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Angiotensin II–Mediated Oxidative DNA Damage Accelerates Cellular Senescence in Cultured Human Vascular Smooth Muscle Cells via Telomere-Dependent and Independent Pathways

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Abstract—Angiotensin II (Ang II) induces reactive oxygen species (ROS) production by human vascular smooth muscle cells (hVSMCs). ROS have been implicated in the development of both acute stress-induced premature senescence (SIPS) and chronic replicative senescence. Global oxidative DNA damage triggers SIPS and telomere DNA damage accelerates replicative senescence, both mediated via p53. This study tests the hypothesis that DNA is an important target for Ang II–induced ROS leading to senescence via telomere-dependent and independent pathways. DNA damage was quantified using the Comet assay, telomere DNA length by Southern blotting and hVSMC senescence by senescence-associated β-galactosidase staining. Exposure to Ang II increased DNA damage in hVSMCs within 4 hours. Inhibition by an AT_1 receptor antagonist (losartan metabolite: E3174) or catalase, confirmed that Ang II–induced DNA damage was AT_1 receptor-mediated, via the induction of ROS. Acute exposure to Ang II resulted in SIPS within 24 hours that was prevented by coincubation with E3174 or catalase. SIPS was associated with increased p53 expression but was not dependent on telomere attrition because overexpression of human telomerase did not prevent Ang II–induced SIPS. Exposure to Ang II over several population doublings accelerated the rate of telomere attrition (by $>$2-fold) and induced premature replicative senescence of hVSMCs—an effect that was also attenuated by E3174 or catalase. These data demonstrate that Ang II–induced ROS-mediated DNA damage results in accelerated biological aging of hVSMCs via 2 mechanisms: (1) Acute SIPS, which is telomere independent, and (2) accelerated replicative senescence which is associated with accelerated telomere attrition. **(*Circ Res*. 2008;102:201-208.)**

Key Words: vascular smooth muscle cell ■ senescence ■ angiotensin II ■ reactive oxygen species ■ DNA damage

Angiotensin II (Ang II) has been widely implicated in the pathogenesis of cardiovascular disease and the vascular smooth muscle cell (VSMCs) is an important target for its actions. Numerous cell signaling pathways have been implicated, several of which involve reactive oxygen species (ROS) generation via NAD(P)H oxidase activity.[1–4] Many previous studies have shown that Ang II stimulates the intracellular accumulation of ROS, particularly H_2O_2, in VSMCs which play a critical role in cell signaling.[2,5,6] In the vascular system, the cell signaling functions of H_2O_2 account for the pathological effects on growth, angiogenesis, hypertrophy and inflammation of Ang II.[3]

DNA is particularly susceptible to damage by ROS but surprisingly this has received little attention in the context of cardiovascular disease. In cultured cells, critical DNA damage induces rapid "stress induced premature senescence" (SIPS).[7] In addition to SIPS, vascular cells may also undergo premature replicative senescence via accelerated attrition of telomeres.[8,9] The telomeres are chromosomal protective cap structures, characterized by long stretches of DNA repeat sequences (TTAGGG) that shorten with replication and cell aging. The high GGG content renders telomeres particularly susceptible to oxidative damage by ROS and this is an important cause of accelerated telomere attrition.[10] Cells with critically shortened or damaged telomeres enter a state of "replicative senescence".[11,12] Unlike SIPS which is rapid (within days), primary cultures of human cells undergo replicative senescence over several weeks or months in culture, depending on their original telomere length, the magnitude of stress, and their rate of proliferation. The states of SIPS and replicative senescence share common molecular signals which usually involve stabilization of p53, increased expression of p21, and subsequent hypophosphorylation of Rb protein which mediates growth arrest.[7] The cyclin-dependent kinase inhibitor,

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p16, may also play a role in mediating growth arrest by some stressors.[7] It is likely that both SIPS and replicative senescence occur simultaneously in populations of vascular cells and their progenitors and that this contributes to manifestations of disease in aging vascular tissues.[13–16]

The senescent cell phenotype and shortened telomeres have both been strongly implicated in the pathogenesis of vascular disease.[13,16] Senescent cells are commonly observed in the vascular wall at sites prone to atheroma in both humans and animal models of disease.[8,17–20] Moreover, VSMCs within atherosclerotic plaques show morphological and biochemical features of senescence,[8,17] oxidative DNA damage, shorter telomeres, and activation of the p53-p21 axis[9] suggesting that human cells undergo accelerated replicative senescence mediated by ROS. In addition, recent studies have emphasized a strong link between shortened telomeres and an increased risk of premature cardiovascular morbidity and mortality.[21,22] Consequently, understanding the key mediators and mechanisms for vascular cell senescence has become an area of great interest.

In this study, we examined the hypothesis that Ang II induces human VSMC senescence via ROS-mediated DNA damage. We also hypothesized that senescence would occur via 2 DNA damage-dependent pathways, ie, SIPS—a telomere independent pathway, and accelerated replicative senescence, associated with accelerated telomere attrition.

Materials and Methods

hVSMCs were obtained from explants of human saphenous vein[23] and hTERT-SMC generated from normal hVSMCs as described.[24,25] The effect of Ang II on ROS production, DNA damage, telomere attrition, and senescence was evaluated by supplementing culture medium with Ang II. For chronic exposure experiments, ie, up to 30 days, the medium overlying cells was supplemented with Ang II on alternate days.

Superoxide production was determined by lucigenin chemiluminescence[1] and nuclear DNA damage was measured using Comet assay.[26] Telomere DNA length was estimated by measuring the Terminal Restriction Fragment (TRF) length via Southern blotting.[27] Senescence-associated β-galactosidase (SA β-gal) activity was measured as originally described.[28] An expanded Materials and Methods section is available in the supplemental materials (available online at http://circres.ahajournals.org).

