Aspirin Downregulates Angiotensin Type 1 Receptor Transcription Implications in Capillary Formation From Endothelial Cells

Sona Mitra, MD, Xianwei Wang, MD, PhD, Magomed Khaidakov, MD, PhD, Zufeng Ding, PhD, Srinivas Ayyadevera, PhD, Emily Hearnsberger, MS, Tanu Goyal, MD, and Jawahar L. Mehta, MD, PhD

Abstract: Aspirin [acetyl salicylic acid (ASA)] inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reactive oxygen species generation, a pathway that underlies formation of new capillaries (angiogenesis). Angiotensin II (Ang II) participates in angiogenesis by activating type 1 receptor (AT1R). We examined if ASA would inhibit AT1R transcription, which requires NADPH oxidase, and thereby new capillary formation. Human umbilical vein endothelial cells were cultured in Matrigel and treated with Ang II with and without ASA. Expression of AT1R and NADPH oxidase was measured by quantitative polymerase chain reaction. Ang II in low concentrations induced AT1R messenger RNA and new capillary formation. ASA and its salicylic acid (SA) moiety both suppressed Ang II-mediated AT1R and vascular endothelial growth factor expression and the subsequent new capillary formation. Of note, the AT1R blocker losartan prevented new capillary formation. ASA and SA also suppressed NADPH oxidase ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and gp91 messenger RNA) expression. These observations suggest that ASA can inhibit Ang II–induced capillary formation in part via blocking NADPH oxidase and AT1R transcription. Because SA moiety had similar effect as ASA on AT1R expression, we suggest that the effect of ASA on new capillary formation is mediated by its SA moiety.

Key Words: aspirin, angiotensin II, endothelial cells, NADPH oxidase

(*J Cardiovasc Pharmacol*™ 2012;60:187–192)

INTRODUCTION

Aspirin [acetyl salicylic acid (ASA)] is an old drug used for its analgesic and antipyretic effects for over 100 years. Since the discovery of its inhibitory effect on cyclooxygenase enzyme and antiplatelet aggregatory effect, this drug is used in the primary and secondary prevention of cardiovascular events.[1–3] In the recent past, several platelet-independent effects of ASA, such as inhibition of smooth muscle proliferation, endothelial proliferation and resultant angiogenesis, and proinflammatory cytokine expression, have been described.[4–7] Others[6–8] and we have shown that ASA via its salicylic acid (SA) moiety can inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and downstream activation of redox-sensitive transcription factors. This pathway underlies transcription of a host of proinflammatory genes, such as vascular cell adhesion molecule-1 (VCAM-1) and low-density lipoprotein receptor 1 (LOX-1).[9,10] ASA has also been shown to have mild antihypertensive effect,[11] modify impaired endothelium-dependent vasodilatation,[12] and have a small life span–prolonging effect in male mice.[13]

Renin–angiotensin system activation results in the formation of a potent hormone angiotensin II (Ang II), which has varied effects in several biological systems. The effect of Ang II in adult tissues is mediated primarily by expression and activation of its type 1 receptor (AT1R). Ang II via AT1R induces NADPH oxidase activation and reactive oxygen species generation followed by activation of mitogen-activated protein kinases (MAPKs) and redox-sensitive transcription factors, a pathway that underlies pathogenesis of several ailments, including hypertension, atherosclerosis, myocardial ischemia, and tissue remodeling after injury. In recent past, AT1R expression has been shown to be a marker of aging process.[14] Recent studies[15–17] suggest that Ang II in small concentrations via AT1R and NADPH oxidase activation can induce angiogenesis.

This study was designed to test the hypothesis that downregulation of AT1R transcription may be a component of the antiangiogenic effects of ASA, mediated via its inhibitory effects on NADPH oxidases and MAPKs.

MATERIALS AND METHODS

Materials and Reagents

Aspirin, SA, Ang II, AT1R blocker losartan (LOS), and NADPH oxidase inhibitor apocynin were purchased from Sigma-Aldrich (St Louis, MO). Matrigel (with reduced growth factor) was purchased from BD Biosciences (Bedford, MA) and RNeasy Mini-Kit, DNase I kit, SuperScript II 1st Strand complementaryDNA (cDNA) Synthesis Kit, and ethidium

Received for publication January 27, 2012; accepted April 19, 2012.
From the Division of Cardiology, The Central Arkansas Veterans Healthcare System, University of Arkansas for Medical Sciences, Little Rock, AR.
Supported in part by funds from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development, Washington, DC, and a grant from Bayer-Schering Pharma.
The authors report no conflicts of interest.
Drs. Mitra and Wang contributed equally to this manuscript.
The contents of this article do not represent the views of the Department of Veterans Affairs or the US Government.
Reprints: Jawahar L. Mehta, MD, PhD, Cardiovascular Division, University of Arkansas for Medical Sciences, Little Rock, AR 72212 (e-mail: MehtaJL@UAMS.edu).

