Original Article

Adrenomedullin Inhibits Angiotensin II-Induced Oxidative Stress and Gene Expression in Rat Endothelial Cells

Takanobu YOSHIMOTO, Naoki GOCHOU, Nozomi FUKAI,
Toru SUGIYAMA, Masayoshi SHICHIRI, and Yukio HIRATA

Adrenomedullin (AM), a potent vasodilator peptide, has recently been suggested to function as an endogenous antioxidant. However, its potential site of action at the cellular level has not been clarified. The present study was undertaken to investigate whether AM directly inhibits intracellular reactive oxygen species (ROS) generation and redox-sensitive gene expression stimulated by angiotensin (Ang) II in rat aortic endothelial cells (ECs). Ang II (10^{-7} mol/l) significantly increased intracellular ROS levels in ECs as measured by dichlorofluorescein (DCF) fluorescence. AM inhibited Ang II-stimulated ROS generation in a dose-dependent manner and this effect was abolished by a superoxide radical scavenger, NAD(P)H oxidase inhibitor, and a protein kinase A (PKA) inhibitor, and mimicked by a cell-permeable cAMP analog. A real-time reverse transcription–polymerase chain reaction (RT-PCR) study showed that Ang II significantly upregulated a set of redox-sensitive genes (ICAM-1, VCAM-1, PAI-1, tissue factor, MCP-1, osteopontin), and these effects were blocked by an antioxidant, *N*-acetyl cysteine (NAC). AM similarly and dose-dependently inhibited the Ang II-induced upregulation of the entire set of these genes *via* a receptor-mediated and PKA-dependent pathway, and the degrees of inhibition were similar to those by NAC. In conclusion, the present study demonstrated that AM potently blocked the Ang II-stimulated intracellular ROS generation from NAD(P)H oxidase and the subsequent redox-sensitive gene expression *via* a cAMP-dependent mechanism in ECs, suggesting that AM has vasculoprotective effects against pro-oxidant stimuli. (*Hypertens Res* 2005; 28: 165–172)

***Key Words*: adrenomedullin, angiotensin II, reactive oxygen species, redox**

Introduction

Accumulating lines of evidence suggest that reactive oxygen species (ROS) formation and the subsequent inflammatory processes induced by humoral factors and mechanical forces play an integral role in the pathogenesis of vascular diseases, such as atherosclerosis and hypertension (*1*, *2*). Among such humoral factors, angiotensin (Ang) II is the most extensively studied and characterized (*1*, *3*, *4*). Ang II, *via* its type 1 (AT1) receptor, activates β-nicotinamide adenine dinucleotide (phosphate)-reduced (NAD(P)H) oxidase to increase intracellular ROS production in vascular cells, which in turn contributes to vascular inflammation (*5*) by upregulating an entire set of redox-sensitive genes, including adhesion molecules (intercellular adhesion molecule-1: ICAM-1; vascular adhesion molecule-1: VCAM-1), pro-inflammatory molecules (monocyte chemoattractant protein-1: MCP-1; osteopontin) and coagulation/antifibrinolytic factors (plasminogen activator-1: PAI-1; tissue factor) (*6*, *7*). These events lead to mono-

From the Department of Clinical and Molecular Endocrinology, Tokyo Medical and Dental University Graduate School, Tokyo, Japan.
This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.
Address for Reprints: Takanobu Yoshimoto, M.D., Ph.D., Department of Clinical and Molecular Endocrinology, Tokyo Medical and Dental University Graduate School, 1−5−45 Yushima, Bunkyo-ku, Tokyo 113−8513, Japan. E-mail: tyoshimoto.cme@tmd.ac.jp
Received May 31, 2004; Accepted in revised form November 2, 2004.

Table 1. Sequences of the PCR Primers for Real-Time RT-PCR

PCR product	Primer sequence	PCR product size
ICAM-1	F: 5′-agctcttcaagctgagcgacat-3′	118
	R: 5′-actcgctctgggaacgaataca-3′	
VCAM-1	F: 5′-gcgaaggaaactggagaagaca-3′	128
	R: 5′-acacattagggaccgtgcagtt-3′	
PAI-1	F: 5′-tggagaggcacaccaaaggtat-3′	126
	R: 5′-cctctaagaaggggtcttcca-3′	
Tissue factor	F: 5′-ttgtgggagcagtggtgttc-3′	138
	R: 5′-gcgtcagcctcctcgtctat-3′	
MCP-1	F: 5′-tctcttcctccaccactatgca-3′	91
	T: 5′-tcacgcttctgggcctgttgttca-3′	
	R: 5′-ggctgagacagcacgtggat-3′	
Osteopontin	F: 5′-agtggtttgcctttgcctgtt-3′	122
	R: 5′-tcagccaagtggctacagcat-3′	
ARPP P0	F: 5′-tagagggtgtccgcaatgtg-3′	107
	T: 5′-acccgactgttgcctcagtgcctcactcca-3′	
	R: 5′-gacaaagccaggacccttttgt-3′	

PCR, polymerase chain reaction; RT-PCR, reverse tanscription–PCR; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular adhesion molecule-1; PAI-1, plasminogen activator-1; MCP-1, monocyte chemoattractant protein-1; ARPP P0, acid ribosmal phosphoprotein P0. F: forward primer; R: reverse primer; T: TaqMan probe.

cyte adhesion, activation, and thrombosis, thereby resulting in endothelial dysfunction and progression of atherosclerosis (*6*–*8*).

