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PRODUCTION AND SECRETION OF ADRENOMEDULLIN FROM VASCULAR SMOOTH MUSCLE CELLS: AUGMENTED PRODUCTION BY TUMOR NECROSIS FACTOR-α

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SUMMARY: In this study, we demonstrate production and secretion of adrenomedullin (AM) from cultured vascular smooth muscle cells (VSMCs). In addition to endothelial cells (ECs), we found immunoreactive (ir-) AM in culture media of rat and bovine VSMCs in the survey for AM-producing cells. Although the secretion level of ir-AM was at most 1/6 that of rat ECs, all the examined VSMCs were shown to produce AM. By gel filtration, reverse phase high-performance liquid chromatography, and biological characterization, ir-AM in the culture medium was identified to be rat AM of 50 residues. By RNA blot analysis, a positive band of AM mRNA was detected in cultured VSMC at an intensity 3-4 fold higher than that in adrenal gland. Gene expression and production of AM were markedly augmented by tumor necrosis factor-α. Based on these data as well as the presence of AM specific receptors on VSMCs, AM secreted from VSMC is deduced to function as an autocrine or paracrine regulator in vascular cell communication.

Vascular smooth muscle cells (VSMCs), which comprise the bulk of arteries and veins, have been recoginzed as structural and contractile components of the blood vessel, in contrast with endothelial cells (ECs) which have recently been shown to serve as the crucial blood-tissue interface (1,2). VSMCs are known to have a variety of receptors for vasoactive substances and cytokines, but they have long been thought to lack the ability to produce these molecules. However, recent studies have demonstrated that VSMCs produce cytokines and vasoactive peptides such as interleukin-1, tumor necrosis factor-α (TNF-α) and endothelin-1 along with their receptors, although not as many factors have been identified as are produced by ECs (3-5). These data suggest that VSMCs produce bioactive substances which in turn regulate vascular functions.

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Abbreviations: AM; adrenomedullin; VSMC, vascular smooth muscle cell; EC, endothelial cell; ir, immunoreactive; RIA, radioimmunoassay; TNF-α, tumor necrosis factor-α, CGRP, calcitonin gene-related peptide; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, Sprague-Dawley; cAMP, cyclic AMP.

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Adrenomedullin (AM) is a newly isolated peptide, eliciting potent vasorelaxant activity comparable to that of calcitonin gene-related peptide (CGRP)(6-8). VSMCs have AM specific receptors, which increase cellular level of cyclic AMP (cAMP)(9,10). AM is shown to circulate in the blood, and its plasma levels in patients with hypertension were higher than those of normotensive volunteers (11,12). These data suggest that AM participates in the physiological regulation of blood pressure. During the course of identifying AM-producing cells, we have recently found active production of AM, comparable to that of endothelin-1, in cultured vascular ECs (13). In a further survey for AM-producing cells, we detected a significant concentration of ir-AM in the culture medium of VSMCs. In the present paper, we report production and secretion of AM from rat and bovine VSMCs as well as its augmentation by TNF-α.