bromide from Invitrogen (Carlsbad, CA). GoTaq quantitative polymerase chain reaction (qPCR) Master Mix was purchased from Promega (Madison, WI). Polyvinyl difluoride (PVDF) membranes, nonfat milk, and 12% 10-well gels were purchased from Bio-Rad (Hercules, CA). AT1R, p-P44/42 MAPK, and p-p38 MAPK antibodies were purchased from ABcam (Cambridge, MA), and β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Western blotting substrate was purchased from Thermo Scientific (Rockford, IL). Human umbilical vein endothelial cells (HUVECs) and vascular endothelial growth medium complemented with vascular endothelial growth factor (VEGF) kit were purchased from American Tissue Culture Collection (Manassas, VA).

Capillary Tube Formation

Matrigel was thawed on ice overnight and spread evenly over each well (30 μL) of a 24-well plate. The plates were incubated for 1 hour at 37°C to allow Matrigel to polymerize. HUVECs were seeded at 3×10^4 per well and grown in 500 μL of endothelial cell basal medium 2 supplemented with 5% fetal bovine serum (FBS) and without endothelial cell growth supplement for 24 hours in a humidified 37°C, 5% CO_2 incubator, as described previously.[17] In some experiments, endothelial cells were cultured in the presence or absence of different chemicals or antibodies. To view the capillary formation, cells were loaded with 10 μM calcein AM (Invitrogen, Carlsbad, CA) for 30 minutes, washed with PBS, and imaged using a fluorescence microscopy.

Endothelial Cell Culture and Study Protocol

In preliminary experiments, cells were treated with Ang II in a wide range of concentrations (0, 0.1, 1, 5, 10, 20, 50, and 100 nmol/L) for 24 hours, to assess the concentrations that would cause most number of capillaries to form (data not shown). As described previously,[17] low concentrations of Ang II (1–10 nM) resulted in a stable, consistent, and clearly discernible response. The 10 nM concentration was used in subsequent experiments. Passage 3 cells were used for the above experiments.

Similarly, preliminary studies were carried out to determine the concentrations of ASA and SA that resulted in stable and consistent response in terms of inhibition of capillary formation (data not shown). These concentrations were established to be 0.5 and 1.0 mM (approximate median of therapeutic range), respectively. Passage 4 cells were used to conduct these experiments.

When HUVECs reached 70% confluence, they were exposed to 0.5 mM ASA or 1 mM SA for 30 minutes and then exposed to 10 nM Ang II for 24 hours. To check the role of AT1R in this process, some HUVEC cultures were treated with 10 μM losartan and then exposed to Ang II. ASA and SA were dissolved in dimethyl sulfoxide (DMSO), and DMSO alone was found to have no effect on cell growth or capillary formation.

As control, parallel aliquots of cells were treated with ASA, SA, and losartan but not Ang II. All experiments were repeated at least 4 times.

Real-time qPCR

Total RNA was isolated from HUVECs using RNeasy Mini-Kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Before using, RNA was treated with DNase I and diluted to 100 μg/mL. One microgram RNA was applied to synthesize cDNA with SuperScript II 1st Strand cDNA Synthesis Kit. All qPCR reactions were carried out in a final volume of 15 μL containing 1X of SYBR Green PCR Master (Applied Biosystems, Carlsbad, CA), 300 nM of each gene-specific primers, and 100 ng cDNA, in sterile deionized water. The standard cycling condition was 50°C for 2 minutes, 90°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 62°C for 1 minute. The results were analyzed using SDS 2.3 relative quantification manager software. The comparative threshold cycle values were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference genes. qPCR was performed in triplicate to ensure quantitative accuracy. qPCR-specific primers (Table) were designed using Probe-Finder (http://www.roche-applied-science.com), web based.

Western Blotting

Proteins were extracted from different HUVEC aliquots using radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Inc). Lysates (20 mg of protein) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Millipore, Bedford, MA). After blocking with phosphate buffered saline with Tween 20 (PBST) containing 5% bovine serum albumin (BSA) or nonfat milk, membranes were sequentially incubated with primary antibodies (overnight at 4°C), washed with PBST (3 times, 10 min each), incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature, washed, treated with the ECL western blotting substrate (Promega), and imaged. Relative expression of proteins was evaluated in relation to β-actin.