Adrenomedullin (AM), a potent vasodilator peptide originally isolated from human pheochromocytoma (*9*), has recently been shown to be abundantly expressed by and secreted from vascular endothelial cells (ECs) in an autocrine/paracrine fashion (*10*). AM has pleiotropic effects, including cell growth, migration, apoptosis, inflammation, angiogenesis, and hormone secretion (*11*, *12*). AM-deficient mice loaded with Ang II and high-salts have recently been shown to exhibit perivascular inflammation in the coronary arteries with increased oxidative stress, which effects are reversed by AM supplementation (*13*), suggesting that AM may protect against oxidative stress *in vivo*. However, the underlying cellular mechanism of the antioxidant effects of AM remains to be clarified.

These observations led us to examine whether AM directly affects the intracellular ROS generation stimulated by Ang II in rat aortic ECs *in vitro*, and whether AM has any antioxidant effect on Ang II-induced redox-sensitive gene expression.

Methods

Materials

Ang II, rat AM and calcitonin gene related peptide (CGRP) 8-37 were purchased from the Peptide Institute (Osaka, Japan). Medium199 (M199) was from Invitrogen (Carlsbad, USA), and fetal bovine serum (FBS) and calf serum (CS) were purchased from Cell Culture Laboratories (Cleveland, USA). *N*-(2-[*p*-Bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89), diphenyleneiodonium chloride (DPI), tiron, *N*-acetyl-L-cysteine (NAC), *N*,*N*′-dimethyl-9,9′-biacridiniumdinitrate (lucigenin), and 8-bromoadenosine-3′,5′-cyclic monophophate (8-bromo-cAMP) were from Sigma (St. Louis, USA). Polymerase chain reaction (PCR) primers were synthesized by JbioS Inc. (Saitama, Japan).

Cell Culture

Rat aortic ECs were prepared from the thoracic aorta of 6-week-old male Sprague-Dawley (SD) rats using the explant method and cultured as previously described (*14*). Confluent cells from passages 5 to 7 were subjected to serum starvation (M199 with 0.5% CS) for 24 h and then used for experiments.

Measurement of Intracellular ROS Levels

Intracellular ROS generation was measured in live rat aortic ECs by the method of Ushio-Fukai *et al*. (*4*) with some modifications. Briefly, starved cells grown on 35 mm glass-bottom dishes (Matsunami, Tokyo, Japan) were pretreated with test compounds or left untreated for 1 h, and then cultured in the presence or absence of 10^{-7} mol/l Ang II. In some experiments, H-89 had been applied to the medium 15 min prior to the AM addition. After 2 h incubation with or without Ang II, cells were incubated with 8×10^{-6} mol/l hydrogen peroxide (H_2O_2)-sensitive fluorophore 5 (and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes Inc., Eugene, USA) for 30 min at 37°C and imaged by inverted fluorescence microscopy (IX71; Olympus, Tokyo, Japan). The relative fluorescent intensities were recorded and analyzed by a charge-coupled device (CCD) Camera (CoolSNAP HQ; Nippon Roper, Chiba, Japan) with an image analysis system (MetaMorph; Nippon Roper).

NAD(P)H Oxidase Activity

NAD(P)H oxidase activity in rat ECs was analyzed by the methods described by Xie *et al*. (*15*) with some modifications. Briefly, starved cells grown on 10 cm dishes were pretreated with test compounds or left untreated for 1 h, and then cultured in the presence or absence of 10^{-7} mol/l Ang II for 2 h. Then cells were harvested, resuspended in lysis buffer composed of 5×10^{-2} mol/l phosphate buffer (pH 7.0), 10^{-3} mol/l ethyleneglycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 μg/ml aprotinin, and 10^{-3} mol/l phenylmeththyl sulfonylfluoride (PMSF), and dounced 100 times on ice. The lysates were centrifuged at $16{,}000\times g$ for 20 min. The membrane fractions (pellet) were resuspended in lysis