Results

ROS Production in Human Saphenous Vein Smooth Muscle Cells

Ang II–induced ROS generation via NADPH oxidase activity has been reported in cultured VSMCs,[1,2,29] although not for cells derived from human saphenous veins. In lysates of these cells, NADPH induced a 3- to 4-fold increase in lucigenin chemiluminescence (see supplemental Figure). In contrast, NADH was relatively ineffective. Similarly, cyclooxygenase and xanthine oxidoreductase were not significant sources of ROS under these conditions, because neither antimycin A/succinate nor xanthine increased chemiluminescence (see supplemental Figure). However, ROS production was significantly inhibited by prior incubation with diphenylene iodonium (DPI) (flavoprotein inhibitor; 83% inhibition) and apocynin (specific inhibitor of NADPH oxidase sub-unit assembly; 25%) confirming involvement of NADPH oxidase.

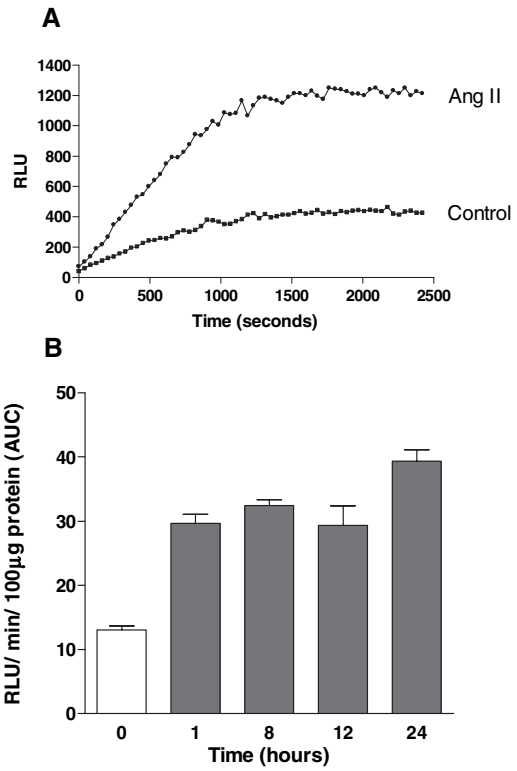

Figure 1. Ang II induces NADPH-mediated superoxide generation in hVSMCs. A, NADPH-mediated superoxide production by Ang II (10^{-7} mol/L) treated cells (24 hours) relative to control cells. Superoxide generation determined at 40 second intervals was plotted over a total period of 40 minutes. Data are representative of 4 separate experiments. B, Time-dependent increase in superoxide production in Ang II–treated cells. Bars represent mean+SEM; n=5 ($P<0.01$ for all time points compared with control). AUC indicates total area under curve.

Incubation with tiron (superoxide scavenger; 73% inhibition) (see supplemental Table) confirmed the detection of superoxide. Mechanisms involving mitochondrial electron transport and cyclooxygenase did not contribute to ROS generation by NADPH as neither rotenone (2μmol/L) nor indomethacin (20μmol/L) significantly decreased chemiluminescence (see supplemental Table). Together these data demonstrate for the first time, that NADPH oxidase–mediated superoxide production is detectable in saphenous vein hVSMCs.

Effect of Ang II on Superoxide Production by hVSMCs

Serum-deprived cells were incubated with Ang II for up to 24 hours before measurement of superoxide production. Ang II (10^{-7}mol/L) greatly potentiated NADPH-stimulated superoxide production in VSMCs (Figure 1A). Quantitation of the area under the curve showed that the Ang II effect was rapid (within 1 hour), consistent with previous reports using human arterial VSMCs,[29] and persisted for at least 24 hours (Figure 1B).

Real-time determination of lucigenin chemiluminescence demonstrated that Ang II–induced ROS production by hVSMCs was mediated via the AT_1 receptor, as effects were blocked by preincubation with the Losartan metabolite, E3174 (Merck; 10^{-5} mol/L), a potent, selective, and specific AT_1

