Angiotensin II Increases the Expression of Lectin-like Oxidized Low-Density Lipoprotein Receptor–1 in Human Vascular Smooth Muscle Cells via a Lipoxygenase-Dependent Pathway

Rona Limor, Marielle Kaplan, Tatsuya Sawamura, Orli Sharon, Shlomo Keidar, Gary Weisinger, Esther Knoll, Michal Naidich, and Naftali Stern

Background: Lectin-like oxidized low-density lipoprotein receptor–1 (LOX-1) is a membrane protein that can act as a surface endocytosis receptor for oxidized LDL (ox-LDL). As increased cellular uptake of ox-LDL by macrophages and activated smooth muscle cells may transform these cells into foam cells, potential interactions among LDL oxidation, ox-LDL uptake, and regulators of vascular smooth muscle cell function are of obvious interest. The objective of this study was to examine the effect of angiotensin II (AII) on the expression of LOX-1 and ox-LDL degradation in human vascular smooth muscle cells (VSMC).

Methods: We performed in vitro experiments in a human VSMC line (T/G HA-VSMC) derived from normal aortic VSMC, using standard methods.

Results: We found that AII (10^{-7}mol/L) increased the expression of LOX-1 (~2.5-fold, P < .0001) in association with higher degradation of ox-LDL by HA-SMC (from 4019 ± 529 ng/mg cell protein to 6207 ± 287ng/mg cell protein; P = .0033). AII also increased the expression of 12-lipoxygenase (12-LO) and 15-lipoxygenase (15-LO) by ~2.2-fold (P = .03) and ~3-fold (P = .006), respectively. In addition, AII (10^{-7}mol/L) increased the release of 12- and 15-hydroxyeicosatetraenoic acid from VSMC within 10 min ~3-fold (P = .03) and 50% (P < .05), respectively.

Conclusions: Our study findings provide evidence that angiotensin II upregulates LOX-1 and 12-LO and 15-LO expression in human VSMC, thereby potentially providing mechanisms for both accelerated LDL oxidation within the cell and the internalization of exogenous ox-LDL, two processes that could increase the susceptibility of human VSMC to further transformation into foam cells.

Key Words: 12-LO, 15-LO, LOX-1, human smooth muscle cells.

There is growing evidence for interaction among blood pressure, the renin–angiotensin system, and lipid metabolism at the vasculature. Angiotensin-converting enzyme (ACE) is present in sclerotic aortic valves where it colocalizes with angiotensin II (AII). Circulating low-density lipoprotein (LDL) may carry and deliver ACE to atherosclerotic lesions. AII increases the synthesis of proteoglycans with enhanced LDL binding capacities. Both native LDL and oxidized LDL (ox-LDL) increase the expression of the AII type 1 receptor (AT1R), which mediates most of the recognized cardiovascular effects of AII. On the other hand, AII also activates several lipoxygenase (LO) enzymes, products of which (such as 12-hydroxyeicosatetraenoic acid [HETE]) have been implicated in cell signaling controlling contraction, growth, migration, and proliferation of VSMC. The LO enzymes are also involved in LDL oxidation, and these effects are subject to acceleration by AII in macrophages. Ox-LDL deposited at the vessel walls can decrease the generation of nitric oxide (NO) and cause endothelial dysfunction and also induce apoptosis in endothelial cells and VSMC. The uptake of ox-LDL in
vascular cells is mediated by scavenger receptors; among these, the expression of lectin-like oxidized low-density lipoprotein receptor–1 (LOX-1) is upregulated in VSMC by high blood pressure.17,18 Because oxidative modification of LDL plays a role in the initiation and progression of atherosclerosis,19 potential interaction among AII, LOs, and ox-LDL are of obvious interest. In this study, we examined the effects of AII on LOX-1 expression in association with its effects on LO enzymes in human VSMC. The results suggest that AII up regulates both LOX-1 and LO enzyme expression, and that the increase in LOX-1 depends on LO activation and is associated with increased uptake of ox-LDL in VSMC.

Methods

Cell Culture

A line of human vascular smooth muscle cells T/G HAVSMC (VSMC; American Type Culture Collection, Manassas, VA) established from a normal aorta was cultured in Ham’s F12K medium with 2 mmol/L L-glutamine, 10 mmol/L HEPES, 10 mmol/L N-[Tris(hydroxymethyl)-methyl]-2-aminoethane-sulfonic acid (TES), 0.05 mg/mL ascorbic acid, 0.01 mg/mL insulin, 0.01 mg/mL transferrin, 10 ng/mL sodium selenite, 10% fetal calf serum (FCS), and 0.03 mg/mL endothelial cell growth supplement. Cells were initially trypsinized, transferred to 10-cm tissue culture dishes, and re-cultured to subconfluence, at which time they were used for RNA and protein preparation. In some experiments, cells were first incubated with AII (10^{-7} mol/L) or losartan (10^{-5} mol/L), an antagonist of the AII type 1 receptor (AT1R).

