Adrenomedullin suppresses interleukin-1β-induced tumor necrosis factor-α production in Swiss 3T3 cells

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Abstract We demonstrated that adrenomedullin (AM) inhibited interleukin-1β-induced tumor necrosis factor-α (TNF-α) secretion and gene transcription in Swiss 3T3 fibroblasts maximally to 23% and 18% of control, while the other peptides elevating intracellular cAMP levels elicited much weaker effects. AM rapidly reduced the gene transcript level of TNF-α, inducing a maximal effect within 1 h. The inhibitory effect of AM was restored with an AM receptor antagonist as well as a cAMP-dependent protein kinase inhibitor. These findings indicate that AM is a potent and quick suppressor of TNF-α production in Swiss 3T3 cells acting through the cAMP protein kinase A pathway. As TNF-α is a major inflammatory cytokine and stimulates AM production in fibroblasts, AM is deduced to be an autocrine or paracrine factor suppressing inflammation through the inhibition of TNF-α production.
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Key words: Adrenomedullin; Tumor necrosis factor-α; Interleukin-1β; Fibroblast

1. Introduction

Adrenomedullin (AM) is a potent vasorelaxant peptide first isolated from extracts of human pheochromocytoma [1]. This peptide of 52 residues structurally belongs to the calcitonin gene-related peptide (CGRP) family [2]. In our systematic survey for AM producing cells, vascular smooth muscle cells (VSMCs), endothelial cells (ECs), monocytes, and macrophages were shown to actively produce and secrete AM [3–6]. The production of AM in VSMCs and ECs is strongly augmented by tumor necrosis factor (TNF), interleukin-1 (IL-1), and lipopolysaccharide (LPS) [3,7,8]. We have recently demonstrated that fibroblasts actively synthesize and secrete AM at a rate comparable to or higher than that of ECs and VSMCs [9]. TNF-α, IL-1β, and LPS are also potent stimulants of AM production in the fibroblasts [9]. In the in vivo study, markedly elevated levels of AM were observed in the plasma of LPS-injected rats and patients with septic shock [10,11]. These data suggest that AM secreted from fibroblasts significantly contributes to the elevation of plasma AM concentration in septic shock and inflammation. On the other hand, fibroblasts express receptors for AM, and AM is a powerful stimulator of cAMP production especially in Swiss 3T3 fibroblasts [9]. In fact, AM stimulates proliferation as well as IL-6 production in Swiss 3T3 cells through its receptor and cAMP-dependent protein kinase (PKA) pathway [9,12]. Based on the data described above, AM is expected to participate in the mediation of inflammation and septic shock in addition to vasodilation.

TNF-α is well known as a major factor inducing inflammation and immune responses and is secreted abundantly from monocytes and macrophages, especially under the stimulation with IL-1 and LPS [13,14]. Fibroblasts, in addition to macrophages and monocytes, have been recognized as one of the possible sources of TNF-α in septic shock and inflammation [15–17]. To elucidate the functions of AM secreted from fibroblasts and assess its contribution to the inflammation, we measured the effects of AM on the basal and IL-1β-induced TNF-α secretion from Swiss 3T3 fibroblasts.

2. Materials and methods

2.1. Materials

The following materials were used: mouse recombinant IL-1β (Intergen), mouse recombinant TNF-α (Boehringer Mannheim), H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide) and H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) (Seikagaku Corp.), genistein (Calbiochem), PD98059 (2′-amino-3′-methoxyflavone) (New England Biolabs). Peptides were obtained from Peptide Institute, and all of them used were of human origin except for those specifically identified by the species name. All substances were dissolved according to the producer's manuals and diluted with an incubation medium, Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal calf serum (FCS) (Filtron).

2.2. Cell culture

3T3 Swiss albino (Swiss 3T3) cells and NCTC clone 929 (L929) cells were purchased from American Type Culture Collection. Swiss 3T3 cells were maintained in DMEM containing 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2. L929 cells were maintained in minimum essential medium (MEM) containing 10% horse serum at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of conditioned medium

Swiss 3T3 cells, grown to confluence in a 6-well plate (Iwaki Glass), were washed twice with DMEM and incubated with the incubation media containing reagents to be tested at 37°C for 0–48 h in a CO_2 incubator. The culture media were collected and kept at −85°C until analysis. More than 95% of the cells were viable under these incubation conditions, estimated by the trypan blue exclusion assay.

