Blockade of Angiotensin II Receptors Reduces the Expression of Receptors for Advanced Glycation End Products in Human Endothelial Cells

Masashi Fujita, Hiroko Okuda, Osamu Tsukamoto, Yoshihiro Asano, Yulin Liao Akio Hirata, Jiyoong Kim, Takeshi Miyatsuka, Tetsuo Minamino, Hitonobu Tomoike, Masafumi Kitakaze

Objectives—Receptors for advanced glycation end products (RAGEs) play crucial roles in atherogenesis. Because tumor necrosis factor α (TNFα) is expressed and upregulates RAGE expression in atherosclerotic lesions, the TNFα-RAGE interaction might be involved in the inflammatory process of atherogenesis. On the other hand, an angiotensin II type-1 receptor blocker (ARB), widely used as an antihypertensive drug, has been reported to have also antiatherosclerotic effects. Thus we investigated whether an ARB exerts antiatherosclerotic effects via inhibiting the TNFα-RAGE interaction.

Methods and Results—Stimulation of human endothelial cells with candesartan as well as olmesartan decreased TNFα-induced RAGE expression in both mRNA and protein levels along with the decrease in the activity of nuclear factor κB and the expression of inflammatory mediators such as vascular cell adhesion molecule (VCAM)-1. Both candesartan and olmesartan inhibited the binding of nuclear factor κB to the RAGE gene promoter. Furthermore, gene silencing of RAGE by RNA interference decreased the expression of TNFα-induced VCAM-1 in both mRNA and protein levels.

Conclusions—RAGE contributes at least partially to the TNFα-induced VCAM-1 expression in both mRNA and protein levels. Blockade of angiotensin II receptors might exert antiatherosclerotic effects via reducing TNFα-RAGE interaction. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: angiotensin II type-1 receptor blocker (ARB)  receptors for advanced glycation end products (RAGEs)  endothelial cell

The cell surface receptor for advanced glycation end products (RAGEs) is a multiligand member of the immunoglobulin superfamily of molecules and has been reported to play crucial roles in atherogenesis. Engagement of RAGEs in endothelial cells leads to the increase in the expression of inflammatory mediators such as monocyte chemoattractant protein (MCP)-1 or vascular cell adhesion molecule (VCAM)-1.2–5 Because tumor necrosis factor (TNF)-α is expressed and upregulates RAGE expression in atherosclerotic lesions,6,7 the TNFα-RAGE interaction might be involved in the mechanisms of the inflammatory process of atherogenesis.

On the other hand, an angiotensin II type-1 receptor blocker (ARB), widely used as an antihypertensive drug, has been reported to have also antiatherosclerotic such as attenuating neointimal formation, decreasing vascular smooth muscle cell (VSMC) proliferation and diminishing vascular inflammation.8 Considering these results, we hypothesized that an ARB has antiatherosclerotic effects via inhibiting TNFα-RAGE interaction, thus leading to the decrease in the inflammatory process of atherosclerosis. We tested this hypothesis using human endothelial cells.

Materials and Methods

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were purchased from Biochrom and cultured at 37°C, under a humidified atmosphere containing 5% CO₂ in EBM-2 medium (Clonetics) supplemented with EGM-2 Single Quotes containing 10% FBS, human fibroblast growth factor-1, vascular endothelial growth factor, ascorbic acid, heparin, and human epidermal growth factor and GA-1000. These cells were used at passages 2 to 10 for all experiments.

Western Blot Analysis
Standard Western blot analysis on total cell lysates was performed using mouse anti-RAGE, VCAM-1, IκBα antibodies (Santa Cruz).
Candesartan and olmesartan were gifts from Takeda Pharm Co Ltd (Osaka, Japan) and Sankyo Co Ltd (Tokyo, Japan), respectively. To ensure equal loading of intact protein, membranes were stripped and retranslated with antibodies against actin. Densitometric analysis was performed using Scanning Imager (Molecular Dynamics).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from HUVECs was extracted using RNA-Bee-RNA Isolation Reagent (Tel-Test). Then 1000 ng of total RNA was reverse transcribed and amplified using an Omniscript RT Kit (Qiagen) according to the protocol of the manufacturer. Oligonucleotide primers and TaqMan probes for human RAGE (assay no. Hs00153957_m1), VCAM-1 (Hs00174239_m1), and GAPDH were designed and purchased from Applied Biosystems. Quantitative real-time PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) by the relative standard curve method.

Chromatin Immunoprecipitation Assay

Either TNFα (10 ng/mL) or vehicle was exposed to HUVECs with and without either candesartan (1 nmol/L) or olmesartan (10 nmol/L) for 2 hours. ChIP assays were performed with a chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology) according to the protocol of the manufacturer with some modifications. The primers for the nuclear factor (NF)-κB–binding site in the RAGE gene promoter were: forward primer, 5'-GGAGGGGTTGCAAAAAGCAGT-3'; reverse primer, 5'-CATCACCCTCTCAACCTCC-3'.

