Production of Adrenomedullin in Macrophage Cell Line and Peritoneal Macrophage*

Atsushi Kubo‡§, Naoto Minamino‡§, Yoshitaka Isumi, Takeshi Katafuchi, Kenji Kangawa‡, Kazuhiro Dohi‡, and Hisayuki Matsuo‡

From the ‡National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka 565-8565 and the §First Department of Internal Medicine, Nara Medical University, Shijo, Kashihara, Nara 634-8013, Japan

We demonstrate that adrenomedullin (AM) is produced and secreted from cultured murine monocyte/macrophage cell line (RAW 264.7) as well as mouse peritoneal macrophage. Immunoreactive (IR) AM secreted from RAW 264.7 cells was chromatographically identified to be native AM. To elucidate the regulation mechanism of AM production in macrophage, we examined the effects of various substances inducing differentiation or activation of monocyte/macrophage. Phorbol ester (TPA), retinoic acid (RA), lipopolysaccharide (LPS), and interferon-γ (IFN-γ) increased AM production 1.5–7-fold in RAW 264.7 cells in a dose- as well as time-dependent manner. By LPS stimulation, the AM mRNA level in RAW 264.7 cells was augmented up to 7-fold after 14 h incubation. RA exerted a synergistic effect when administered with TPA, LPS, or IFN-γ, whereas IFN-γ completely suppressed AM production in RAW 264.7 cells stimulated with LPS. Dexamethasone, hydrocortisone, estradiol, and transforming growth factor-β dose-dependently suppressed AM production in RAW 264.7 cells. AM production was also investigated in mouse peritoneal macrophage. Primary mouse macrophage secreted IR-AM at a rate similar to that of RAW 264.7 cells, and its production was enhanced 9-fold by LPS stimulation. AM was found to increase basal secretion of tumor necrosis factor α (TNF-α) from RAW 264.7 cells, whereas AM suppressed the secretion of TNF-α and interleukin-6 from that stimulated with LPS. Thus, macrophage should be recognized as one of the major sources of AM circulating in the blood. Especially in cases of sepsis and inflammation, AM production in macrophage is augmented, and the secreted AM is deduced to function as a modulator of cytokine production.

Adrenomedullin (AM) is a potent vasorelaxant peptide originally isolated from extracts of human pheochromocytoma by monitoring the elevating activity of platelet cAMP (1). AM shows slight homology with calcitonin gene-related peptide (CGRP) and has a potent and long-lasting depressor effect when injected intravenously into anesthetized rats (1, 2). We have shown that cultured endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) produce and secrete AM into culture medium (3, 4). The production and secretion of AM in VSMC and EC were augmented by interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and lipopolysaccharide (LPS) (5, 6), which are known to be major factors inducing septic shock (7–9). In the in vivo study, intravenous administration of LPS into rats actually elevated plasma AM concentration 20-fold and augmented AM gene expression in blood vessels, lung, and intestine (10). Plasma AM levels were also remarkably increased in patients with septic shock compared with those in healthy volunteers (11, 12). These data suggest the possibility that AM contributes to induction of refractory hypotension in septic shock.

On the other hand, macrophages are activated by exposure to stimuli of foreign bodies such as LPS and then start to produce and secrete various cytokines, such as IL-1 and TNF-α. These data suggest that macrophage is presumed to be another candidate for AM-producing cells in sepsis in addition to VSMC and EC. In fact, Miller et al. (13) have shown the expression of the AM gene in various tumor cell lines, including histiocyte lymphoma cell line (U937), by using the reverse transcriptase-PCR method. Recent reports have also demonstrated that AM or its mRNA is significantly detected in the alveolar macrophages and endometrial macrophages (14, 15). However, the secretion of AM from macrophage has not yet been studied. In this study, we demonstrate that AM is produced and secreted from cultured murine macrophage cell line (RAW 264.7) as well as murine peritoneal macrophage.