2.4. Measurement of TNF-α

Biologically active TNF-α in the conditioned medium was measured by the cell cytotoxicity assay, using mouse fibroblast L929 cells [18]. L929 cells were cultured in a 24-well plate at a concentration of 5×10^4 cells/well and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then incubated with the conditioned media or mouse TNF-α as a standard in the medium

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containing 5-[^{125}I]-Iodo-2'-deoxyuridine (^{125}I-DU, 0.1 μCi/ml) (Amersham) and 0.5 μg/ml actinomycin D (Sigma). After a 24-h incubation, the L929 cells were washed twice with ice-cold PBS, and the radioactivity of ^{125}I-DU incorporated into nuclei was extracted and counted by the method reported previously [9]. A monoclonal antibody against mouse TNF-α (Genzyme) was used to determine whether or not the cytotoxic activity observed was derived from TNF-α.

2.5. Quantification of TNF-α gene transcript level by real time quantitative PCR

Swiss 3T3 cells, grown to confluence in a 6-well plate, were washed twice with DMEM and incubated with the incubation media containing stimulants for 0–8 h. Total RNA was extracted and reverse-transcribed into cDNA with SuperScript II (Life Technologies). Mouse TNF-α cDNA and GAPDH cDNA were each amplified with respective pairs of the following oligonucleotides: GTGATC-GGTCCCCAAAGG (base 326–343, sense) and GGGTCTGGGCCA-TAGAACTG (base 394–414, antisense) for TNF-α [6], ACATGTTC-CAGTATGACTCCACTCAC (base 174–199, sense) and TCTCG-CTCCTGGAAGATGGT (base 282–301, antisense) for GAPDH [6]. To measure mouse TNF-α and GAPDH gene transcript levels, real time quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems) was performed as reported previously [6]. We used the following oligonucleotide probes labeled with the reporter and quencher fluorescence: ATGAGAAGTTCCCAAATGGCCTCCCT (base 346–369) for TNF-α, and AACGGCACAGTCAAGGCCGAGAAT (base 209–233) for GAPDH. TNF-α gene transcript levels were compared after correcting them by a GAPDH gene transcript level as an internal standard. Known amounts of cDNA fragments of mouse TNF-α (base 326–414) and GAPDH (base 174–301) were used as standards. The data were averages of four experiments.

2.6. Statistics

Values were expressed as mean ± S.E.M. Statistical analysis of the results was performed with a one-way analysis of variance (ANOVA), followed by a multiple comparison test (Dunnett's test), and $P < 0.05$ was considered significant.

3. Results

3.1. Effect of AM on TNF-α secretion and gene transcription in Swiss 3T3 cells

TNF-α concentration in the culture medium of non-stimulated Swiss 3T3 cells was determined to be 4.1 ± 0.14 pg/ml (basal) after a 24-h incubation. AM alone was of no effect on the basal TNF-α secretion level from Swiss 3T3 cells in a range of 10^{-10}–10^{-7} M (Fig. 1a). As TNF-α production in macrophages, astrocytes, and fibroblasts has been reported to be stimulated with IL-1 [13,19,20], we examined whether AM affects TNF-α synthesis and secretion from Swiss 3T3 cells in the presence of IL-1β. TNF-α concentration in the medium of Swiss 3T3 cells after a 24-h stimulation with IL-1β (1 ng/ml) was determined to be 14.9 pg/ml (control), which was 3.6-fold higher than the basal level. AM dose-dependently inhibited TNF-α secretion from IL-1β-stimulated Swiss 3T3 cells in a physiological concentration range of 10^{-9}–10^{-7} M, and TNF-α concentration was decreased to 23% of the control after a 24-h incubation. The 50% inhibitory concentration (IC_{50}) value for AM was 1×10^{-9} M. The cytotoxic activity in the conditioned media of Swiss 3T3 cells in the presence (control) or absence (basal) of IL-1β was inhibited by 91% and 88% with the neutralizing monoclonal antibody against mouse TNF-α (25 μg/ml). These findings indicate that the cytotoxic activity in the conditioned medium of Swiss 3T3 cells was almost completely derived from TNF-α secreted from Swiss 3T3 cells. We also examined the effects of AM on the TNF-α gene transcription in Swiss 3T3 cells using the real time quan-
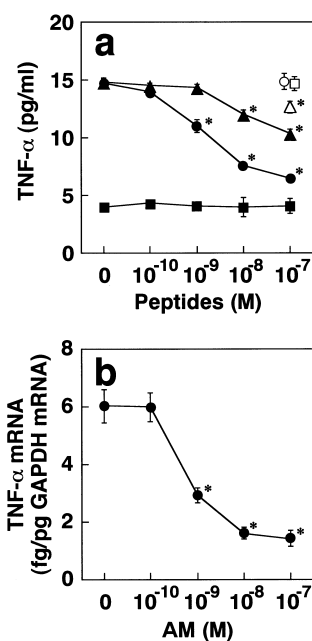

