Adrenomedullin Reduces VEGF-Induced Endothelial Adhesion Molecules and Adhesiveness Through a Phosphatidylinositol 3'-Kinase Pathway

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Objective—In the initial phase of inflammation, vascular endothelial growth factor (VEGF) can act as a proinflammatory cytokine by inducing adhesion molecules that bind leukocytes to endothelial cells. Adrenomedullin (AM) is known to act as either a proinflammatory or an anti-inflammatory agent. In this study, we examined the effects of AM on adhesion molecule expression and leukocyte adhesiveness in VEGF-stimulated human umbilical vein endothelial cells.

Methods and Results—When stimulated with VEGF, the mRNAs of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin were dose-dependently upregulated. AM inhibited the VEGF-induced protein and mRNA expression of ICAM-1, VCAM-1, and E-selectin. Phosphatidylinositol 3'-kinase inhibitor and a dominant-negative form of Akt significantly inhibited the suppressive effect of AM on VEGF-induced adhesion molecule expression. Thus, AM inhibits VEGF-stimulated ICAM-1 and VCAM-1 expression through a phosphatidylinositol 3'-kinase/Akt pathway. AM reduced VEGF-induced endothelial adhesiveness for leukocytes.

Conclusions—These results suggest that AM might have an anti-inflammatory role in controlling VEGF-induced adhesion molecule gene expression and adhesiveness toward leukocytes in endothelial cells. (Arterioscler Thromb Vasc Biol. 2003;23:1377-1383.)

Key Words: adrenomedullin • vascular endothelial growth factor • adhesion molecules • endothelial cells • inflammation

An important feature of the inflammatory process is the localization of leukocytes. During the initial phase of inflammation, endothelial cells abundantly express adhesion molecules that regulate leukocyte adhesion and also the adhesion of endothelial cells to the extracellular matrix.1,2 These adhesion molecules have a role first in the recruitment and then in the consequent adhesion and transmigration of leukocytes. Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectins are important adhesion molecule families that play a role in leukocyte-endothelial adhesion.3 Thus, cytokines and growth factors that downregulate the expression of cell adhesion molecules might have an anti-inflammatory effect.

Adrenomedullin (AM) is a pluripotent regulatory peptide initially isolated from a human pheochromocytoma.4 Circulating concentrations of AM increase in a variety of experimental conditions and inflammatory diseases.5–9 During the inflammatory process, most AM is derived from fibroblasts, vascular smooth muscle cells, vascular endothelial cells, monocytes, macrophages, and neutrophils.10–15 Increased AM has multiple effects in inflammatory disease.16 AM can induce ocular inflammation in the rabbit.17 However, AM also has an anti-inflammatory effect in acetic acid–induced peritonitis.18 Thus, the role of AM might change according to the cell type and time after onset of inflammatory challenge. Because the actions or changes of AM during inflammation might play a pivotal role in immune function, it is important to measure site-specific tissue responses to AM in specific inflammatory processes.

The regulation of adhesion molecule expression in endothelial cells is important in the prevention and treatment of the initial phase of inflammation. However, the regulatory role of AM on the endothelial expression of adhesion molecules during inflammation has not been investigated thoroughly. We have previously shown that vascular endothelial growth factor (VEGF) increases ICAM-1, VCAM-1, and E-selectin mRNA and protein expression in endothelial cells through the Flk-1/kinase insert domain containing receptor (KDR) receptor.19,20 This stimulation is mediated through nuclear factor-κB activation and is suppressed by phosphatidylinositol (PI) 3'-kinase.19 Recently, we also reported that AM activates the PI 3'-kinase/Akt pathway in human umbilical vein endothelial cells (HUVECs).21 In the current study, we examined whether AM reduces VEGF-induced expres-
sion of adhesion molecules in HUVECs. Interestingly, we found that AM suppressed VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin. Furthermore, AM reduced VEGF-induced endothelial adhesiveness for leukocytes. Therefore, AM could have potential as an agent to treat or prevent endothelial cell injury before or during inflammation.