MATERIALS AND METHODS

Materials: Rat and human AMs were synthesized by the Peptide Institute (Osaka). Human AM[40-52]-NH_2 and its N-Tyr derivative were synthesized by a peptide synthesizer (Applied Biosystems, 431A). Mouse TNF-α was purchased from Boehringer Mannheim Biochemica.
Cell culture: Rat VSMC-1 and -2 were isolated from Sprague-Dawley (SD) rat thoracic aorta by the explant method and cloned (14), while VSMC-3 and -4 were prepared by the enzyme dispersion method (15). The isolated VSMCs were identified by positive immunostaining with monoclonal anti-α smooth muscle actin antiserum (Clone 1A4, Sigma)(16) and undetectable uptake of fluorescent acetylated low density lipoprotein (17). Wistar rat VSMCs and bovine arterial VSMCs were donated by Dr. T. Iwamoto (National Cardiovascular Center) and Dr. Y. Morishita (Kyowa Hakko Kogyo). All VSMCs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37℃ in a humidified atmosphere containing 5% CO_2. Rat aortic ECs were isolated and maintained as reported (13). All the cells were used at passages 8-18 in the present study.
Preparation of conditioned medium: VSMCs, grown to confluence, were washed twice with DMEM and incubated in DMEM containing 0.01% bovine serum albumin (BSA) for 2 h. The media were then replaced with DMEM containing 0.01% BSA or 0.01% BSA and 0.1-30 ng/ml of TNF-α, and incubated at 37℃ for 1-48 h. Viability of rat VSMCs after 24h incubation was estimated to be more than 97% by trypan blue staining. Culture media (1-5 ml) were acidified with acetic acid (final concentration: 1M), heated at 100℃ for 10 min to inactivate proteases, and lyophilized. The lyophilizates were dissolved in a radioimmunoassay (RIA) buffer and submitted to RIAs for AM and CGRP. For measurements of cellular AM contents, VSMCs were washed twice with phosphate-buffered saline, scraped in 1M acetic acid, and collected. After heat treatment, the cell lysates were sonicated and centrifuged, and the resulting supernatants were condensed with Sep-pak C_{18} cartridges and submitted to RIAs.
RIAs for AM, CGRP and cAMP: Details of the RIA system using antiserum #172CI-7 against human AM[40-52]-NH_2 will be reported in a separate paper. The antiserum recognizes the C-terminal amide structure common to AMs of different species and shows the same affinity to human, rat and bovine AMs (unpublished data). RIA was performed as described for human AM (11). Monoiodinated N-Tyr-AM[40-52]-NH_2, isolated by reverse phase high performance liquid chromatography (HPLC), was used for a tracer. Antiserum against rat CGRP was purchased from Peninsula Lab (USA). Cyclic AMP was measured by RIA as reported (18).
Characterization of ir-AM in culture medium of rat VSMCs: Culture medium (120 ml) of rat VSMCs was acidified with acetic acid (final concentration: 1M), and boiled for 10 min. The peptide fraction of the medium was condensed with Sep-pak C_{18} ENV cartridge (Waters) as reported previously (13), and was subjected to gel filtration on a Sephadex G-50 column (fine, 1.5 x 100 cm). Peak fraction of ir-AM was separated by reverse phase HPLC on a μ-Bondasphere 5μ C_{18} column (300A, 3.9 x 150 mm, Waters) using a linear gradient elution of CH_3CN for 10% to 60% in 0.1% trifluoroacetic acid (TFA) over 60 min. Culture medium (20 ml), stimulated with TNF-α (20 ng/ml), was condensed with Sep-pak C_{18} cartridge, and separated by reverse phase HPLC. Oxidation of methionine residues of the extracts or rat AM was performed with 0.05% H_2O_2 in 1M formic acid for 30 min (19).
RNA blot analysis: Tissues were collected from male SD rats (11 week old) and immediately frozen in liquid nitrogen. Total RNA were extracted by the acid guanidium thiocyanate-phenol-

chloroform method (20) and repeated ethanol precipitation three times. For cultured VSMCs and ECs, 4M guanidium thiocyanate was directly applied to culture dishes, and RNA was extracted by the same method. Poly(A^+)RNA was prepared by using Oligotex dT-30 Super (Daiichi Pure Chemicals). Poly(A^+)RNA (5 μg) or total RNA (25 μg), was denatured with formaldehyde and formamide, and electrophoresed on a 1% agarose gel containing formaldehyde. RNA was then transferred to Zeta probe membrane (Bio-Rad) and fixed with ultraviolet irradiation. Hybridization and washing of the membrane were carried out as reported (13). Eco RI-Bgl I cDNA fragment of rat AM corresponding to nucleotide -153～422 was radiolabeled by the random primed method and used for hybridization (21). For comparison of mRNA contents in each tissue, the membrane used for tissue distribution was re-hybridized to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Eco RI-Bam HI fragment; 492～799)(22). Band intensity was estimated by a radioimage analyzer (Fuji, BAS 2000).

