

Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Learn and Live SM

Angiotensin II Induces Phenotype-Dependent Apoptosis in Vascular Smooth Muscle Cells

Jean-Loup Bascands, Jean-Pierre Girolami, Muriel Troly, Isabelle Escargueil-Blanc, Dani Nazzal, Robert Salvayre and Nelly Blaes

Hypertension 2001;38;1294-1299

DOI: 10.1161/hy1201.096540

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2001 American Heart Association. All rights reserved. Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://hyper.ahajournals.org/cgi/content/full/38/6/1294>

Subscriptions: Information about subscribing to Hypertension is online at
<http://hyper.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
<http://www.lww.com/reprints>

Angiotensin II Induces Phenotype-Dependent Apoptosis in Vascular Smooth Muscle Cells

Jean-Loup Bascands, Jean-Pierre Girolami, Muriel Troly, Isabelle Escargueil-Blanc, Dani Nazzal, Robert Salvayre, Nelly Blaes

Abstract—Angiotensin II regulates vascular structure through growth and apoptosis, with implications in pathophysiology. Subtypes of vascular smooth muscle cells with specific morphology, growth, or apoptotic features have been isolated. Here, we investigated the effects of angiotensin II on apoptosis of 2 morphologically different rat aortic smooth muscle cell phenotypes. Spindle and epithelioid cell lines cultured under low serum conditions were stimulated by angiotensin II. Responsiveness was evaluated by calcium signaling. In both phenotypes, an angiotensin II type 1 receptor–mediated transient intracellular calcium peak arose from intracellular pools. However, a sustained nifedipine-sensitive calcium entry occurred specifically in epithelioid cells. Angiotensin II did not impair spindle cell survival, whereas a delayed reduction in cell number occurred in epithelioid cells. Cell death through apoptosis was characterized by cellular and nuclear morphology. Consistently, DNA fragmentation, evaluated by biochemical quantification, nuclei staining, and ladders, and caspase 3-like activity were promoted by angiotensin II in epithelioid cells. Kinetics of annexin V binding showed that apoptosis was a delayed process. Angiotensin II–induced apoptosis of epithelioid cells was prevented by angiotensin II type 1 but not type 2 receptor antagonists and was inhibited by a calcium chelator or calcium antagonist. Conversely, epithelioid cell apoptosis could be induced by a calcium ionophore. Thus, the death signaling promoted by angiotensin II in epithelioid cells involves type 1 receptor–mediated calcium entry. These data suggest that angiotensin II can promote angiotensin II type 1 receptor–mediated apoptosis in vascular smooth muscle cells, depending on their phenotype. This process may play a role in vascular remodeling in cardiovascular diseases. (*Hypertension*. 2001;38:1294-1299.)

Key Words: angiotensin II ■ apoptosis ■ calcium ■ muscle, smooth, vascular ■ cells

Vascular smooth muscle cells (VSMCs) maintain vascular tone mainly through coordinated contraction/relaxation, and they play a role in arterial wall remodeling through proliferation, hypertrophy, and apoptosis.¹ The arterial tree exhibits heterogeneity in response to vasoactive stimuli and in alterations induced by hypertension or atherosclerosis. The concept that distinct VSMC subtypes may play specific roles, at different locations or pathophysiological situations, is well documented in animals,²⁻⁶ and VSMC heterogeneity also exists in human arteries.⁷ In the rat arterial model, 2 major VSMC subtypes have been discerned on the basis of stable distinct morphologies in culture: spindle versus epithelioid.^{2,8,9} They also differ in growth properties or protein synthesis^{2,8-11}; however, their responses to vasoactive stimuli are barely known.¹²

Angiotensin (Ang) II, the main effector peptide of the renin-angiotensin system, plays an important role in normal vascular physiology and cardiovascular diseases,¹³ mostly through the Ang II type 1 (AT₁) receptor and partly through the Ang II type 2 (AT₂) receptor. AT₁ receptor–mediated intracellular calcium concentration ([Ca²⁺]_i) signaling is a

major determinant of VSMC contraction.¹⁴ Ang II is a modulator of VSMC growth with proliferative/hypertrophic effects mediated by the AT₁ receptor through complex, partly calcium-dependent, signaling.¹⁴ Heterogeneity exists in Ang II calcium signaling¹⁴⁻¹⁶ and long-term responses¹⁷⁻¹⁹ between individual VSMCs, arteries, or arterial layers. In vivo, Ang II can induce a delayed apoptosis through AT₁ receptor activation.^{20,21} Inversely, in vitro, Ang II induces apoptosis through AT₂ receptor activation in AT₂-transfected VSMCs,²² whereas AT₁ receptors might also protect native VSMCs against acute NO-induced apoptosis.²³

The question of whether the diversity in Ang II biological effects could originate from variations in the different VSMC subtypes has not been addressed. Therefore, the aim of the present study was to investigate whether VSMC subtypes responded differently to Ang II in [Ca²⁺]_i signaling and long-term survival. We used 2 stable cell lines, Sp-SMC and Ep-SMC, as respective models of spindle and epithelioid rat arterial VSMC subtypes. The results show dramatically different Ang II responses in the 2 VSMC lines. A delayed AT₁ receptor–mediated calcium-dependent apoptosis was

Received May 31, 2001; first decision June 13, 2001; accepted June 13, 2001.