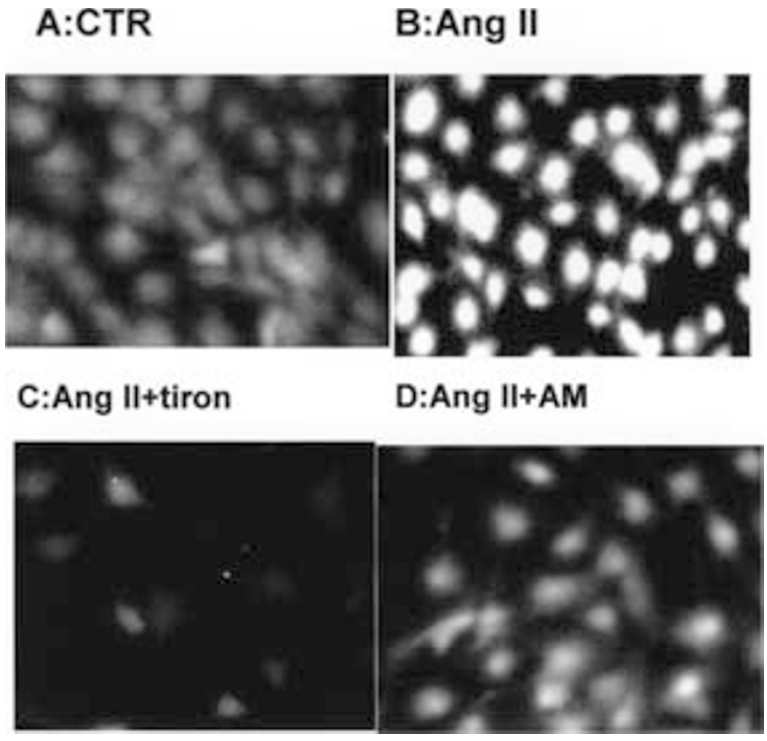

Fig. 1. *AM inhibits Ang II-stimulated ROS generation in rat ECs. Representative microscopic images of DCF fluorescence (magnification: ×400) in non-treated cells (control: CTR) (A), Ang II-stimulated cells (B), and Ang II-stimulated cells pretreated with tiron (10^{-2} mol/l) (C) or AM (10^{-7}mol/l) (D) are shown.*

buffer and stored on ice until use. An aliquot (50 μg) of the protein was added to an assay buffer composed of 5×10^{-2} mol/l phosphate buffer (pH 7.0), 10^{-3} mol/l EGTA, 1.5×10^{-1} mol/l sucrose, 2×10^{-5} mol/l dark-adapted lucigenin with or without 1×10^{-4} mol/l DPI in a 96-well white assay plate (Corning, Rochester, USA). After preincubation at 37°C for 5 min, NADH (10^{-4} mol/l) was added and luminescence was measured with a microplate luminometer (LB96V; Berthold Japan, Tokyo) over 1-min intervals for a total of 15 min. The NAD(P)H oxidase-dependent superoxide anion (O_2^-) productions with and without DPI were measured over a period of 15 min by lucigenin chemiluminescence, and NAD(P)H oxidase activity was taken as the difference between the production with DPI and that without DPI.

Quantification of mRNA

Rat ICAM-1, VCAM-1, PAI-1, tissue factor, and osteopontin mRNA levels were quantified with a real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) using fluorescent SYBR green technology (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) as described previously (*13*). Rat MCP-1 and acid ribosomal phosphoprotein P0 (ARPP P0) mRNA levels were quantified by TaqMan fluorescence methods as described previously (*16*), except using a QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany) and LightCycler. Total RNA was extracted, first-strand cDNA synthesized, and the amplification reaction performed as described previously (*16*). PCR primers, TaqMan probes and the size of each PCR product are listed in Table 1.

The mRNA levels of the target sequence were normalized by those of ARPP P0 used as an endogenous internal control; the relative levels of each mRNA to that of ARPP P0 were calculated as shown in each figure.

Statistical Analysis

Data were expressed as the mean±SEM. Differences between groups were examined for statistical significance using either unpaired *t*-test or one-way analysis of variance (ANOVA) with Dunn's post hoc test as appropriate. *p* values less than 0.05 were considered statistically significant.

Results

AM Inhibits Ang II-Stimulated ROS Generation

We first examined the effect of AM on ROS generation stimulated by Ang II in rat aortic ECs by measurement of dichlorofluorescein (DCF) fluorescence using microfluorometry. Stimulation with Ang II for 2 h caused a greater increase in

