM to 10 μM) reduced DNA synthesis in a concentration-dependent manner (Fig. 4B), the sensitivity of transgenic cultures to the effects of nifedipine being significantly higher than that observed in control Sprague-Dawley cells. The values of EC_{50} were of 2.43 ± 0.32 μM versus 1.0 ± 0.17 μM in Sprague-Dawley- and transgenic-derived cultures, respectively ($P < 0.05$). The treatment with nitrendipine (10 μM) or nisoldipine (10 μM) yielded results analogous to that obtained with nifedipine at the same concentration (data not shown).

To discard that the lack of action of captopril and losartan was due to inability of the drugs to exert its pharmacological actions in the present experimental conditions, we performed experiments in which protein synthesis, determined by the uptake of [^{14}C]leucine, was used as a marker of activity. Indeed, when angiotensin II (100 nM) was added to serum-free vehicle medium, there was a significant increase in [^{14}C]leucine uptake both in Sprague-Dawley- (from 10 358 ± 939 to 16 460 ± 636 dpm/well, i.e., by 58.9 ± 6.1%; $P < 0.05$) and transgenic-derived cultures (from 13 820 ± 297 to 22 460 ± 301 dpm/well, i.e. by 62.5 ± 2.1%; $P < 0.05$). The protein synthesis induced by angiotensin II was abolished by the addition of 10 μM losartan (the [^{14}C]leucine uptake decreased by 57.5 ± 4.5 and 60.3 ± 3.3% in Sprague-Dawley- and transgenic-derived cultures, respectively). Similar effects were obtained with saralasin (10 μM; data not shown). Furthermore, when cells from transgenic rats were treated with angiotensin I (100 nM) there was an increase in [^{14}C]leucine uptake similar to that observed with 100 nM angiotensin II (60.1 ± 4.5% increase). Captopril (10 μM) reduced the effect of angiotensin I by 30 ± 1.2%, this effect being completely abolished when losartan (10 μM) was employed in addition to captopril.

4. Discussion

The Ren-2 transgenic rat is a genetic model of fulminant hypertension that differs from normotensive Sprague-Dawley rats by a single gene defect, i.e., the insertion of the mouse renin Ren-2 gene into the rat genome, which is expressed, among other tissues, in the vasculature (Bader et al., 1992; Hilgers et al., 1992). In the present culture conditions, significant radioimmunoassayable angiotensin II was detected in the cell extracts from transgenic-derived cells, while no traces of angiotensin II were observed in Sprague-Dawley-derived cultures. It seems reasonable to propose that these differences in angiotensin II content may be due to the expression of the Ren-2 gene in cultures from transgenic rats, which is activating an in situ renin-angiotensin system and synthesizing angiotensin II. Studies in vivo and in vitro suggest an increased renin-angiotensin system activity in the vessels of Ren-2 transgenic rats (Hilgers et al., 1992; Arribas et al., 1994), which is consistent with a paracrine or autocrine regulation of the vascular tone by tissular renin-angiotensin system through the local production of angiotensin II (Arribas et al., 1994).

In the present work, in agreement with our previously reported results (Peiró et al., 1992), we found that vascular smooth muscle cells isolated from the transgenic animals proliferate significantly faster than cells from normotensive Sprague-Dawley rats cultured in identical conditions, namely, in the presence of 10% fetal calf serum. The fact that such abnormality in the proliferative characteristics is observed in vitro is likely reflecting the existence of intrinsic altered growth mechanisms in this cellular strain. The enhanced growth response of transgenic-derived cells depended on the amount of fetal calf serum in the culture medium. The hyperresponsiveness of these cells to fetal calf serum was further confirmed by the fact that basal DNA synthesis was also enhanced in cultures grown for several days in the presence of 10% fetal calf serum before growth arrest.

Among the vascular trophic substances, the possible effect of angiotensin II has been widely analyzed. In rat cultured vascular smooth muscle cells, the reported results are controversial, although most studies using cultures from the normotensive rat strains Wistar-Kyoto, Wistar and Sprague-Dawley do not find mitogenic effects to angiotensin II but rather support that this agent produces cell hypertrophy (Geisterfer et al., 1988; Berk et al., 1989; Millet et al., 1992). However, it has been reported in aortic smooth muscle from Sprague-Dawley rats that angiotensin II can potentiate the mitogenic effects of fetal calf serum or growth factors such as platelet-derived growth factor BB isoform, although it does not augment DNA synthesis by itself (Lyall et al., 1988; Bobik et al., 1990).

As radioimmunoassayable angiotensin II was found in the cultured vascular smooth muscle cells from transgenic rats, we tested whether the blockade of the renin-angiotensin system could interfere with the increased growth rate observed in these cells. Neither the growth curves for Sprague-Dawley- nor transgenic-derived cultures were modified by the addition of high concentrations of captopril, losartan, saralasin, or PD123319 to the culture medium during 4 days. It is worth to remark that these growth

curves were performed in the presence of 10% fetal calf serum, which has been described as an optimal condition for detecting the mitogenic action of angiotensin II on smooth muscle cells cultured from Sprague-Dawley mesenteric arteries (Lyall et al., 1988). In addition, both in Sprague-Dawley- and transgenic-derived cultures, DNA synthesis induced by fetal calf serum was unaffected by either captopril, losartan, saralasin, or PD123319. Our data agree with those works employing vascular smooth muscle cells derived from Sprague-Dawley rat strains in which angiotensin II has not been found to be a proliferative factor (Geisterfer et al., 1988; Berk et al., 1989; Stouffer et al., 1993). It is worth to remember that Ren-2 transgenic animals have been developed from a Sprague-Dawley strain (Mullins et al., 1990).