From INSERM U388 (J.-L.B., J.-P.G.), INSERM U397 (N.B.), and INSERM U466 (M.T., I.E.-B., D.N., R.S., N.B.), Institut Louis Bugnard, Toulouse, France.

Correspondence to Nelly Blaes, PhD, INSERM U466, CHU Rangueil, 31403 Toulouse Cedex 4, France. E-mail blaes@rangueil.inserm.fr

© 2001 American Heart Association, Inc.

Hypertension is available at <http://www.hypertensionaha.org>

elicited in Ep-SMCs only; it was related to specific calcium signaling. This strongly suggests a phenotype-dependent apoptotic sensitivity of VSMCs to Ang II through AT₁ receptors.

Methods

Materials

Ang II, nifedipine, N^G nitro-L-arginine methyl ester (L-NAME), thapsigargin (TG), and propidium iodide (Sigma Chemical Co), EXP3174 (active metabolite of losartan), PD 123,177, and DUP753 (losartan) (Merck Sharp & Dohme), nisoldipine (Bayer), CGP42112A (RBI), fura 2-AM and SYTO-16 (Molecular Probes), polyclonal anti-AT₁ and anti-AT₂ antibodies (Santa Cruz), monoclonal anti-myosin (smooth) and anti- α -actin (Sigma), and annexin V-FITC (Immunotech) were used.

SMC Cultures

Sp-SMCs and Ep-SMCs, initially named V6-SMCs and V8-SMCs, respectively, have been isolated from Wistar rat aortic media as stable cell lines.⁸ Standard primary VSMCs from rat aorta,²⁴ heterogeneous, although mostly spindle cells,^{9,12} were used as a reference. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 93-23, revised 1985) and were approved by the Institutional Care and Use Committee. Sp-SMCs and Ep-SMCs were characterized by expression of smooth muscle markers in 0.2% serum and by serum-independent (0.0% serum) growth. For apoptosis experiments, cells cultured 24 hours in MEM plus 10% newborn calf serum were serum-deprived (0.2% serum) 24 hours before stimulation.

Measurement of [Ca²⁺]_i

Cells in Krebs-Ringer buffer (1 mmol/L CaCl₂) were loaded with the permeant calcium-sensitive fluorescent probe fura 2-AM and washed before Ang II perfusion. Fluorescence was measured with a Spex Fluorilog spectrofluorometer. [Ca²⁺]_i was determined as previously described.²⁵

Viability

Whole viability of a culture was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test²⁶ or by counting cell number (Coulter counter, Coultronics).²⁴

Morphology of Apoptosis or Necrosis

For nuclear staining, monolayers were coincubated with 1 μ g/mL SYTO-16 and 4 μ g/mL propidium iodide fluorescent DNA-binding dyes.²⁶ Dead cells, permeable for propidium iodide, stained red. Apoptosis (green condensed or fragmented nuclei), primary necrosis (red nuclei with loose chromatin), postapoptotic necrosis (red apoptotic nuclei), and normal cells (green nuclei with loose chromatin) were discriminated.

Annexin V-FITC Staining

Trypsinized VSMCs washed in binding buffer were incubated in FITC-labeled annexin V (annexin kit, Immunotech) for 10 minutes. Cells were analyzed by flow cytometry by using a single cell gate, and the percentage of annexin-positive cells was evaluated.

DNA Fragmentation

DNA fragmentation was visualized by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) procedure (In Situ Cell Death Detection POD kit, Boehringer). For quantification of DNA fragmentation, cell lysates were centrifuged (27 000g). Separated chromatin pellet and DNA cleavage products were quantified by the fluorometric 4,6-diamidino-2-phenylindole procedure.²⁶ DNA ladders were visualized by ethidium bromide after DNA electrophoresis (Suicide-Track DNA Ladder Isolation Kit, Oncogene Research Products).

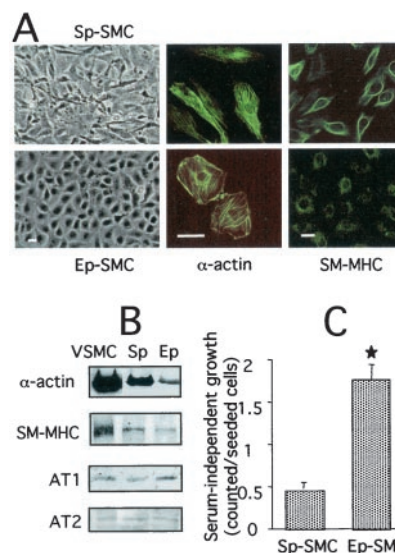


Figure 1. Phenotypes of Sp-SMCs and Ep-SMCs. A, Phase-contrast microscopy (magnification, $\times 100$) and immunofluorescence staining for smooth muscle α -actin (magnification, $\times 320$) and smooth muscle myosin heavy chain (SM-MHC) (magnification, $\times 200$). Bars represent 10 μ m. B, Representative Western blots of α -actin, SM-MHC, and AT₁ and AT₂ receptor proteins in 0.2% serum. Sp and Ep indicate Sp-SMCs and Ep-SMCs, respectively. C, Serum-independent growth in 0.0% serum. Results are presented as number of cells after 7 days divided by seeded cells. Values are mean \pm SEM of 3 experiments.

Caspase 3-Like Activity

Caspase 3-like activity was determined by detecting chromophore *p*-nitroanilide after cleavage from the labeled substrate ac-DEVD-*p*-nitroanilide (Bachem).