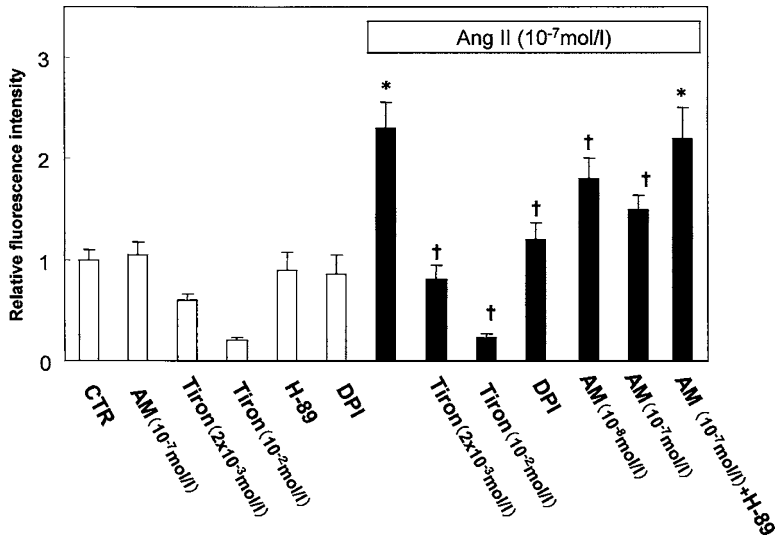

Fig. 2. *AM inhibits Ang II-stimulated ROS generation from NAD(P)H oxidase in rat ECs. ECs were pretreated with AM (10^{-8}–10^{-7} mol/l), tiron (2×10^{-3}–10^{-2} mol/l), H-89 (10^{-5} mol/l), or DPI (10^{-5} mol/l) for 1 h or left untreated, and then stimulated with Ang II for 2 h or left unstimulated. In some experiments, H-89 was applied 30 min before AM pretreatment. The data in the columns are the means±SEM of quantified DCF fluorescence intensity quantified in more than 50 cells from three independent experiments, and are expressed as the fold-increase over the control (CTR).* *p < 0.05 vs. CTR; †p < 0.05 vs. Ang II.*

DCF fluorescence compared to that in untreated cells, and this effect was abolished by pretreatment with tiron (10^{-2} mol/l), a cell-permeable superoxide radical scavenger, and AM (10^{-7} mol/l) (Fig. 1). As summarized in Fig. 2, Ang II-stimulated ROS generation was blocked by pretreatment with DPI (10^{-5} mol/l), a flavin-containing oxidase inhibitor, and tiron in a dose-dependent (2×10^{-3}–10^{-2} mol/l) manner. Pretreatment with AM caused a dose-dependent (10^{-8}–10^{-7} mol/l) inhibition of the Ang II-stimulated ROS generation, and this effect was completely abolished by H-89 (10^{-5} mol/l), a protein kinase A (PKA) inhibitor (Fig. 2). Treatment with either AM (10^{-7} mol/l), DPI (10^{-5} mol/l) or H-89 (10^{-5} mol/l) alone did not show any effect on basal ROS generation, whereas tiron decreased basal ROS generation in a dose-dependent manner. These data suggest that AM exerts its antioxidant effect by inhibiting Ang II-induced intracellular ROS generation *via* a PKA-dependent pathway in rat ECs.

AM Directly Inhibits NAD(P)H Oxidase Activity

In order to confirm that the Ang II-stimulated ROS molecules were derived from NAD(P)H oxidase, we measured NAD(P)H oxidase activity by a lucigenin chemiluminescence assay (Fig. 3). Membrane fractions from rat ECs treated with Ang II showed an approximately 2-fold increase in NAD(P)H oxidase activity compared to those from non-treated cells. Pretreatment with AM induced a dose-dependent (10^{-8}–10^{-7} mol/l) inhibition of Ang II-stimulated NAD(P)H oxidase activity, and this effect was mimicked by 10^{-4} mol/l of 8-bromo-cAMP (Fig. 3), a dose which increased intracellular cAMP to levels comparable to those stimulated by 10^{-7} mol/l of AM (data not shown).

To study whether the AM produced by rat ECs in an autocrine/paracrine fashion affects the Ang II-stimulated NAD(P)H oxidase activity, we also examined the effect of CGRP8-37, an AM/CGRP receptor antagonist, on Ang II-induced NAD(P)H oxidase activity. The pretreatment with CGRP8-37 (3×10^{-6} mol/l) tended to increase Ang II-stimulated NAD(P)H oxidase activity compared to that by Ang II without CGRP8-37 pretreatment, although the difference was not statistically significant (Ang II without CGRP8-37: 1.9±0.17; Ang II with CGRP8-37: 2.3±0.15; data are shown as the fold-increase over controls).

AM Inhibits Ang II-Induced Redox-Sensitive Gene Expression

We next examined whether AM affects Ang II-induced gene expression (ICAM-1, VCAM-1, PAI-1, tissue factor, MCP-1, and osteopontin) in ECs using real time RT-PCR. Time course experiments revealed that the maximum mRNA inductions of all the genes in the set were observed at 3 h after Ang II stimulation (data not shown). AM significantly and dose-dependently (10^{-8}–10^{-7} mol/l) ($p<0.05$) blocked the Ang II-induced gene expressions of ICAM-1, VCAM-1, PAI-1 (Fig. 4), tissue factor, MCP-1, and osteopontin (Fig. 5), and these inhibitory effects were similarly reversed by pretreatment with either H-89 or CGRP8-37. In addition, the inhibitory effects of AM on the Ang II-induced gene expressions were similar to those by NAC (Figs. 4 and 5). It is of note that