RESULTS

In our survey for AM-producing cells, we have found active production of AM in cultured vascular ECs, as reported previously (13). Compared to ECs, we have detected a much lower level of AM production in cultured rat VSMCs. The extremely high level of AM production in ECs led to this detailed examination of production of AM by cultured VSMCs.

We first measured production of AM in several cultured rat VSMCs and bovine VSMC. As shown in Fig. 1, all of the 6 examined VSMCs were shown to secrete ir-AM into the culture media. Total ir-AM secreted from VSMC-1 and VSMC-2, isolated by the explant method from SD rat aorta, was 9.5 and 1.7 fmol/10^5cells/24 h, respectively. Ir-AM accumulated in the culture media of SD rat VSMC-3, VSMC-4, and Wistar rat VSMC, prepared by enzyme dispersion, were measured to be 8.6, 5.8 and 2.7 fmol/10^5cells/24 h. Bovine VSMC secreted ir-AM at a concentration of 1.1 fmol/10^5 cells/24 h. Under the same conditions, SD rat ECs secreted a much higher level of ir-AM (60.4 fmol/10^5 cells/24 h). VSMC-1 of the highest AM-secretion level was used for the following experiments.

Ir-AM concentration in the culture medium increased linearly up to 12 h. Its secretion rate was almost constant during this period (0.6 fmol/10^5 cells/h). The secretion rate of ir-AM

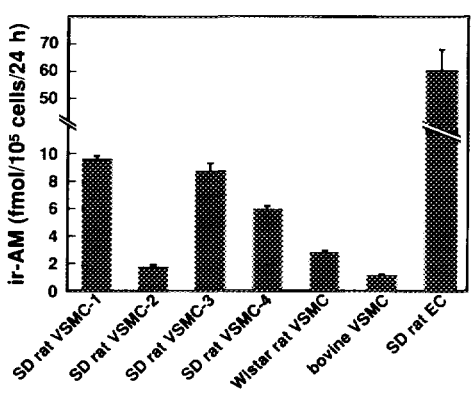

Figure 1. Production of ir-AM by rat VSMCs, bovine VSMC and rat vascular EC. Each value represents mean ± SEM of four separate dishes. VSMC-1 and -2 were prepared by the explant method, and VSMC-3, -4 and Wistar rat VSMC by the enzyme dispersion method.

gradually decreased to 0.26 fmol/10^5 cells/h in 24-48 h (Fig. 2a). Cellular content of ir-AM was measured to be 0.23 fmol/10^5 cells after 24 h incubation, corresponding to 2.4% of the total ir-AM in the culture medium. The low level of ir-AM contents in VSMCs suggests that AM produced by VSMC is constitutively secreted into the culture medium, as in the case of ECs. We also measured ir-CGRP concentration, but failed to detect a significant level of ir-CGRP either in culture medium (<0.004 fmol/10^5 cells/24 h) or in cell extract (<0.01 fmol/10^5 cells) of rat VSMCs.

Ir-AM secreted from cultured rat VSMCs was characterized by gel filtration and reverse phase HPLC. In Sephadex G-50 gel filtration, more than 80% of total ir-AM in the culture medium was eluted at the molecular weight region of 6K (Fig. 3a). Fractions #24-26 of 6K ir-AM were pooled and a portion of the fractions was analyzed by reverse phase HPLC, either directly or after oxidation. Ir-AM was eluted at retention times identical to that of rat AM or its methionine sulfoxide form (Figs. 3b and 3d). Another portion of fractions #24-26 was submitted to the cAMP production assay using VSMC-4 and was shown to increase cAMP level significantly (data not shown). Based on these data, we conclude that ir-AM secreted from rat VSMC is identical to rat AM of 50 residues.

