Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 567 (2007) 231–239
www.elsevier.com/locate/ejphar

Importance of intracellular Angiotensin II in vascular smooth muscle cell apoptosis: Inhibition by the Angiotensin AT_1 receptor antagonist irbesartan

Emilio Ruiz [a,1], Santiago Redondo [a,1], Eugenia Padilla [a], Antonio Gordillo-Moscoso [a], Mercedes Salaices [b], Gloria Balfagón [c], Teresa Tejerina [a,*]

[a] *Department of Pharmacology, School of Medicine, Universidad Complutense de Madrid, Av. Complutense, s/n. 28040 Madrid, Spain*
[b] *Department of Pharmacology and Therapeutics, School of Medicine, Universidad Autónoma de Madrid, Madrid, Spain*
[c] *Department of Physiology, School of Medicine, Universidad Autónoma de Madrid, Madrid, Spain*

Received 4 December 2006; received in revised form 28 February 2007; accepted 13 March 2007
Available online 6 April 2007

Abstract

The intracellular uptake of Angiotensin II has been described, although its physiological role is not yet understood. We aimed to study the role of Angiotensin II internalization in Angiotensin II-induced apoptosis. Vascular smooth muscle cells were cultured from male Wistar–Kyoto rats and treated with Angiotensin II (1 μM, 48 h). Apoptosis was assessed by DNA fragmentation, cell cytometry and caspase-3 activity. The Angiotensin AT_1 receptor antagonist irbesartan (0.1–10 μM) and the inhibitors of Angiotensin II internalization phenylarsine oxide (PAO, 20 μM), but not the AT_2 receptor antagonist PD123319 (*S*-(+)-1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid di(trifluoroacetate) salt), decreased Angiotensin II-mediated apoptosis. Pre-treatment with irbesartan, but not with PD123319, blocked Angiotensin II internalization. We found a strong correlation between intracellular Angiotensin II staining and Angiotensin II-induced apoptosis for all compared groups. We therefore conclude that internalization of Angiotensin II is involved in apoptosis of vascular smooth muscle cells induced by this peptide.

Keywords: Vascular smooth muscle cell; Angiotensin; Apoptosis; Internalization; Cell trafficking

1. Introduction

The octapeptide Angiotensin II is considered as the main effector of the renin–angiotensin system. This peptide plays an important role in normal vascular physiology as well as in cardiovascular disease, mostly through the Angiotensin II type 1 receptor (AT_1) and partially through the Angiotensin II type 2 receptor (AT_2) (Andresen et al., 2004). Angiotensin II acts as a potent vasoconstrictor (Vanhoutte et al., 2005), comitogen (Xiao et al., 2004) and it also induces apoptosis (Bascands et al., 2001). On the other hand, in atherosclerosis and hypertensive vascular remodelling, Angiotensin II is increasingly regarded as an accelerator of vascular cell damage, not only by means of shear stress but also through direct tissue damage at the vessel wall level (Landmesser et al., 2002). Interestingly, this effect seems to be mediated by the Angiotensin AT_1 receptor (Daugherty et al., 2001).

Selective non-peptidic Angiotensin AT_1 receptor antagonists have been developed for the clinical treatment of hypertension and there is intense research of its putative antiatherosclerotic or plaque stabilizing effects. Some studies have revealed that Angiotensin AT_1 receptor antagonists, such as losartan, prevented apoptotic cell death induced by Angiotensin II in vascular smooth muscle cells in several *in vitro* assays (Bascands et al., 2001; Siegert et al., 1999). At the same time, Angiotensin AT_1 receptor antagonists have been described to decrease hypertensive oxidative damage in *in vivo* studies (Daugherty et al., 2001; Landmesser et al., 2002), as well as decrease plaque vulnerability in clinical series (Cipollone et al., 2004).

The classical pathway for Angiotensin II signaling by induction of second messengers has been complemented by the mechanisms which lead to Angiotensin II internalization (Cook et al., 2001). It is generally accepted that Angiotensin II binding to the Angiotensin AT_1 receptor causes rapid internalization of the

* Corresponding author. Tel./fax: +34 913941476.
E-mail address: teje@med.ucm.es (T. Tejerina).
[1] These two authors have contributed equally to this work.

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2007.03.053

AT_1–Angiotensin II complex (Anderson et al., 1993). The uptake of Angiotensin II into vascular smooth muscle cells *in vivo* was demonstrated more than 30 years ago (Robertson et al., 1971). The mechanism of Angiotensin II internalization is not fully understood. It is thought to be mediated, in part, by β-arrestins. β-arrestins have been shown to link receptors to the cytoplasmic clathrin-binding protein (Kule et al., 2004). Clathrin forms a cage-like structure that forms coated vesicles, which invaginate from the cell surface (Trowbridge et al., 1993). Disruption of the structure of the clathrin coat by treatment with phenylarsine oxide (PAO) markedly inhibits Angiotensin II internalization in vascular smooth muscle cells (Ullian et al., 1989).

The potential importance for intracellular Angiotensin II for cardiovascular medicine is highlighted by the finding of an association between intracellular Angiotensin II and oxidative damage in pathological specimens from diabetic hearts (Frustaci et al., 2000). It has been postulated that the ability of AT_1 antagonists to inhibit intracellular functions of Angiotensin II may depend on their liposolubility (Cook et al., 2001). Since irbesartan is a highly liposoluble molecule, we aimed to study the physiological role of Angiotensin II internalization on the apoptosis induced by Angiotensin II and the preventive role of irbesartan concerning this phenomenon.

2. Methods

2.1. Cell cultures

Male Wistar–Kyoto rats (230±20 g) were used. The investigation followed the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Cultures of rat vascular smooth muscle cells were obtained from enzymatically dissociated rat thoracic aorta. Cells showed the typical hill-and-valley phenotype and stained positive for α-actin. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (Gibco®, Madrid, Spain), supplemented with 100 IU/ml penicillin G (sodium salt) and 100 μg/ml streptomycin (Gibco, Madrid, Spain). Experiments were conducted with cells at passages 3–5. The cells showed a spindle-like morphology.

We set the cell culture conditions for Angiotensin II-induced apoptosis as a low density seed (20,000 cells/cm^2, which yields 70% confluence) and low-serum cell culture medium (0.4% foetal calf serum). These conditions are similar to the ones reported by other groups (Qin et al., 2004).