TABLE. Primers Used for qPCR

Primer	Sequence
GAPDH-F	AGA GCC CAA ACA GAT CCC CT
GAPDH-R	GGT CCG AAG CTG CCT GTA TG
VEGF-F	AGG GCA GAA TCA TCA CGA AGT
VEGF-R	GCT GCG CTG ATA GAC ATC CA
P22phox-F	CTG CTT CCT GTG TGT CGC AA
P22phox-R	AGG CAG ATC ATA TAG GCC ACC
P47phox-F	CGT GGT GGA GGT CGT AGA GAA
P47phox-R	TTC CGT CTC GTC AGG ACT GT
P67phox-F	GAG GGA TGC TCT ACT ACC AGA C
P67phox-R	CCT CGA AGC TGA ATC AAG GC
Gp91-F	GAC GCT GCT GTT TGA GAA ATG
Gp91-R	ATC GCT GAA GAA GGG TTT GTG
AT1R-F	GCC CTT TGG CAA TTA CCT ATG T
AT2R-R	CGT ACA GGT TGA AAC TGA CGC
Rel-F	CCT TGC GCT CTA TGA CTT CAC
Rel-R	CCT GCG TGC GAA GAT GTA G
Jun B-F	ACA CAG CTA CGG GAT ACG G
Jun B-R	TGT AGT CGT GTA GAG AGA GGC

Statistical Analysis

Data were analyzed using Microsoft Excel data analysis package. Comparisons between treated and control groups were made by 2-tailed Student *t* test, and a *P* value <0.05 was considered significant. All results are presented as means ± standard error.

RESULTS

Ang II Induces Capillary Formation and ASA and SA Inhibit It

As shown in Figure 1, Ang II induced robust new capillary formation from HUVECs. The capillary formation was evident only in response to low concentrations of Ang II (10 nM) ($P < 0.05$ vs. control). The Ang II–induced capillary formation was inhibited by AT1R blocker losartan ($P < 0.05$ vs. Ang II–treated cells). Importantly, ASA and SA also significantly reduced Ang II–induced capillary formation ($P < 0.05$ vs. Ang II–treated cells). It is of note that ASA, SA, and losartan alone had no effect on Ang II–induced capillary formation (data not shown). Results of representative experiments are shown in Figure 1A, and the data from several experiments are summarized in Figure 1B.

Ang II Induces VEGF Release and ASA and SA Inhibit It

VEGF is the most powerful stimulus for angiogenesis.[17,18] Therefore, we examined VEGF expression and found that low concentrations of Ang II stimulated VEGF expression in HUVECs ($P < 0.05$ vs. control). Importantly,