Fig. 1. Effects of AM, CGRP, amylin, VIP, and PACAP on secretion and gene transcription of TNF-α in Swiss 3T3 cells. (a) Swiss 3T3 cells were stimulated for 24 h with the indicated concentrations of the peptides and IL-1β (1 ng/ml). ■, AM; ●, AM+IL-1β; ▲, CGRP+IL-1β; △, amylin+IL-1β; ○, PACAP+IL-1β; □, VIP+IL-1β. TNF-α concentrations were measured as described in Section 2. Each point represents the mean ± S.E.M. of four separate wells. (b) TNF-α gene transcript levels in Swiss 3T3 cells were measured by real time quantitative PCR after a 1-h incubation with the indicated concentrations of AM and IL-1β (1 ng/ml). TNF-α gene transcript level was corrected using a GAPDH gene transcript level as an internal standard. Each point represents the mean ± S.E.M. of four separate measurements. The basal TNF-α mRNA level was 0.44 fg/pg GAPDH mRNA. *$P < 0.01$ vs. TNF-α level stimulated with IL-1β (■, excluded from comparison).

titative PCR. AM dose-dependently decreased a TNF-α gene transcript level in Swiss 3T3 cells stimulated with IL-1β (1 ng/ml) to 18% of the control in a manner similar to the protein level after a 1-h incubation (Fig. 1b). CGRP also inhibited TNF-α secretion from IL-1β-stimulated Swiss 3T3 cells, with the IC_{50} value (1×10^{-8} M) being 10 times higher than AM. Rat amylin weakly suppressed TNF-α secretion from IL-1β-stimulated Swiss 3T3 cells at a concentration of 10^{-7} M, while 10^{-7} M of vasoactive intestinal peptide (VIP) and rat pituitary adenylate cyclase-activating polypeptide (PACAP) did not alter the TNF-α secretion under the stimulation with IL-1β.

3.2. Effects of AM receptor antagonists and protein kinase inhibitors on TNF-α secretion from Swiss 3T3 cells stimulated with AM and IL-1β

AM[22–52], an AM receptor antagonist, dose-dependently restored an AM-suppressed TNF-α concentration in the medium of Swiss 3T3 cells stimulated with IL-1β (1 ng/ml). TNF-α concentration in the medium was completely returned to the control level at 10^{-5} M of AM[22–52], and this dose of AM[22–52] alone did not alter the control TNF-α concentration at all. CGRP[8–37], a CGRP receptor antagonist, also restored the AM-suppressed TNF-α concentration in the medium of IL-1β-stimulated Swiss 3T3 cells in a manner and potency similar to that of AM[22–52]. The effects of AM and CGRP receptor antagonists on AM-suppressed TNF-α

production were quite similar to those reported for the inhibition on AM-induced cAMP production in Swiss 3T3 cells [9].

AM has been reported to induce its effects through the cAMP-, Ca^{2+}-, or mitogen-activating protein kinase-mediated pathway by a series of studies [8,9,21,22]. To identify the signal transduction mechanism involved in the inhibitory activity of AM, we administered four kinds of protein kinase inhibitors to Swiss 3T3 cells in the presence of AM (10^{-8} M) and IL-1β (1 ng/ml) and measured TNF-α concentration in the culture media. As shown in Fig. 2, PD98059 (mitogen-activated protein kinase inhibitor), H-7 (protein kinase C inhibitor), and genistein (tyrosine kinase inhibitor) were not effective at all. H-89, an inhibitor for PKA, restored AM-suppressed TNF-α concentration in the medium of IL-1β-stimulated Swiss 3T3 cells to 77% of the control, while the same dose of H-89 did not alter TNF-α secretion in the presence of IL-1β.

3.3. Time-dependent effects of AM and IL-1β on TNF-α secretion and gene transcription in Swiss 3T3 cells

TNF-α concentrations in the culture media of Swiss 3T3 cells were measured up to 48 h under basal (no stimulation), control (IL-1β, 1 ng/ml), and AM-suppressed (AM, 10^{-8} M; IL-1β, 1 ng/ml) conditions (Fig. 3a). Compared to the time-course of the basal TNF-α level, IL-1β stimulated TNF-α secretion from Swiss 3T3 cells over 48 h, where TNF-α concentration was 3.4-fold higher than the basal level. AM (10^{-8} M) suppressed the TNF-α concentration to 34% and 24% of the control after 1 h and 24 h, respectively. We also examined from 0 to 8 h the effect of AM (10^{-8} M) on TNF-α gene transcription in IL-1β-stimulated Swiss 3T3 cells (Fig. 3b). TNF-α gene transcript level reached a maximum after a 1-h stimulation with IL-1β, and then decreased to the basal level. AM suppressed IL-1β-elevated TNF-α gene transcript level in Swiss 3T3 cells from 0.5 to 4 h, particularly after a 1-h incubation to 22% of the control.