2.2. Measurement of apoptosis by DNA fragmentation

The cells (5000 cells/well) were plated on 96-well plates and allowed to attach for 24 h. Angiotensin II (1 μM) was added for 48 h (0.4% foetal calf serum). To establish the role of the Angiotensin AT_1 receptor in the apoptosis induced by Angiotensin II, the cells were preincubated with the Angiotensin AT_1 receptor antagonist irbesartan (0.1–10 μM) or with the AT_2 receptor antagonist PD123319 (*S*-(+)-1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid di(trifluoroacetate) salt) at 1–10 μM for 30 min prior to Angiotensin II treatment. We also blocked Angiotensin II internalization with phenylarsine oxide (PAO, 20 μM, 30 min preincubation) which disrupts clathrin-coated pits. Cellular DNA fragmentation was measured with a commercially available cellular DNA fragmentation Enzyme-linked Immunosorbent Assay (ELISA) kit from Roche-Boerhinger®, Spain. After treatment, the cells were washed with phosphate buffer saline (PBS) and incubated with the kit lysis buffer composed by bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), and Tween®20 for 30 min at room temperature. Soluble Bromodeoxyuridine (BrdU)-labelled DNA fragments present in the buffer were quantified using the ELISA kit. DNA fragmentation was expressed as fold increase of the control values.

2.3. Western blotting

Protein expression induced by cell treatments was assessed by Western blotting, according to a method previously described (Redondo et al., 2005). Cells (80,000 cells/well) were plated onto 6-well plates and allowed to attach for 24 h. For Bcl-x_s detection, the cells were treated with Angiotensin II 1 μM for 3–18 h in 0.4% foetal calf serum containing medium. The cells were washed with ice-cold PBS, and lysed on ice with 200 μl lysis buffer (10% glycerol, 2.3% sodium dodecyl sulphate, 62.5 mM Trizma base–HCl, pH 6.8; 150 mM NaCl, 10 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml chymostatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride) and boiled for 5 min. Equal amounts of protein were run on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Amersham®, Madrid, Spain), and blocked overnight at 4 °C in blocking solution (5% bovine serum albumin in TBS-T: 25 mM Trizma base, 75 mM NaCl, pH=7.4, 0.1% v/v Tween®20). For analysis of Bcl-x_s, blots were incubated with mouse monoclonal anti Bcl-x_s (1:500) (Transduction Labs®, Madrid, Spain). After washing in TBS-T solution, the blots were further incubated for 1 h at room temperature with the horseradish peroxidase conjugated anti-mouse antibodies diluted at 1:10,000 (Santa Cruz Biotechnology®, CA, USA) in the blocking solution. The blots were then washed 5 times in TBS-T, and antibody-bound protein was visualized with Enhanced Chemiluminescence's (ECL) kit (Amersham Biosciences®, Barcelona, Spain). Smooth muscle α-actin was used as a housekeeping protein, and was determined following the same procedure as mentioned above, using a specific anti-α-actin mouse monoclonal antibody (Sigma-Aldrich®, Madrid, Spain), at 1:1000 in TBS-T.

2.4. Measurement of apoptosis by flow cytometry

Cellular DNA content was measured by fluorescence-assessed flow cytometry (FACS). The cells (10^5 cells/flask) were plated and allowed to attach for 24 h and then treated with Angiotensin II 1 μM for 24 or 48 h in 0.4% foetal calf serum containing medium. The cells were then harvested by

trypsinization, washed with PBS, pelleted, and resuspended in PBS containing 0.6% Nonidet P-40 and 100 g/ml propidium iodide, to which RNase was added to a final concentration of 100 μg/ml. Flow cytometric analysis was carried out with a FACScan (Becton-Dickinson®) flow cytometer equipped with a 15-mW Argon laser emitting at 488 nm. Propidium iodide fluorescence was determined through a 575/24-BP filter; 10,000 cells were acquired per sample, and a double discriminator module was used to ensure detection of single cells.

2.5. Analysis of caspase-3 activity

Vascular smooth muscle cells were plated on 90 mm Petri dishes (5×10^5 cells/dish) and allowed to attach for 24 h. The cells were then pre-treated with irbesartan (10 μM) or PAO (20 μM) for 30 min and then with Angiotensin II 1 μM for 24 h in 0.4% foetal calf serum containing medium. Caspase-3 activity was measured spectrophotometrically using a commercially available kit (Calbiochem®) following the manufacturer's instructions.

2.6. Immunofluorescence staining

Subcellular locations of Angiotensin II and Angiotensin AT_1 receptor were analyzed by confocal images of immunofluorescence stained samples. The cells were plated onto cover slips and allowed to attach for 24 h. The culture medium was changed for DMEM containing 0.4% foetal calf serum and 1 μM Angiotensin II for 5 min to 3 h to determine the time-dependency of Angiotensin II internalization and nuclear translocation. Once the conditions were set, the cells were pre-treated for 30 min in the presence or absence of irbesartan at 0.1, 1 or 10 μM, PD123319 at 1 or 10 μM, or the internalization inhibitor PAO (20 μM) and then with Angiotensin II at 1 μM for 60 min. The cells were washed with PBS and fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature and permeabilized with 0.4% triton-X100 for 30 min at room temperature. After blocking with 3% bovine serum albumin (BSA) in PBS, the cells were then incubated with goat polyclonal anti-Angiotensin II (1:100) for 1 h. The Angiotensin I/Angiotensin II antibody (sc-7419) is a goat polyclonal antibody, and therefore cross-reactive for Angiotensin I and Angiotensin II (Santa Cruz Biotechnologies®, Santa Cruz, USA), as previously described (Thomas et al., 2004). To test the specificity of this antibody in our experimental model, we ran a control experiment in which the primary antibody was blocked with the Angiotensin II peptide. The antibody against the Angiotensin AT_1 receptor was purchased by Acris Antibodies GmbH® (Germany) and diluted 1:100. Excess of the primary antibody was removed by washing with blocking solution, followed by incubation with donkey anti-goat Alexa 468® (1:100, Molecular, Probes) for 1 h. The cells were then

