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

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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 animals2–6 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 synthesis2,8–11; 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
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, N6 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), CGP42112 A (RBI), fura 2-AM and SYITO-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 standard cell lines.8 Standard primary VSMCs from rat aorta, heterogenous, 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 Fluorolog 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) test and by seeding cell number (Coulter counter, Coultronics).24

Morphology of Apoptosis or Necrosis
For nuclear staining, monolayers were coincubated with 1 μg/mL SYTO-16 and 4 μg/mL propidium iodide fluorescent DNA-binding dyes.20 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).

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).
Ang II–Stimulated \([\text{Ca}^{2+}]_i\) Signaling in VSMC Phenotypes

In VSMC cultures, Ang II elicited a classic biphasic \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) and plateau levels were higher in Ep-SMCs (Figure 2B). All responses were dose dependent (Figure 2C). Pretreatment with the AT1 receptor antagonists EXP3174 or DUP735, but not with the AT2 antagonist PD 123,177, prevented the Ang II–induced \([\text{Ca}^{2+}]_i\) responses (Figure 2A). The results indicate that in both Sp-SMCs and Ep-SMCs, functional AT1 receptors are coupled with \([\text{Ca}^{2+}]_i\) signaling, but with distinct patterns.

Calcium Sources of Ang II–Induced \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) peak resulted mainly from internal calcium stores, whereas the plateau in Ep-SMCs required calcium entry. Pretreatment with TG (1.5 \(\mu\)mol/L, 7 minutes before Ang II), an inhibitor of the sarcoplasmic reticulum calcium-ATPase pump, increased \([\text{Ca}^{2+}]_i\), 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 \(\mu\)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). NiCl2 (100 \(\mu\)mol/L) abolished the Ep-SMC response, suggesting the participation of calcium-induced calcium release. 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...
characterized. Examination of Ep-SMC morphology showed that Ang II promoted apoptosis, i.e., nuclear condensation, apoptotic bodies, chromatin fragmentation, and postapoptotic necrosis (Figure 4C; 40 ± 18% apoptotic figures in Ang II–treated Ep-SMCs versus 8 ± 3% apoptotic figures in control 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 ± 5.8% in Ep-SMCs versus 7 ± 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 expose 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.

AT\(_1\)-Receptor–Induced and Calcium-Dependent Apoptosis in Ep-SMCs

Apoptosis was abolished by AT\(_1\), (EXP3174) but not by AT\(_2\), (PD 123,177) receptor antagonists (Figure 6A, 6B, and 6C)
and was not induced by the AT$_2$ agonist CGP42112A (not shown). We tested whether NO, a known inducer of VSMC apoptosis, could mediate apoptosis. L-NAME (10 μmol/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 mM, 20 minutes; Figure 6A and 6C), nifedipine (10 μmol/L, Figure 6A), or nisoldipine (10 μmol/L, Figure 6B and 6C). Conversely, A23187 (10 μmol/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 AT$_1$ receptors but not by AT$_2$ 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 AT$_1$ 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 phosphatidylerine 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 AT$_1$ (antia apoptotic) or AT$_2$ (proapoptotic) receptors.\[^{22}\] Previous in vitro results have shown that Ang II can induce apoptosis in AT$_2$ receptor–transfected VSMCs\[^{23}\] and protect native VSMCs, through AT$_1$ 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 manner\[^{20}\] in rat arteries via the AT$_1$ receptor.\[^{20,21}\] The present AT$_1$ receptor–induced apoptosis extends the possible cellular mechanisms of VSMC growth regulation by Ang II. Summarizing, AT$_1$ receptor activation may control VSMC growth through either proliferation or delayed apoptosis, whereas AT$_2$ 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 AT$_1$/AT$_2$ receptor expression in Sp-SMCs versus Ep-SMCs. Both cell lines expressed AT$_1$ and AT$_2$ receptors, but apoptosis was clearly AT$_1$ receptor–mediated, inasmuch as it was prevented by AT$_1$ but not by AT$_2$ receptor antagonists and was not induced by an AT$_2$ 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 AT$_1$ receptor–mediated calcium entry seems causally involved in apoptosis. A role for calcium signaling is documented in apoptosis\[^{28}\] and in AT$_1$ 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 AT$_1$ 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.\[^{7,8,11,33}\] In the present study, VSMC lines of spindle or epithelioid phenotypes, both from healthy adult rat aortas, 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 to fulfill specific roles.\[^{2–6}\] Heterogeneity was mostly studied with VSMC cell lines originating from different rat arteries.\[^{7,8,11,33}\] In the present study, AT$_1$ receptors were functional in both phenotypes but were coupled to distinct AT$_2$ receptor [Ca$^{2+}$] 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 (i.e., potential Ang II formation) was found in epithelioid compared with elongated embryonic VSMCs. The renin-angiotensin system could be a determinant of VSMC subtype–specific behavior.

VSMC apoptosis occurs in all cardiovascular diseases, hypertension, atherosclerosis, and restenosis. 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, in aneurysms, in narrowed peripheral arteries, or within atherosclerotic lesions. With respect to atherosclerosis, human plaques contain a VSMC subtype with apoptotic-prone behavior and have increased ACE activity. 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.

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References

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