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INTERLEUKIN-1, TUMOR NECROSIS FACTOR AND LIPOPOLYSACCHARIDE ADDITIVELY STIMULATE PRODUCTION OF ADRENOMEDULLIN IN VASCULAR SMOOTH MUSCLE CELLS

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SUMMARY: To elucidate physiological functions of adrenomedullin (AM) secreted from vascular smooth muscle cells (VSMCs), we examined the effect of cytokines, growth factors and related substances on AM production in cultured rat VSMC. Among them, interleukin-1α (IL-1α), IL-1β, tumor necrosis factor-α (TNF-α) and TNF-β, as well as lipopolysaccharide (LPS), markedly augmented production and gene expression of AM. Although maximal stimulation levels of these substances were not greatly different, ED_{50} values of IL-1s (0.3 ng/ml) were about 1/10 that of TNFs and LPS. AM mRNA levels maximized at 3-6 h after stimulation with IL-1β and LPS, while TNF-α increased the AM mRNA level up to 48 h. Furthermore, IL-1α, TNF-α and LPS additively increased AM production in VSMC. AM production was slightly augmented by fibroblast, epidermal and platelet derived growth factors. These results suggest that AM secreted from VSMC actually exerts a vasorelaxant effect under physiological conditions such as endotoxin shock, atherosclerosis and inflammation.

Adrenomedullin (AM) is a new peptide first isolated from human pheochromocytoma and is structurally a member of the calcitonin gene-related peptide (CGRP) superfamily (1). The peptide elicits potent vasorelaxant activity comparable to that of CGRP (1-3). Plasma levels of AM in patients with hypertension and renal failure have been reported to be higher than those of normal volunteers, suggesting that AM participates in blood pressure regulation (4,5). Recently, we have demonstrated that cultured endothelial cell (EC) actively produces AM and that the AM gene is also expressed in intact rat aorta (6). Since vascular smooth muscle cell (VSMC) has AM specific receptors coupled with the adenylate cyclase system (7,8), AM is thought to function as an endothelium-derived relaxation factor. Furthermore,

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Abbreviations: AM, adrenomedullin; VSMC, vascular smooth muscle cell; EC, endothelial cell; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; PDGF, platelet derived growth factor; FGF, fibroblast growth factors; EGF, epidermal growth factor; TGF, transforming growth factor; NO, nitric oxide; ir, immunoreactive; RIA, radioimmunoassay; CGRP, calcitonin gene-related peptide; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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VSMC has very recently been shown to produce and secrete AM in a significant concentration (9). VSMCs have long been recognized as a structural and contractile component of the blood vessel, being regulated by vasoactive substances brought from the blood stream, ECs or perivascular nerves (10,11). Our data on AM production and secretion from VSMCs afforded solid evidence that VSMC produces substances regulating vascular function. To elucidate physiological functions of AM in the vascular wall, it is essential to understand the regulation mechanism of AM production in VSMC. Thus, we investigated the effects of cytokines, growth factors and LPS on AM production and gene expression in cultured VSMC, since we have recently found that TNF-α highly augments AM production in VSMC (9).