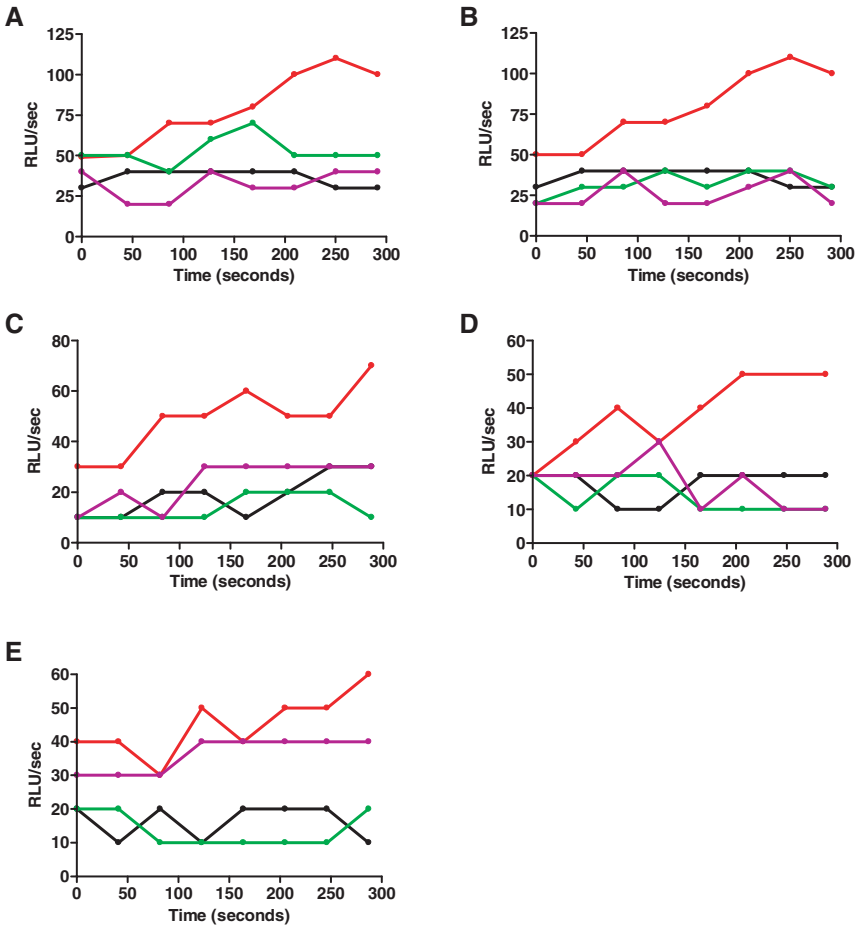

Figure 2. Ang II induces superoxide production in hVSMCs via the AT_1 receptor and NADPH oxidase. VSMCs were preincubated with Ang II for 1 hour then lysates used for measurement of superoxide by real-time lucigenin chemiluminescence. This was measured at 40-second intervals and plotted over 5 minutes. In A, cells were preincubated with E3174 before Ang II. In B through E, inhibitors were added to VSMC lysates. For all panels: black indicates control; red, Ang II; green, inhibitor alone; purple, Ang II+inhibitor. E3174 (A, 10^{-5} mol/L), tiron (B, 10×10^{-3} mol/L), apocynin (C, 100×10^{-6} mol/L) and DPI (D, 50×10^{-6} mol/L) inhibited superoxide production in cells treated with Ang II (10^{-7} mol/L) for 60 minutes. Rotenone (E, 2×10^{-6} mol/L) did not affect superoxide production. Data are representative of 3 to 5 experiments.

receptor antagonist (Figure 2A). In cell lysates derived from Ang II–treated cells, addition of tiron (10mmol/L), a relatively selective and nontoxic superoxide scavenger, inhibited chemiluminescence confirming that superoxide was being detected (Figure 2B). Both apocynin and DPI completely blocked the Ang II–dependent chemiluminescence, strongly suggesting superoxide was derived from NADPH-oxidase activity (Figure 2C and 2D). Although DPI is an effective, yet nonspecific flavoprotein inhibitor, apocynin specifically inhibits NADPH oxidase subunit assembly in cells. Rotenone did not block superoxide production in lysates derived from Ang II–treated VSMCs, confirming the lack of mitochondrial involvement (Figure 2E).

Ang II Induces DNA Damage in hVSMCs via the AT_1 Receptor

The "Comet assay"[26] has been used extensively as a very sensitive measure of DNA damage in cells and was used to investigate nuclear DNA damage induced by Ang II (Figure 3A through 3C). The culture medium overlaying hVSMCs was supplemented with Ang II for 4 or 24 hours before measurement of damage. Ang II induced DNA damage in hVSMCs which was significant by 24 hours (Figure 3D). Allowing cells to take up catalase by preincubation with the enzyme (300 U/mL for 3 hours at 37°C) inhibited Ang II–induced DNA damage suggesting that damage was ROS-mediated and more specifically required hydrogen peroxide generation. This result does not imply necessarily that hydrogen peroxide persists for 24 hours, because downstream mediators may actually mediate damage. Furthermore, endogenous peroxidases and DNA repair may also explain the delayed nature of DNA damage detection. Nevertheless, however, the catalase data show that the production of hydrogen peroxide was necessary for the DNA damage observed at 24 hours after Ang II treatment of hVSMCs. Moreover, the DNA damage was AT_1 receptor-mediated as it was completely prevented by coincubation with E3174 (Figure 3E). Together these data confirm that Ang II, via the AT_1 receptor and hydrogen peroxide production results in DNA damage in hVSMCs. Because ROS production and DNA damage have the capacity to profoundly influence cell viability, we next investigated whether Ang II could induce either SIPS and/or accelerated replicative senescence via telomere attrition.

Ang II Induces SIPS in Cultured hVSMCs

SIPS has been demonstrated in several cell types, including hVSMCs.[30] SA-β-gal activity is widely used as a marker of senescence of cells in culture and in tissues.[28] In this study, VSMCs were treated with Ang II for 24 hours and stained for SA-β-gal activity 24 hours after return to normal media. Ang II caused a dose-dependent increase in senescence, maximal at 10^{-8} mol/L (Figure 4A). Coincubation with either E3174 or catalase prevented Ang II–induced SIPS, suggesting this was