The gene expression level of AM in VSMC was compared to that in other rat tissue by RNA blot analysis (Fig. 4). The strongest band hybridizing to AM cDNA probe was found in EC lane as reported in our previous paper (13). Although intensity was 1/5-1/10 that of EC, the second strongest band was observed in VSMC lane. The band intensity of VSMC was several times higher than that of cardiac atrium, adrenal gland and lung, even when intensity was corrected based on GAPDH band intensity. In thoracic aorta lane, 25 μg of total RNA corresponding to only 0.25-0.5 μg of poly(A^+)RNA was loaded, but a positive band was clearly observed. The high AM mRNA levels in VSMC and EC as well as significant gene expression in intact aorta indicate that vascular cells actively express AM gene in both normal and cultured conditions.

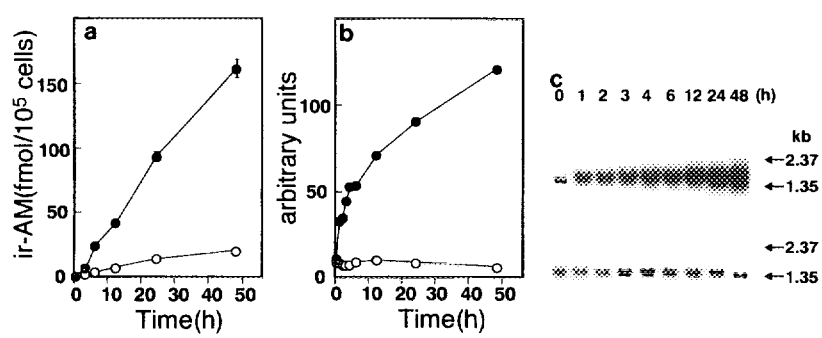

Figure 2. (a) Accumulation of ir-AM in culture media of rat VSMCs with TNF-α stimulation (20 ng/ml)(closed circle) and without stimulation (open circle). Each point represents mean ± SEM of four separate dishes.
(b) AM gene expression level of rat VSMCs with TNF-α stimulation (20 ng/ml) (closed circle) and without stimulation (open circle).
(c) RNA blot analysis of AM transcripts in rat VSMCs after TNF-α stimulation (20 ng/ml) (upper panel), without stimulation (lower panel).