MATERIALS AND METHODS

Materials: Human AM[40-52]-NH_2 and its N-Tyr derivative were synthesized by a peptide synthesizer (Applied Biosystems, 431A). Mouse recombinant IL-1α, IL-2, IL-3, IL-4, IL-6, human recombinant TNF-β and platelet-derived growth factor-AA homodimer (PDGF-AA) were purchased from Genzyme. Mouse recombinant TNF-α and bovine recombinant basic fibroblast growth factor (bFGF) were obtained from Boehringer Mannheim Biochemica, and murine recombinant IL-1β from Intergen. Human recombinant transforming growth factor β_1 (TGF-β_1) and bovine brain acidic FGF were products of Wako Pure Chemicals. Human recombinant epidermal growth factor (EGF) and PDGF-BB homodimer were purchased from Biomedical Technologies and Austral Biologicals, respectively. Lyophilized substances were dissolved according to the producer's manuals and diluted with Dulbecco's modified Eagle's medium (DMEM) containing 0.1% bovine serum albumin (BSA). *Escherichia coli* LPS (serotype 026:B6) was purchased from Paesel+Lorei and was dissolved in 0.9% NaCl solution.
Cell culture: Rat VSMC were isolated from Sprague-Dawley rat thoracic aorta by the explant method and cloned (9). The isolated VSMCs were identified by positive immunostaining with monoclonal anti-α smooth muscle actin antiserum (Clone 1A4, Sigma) and undetectable uptake of fluorescent acetylated low density lipoprotein (9). VSMC was maintained in DMEM containing 10% fetal calf serum (FCS) at 37℃ in a humidified atmosphere containing 5% CO_2, and was used at passages 8-20 in the present study.
Preparation of conditioned medium: VSMCs, grown to confluence in a 6-well plate, were washed twice with DMEM and incubated in DMEM containing 0.1% BSA for 2 h. The media were then replaced with DMEM containing 0.1% BSA and stimulants, and incubated at 37℃ for 0.25-48 h. Viability of rat VSMCs after 24 h incubation was more than 97% by trypan blue staining. Culture media (1 ml) were acidified with acetic acid (final concentration: 0.5 M), 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.
RIA for AM: Details of the RIA system using antiserum #172CI-7 against human AM[40-52]-NH_2, which recognizes the C-terminal amide structure common to mammalian AMs, will be reported in a separate paper. RIA was performed as described for human AM (12). Monoiodinated N-Tyr-AM[40-52]-NH_2 was used for a tracer (6).
RNA blot analysis: RNA blot analysis for AM was performed as reported in our previous paper (6). In brief, total RNA (25 μg), extracted by the acid guanidium thiocyanate-phenol-chloroform method, was denatured, electrophoresed, and was then transferred to Zeta probe membrane (Bio-Rad). Hybridization and washing of the membrane were carried out as reported (6). Eco RI-Bgl I cDNA fragment of rat AM (nucleotide -153～422) was used as a probe. After removing radioactive probes, the membrane was re-hybridized to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Eco RI-Bam HI fragment; 492～799). Band intensity was estimated by a Bioimage analyzer (Fuji, BAS 2000), and was compared after correction and normalization with each 0 time band intensity and GAPDH band intensity.
Statistics: Values were expressed as mean±SEM. Statistical analysis of data was performed by Student's *t* test, and $P<0.01$ or $P<0.03$ was considered statistically significant.

RESULTS

We measured immunoreactive (ir-) AM concentrations in culture media of rat VSMC after 24 h stimulation with 14 cytokines and growth factors, since AM was shown to be

Table 1. Effects of various substances on ir-AM production of rat VSMC

substance	concentration	ir-AM (fmol/10^5 cells/24 h)
control		10.37 ± 0.30
IL-1 α	20 ng/ml	35.38 ± 0.94*
IL-1 β	10 ng/ml	42.76 ± 0.91*
IL-2	500 unit/ml	13.99 ± 0.69*
IL-3	200 unit/ml	10.91 ± 0.39
IL-4	20 ng/ml	8.57 ± 0.19*
IL-6	4 ng/ml	10.75 ± 0.57
TNF- α	20 ng/ml	48.92 ± 0.83*
TNF- β	20 ng/ml	33.13 ± 2.57*
TGF- β 1	20 ng/ml	11.25 ± 0.20
EGF	25 ng/ml	14.29 ± 0.32*
PDGF-AA	10 ng/ml	10.15 ± 0.97
PDGF-BB	25 ng/ml	15.79 ± 0.58*
acidic-FGF	20 ng/ml	15.60 ± 0.19*
basic-FGF	20 ng/ml	14.07 ± 0.20*
LPS	10 ng/ml	33.92 ± 2.31*

Each value represents mean ± SEM of 3 or 6 separate dishes.
Asterisk indicates statistically significant difference from control (P<0.01).

