Shear Stress Regulates Angiotensin Type 1 Receptor Expression in Endothelial Cells

Bhama Ramkhelawon, Jose Vilar, Daniel Rivas, Barend Mees, Rini de Crom, Alain Tedgui, Stéphanie Lehoux

Rationale: Shear stress (SS) has an established role in atherosclerotic plaque localization, but how it exerts its protective effect is not fully understood.

Objective: To test the hypothesis that SS may downregulate angiotensin type 1 receptors (AT1Rs). Angiotensin II has been shown to be proinflammatory and to promote atherosclerosis.

Methods and Results: Using immunohistochemistry, we found a pronounced expression of AT1R in the inner, atheroprotec regions of the aortic arch of C57BL/6 and endothelial NO synthase–deficient (eNOS−/−) mice but not eNOS-overexpressing mice. In human umbilical vein endothelial cells (HUVECs), laminar SS (15 dyn/cm²) induced a biphasic decrease in AT1R protein expression characterized by a first reduction at 1 hour (31±4% of static control, P<0.01), partial recovery at 3 hours (65±9%), and a second more prolonged decline at 6, 12, and 24 hours (48±9%, 36±9%, 33±5%, respectively, P<0.05). One and 24 hours of SS significantly reduced fluorescent angiotensin binding compared to static HUVECs. Shear-induced downregulation of AT1R was abolished by treatment with protein kinase A and G inhibitors or Nω-nitro-l-arginine methyl ester (L-NAME). Fittingly, stimulating static HUVECs with an NO donor decreased AT1R protein levels. RT-PCR revealed a significant (P<0.05) decrease of AT1R mRNA in HUVECs exposed to SS during 3 (6±2% of static control), 6 (4±1%), 12 (4±1%), and 24 hours (15±4%), suggesting a transcriptional downregulation of AT1R at length. Finally, angiotensin-induced vascular cell adhesion molecule was abated in HUVECs exposed to SS and in the outer aortic arch of mice.

Conclusions: Our results demonstrate that SS may convey some of its atheroprotective effects through downregulation of AT1R in endothelial cells. (Circ Res. 2009;105:869-875.)

Key Words: shear stress □ angiotensin □ nitric oxide □ endothelium □ atherosclerosis

Hemodynamic forces are major determinants of atherosclerotic plaque localization. Plaques tend to form at arterial bifurcations, branch points, and curvatures where blood flow is low and oscillatory, whereas blood vessels exposed to high laminar shear stress (SS) remain comparatively devoid of plaques.1–3 The protective effect of SS is largely attributable to the local release of nitric oxide (NO), an important vasoprotective agent, known to reduce endothelial permeability, leukocyte adhesion, vascular smooth muscle cell proliferation, and thrombosis, while favoring endothelial survival.1–6 In contrast, oscillatory blood flow or low SS induces the expression of adhesion molecules (intracellular adhesion molecule-1, vascular cell adhesion molecule-1), chemokines (monocyte chemoattractant protein-1, interleukins), and growth factors that contribute to leukocyte recruitment and infiltration,7,8 which constitute the early steps of plaque formation. Appropriately, the knockout (KO) or pharmaceutical inhibition of endothelial NO synthase (eNOS) is associated with enhanced lesion growth in animal models of atherosclerosis.9,10 Opposite to the NO/NO synthase system, the renin–angiotensin system, which participates in vasoconstriction and arterial remodeling, has rather proatherosclerotic effects in the vascular wall.11 In animal models of atherosclerosis, angiotensin II (Ang II) perfusion exacerbates the development of atherosclerotic plaques.12 Conversely, angiotensin type 1 receptor (AT1R) antagonists show a beneficial effect on plaque regression,12 caused by a decrease in the inflammatory properties of the plaque.13 The atherogenic effects of Ang II can be explained not only by its impact on blood pressure but also by a direct inflammatory action of the hormone on vascular cells, inducing the expression of adhesion molecules and chemokines.14
In spite of the fact that the roles of SS and Ang II in the blood vessel wall are well established, the interaction between these 2 factors remains poorly investigated. However, indirect evidence exists showing that NO, which is produced by SS, modulates the biological functions of Ang II such as vascular smooth muscle cell migration and vascular reactivity. Moreover, the expression of AT1R is reduced in vascular smooth muscle cells exposed to an NO donor, suggesting that SS may not only modulate the vascular response to Ang II through the opposing effects of NO but may also directly influence AT1R expression in vascular cells. In the present study, we hypothesized that SS may regulate the expression of AT1R in endothelial cells (ECs), which could account at least in part for the protective, antiatherogenic effects of SS.