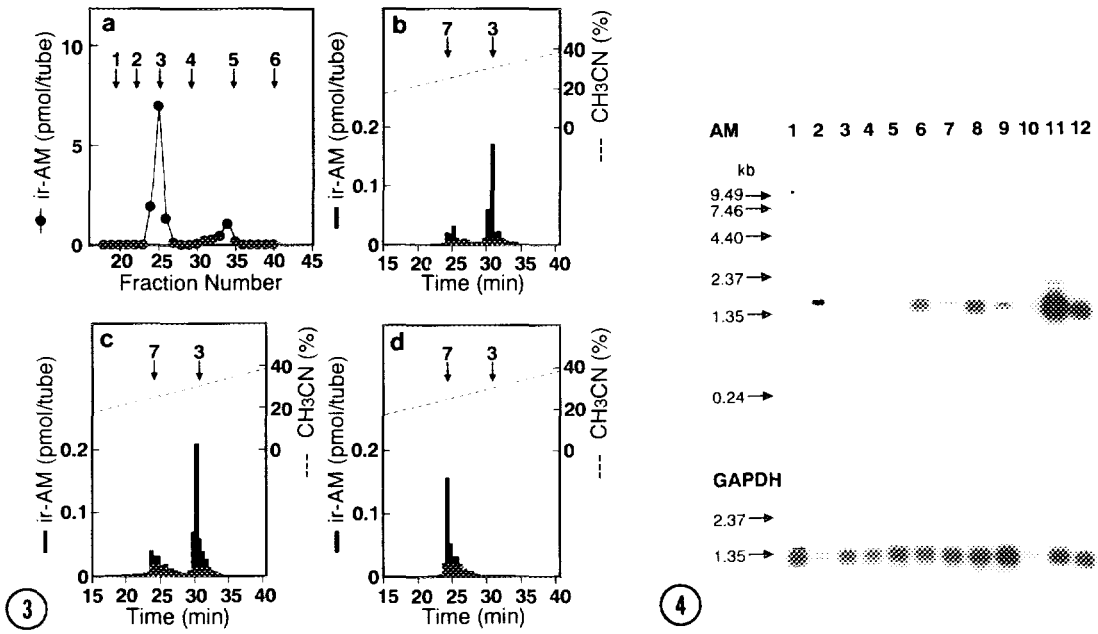

Figure 3. Characterization of ir-AM produced by rat VSMCs.
(a) Sephadex G-50 gel filtration of ir-AM in culture medium of VSMCs. Sample: 120 ml equivalents of culture medium. Column: 1.5 x 100 cm. Solvent: 2M acetic acid. Fraction size: 4.0 ml/tube. Flow rate: 5.0 ml/h.
(b,c,d) Reverse phase HPLC of ir-AM from culture medium of rat VSMCs. Sample: (b) fractions #24-26 in Fig. 3a, (c) ir-AM from TNF-α stimulated culture medium, (d) oxidized fractions #24-26 in Fig. 3a.
Column: μ-Bondasphere 5μ C18 column (300A, 3.9 x 150 mm). Flow rate: 1.0 ml/min. Fraction size: 0.5 ml/tube. Solvent system: Linear gradient elution from 10% to 60% CH_3CN in 0.1% TFA over 60 min.
Arrows indicate elution positions of (1) BSA, (2) ribonuclease A, (3) rat AM, (4) big endothelin-1, (5) endothelin-1, (6) NaCl and (7) methionine-sulfoxide form of AM.

Figure 4. RNA blot analysis of AM transcripts in rat tissue, cultured rat EC and VSMC. Each lane contains 5 μg of poly(A^+)RNA, except for thoracic aorta (25 μg of total RNA). Lanes: (1) brain, (2) lung, (3) liver, (4) stomach, (5) jejunum/ileum, (6) adrenal gland, (7) kidney, (8) cardiac atrium, (9) ventricle, (10) thoracic aorta, (11) cultured EC, (12) cultured VSMC. RNA molecular size standards are shown at left in kilobases. Autoradiography was -80℃ for 24 h. Lower panel indicates RNA blot analysis of GAPDH transcripts.

We examined effects of vasoactive substances, cytokines, growth and differentiation factors on the production of AM by rat VSMCs. Among them, TNF-α was found to markedly stimulate production and secretion of ir-AM. Figure 2a shows time-dependent accumulation of AM in the culture medium after TNF-α stimulation. Ir-AM content in the culture medium increased linearly for 48 h, with the secretion rate being almost constant (about 3.5 fmol/10^5 cells/h). The cellular/secreted ratio of ir-AM of VSMCs was unchanged by TNF-α stimulation. The AM gene expression level had already increased after 1 h of TNF-α stimulation, and reached an 11 fold-higher level after 48 h, in contrast to the level of AM mRNA in VSMC which gradually decreased without stimulation (Figs. 2b and 2c). Secretion of ir-AM into the culture medium as well as the gene expression level of AM was increased in a dose-dependent manner by TNF-α stimulation, as shown in Fig. 5. After 14 h incubation, both ir-AM concentration

