Adrenomedullin Inhibits Angiotensin II–Induced Expression of Tissue Factor and Plasminogen Activator Inhibitor-1 in Cultured Rat Aortic Endothelial Cells


Abstract—Adrenomedullin (AM) is a potent vasodilating peptide having a variety of pharmacological properties mainly in respect to vascular pathophysiology. We have previously demonstrated that angiotensin II (Ang II) or natriuretic peptides have influence on the expression of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) in vascular endothelial cells. The aim of this study was to elucidate the effects of AM on TF and PAI-1 mRNA and protein expression in endothelial cells. As a result, AM inhibited Ang II–induced TF and PAI-1 mRNA expression in a dose- and time-dependent manner. Because the expression of TF and PAI-1 mRNA induced by Ang II was attenuated by the increase of intracellular concentrations of cAMP by forskolin and 8-bromo-cAMP and because AM increased the intracellular level of cAMP in rat aortic endothelial cells, it was indicated that the inhibitory effect of AM on the expressions of TF and PAI-1 was mainly mediated by the cAMP-dependent signal transduction. Furthermore, the inhibitory effect of AM on TF and PAI-1 expression was partly attenuated by an NO synthase inhibitor, N\textsuperscript{G}-nitro-L-arginine methyl ester. In conclusion, AM is shown to contribute to the regulation of blood coagulation and fibrinolysis by vascular endothelial cells mainly via the cAMP pathway. (Arterioscler Thromb Vasc Biol. 2001;21:1078-1083.)

Key Words: adrenomedullin ■ angiotensin II ■ plasminogen activator inhibitor-1 ■ tissue factor ■ vascular endothelial cells

Adrenomedullin (AM), a potent 52-amino acid vasodilating peptide, was originally isolated from human pheochromocytoma cells as a substance that increases platelet cAMP concentration.\(^1,2\) In addition to its vasodilatory activity, AM has various biological activities, including diuresis,\(^3\) inhibition of aldosterone secretion,\(^4\) and inhibition of the proliferation and migration of vascular smooth muscle cells (VSMCs).\(^5\) The plasma AM concentration in patients with hypertension or congestive heart failure is significantly higher than that in healthy individuals.\(^6-8\) This elevated AM concentration is believed to attenuate the effects of activation of the renin-angiotensin system in these diseases. We have previously demonstrated that angiotensin II (Ang II) induces the expression of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1)\(^9\) in vascular endothelial cells. Enhanced expressions of TF and PAI-1 may promote thrombus formation and the development of ischemia, respectively, by initiating extrinsic blood coagulation and by inhibiting fibrinolytic activation.\(^10-14\) We also reported that the enhanced expression of TF and PAI-1 by Ang II is inhibited by natriuretic peptides (NPs),\(^15\) which are important endogenous vasodilatory substances. In the present study, we determined the pathophysiological significance of AM, another counter-regulator of Ang II, on the coagulation and fibrinolytic systems regulated by vascular endothelial cells.

Methods

Reagents

Endothelial cell growth supplement was obtained from Becton Dickinson Labware. DMEM and heat-inactivated FBS were from GIBCO-BRL Life Technologies Inc. Rat AM, Ang II, pro-AM NH\textsubscript{2}-terminal 20 peptide (PAMP), and AM-[22-52] were purchased from Peptide Institute, Inc. BSA, forskolin, 8-bromo-cAMP, \(N\textsuperscript{G}\)-nitro-L-arginine methyl ester (L-NAME), and antibiotics were purchased from Sigma Chemical Co.

Cell Cultures

Rat aortic endothelial cells (RAECs) were isolated from male Sprague-Dawley rats (200 to 250 g) by using a primary explant technique previously described.\(^16-17\) RAECs were harvested 4 to 8 passages after primary culture. After reaching confluence, RAECs were cultured in a conditioned medium (DMEM containing 0.1% BSA and antibiotics) for 48 hours before the experiments. Vascular endothelial cells were identified by their cobblestone appearance and confirmed by the uptake of fluorescein isothiocyanate–labeled acetyl-LDL as previously described.\(^18\)

Measurements of TF and PAI-1 mRNA Expression

After RAECs were stimulated with conditioned medium containing varying concentrations of Ang II and AM, total RNA was prepared
from RAECs by use of RNeasy kits (Qiagen GmbH) in accordance with the manufacturer’s instructions. Total RNA samples were fractionated on 1% agarose/formaldehyde gels and capillary-transferred to nitrocellulose membranes (Hybond-N’, Amersham International plc). After the membranes were baked, they were prehybridized and hybridized overnight with specific cDNA probes and then washed with 2× SSC/0.1% SDS at room temperature, 1× SSC/0.1% SDS at 65°C, and then 0.1× SSC/0.1% SDS at 65°C. The membranes were air-dried and exposed to X-Omat AR film (Eastman Kodak Co) at −80°C without intensifying screens.