Immunofluorescence Microscopy

Cells on coverslips, fixed with methanol, were incubated with primary antibody (1:50 dilution) and then with the appropriate fluorescein-conjugated secondary antibody.⁸

Western Blot Analysis

Proteins (40 μ g) were subjected to SDS-PAGE, transferred to membranes (Amersham), incubated with primary antibody (1:200 dilution) and the appropriate secondary antibody,²⁶ and visualized by ECL kit (Amersham).

Data Analysis

Values are presented as mean \pm SEM. Groups were compared by ANOVA, and means were compared by the Student's *t* test. Values of *P* < 0.05 were considered statistically significant.

Results

VSMC Phenotypes

The stable morphologically distinct Sp-SMC and Ep-SMC cell lines (Figure 1A) expressed both smooth muscle α -actin (43 kDa) and myosin heavy chain (205 kDa) under low serum conditions (0.2%), but expression was markedly lower in Ep-SMCs (Figure 1B). Only Ep-SMCs were capable of serum-independent growth (Figure 1C), as were other epithelioid smooth muscle cell models.^{9,10} AT₁ receptor (41 kDa) expression was higher in Ep-SMCs. AT₂ receptor (44 kDa) expression was comparable between both cell lines (Figure 1B).

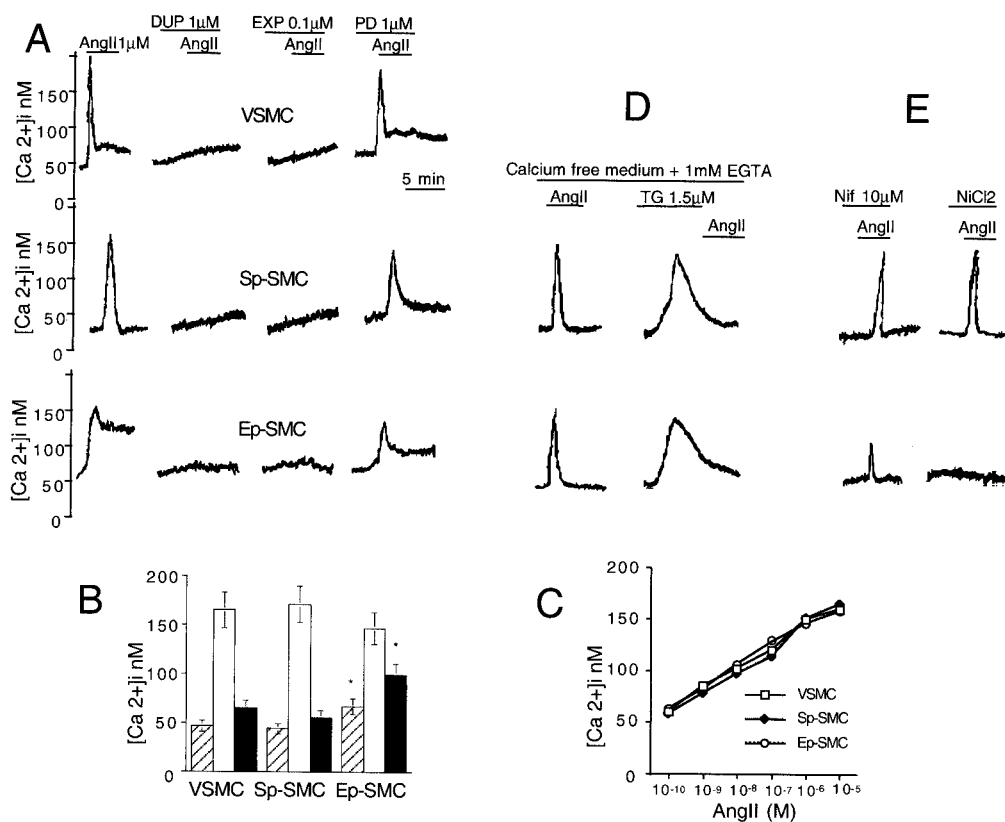


Figure 2. Ang II-elicited $[Ca^{2+}]_i$ signaling. A, Representative tracings of $[Ca^{2+}]_i$ responses to Ang II ($1 \mu\text{mol/L}$) in primary VSMC, Sp-SMC, or Ep-SMC cultures in 1 mmol/L extracellular calcium concentration ($[Ca^{2+}]_o$). Effect of AT_1 (DUP735 [DUP], EXP3174 [EXP]) or AT_2 (PD 123,177 [PD]) receptor antagonists 2 minutes before and throughout Ang II administration. B, Averaged $[Ca^{2+}]_i$ levels: baseline (hatched bars), $[Ca^{2+}]_i$ peaks (open bars), and 5 minutes after stimulation (solid bars). Bars represent mean \pm SEM ($n=8$). $*P<0.05$ vs VSMCs. C, Dose response of Ang II-elicited peak. D, Responses in absence of $[Ca^{2+}]_o$, without and with thapsigargin (TG, $1.5 \mu\text{mol/L}$) pretreatment (7 minutes) before Ang II. E, Responses in 1 mmol/L $[Ca^{2+}]_o$ with nifedipine (Nif, $10 \mu\text{mol/L}$) or $NiCl_2$ ($100 \mu\text{mol/L}$) pretreatment.