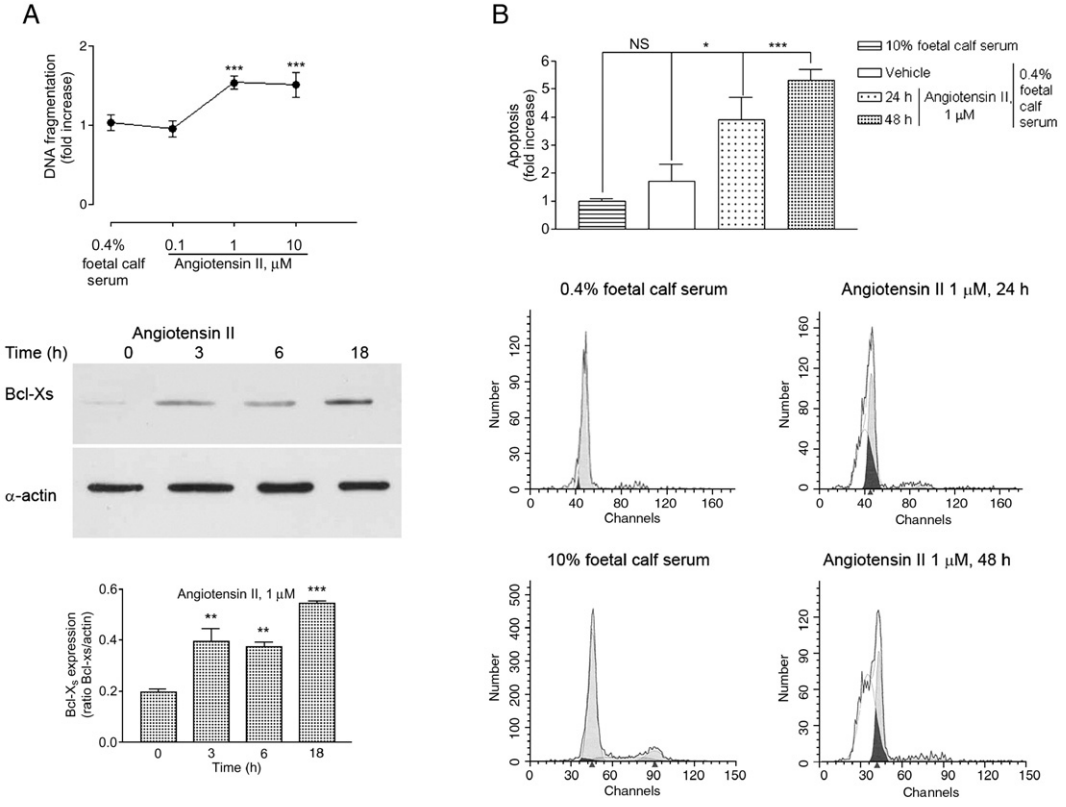

Fig. 1. Angiotensin II induces apoptosis in rat aortic vascular smooth muscle cells. Panel A: analysis of DNA fragmentation after 48 h treatment with Angiotensin II (0.1, 1 or 10 μM in 0.4% foetal calf serum containing medium). Panel B: flow cytometry study of the apoptosis. Upper panel: bar graph shows the increase in the apoptosis of three independent experiments, each in duplicate. Lower panel: representative experiment. NS = Non-significant, $*P<0.05$, $**P<0.01$, $***P<0.001$. Panel C: Upper panel: representative Western blot shows the expression of the proapoptotic protein Bcl-x_s induced by Angiotensin II (1 μM in 0.4% foetal calf serum containing medium). Lower panel: Bar graph shows densitometric analysis of Bcl-x_s expression of 3 different experiments with respect to Control (time 0).

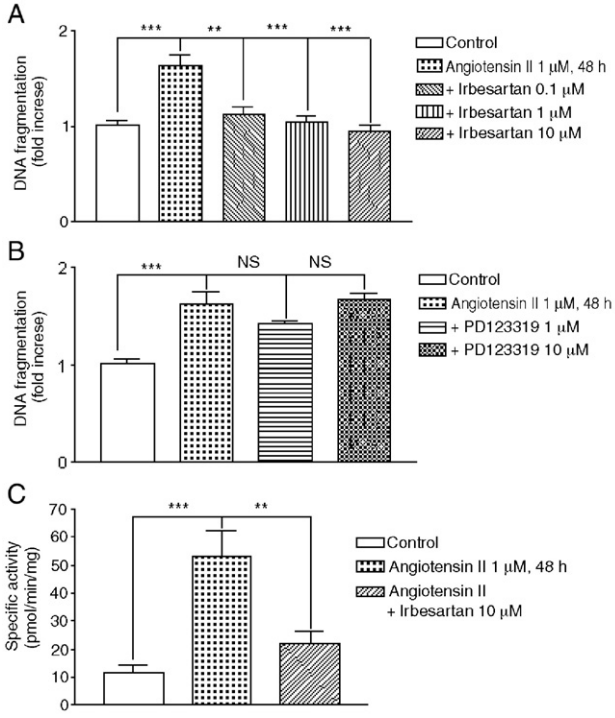

Fig. 2. Irbesartan inhibits apoptosis induced by Angiotensin II in vascular smooth muscle cells. Panel A: Preventive effect of the AT_1 antagonist irbesartan (0.1 to 10 μM) on apoptosis induced by Ang II in vascular smooth muscle cells (1 μM, 48 h). Panel B: Effect of the AT_2 receptor antagonist PD123319. Apoptosis was measured as fold increase of DNA fragmentation of 3 different experiments, each in hexuplicate. Panel C: Caspase-3 activity in cells treated with Angiotensin II (1 μM, 24 h) in the presence or absence of the Angiotensin AT_1 receptor antagonist irbesartan 10 μM. Bar graphs show specific activity of caspase-3 as pmol of *P*-nitroaniline/min/mg of protein in 3 different experiments, each in duplicate. NS=Non-significant, $^{**}P<0.01$, $^{***}P<0.001$.

washed four times with a blocking buffer every 5 min. Images were captured using a Leica TCS SP2 inverted microscope. Quantification of the fluorescence was performed using the Image J 1.33 software (US National Institutes of Health).

2.7. Transfection of AT_1R–EYFP

Rat vascular smooth muscle cells were plated onto coverslips and allowed to attach for 24 h. The cells were transfected with a AT_1R–EYFP construct kindly donated by Dr. Julia Cook (Division of Research, Ochsner Clinic Foundation, New Orleans, USA), as described (Cook et al., 2004). The expression plasmid (2 μg of pcDNA) was transfected into donor cells by a lipid-mediated method using a Fugene 6® (Roche-Boerhinger®, Barcelona, Spain) for 3 h. Two days after transfection, the transfected cells were treated with Angiotensin II 1 μM, fixed with formaldehyde at 4% in PBS and examined by confocal microscopy.