constitutively produced and secreted from VSMC into the culture medium (9). Among them, IL-1α, IL-1β, TNF-α and TNF-β stimulated AM production and secretion from VSMC and increased ir-AM concentration to 341%, 412%, 472% and 319% of the control, respectively (Table 1). In contrast, other cytokines, such as IL-2, IL-3, IL-6 and TGF-β, had no apparent effect on the production of AM. PDGF-BB, the most effective growth factor for VSMC, as well as EGF, acidic and basic FGFs only slightly enhanced production of AM in VSMC, while

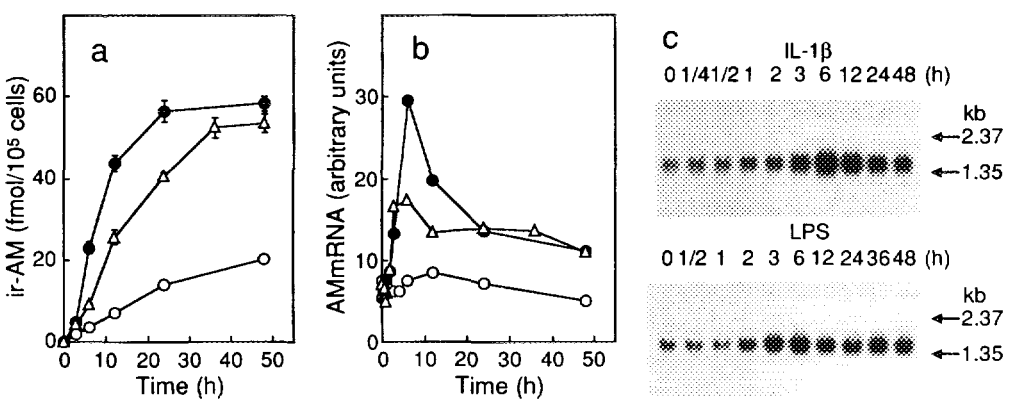

Figure 1. Time-dependent AM production and gene expression in cultured rat VSMC by IL-1β and LPS stimulation.
(a) Immunoreactive AM concentration in culture medium of rat VSMC. Closed circle, IL-1β, 10 ng/ml; open triangle, LPS, 30 ng/ml; open circle, control (without stimulation). Each point represents mean ± SEM of four separate dishes.
(b) AM mRNA level in rat VSMC. Closed circle, IL-1β (10 ng/ml); open triangle, LPS (30 ng/ml); open circle, control (without stimulation).
(c) RNA blot analysis of AM transcripts in rat VSMCs after IL-1β stimulation (10 ng/ml) (upper panel) and LPS stimulation (30 ng/ml) (lower panel).