Ang II–Stimulated $[Ca^{2+}]_i$ Signaling in VSMC Phenotypes

In VSMC cultures, Ang II elicited a classic biphasic $[Ca^{2+}]_i$ signal with a transient peak, followed by a stationary phase slightly above the baseline (Figure 2A). In Sp-SMCs, the response was a single transient peak. In Ep-SMCs, the signal was biphasic, with a high plateau. The basal $[Ca^{2+}]_i$ and plateau levels were higher in Ep-SMCs (Figure 2B). All responses were dose dependent (Figure 2C). Pretreatment with the AT_1 receptor antagonists EXP3174 or DUP753, but not with the AT_2 antagonist PD 123,177, prevented the Ang II-induced $[Ca^{2+}]_i$ responses (Figure 2A). The results indicate that in both Sp-SMCs and Ep-SMCs, functional AT_1 receptors are coupled with $[Ca^{2+}]_i$ signaling, but with distinct patterns.

Calcium Sources of Ang II–Induced $[Ca^{2+}]_i$ Signaling in VSMC Phenotypes

In calcium-free medium, the peak responses to Ang II were similar in Sp-SMCs and Ep-SMCs, whereas the plateau phase was abolished in Ep-SMCs (Figure 2D). This suggests that the Ang II-induced transient $[Ca^{2+}]_i$ peak resulted mainly from internal calcium stores, whereas the plateau in Ep-SMCs required calcium entry. Pretreatment with TG ($1.5 \mu\text{mol/L}$, 7 minutes before Ang II), an inhibitor of the sarcoplasmic

reticulum calcium-ATPase pump, increased $[Ca^{2+}]_i$ by depletion of TG-sensitive stores. In both lines, TG prevented, in calcium-free medium, a subsequent Ang II-induced signal (Figure 2D). This suggests no difference in the intracellular pools (TG sensitive) rapidly mobilized by Ang II in the 2 phenotypes. Preincubation with nifedipine ($10 \mu\text{mol/L}$, dihydropyridine blocker of L-type voltage-operated channels) did not alter the Sp-SMC response but reduced the peak and abolished the plateau in Ep-SMCs (Figure 2E). $NiCl_2$ ($100 \mu\text{mol/L}$) abolished the Ep-SMC response, suggesting the participation of calcium-induced calcium release.²⁷ In summary, in both Sp-SMCs and Ep-SMCs, calcium was rapidly mobilized by Ang II from TG-sensitive intracellular pools. In Ep-SMCs, an additional calcium entry occurred, mostly through L-type voltage-operated channels.

Ang II–Induced VSMC Phenotype–Dependent Apoptosis

Under low serum conditions (0.2%), Ang II dramatically reduced Ep-SMC but not Sp-SMC number (Figure 3A), suggesting that Ang II differently altered cell survival. Reduction in Ep-SMC number was dose dependent (Figure 3B) and occurred 3 days after stimulation (not shown). At 10 minutes, Ang II incubation was enough to reduce the cell number 3 days later (Figure 3B). The nature of cell death was

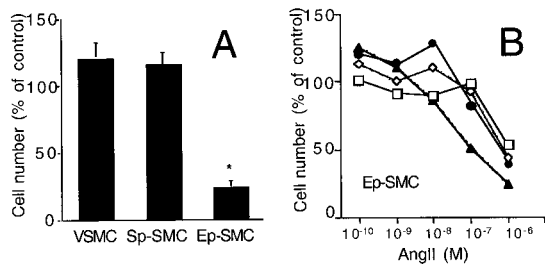


Figure 3. Ang II and survival of VSMC phenotypes. A, VSMC, Sp-SMC, or Ep-SMC numbers counted 96 hours after addition of Ang II (1 $\mu\text{mol/L}$) in low (0.2%) serum. Results are expressed as percentage of control without Ang II and are mean \pm SEM of 4 experiments. * $P < 0.05$ vs Ang II-treated VSMCs or Sp-SMCs. B, Concentration response curves of Ep-SMC numbers in cultures stimulated with Ang II for variable times (10 minutes [□], 20 minutes [◇], 48 hours [●], and 96 hours [▲]) and thereafter cultured up to 96 hours. Results are presented as percentage of control without Ang II and are mean \pm SEM of 3 experiments.

characterized. Examination of Ep-SMC morphology showed that Ang II promoted apoptosis, ie, nuclear condensation, apoptotic bodies, chromatin fragmentation, and postapoptotic necrosis (Figure 4C; 40 \pm 18% apoptotic figures in Ang II-treated Ep-SMCs versus 8 \pm 3% apoptotic figures in con-