2.8. Drugs and reagents

Irbesartan was a generous gift by Bristol-Myers-Squibb. It was diluted in DMSO and added to the samples at a constant ratio of 1:1000. Phenylarsine oxide (PAO) was purchased from Sigma (Madrid, Spain) and diluted in dimethyl sulfoxide (DMSO), at 1:1000 final concentration of DMSO in the culture medium. PD123319 was purchased from Sigma® and dissolved in PBS.

2.9. Statistical analysis

The results are expressed as the mean±S.E.M and accompanied by the number of observations. A statistical analysis of the data was carried out by a one way analysis of variance (ANOVA) test followed by a post-hoc Tukey multiple comparison procedure. Differences with a *P* value of less than 0.05 were considered statistically significant.

3. Results

3.1. Apoptotic effect of Angiotensin II under controlled experimental conditions

We first performed a concentration–response curve in order to assess whether Angiotensin II was able to induce apoptosis (measured as DNA fragmentation) when added to 0.4% foetal calf serum medium. As shown in Fig. 1, panel A (upper panel), Angiotensin II was able to trigger apoptotic cell death when incubated for 48 h at 1 μM. The apoptotic effect under these conditions was equally assessed by expression of the apoptotic inductor Bcl-x_s (Fig. 1, panel A, lower panel) and by assessment of hypoploid nuclei in cell cytometry assays (Fig. 1, panel B).

For the tested experimental conditions, proliferation induced by Angiotensin II was not observed (data not shown).

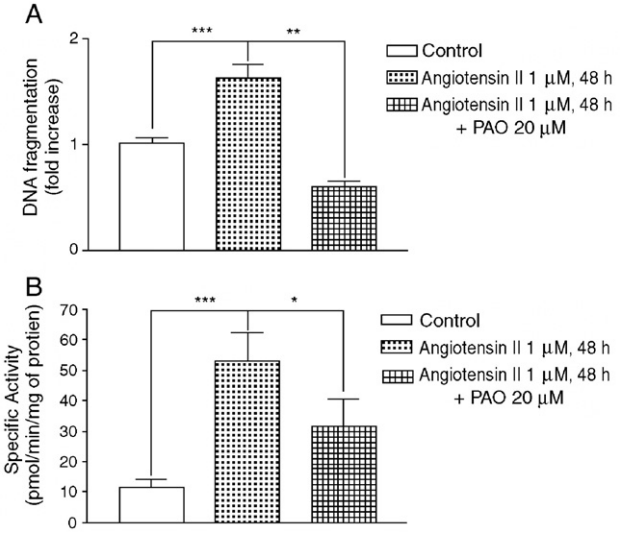

Fig. 3. Inhibitors of Angiotensin II internalization decreased Angiotensin II-induced apoptosis. Panel A: Effect of the inhibitor of Angiotensin II internalization phenylarsine oxide (PAO, 20 μM) on the apoptosis induced by Angiotensin II (1 μM in 0.4% foetal calf serum containing medium, 48 h). Apoptosis was measured as fold increase of DNA fragmentation in three different sets of experiments, each in hexuplicate. Panel B: Caspase-3 activity in cells treated with Angiotensin II (1 μM in 0.4% foetal calf serum containing medium, 24 h) in the presence or absence of PAO 20 μM. Bar graphs show specific activity of caspase-3 as pmol of *P*-nitroaniline/min/mg of protein in 3 different experiments, each in duplicate. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

3.2. Apoptosis induced by Angiotensin II depends on the interaction of angiotensin with the Angiotensin AT_1 receptor

To study the role of the Angiotensin AT_1 receptor on the apoptosis induced by Angiotensin II in rat vascular smooth muscle cells, cellular DNA fragmentation induced by Angiotensin II was analyzed in the presence or absence of increasing concentrations of irbesartan from 0.1 to 10 μM or the AT_2 receptor antagonist PD123319 (1 or 10 μM). As shown in Fig. 2, panel A, treatment with Angiotensin II generates an increase in DNA fragmentation compared to control cells. Apoptosis induced by Angiotensin II was diminished in a concentration-dependent manner by pre-treatment with irbesartan. However, PD123319 did not exert any effect on DNA fragmentation (Fig. 2, panel B). We also analyzed the caspase-3 activity in the presence of Angiotensin II and found that Angiotensin II increased the activity of caspase-3 in rat vascular smooth muscle cells. This effect was diminished by pre-treatment with irbesartan (Fig. 2, panel C).

3.3. Role of internalization inhibition on Angiotensin II-induced apoptosis

To assay whether internalization of Angiotensin II plays a role on its apoptotic effect, we studied the apoptosis induced by Angiotensin II in the presence or absence of PAO. Fig. 3 panel A shows that pre-treatment with PAO for 30 min prior to Angiotensin II treatment significantly reduced the apoptosis induced by Angiotensin II, measured as DNA fragmentation. The internalization inhibitor did not exert a significant effect when added in the absence of Angiotensin II.