Materials and Methods

Materials

Fetal bovine serum and Hanks’ balanced salt solution were purchased from Life Technologies, Inc. Collagenase type II and elastase were purchased from Worthington Biomedical. Recombinant human VEGF165 was obtained from R&D Systems. Antibody for phospho-Akt (Ser473) was purchased from Cell Signaling Technology Inc. Collagenase type II and elastase were purchased from Sigma Chemical Co.

Endothelial Cell Culture

HUVECs were isolated by methods previously described. The isolated HUVECs were incubated and maintained in medium containing 20% fetal bovine serum at 37°C in 5% CO₂. The primary cultured cells used in this study were between passages 3 and 5.

Application of VEGF and AM

HUVECs were plated onto gelatinized Petri dishes in medium containing 20% serum and then switched to a medium containing 1% serum with various concentrations of VEGF. We previously reported that VEGF maximally induced ICAM-1, VCAM-1, and E-selectin mRNA expression at 4 hours, so this treatment time was used for all experiments. In experiments involving AM, various concentrations of AM (10⁻¹² to 10⁻⁴ mol/L) or control buffer were added to the cell medium with VEGF (20 ng/mL) treatment. Control samples received buffer in place of AM and/or VEGF.

PI 3'-Kinase Assay

HUVECs were incubated in medium containing 1% serum with various concentrations of AM (10⁻¹², 10⁻¹⁰, and 10⁻⁸ mol/L) for various times (0, 1, 2, 3, and 5 minutes), with or without wortmannin (30 nmol/L), and then immediately lysed in a lysis buffer. Insoluble materials were removed by centrifugation. PI 3'-kinase activity was assayed in the immunoprecipitates as described previously.

Application of PI 3'-Kinase Inhibitor and Transfection

HUVECs were first treated for 30 minutes with wortmannin (30 nmol/L), a potent PI 3'-kinase inhibitor. After incubation, the cells were washed and given fresh medium with AM and VEGF. Control cultures received equivalent amounts of buffer. Mammalian expression vector containing the cDNA of the Lys197Met dominant-negative mutant of Akt1 (Upstate Biotechnology) or its control empty vector (Upstate Biotechnology) was transfected into HUVECs with the use of a commercially available system (LipofectAMINE Plus, Gibco-BRL) and incubated for 24 hours in medium 199 with 10% fetal bovine serum. After incubation, the cells were washed and given fresh medium with VEGF and AM. Cells were assayed as described in subsequent paragraphs.

RNase Protection Assay (RPA) for Expression Analysis of ICAM-1, VCAM-1, and E-Selectin mRNA Transcripts

Measurements of mRNA levels of ICAM-1, VCAM-1, and E-selectin were performed as previously described.

Immunoblot Analysis and Radioimmunoassay

The amounts of ICAM-1, VCAM-1, and E-selectin proteins were measured as described by Kim et al. To quantify the amount of AM protein secreted by HUVECs, cells were incubated in a previously described method. Concentrated eluates were assayed with a specific and sensitive human AM 1-52 radioimmunoassay kit (Phoenix Pharmaceuticals).

Adhesion Assay

Leukocyte-endothelial adhesion was measured by fluorescent labeling of leukocytes by methods previously described. The percentage of leukocytes adhering to HUVECs was calculated by the formula: % adherence=(adherent signal/total signal)×100.

Statistics

All signals were visualized and analyzed by densitometric scanning (LAS-1000, Fuji Film). Data are expressed as mean±SD. Statistical significance between 2 groups was calculated with the unpaired Student’s t test. Statistical significance was tested by 1-way ANOVA, followed by the Student-Newman-Keuls test. Statistical significance was set at P<0.05. An expanded Methods section can be found in an online data supplement (see http://www.ahajournals.org).