Preparation of cDNA Probes
TF, PAI-1, and GAPDH cDNA probes were generated by a reverse transcription–polymerase chain reaction with the use of synthesized oligonucleotide primers as previously described.\(^{16-21}\) \(\beta\)-labeled cDNA probes were generated by a random primer method with \(\alpha\)-\(\beta\)-dNTP (111 mBq/mmols, NEN Research Products) and a Random Primer DNA Labeling Kit (Takara Biomedicals). The labeled probes were denatured in boiling water and chilled on ice just before hybridization.

Measurement of TF and PAI-1 Proteins
TF activity on the RAEC surface was measured by using a chromogenic assay with S-2765 (Chromogenix AB) as described previously.\(^9\) The conditioned media were collected immediately, and the active PAI-1 level was measured by an ELISA kit (RPAIKT, Molecular Innovations Inc) according to the manufacturer’s instructions.

Measurements of Intracellular Concentrations of cAMP and cGMP
After RAECs were incubated with AM and Ang II for the indicated times and concentrations, 0.1 mol/L HCl was added to the dishes for 10 minutes to lyse the adhering cells. The cell lysates were centrifuged for 10 minutes, and the supernatants were assayed in enzyme immunoassay kits (cAMP, cGMP Elia Kit, Biomol Research Laboratories) according to the manufacturer’s instructions.

Data Analysis
The intensity of detected bands from Northern blotting analysis was quantified by densitometry. In the present study, standardized TF and PAI-1 mRNA expression refers to the quotient of respective intensities divided by that of internal standard GAPDH mRNA. Relative TF and PAI-1 mRNA expression levels are presented as the fold increase over corrected PAI-1 and TF mRNA expression compared with levels in nontreated or quiescent RAECs. All values are expressed as mean±SEM of 3 independent experiments. Statistical analysis was performed by ANOVA and Fisher protected least significant differences. Values of \(P<0.05\) were considered statistically significant.

Results
Effects of AM on TF mRNA Expression and Activity Induced by Ang II
When RAECs were incubated for 1 hour with a vehicle or various concentrations of AM (0 to 1000 nmol/L), it was shown that AM solely does not affect the expression of TF or PAI-1 mRNA (data not shown).

We have previously reported that the expressions of TF and PAI-1 mRNA increase significantly in RAECs with Ang II (0.1 nmol/L). In the present study, the mRNA expression of TF increased to 2.86±0.04 after incubation with Ang II (1 nmol/L) for 3 hours compared with control. Pretreatment of the RAECs for 1 hour with AM significantly inhibited this induction in a dose-dependent manner (Figure 1a). The cell-surface TF activity on RAECs significantly increased with the stimulation of Ang II (100 nmol/L) at 8 hours. Pretreatment of RAECs with AM for 1 hour significantly inhibited Ang II–induced TF activity in a dose-dependent manner (Figure 1b).

Effects of AM on PAI-1 mRNA and Protein Expression Induced by Ang II
AM also had an inhibitory effect on Ang II–induced PAI-1 mRNA expression. Pretreatment of the RAECs for 1 hour with AM significantly inhibited the PAI-1 mRNA expression induced by Ang II (1 nmol/L, 3 hours) in a dose-dependent fashion (Figure 2a). Active PAI-1 levels in the supernatants significantly increased to 18.43±2.24 with the stimulation of Ang II (100 nmol/L) at 8 hours. Pretreatment of RAECs with AM for 1 hour dose-dependently inhibited the Ang II–induced PAI-1 level (Figure 2b).

Effect of Antagonist of AM
To elucidate whether these effects of AM are mediated through its specific receptor, AM-[22-52] (a selective AM receptor antagonist)\(^23\) was added to the culture medium 30 minutes before the AM treatment. The inhibitory effects of AM on PAI-1 mRNA expression were partly cancelled by the pretreatment with AM-[22-52] (Figure 3). Thus, the effects of AM on RAECs are suspected to be related to its specific cellular receptor.

Signal Transduction With AM in RAECs
It has been reported that many of the AM-related cellular responses are mediated through the cAMP-dependent path-
way in vascular endothelial cells. Therefore, first, we investigated whether elevation of cAMP in RAECs would mimic the effect of AM. Forskolin (an activator of adenylate cyclase) and 8-bromo-cAMP (a cAMP analogue) were respectively added to RAECs for 30 minutes before the stimulation with Ang II (1 nmol/L) for 3 hours. Forskolin and 8-bromo-cAMP significantly inhibited Ang II–induced PAI-1 mRNA expression in a dose-dependent manner (Figure 4a and 4b, respectively). Second, the intracellular concentrations of cAMP in RAECs were measured with respect to the stimulation with/without AM and Ang II. The level of cAMP was significantly increased nearly 3-fold compared with the control level after incubation with AM; however, Ang II had no influence on the concentration of cAMP (Figure 5a). From
these results, it was indicated that the inhibitory effect of AM on the expression of PAI-1 is mainly mediated through a cAMP-dependent mechanism.

Recently, reports have indicated that the vasodilating effect of AM is also mediated by NO released from vascular endothelial cells. When RAECs were pretreated with the NO synthase inhibitor L-NAME for 36 hours before the addition of AM, the inhibitory effect of AM on PAI-1 mRNA expression was partly but significantly attenuated (Figure 6). Therefore, it was speculated that NO might contribute in some way to the inhibitory effect of AM on PAI-1 expression, but it was not possible to demonstrate a significant increase of intracellular cGMP concentration in RAECs with AM (Figure 5b); hence, a mechanism independent of cGMP is speculated.