Contrasting with the lack of activity of the renin-angiotensin system inhibitors, the proliferation rate of either Sprague-Dawley- or transgenic-derived cultures was significantly reduced by the addition of a dihydropyridine Ca^{2+} channel antagonist to the culture medium. These results were consistent with previous data from different authors (see review by Jackson and Schwartz, 1992) and, in agreement with most of those studies, rather high concentrations (10 μM) of nifedipine were required for inhibiting growth of proliferating vascular smooth muscle cells (Kuriyama et al., 1988; Sato et al., 1989). DNA synthesis induced by fetal calf serum was also inhibited by nifedipine in a concentration-dependent manner. In Sprague-Dawley-derived cultures, this effect was observed at 1 μM, a concentration range similar to that previously reported for dihydropyridine Ca^{2+} channel antagonists to decrease rat vascular smooth muscle cell DNA synthesis (Nilsson et al., 1985; Tomita et al., 1987; Thyberg and Palmberg, 1987). However, cells from transgenic animals were more sensitive to nifedipine effects, as reduction of DNA synthesis could be observed at concentrations of 100 nM. In addition, the EC_{50} values for nifedipine inhibition of DNA synthesis were also significantly lower in transgenic-derived cultures.

In growth curves, the antiproliferative actions of nifedipine were observed, in both cell types, at a concentration of 10 μM, with no effects obtained at lower concentrations. On the contrary, DNA synthesis could be reduced with a 10- to 100-fold lower concentration. This observation is in agreement with previous reports (see Jackson and Schwartz, 1992), and it is likely due to the fact that growth curves were performed with proliferating smooth muscle cells whereas the experiments analyzing DNA synthesis were performed in quiescent cells after a growth arrest of 24 h. In this way, it has been reported that L-type Ca^{2+} channels are needed for transition from quiescence to growth (Jackson et al., 1989). In addition, a reduction in the number of voltage-dependent Ca^{2+} channels in proliferating cells has been described (Dudkin et al., 1988; Rampe et al., 1988). However, despite this lower effectiveness on proliferating cells, the inhibitory effects of 10 μM nifedipine on the growth curves were also significantly higher in cultures from Ren-2 transgenic rats, reducing to half the cell number obtained after 4 days of treatment. In control Sprague-Dawley-derived cultures, nifedipine did not reduce by more than 20% the cell number observed in control situation. Moreover, the population doubling time was only slightly increased by about 6 h in Sprague-Dawley-derived cultures, while it was augmented by 28 h in the case of transgenic-derived ones. Indeed, the enhanced growth rate of transgenic cultures was reduced by nifedipine to values even lower than those observed in untreated control Sprague-Dawley-derived cells. These results suggest that the hyperresponsiveness of vascular smooth muscle cells from transgenic rats to the serum growth factors depends on a higher role for extracellular Ca^{2+}, which enters through L-type dihydropyridine-sensitive Ca^{2+} channels.

The importance of intracellular concentrations of free Ca^{2+} in the growth of vascular smooth muscle, either from normotensive or hypertensive strains, is well established (Jackson and Schwartz, 1992). As shown by the present data, Ca^{2+} seems to play also an important role in the proliferation of vascular smooth muscle cells from the Ren-2 transgenic rat hypertensive model. We speculate a possible explanation for the present results, based on the intracellular effects of angiotensin II. Recently, Haller et al. (1996) indicated that microinjection of angiotensin II in cultured vascular smooth muscle cells enhances cytosolic and nuclear levels of Ca^{2+}, which is mainly dependent on extracellular Ca^{2+} as it is reduced with dihydropyridines. Interestingly, the effects of angiotensin II on Ca^{2+} influx were unaffected by extracellular treatment with an angiotensin AT_1 receptor antagonist but mediated by intracellular angiotensin II receptors that can lead to translocation of Ca^{2+}-sensitive protein kinase C towards the nucleus, maybe affecting cell proliferation (Haller et al., 1996). Therefore, we propose that the presence of angiotensin II in cells from Ren-2 transgenic rats, even without release to extracellular space, could be on the basis of its enhanced proliferation by a mechanism involving extracellular Ca^{2+} entry. Supporting this hypothesis, we have immunocytochemical evidence (unpublished results) that angiotensin II is contained in granule-like structures into the cytoplasm of vascular smooth muscle cells derived from transgenic rats.

In conclusion, our results indicate that vascular smooth muscle cultures from hypertensive transgenic rats exhibit cell hyperplasia in response to fetal calf serum that seems to be independent on the local release of angiotensin II and resistant to the blockade of angiotensin-converting enzyme and angiotensin receptors. However, this increased proliferation can be abolished by L-type Ca^{2+} channel blockade. The presence of intracellular angiotensin II in transgenic vascular smooth muscle cells may provide a clue for better understanding the mechanism involved in the enhanced proliferative responses of this cell type.

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