Total RNA Preparation

Before RNA preparation, cultured cells were incubated for 24 h in Ham’s F12K medium containing 0.4% fetal calf serum, 2 mmol/L L-glutamine, 10 mmol/L HEPES,

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human LOX-1</td>
<td>5’ Primer TTACTCTCCATGGTGCTGCC</td>
</tr>
<tr>
<td></td>
<td>3’ Primer AGCTCTTCTGCCGTTGCCC</td>
</tr>
<tr>
<td>Human 12-LO platelet type</td>
<td>5’ Primer GATGATCTACCTCCAATATG</td>
</tr>
<tr>
<td></td>
<td>3’ Primer CTGGCCCAGAAGATCTG</td>
</tr>
<tr>
<td>Human cyclophillin</td>
<td>5’ Primer TCCTAAAGCATAAGGCTCCTGAGCAT</td>
</tr>
<tr>
<td></td>
<td>3’ Primer CGCTCAGGCGCAACTATATCA</td>
</tr>
<tr>
<td>Human 15-LO type II</td>
<td>5’ Primer AACTCAAGGTGGAAGTACCCAGGAG</td>
</tr>
<tr>
<td></td>
<td>3’ Primer ATATAAGTTGGCCACGCAATATTC</td>
</tr>
</tbody>
</table>

LO = lipoxygenase; LOX-1 = lectin-like oxidized low-density lipoprotein receptor–1.
10 mmol/L TES, 0.05 mg/mL ascorbic acid, 0.01 mg/mL insulin, 0.01 mg/mL transferrin, and 10 ng/mL sodium selenite, without endothelial cell growth supplement. Total RNA from cultured HA-VSMC was extracted using Trizol reagent (GibcoBRL, Life Technologies Ltd., Paisley, United Kingdom).

Amplification of Reverse-Transcribed RNA by Polymerase Chain Reaction

All oligonucleotides were synthesized by Sigma-Aldrich (St. Louis, MO) and were purified by high-performance liquid chromatography (HPLC). The sequences of nucleotides (provided in Table 1) were designed from the reported sequences of the various genes. A 1-μg quantity of total RNA underwent reverse-transcription (RT) using a commercially available kit (Clontech Laboratories, Palo Alto, CA) and further amplified by polymerase chain reaction (PCR). The PCR consisted of 40 cycles of 94°C for 40 sec, 55°C for 1 min and 72°C for 1 min. Human cyclophillin primers were used for normalization of the PCR reaction. Blank reaction controls with no RNA template or no Moloney-murine leukemia virus (MMLV-RT) were performed through the RT and PCR steps.

Cloning and Sequencing

The PCR products were extracted from the agarose gel with a commercial kit (Jetsorb, Genomed, GmbH, Bad Oeynhausen, Germany). The DNA fragments obtained were PCR-amplified and sequenced at the Weizman Institute Sequencing Facility (Rehovot, Israel).

Western Immunoblotting

Cultured cells were washed with phosphate-buffered saline (PBS), scraped and lysed on ice in a lysis buffer consisting of PBS (7.4), supplemented with 1% Triton X-100, 1 tablet of Boehringer Proteinase Inhibitors (Boehringer-Mannheim, Mannheim, Germany), and 0.1% sodium dodecyl sulfate (SDS). This was followed by mild glass homogenization (10

---

FIG. 2. A) Effect of angiotensin II (AII) (10⁻⁷ mol/L) on LOX-1 expression. Lanes 1 to 3: control; lanes 4 to 6: AII. B) Densitometric analysis of LOX-1 mRNA expression in untreated VSMC and of cells treated for 1 h with AII (10⁻⁷ mol/L) alone or in the presence of losartan (10⁻⁵ mol/L) or baicalein (10⁻⁷ mol/L). Results are expressed in arbitrary units relative to control incubates and are means ± SEM of six separate experiments. ***P < .0001 v control; #P < .05 v AII; ####P < .0001 v AII. Abbreviations as in Fig. 1.
strokes) and centrifugation at 10,000 g for 10 min at 4°C. Aliquots of the supernatant were saved for protein estimation and Western blot analysis. The SDS protein electrophoresis was performed on a 8% SDS polyacrylamide gel. For Western transfer of protein to Protean nitrocellulose (BA85) (S&S, Dassel, Germany), standard methods were followed using a human anti LOX-1 antibody (1:1000) generated by one of the investigators (T.S.).