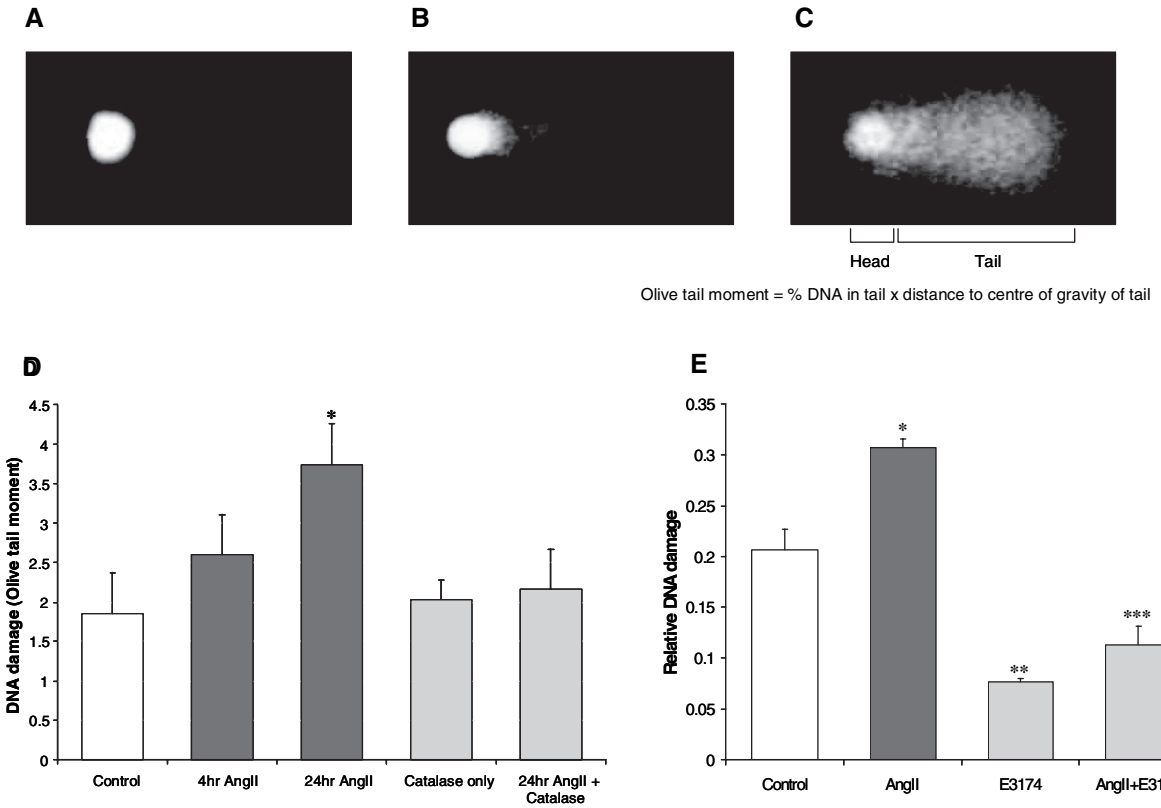

Figure 3. Ang II–induced DNA damage in hVSMCs. A, Image of a cell nucleoid using the Comet assay. Limited migration of DNA toward the anode indicates the cell has low level DNA damage. B, Nucleoid showing more significant migration of DNA into the tail of the Comet indicates greater DNA damage. C, DNA breaks within long loops of DNA allow them to migrate toward the anode, giving rise to a Comet-like appearance in heavily damaged nucleoids. D, DNA damage was measured using the Comet assay in hVSMCs treated with Ang II (10^{-6} mol/L)±catalase preincubation (300 Units/mL; n=3). Bars represent mean+SEM. (*$P<0.05$ vs control). E, Ang II induces DNA damage in hVSMCs via the AT_1 receptor. Cells were treated with Ang II (10^{-6} mol/L)±E3174 (10^{-5} mol/L) for 20 hours. Bars represent mean+SEM. (*$P<0.01$ and **$P<0.001$ vs control; ***$P<0.001$ vs Ang II–treated cells; n=3).

AT_1 receptor-mediated and dependent on hydrogen peroxide (Figure 4B). This rapid induction of senescence is characteristic of SIPS and a ≈1.5-fold increase in p53 and in p21 was consistently observed (Figure 4C) suggesting that cell cycle arrest and senescence occurred in response to DNA damage. Using Western blotting we were unable to detect significant changes in expression of the CDK inhibitor, $p16^{INK4a}$ (data not shown).

Ang II–Induced SIPS Occurs via Telomere-Independent Mechanism

We repeated the acute exposure to Ang II using hVSMCs transfected with TERT to preserve telomeres.[24] Overexpression of TERT in these cells was confirmed by Western blotting (Figure 4D). Importantly, Ang II–induced SIPS was not prevented by TERT-overexpression, suggesting that SIPS was not mediated by telomere damage and was thus telomere-independent (Figure 4E).

Ang II Accelerates Telomere Loss in hVSMCs

Telomere attrition has been described as a consequence of oxidative DNA damage[10] and promotes premature replicative senescence. Not all cells exposed to Ang II undergo SIPS. As the telomere is a vulnerable target for ROS[10] we considered whether continuing exposure to Ang II would result in accelerated telomere attrition and premature replicative senescence. Using Southern blotting,[27] we showed that Ang II markedly accelerated the rate of telomere loss (>2-fold versus control) in a dose-dependent manner (Figure 5A). Coincubation with E3174 markedly attenuated Ang II–induced telomere attrition, confirming this effect was mediated via the AT_1 receptor (Figure 5B).

Telomerase activity preserves telomere DNA length in some cells, and inhibition of telomerase has been proposed as a mechanism for telomere loss in response to Ang II–derived ROS.[31] Using a sensitive PCR ELISA we were unable to detect telomerase activity in cultured hVSMCs, either before or after exposure to Ang II, thereby discounting modulation of telomerase activity as a primary mechanism for Ang II–induced telomere loss in adult hVSMC (data not shown). These data suggest that Ang II promotes accelerated attrition of telomere DNA in adult hVSMCs via ROS-mediated DNA damage, rather than by inhibition of telomerase activity.

Ang II Induces Replicative Senescence in hVSMCs

Critical loss of telomere DNA has been associated with reduced cellular proliferation and premature cell senescence.[27] SA-β-gal activity was used to enumerate senescent hVSMCs after exposure to Ang II over a period of 30 days in culture. Ang II induced a dose-dependent increase in cell senescence with a maximal effect at 10^{-8} mol/L (Figure 6).