Discussion

In the present study, sole incubation with AM at a concentration of 1 nmol/L did not alter the basal expressions of TF or PAI-1 mRNA, but it significantly inhibited those inductions by Ang II in RAECs. This concentration of AM was 1000 times greater than the reported physiological plasma concentration in rats (3.6 ± 0.6 pmol/L). However, local levels of AM in coronary vascular tissue may be much greater than plasma concentrations of AM, because it has recently been shown that a considerable amount of AM is synthesized in and secreted from vascular endothelial cells. In addition, plasma concentrations of AM are found to be high in hypertensive individuals and in individuals with severe congestive heart failure. With this matter taken into account, our results suggest that AM may counteract the effect of Ang II, in respect not only to the regulation of vascular tone but also to the regulation of hemostatic responses in certain pathophysiological conditions. Nonetheless, the present experiment was performed on cultured endothelial cells; therefore, extrapolation to in vivo conditions should be made with caution.

PAMP is the 20–amino terminal portion of pro-AM, which is found not only in the adrenal medulla, heart, lungs, and kidneys but also in vascular wall cells. Because PAMP receptors are reported to be present on vascular endothelial cells and VSMCs and because PAMP exerts a hypotensive effect similar to that of AM, we speculated that PAMP might also exhibit an effect similar to that of AM in respect to the hemostatic regulation. However, it did not show any inhibitory effect on the expression of TF or PAI-1 induced by Ang II in the present study (data not shown). The reason for their difference is unclear, but the cultured cells used in the present experiments might be lacking the receptors for PAMP.

Recently, a family of receptor activity–modifying proteins (RAMPs 1 to 3) has been identified in humans. Associated with the calcitonin receptor–like receptor, RAMP2 or RAMP3 is considered to be the specific receptor for AM. Although which type of receptor(s) is engaged in the present study is not clear, the inhibitory effect of AM on PAI-1 expression is considered to be receptor-mediated, inasmuch as it was canceled by the pretreatment with AM-[22-52], which is known as a selective AM receptor antagonist.

When AM binds to its specific receptor, it is reported to evoke a rise in the levels of cAMP within mammalian endothelial cells, VSMCs, and mesangial cells. Various physiological actions of AM, such as the suppression of VSMC proliferation, migration, and construction and the inhibition of endothelin expression in vascular wall cells, are considered to be mediated by the cAMP-dependent mechanism. In consideration of the fact that the expression of PAI-1 mRNA induced by Ang II was attenuated by the increase of intracellular concentrations of cAMP by forskolin or 8-bromo-cAMP and that the intracellular concentration of cAMP in RAECs was indeed increased by AM, it was indicated that the inhibitory effect of AM on the expression of PAI-1 was mainly mediated by the cAMP-dependent signal transduction.

Recently, AM has been reported to enhance NO production through the upregulation of NO synthase in VSMCs and cardiomyocytes and has also been reported to promote cGMP production in bovine aorta–derived cultured endothelial cells. These reports suggest that NO may transmit the biological effects of AM. In the former study that we performed with NPs and NO donors, enhanced production of NO was proven to suppress the expressions of TF and PAI-1 induced by Ang II in RAECs. It has also been indicated that the activation of protein kinase G and the consequent elevation of cGMP are involved in its mechanism, inasmuch as the pretreatment of RAECs with Rp-8-bromo-cGMP significantly attenuated the inhibitory effects of NPs against Ang II–induced TF and PAI-1 expressions. Taking these facts into account, we speculated that besides the cAMP-dependent pathway, NO might also contribute to the suppression of TF and PAI-1 by AM in the present study. As a result, the inhibitory effect of AM on PAI-1 mRNA expression was partly attenuated by the pretreatment with L-NAME; hence, a contribution of NO was indicated. It is well known that cGMP mediates many of the biological functions of NO. However, intracellular cGMP in our model was not increased by AM; therefore, a mechanism that is independent of cGMP is speculated. A precise mechanism by which NO mediates the function of AM needs to be further elucidated.

TF is a cell membrane–associated protein that is not expressed in the intact endothelium. TF plays an important role in physiological hemostasis by initiating extrinsic blood coagulation. PAI-1 is a serine protease inhibitor that regulates fibrinolysis by inhibiting activation by the tissue plas-
minogen activator and, consequently, suppressing the conversion of plasminogen to plasmin.\(^\text{43,45}\) We have previously demonstrated that Ang II induces expressions of TF and PAI-1,\(^\text{1}\) whereas pretreatment with NPs attenuates those inductions.\(^\text{15,42}\) In the present study, AM had inhibitory induction of plasminogen to plasmin.\(^\text{44,45}\) We have previously shown that AM inhibits tissue factor pathway inhibitor 1 induced by angiotensin II in cultured rat aortic endothelial cells. \textit{Thromb Haemost}. 1998;79:631–634.


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