Fig. 3. Time-course of TNF-α secretion and gene transcription in Swiss 3T3 cells. (a) TNF-α concentrations in the medium were measured after a 0–48-h incubation of Swiss 3T3 cells with IL-1β (1 ng/ml) alone (■), AM (10^{-8} M) and IL-1β (●), or without any stimulation (▲). Each point represents the mean ± S.E.M. of four separate wells. TNF-α concentration stimulated with AM and IL-1β is significantly lower than that stimulated with IL-1β after a 1-h incubation ($P < 0.01$). (b) TNF-α gene transcript levels in Swiss 3T3 cells were measured after a 0–8-h incubation of the cells with IL-1β (1 ng/ml) alone (■), or AM (10^{-8} M) and IL-1β (●). TNF-α gene transcript levels were plotted after correcting them based on the GAPDH gene transcript levels. Each point represents the mean ± S.E.M. of four separate measurements. TNF-α mRNA level stimulated with AM and IL-1β is significantly lower than that stimulated with IL-1β between 0.5-h and 2-h incubation ($P < 0.01$).

4. Discussion

AM potently inhibited TNF-α secretion and gene transcription in IL-1β-stimulated Swiss 3T3 cells to 23% and 18% of the control, respectively. This inhibitory effect is dose-dependent within a physiological concentration range (Fig. 1). The TNF-α concentration in the culture medium of IL-1β-stimulated Swiss 3T3 cells was significantly decreased already after a 1-h incubation with AM and was continually suppressed up to 48 h. The TNF-α gene transcript level was elevated maximally 13-fold with IL-1β after a 1-h stimulation, and AM strongly suppressed this quick increase of the TNF-α gene transcript level (Fig. 3). These results suggest that AM is a potent suppressor of TNF-α production in Swiss 3T3 cells.

H-89, a specific inhibitor of PKA, restored AM-suppressed TNF-α production to 77% of the control in the presence of IL-1β (Fig. 2), while the inhibitors for the mitogen-activated protein kinase, protein kinase C and tyrosine kinase were of no effect. IL-1β-induced TNF-α production in astrocytoma and zona glomerulosa cells has also been shown to be regulated by the cAMP-mediated pathway [23]. Taken together, AM is found to inhibit IL-1β-induced TNF-α production through the cAMP-PKA pathway by activating the receptor-coupled adenylate cyclase.

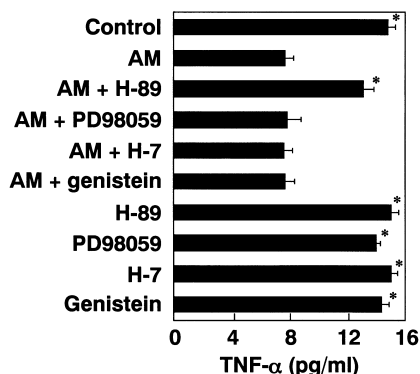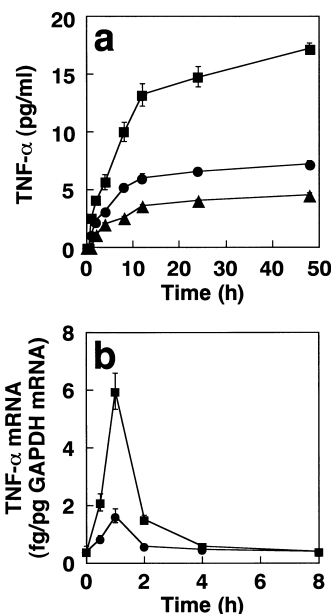Among the other examined peptides that are known to elevate intracellular cAMP, CGRP and amylin weakly inhib-

Fig. 2. Effects of protein kinase inhibitors on AM-suppressed TNF-α secretion from Swiss 3T3 cells. Swiss 3T3 cells were incubated for 24 h with the indicated combinations of AM (10^{-8} M), H-89 ($5×10^{-6}$ M), PD98059 ($5×10^{-6}$ M), H-7 ($5×10^{-6}$ M), and genistein ($5×10^{-6}$ M) under the stimulation of IL-1β (1 ng/ml). TNF-α concentrations in the media were measured as described in Section 2. Control indicates TNF-α concentration in the medium of Swiss 3T3 cells stimulated with IL-1β. Each column represents the mean ± S.E.M. of four separate wells. *$P < 0.01$ vs. TNF-α concentration stimulated with AM and IL-1β.