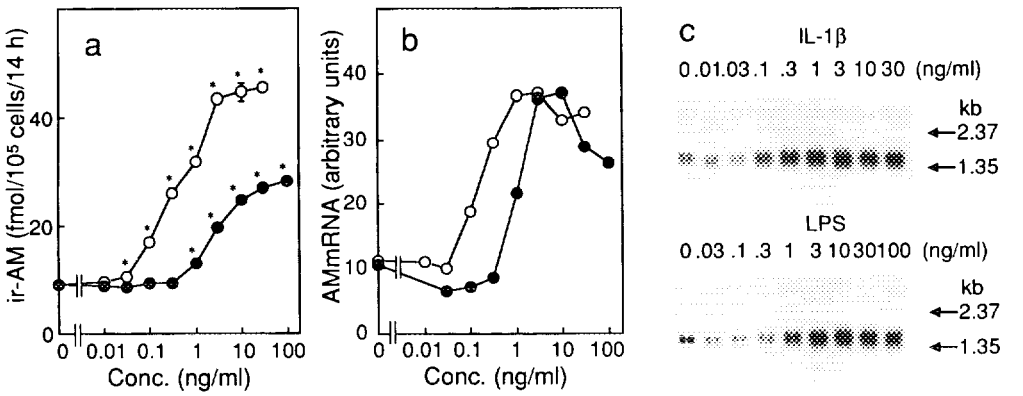

Figure 2. Dose-dependent AM production and gene expression in cultured rat VSMC by IL-1β and LPS stimulation.
(a) Immunoreactive AM concentration in culture medium of rat VSMCs after 14 h stimulation. Open circle, IL-1β; closed circle, LPS. Each point represents mean ± SEM of six separate dishes. *) $P<0.01$.
(b) AM mRNA level in rat VSMC stimulated with IL-1β (open circle) and LPS (closed circle) for 14 h.
(c) RNA blot analysis of AM transcripts in rat VSMC stimulated with IL-1β (upper panel) and LPS (lower panel) for 14 h. Each lane contains 25 μg of total RNA.

PDGF-AA did not stimulate it at all. In addition to these substances, we checked the effect of LPS, since LPS induces biological effects similar to those of IL-1 and TNF. LPS augmented production and secretion of AM by 327%, which was comparable to that of IL-1 and TNF.

Since we have reported the stimulatory effect of TNF-α on AM production in VSMC (9), we examined in detail the effect of IL-1α, IL-1β, TNF-β and LPS on AM production in this study. As shown in Fig. 1a, ir-AM accumulated in the culture medium of the IL-1β and LPS group at a rate 6.1 times and 3.6 times higher than that in the control group after 12 h incubation. After 36 h incubation, the secretion rate of AM in the IL-1β and LPS group decreased to the level of the control group. Time-dependent changes of AM gene transcription in VSMC supported these results (Figs. 1b and 1c). Since specific radioactivity of the probe is different in each RNA blot analysis, only relative band intensity is comparable after correction and normalization. By IL-1β stimulation, AM gene transcription increased 5.5 fold after the first 6 h stimulation, and then gradually decreased to a level twice as high as that of the control at 48 h. In the case of LPS stimulation, AM gene transcription was augmented 2.5 fold in the first 3 h and was maintained at a level 2-2.5 fold higher than the control during stimulation. Although the effect of IL-1β on AM gene transcription was stronger than that of LPS at 6 h, prolonged effects of LPS increased AM concentration in the culture medium comparable to that of IL-1β at 48 h. In contrast, TNF-α continuously stimulated AM gene transcription and increased AM mRNA level up to 48 h, and total AM produced by TNF-α stimulated VSMC was several times higher than that of IL-1β and LPS after 48 h incubation (9).

IL-1β and LPS stimulated gene expression and production of AM in a dose-dependent manner, as shown in Fig. 2. After 14 h stimulation, ir-AM concentration in the culture medium and AM mRNA in VSMC increased 4.8 fold and 3.3 fold at 1-3 ng/ml of IL-1β. Its ED_{50} value was estimated to be 0.2-0.5 ng/ml. LPS also dose-dependently augmented gene expression and production of AM 3.2 fold and 3.5 fold with an ED_{50} value of 1-3 ng/ml.