Methods

Immunohistochemical Analysis

Experiments were performed in accordance with the European Community Standards for the Care and Use of Laboratory Animals and were approved by the local ethics committee. Eight-week-old C57BL/6 mice, eNOS-overexpressing transgenic mice (eNOS KO), and wildtype mice were used. HUVECs were either maintained in static conditions alone, static + AT1R blocker (ARB) (Losartan, 10⁻⁶ mol/L) or exposed to a shear stress stimulus of 0.6 dyn/cm² at 1 Hz. Two successive longitudinal 7-μm sections were cut using a cryostat, and transferred onto gelatin-coated slides. Nonspecific binding sites on the tissue sections were blocked with 10% goat serum for 30 minutes at room temperature. Thereafter, anti-AT1R antibody (Abcam) and anti-CD31 or anti–VCAM-1 (Santa Cruz) antibodies were incubated overnight at 4°C. The sections were washed twice with cold PBS and scraped off in 200 μL of RIPA buffer (50 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L glycerophosphate, 0.1% SDS, 0.1% deoxycholate, 1% Triton, complete protease inhibitor cocktail tablet [Roche]). Protein content was quantified using the Bradford (Bio-Rad) protein assay. Thirty micrograms of lysate were mixed with reducing sample buffer for electrophoresis and subsequent transfer onto nitrocellulose membranes (Amersham), and equal loading was verified using Ponceau red solution. Membranes were incubated with anti-AT1R or anti-AT2R antibodies (Abcam) or anti–VCAM-1 (Santa Cruz). After secondary antibody incubation (Amersham), immunodetection proceeded using an enhanced chemiluminescence kit (ECL Plus, Amersham), and bands were revealed using the Las1000 imaging system and Image Gauge software (Fuji).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) (Promocell) were grown to confluence on 0.2% gelatin-coated culture slides at 37°C in a humidified 5% CO₂ incubator. Cells were cultured in endothelium cell basal medium containing growth factors (human epidermal growth factor, human basic fibroblast growth factor, and human EC growth supplement; Promocell), 5% FCS (Boehringer–Mannheim) supplemented with streptomycin (100 μg/mL), penicillin (100 U/mL), and 10 μg/L Fungizone. In some experiments, cells were treated with Ang II (10⁻⁸ mol/L), the NO synthase inhibitor N⁶-nitro-L-arginine methyl ester (L-NNAME) (10⁻⁴ mol/L), the NO donor S-nitroso-L-acetyl penicillamine (SNAP) (10⁻⁶ mol/L), a protein kinase (PKG) inhibitor (KT5720, 10⁻⁶ mol/L), a PKG inhibitor (KT5823, 10⁻⁶ mol/L), an AT1R antagonist (Losartan, 10⁻⁶ mol/L), an AT2R antagonist (PD123319, 10⁻⁶ mol/L), or actinomycin D (5 μg/mL). For each figure, each individual n corresponds to data obtained from an individual batch of HUVECs processed separately from the next.

Fluorescent–Angiotensin II Binding Assay

HUVECs were washed twice with cold PBS and scraped off in 200 μL of RIPA buffer (50 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L glycerophosphate, 0.1% SDS, 0.1% deoxycholate, 1% Triton, complete protease inhibitor cocktail tablet [Roche]). Protein content was quantified using the Bradford (Bio-Rad) protein assay. Thirty micrograms of lysate were mixed with reducing sample buffer for electrophoresis and subsequent transfer onto nitrocellulose membranes (Amersham), and equal loading was verified using Ponceau red solution. Membranes were incubated with anti-AT1R or anti-AT2R antibodies (Abcam) or anti–VCAM-1 (Santa Cruz). After secondary antibody incubation (Amersham), immunodetection proceeded using an enhanced chemiluminescence kit (ECL Plus, Amersham), and bands were revealed using the Las1000 imaging system and Image Gauge software (Fuji).