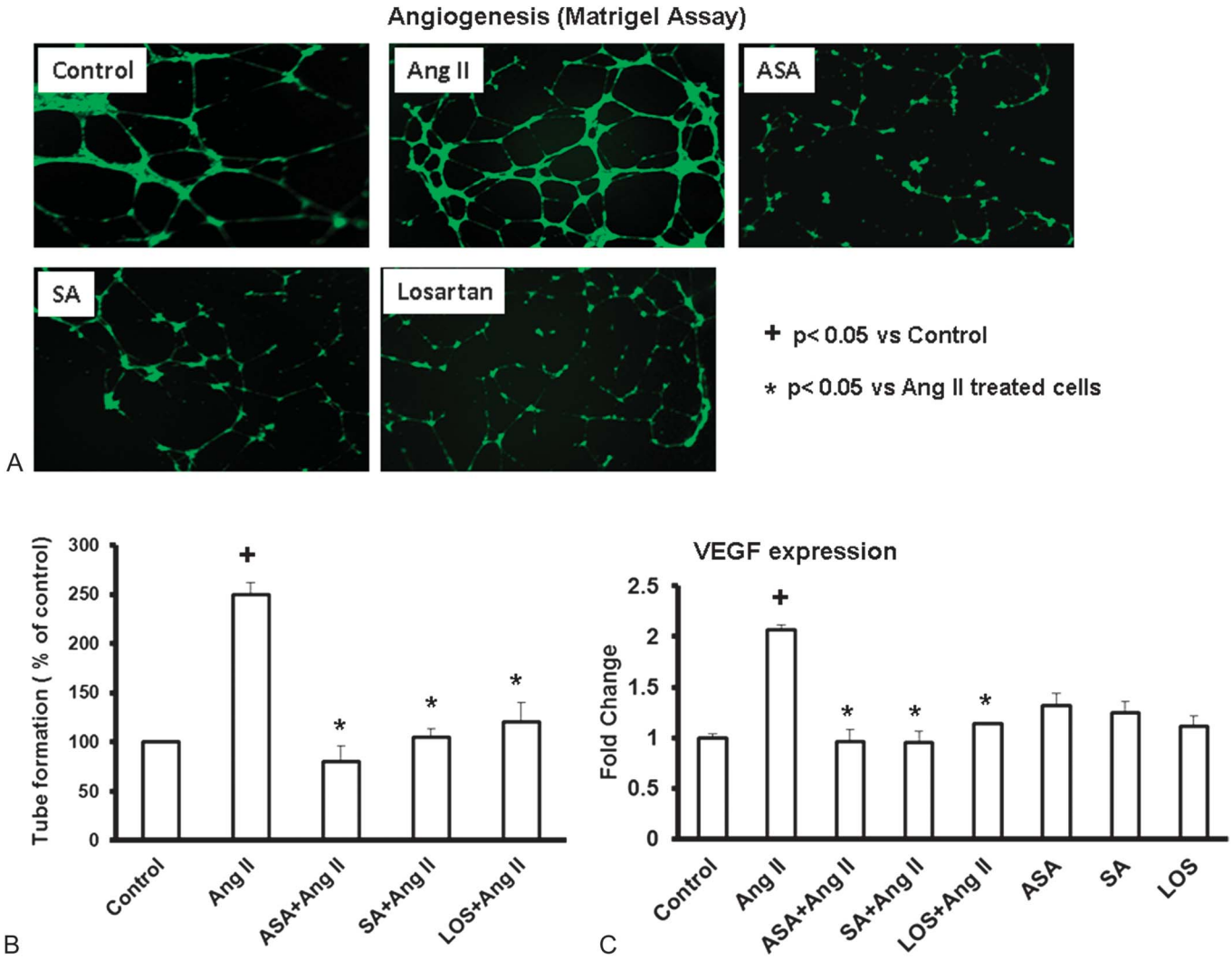

FIGURE 1. A, Representative images show capillary tube formation from HUVECs treated with Ang II alone and from cells pretreated with 0.5 mM ASA, 1 mM SA, and 10 μM losartan followed by incubation with Ang II. B, Bar graph shows summary of data in mean ± SE from 3 separate experiments. C, VEGF mRNA expression in cells treated with Ang II alone and in cells pretreated with 0.5 mM ASA, 1 mM SA, 10 μM losartan followed by incubation with Ang II. Note that ASA, SA, or losartan alone had no effect on VEGF expression. Bar graphs represent mean ± SE (n = 3 per group). $^{+}P < 0.05$ versus control; $^{*}P < 0.05$ versus Ang II–treated cells. mRNA, messenger RNA.

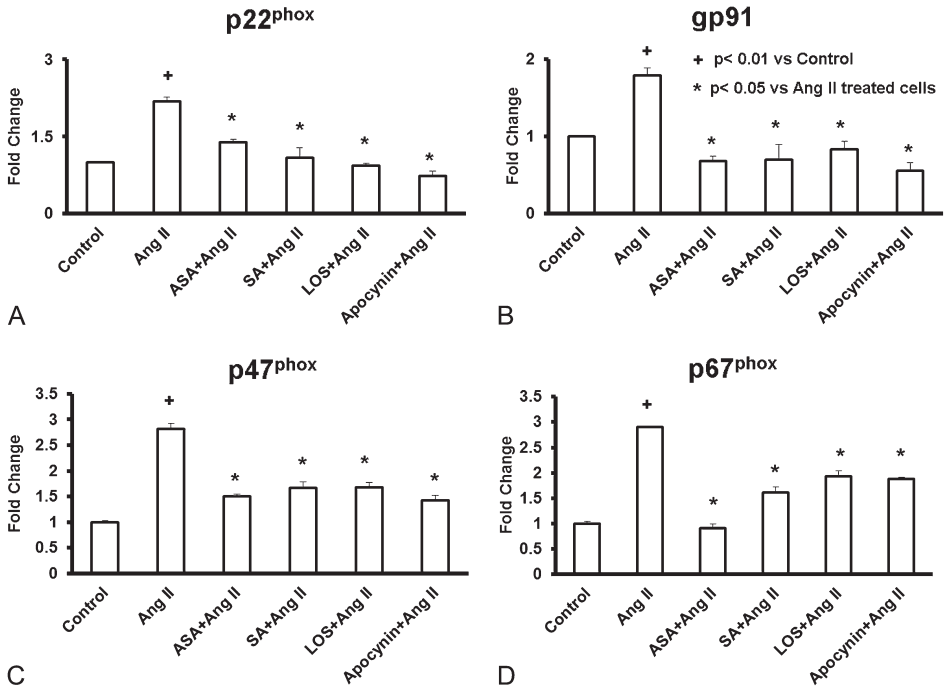

FIGURE 2. mRNA expression of NADPH oxidase subunits (p22^{phox}, p47^{phox}, p67^{phox}, and gp91) in HUVECs pretreated with ASA, SA, losartan, or apocynin followed by incubation with Ang II. Bar graphs represent mean ± SE (n = 3 per group). $^{+}P <$ 0.01 versus control; $^{*}P < 0.05$ versus Ang II–treated cells. mRNA, messenger RNA.

losartan, ASA, and SA reduced Ang II–induced VEGF expression significantly (all $P < 0.05$ vs. Ang II–treated cells). ASA and SA alone had no effect on Ang II–induced VEGF expression. These data are summarized in Figure 1C.