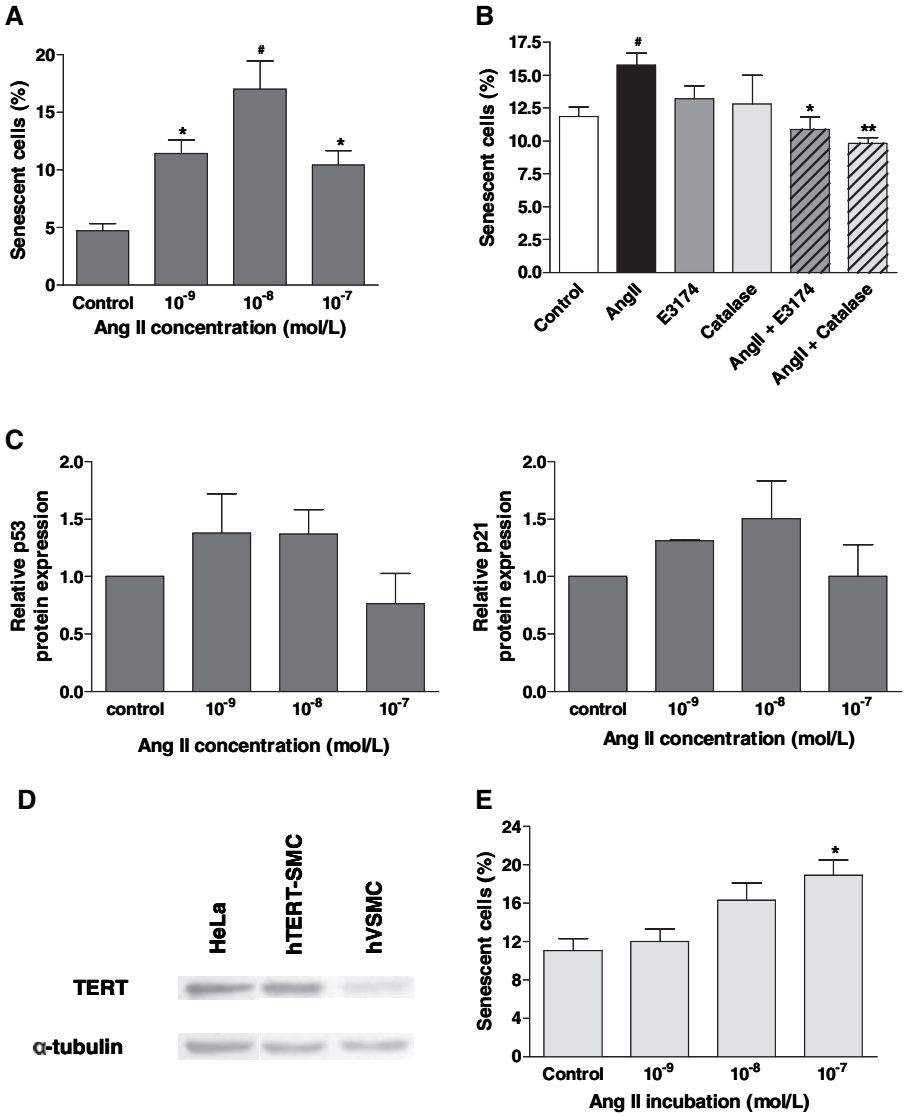

Figure 4. Ang II accelerates SIPS in human VSMCs. A, hVSMCs were treated with Ang II for 24 hours, then replated at 1×10^5 cells per well for 24 hours, before staining for SA-β-gal. Data are shown as mean+SEM; n=5 (*$P<0.05$ and #$P<0.01$ compared with control). B, Ang II–induced hVSMC SIPS is dependent on AT_1 receptor and ROS generation. hVSMCs were pretreated with E3174 (10^{-5} mol/L for 1 hour) or catalase (300Units/mL for 3 hours) before induction of senescence with 10^{-8} mol/L Ang II for 24 hours. Bars represent mean+SEM; n=5. (#$P<0.05$ vs control; *$P<0.05$ and **$P<0.01$ vs Ang II alone). C, Ang II induces expression of p53 and p21 within 24 hours. VSMCs were treated with 10^{-8} mol/L Ang II for 24 hours and p53 and p21 detected by Western blotting, compared with β-actin and α-tubulin loading controls. Bars represent mean+SEM of 2 experiments. D, Confirmation of TERT expression in hTERT-SMCs. Whole cell lysates from hTERT-SMCs and hVSMCs were blotted for hTERT using α-tubulin loading control. HeLa cell extract was used as a positive control. E, Ang II dose-dependently increases hTERT-SMC senescence. Quiescent cells were treated with Ang II for 24 hours and reseeded at $5\text{x}10^4$ cells per well (12 well) for 24 hours, before staining for SA-β-gal. Bars represent mean+SEM; n=3 (*$P<0.05$ compared with control).

Taken together with the data on telomere length, this finding suggests that over time in culture, Ang II accelerated the loss of telomere DNA and this was associated with premature replicative senescence.

Discussion

The present study demonstrates that Ang II–induced ROS production leads to DNA damage and the accelerated biological aging of hVSMCs via 2 mechanisms: (1) SIPS, which is telomere independent, and (2) accelerated replicative senescence which is associated with telomere attrition.

A common paradigm for the induction of cell senescence is loss of telomere integrity or critical shortening of telomeres, resulting in a "DNA damage response" and cell cycle arrest via activation of CDKIs.[32] Telomere shortening occurs progressively with every cell division due the "end-replication problem", eventually leading to critical shortening of telomeres which triggers replicative senescence. This process is accelerated by exposure of cells to oxidant stress and DNA damage.[10,33] The telomere region of DNA is especially vulnerable to oxidative damage.[34,35] Moreover, the repair of DNA within the telomere is inefficient relative to the rest of the genome.[36] We show that the average loss of TRF length in hVSMCs in culture is ≈1.0% per population doubling, consistent with our previous data on human vascular endothelial cells[27] and studies using cultured VSMCs.[24,37] We also show that treatment with Ang II markedly accelerated this loss by 2.5-fold, thereby predisposing hVSMCs to accelerated replicative senescence.

Cell senescence may also arise more rapidly, in a manner independent of the slower, replication-dependent attrition of telomere DNA. This mechanism, termed "premature senescence" or SIPS, involves activation of identical CDKIs to those activated in replicative senescence.[7] Induction of SIPS typically arises from treatment of cells with subcytotoxic levels of agents that generate ROS, eg, UV-irradiation, peroxides.[38,39] To date, 2 possible mechanisms have been proposed to account for the induction of SIPS by ROS: (1) oxidant-induced p38 phosphorylation,[40] and (2) critical but sublethal DNA damage with transient activation of p53 and subsequent expression of p21[41]; although the dependence of p21 expression on p53 has recently been disputed.[42] We show