Quantitative RT-PCR

RT-PCR was performed to quantify AT1R mRNA levels using the RNaseasy micro protocol (Qiagen) to isolate total RNA from cells. One microgram of RNA was mixed with random primers and reverse transcribed according to the first-strand method (Supershift, Invitrogen). cDNA thus obtained was amplified by PCR under the following conditions: 30 seconds at 94°C, 30 seconds at 57.6°C, 30 seconds at 72°C, for 40 cycles. PCR primers used were as follows: AT1R sense, 5’-GGT CGG GTG TGC ATT GTA TGG C-3’; AT1R antisense, 5’-CAA AGG GCC AGC GGT ATT CCA TAG-5’; GAPDH sense, 5’-GAA GGT GAA GGT CGG AGT C-3’; GAPDH antisense, 5’-GAA GAT GGT GAT GGG ATT TC-5’.
The same cDNA samples were used for GAPDH and AT1R amplification. PCR amplification resulted in 485 bp fragments originated from AT1R mRNA and 226 bp from GAPDH mRNA. For quantification, the number of PCR cycles was chosen within the linear exponential phase with respect to the amount of cDNA template and the PCR performed.

Statistical Analysis
Data presented as means±SEM were obtained in at least 3 independent experiments, obtained with different sets of cells. Values reported from western blots are expressed with respect to static control experiments. Statistical significance was determined by the nonparametric Mann–Whitney test, with P<0.05 considered as statistically significant.

Results
Differential SS-Dependent Expression of AT1R in the Vascular Wall
To assess the expression of AT1R in arteries, we compared the inner curvature of the aortic arch, where atherosclerotic plaques tend to form, to the neighboring outer curvature of the arch that is comparatively protected. Immunostaining of C57BL/6 mouse aortic arches showed a distinctive endothelial AT1R staining at the inner curvature of the aortic arch but not in the adjoining outer curvature (Figure 1). AT1R expression was similarly absent from the outer curvature of eNOS tg mice, but in these animals, even the inner curvature failed to show endothelial AT1R staining. On the contrary, in vessels of eNOS KO mice, both the inner and outer curvature displayed positive AT1R staining in the endothelium (Figure 1). Hence, endothelial AT1R expression is limited to plaque-prone areas having low SS in wild-type animals and is modulated by endothelial eNOS expression.

Laminar Flow Decreases AT1R Protein Levels in HUVECs
To explain the selective regional distribution of AT1R observed in immunostaining experiments, we examined the effects of SS on AT1R protein expression. HUVECs were exposed to laminar flow with a SS of 0 or 15 dyn/cm² for 1, 3, 6, 12, or 24 hours. Western blotting revealed that AT1R protein levels followed a biphasic regulation, characterized by an initial decrease at 1 hour (31±4% of static control, P<0.001), partial recovery at 3 hours (65±9%, P<0.05), and a second more prolonged decrease at 6, 12, and 24 hours (48±9%, 36±9%, 33±5% of static control, respectively; P<0.01) (Figure 2A). Interestingly, we found that oscillatory flow (0±6 dyn/cm²) had the opposite effect, increasing AT1R levels at 12 hours (240±26%, P<0.01) and 24 hours (185±25, P<0.05) (Figure 2B). On the other hand, AT1R levels increased significantly at 1 hour of SS and remained elevated thereafter (P<0.05) (Figure 2A), suggesting a differential modulation of the Ang II receptors by SS.