**Extraction and measurement of 12-HETE**

Cultured confluent cells were washed twice with medium and treated with AII (10^{-7} mol/L) for 10 min. The incubation was stopped by adding 4 mL of ice-cold ethanol (100%) to the dishes at 0°C. For measurement of 12-HETE, we used a modification of the reverse-phase ultraviolet (UV)–HPLC method of Eskra et al, which has been validated by RIA, as previously described.22

**Gel Analysis**

Aliquots (20 μL) of the PCR products were subjected to electrophoresis in 2.0% commercial agarose E-Gel (In-vitrogen, Carlsbad, CA) and then photographed.

**Cellular Uptake of Lipoproteins**

The LDL was radioiodinated by the iodine monochloride method as modified for lipoproteins.23 Radioiodinated oxidized LDL (Ox-125I-LDL) was prepared from 125I-LDL that was dialyzed against PBS, followed by incubation with 5 μmol/L of CuSO_{4} at 37°C for 24 h. The Ox-125I-LDL (10 mg of protein/L) was incubated with the cells at 37°C for 5 h. Lipoprotein degradation was measured in the collected medium as the trichloroacetic acid (TCA)–soluble, nonlipid radioactivity, which was not due to free iodide.24 Lipoprotein degradation in a cell-free system was measured under identical conditions was minimal (<10%) and was subtracted from the total degradation.

**Statistical Analysis**

Results are expressed as mean ± SEM. Data were assessed by the Student t test or one-way analysis of variance followed by the Newman-Keuls post hoc analysis as appropriate. Statistical significance was defined as $P < .05$. 

**FIG. 3.** Effect of AII treatment on LOX-1 protein expression. The VSMC were treated with AII for 15 min. A) Western blot performed with a polyclonal antibody (1:1000). B) Densitometric analysis based on three separate experiments (mean ± SEM). Abbreviations as in Figs. 1 and 2.
Results

Effect of AII on LOX-1 and ox-LDL degradation

Using specific oligonucleotides (Table 1), mRNA for LOX-1, human platelet type 12-LO the long form and 15-LO type II were consistently detected in VSMC (Fig. 1). After 1 h of incubation with AII (10^{-7} mol/L), mRNA expression of LOX-1 increased significantly ($P < .0001$) as shown by the housekeeping gene–normalized densitometric analyses in Figs. 2A and 2B. Furthermore, the AII-induced increase in LOX-1 mRNA expression was blocked by losartan (10^{-5} mol/L), a specific AT1 receptor blocker and also by baicalein (10^{-5} mol/L), a specific 12-LO blocker (Fig. 2B).

The effect of AII (10^{-7}mol/L) on of LOX-1 protein expression is depicted in Fig. 3. There was a threefold increase in of LOX-1 protein expression, which can be seen in the Western immunoblot of one of the experiments (Fig. 3A) and in the overall densitometric analysis ($n = 3$; Fig. 3B) ($P < .001$).

Treatment of cultured VSMC with AII (10^{-7} mol/L) for 24 h increased the rate of degradation of radiiodinelabeled ox-LDL by $\sim 60\%$ (Fig. 4). This effect was inhibited in the presence of the AT1R antagonist losartan (10^{-5} mol/L).

Effect of AII on Human Platelet Type 12-LO and 15-LO Type II

Treatment with AII for 1 h significantly increased the mRNA expression of human platelet type 12-LO and 15-LO type II in VSMC, which was blocked by both losartan (10^{-5} mol/L) and baicalein (10^{-5} mol/L) (Figs. 5A to 5D). When cultured VSMC were exposed to AII (10^{-7} mol/L) for 10 min, the release of 12-HETE increased by threefold (Fig. 6A) and the release of 15-HETE increased by 50% (Fig. 6B).

Discussion

A member of a family of receptors for ox-LDL, LOX-1 has been initially identified in endothelial cells in which it supports the binding, internalization, and degradation of ox-LDL.\textsuperscript{15,16} The receptor LOX-1 may play a role both in the initiation and the progression of atherosclerosis, as it is expressed both in the initial phases of plaque formation and in established human atheromatous lesions.\textsuperscript{25–27} Activation of LOX-1 leads to the generation of reactive oxygen species, a decrease in the release of NO from endothelial cells, and increased expression of endothelin-1, AT1R, and cell adhesion molecules,\textsuperscript{28} all of which contribute to both hypertension and vascular damage. In endothelial cells, LOX-1 expression is subject to regulation by tumor necrosis factor–$\alpha$ (TNF-$\alpha$), shear stress, lipopolysaccharide (LPS), ox-LDL, and AII.\textsuperscript{29,30–33} Although LOX-1 is apparently expressed in human VSMC also, hypertension and high cholesterol\textsuperscript{34,35} are currently the only recognized endogenous factors that appear to regulate its expression in these cells.