NF-κB p65 Transcription Factor Assay

The activation of NF-κB binding to the nucleus of HUVECs treated with and without TNFα (10 ng/mL) in the absence or presence of either candesartan (1 nmol/L) or olmesartan (10 nmol/L) was determined using the nonradioactive NF-κB p65 transcription factor assay kit (Chemicon International Inc) according to the instructions provided. Nuclear protein extracts were also prepared according to the protocol of the manufacturer.

Gene Silencing via RNA Interference

HUVECs were seeded into P6 dishes coated with human fibronectin (Biocoat, BD-Falcon) and grown until ~70% to 80% confluence, followed by transfection with 30 pmol of the negative control sequence or RAGE-specific small interfering RNA (siRNA) duplex using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. The following siRNA oligonucleotides for this study were purchased from Dharmacon: human RAGE [1] siRNA (sense, 5'-GCC AGA AGG UGG ACG A GU A-3'; antisense, 5'-UAC UGC UCC ACC UUC UGG C-3'); siRNA human RAGE [2] (sense, 5'-CCU CAA AUC CAC UGG AUG A-3'; antisense, 5'-UCA UCC AGU GGA UUU GAG G-3'). As a negative control, cells were transfected with siControl Non-Targeting siRNA No. 1 (Dharmacon). At 24 hours after transfection quantitative real-time polymerase chain reaction (PCR), 48 hours after transfection Western blot analysis were performed.

Statistical Analysis

Results of the experimental studies are reported as the means±SE. Differences were analyzed by ANOVA followed by the appropriate post hoc test. A probability value of <0.05 was regarded as significant.

Results

ARB Reduces the Expression of RAGE Protein and mRNA in Human Endothelial Cells

Because TNFα is known to increase endothelial RAGE protein expression, we first examined the effect of candesartan on TNFα-induced RAGE protein expression by Western blot analysis. Stimulation of HUVECs with TNFα (25 ng/mL) for 24 hours led to a 2.9±0.5-fold increase in cell surface RAGE protein expression compared with control (P<0.05, n=4), and, furthermore, concomitant treatment with candesartan significantly reduced the expression of TNFα-induced RAGE protein in a concentration-dependent manner with a maximal reduction at 1 nmol/L candesartan (Figure 1A). To confirm that this effect is not specific for candesartan alone, we checked the effects of other ARB, olmesartan, on the expression of TNFα-induced RAGE protein. Treatment with olmesartan also reduced the expression of TNFα-induced RAGE protein with a maximal reduction at 10 nmol/L olmesartan (Figure 1B). Furthermore, to examine the decrease in endothelial RAGE protein expression by candesartan resulting from reduced mRNA expression, we investigated the RAGE mRNA levels by the quantitative real-time
PCR. Treatment with candesartan significantly reduced the TNF\(\alpha\)-induced RAGE mRNA expression (Figure 1C).

**ARBS Inhibit the Binding of NF-\(\kappa\)B to the RAGE Promoter**

TNF\(\alpha\)-induced endothelial RAGE expression is regulated by activation of the NF-\(\kappa\)B-binding site (nucleotide number −686 to −678) in the RAGE promoter. To examine whether ARBs may interact with this pathway, we performed ChIP assays to detect the binding of NF-\(\kappa\)B to the RAGE gene promoter. As shown in Figure 1D, both candesartan (1 nmol/L) and olmesartan (10 nmol/L) inhibited the TNF\(\alpha\)-induced binding of NF-\(\kappa\)B to the RAGE promoter.

**ARBS Reduced the Activation of NF-\(\kappa\)B in Human Endothelial Cells**

Either candesartan (1 nmol/L) or olmesartan (10 nmol/L) reduced the TNF\(\alpha\)-induced activation of NF-\(\kappa\)B detected by not only the degradation of I\(\kappa\)B but also the transcriptional factor assay (Figure 2A and 2B).

**ARB-Mediated Reduction of Endothelial RAGE Expression Decreases Proinflammatory Effects in Human Endothelial Cells**

In human endothelial cells, activation of RAGE has previously been shown to upregulate the expression of proinflammatory mediators such as MCP-1 and VCAM-1. To assess the functional relevance of reduced endothelial RAGE expression, human endothelial cells were stimulated with TNF\(\alpha\) (25 ng/mL) in the presence or absence of candesartan (1 nmol/L) or olmesartan (10 nmol/L) for 12 hours before investigating VCAM-1 protein expression and for 4 hours before investigating mRNA expression. Stimulation of endothelial cells with TNF\(\alpha\) (25 ng/mL) increased VCAM-1 protein and mRNA expressions as determined by Western blot analysis and quantitative real-time PCR, respectively. Treatment of cells with either candesartan or olmesartan significantly reduced TNF\(\alpha\)-induced VCAM-1 protein and mRNA expressions (Figure 2C and 2D).