To confirm the decreased protein expression of AT1R observed under flow conditions, we conducted binding assays with fluorescent Ang II in cells exposed to static conditions or 1 or 24 hours of SS. As demonstrated in Figure 3, cells exposed to flow showed a significant decrease in Ang II binding (62±13 at 1 hour, 23±2 at 24 hours, P<0.05), similar to losartan-treated static cells (33±9, P<0.05), compared with untreated static cells (509±88). These results are AT1R-specific because all cells were treated with an AT2R inhibitor just before incubation with the fluorescent Ang II. The punctate Ang II binding pattern corresponded to the surface distribution of the AT1R receptor observed by immunocytochemistry in HUVECs (data not shown). Hence, the decrease in AT1R protein expression observed in cells exposed to SS is associated with a reduction in Ang II binding.

Pathways of SS-Induced AT1R Regulation
It is well known that SS triggers NO formation through the phosphorylation and activation of eNOS. To assess whether this pathway participates in the regulation of AT1R expression, we treated HUVECs with the eNOS inhibitor L-NAME (10⁻⁷ mol/L). Figure 4A demonstrates that the SS-induced decrease of AT1R was completely abolished by this treatment. These data are upheld by the finding that compared with ECs from wild-type mice, binding of Ang II to ECs from eNOS KO mice was enhanced, whereas binding to ECs of eNOS overexpressing mice was diminished (Online Figure I, available at http://circres.ahajournals.org). Furthermore, to further assess whether NO is sufficient for regulation of
AT1R levels, we exposed static HUVECs to SNAP (10^{-6} mol/L), an NO donor. SNAP incubation significantly reduced AT1R protein levels at 1 hour (31\% \pm 3\%) (P<0.05) and 3 hours (44\% \pm 6\%) (P<0.05) but did not show any effect at later time points (Figure 4B). Hence SS regulates AT1R expression in an NO-dependent manner in ECs, and NO release is sufficient for loss of AT1R.

To further investigate the molecular mechanisms underlying the SS-NO–dependent AT1R regulation, we evaluated the potential role of PKA and PKG. Both the PKA (10^{-6} mol/L) and the PKG (10^{-6} mol/L) inhibitors prevented SS-induced AT1R decrease at 1, 3, 6, 12, and 24 hours (Figure 5), indicating a role for both kinases in regulating AT1R levels under shear conditions. Moreover, a significant increase in AT1R levels at 24 hours (148\% \pm 10\%) (P<0.05) was revealed in cells exposed to flow and the PKG inhibitor, indicating that this pathway may have supplemental, lengthy regulatory effects on the receptor. To better define the PKA/PKG pathway linking SS with AT1R loss, we evaluated phosphorylation of eNOS in cells treated with different inhibitors and exposed to SS. In untreated and PKG inhibitor-treated cells, phosphorylation or eNOS was elevated at 1 hour and remained high thereafter. However, this effect was countered by either PKA inhibitor or L-NAME (Online Figure II). Hence, our results suggest a cascade whereby SS activates PKA, leading to eNOS activation and phosphorylation, followed by PKG activation and downstream AT1R loss.

**SS Downregulates AT1R mRNA**

To analyze whether the reduction in AT1R protein levels reflected a transcriptional regulatory event, we determined the AT1R mRNA levels by RT-PCR in cells exposed to Figure 2. Steady SS and oscillatory flow regulate AT1R and AT2R differentially in ECs. A, Western blot showing reduced protein levels of AT1R in HUVECs exposed to a SS of 15 dyn/cm² during 1, 3, 6, 12, or 24 hours compared with control cells (top). In contrast, increased AT2R protein expression was observed under shear conditions at all time points (bottom). B, Oscillatory flow was associated with increased protein levels of AT1R at both 12 and 24 hours. Results are means \pm SEM of n=3 to 5 experiments. The expression level of AT1R and AT2R in static conditions was set as 100%. GAPDH bands indicate equivalent protein loading in each condition. *P<0.05, **P<0.01.