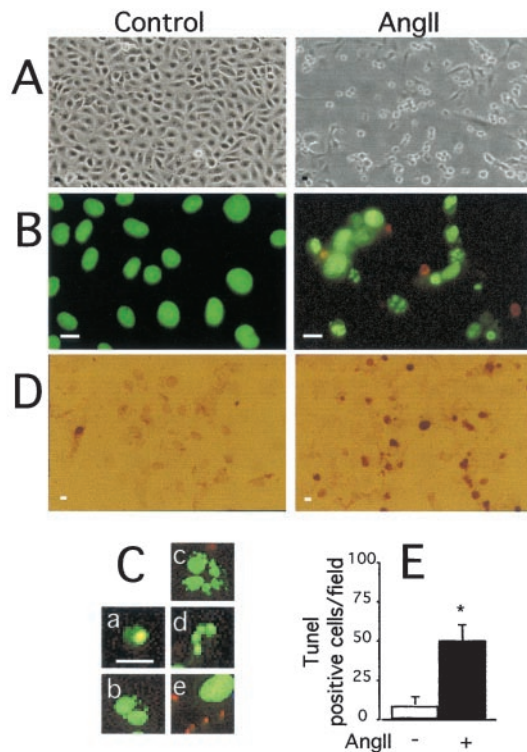


Figure 4. Ang II-induced apoptosis in Ep-SMCs. A, Phase-contrast microscopy of control and Ang II (1 $\mu\text{mol/L}$)-treated Ep-SMCs. Magnification, $\times 100$. B, Nuclear staining with SYTO-16 (green) and propidium iodide (red). Magnification, $\times 200$. C, Apoptotic nuclei in Ang II-treated Ep-SMCs (a indicates nuclear condensation; b, apoptotic bodies; c and d, fragmentation; and e, red postapoptotic bodies near a normal nucleus). Magnification, $\times 600$. D, TUNEL staining in control and Ang II-treated Ep-SMCs. Magnification, $\times 125$. E, Quantification of TUNEL-positive nuclei. Results are presented as number of positive nuclei per microscopic field and are mean \pm SEM of 3 experiments. * $P < 0.05$ vs control. Bars represent 10 μm .

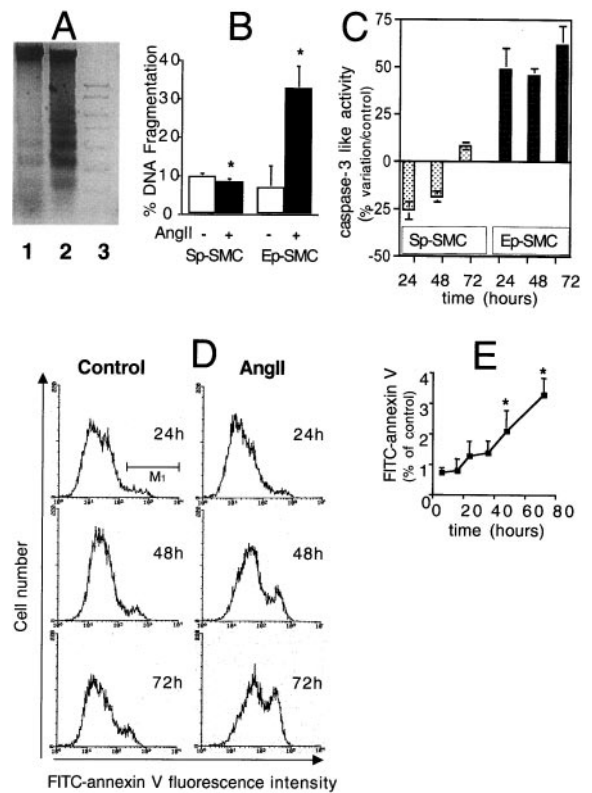


Figure 5. Biochemical features of Ang II-induced apoptosis in Ep-SMCs. A, DNA ladders in Ep-SMCs. Lanes are as follows: 1, control serum deprivation; 2, Ang II treatment (1 $\mu\text{mol/L}$) for 72 hours; and 3, 2-kb ladder marker. B, Quantification of DNA fragments in Sp-SMCs or Ep-SMCs. Results are presented as percent fragments in total DNA and are mean \pm SEM of 4 experiments. * $P < 0.05$ vs control. C, Caspase 3-like activity in Sp-SMCs or Ep-SMCs. Results are expressed as percent variation divided by corresponding control. Values are mean \pm SEM of 4 experiments. D and E, Kinetics of FITC-annexin V labeling of Ep-SMCs analyzed by fluorescence-activated cell sorting. Panel D shows representative histograms of FITC-annexin V fluorescence intensity in control serum-deprived (0.2% serum) and Ang II-treated Ep-SMCs. Panel E shows the percentage of annexin V-positive cells as determined by setting the marker M1 for positive population. Values are mean \pm SEM of 4 experiments. * $P < 0.05$ vs control.

trol Ep-SMCs, $P < 0.05$). Primary necrosis was $< 5\%$ of nuclei. Apoptosis was confirmed by TUNEL staining (Figure 4D and 4E), DNA ladders (Figure 5A), DNA fragmentation (Figure 5B; 33 \pm 5.8% in Ep-SMCs versus 7 \pm 5.6% in controls, $P < 0.05$), and caspase 3-like activity (Figure 5C). Interestingly, opposite effects were induced in Sp-SMCs (Figure 5B and 5C). Kinetics of annexin V-FITC labeling to exposed plasma membrane phosphatidylserine (early apoptosis marker) showed that Ang II promoted not an early (up to 24 to 36 hours) but a delayed increase in the labeled Ep-SMC population (Figure 5D and 5E). Taken together, the results show that Ang II promoted a delayed apoptosis in Ep-SMCs but not in Sp-SMCs.