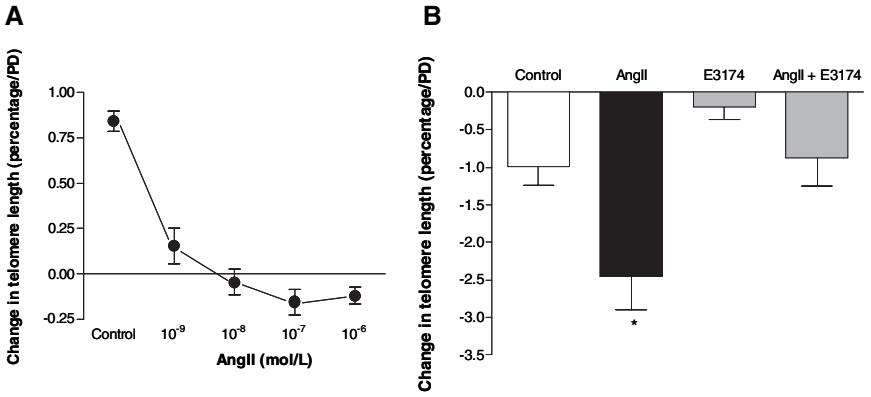

Figure 5. Ang II causes telomere attrition in hVSMCs. A, Dose-response of Ang II on telomere length in hVSMCs, for 24 hours. Mean percentage telomere changes per PD with SEM shown; n=5. ($P<0.001$ for all doses compared with control). B, Ang II treatment accelerates hVSMC telomere attrition via the AT_1 receptor. Telomere length was measured in hVSMCs grown continuously in media supplemented daily for 7 days with Ang II (10^{-6} mol/L)±E3174 (10^{-5} mol/L). *$P<0.05$ vs all other treatments; n=9; n=3 for E3174 alone.

that Ang II rapidly induces oxidative DNA damage in hVSMCs, associated with an increase in p53 and rapid onset of senescence. These observations demonstrate the capacity of Ang II to induce SIPS via a mechanism dependent on the associated DNA damage. Importantly, our data using hTERT-expressing VSMCs confirm that SIPS is independent of telomere attrition—as similarly observed in other cell types.[43,44]

Our data are consistent with the hypothesis that the fate of the hVSMCs exposed to Ang II is dependent on the magnitude of the resulting DNA damage. We propose that severe and lethal DNA damage would result in apoptosis, consistent with reports of Ang II–induced apoptosis in some cell types.[45–48] Sublethal but critical levels of DNA damage would rapidly induce p53-dependent SIPS. Lower levels of DNA damage sustained over several population doublings would target and accelerate the gradual erosion of telomeres, promoting premature replicative senescence. It is likely that all 3 responses would occur in the same cell population, the individual cells' response to Ang II depending on the biological age of the cell, their individual sensitivity to Ang II, and the efficiency of their oxidant defense and DNA repair mechanisms. Further work is required to evaluate this hypothesis.

The development of vascular cell senescence is increasingly recognized as an important mechanism in the pathogenesis of vascular disease.[14,49] Senescent vascular cells are not benign and may undergo a phenotypic shift to a more inflammatory and atherogenic phenotype.[8,50] Cells with a senescence phenotype have been identified in human and experimental animal arteries at sites prone to atherosclerosis.[8,17–20] In rabbits, denuded arteries show evidence of extensive SA-β-gal staining.[20] Similar SA-β-gal staining has been detected in diseased coronary arteries but not in healthier internal mammary arteries in humans.[8] It has also been shown that the senescent cells persist in the vascular wall[14] and so may inhibit natural repair by inhibiting recruitment of progenitor cells. The importance of vascular cell senescence in determining the function of an aging vascular system has stimulated interest in identifying the molecular mediators and pathways for its induction. Our data strongly support the hypothesis that Ang II may play a key role in the development of human vascular cell senescence.

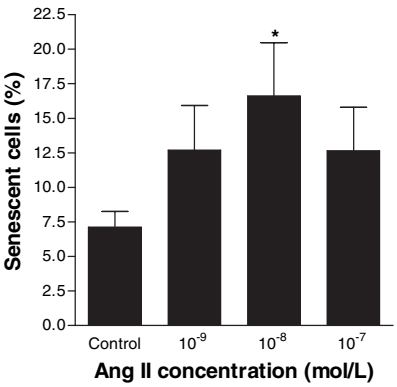

Figure 6. Ang II accelerates replicative senescence in human VSMCs. VSMCs were treated on alternate days for 30 days with Ang II, then replated at $1x10^5$ cells per well for 24 hours. Cells were then stained for SA-β-gal activity. Bars represent mean+SEM, n=6. *$P<0.05$ compared with control.

Telomere length is also used as a marker of vascular cell aging in vivo. When senescence is observed in the vascular wall, those cells often show evidence of telomere attrition. For example, VSMCs with shortened telomeres have been identified in established atherosclerotic plaques[51] and at sites of enhanced vascular wall stress in humans, ie, those sites predisposed to atheroma.[52] Evidence for the clinical significance of shortened telomeres is now emerging albeit fuelled by literature using the leukocyte telomere as a surrogate marker for vascular wall aging.[49] Cawthon and colleagues showed strong associations between reduced telomere DNA length in adults and premature mortality from cardiovascular disease.[21] Furthermore, people with shorter telomeres have a 3-fold higher risk of premature MI[22] and patients with coronary artery disease have shorter leukocyte telomeres compared with disease-free controls.[22,53] Moreover, a recent report links shorter telomere DNA with CVD risk factors and subsequent risk of MI or stroke.[54] The link between oxidative damage, telomeres, and vascular senescence has been further strengthened by studies detailing telomere-dependent senescence of VSMCs within human atherosclerotic plaques via a mechanism dependent on oxidative DNA damage and cell cycle arrest.[9] Our data support and extend this concept by suggesting a pathway from oxidative DNA damage through to senescence, with the hypothesis that the magnitude of DNA damage determines whether cells enter senescence

through a telomere-independent SIPS pathway, or a replication and telomere-dependent pathway.