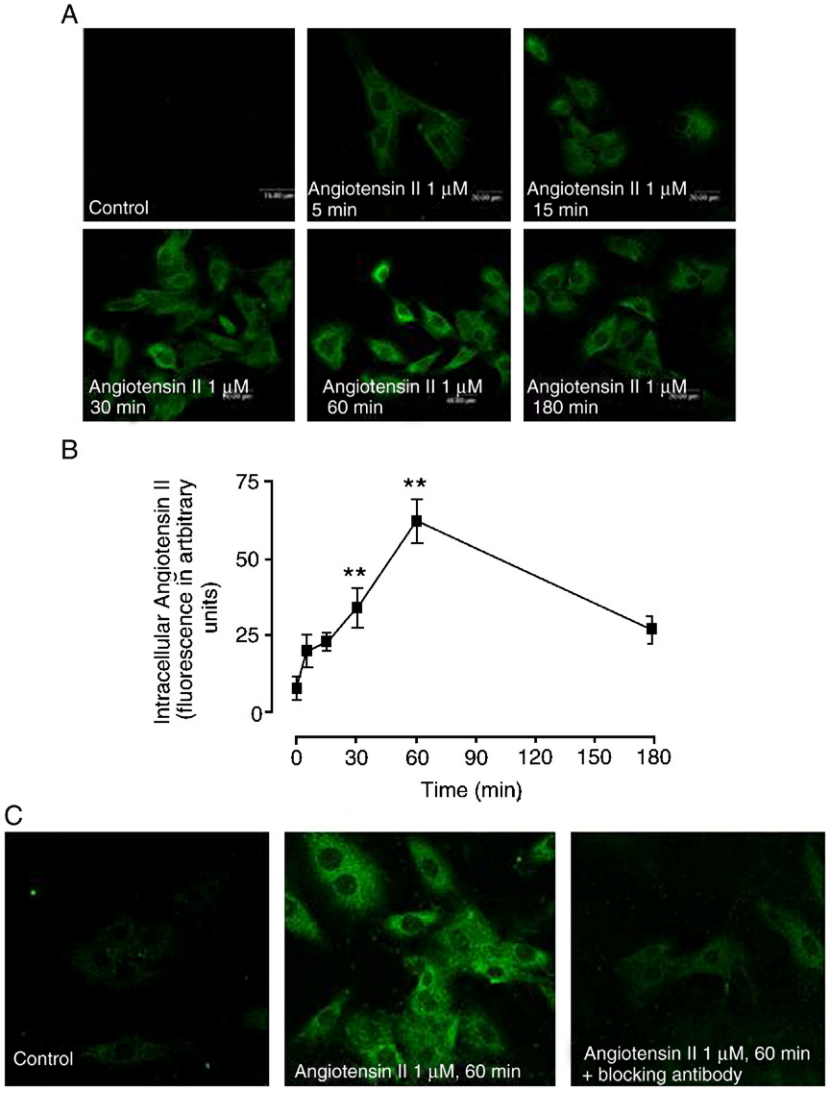

Fig. 4. Time-dependency of Angiotensin II internalization. Panel A: Representative confocal images show intracellular localization of Angiotensin II in cells treated with Angiotensin II 1 μM (1 μM in 0.4% foetal calf serum containing medium) for the time indicated above. Panel B: Graph show data of intracellular Angiotensin II measured as fluorescence in arbitrary units (3 different experiments). Panel C: Control of antibody specificity of staining samples. Primary antibody was blocked with Angiotensin II peptide. $^{**}P<0.01$.

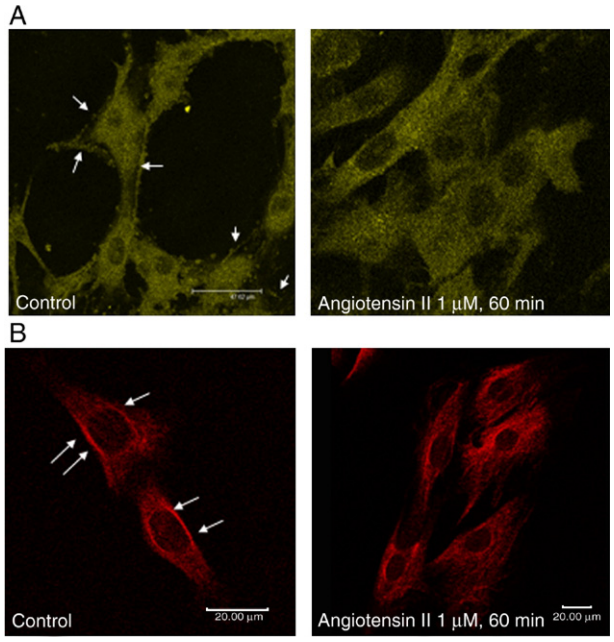

Fig. 5. Internalization of Angiotensin AT_1 receptor. Panel A: Transfection of AT_1R–EYFP in rat vascular smooth muscle cells. VSMC in culture were transfected with a plasmid encoding for the fusion protein Angiotensin AT_1 receptor–EYFP according to Methods. Cells were treated with vehicle (left photograph) or Angiotensin II (1 μM in 0.4% foetal calf serum containing medium) for 1 h (right photograph). The arrows indicate the membrane localization of AT_1R–EYFP in control or the intracellular localization of AT_1R–EYFP in Angiotensin II-treated cells. Panel B: Immunofluorescence staining of Angiotensin AT_1 receptor in rat vascular smooth muscle cells untreated (left) or treated with Angiotensin II (right) at 1 μM in 0.4% foetal calf serum containing medium for 1 h. The arrows indicate the membrane localization of Angiotensin AT_1 receptor in control or the intracellular localization of Angiotensin AT_1 receptor in Angiotensin II-treated cells.

A similar protective effects of both inhibitors were observed when apoptosis was assessed by caspase-3 activity (Fig. 3, panel B).

3.4. Time-dependent analysis of Angiotensin II internalization

Subsequently, cellular internalization of Angiotensin II was analyzed by confocal microscopy. Fig. 4, panel A, shows the time-dependent internalization of Angiotensin II (1 μM) in rat vascular smooth muscle cells. We found that control cells did not show any Angiotensin II staining (as observed by the lack of Angiotensin II green fluorescence). Treatment with Angiotensin II increased the fluorescence signal of intracellular Angiotensin II, reaching the maximum internalization after 60 min treatment. Interestingly, there is a more intense fluorescence signal in the perinuclear region at this time-point.

Quantification of the fluorescence intensity shows a time-dependent increase which reaches statistical significance at 30 and 60 min (Fig. 4, panel B).

In order to provide an internal control for antibody specificity, the Angiotensin II peptide was added to anti-Angiotensin II antibody in another set of experiments. As shown in Fig. 4, panel C, this experimental approach dramatically inhibited the Angiotensin II immunostaining.

3.5. Angiotensin II internalization takes place through Angiotensin AT_1 receptor

To assess whether the Angiotensin AT_1 receptor is also internalized, rat vascular smooth muscle cells were transfected with a plasmid encoding for AT_1R–EYFP. As shown in Fig. 5, panel A, fluorescent AT_1 forms a strong band at the cell surface level (arrows), which is lost after Angiotensin II incubation (1 μM, 1 h).

Similar results were obtained when cells were stained with anti-AT_1 antibody (Fig. 5, panel B). Angiotensin II treatment (1 μM, 1 h) induced the loss of membrane staining which is observed under basal conditions (arrows). Additionally, colocalization for Angiotensin II and Angiotensin AT_1 receptor was observed (data not shown).