AT₁-Receptor-Induced and Calcium-Dependent Apoptosis in Ep-SMCs

Apoptosis was abolished by AT₁ (EXP3174) but not by AT₂ (PD 123,177) receptor antagonists (Figure 6A, 6B, and 6C)

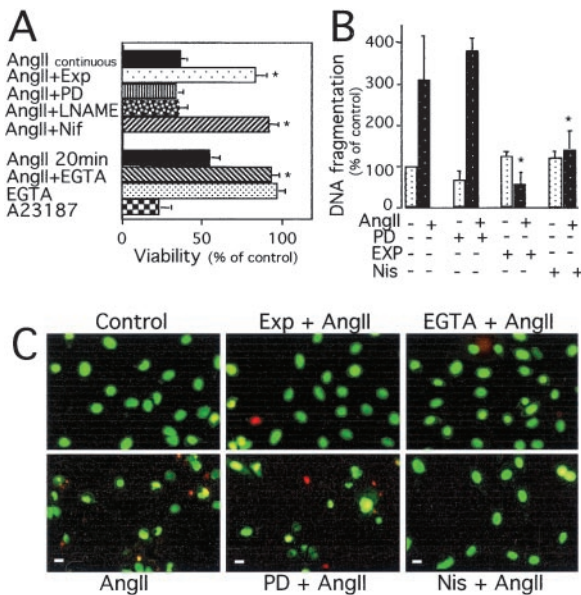


Figure 6. AT₁ receptor mediation, calcium dependence, and lack of NO generation in Ep-SMC apoptosis. Ep-SMCs were pretreated with EXP (0.1 μ mol/L), PD (1 μ mol/L), L-NAME (10 μ mol/L), Nif (1 μ mol/L), or nisoldipine (Nis, 1 μ mol/L) before Ang II (1 μ mol/L) for 72 hours. Ep-SMCs were pretreated for 30 minutes with EGTA (5 mmol/L) or calcium ionophore A23187 (10 μ mol/L) with or without Ang II for 20 minutes and with a subsequent 72-hour culture without drug. A, Cell viability by MTT test. Results are presented as percent viability of control nontreated Ep-SMCs. Values are mean \pm SEM of 3 to 5 experiments. * P <0.05 vs Ang II treatment. B, DNA fragmentation. Results are presented as percent fragmentation in control Ep-SMC DNA. C, Nuclear staining with SYTO-16 and propidium iodide (magnification, \times 200). Bars represent 10 μ m.

and was not induced by the AT₂ agonist CGP42112A (not shown). We tested whether NO, a known inducer of VSMC apoptosis, could mediate apoptosis. L-NAME (10 μ mol/L), an inhibitor of NO generation, did not prevent Ang II-induced Ep-SMC death (Figure 6A). Because Ang II specifically induced calcium entry in Ep-SMCs, we studied whether calcium entry was involved in apoptosis. Ang II-induced Ep-SMC apoptosis was significantly inhibited by EGTA (5 mmol/L, 20 minutes; Figure 6A and 6C), nifedipine (10 μ mol/L, Figure 6A), or nisoldipine (10 μ mol/L, Figure 6B and 6C). Conversely, A23187 (10 μ mol/L, 20 minutes), a calcium ionophore, induced Ep-SMC death (Figure 6A). The results demonstrate that Ang II-induced apoptosis in Ep-SMCs involved calcium entry and was mediated by AT₁ receptors but not by AT₂ receptors.

Discussion

The present study shows for the first time that Ang II can induce apoptosis in VSMCs depending on their phenotype (epithelioid or spindle-shaped) through the activation of AT₁ receptor-mediated calcium entry. Apoptosis was specifically promoted in the epithelioid phenotype (Ep-SMCs). Characterization of cell death was made on the basis of typical morphological features (cell shrinkage, nuclear condensation, and fragmentation) and biochemical changes (DNA fragmentation, caspase 3-like activity, and annexin V binding after

plasma membrane phosphatidylserine translocation). Apoptosis was dose and time dependent and occurred under serum deprivation.

Ang II is well known as a regulator of VSMC proliferation/hypertrophy. More recently, Ang II has been proposed as a bifunctional modulator of VSMC apoptosis through either AT₁ (antiapoptotic) or AT₂ (proapoptotic) receptors.²² Previous in vitro results have shown that Ang II can induce apoptosis in AT₂ receptor-transfected VSMCs²² and protect native VSMCs, through AT₁ receptors, against early (NO-induced) apoptosis.²³ In the present study, in VSMCs of different phenotypes, Ang II did not induce early apoptosis as reported in a previous study.²³ However, in Ep-SMCs, Ang II promoted a delayed apoptosis. Consistently, apoptosis has been reported in a delayed manner²⁰ in rat arteries via the AT₁ receptor.^{20,21} The present AT₁ receptor-induced apoptosis extends the possible cellular mechanisms of VSMC growth regulation by Ang II. Summarizing, AT₁ receptor activation may control VSMC growth through either proliferation or delayed apoptosis, whereas AT₂ receptor activation may induce antigrowth effects (inhibition of proliferation and apoptosis).

The VSMC phenotype specificity in the apoptotic response to Ang II cannot be explained by differential AT₁/AT₂ receptor expression in Sp-SMCs versus Ep-SMCs. Both cell lines expressed AT₁ and AT₂ receptors, but apoptosis was clearly AT₁ receptor-mediated, inasmuch as it was prevented by AT₁ but not by AT₂ receptor antagonists and was not induced by an AT₂ receptor agonist. Apoptosis in Ep-SMCs required only short Ang II exposure with associated calcium influx. Apoptosis was reduced by an extracellular calcium chelator and by calcium antagonists. Furthermore, apoptosis was induced by a calcium ionophore. Thus, the AT₁ receptor-mediated calcium entry seems causally involved in apoptosis. A role for calcium signaling is documented in apoptosis²⁸ and in AT₁ receptor-induced apoptosis.^{29,30} Calcium can directly activate hydrolytic enzymes or signal cell death through calcium-dependent kinases or phosphatases. Further investigation will be necessary to determine precisely the AT₁ receptor downstream apoptotic pathway in Ep-SMCs.