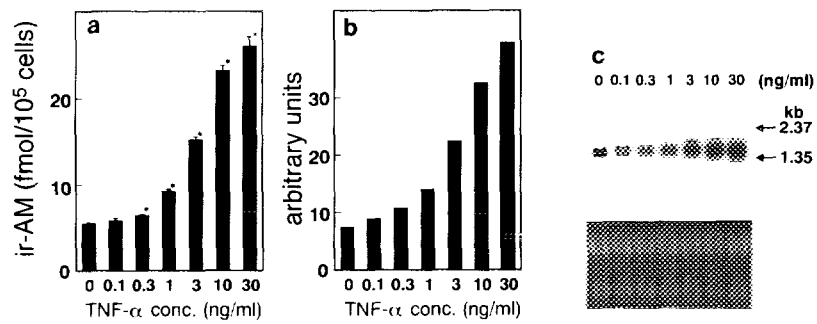

Figure 5. Dose-dependent AM production and gene expression by TNF-α stimulation.
(a) Immunoreactive AM production of rat VSMCs by TNF-α stimulation. Each value represents mean ± SEM of six separate dishes. *) $P<0.01$.
(b) AM mRNA levels of rat VSMC stimulated by TNF-α.
(c) RNA blot analysis of AM transcripts in rat VSMCs. Each lane contains 25 μg of total RNA. Lower panel indicates ethidium bromide staining of agarose gel.

and AM mRNA level increased 5 fold. Significant stimulation was observed from 0.1-0.3 ng/ml of TNF-α with an ED_{50} value of about 3 ng/ml. Ir-AM secreted from TNF-α stimulated VSMCs was also identified to be AM by reverse phase HPLC (Fig. 3c).

DISCUSSION

In this study, we first showed that cultured VSMCs produce and secrete ir-AM. Chromatographic identification as well as biological characterization of ir-AM secreted from VSMCs afforded solid evidence for production of a bioactive AM of 50 residues. The secretion level of ir-AM was different in each cell line, but all the examined VSMCs were found to produce AM (Fig. 1). Active expression of AM in VSMC was also confirmed by RNA blot analysis (Fig. 4). These data demonstrated that VSMCs generally synthesize and secrete AM. Very recently, we have reported that vascular ECs actively produce and secrete AM and that the secretion level of AM is comparable to that of endothelin-1 (13). Although the secretion level of AM from VSMC is at most 1/6 that of EC, the gene expression level of AM in VSMC is higher than that of other rat tissue, such as adrenal gland, which also actively expresses AM gene. Cultured VSMCs or ECs might be transformed from intact cells, but AM gene expression was also detected in intact rat aorta. A high population of VSMC in the aorta as well as a relatively high level of gene expression and secretion of AM from VSMCs suggest that VSMCs may be a major source of vascular AM and another candidate for cells secreting AM into the blood stream. Since AM-specific receptors coupled to the adenylate cyclase system are present on VSMCs (9,10), AM secreted from VSMCs is deduced to function as an autocrine or paracrine regulator in the vascular cell communication.

Among the substances and factors examined, TNF-α was found to most strongly stimulate the production of AM. As shown in Figs. 2 and 5, secretion and gene expression of AM were increased about 9 and 16 fold by TNF-α stimulation as compared to controls, and significant stimulation was observed as low as 0.3 ng/ml of TNF-α. TNF-α is a major inflammatory cytokine mainly produced by infiltrating mononuclear cells and is the main factor in the

development of endotoxin shock (23). In most cases, endotoxic shock induces alterations of vascular function, such as vasodilation. Since AM is one of the most potent vasorelaxant peptides, AM secreted from TNF-stimulated VSMCs may induce hypotension in endotoxin shock. In the atherosclerotic region, TNF-α secreted from macrophages possibly stimulates production of AM, which might in turn act as a regulator in the vascular wall (24).

Production of AM by VSMC provides new insight into VSMC functions. VSMC has been recognized as a target of substances delivered from blood, ECs and perivascular nerves, since it has receptors for these molecules. In fact, a limited number of bioactive peptides have been identified in VSMC (5). However, recent studies have verified that VSMC elaborates polypeptides influencing endothelium and vascular smooth muscle such as interleukin-1, TNF-α and colony stimulating factors (3,4,25). The present identification of AM in VSMCs supports the assumption that VSMC has the potential to produce factors regulating vascular function.

In conclusion, this study demonstrates that AM is synthesized and secreted from VSMCs. This fact, as well as augmented production of AM by TNF-α, strongly suggests that AM is a local regulator in controlling vascular cell function.

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