Results

AM Decreased VEGF-Induced ICAM-1, VCAM-1, and E-Selectin mRNA Expression

We previously reported that VEGF produced a maximal effect on expression of ICAM-1, VCAM-1, and E-selectin mRNA in HUVECs at 4 hours. In this experiment, we examined the effect of various concentrations of VEGF at this time point. VEGF increased ICAM-1, VCAM-1, and E-selectin mRNA expression in a dose-dependent manner (Figure 1A). VEGF at 1 ng/mL significantly increased ICAM-1, VCAM-1, and E-selectin mRNA levels. VEGF at 20 ng/mL further increased these mRNAs.

AM (10⁻⁸ mol/L) significantly inhibited VEGF-induced ICAM-1, VCAM-1, and E-selectin mRNA expression (Figure 1B). AM (10⁻¹² to 10⁻¹⁰ mol/L) significantly decreased VEGF (20 ng/mL)-induced mRNA expression of these 3 adhesion molecules in a dose-dependent manner (Figure 1B). However, AM (10⁻⁸ mol/L) alone did not have any significant effect on the mRNA levels of these adhesion molecules. These results suggested that AM inhibited the VEGF-induced mRNA of adhesion molecules in HUVECs.

AM Activated PI 3'-Kinase in a Dose- and Time-Dependent Manner, and HUVECs Produced AM Protein

To evaluate whether the PI 3'-kinase pathway was involved, we performed a PI 3'-kinase assay in HUVECs. AM (10⁻⁸ mol/L) stimulated PI 3'-kinase activity in a time-dependent manner, and the PI 3'-kinase inhibitor wortmannin (30 nmol/L) inhibited the AM-stimulated PI 3'-kinase activity (Figure 2A). AM-stimulated PI 3'-kinase activity peaked at 3 minutes. AM also increased PI 3'-kinase activity in a dose-dependent manner (Figure 2B).

A functional AM receptor consists of 2 proteins, the calcitonin receptor–like receptor (CRLR) and the receptor activity–modifying proteins (RAMPs) that couple the receptor to the cellular signal transduction pathway. Because CRLR is a G protein–coupled receptor, we would like to
AM Decreased VEGF-Induced Adhesion Molecule mRNAs Through a PI 3'-Kinase/Akt Pathway

The specific PI 3'-kinase inhibitor wortmannin (30 nmol/L) increased expression of ICAM-1, VCAM-1, and E-selectin mRNAs (Figure 3A). Moreover, pretreatment with wortmannin (30 nmol/L) enhanced VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin mRNAs (Figure 3A). These results suggested that endogenous PI 3'-kinase activity acts as a negative regulator for the expression of ICAM-1, VCAM-1, and E-selectin mRNAs. Because AM is a positive stimulator of PI 3'-kinase activity, we examined the effect of AM with and without the PI 3'-kinase inhibitor. Wortmannin (30 nmol/L) significantly decreased the suppressive effect of AM in VEGF-induced mRNA expression of the 3 adhesion molecules (Figure 3A). These results indicate that AM decreases VEGF-induced ICAM-1, VCAM-1, and E-selectin mRNA expression through the PI 3'-kinase pathway.

Figure 1. AM suppresses VEGF-induced mRNA levels of ICAM-1, VCAM-1, and E-selectin in HUVECs. A, HUVECs were incubated for 4 hours with the indicated dose of VEGF. To clarify the identity of the bands, ICAM-1 (I), VCAM-1 (V), and E-selectin (E) probes were applied individually to the total RNA from HUVECs to reveal protected bands of 367, 279, and 187 bp, respectively (upper panel). Densitometric analyses are presented as the relative ratio of ICAM-1, VCAM-1, or E-selectin mRNA to cyclinphilin mRNA (lower panel). The relative ratio measured in the control buffer (CB) sample is arbitrarily presented as 1. Bars represent the mean±SD from 4 experiments. *P<0.05 vs CB; +P<0.05 vs VEGF (20 ng/mL) only.