In this study, we provide direct evidence that, in a cell line of VSMC derived from normal human aortic smooth muscle cells, AII upregulates LOX-1 expression in association with increased VSMC degradation of ox-LDL and stimulation of platelet type 12-LO and 15-LO type II expression and activity. That AII in-
12-LO expression in this line of human VSMC is consistent with previous reports. To our knowledge, however, this is the first evidence that 15-LO type II is expressed in human vascular cells and is upregulated by AII. Kim et al identified a leukocyte form of 12-LO in human aortic VSMC and this isoform is known to generate 15- and 12-HETE. In previous studies, we were unable to identify 15-LO mRNA type I expression in human umbilical artery smooth muscle cells. In contrast, we have now used primers designed to identify type II 15-LO and have observed AII-inducible expression of this isoform. Furthermore, 12- and 15-LO mRNA were induced within 1 h of exposure to AII, which resembles the early effect of AII on classical “immediate response genes” such as c-fos, c-myc, and c-jun as well as on recently identified growth-related peptides such as the angiogenic factor CYR61 and the connective tissue growth factor CTGF.

In a previous report, the AT1R antagonist losartan was found to reduce LOX-1 expression in aortic neointima of hypercholesterolemic rabbits, suggesting that AII may be involved in increased LOX-1 expression in proliferating VSMC comprising atheromatous lesions. The observation that the AT1R antagonist losartan blocked AII-induced LOX-1 in our study and reduced LOX-1 expression in modified (neointimal) VSMC of hypercholesterolemic rabbits suggest that AII may be an inducer of LOX-1 expression in VSMC in vivo. Furthermore, taken together with our results, observations that losartan inhibits cellular uptake of ox-LDL by macrophages from hypercholesterolemic patients are consistent with the concept that AII affects ox-LDL trafficking at least in part via modulation of LOX-1 expression in humans in vivo.

The finding that the specific 12-LO inhibitor baicalin blocked AII-induced LOX-1 in our study and reduced LOX-1 expression in modified (neointimal) VSMC of hypercholesterolemic rabbits suggest that AII may be an inducer of LOX-1 expression in VSMC in vivo. Furthermore, taken together with our results, observations that losartan inhibits cellular uptake of ox-LDL by macrophages from hypercholesterolemic patients are consistent with the concept that AII affects ox-LDL trafficking at least in part via modulation of LOX-1 expression in humans in vivo.
was blocked by the LO inhibitor baicalein suggests that these rapidly generated eicosanoid products may have a role in downstream signaling, which eventually leads to increased LOX-1 expression.

In addition to the rapid AII-dependent induction of 12- and 15-HETE generation, longer exposure to AII also increased mRNA expression of human platelet type 12-LO and 15-LO type II 2. Because LO enzymes (particularly 15-LO) comprise a key mechanism by which LDL can be oxidized in vivo, our results are compatible with the function of AII-driven machinery for ox-LDL trafficking and generation in human VSMC. On one hand, AII increases LOX-1 expression, which facilitates the uptake of extracellular ox-LDL into VSMC. Our finding that AII increases cell-dependent ox-LDL degradation supports this notion, as it indicates

that more ox-LDL was taken up by the cells after AII treatment, presumably secondary to enhanced LOX-1 expression. On the other hand, AII induces increases in the activity and expression of human platelet type 12-LO and 15-LO type II 2, thus providing a pathway by which native LDL taken up by VSMC can be oxidized by either juxta- or intracellular means.

The evidence that hypertension, hypercholesterolemia, and AII are each linked to increased LOX-1 expression in VSMC provides an additional pathway by which hypertension and the renin-angiotensin system may contribute to atherogenesis. An AII-dependent concomitant acceleration of ox-LDL uptake and LDL oxidation, as shown in the present report, may accelerate transformation of VSMC into foam cells and thus contribute to plaque instability.43 As hypertension per

---

FIG. 6. Effect of AII treatment on the release of 12- and 15-HETE. The VSMC were treated with AII (10⁻⁷ mol/L) for 10 min. The reaction was stopped with cold ethanol, and 12- and 15-HETE were extracted and measured as described in Methods. Results are expressed as means ± SEM of three separate experiments. A) 12-HETE release; *P < .05. B) 15-HETE release; *P < .05. Abbreviations as in Figs. 1 and 2.
se also increases LOX-1 expression but not LO enzymes, the relative contribution of high blood pressure and AII to LOX-1–related alterations in VSMC comprising the arterial wall remains subject to further investigation.

References