**Gene Silencing of RAGE via RNA Interference**

Adversely, to confirm the involvement of RAGE in the TNF\(\alpha\)-mediated VCAM-1 expression, we performed gene silencing of RAGE via RNA interference. We checked the inhibitory effects of siRNA on the expression of RAGE mRNA levels by quantitative real-time PCR. siRNA against RAGE [1] and [2] (siRAGE [1] and [2]) decreased the expression of RAGE in mRNA levels compared with that of siRNA control (18.8±1.2% and 32.4±2.6%, respectively; Figure 3A). In the transfected cells with siRNA against RAGE using siRAGE [1] and [2], the expression of TNF\(\alpha\)-induced VCAM-1 protein were decreased compared with that...
of the nontransfected cells (Figure 3B). Furthermore, in the transfected human endothelial cells with siRAGE [1] and [2], TNFα-induced VCAM-1 mRNA expressions were also significantly decreased compared with the nontransfected cells (Figure 3C). These data suggest that RAGE is involved in the TNFα-induced VCAM-1 expression in both mRNA and protein levels.

Discussion

We showed here that angiotensin II receptors blockers (ARBs), such as candesartan or olmesartan suppressed, TNFα-induced RAGE protein and mRNA expressions at least partially through the inhibition of the binding of NF-κB to the RAGE gene promoter in human endothelial cells with subsequently reduced the expression of inflammatory mediators such as VCAM-1. Furthermore, we clearly showed that RAGE were involved in the process of TNFα-induced VCAM-1 mRNA and protein expression using RNA interference technique. These results may propose the novel mechanisms that ARBs exert the antiatherosclerotic effects.

RAGE Are Involved in TNFα-Induced Inflammatory Process of Atherogenesis

In the atheromatous plaque, TNFα is released from inflammatory cells.10 TNFα activates NF-κB sites in the RAGE promoter to induce endothelial RAGE expression.6 Stimulation of RAGE activates a key signal transduction and is believed to lead to a vicious circle that enhances atherosclerosis. Interestingly, blockade of RAGE with the soluble extracellular domain of RAGE completely suppressed atherosclerosis in diabetic mice.11 Furthermore, blockade of RAGE in the established atherosclerotic plaques suppressed the further progression of atherosclerotic lesion area.12 Taken together, in this study we focused on the TNFα-RAGE interaction in the vicious circles in the atherosclerosis and we showed here that TNFα increased the RAGE expression in protein and mRNA levels in human endothelial cells, along with the activation of NF-κB and the increase in VCAM-1 protein and mRNA expression. Furthermore, we demonstrated that gene silencing of RAGE via RNA interference decreased the expression of VCAM-1 protein and mRNA induced by TNFα. These results suggested that TNFα-RAGE interaction was involved in the inflammatory process of atherogenesis.

ARBs Reduce the TNFα-Induced RAGE Protein and mRNA Expression

An ARB was reported to reduce RAGE expression in the kidney of diabetic KK/Ta mice.13 However, to our knowledge, this is the first report that ARBs inhibit TNFα-induced RAGE expression in human endothelial cells, supporting that ARBs have antiatherogenic effects. TNFα-induced endothelial RAGE expression is regulated by the activation of the NF-κB site in the RAGE promoter.6 In this study, we showed that both candesartan and olmesartan inhibited the binding of NF-κB to the RAGE gene promoter from ChIP assay. These results suggested that ARBs generally reduced TNFα-induced RAGE protein and mRNA expression via the inhibition of the binding of NF-κB to the RAGE gene promoter. Thus, we propose the novel mechanisms that ARBs have been demonstrated to attenuate the degree of atherosclerosis and suggest that the reduction of RAGE expression by ARBs might represent a novel strategy to limit RAGE-mediated inflammatory processes in the vessel wall.

Clinical Implications

Our findings might have important pathophysiological and clinical implications for the high-risk patients with atherosclerosis, because these patients have enhanced RAGE expression in the vasculature and TNFα-RAGE interaction leads to the expression of proatherogenic mediators, such as MCP-1 or VCAM-1. Although further investigation is needed to clarify the precise mechanisms by which ARB inhibit RAGE expression, we believe that this study will throw light on the treatment of the patients with atherosclerosis.

Acknowledgments

We thank Yoko Nagamachi and Akiko Ogai for technical assistance and Yukari Arino for secretarial work.
Sources of Funding
O.T. and Y.A. are Research Fellows of Japan Society for the Promotion of Science for Young Scientists. This study was supported by a grant from the Japan Cardiovascular Research Foundation.

Disclosure(s)
None.

References
Blockade of Angiotensin II Receptors Reduces the Expression of Receptors for Advanced Glycation End Products in Human Endothelial Cells
Masashi Fujita, Hiroko Okuda, Osamu Tsukamoto, Yoshihiro Asano, Yulin Liao, Akio Hirata, Jiyoong Kim, Takeshi Miyatsuka, Seiji Takashima, Tetsuo Minamino, Hitonobu Tomoike and Masafumi Kitakaze

Arterioscler Thromb Vasc Biol. published online August 3, 2006;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2006/08/03/01.ATV.0000239569.99126.37.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/