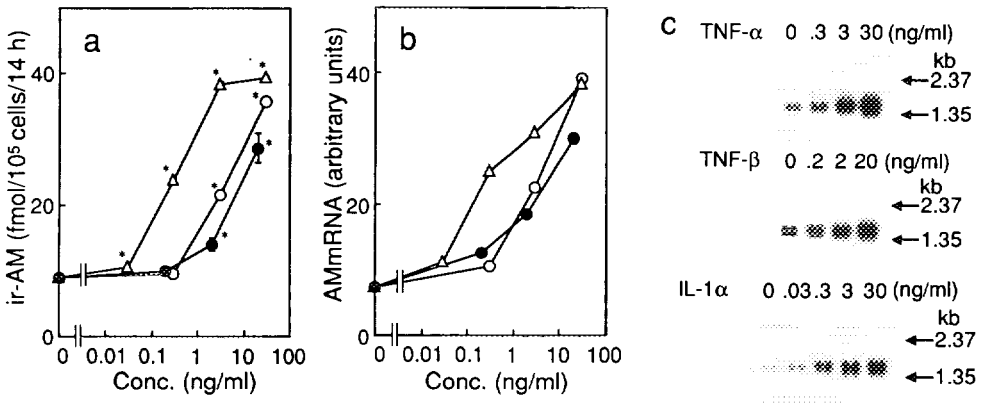

Figure 3. Dose-dependent AM production and gene expression in cultured rat VSMC by TNF-α, TNF-β and IL-1α stimulation.
(**a**) Immunoreactive AM concentration in culture medium of rat VSMC. Open circle, TNF-α; closed circle, TNF-β; open triangle, IL-1α. Culture medium was collected after 14 h stimulation. Each point represents mean±SEM of six separate dishes. *) $P<0.01$.
(**b**) AM mRNA level in rat VSMC stimulated with IL-1α (open circle), TNF-β (closed circle) and IL-1α (open triangle) for 14 h.
(**c**) RNA blot analysis of AM transcripts in rat VSMC stimulated with TNF-α (top), TNF-β (middle) and IL-1α (bottom) for 14 h. Each lane contains 25 μg of total RNA.

We examined effects of IL-1α and TNF-β on AM gene expression and production of AM, along with those of TNF-α (Fig. 3). After 14 h stimulation, IL-1α, TNF-α and TNF-β showed a dose-dependent increase in AM production and gene transcription, and ED_{50} values of IL-1α, TNF-α and TNF-β were estimated to be about 0.3 ng/ml, 3 ng/ml and 3-5 ng/ml, respectively, both in AM production and gene transcription. Based on the ED_{50} values, IL-1α and IL-β were found to be about 10 times more potent than TNF-α and TNF-β in stimulating AM gene transcription and production in cultured VSMC, although the maximal stimulation levels of all four substances were comparable (Table 1, Figs. 2 and 3).

Since IL-1β, TNF-α and LPS are often known to cooperatively exert physiological action, ir-AM concentration in the culture medium of VSMC was measured by combining these substances (Fig. 4). By combined administration of submaximal doses of IL-1β, TNF-α

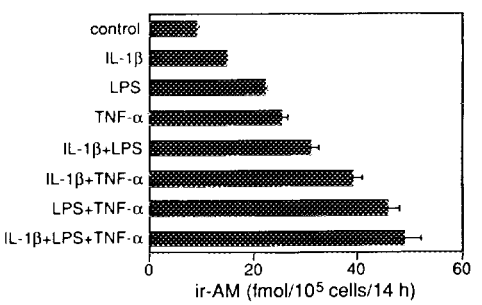

Figure 4. Additive effect of IL-1β, LPS and TNF-α on AM production in cultured rat VSMC. Stimulants were added and incubated for 14 h in the following concentrations; IL-1β, 0.1 ng/ml; LPS, 5 ng/ml; TNF-α, 5 ng/ml. Each value represents mean±SEM of six separate dishes. Significant difference ($P<0.03$) was observed between all combinations, except between LPS+TNF-α and IL-1β+LPS+TNF-α.

and LPS to cultured VSMC, AM production was augmented and ir-AM concentration in the medium reached a level about 5 times higher than that of the control by simultaneous addition of the three substances. Submaximal concentration of IL-1β or LPS acted in more than an additive manner with TNF-α to increase AM production, and co-administration of IL-1β and LPS or the three substances to VSMC exerted at least an additive effect on AM production.

DISCUSSION

We have reported that AM is produced and secreted from cultured rat EC and VSMC and that secreted AM was identical to native AM chromatographically and biologically (6,9). Although the secretion rate of AM from VSMC was lower than that of EC, AM is constitutively secreted from VSMC and AM receptors are present on VSMC, suggesting that AM secreted from VSMC regulates VSMC (6-9). To realize the function of AM in the vascular wall, the regulation mechanism of AM gene expression and production should be elucidated. Since TNF-α was found to highly augment AM production in VSMC (9), we surveyed a series of cytokines and growth factors to identify substances affecting AM production in rat VSMC.