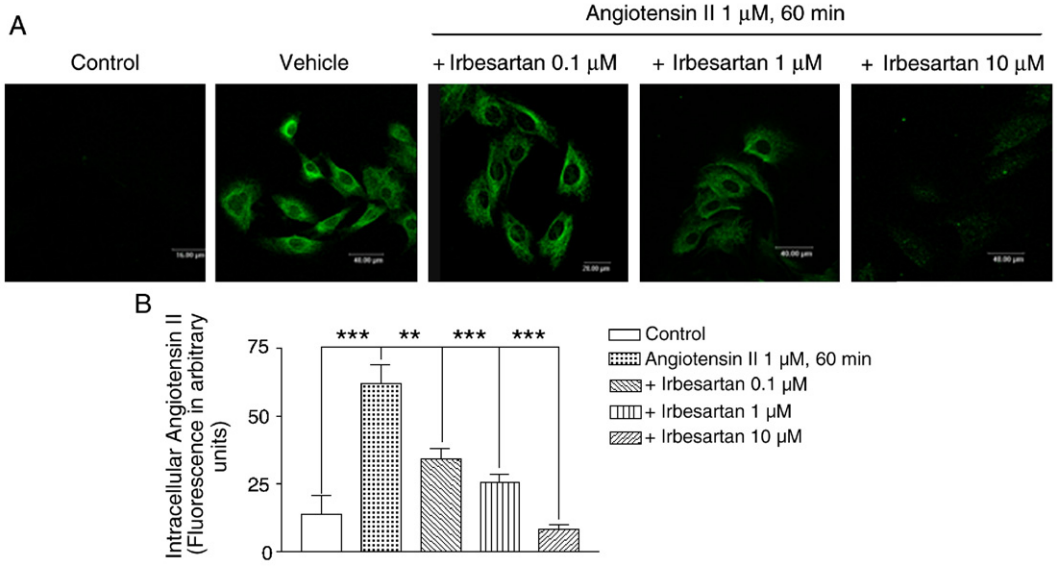

Fig. 6. Inhibition of internalization of Angiotensin II by irbesartan. Irbesartan (0.1 to 10 μM) was preincubated for 30 min prior to the addition of Angiotensin II 1 μM for further 1 h. Panel A: Representative confocal images show intracellular localization of Angiotensin II in vascular smooth muscle cells treated with Angiotensin II 1 μM. Panel B: Bar graphs show the intracellular Angiotensin II measured as fluorescence in arbitrary units of three different experiments. $^{**}P<0.01$, $^{***}P<0.001$.

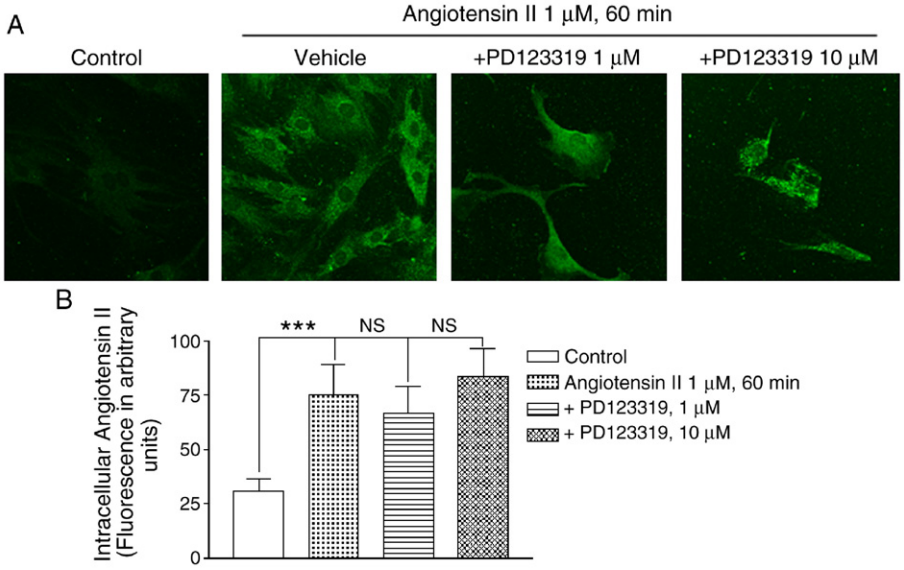

Fig. 7. Effect of PD123319 on Angiotensin II-internalization. PD123319 (1–10 μM) was preincubated for 30 min prior to the addition of Angiotensin II 1 μM for further 1 h. Panel A: Confocal images show intracellular localization of Angiotensin II in vascular smooth muscle cells treated with Angiotensin II 1 μM. Panel B: Bar graphs show the intracellular Angiotensin II measured as fluorescence in arbitrary units. NS=Non-significant, ***$P<0.001$.

3.6. Irbesartan inhibits Angiotensin II staining

Since we have shown that AT_1 is co-internalized with exogenously added Angiotensin II from cell membrane to cytoplasm, the question arises whether pharmacological AT_1 blockade is able to inhibit Angiotensin II internalization which takes place after binding to the Angiotensin AT_1 receptor. Fig. 6 shows that pre-treatment with increasing concentrations of irbesartan (0.1–10 μM) caused a concentration-dependent reduction in the Angiotensin II internalization.

3.7. Pharmacological AT_2 blockade does not affect Angiotensin II internalization

As shown in Fig. 7, panel A, preincubation of cell cultures with the AT_2 receptor antagonist PD123319 at 1 and 10 μM

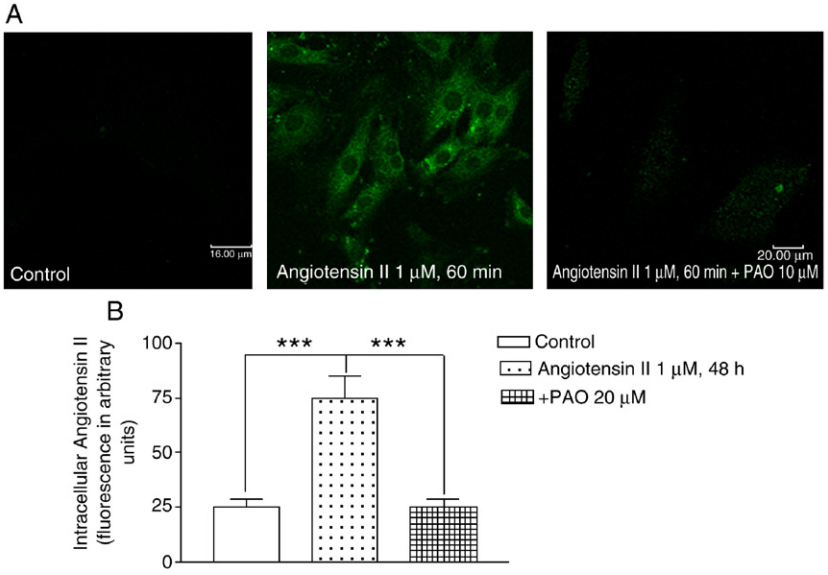

Fig. 8. Inhibition of Angiotensin II internalization by phenylarsine oxide (PAO). PAO (20 μM) was preincubated for 30 min prior to the addition of Angiotensin II 1 μM for further 1 h. Panel A: Confocal images show intracellular localization of Angiotensin II in vascular smooth muscle cells treated with Angiotensin II in the presence or absence of inhibitors. Panel B: Bar graphs show the intracellular Angiotensin II staining of three different experiments. ***$P<0.001$.