Ang II Induces NADPH Oxidase and ASA and SA Inhibit It

Previous studies have shown that NADPH oxidase is the major mediator of oxidant stress that leads to angiogenesis.[17,19,20] Therefore, we examined expression of NADPH oxidase and found that low concentrations of Ang II stimulated the expression of several NADPH oxidase subtypes (p22^{phox}, p47^{phox}, p67^{phox}, and gp91) in HUVECs ($P < 0.01$ vs. control). Importantly, losartan, ASA, and SA and NADPH oxidase inhibitor apocynin (600 μmol/L) all reduced Ang II–induced expression of these NADPH oxidase subtypes ($P < 0.05$ vs. Ang II alone–treated cells). These data are summarized in Figure 2.

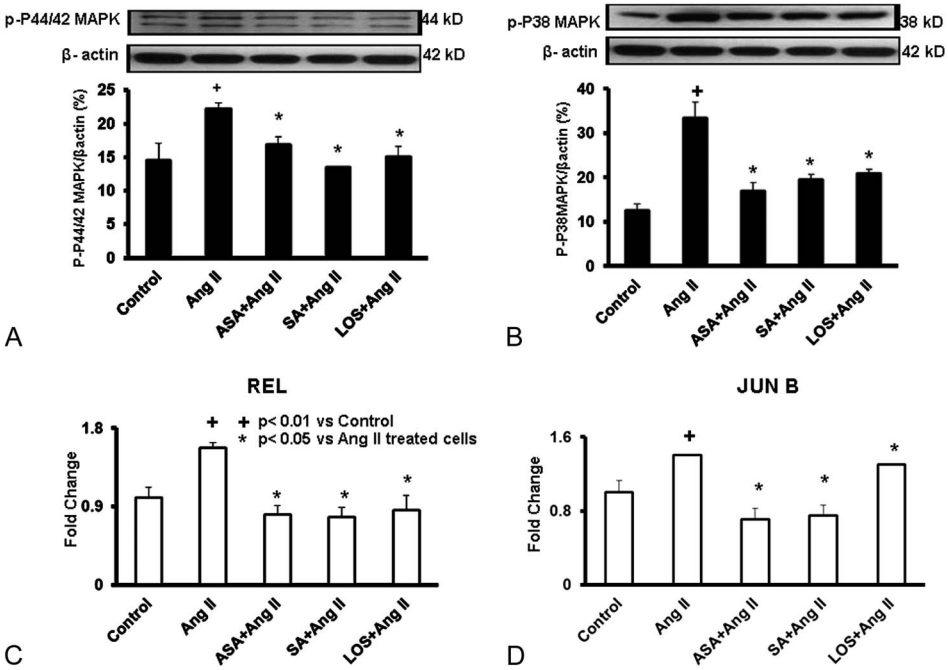

FIGURE 3. A, Western blotting shows phos-p38 MAPK and phos-p44/42 MAPK protein expression in HUVECs treated with Ang II or pretreated with 0.5 mM ASA, 1 mM SA, or 10 μM losartan followed by incubation with Ang II. B, Rel and Jun B mRNA expression in cells treated with Ang II alone and in cells pretreated with 0.5 mM ASA, 1 mM SA, and 10 μM losartan followed by incubation with Ang II. Bar graphs represent mean ± SE (n = 3 per group). $^{+}P < 0.01$ versus control; $^{*}P < 0.05$ versus Ang II–treated cells. mRNA, messenger RNA.

Ang II Induces Jun, Rel, and p-p38 and p-p44/42 MAPKs and ASA and SA Inhibit It

MAPK activation is a downstream of NADPH oxidase, and others and we have shown that inhibition of p38 and p44/42 MAPKs reduces angiogenesis.[17,21] In this study, we examined the expression of p38 and p44/42 MAPKs and Rel and Jun B and found that low concentrations of Ang II stimulated the expression of the p-p38 and p-p44/42 MAPKs, Rel, and Jun B in HUVECs. There was no effect of Ang II on p38 or p44/42 MAPK protein (data not shown). Importantly, losartan, ASA, and SA reduced Ang II–induced expression of p-p38 and p-p44/42 MAPKs, Rel, and Jun B (all $P < 0.05$ vs. Ang II–treated cells). These data are summarized in Figure 3.

ASA and SA Decrease AT1R Expression

Ang II, as reported previously,[22] increases AT1R expression in HUVECs. This was confirmed in the present study. Both ASA and SA suppressed Ang II–mediated AT1R expression at messenger RNA and protein levels ($P < 0.01$ vs. Ang II–treated cells; Fig. 4C), without a significant effect on the basal levels of AT1R. Losartan also significantly reduced Ang II–mediated AT1R expression ($P < 0.01$ vs. Ang II–treated cells).