Our data demonstrate Ang II–dependent induction of senescence in hVSMCs via the induction of ROS from NADPH oxidase and subsequent DNA damage. These findings are supported by a recent report suggesting that Ang II induced premature senescence in human aortic smooth muscle cells over a period of 3 days.[30] These authors suggested that telomere attrition was not involved because telomere length did not change over 3 days in culture. Our current observations, using hTERT-expressing cells provide much more conclusive evidence that Ang II induces SIPS via a telomere-independent pathway which is ROS and DNA damage dependent. Both studies support the importance of the p53-p21 pathway in the development of Ang II–induced SIPS. Consistent with our findings, Ang II was recently reported to accelerate endothelial progenitor cell senescence via ROS generation and downregulation of telomerase.[31] In contrast, our data suggest that Ang II–induced DNA damage, rather than its impaired repair, is the primary mechanism for accelerated telomere loss in adult human cells. This is not surprising mindful of the much greater telomerase activity in progenitor cells. Nevertheless, the senescence of both resident mature vascular cells and progenitor cells may play a role in the biological aging and associated pathology of vascular tissues.

Our hypothesis that Ang II may play a direct role in accelerating vascular aging processes is also supported by reports showing that treatment of experimental animals with drugs that inhibit the renin-angiotensin system, markedly increases survival and prevents many of the pathological changes associated with cardiovascular aging.[55] Our studies extend these observations and provide the first direct evidence that Ang II acting via its AT_1 receptor can induce DNA damage and senescence, either with or without accelerated telomere attrition, in adult hVSMCs.

Our study has some limitations. Firstly, our model uses human saphenous vein SMCs. Although ROS production by Ang II has been studied in both human and murine VSMCs taken from different vascular sites,[1,2,56] ours is the first data on induction of DNA damage and senescence by Ang II using saphenous vein cells. However, it is likely that these data are applicable to other sources of VSMCs that respond to Ang II. Secondly, this study uses cultured cells in vitro to investigate mechanisms of senescence rather than a direct examination of senescence in vivo. However, the evidence cited above suggests that the mechanisms of senescence are likely to be similar in both settings. Finally, Dikalov et al[57] recently critically reviewed the sensitivity and applicability of lucigenin chemiluminescence in the detection of ROS in vascular cells. Potential artifacts arising from lucigenin auto-oxidation were thought, on balance of the evidence in the literature, to be insignificant. Although this remains a possibility, in many cases lucigenin detection of ROS in vascular cells has been corroborated by alternate assays and our data are consistent with these findings.

In conclusion, our data show that Ang II causes DNA damage in hVSMCs via NADPH oxidase-derived ROS and that this results in SIPS or replicative senescence. These findings suggest novel mechanisms to directly implicate Ang II in the pathogenesis of human vascular cell aging and vascular disease.

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Disclosures

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Supplemental Materials and Methods

hVSMC Culture and Treatments: hVSMC were obtained by primary culture of human saphenous vein explants and used from passages 2 to 7, as previously described.[1] Cells were cultured in RPMI 1640 media with additional supplements [10% (v/v) smooth muscle cell growth supplement (TCS Cellworks, Buckingham), 100µg/ml glutamine, 100µg/ml penicillin/streptomycin, 20mM HEPES buffer] and incubated at 37^{o}C with 5% (v/v) CO_2.

The effect of Ang II (Sigma-Aldrich) was evaluated by supplementing the culture medium with Ang II. For chronic exposure experiments, i.e. up to 30 days, the medium overlying cells was supplemented with Ang II on alternate days. Cells were passaged a total of 8 times during this experiment.

hTERT-SMC were generated from normal human smooth muscle cells as previously described.[2] hTERT-infected SMC have population doubling times that are similar to, or slightly longer than, control SMC mass cultures from the same donor.[3] Lifespan of human hTERT-SMC is significantly longer than control cells, extending to 80 population doublings or longer, even when cells are derived from elderly donors.[3] However, most hTERT-SMC populations do senesce in culture after many population doublings, likely due to influences of prolonged *in vitro* cultures as opposed to telomeric erosion.[3]

Population Doublings (PD) of hVSMC: Cells were cultured in six-well plates, at a seeding density of $1x10^5$ cells/well. Confluence was typically reached within 3-4 days. With every passage, cells were counted using a hemocytometer. The number of PD prior to each passage was calculated using the formula:

$$PD = \log_{10}(\text{cells harvested}/\text{cells seeded})/\log_{10}(2)$$

Measurement of Superoxide Production: Serum-deprived hVSMCs were exposed to Ang II (10^{-7} mol/L) in the culture media, for up to 24 hours. The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase-mediated superoxide anion levels in cell lysates, as previously described by Griendling *et al*[4] Lucigenin was used at a concentration of 5μmol/L, which specifically detects superoxide and does not induce redox cycling.[5] Protein concentration of cell lysates was determined by the Bradford assay[6] and 100μg used per reaction. Luminescence was measured every 40 seconds for a total of 41 minutes, in an EG&G Berthold LB96V luminometer (Berthold Technologies, Redbourn, UK). Activity was expressed as relative light units (RLU) per minute per 100μg protein. To identify the source of receptor-induced superoxide production in these cells, the effects of ROS activators were determined: antimycin A (30μmol/L)/ succinate (5mmol/L) (mitochondrial complex III), xanthine (1mmol/L) (xanthine oxidoreductase), NADH and NADPH (400μmol/L for both) (NAD(P)H oxidase). ROS inhibitors were also used: diphenylene iodonium (DPI) (50μmol/L) (flavoprotein including NADPH oxidase), apocynin (100μmol/L) (rotenone (2μmol/L)

(mitochondrial complex I), indomethacin (20μmol/L) (cyclooxygenases), and tiron (10mmol/L) (superoxide scavenger).