We examined Akt phosphorylation in transfected endothelial cells. Compared with control vector, HUVECs transfected with the Lys179Met Akt mutant showed decreased Akt phosphorylation in HUVECs. Akt acts downstream of the PI 3'-kinase pathway.24 We also examined whether the Akt pathway was involved in the suppressive effect of AM in VEGF-induced adhesion molecule expression. Because the expression of the Lys179Met Akt mutant causes a loss of Akt kinase activity (Figure 3B), we used a mammalian expression vector containing the Lys179Met dominant-negative mutant of Akt cDNA or its control empty vector. We were able to achieve a transfection efficiency of approximately 30%, as assessed by immunofluorescent detection of the tagged protein of Lys179Met Akt mutant, c-Myc, with an anti-c-Myc antibody (Invitrogen; data not shown). After incubation with expression vectors, HUVECs were treated with VEGF and AM as described earlier.

We examined Akt phosphorylation in transfected endothelial cells. Compared with control vector, HUVECs transfected with the Lys179Met Akt mutant showed decreased Akt-Ser473 phosphorylation in response to VEGF (Figure 3B). Gene transfer with the Lys179Met Akt mutant also increased the protein level of Akt above control levels. The blots were reprobed with an anti-actin antibody to verify equivalent protein loading. These results showed that expression of a dominant-negative Akt mutant significantly decreased Akt phosphorylation in HUVECs. Compared with control vector, the Lys179Met Akt mutant significantly decreased the suppressive effect of AM on VEGF-induced mRNA expression of ICAM-1, VCAM-1, and E-selectin (Figure 3B, lower panel). Taken together, these results suggest that the Akt pathway participates in the suppressive effect of AM in HUVECs.

AM Decreased VEGF-Induced ICAM-1 and VCAM-1 Protein Expression

We further examined the VEGF-induced protein levels of ICAM-1 and VCAM-1 in HUVECs after treatment with AM.
AM Suppresses VEGF-Induced Leukocyte Adhesiveness to HUVECs

Because the induction of adhesion molecules in endothelial cells induces leukocyte adhesion, we examined whether AM decreases VEGF-stimulated leukocyte adhesion to HUVECs. Cells treated with VEGF bound more leukocytes, and AM decreased this effect dramatically (Figure 5A). For quantification, the number of leukocytes that adhered to quiescent HUVECs was taken to be 100% adherence (Figure 5B). Stimulation of HUVECs with VEGF (20 ng/mL) for 8 hours induced a significant (≈4-fold) increase in the adhesion of leukocytes compared with treatment by control buffer. However, treatment of VEGF-stimulated cells with AM led to a 40% decrease in leukocyte adhesion. AM (10⁻⁸ mol/L) by itself did not significantly increase endothelial adhesiveness for leukocytes. These findings suggest that AM decreases VEGF-stimulated leukocyte adhesion to HUVECs.

Discussion

AM gene expression and level in the systemic circulation or localized tissue increase in inflammatory states. However, there are conflicting data on the action of AM on inflammation in vivo. Although AM might induce an inflammatory reaction in some cases, AM also decreases inflammation in other cases. Thus, AM can play a role in differential regulation among tissues during inflammation because of its capacity to bind to multiple classes of receptors and elicit different responses at specific tissue sites. In this experiment, we investigated the effect of AM on VEGF-induced inflammatory responses in endothelial cells.

The expression of endothelial adhesion molecules is important in leukocyte recruitment and migration in inflammatory disease. However, the regulatory role of AM in the endothelial expression of adhesion molecules has not been investigated so far. Previously, we reported that VEGF increased ICAM-1, VCAM-1, and E-selectin protein and mRNA levels and increased leukocyte adhesiveness to HUVECs. In this study, we used AM (10⁻⁷ mol/L) with VEGF treatment to evaluate whether AM decreases VEGF-induced ICAM-1, VCAM-1, and E-selectin expression in HUVECs.