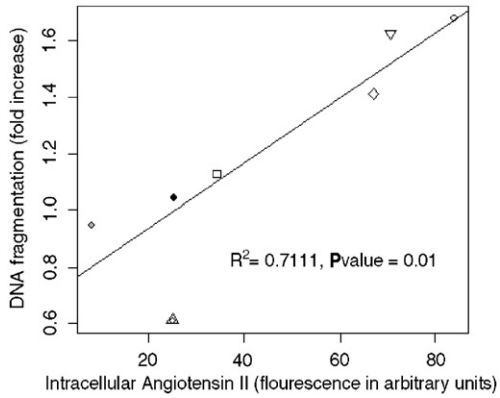

Fig. 9. Angiotensin II-induced apoptosis and internalization correlate each other for all analyzed groups. Figure shows a high correlation between Angiotensin II-induced apoptosis (measured as mean of fold increase of DNA fragmentation) and intracellular Angiotensin II (mean of fluorescence intensity, in arbitrary units). Inverted triangle: Angiotensin II, 1 μM, 48 h. Square: +Irbesartan 0.1 μM. Black circle: +Irbesartan 1 μM. Grey circle: +Irbesartan 10 μM. Rhomboid: +PD123319 1 μM. White circle: +PD123319 10 μM. Circle inside a triangle: +phenylarsine oxide (PAO) 20 μM.

proved unable to block the internalization of Angiotensin II. Quantitative analysis did not show a significant decrease of fluorescence intensity compared to the Angiotensin II group (Fig. 7, panel B).

3.8. Role of PAO on Angiotensin II internalization

Pre-treatment with PAO completely blocked cellular internalization of Angiotensin II (Fig. 8, panel A). When quantified, the inhibitory effect of these compounds reached statistical significance (Fig. 8, panel B).

3.9. Correlation between Angiotensin II-induced internalization and apoptosis

As shown in Fig. 9, for all treated groups, a strong and significant correlation for these two parameters was found.

4. Discussion

The present study describes for the first time the obligatory role of intracellular uptake of Angiotensin II into vascular smooth muscle cells for the induction of apoptotis, as well as its dependence of Angiotensin AT_1 receptor.

The effects of Angiotensin II on cultured rat vascular smooth muscle cells are pleiotropic, including cell proliferation, hypertrophy and apoptosis. The role of Angiotensin II in cell proliferation has been described in the presence of other proliferative factors such as 20% foetal calf serum (Xiao et al., 2004), which functions as a comitogen. However, since the role of Angiotensin II in vascular cell toxicity and plaque vulnerability has been described in experimental animals (Landmesser et al., 2002) and patients (Cipollone et al., 2004) we aimed to assess whether an induction of cell death by Angiotensin II took place. In our first set of experiments, we found that 1 μM was the lowest concentration to induce apoptosis in our cell culture model (0.4% foetal calf serum). This concentration is higher than the one found in plasma from hypertensive patients (Laviades et al., 2000) although *in vitro* induction of apoptosis in the absence of other comitogen or co-apoptotic stimuli may need higher concentrations than the ones found in plasma. This same concentration has been reported to induce vascular smooth muscle cell apoptosis under low serum conditions (Bascands et al., 2001; Siegert et al., 1999).

Controversy exists regarding which type of Angiotensin II receptor is responsible for its apoptotic effect. It has been suggested that AT_1 mediates cell growth while AT_2 mediates apoptosis, although the importance of the AT_2 receptor has been described to be reduced in postnatal life and, to add more complexity, AT_1 and AT_2 receptors may be both implicated on the Angiotensin II-induced cellular inflammation (Andresen et al., 2004). One possible explanation for the lack of a AT_2-mediated effect which has been reported here (Fig. 2, panel B) may be the low AT_2 expression in smooth muscle cell culture models which are obtained from adult animals (De Gasparo et al., 2000). Our *in vitro* model establishes an AT_1-dependent apoptosis. This model may be related to the *in vivo* effects of AT_1-dependent aneurysm formation in response to high-dose Angiotensin II infusion in experimental animals (Daugherty et al., 2001) as well as the clinical observation of plaque stabilization after AT_1 blockade (Cipollone et al., 2004).

We found that the AT_1 antagonist irbesartan reduced DNA fragmentation in a concentration-dependent manner. The concentrations of irbesartan used were in the range of concentrations used in other *in vitro* studies (Bascands et al., 2001). Interestingly, the 1 and 10 μM concentrations of the drug were able to inhibit both the apoptotic effect (Fig. 2, panel A) and the internalization of Angiotensin II (Fig. 6, panel B).

The importance of Angiotensin II internalization regarding its apoptotic effect is highlighted by the fact that pre-treatment of rat vascular smooth muscle cells with PAO, an inhibitor of Angiotensin II internalization, decreased apoptosis induced by Angiotensin II (Fig. 3, panels A and B). Clathrin forms a cage-like structure which invaginates from the cell membrane (Trowbridge et al., 1993). Phenylarsine oxide (PAO) inhibits clathrin-mediated endocytosis (Ullian et al., 1989). We found that PAO inhibited both apoptosis (Fig. 3, panels A and C) and internalization of Angiotensin II (Fig. 7, panels A and B).

Internalization of Angiotensin II has been reported in numerous cells of vascular (Cook et al., 2006) origin that express the Angiotensin AT_1 receptor. It has been reported that Angiotensin II is internalized by cultured proximal tubular cells and seems to be involved in sodium transport in the kidney (Thekkumkara et al., 2002). We found enhanced perinuclear localization of Angiotensin II after 60 min treatment (Fig. 4, panels A and B). Early studies also reported perinuclear localization of Angiotensin II in rat cardiac myocites and vascular smooth muscle cells (Robertson et al., 1971).

Molecular pathways which regulate Angiotensin II-induced apoptosis are not yet completely understood. A recent publication (Li et al., 2006) reports the essential roles of Akt phosphorylation and membrane FasL expression in this process. Since these pathways are related to intracellular trafficking, their role is likely

associated with Angiotensin II internalization, which is instrumental in the eventual DNA fragmentation.