Regional heterogeneity exists in response to vasoactive stimuli in normal and diseased vessels. One hypothesis is that arterial walls vary in composition of different VSMC subtypes to fulfill specific roles.²⁻⁶ Heterogeneity was mostly studied with VSMC cell lines originating from different rat arteries.^{2,8-11,31} In the present study, VSMC lines of spindle or epithelioid phenotypes, both from healthy adult rat aortas,⁸ were used. Similarity in their culture history allowed comparison of the intrinsic properties of the phenotypes, excluding the influence of environmental factors.^{10,11,31} Although transformed, these cell lines can be compared with other models of VSMC subtypes^{9,10}; the 2 VSMC lines differ in serum-independent growth (specific to epithelioid cells) and in differentiation state (lower for epithelioid cells) as in other models.^{9,10} In the present study, AT₁ receptors were functional in both phenotypes but were coupled to distinct AT₁ receptor [Ca²⁺]_i signals. The sustained calcium entry specific to Ep-SMCs has been observed in epithelioid VSMC-enriched cultures stimulated with other vasoconstrictors,¹² and it

could be a feature of the epithelioid subtype. VSMC heterogeneity might participate in regional differences in contractile or pathological alterations. As a major point, the present study suggests a susceptibility of epithelioid VSMCs to Ang II-induced apoptosis. Interestingly, a higher ACE activity (ie, potential Ang II formation) was found in epithelioid compared with elongated embryonic VSMCs.⁷ The renin-angiotensin system could be a determinant of VSMC subtype-specific behavior.

VSMC apoptosis occurs in all cardiovascular diseases, hypertension, atherosclerosis, and restenosis.¹ The present Ang II effects on VSMC subtypes could have pathophysiological relevance. An AT₁ receptor-mediated remodeling of vascular wall mass by apoptosis of a peculiar VSMC subtype may occur. This process could regulate proliferation during healing,¹⁹ in aneurisms, in narrowed peripheral arteries, or within atherosclerotic lesions.¹ With respect to atherosclerosis, human plaques contain a VSMC subtype³² with apoptotic-prone behavior³³ and have increased ACE activity.³⁴ Therefore, Ang II could locally contribute to plaque weakening and rupture by promoting apoptosis. Finally, the present study points out the importance of extending the knowledge on VSMC heterogeneity to better understand vascular pathology.

Acknowledgments

The present study was supported by funds from INSERM, the Merck Sharp & Dohme laboratory, and Ligue Nationale Contre le Cancer. We thank Dr Joost Peter Schanstra for revising the English version.