In preliminary studies, we found that a maximal dose of AM (10⁻⁷ mol/L) produced a further but slight decrease in VEGF-induced ICAM-1, VCAM-1, and E-selectin expression. Densitometric analyses yielded the following data, presented as the relative ratio of ICAM-1, VCAM-1, and E-selectin to actin: VEGF, 5.2 ± 1.2; 11.2 ± 1.7; 7.5 ± 1.9; VEGF plus AM (10⁻⁸ mol/L), 1.5 ± 0.7; 1.9 ± 0.5; 1.7 ± 0.4; and VEGF plus AM (10⁻⁷ mol/L), 1.3 ± 0.5; 1.8 ± 0.6; 1.7 ± 0.5. Thus, use of the submaximal concentration (10⁻⁸ mol/L) of AM is considered to be rational in this experiment. AM significantly decreased VEGF-induced mRNA expres-
sion of ICAM-1, VCAM-1, and E-selectin (Figure 1). These data suggest that treatment with AM could decrease VEGF-induced adhesion molecule expression in vascular endothelial cells during inflammation. Because most anti-inflammatory drugs suppress adhesion molecule expression in endothelial cells, AM might have an anti-inflammatory role.

Whereas the mRNA levels of ICAM-1 and VCAM-1 underwent dynamic changes, their protein levels measured by immunoblot analyses showed less change. It is well known that gene expression is regulated at multiple levels. Although the expression of most genes is regulated primarily at the transcriptional level, gene expression can also be controlled at the level of translation. In addition, the level of protein within cells can be controlled by different rates of protein degradation. Thus, ICAM-1 and VCAM-1 might have relatively stable protein levels because of controls at the level of translation or degradation.

Figure 3. AM suppresses VEGF-induced mRNA levels of ICAM-1, VCAM-1, and E-selectin through the PI 3'-kinase/Akt pathway. A, HUVECs were incubated for 4 hours with control buffer (CB), wortmannin (WT, 30 nmol/L), VEGF165 (VE 20 ng/mL), wortmannin plus VEGF165 (WV), AM plus VEGF165 (AV), or AM plus VEGF165 plus wortmannin (AVW) (upper panel). Densitometric analyses are presented as the relative ratio of each mRNA to cyclophilin mRNA (lower panel). The relative ratio measured in the CB sample is arbitrarily presented as 1. B, HUVECs were transfected with the Lys179Met dominant-negative mutant of Akt cDNA (DNAkt) or control vector (CV). Then HUVECs were incubated with control buffer (CB), VEGF165 (VE 20 ng/mL), or AM plus VEGF165 (AV) for 15 minutes. Cell lysates (20 µg) were immunoblotted with phospho-Akt antibody and reprobed with Akt (upper panels). The blot was reprobed with actin antibody to demonstrate equal loading of protein. ICAM-1, VCAM-1, and E-selectin mRNAs were also assessed by RPA, as shown. Densitometric analyses are presented as described in A. Bars represent the mean±SD from 3 experiments. *P<0.05 vs CB; **P<0.05 vs VE; ***P<0.05 vs CB or CV or DNAkt; #P<0.05 vs VE or CV.

Figure 4. AM suppresses VEGF-induced protein levels of ICAM-1 and VCAM-1 in HUVECs. A, HUVECs were incubated for 6 hours with control buffer (CB), AM (10^-8 mol/L), VEGF165 (VE 20 ng/mL), or AM plus VE (AV) (upper panels). Densitometric analyses are presented as the relative ratio of each protein to actin (lower panel). The relative ratio in CB is arbitrarily presented as 1. B, HUVECs were incubated for 6 hours with CB, wortmannin (WT, 30 nmol/L), VE (20 ng/mL), WT plus VE (WV), AM plus VE (AV), or AM plus VE plus WT (AVW). Total protein (50 µg) from the cells was blotted and probed with the indicated antibodies (upper panels). The blots were reprobed with an anti-actin antibody. Densitometric analyses are presented as described in A. Bars represent mean±SD from 3 experiments. *P<0.05 vs CB; **P<0.05 vs VE.
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there are few reports about the signal-through a G protein–receptor binding. Furthermore, we showed that AM acts partially exert its effect on endothelial cells through AM (data not shown). These findings suggested that AM might either a calcitonin gene-related peptide receptor or an AM expression of endothelial adhesion molecules.