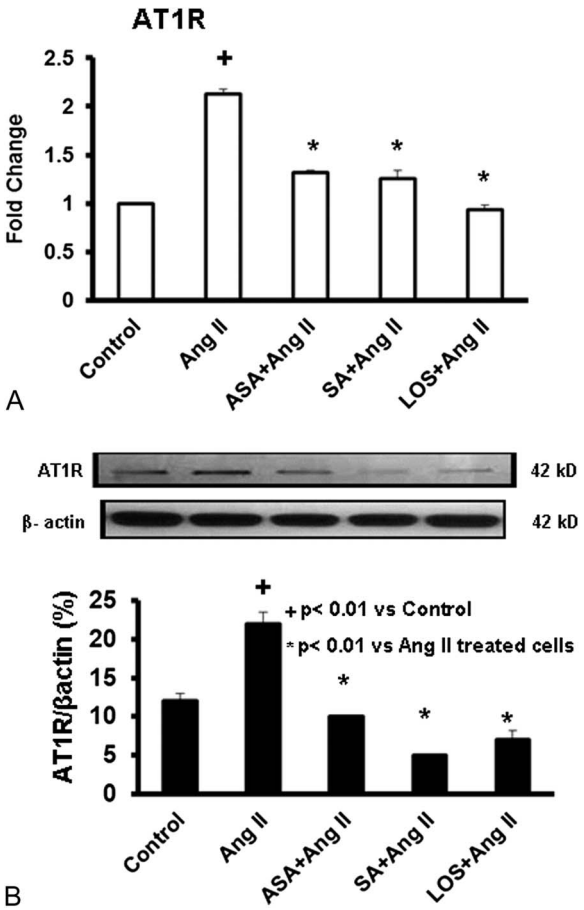

FIGURE 4. AT1R expression in HUVECs treated with Ang II or pretreated with 0.5 mM ASA, 1 mM SA, and 10 μM losartan followed by treatment with Ang II. A, AT1R mRNA expression. B, AT1R protein expression. Bar graphs represent mean ± SE (n = 3 per group). $^{+}P < 0.01$ versus control; $^{*}P < 0.01$ versus Ang II–treated cells. mRNA, messenger RNA.

DISCUSSION

The most important observation in this study is that ASA in therapeutically achieved concentration of 0.5 mM decreased Ang II–mediated AT1R expression in HUVECs at transcriptional level. The protein levels of AT1R also decreased proportionately. The SA moiety of ASA had a similar effect as ASA. The decrease in AT1R expression translated into inhibition of its biological effects, that is, new capillary formation from endothelial cells.

The inhibitory effect of ASA and SA on AT1R transcription identified in this study is novel and interesting. Normally, treatment of endothelial cells, fibroblasts, and vascular smooth muscle cells with Ang II increases AT1R expression, and this process is thought to involve NADPH oxidase–MAPK pathway.[17,22–24] Because ASA and its SA moiety significantly blocked the expression of several subtypes of NADPH oxidases (Fig. 2), it is not surprising that Ang II–mediated AT1R upregulation was blocked. Furthermore, both ASA and SA blocked Ang II–mediated upregulation of Jun B, Rel, and the phosphorylated forms of p38 and p44/42 MAPKs. Recent work from our laboratory shows that ASA reduces AT1R transcription in mouse cardiac fibroblasts and that this process also involves activation of NADPH oxidase–MAPK–nuclear factor kappa B pathway, and this translates into inhibition of collagen generation.[25]

It is of note that the overall NADPH oxidase inhibitor apocynin also decreased Ang II–mediated AT1R expression, suggesting a key role of NADPH oxidases in AT1R expression. ASA and SA reduced Ang II–mediated AT1R expression in a manner similar to apocynin, implying that ASA and SA exert their effect most likely at the level of NADPH oxidases.

Previous studies have documented that Ang II, in low concentrations, stimulates formation of new capillaries from endothelium.[17] Atherosclerotic lesions express all components of renin–angiotensin system, particularly AT1R.[24] The advancing atherosclerotic lesion contains large number of capillary channels (vasa vasorum), which are believed to contribute to the rupture of the plaque and result in acute ischemic syndromes.[25,26] AT1R activation, besides being a proangiogenic signal, is also a proinflammatory stimulus.[27] Accumulation of foam cells and immune cells and thin cap, probably as a result of release of matrix metalloproteinases (MMPs), are other key features of "vulnerable" plaque.[28–30] AT1R blocker therapy, with agents like losartan, reduces neovascularization and the atherosclerotic burden and modifies the molecular characteristics of advancing plaque.[31,32] Profibrotic effects of Ang II translate into fibrosis and reduction in compliance after tissue injury in animal models and in humans.[33–36] Upregulation of AT1R expression has been identified in aging arteries.[37] It is interesting that ASA replicates many manifestations of AT1R blockade, such as stabilization of atherosclerotic plaque,[38] decrease in VEGF expression and subsequent angiogenesis,[6,39] and tissue fibrosis.[8] This study clearly demonstrates that treatment of endothelial cells with ASA in therapeutically achieved concentration is associated with reduction in NADPH oxidase–MAPK pathways and AT1R