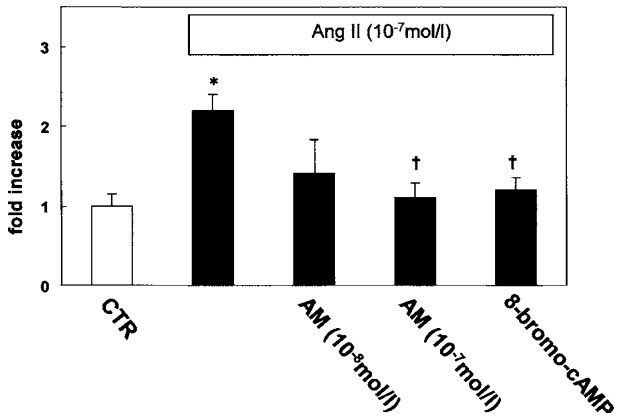

Fig. 3. *AM inhibits Ang II-stimulated NAD(P)H oxidase activity in rat ECs. NAD(P)H oxidase activity was determined by the lucigenin chemiluminescence method as described in Methods. Ang II-stimulated rat ECs were pretreated with AM (10^{-8}, 10^{-7} mol/l) or 8-bromo-cAMP (10^{-4} mol/l) or left untreated. The data in the columns are the means±SEM from five independent experiments, and are expressed as the fold-increase over the control (CTR).* *p < *0.05* vs. *CTR;* †p < *0.05* vs. *Ang II.*

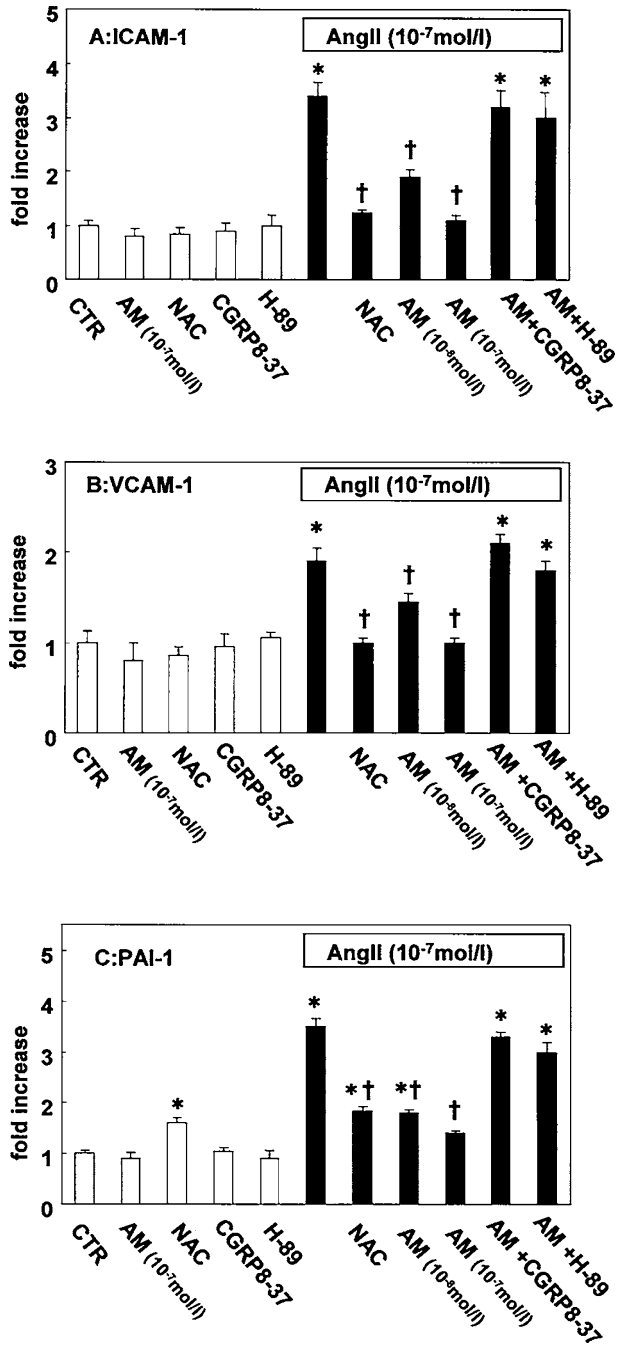

Fig. 4. *AM inhibits Ang II-stimulated ICAM-1, VCAM-1 and PAI-1 gene expressions in rat ECs. ECs pretreated with AM (10^{-8} or 10^{-7} mol/l) or NAC (10^{-2} mol/l) for 1 h or left untreated were stimulated with Ang II (10^{-7} mol/l) for 3 h (open column) or not stimulated (closed column). In some experiments, CGRP8-37 or H-89 was applied 30 min before AM pretreatment. Cells were harvested and mRNA levels were quantified for ICAM-1 (A), VCAM-1 (B), and PAI-1 (C) by real-time RT-PCR as described in Methods. Data in the columns are the means±SEM from five independent experiments, and are shown as the fold-increase over the control (CTR).* *p < *0.05* vs. *the CTR;* †p < *0.05* vs. *Ang II.*