McLatchie et al reported that mRNAs for CRLR and RAMP2 were expressed in HUVECs. Thus, the CRLR-RAMP2 complex is a functional AM receptor in HUVECs. In this study, we tested a putative AM receptor blocker, AM22-52. AM22-52 (10−8 mol/L) partially blocked the suppressive effect of AM in HUVECs (data not shown). These findings suggested that AM might partially exert its effect on endothelial cells through AM receptor binding. Furthermore, we showed that AM acts through a G protein–coupled receptor in HUVECs (Figure 2C).

The intracellular signaling mechanism of AM appears to be cAMP, Ca2+ mobilization, inositol-1,4,5-triphosphate, protein kinase A, and mitogen-activated protein kinase pathways. However, there are few reports about the signaling pathways of the suppressive effect of AM on the expression of endothelial adhesion molecules.

The PI 3′-kinase inhibitor wortmannin significantly increased mRNA expression of ICAM-1, VCAM-1, and E-selectin in the absence of VEGF or AM treatment (Figure 3A). These results suggest that expression of these adhesion molecules is negatively regulated by a PI 3′-kinase pathway. Pretreatment with wortmannin significantly decreased the suppressive effect of AM on expression of the 3 adhesion molecule mRNAs (Figure 3A). These data indicate that AM exerts its suppressive effect in VEGF-induced adhesion molecule expression in HUVECs through the PI 3′-kinase pathway. Other studies have suggested that a PI 3′-kinase–dependent pathway is associated with stimulated neutrophil adhesion to endothelial cells. Our findings are in agreement with those studies.

Akt acts downstream of the PI 3′-kinase pathway. We also examined whether the Akt pathway was involved in the suppressive effect of AM in VEGF-induced ICAM-1, VCAM-1, and E-selectin expression. We previously reported that AM increased Akt phosphorylation in a dose- and time-dependent manner. In this experiment, we found that a dominant-negative form of Akt significantly reversed the suppressive effect of AM on VEGF-induced adhesion molecule expression (Figure 3B). Therefore, all of these results suggest that the PI 3′-kinase/Akt pathway could be an essential step in the anti-inflammatory effect of AM. Because the PI 3′-kinase/Akt signal pathway can stimulate cells to survive and grow, AM can act as a survival and growth factor in endothelial cells through this pathway. Furthermore, because endothelial cell apoptosis contributes to the pathogenesis of atherosclerosis and PI 3′-kinase/Akt is an antiapoptotic signal pathway, AM might play a role in the regulation of atherosclerosis in vascular lesions. In this study, we provide the first demonstration that an AM-induced anti-inflammatory effect depends on a G protein–coupled receptor and the PI 3′-kinase/Akt pathway. However, there is a need for further evaluation to identify the molecules downstream of the PI 3′-kinase/Akt pathway.

Because adhesion molecule expression on leukocytes and endothelial cells is closely associated with leukocyte adhesiveness, we further evaluated the effect of AM on VEGF-stimulated endothelial adhesiveness for leukocytes. In this study, our data indicated that AM suppressed VEGF-stimulated endothelial adhesiveness. These data are in complete agreement with the suppressive effect of AM on VEGF-induced adhesion molecule expression.

All of our data suggest that AM might have an anti-inflammatory effect, especially in VEGF-induced adhesion molecule expression and leukocyte adhesiveness. In addition, the effects of AM are mediated through the AM receptor and the PI 3′-kinase/Akt signal transduction pathway. In the future, it might be possible to exploit the anti-inflammatory effects of AM to control inflammation caused by certain diseases or treatments. However, further in vivo studies will be required before clinical application can be tested.

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