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different periods of SS. AT1R mRNA levels were equivalent in unstimulated controls and HUVECs exposed to 1 hour (115±8%) of SS. However, a significant (P<0.05) decrease of AT1R mRNA was observed in HUVECs exposed to SS for 3 hours (6±2% of static control), 6 hours (4±1%), 12 hours (4±1%), and 24 hours (15±4%) (Figure 6A). To determine whether this reduction in AT1R by SS was dependent on changes in AT1R mRNA stability, we treated cells with actinomycin D, an inhibitor of gene transcription. Actinomycin D was added to cell medium in either static or SS conditions, and AT1R mRNA was examined 1, 3, 6, 12, and 24 hours thereafter. As shown in Figure 6B, no significant difference was observed in the time-dependent decrease of AT1R mRNA between the static group and SS-induced group. These results support the notion that SS primarily decreases the transcription rate of the AT1R gene, rather than altering mRNA stability.

Lower AT1R Expression Is Associated With a Reduced Proinflammatory Response

To verify that the loss of AT1R expression in ECs could translate into reduced atherosclerotic potential, wild-type mice were injected with Ang II and the vascular expression of VCAM-1 was evaluated after 6 hours. As demonstrated in Figure 7A, both AT1R and VCAM-1 expression could be readily detected in ECs in the inner curvature of the aortic arch, whereas the outer curvature displayed little VCAM-1 staining and no endothelial AT1R. VCAM-1 could not be detected in vessels from control mice not injected with Ang II (data not shown). These results were confirmed in HUVECs; cells were maintained in static conditions or exposed to SS during 24 hours, then stimulated with Ang II (10−6 mol/L) during 6 hours, with or without concomitant treatment with losartan or PD123319. VCAM-1 was upregulated significantly (P<0.05) in static HUVECs exposed to Ang II alone (164±20% of unstimulated control) or in combination with PD123319 (201±36%) but not in cells treated with the ARB (Figure 7B). In comparison, Ang II failed to induce VCAM-1 expression in all cells exposed to SS.

Discussion

Laminar SS plays a fundamental role in the regulation of EC functions. The results presented herein identify for the first time a new mechanism whereby SS may convey some of its atheroprotective effects. Firstly, we demonstrated that endothelial AT1R expression in whole vessels was only localized in the inner aortic arch, characterized by disturbed or oscillatory SS, but not in the outer aortic arch exposed to high SS. Secondly, in vitro assays revealed that SS was associated with the downregulation of AT1R both at the protein and at the mRNA levels in ECs, in an NO-dependent manner. PKA and PKG were implicated in this regulatory mechanism as well. Finally, absence of endothelial AT1R expression was associated with lower VCAM-1 induction by Ang II, both in vivo and in vitro.

We found that exposure to SS was associated with a biphasic reduction in AT1R protein expression, characterized by a transient decrease at 1 hour and a more prolonged loss at 24 hours. Reduced binding of fluorescent Ang II to the AT1R observed in HUVECs exposed to SS uphold these findings. The rapid decrease in receptor number after SS exposure suggests that the protein half-life is short and could be explained by an intracellular degradation of the receptor following its activation by SS conditions. The rise in AT1R expression observed at 3 hours could result from a lower rate of endocytosis, reduced AT1R vesicle lysis in the favor of enhanced recycling, or posttranscriptional regulation. At later time points, from 3 hours of SS onwards, RT-PCR revealed that AT1R mRNA expression was downregulated. Experiments with actinomycin D suggest that the long-term reduc-
pronounced endothelial staining for AT1R in the inner curvature of the aortic arch of wild-type and eNOS KO mice but not eNOS tg mice. Conversely, AT1R could be detected in the outer curvature of eNOS KO mice, despite elevated SS in that segment, and L-NAME blocked the downregulation of AT1R by SS in HUVECs. To gain insight into the molecular mechanisms by which SS-induced NO controls AT1R expression, we investigated the implication of either PKA, which lies upstream of eNOS activation by SS\textsuperscript{22,23} and PKG, the primary downstream signaling effector of NO\textsuperscript{24–26}. Our results show that the PKA inhibitor blocked eNOS phosphorylation and prevented the decrease in AT1R expression under flow conditions. Downstream of eNOS, PKG inhibition likewise abolished the loss of AT1R. These latter results actually differ from a previous work\textsuperscript{19} showing that AT1R reduction by an NO donor occurred independently of cGMP in vascular smooth muscle cells. These contradictory results suggest that NO may elicit different downstream effectors targeting the AT1R according to cell type.