References

- Haunstetter A, Izumo S. Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circ Res*. 1998;82:1111-1129.
- Schwartz SM, deBlois D, O'Brien ERM. The intima: soil for atherosclerosis and restenosis. *Circ Res*. 1995;77:445-465.
- Daemen MJ, De Mey JG. Regional heterogeneity of arterial structural changes. *Hypertension*. 1995;25:464-473.
- Frid MG, Dempsey EC, Durmowicz AG, Stenmark KR. Smooth muscle cell heterogeneity in pulmonary and systemic vessels: importance in vascular disease. *Arterioscler Thromb Vasc Biol*. 1997;17:1203-1209.
- Seidel CL. Cellular heterogeneity of the vascular tunica media: implications for vessel wall repair. *Arterioscler Thromb Vasc Biol*. 1997;17:1868-1871.
- Topouzis S, Catravas JD, Ryan JW, Rosenquist TH. Influence of vascular smooth muscle heterogeneity on angiotensin converting enzyme activity in chicken embryonic aorta and in endothelial cells in culture. *Circ Res*. 1992;71:923-931.
- Mironov AA, Rekhter MD, Kolpakov VA, Andreeva ER, Polishchuk RS, Bannykh SI, Filippov SV, Peretjatko LP, Kulida LV, Orekhov AN. Heterogeneity of smooth muscle cells in embryonic human aorta. *Tissue Cell*. 1995;27:31-38.
- Blaes N, Bourdillon MC, Daniel-Lamaziere JM, Michaille JJ, Andujar M, Covacho C. Isolation of two morphologically distinct cell lines from rat arterial smooth muscle expressing high tumorigenic potentials. *In Vitro Cell Dev Biol*. 1991;27A:725-734.
- Bochaton-Piallat ML, Ropraz P, Gabbiani F, Gabbiani G. Phenotypic heterogeneity of rat arterial smooth muscle cell clones: implications for the development of experimental intimal thickening. *Arterioscler Thromb Vasc Biol*. 1996;16:815-820.
- Lemire JM, Potter-Perigo S, Hall KL, Wight TN, Schwartz SM. Distinct rat aortic smooth muscle cells differ in versican/PG-M expression. *Arterioscler Thromb Vasc Biol*. 1996;16:821-829.
- Bochaton-Piallat ML, Gabbiani G, Pepper MS. Plasminogen activator expression in rat arterial smooth muscle cells depends on their phenotype and is modulated by cytokines. *Circ Res*. 1998;82:1086-1093.
- Neylon CB, Avdonin PV, Dilley RJ, Larsen MA, Tkachuk VA, Bobik A. Different electrical responses to vasoactive agonists in morphologically distinct smooth muscle cell types. *Circ Res*. 1994;75:733-741.
- Levy BI. The potential role of angiotensin II in the vasculature. *J Hum Hypertens*. 1998;12:283-287.
- Hughes AD. Molecular and cellular mechanisms of action of angiotensin II (AT₁) receptors in vascular smooth muscle. *J Hum Hypertens*. 1998;12:275-281.
- Johnson EM, Theler JM, Capponi AM, Vallotton MB. Characterization of oscillations in cytosolic free Ca²⁺ concentration and measurement of cytosolic Na⁺ concentration changes evoked by angiotensin II and vasopressin in individual rat aortic smooth muscle cells: use of microfluorometry and digital imaging. *J Biol Chem*. 1991;266:12618-12626.
- Helou CM, Marchetti J. Morphological heterogeneity of renal glomerular arterioles and distinct [Ca²⁺]_i responses to Ang II. *Am J Physiol*. 1997;273(pt 2):F84-F96.
- Geisterfer AA, Peach MJ, Owens GK. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res*. 1988;62:749-756.
- Touyz RM, Deng LY, He G, Wu XH, Schiffrin EL. Angiotensin II stimulates DNA and protein synthesis in vascular smooth muscle cells from human arteries: role of extracellular signal-regulated kinases. *J Hypertens*. 1999;17:907-916.
- deBlois D, Viswanathan M, Su JE, Clowes AW, Saavedra JM, Schwartz SM. Smooth muscle DNA replication in response to angiotensin II is regulated differently in the neointima and media at different times after balloon injury in the rat carotid artery: role of AT₁ receptor expression. *Arterioscler Thromb Vasc Biol*. 1996;16:1130-1137.
- Diep QN, Li JS, Schiffrin EL. In vivo study of AT₁ and AT₂ angiotensin receptors in apoptosis in rat blood vessels. *Hypertension*. 1999;34:617-624.
- Nakamura M, Tanaka M, Abe S, Fujiwara H. Sudden pressure elevation can trigger acute muscle cell death of the heart and aorta. *Atherosclerosis*. 1999;146:25-32.
- Yamada T, Akishita M, Pollman MJ, Gibbons GH, Dzau VJ, Horiuchi M. Angiotensin II type 2 receptor mediates vascular smooth muscle cell apoptosis and antagonizes angiotensin II type 1 receptor action: an in vitro gene transfer study. *Life Sci*. 1998;63:PL289-PL295.
- Pollman MJ, Yamada T, Horiuchi M, Gibbons GH. Vasoactive substances regulate vascular smooth muscle cell apoptosis: countervailing influences of nitric oxide and angiotensin II. *Circ Res*. 1996;79:748-756.
- Blaes N, Boissel JP. Growth-stimulating effect of catecholamines on rat aortic smooth muscle cells in culture. *J Cell Physiol*. 1983;116:167-172.
- Schanstra JP, Bataille E, Marin Castano ME, Barascud Y, Hirtz C, Pesquero JB, Pecher C, Gauthier F, Girolami JP, Bascands JL. The B1-agonist [des-Arg10]-kallidin activates transcription factor NF-kappaB and induces homologous upregulation of the bradykinin B1-receptor in cultured human lung fibroblasts. *J Clin Invest*. 1998;101:2080-2091.
- Meilhac O, Escargueil-Blanc I, Thiers JC, Salvayre R, Negre-Salvayre A. Bcl-2 alters the balance between apoptosis and necrosis, but does not prevent cell death induced by oxidized low density lipoproteins. *FASEB J*. 1999;13:485-494.
- Smith JB. Calcium homeostasis in smooth muscle cells. *New Horiz*. 1996;4:2-18.
- Nicotera P, Orrenius S. The role of calcium in apoptosis. *Cell Calcium*. 1998;23:173-180.
- Li D, Yang B, Philips MI, Mehta JL. Proapoptotic effects of Ang II in human coronary artery endothelial cells: role of AT₁ receptor and PKC activation. *Am J Physiol*. 1999;276:H786-H792.
- Kajstura J, Cigola E, Malhotra A, Li P, Cheng W, Meggs LG, Anversa P. Angiotensin II induces apoptosis of adult ventricular myocytes in vitro. *J Mol Cell Cardiol*. 1997;29:859-870.
- Li WG, Miller FJ Jr, Brown MR, Chatterjee P, Aylsworth GR, Shao J, Spector AA, Oberley LW, Weintraub NL. Enhanced H₂O₂-induced cytotoxicity in "epithelioid" smooth muscle cells: implication for regression of neointima. *Arterioscler Thromb Vasc Biol*. 2000;20:1473-1479.
- Chung IM, Schwartz SM, Murry CE. Clonal architecture of normal and atherosclerotic aorta: implications for atherogenesis and vascular development. *Am J Pathol*. 1998;152:913-923.
- Bennett MR, Littlewood TD, Schwartz SM, Weissberg PL. Increased sensitivity of human vascular smooth muscle cells from atherosclerotic plaques to p53-mediated apoptosis. *Circ Res*. 1997;81:591-599.
- Diet F, Pratt RE, Berry GJ, Momose N, Gibbons GH, Dzau VJ. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation*. 1996;94:2756-2767.