transcription. At the same time, there is inhibition of angiogenic effects of Ang II on endothelial biology, that is, formation of capillaries. It is conceivable that the effects of ASA on angiogenesis are related, at least in part to the reduction in NADP oxidase–MAPK pathways and AT1R transcription.

In essence, this study provides evidence that ASA treatment of endothelial cells results in a decrease in AT1R expression and capillary formation from endothelial cells. Reduction in capillary formation may be the basis for a modest antiatherosclerotic[40,41] and antiaging[42] effect of ASA. Because the effects of ASA were seen in therapeutically achieved concentrations, these data may be clinically relevant. Last, because the effects of SA were similar to those of ASA, it seems that it is the SA moiety that is responsible for the inhibitory effects of ASA on AT1R transcription.

REFERENCES

1. Antithrombotic Trialists' (ATT) Collaboration, Baigent C, Blackwell L, et al. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet*. 2009;373:1849–1860.
2. Sanchez-Ross M, Waller AH, Maher J, et al. Aspirin for the prevention of cardiovascular morbidity. *Minerva Med*. 2010;101:205–214.
3. Elwood PC, Stillings MR. Use of aspirin in cardiovascular prophylaxis. *Cardiovasc J S Afr*. 2000;11:155–160.
4. Khan Q, Mehta JL. Relevance of platelet-independent effects of aspirin to its salutary effect in atherosclerosis-related events. *J Atheroscler Thromb*. 2005;12:185–190.
5. Brooks G, Yu XM, Wang Y, et al. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit vascular smooth muscle cell proliferation via differential effects on the cell cycle. *J Pharm Pharmacol*. 2003;55:519–526.
6. Khaidakov M, Szwedo J, Mitra S, et al. Angiostatic effects of aspirin in hypoxia-reoxygenation are linked to modulation of TGFβ1 signaling. *J Cardiovasc Pharmacol Ther*. 2011;16:105–110.
7. Voisard R, Fischer R, Osswald M, et al. Aspirin (5 mmol/L) inhibits leukocyte attack and triggered reactive cell proliferation in a 3D human coronary in vitro model. *Circulation*. 2001;103:1688–1694.
8. Muller DN, Heissmeyer V, Dechend R, et al. Aspirin inhibits NF-kappaB and protects from angiotensin II-induced organ damage. *FASEB J*. 2001; 15:1822–1824.
9. Li D, Chen H, Romeo F, et al. Statins modulate oxidized low-density lipoprotein-mediated adhesion molecule expression in human coronary artery endothelial cells: role of LOX-1. *J Pharmacol Exp Ther*. 2002; 302:601–605.
10. Mehta JL, Chen J, Hermonat PL, et al. Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc Res*. 2006;69:36–45.
11. Bautista LE, Vera LM. Antihypertensive effects of aspirin: what is the evidence? *Curr Hypertens Rep*. 2010;12:282–289.
12. Magen E, Viskoper JR, Mishal J, et al. Effects of low-dose aspirin on blood pressure and endothelial function of treated hypertensive hypercholesterolaemic subjects. *J Hum Hypertens*. 2005;19:667–673.
13. Strong R, Miller RA, Astle CM, et al. Nordihydroguaiaretic acid and aspirin increase lifespan of genetically heterogeneous male mice. *Aging Cell*. 2008;7:641–650.
14. Imanishi T, Hano T, Nishio I. Angiotensin II accelerates endothelial progenitor cell senescence through induction of oxidative stress. *J Hypertens*. 2005;23:97–104.
15. Skultetyova D, Filipova S, Riecansky I, et al. The role of angiotensin type 1 receptor in inflammation and endothelial dysfunction. *Recent Pat Cardiovasc Drug Discov*. 2007;2:23–27.
16. Ushio-Fukai M. Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc Res*. 2006;71:226–235
17. Hu C, Dandapat A, Mehta JL. Angiotensin II induces capillary formation from endothelial cells via the LOX-1 dependent redox-sensitive pathway. *Hypertension*. 2007;50:952–957.
18. Khurana R, Simons M, Martin JF, et al. Role of angiogenesis in cardiovascular disease: a critical appraisal. *Circulation*. 2005;112:1813–1824.
19. Ushio-Fukai M, Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. *Mol Cell Biochem*. 2004;264:85–97.