The major finding of the present work is the significant role of Angiotensin II internalization in its apoptotic effect (Fig. 9). Future studies will broaden our knowledge of the intracellular trafficking of Angiotensin II, its pharmacological modulation and its implication for therapeutics in atherosclerosis.

Acknowledgments

This work was supported in part by the FISS grant (01-0815 and RECAVA) and by a grant from Bristol-Meyers-Squibb. We are grateful to Dr. Julia Cook for providing us the AT_1R–EYFP construct and for her useful comments.

References

Anderson, K.M., Murahashi, T., Dostal, D.E., Peach, M.J., 1993. Morphological and biochemical analysis of angiotensin II internalization in cultured rat aortic smooth muscle cells. Am. J. Cell Physiol. 264, C179–C188.

Andresen, B.T., Romero, G.G., Jackson, E.K., 2004. AT_2 receptors attenuate AT_1receptor-induced phospholipase D activation in vascular smooth muscle cells. J. Pharmacol. Exp. Ther. 309, 425–431.

Bascands, J.L., Girolami, J.P., Troly, M., Escargueil-Blanc, I., Nazzal, D., Salvayre, R., Blaes, N., 2001. Angiotensin II induces phenotype-dependent apoptosis in vascular smooth muscle cells. Hypertension 38, 1294–1299.

Cipollone, F., Fazia, M., Iezzi, A., Pini, B., Cuccurullo, C., Zucchelli, M., de Cesare, D., Ucchino, S., Spigonardo, F., De Luca, M., Muraro, R., Bei, R., Bucci, M., Cuccurullo, F., Mezzetti, A., 2004. Blockade of the angiotensin II type 1 receptor stabilizes atherosclerotic plaques in humans by inhibiting prostaglandin E2-dependent matrix metalloproteinase activity. Circulation 109, 1482–1488.

Cook, J.L., Zhang, Z., Re, R.N., 2001. In vitro evidence for an intracellular site of angiotensin action. Circ. Res. 89, 1138–1146.

Cook, J., Re, R., Alam, J., Hart, M., Zhang, Z., 2004. Intracellular angiotensin II protein alters Angiotensin AT_1 receptor fusion protein distribution and activates CREB. J. Mol. Cell. Cardiol. 36, 75–90.

Cook, J.L., Mills, S.J., Naquin, R., Alam, J., Re, R.N., 2006. Nuclear accumulation of the AT(1) receptor in a rat vascular smooth muscle cell line: effects upon signal transduction and cellular proliferation. J. Mol. Cell. Cardiol. 40, 696–707.

Daugherty, A., Manning, M.W., Cassis, L.A., 2001. Antagonism of AT_2 receptors augments angiotensin II-induced abdominal aortic aneurysms and atherosclerosis. Br. J. Pharmacol. 134, 865–870.

De Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W., Unger, T., 2000. International Union of Pharmacology XXIII. The angiotensin II receptors. Pharmacol Rev. 52, 415–472.

Frustaci, A., Kajstura, J., Chimenti, C., Jakoniuk, I., Leri, A., Maseri, A., Nadal-Ginard, B., Anversa, P., 2000. Myocardial cell death in human diabetes. Circ. Res. 87, 1123–1132.

Kule, C., Karoor, V., Day, J.N.E., Thomas, W.G., Baker, K.M., Dinh, D., Acker, K.A., Booz, G.W., 2004. Agonist-dependent internalization of the angiotensin II type one receptor (AT_1): role of C-terminus phosphorylation in recruitment of β-arrestins. Regulatory Pept. 120, 141–148.

Landmesser, U., Cai, H., Dikalov, S., McCann, L., Hwang, J., Jo, H., Holland, S.M., Harrison, D.G., 2002. Role of p47(phox) in vascular oxidative stress and hypertension caused by angiotensin II. Hypertension 40, 511–515.

Laviades, C., Varo, N., Diez, J., 2000. Transforming growth factor beta in hypertensives with cardio renal damage. Hypertension 36, 517–522.

Li, Y., Song, Y.H., Mohler, J., Delafontaine, P., 2006. ANG II induces apoptosis of human vascular smooth muscle via extrinsic pathway involving inhibition of Akt phosphorylation and increased FasL expression. Am. J. Physiol, Heart Circ. Physiol. 290, H2116–H2123.

Qin, X.-P., Ye, F., Hu, C.-P., Liao, D.-F., Deng, H.-W., Li, Y.-J., 2004. Effect of calcitonin gene-related peptide on angiotensin II-induced proliferation of rat vascular smooth muscle cells. Eur. J. Pharmacol. 488, 45–49.

Redondo, S., Ruiz, E., Santos-Gallego, C.G., Padilla, E., Tejerina, T., 2005. Pioglitazone induces vascular smooth muscle cell apoptosis through a peroxisome proliferator-activated receptor-gamma, transforming growth factor-beta1, and a Smad2-dependent mechanism. Diabetes 54, 811–817.

Robertson Jr., A.L., Beaulnes, A., 1971. Angiotensin II: rapid localization in nuclei of smooth muscle cells. Science 172, 1138–1139.

Siegert, A., Ritz, E., Orth, S., Wagner, J., 1999. Differential regulation of transforming growth factor receptors by angiotensin II and transforming growth factor-beta1 in vascular smooth muscle. J. Mol. Med. 77, 437–445.

Thekkumkara, T., Linas, S.L., 2002. Role of internalization of AT_1A receptor function in proximal tubule epithelium. Am. J. Physiol., Renal Fluid Electrolyte Physiol. 282, 623–629.

Thomas, M.A., Fleissner, G., Stohr, M., Hauptfleisch, S., Lemmer, B., 2004. Localization of components of the rennin–angiotensin system in the suprachiasmatic nucleus of normotensive Sprague–Dawley rats: Part A. Angiotensin I/II, a light and electron microscopic study. Brain Res. 1008, 212–223.

Trowbridge, I.S., Collawn, J.F., Hopkins, C.R., 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. Annu. Rev. Cell Biol. 9, 129–161.

Ullian, M.E., Linas, S.L., 1989. Role of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. J. Clin. Invest. 84, 840–846.

Vanhoutte, P.M., Feletou, M., Taddei, S., 2005. Endothelium-dependent contractions in hypertension. Br. J. Pharmacol. 144, 449–458.

Xiao, F., Puddefoot, J.R., Barker, S., Vinson, G.P., 2004. Mechanism for aldosterone potentiation of angiotensin II-stimulated rat arterial smooth muscle cell proliferation. Hypertension 44, 340–345.