treatment with NAC alone significantly ($p<0.05$) increased the steady-state mRNA levels of PAI-1 (Fig. 4) and osteopontin (Fig. 5), but not those of other genes, while combined treatment with AM and NAC inhibited the Ang II-induced expressions of the full set of redox-sensitive genes to levels comparable to those by NAC alone (Figs. 4 and 5). Treatment with AM alone, however, did not affect the steady-state mRNA levels of any of these genes. These results suggest that AM inhibits the Ang II-induced upregulation of the full set of redox-sensitive genes in the same manner as NAC.

Discussion

Ang II stimulates NAD(P)H oxidase to generate O_2^-, which is promptly converted by superoxide dismutase (SOD) to a more stable molecule, H_2O_2. H_2O_2 is a major determinant of the intracellular redox-state; it functions as a signaling molecule and is subsequently scavenged by catalase or glutathione peroxidase (*6*, *17*). The present study showed that tiron potently inhibited both basal and Ang II-stimulated ROS generation and DPI inhibited Ang II-stimulated ROS generation, suggesting that intracellular H_2O_2 molecules detected by DCF fluorescence are largely derived from O_2^-, and Ang II-stimulated ROS molecules are largely derived from NAD(P)H oxidase.

The present findings demonstrated for the first time that AM inhibits NAD(P)H oxidase-derived O_2^- generation stimulated by Ang II in rat ECs. These results are consistent with a recent study showing that AM gene transfer inhibits NAD(P)H oxidase activity and decreases superoxide genera-

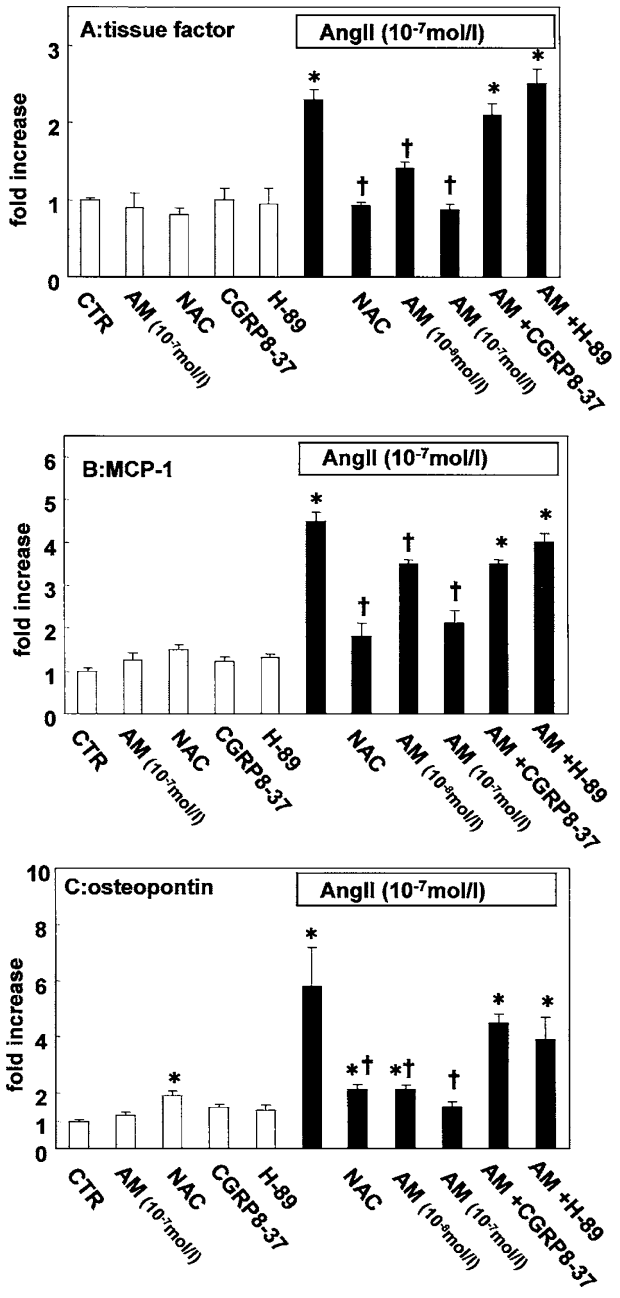

Fig. 5. *AM inhibits Ang II-stimulated tissue factor, MCP-1, and osteopontin gene expressions in rat ECs. ECs pretreated with AM (10^{-8} or 10^{-7} mol/l) or NAC (10^{-2} mol/l) for 1 h or left untreated were stimulated with Ang II (10^{-7} mol/l) for 3 h (open column) or not stimulated (closed column), and mRNA levels were quantified for tissue factor (A), MCP-1 (B), and osteopontin (C) by real-time RT-PCR. The data were calculated and plotted as described in Fig. 3.* *$p < 0.05$ vs. *the control (CTR);* †$p < 0.05$ vs. *Ang II.*

tion in rat myocardial tissues after ischemia and reperfusion injury (*18*). Taken together, these results suggest that the O_2^- generated from NAD(P)H oxidase is one of the major molecules targeted by AM in exerting its cardiovascular protective effects.