20. Ushio-Fukai M. VEGF signaling through NADPH oxidase-derived ROS. *Antioxid Redox Signal*. 2007;9:731–739.
21. Seeger FH, Sedding D, Langheinrich AC, et al. Inhibition of the p38 MAP kinase in vivo improves number and functional activity of vasculogenic cells and reduces atherosclerotic disease progression. *Basic Res Cardiol*. 2010;105:389–397.
22. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82–C97.
23. Berk BC. Angiotensin II signal transduction in vascular smooth muscle: pathways activated by specific tyrosine kinases. *J Am Soc Nephrol*. 1999; 10(suppl 11):S62–S68.
24. Yang BC, Phillips MI, Zhang YC, et al. Critical role of AT1 receptor expression after ischemia/reperfusion in isolated rat hearts: beneficial effect of antisense oligodeoxynucleotides directed at AT1 receptor mRNA. *Circ Res*. 1998;83:552–559.
25. Wang X, Lu J, Khaidakov M, et al. Aspirin suppresses cardiac fibroblast proliferation and collagen formation through downregulation of angiotensin type 1 receptor transcription. *Toxicol Appl Pharmacol*. 2012;259:346–354.
26. Di Stefano R, Felice F, Balbarini A. Angiogenesis as risk factor for plaque vulnerability. *Curr Pharm Des*. 2009;15:1095–1106.
27. Sluimer JC, Daemen MJ. Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J Pathol*. 2009;218:7–29.
28. Phillips MI, Kagiyama S. Angiotensin II as a pro-inflammatory mediator. *Curr Opin Investig Drugs*. 2002;3:569–577.
29. Newby AC, George SJ, Ismail Y, et al. Vulnerable atherosclerotic plaque metalloproteinases and foam cell phenotypes. *Thromb Haemost*. 2009; 101:1006–1011.
30. Shah PK. Mechanisms of plaque vulnerability and rupture. *J Am Coll Cardiol*. 2003;41(suppl):15S–22S.
31. Fukuhara M, Geary RL, Diz DI, et al. Angiotensin-converting enzyme expression in human carotid artery atherosclerosis. *Hypertension*. 2000; 35:353–359.
32. Graninger M, Reiter R, Drucker C, et al. Angiotensin receptor blockade decreases markers of vascular inflammation. *J Cardiovasc Pharmacol*. 2004;44:335–339.
33. Suganuma E, Babaev VR, Motojima M, et al. Angiotensin inhibition decreases progression of advanced atherosclerosis and stabilizes established atherosclerotic plaques. *J Am Soc Nephrol*. 2007;18:2311–2319.
34. Chen K, Chen J, Li D, et al. Angiotensin II regulation of collagen type I expression in cardiac fibroblasts: modulation by PPAR-gamma ligand pioglitazone. *Hypertension*. 2004;44:655–661.
35. Sun Y, Weber KT. Angiotensin II receptor binding following myocardial infarction in the rat. *Cardiovasc Res*. 1994;28:1623–1628.
36. Kurdi M, Booz GW. New take on the role of angiotensin II in cardiac hypertrophy and fibrosis. *Hypertension*. 2011;57:1034–1038.
37. D'Amario D, Cabral-Da-Silva MC, Zheng H, et al. Insulin-like growth factor-1 receptor identifies a pool of human cardiac stem cells with superior therapeutic potential for myocardial regeneration. *Circ Res*. 2011;108:1467–1481.
38. de Cavanagh EM, Inserra F, Ferder L. Angiotensin II blockade: a strategy to slow ageing by protecting mitochondria? *Cardiovasc Res*. 2011;89:31–40.
39. Khaidakov M, Szwedo J, Mitra S, et al. Antiangiogenic and antimitotic effects of aspirin in hypoxia—reoxygenation modulation of the LOX-1-NADPH oxidase axis as a potential mechanism. *J Cardiovasc Pharmacol*. 2010;56:635–641.
40. Lu L, Liu H, Peng J, et al. Regulations of the key mediators in inflammation and atherosclerosis by aspirin in human macrophages. *Lipids Health Dis*. 2010;9:16.
41. Yamamoto Y, Yamashita T, Kitagawa F, et al. The effect of the long term aspirin administration on the progress of atherosclerosis in apoE-/- LDLR-/- double knockout mouse. *Thromb Res*. 2010;125:246–252
42. Kouraklis G, Patapis P, Misiakos E, et al. Effects of acetylsalicylic acid on experimental atherogenesis induced in rabbits. *Int Angiol*. 2004;23: 139–143.
43. Waldstein SR, Wendell CR, Seliger SL, et al. Nonsteroidal anti-inflammatory drugs, aspirin, and cognitive function in the Baltimore longitudinal study of aging. *J Am Geriatr Soc*. 2010;58:38–43.