Several authors have advocated that Ang II participates in atherosclerosis through its proinflammatory actions. On the one hand, treatment of hypercholesterolemic rabbits, ApoE\textsuperscript{−/−} mice, and nonhuman primates with AT1R antagonists decreased vascular inflammation and reduced progression of atherosclerosis.\textsuperscript{13,27–29} On the other hand, Ang II infusion was found to promote the rapid formation of atherosclerotic lesions in low-density lipoprotein receptor–deficient (LDLR\textsuperscript{−/−}) and ApoE\textsuperscript{−/−} mice.\textsuperscript{30,31} Most importantly, a recent study using bone marrow transplantation strategies showed that AT1a receptor expression in the vascular tissues of recipient mice, rather than in donor-infiltrating cells, was required for Ang II–induced atherosclerosis.\textsuperscript{32} Our finding that AT1R is expressed by ECs that lie in low SS, atheroprotective vessel segments provides a functional link between the known proatherosclerotic properties of Ang II and the focality of atherosclerotic plaque localization. Furthermore, we found that Ang II–induced VCAM-1 expression coincided with sites where endothelial AT1R expression was enhanced, in the inner curvature of the aortic arch. A similar VCAM-1 distribution was previously reported in mice administered proinflammatory lipopolysaccharide\textsuperscript{33,34} and in ApoE-deficient animals.\textsuperscript{33,34} Our results were confirmed in cultured HUVECs, where Ang II–induced, AT1R-dependent VCAM-1 expression was abolished by SS. Hence, the presence of AT1R in ECs could be particularly detrimental because this receptor may directly mediate Ang II–induced expression of VCAM-1 in ECs and also that of P-selectin and intracellular adhesion molecule-1,\textsuperscript{35} facilitating monocyte adhesion. Ang II was also found to reduce the availability of NO in ECs through an AT1R-dependent increment in ROS generation,\textsuperscript{36} further emphasizing the role of AT1R and NO in the pro- and anti-atherogenic balance controlled by SS. Taken together, these findings might help to explain the antiatherosclerotic effects of ACE inhibitors and AT1R antagonists and suggest that endothelial AT1R expression may contribute not only to atherosclerotic lesion development but also to plaque progression.

In conclusion, we propose that laminar SS selectively downregulates AT1R expression in ECs. Because AT1R can engender endothelial activation, dysfunction, and atherosclerosis, our findings suggest that SS-mediated inhibition of AT1R expression contributes to antiatherogenic and vasoprotective effects exerted by laminar SS.
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Disclosures
None.

References
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Online Figure I. Differential fluorescent AngII binding to ECs from wild-type (C57BL/6), eNOS-transgenic (eNOS tg) and eNOS knockout mice. Mice were killed by a lethal dose of sodium pentobarbital (100 mg/kg). The lungs were rapidly excised, rinsed with ice cold PBS, diced, and digested in 0.1% collagenase (Roche) for 60 min. Undigested tissue fragments were filtered out and the cells were grown for 2-4 days at 37°C. Upon reaching confluence, endothelial cells were selected using 10µg of anti-mouse CD102 antibody (Pharmigen) coupled to 10µL of Dynabeads (Invitrogen). The retained cells were grown until confluence and a second positive selection carried out. Endothelial cell purity averaged at 95%. Binding was carried out as described in the main document, in the presence of AT2R inhibitor (PD123319, 10^-6 mol/L) added 20 min before fluo-AngII. Results are mean±SEM of n=4 experiments. *p<0.05. Scale bar = 50 µm.
Online Figure II. eNOS was phosphorylated in untreated HUVECs exposed to shear stress during 1, 3, 6, 12, and 24 hrs. eNOS phosphorylation was abolished by L-NAME (10^{-4}mol/L) and PKA inhibitor treatment (KT5720; 10^{-6}mol/L), but not by the PKG inhibitor (KT5823; 10^{-6}mol/L). Western blotting was conducted as described in the main document using anti-eNOS (BD Transduction Laboratories) or anti-phospho-ser1177 eNOS (Cell Signaling) antibodies. Results are representative of n=3 experiments.