It has been suggested that the nitric oxide/cyclicGMP pathway is involved in the cardioprotective mechanism by AM (*18*). In contrast, our present study showed that a PKA inhibitor (H-89) completely abolished the AM-induced inhibition of Ang II-stimulated intracellular ROS generation and a cell-permeable cAMP analog (8-bromo-cAMP) mimicked the AM-mediated inhibition of NAD(P)H oxidase. Thus our results strongly suggest that the antioxidant action of AM is mediated *via* a cAMP/PKA-dependent pathway in rat ECs.

It is now well established that intracellular ROS plays a pivotal role in the regulation of gene expression under different redox states within cells (*6*, *7*). Furthermore, it has been shown that the gene expression of many pro-inflammatory mediators is regulated in a redox-sensitive manner (*1*, *6*, *7*). In this study, we clearly demonstrated that AM inhibits Ang II-induced expression of a complete set of redox-sensitive genes, as does a potent antioxidant, NAC. Furthermore, AM suppressed the Ang II-induced expression of all genes in the set *via* a receptor-mediated and PKA-dependent pathway, because an AM/CGRP receptor antagonist and a PKA inhibitor reversed the inhibitory effect by AM on the Ang II-induced redox-sensitive genes. It has recently been shown that perivascular inflammation in response to Ang II and salt-loading is more markedly induced in AM-deficient mice than in wild-type mice (*13*, *19*). Conversely, it has been shown that oxidative stress increases AM gene expression in vascular smooth muscle cells, suggesting that AM may function as a counter-regulator to oxidative stress (*20*). In accordance with the present results, we have very recently reported that AM inhibits Ang II-induced oxidative stress, redox-sensitive c-Jun N-terminal kinase (JNK) activation, and gene expression in vascular smooth muscle cells *via* a cAMP/PKA-dependent pathway (*21*). Taken together, these results strongly suggest that AM, through its antioxidant properties, exerts a vasculoprotective effect against Ang II-induced vascular injury, possibly by blocking upregulation of most, and possibly all, pro-inflammatory mediators in vascular cells.

In the present study, pretreatment with CGRP8-37 tended to increase Ang II-stimulated NAD(P)H oxidase activity, though not to a statistically significant degree, indicating that steady state AM production from rat ECs under the present cell culture conditions is not sufficient to counteract exogenous Ang II-stimulated NAD(P)H oxidase activation. Nevertheless, AM production is known to be enhanced under inflammation, oxidant stress, and atherogenic stimuli (*10*, *20*), and thus it is possible to speculate that locally increased AM may play a protective role in the cardiovascular injury caused by oxidant stress. However, further studies will be needed to examine this possibility.

In the present study, although both NAC and AM showed almost similar inhibitory effects on Ang II-induced PAI-1 and osteopontin gene expression, NAC by itself somehow induced both gene expressions. It has been shown that certain

antioxidants, such as pyrrolidinedithiocarbamate (PDTC) and NAC, paradoxically enhance the activity of some redox-sensitive transcriptional factors, such as AP-1, which is generally activated under various pro-oxidant conditions (*22*, *23*). Thus, the inhibitory mechanisms responsible for PAI-1 and osteopontin gene expression by AM and NAC may differ from those of other genes.

It remains unknown whether the inhibitory effect of AM on Ang II-stimulated redox-sensitive gene expression is related to the cell growth and apoptosis of ECs. We have previously reported that AM had an anti-apoptotic effect on rat ECs, although it did not show any effect on DNA synthesis, cell proliferation, or mitogen-activated protein kinase (MAPK) activation in rat ECs (*24*). There have been contradictory reports regarding the effect of AM on endothelial cell growth. Two studies have reported that AM promotes endothelial cell growth (*25*, *26*), and another has reported that it inhibits such growth (*27*). The effect of AM on cellular proliferation varies among different cell types and species, and even within individual cells depending on the cell cycle status (*12*). A diversity of signaling pathways by AM in a variety of cells, including cAMP/PKA, MAPK, phosphatidyl inositol-3 kinase, and nitric oxide/cyclicGMP, may also contribute to the different cellular responses.