ited IL-1β-induced TNF-α production, while VIP and PACAP elicited no effect (Fig. 1a). We have reported that CGRP and amylin weakly inhibit ^{125}I-labeled AM binding to Swiss 3T3 cells and increase cAMP production at a concentration higher than 10^{-8} or 10^{-7} M [9]. Thus, the inhibitory effects of CGRP and amylin on IL-1β-induced TNF-α secretion were thought to be mediated by their binding to the AM-specific receptor and low elevation of the intracellular cAMP in Swiss 3T3 cells. Although PACAP and VIP were reported to inhibit LPS-induced TNF-α production in macrophages [24], these two peptides were able to elevate cAMP production in Swiss 3T3 cells only to 1/30 that stimulated with AM, resulting in no significant alteration of TNF-α secretion. In fact, AM most potently stimulated cAMP production in Swiss 3T3 cells among the peptides so far examined [9]. Based on these findings, AM is concluded to be the most potent peptidergic suppressor of IL-1β-induced TNF-α production in Swiss 3T3 cells.

McLatchie et al. have demonstrated that AM and CGRP share a core receptor, calcitonin receptor-like receptor (CRLR) [25]. The CRLR is specific for AM when it is co-expressed with receptor activity-modifying protein (RAMP)-2 or RAMP-3, while this receptor turns to be specific for CGRP when co-expressed with RAMP-1. AM, CGRP, AM[22–52] and CGRP[8–37] have been reported to inhibit ^{125}I-labeled rat AM binding to the membrane preparation of HEK293T cells expressing CRLR and RAMP-2 with the IC_{50} values of 7.5×10^{-10}, 1.3×10^{-6}, 1.5×10^{-8} and 7.5×10^{-8} M, respectively [25]. As for Swiss 3T3 cells, we have reported that these four peptides inhibit its binding to the cells with the IC_{50} values of 4.1×10^{-9}, 5.1×10^{-7}, 9.0×10^{-7} and 6.0×10^{-8} M [9], and AM[22–52] and CGRP[8–37] elicited the comparable inhibitory effects in this study. Based on these data, it is rather hard to deduce that the action of AM on Swiss 3T3 cells is induced simply through the CRLR and RAMP-2, even though we used the receptor system of different species. Bühlmann et al. have recently found that the specificity of the CRLR is shifted when two RAMPs are expressed together [26]. Our preliminary studies indicate that more than two types of the RAMPs are usually expressed at the different ratios in each cell line. Taken together, it is necessary to quantitatively measure the expression levels of CRLR and RAMPs to elucidate the signal transduction of AM and CGRP through the cell membrane.

In addition to fibroblasts, ECs and VSMCs, we have reported that AM is secreted from monocytes, macrophages, and related cell lines especially under inflammatory conditions. In the case of macrophage-derived RAW264.7 cells, AM apparently suppressed LPS-induced TNF-α production [6]. In this and preceding studies, we demonstrated that AM was actively secreted from fibroblasts and suppressed IL-1β-induced TNF-α production. These results suggest that AM can generally suppress TNF-α production. On the other hand, we have reported that AM stimulates a basal as well as TNF-α-, IL-1β-, or LPS-induced IL-6 production in Swiss 3T3 cells [12]. Although IL-6 is classified into the proinflammatory cytokine, it has been reported that IL-6 inhibits TNF-α production in vitro and in vivo [27–29]. Thus, both AM and IL-6 are deduced to alter TNF-α production in the same direction. TNF-α is well known as a major inflammatory cytokine in inflammatory responses, because the administration of monoclonal antibody against TNF-α or soluble TNF receptor blocks various acute and chronic responses in animal models of inflammatory diseases [13]. Based on these data, AM is deduced to function as an autocrine or paracrine factor suppressing the propagation of inflammation through the inhibition of TNF-α production, and the inflammatory cytokines may utilize AM as a feedback regulator of their production. Glucocorticoid is a potent anti-inflammatory drug, but it stimulates AM production in many types of cells [8,9,30]. The suppressive effect of AM on the cytokine-induced TNF-α production might be involved in a part of the anti-inflammatory action of the glucocorticoid.

In conclusion, we demonstrated that AM potently inhibited IL-1β-induced TNF-α gene transcription and secretion from Swiss 3T3 fibroblasts through the cAMP-PKA pathway. As fibroblasts actively synthesize and secrete AM, these findings suggest that AM is an autocrine or paracrine anti-inflammatory factor in the mesenchymal tissue.

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