Among the cytokines and growth factors examined, TNF-α, TNF-β, IL-1α and IL-1β markedly stimulated AM production in cultured rat VSMC (Table 1), while other cytokines and growth factors stimulated AM production weakly or not at all. Activation of AM production by PDGF-BB, EGF and FGFs may be due to indirect effects of their growth stimulation activity, since, for example, FGF slightly increased GAPDH gene transcription in a similar manner. Maximal stimulation levels of AM production by TNFs and IL-1s were comparable, but ED_{50} values of IL-1α and IL-1β (0.3 ng/ml) were 1/10-1/15 that of TNF-α and TNF-β, indicating that IL-1s are the most potent stimulants of AM production so far examined.

Stimulatory effect of LPS on AM production was as strong as that of TNF on a weight basis. Since VSMC has been reported to produce IL-1 and TNF-α in response to LPS stimulation, there is a possibility that IL-1 and TNF-α induced by LPS act on VSMC and activate AM gene transcription (13,14). However, TNF-α stimulated AM gene expression up to 48 h (9), and IL-1β or LPS induced AM gene expression reached a maximal level after 3-6 h stimulation (Fig. 1). These results indicate that TNF-α, IL-1β and LPS activate AM gene expression through different mechanisms and that LPS action is likely to be initiated via binding to its receptor, although an LPS receptor has not been identified in VSMC (15,16).

As shown in Fig. 4, IL-1β, TNF-α and LPS were found to cooperatively stimulate AM production in VSMC. These effects were more than simply additive, but not so high as that expected from synergism. LPS is a main component of bacterial endotoxin, and TNF-α and IL-1β are the major inflammatory cytokines in the development of endotoxin shock (17,18). The fact that IL-1, TNF and LPS are the major activators of AM production in VSMC and that specific AM receptors are present on VSMC, is consistent with the hypothesis that AM contributes to hypotension in endotoxin shock. TNF and IL-1 are also known to participate in generating atheroma and inflammation in blood vessels. In such regions, activated macrophage as well as VSMC and EC have been shown to produce TNF and IL-1 (19-21). Thus, in areas where TNF and IL-1 are induced and secreted, it is likely that AM production in VSMC is highly augmented. This augmented AM in turn regulates VSMC functions, such as vasorelaxation.

Recent studies have verified that VSMC produces IL-1 and TNF-α in response to IL-1s, TNFs and LPS, suggesting that AM production in VSMC was amplified through the

activation of the LPS/TNF/IL-1 system (13,14,22,23). Inducible nitric oxide (NO) synthase gene expression in VSMC is also induced by IL-1β, TNF-α and LPS (24). Submaximal levels of IL-1β and TNF-α synergistically induce NO synthase gene expression, which results in relaxation of vascular smooth muscle. In this regard, LPS-induced inhibition of rat aortic ring contraction, previously attributed to induction of NO synthase through generation of IL-1 and TNF-α within tissue (24,25), may be mediated through both AM production and induction of NO synthase. Based on these data, IL-1s, TNFs and LPS concertedly increase production of AM and NO in VSMC, which may cause serious hypotension in endotoxin shock.

Very recently, we have cloned and sequenced the human AM gene (26). In the 5'-upstream sequence, cAMP-regulated enhancer, AP-1 site, AP-2 site, GC-box and shear stress response element were observed. Typical NF-κB site as well as some other sequences reported to be required for gene activation by TNF-α, IL-1β and LPS are not found in the sequence up to -2355 base from initiating ATG, although several homologous sequences are present (27-31). AM gene expression may be regulated by a mechanism different from that so far reported or by a mechanism using these homologous sequences. The nature of the regulatory mechanism is a problem still to be solved.

In conclusion, AM production in VSMC is highly augmented by TNF, IL-1 and LPS, suggesting that AM participates in the local regulation of VSMC function, especially in the cases of endotoxin shock, atherosclerosis and inflammation.

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