In conclusion, the present study revealed for the first time that AM directly inhibits Ang II-stimulated NAD(P)H oxidase-derived O_2^- generation and subsequent redox-sensitive gene expression *via* a receptor-mediated and cAMP/PKA-dependent mechanism in rat ECs. Thus, our present *in vitro* data support the contention that AM with its antioxidant actions plays a vasculoprotective role in Ang II-induced vascular injury *in vivo* (*13*, *18*). However, the pathophysiological significance of AM as an endogenous antioxidant remains to be determined in oxidative stress-related cardiovascular diseases.

References

1. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000; **86**: 494–501.
2. Alexander RW: Theodore Cooper Memorial Lecture. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension* 1995; **25**: 155–161.
3. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994; **74**: 1141–1148.
4. Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK: p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *J Biol Chem* 1996; **271**: 23317–23321.
5. Dohi Y, Ohashi M, Sugiyama M, Takase H, Sato K, Ueda R: Candesartan reduces oxidative stress and inflammation in patients with essential hypertension. *Hypertens Res* 2003; **26**: 691–697.
6. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M: Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* 2000; **20**: 2175–2183.
7. Kunsch C, Medford RM: Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 1999; **85**: 753–766.
8. Tomiyama H, Kushiro T, Okazaki R, Yoshida H, Doba N, Yamashina A: Influences of increased oxidative stress on endothelial function, platelets function, and fibrinolysis in hypertension associated with glucose intolerance. *Hypertens Res* 2003; **26**: 295–300.
9. Kitamura K, Kangawa K, Kawamoto M, *et al*: Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993; **192**: 553–560.
10. Minamino N, Kikumoto K, Isumi Y: Regulation of adrenomedullin expression and release. *Microsc Res Tech* 2002; **57**: 28–39.
11. Minamino N, Kangawa K, Matsuo H: Adrenomedullin: a new peptidergic regulator of the vascular function. *Clin Hemorheol Microcirc* 2000; **23**: 95–102.
12. Shichiri M, Hirata Y: Regulation of cell growth and apoptosis by adrenomedullin. *Hypertens Res* 2003; **26**: S9–S14.
13. Shimosawa T, Shibagaki Y, Ishibashi K, *et al*: Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. *Circulation* 2002; **105**: 106–111.
14. McGuire PG, Orkin RW: Isolation of rat aortic endothelial cells by primary explant techniques and their phenotypic modulation by defined substrata. *Lab Invest* 1987; **57**: 94–105.
15. Xie Z, Pimental DR, Lohan S, *et al*: Regulation of angiotensin II-stimulated osteopontin expression in cardiac microvascular endothelial cells: role of p42/44 mitogen-activated protein kinase and reactive oxygen species. *J Cell Physiol* 2001; **188**: 132–138.
16. Shichiri M, Hirata Y: Antiangiogenesis signals by endostatin. *FASEB J* 2001; **15**: 1044–1053.
17. Fridovich I: Superoxide anion radical ($O_2^{\cdot-}$), superoxide dismutases, and related matters. *J Biol Chem* 1997; **272**: 18515–18517.
18. Kato K, Yin H, Agata J, Yoshida H, Chao L, Chao J: Adrenomedullin gene delivery attenuates myocardial infarction and apoptosis after ischemia and reperfusion. *Am J Physiol* 2003; **285**: H1506–H1514.
19. Shimosawa T, Matsui H, Xing G, Itakura K, Ando K, Fujita T: Organ-protective effects of adrenomedullin. *Hypertens Res* 2003; **26** (Suppl): S109–S112.
20. Ando K, Ito Y, Kumada M, Fujita T: Oxidative stress increases adrenomedullin mRNA levels in cultured rat vascular smooth muscle cells. *Hypertens Res* 1998; **21**: 187–191.
21. Yoshimoto T, Fukai N, Sato R, *et al*: Antioxidant effect of adrenomedullin on angiotensin II-induced reactive oxygen species generation in vascular smooth muscle cells. *Endocrinology* 2004; **145**: 3331–3337.
22. Meyer M, Schreck R, Baeuerle PA: H_2O_2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1

in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J* 1993; **12**: 2005–2015.
23. Gomez del Arco P, Martinez-Martinez S, Calvo V, Armesilla AL, Redondo JM: Antioxidants and AP-1 activation: a brief overview. *Immunobiology* 1997; **198**: 273–278.
24. Kato H, Shichiri M, Marumo F, Hirata Y: Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology* 1997; **138**: 2615–2620.
25. Miyashita K, Itoh H, Sawada N, *et al*: Adrenomedullin promotes proliferation and migration of cultured endothelial cells. *Hypertens Res* 2003; **26** (Suppl): S93–S98.
26. Nishimatsu H, Hirata Y, Shindo T, *et al*: Endothelial responses of the aorta from adrenomedullin transgenic mice and knockout mice. *Hypertens Res* 2003; **26** (Suppl): S79–S84.
27. Michibata H, Mukoyama M, Tanaka I, *et al*: Autocrine/paracrine role of adrenomedullin in cultured endothelial and mesangial cells. *Kidney Int* 1998; **53**: 979–985.