Measurement of DNA damage: Genomic DNA damage was measured using Single Cell Gel Electrophoresis (Comet Assay).[7] Briefly, trypsinised cells were embedded in 0.6% ($^{w}/_{v}$) low melting point agarose then lysed in 2.5mol/L NaCl, 100mmol/L EDTA, 10mmol/L Tris-HCl, pH 10, 1% ($^{v}/_{v}$) Triton-X-100 at 4^{o}C overnight. Denaturation (20 minutes) followed by electrophoresis (20 minutes at 25V and 300mA) was carried out in 0.3mol/L NaOH, 1mmol/L EDTA, pH >13. Slides were then washed and stained with SyBr Green (Molecular Probes) and observed by fluorescence microscopy. Fifty cells per slide and three slides per treatment were analysed using the Komet 5 analysis software (Kinetic Imaging, Liverpool, UK; see Figure 1).

Determination of telomere DNA length: Human VSMC (0.25 x 10^6) were seeded in 75cm^2 flasks and grown for approximately 7 days. Telomere length was measured using the Terminal Restriction Fragment (TRF) length via Southern blotting as previously described[8] and utilizing a ^{32}P-labelled telomere specific probe $(TTAGGG)_4$. The average TRF length was calculated using the following equation where OD_i is the optical density of the telomeric smear at a particular molecular weight (MW_i): $\sum(OD_i)/ \sum (OD_i/MW_i)$

Measurement of Telomerase Activity: A photometric enzyme immunoassay was used for detecting the telomerase activity, incorporating the telomeric repeat amplification protocol (TRAP). Whole cell extracts equivalent to 1 X 10^3

cells/ reaction were assayed for telomerase activity using the TeloTAGGG Telomerase PCR ELISA (Roche Applied Science).

Senescence-associated β-galactosidase (SA-β-gal) activity: SA-β-gal activity was measured using the Senescent Cells Staining Kit (Sigma-Aldrich), as similarly described.[9] hVSMC were rinsed with PBS, fixed for 6 to 7 minutes at room temperature in fixation buffer (2% formaldehyde/0.2% glutaraldehyde), rinsed three times with PBS, then incubated with fresh SA-β-gal staining solution, pH 6.0, for 24 hours at 37^{o}C (no CO_2).

SDS-PAGE and Western blotting: Samples were stored in lysis buffer (5mM Magnesium acetate, 50mM KCl, 50mM Tris, pH 7.4, 3mM EDTA, 3mM β-mercaptoethanol, 10% Sigma protease inhibitor cocktail) at -80°C overnight. Once thawed on ice, samples were homogenized using micropestles in 1.5ml eppendorf tubes (25 strokes per sample) and total protein concentration was determined using the Bradford assay.[6] Total protein extracts (40μg) were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels, by electrophoresis. Proteins were transferred to nitrocellulose membranes (Amersham Biosciences) and blocked with 10% non-fat dry milk in 0.1% Tween-TBS for 1 hour. Membranes were incubated with anti-p53 antibody, anti-p21 or anti-p16 (Santa Cruz, Santa Cruz, CA). Anti-α-tubulin (Sigma) or anti-β-actin antibody (Sigma) was used to correct for small differences in protein loading. This was followed by 1 hour incubation with an appropriate HRP-labelled secondary antibody (Abcam, Cambs, UK or Sigma). Bands were detected using enhanced chemiluminescence. Semi-quantitation was

performed on ECL films by densitometry using an AlphaImager documentation and analysis system (Alpha Innotech Ltd, Cannock, UK).

Statistical Analysis: All data are presented as a mean + SEM. Comparisons between groups were made using one-way ANOVA with either a Bonferroni multiple comparison post test or Dunnett's post test. A p-value of <0.05 was considered to be statistically significant.

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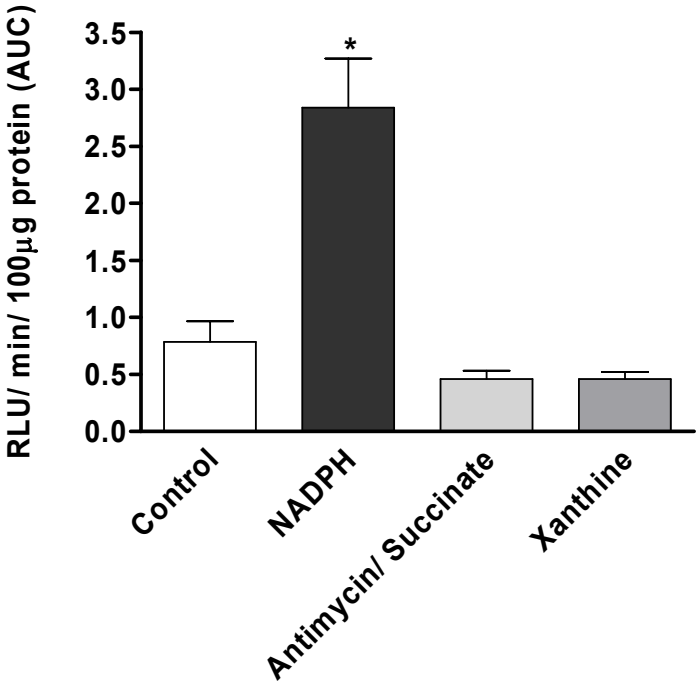
Legend to Supplemental figure

Supplemental Figure. NADPH oxidase activity is the major source of superoxide production in cultured venous human VSMC.

ROS generation was predominantly stimulated by NADPH in cell lysates. Bars represent mean+SEM. n=4 except for NADH n=3 (*p<0.01 compared with control).

Supplemental Figure



Supplemental Table: NADPH oxidase activity is a major source of superoxide production in lysates of cultured human VSMC.

Inhibitor	% Inhibition of lucigenin chemiluminescence
DPI (50µmol/L)	83 ± 2***
Apocynin (100µmol/L)	25 ± 4*
Tiron (10mmol/L)	73 ± 2**
Indomethacin (20µmol/L)	15 ± 14
Rotenone (2µmol/L)	13 ± 2

DPI; diphenyleneiodonium. Values are mean (± sem) of percentage inhibition of chemiluminescence due to NADPH alone. n = 4 or 5.

*, p<0.05; **p<0.01; ***p<0.001 compared to NADPH alone.