EXPERIMENTAL PROCEDURES

Materials—Human AM (40–52) and its N-Tyr derivative were synthesized by peptide synthesizer 431A (Applied Biosystems, Foster City, CA). The following materials were used: rat interferon-γ (IFN-γ) (Life Technologies, Inc.), murine recombinant IL-1β (Intragen, Purchase, NY), mouse recombinant TNF-α (Boehringer Mannheim, Mannheim, Germany), dexamethasone, human recombinant transforming growth factor-β (TGF-β), 12-O-tetradecanoylphorbol-13-acetate (TPA), forskolin, hydrocortisone (Wako Pure Chemical, Osaka, Japan), 17-estradiol, hyoxanthine (Nacalai Tesque, Kyoto, Japan), Escherichia coli LPS (serotype O26: B6) (Parsel+Lorei, Frankfurt, Germany), all-trans-retinoic acid, calcium gene-related peptide; EC, endothelial cell; VSMC, vascular smooth muscle cell; ELISA, enzyme-linked immunoadsorbent assay; NO, nitric oxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPA, 12-O-tetradecanoylphorbol-13-acetate; RA, retinoic acid; LPS, lipopolysaccharide; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α; IL-1, interleukin-1; IL-6, interleukin-6; TGF-β, transforming growth factor-β; LDL, low density lipoprotein; ox-LDL, oxidized LDL; ac-LDL, acetylated LDL; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; FCS, fetal calf serum; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; RIA, radiimmunoassay; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate.
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Adrenomedullin production in RAW 264.7 macrophages was characterized by gel filtration and reverse phase high performance liquid chromatography (HPLC) on a Biobeads S-X1 column. The chromatography was performed using an elution buffer containing 0.1 M sodium acetate (pH 5.2) and 0.1% (v/v) Triton X-100. The peaks were pooled and separated by reverse phase HPLC on a C18 column. The fraction containing adrenomedullin (AM) was collected and lyophilized. The lyophilized sample was dissolved in 0.9% saline and diluted with the incubation medium (Dulbecco's modified Eagle's medium). The concentration of AM in the incubation medium was determined using radioimmunoassay (RIA) for AM.

Preparation of Conditioned Medium—RAW 264.7 cells, grown to confluence in a 6-well dish, were washed twice with DMEM, replaced with 1 ml of DMEM containing 0.1% BSA and serum-free, and incubated for 7 days. The media were then replaced with 10% FCS DMEM and incubated for 4–14 h. The media were then collected, centrifuged, and ultracentrifuged to remove cell debris. The supernatant was lyophilized and used as a conditioned medium (CM).

Cell Culture—RAW 264.7 macrophages (an Abelson leukemia virus-transformed macrophage cell line of Balb/c mouse origin) were obtained from American Type Culture Collection (Rockville, MD) and were cultured in DMEM containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO2.

Preparation of Conditioned Medium—RAW 264.7 cells, grown to confluence in a 6-well dish, were washed twice with DMEM, replaced with 1 ml of DMEM containing 0.1% BSA and stimulants, and incubated for 1 h (n = 6). The media were then collected, centrifuged, and ultracentrifuged to remove cell debris. The supernatant was lyophilized and used as a conditioned medium (CM).

Preparation of Microperitoneal Macrophages—Thioglycolate-elicited macrophages were collected by peritoneal lavage with pyrogen-free saline at 7 days after intraperitoneally injecting 2 ml of sterile thioglycolate to 6-week-old C57/He mice. The macrophages were incubated in culture medium for 4–14 h. The media were then collected, centrifuged, and ultracentrifuged to remove cell debris. The supernatant was lyophilized and used as a conditioned medium (CM).

RESULTS

Production and Secretion of AM from RAW 264.7 Cells and Its Chromatographic Identification—We measured AM content in culture medium of RAW 264.7 cells after incubation for 4, 8, or 14 h by using RIA specific to AM. As shown in Fig. 1, AM content in culture medium increased almost linearly up to 14 h without any stimulation. Intracellular AM content of RAW 264.7 cells was also measured after incubation for 8 h and treatment with TNF-α, IL-6, and LPS. The intracellular level of AM was constant and much lower (less than 10%) than that in culture medium after 14 h incubation (data not shown). This result suggests that intracellular AM synthesis in RAW 264.7 cells is not stored in the cells but is secreted constitutively in the medium after synthesis. Thus, we measured AM content in the culture medium to evaluate AM production in RAW 264.7 cells.

To identify AM secreted into culture medium from RAW 264.7 cells, we characterized AM by gel filtration and reverse phase HPLC. The culture medium was collected after incubation with 10−7 M TPA for 14 h, since the secretion rate of AM from RAW 264.7 cells was low without any stimula-
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**FIG. 1.** Time-dependent AM production in RAW 264.7 cells stimulated with TPA, RA, LPS, and IFN-γ. RAW 264.7 cells were stimulated with TPA, RA, LPS, and IFN-γ for 4, 8, and 14 h at concentrations listed below. After stimulation, IR-AM concentration in culture medium was measured by RIA specific for AM. Closed circles, TPA (10^{-7} M); closed triangle, RA (10^{-5} M); open circles, LPS (100 ng/ml); open triangles, IFN-γ (100 units/ml); closed squares, control (without stimulation). Each point represents the mean ± S.E. of six separate dishes. *, p < 0.05.

**Regulation of IR-AM Production in RAW 264.7 Cells**—We first examined various substances that induced monocyte-macrophage differentiation or activation (26). Among them, TPA, RA, LPS, and IFN-γ significantly increased IR-AM secretion from RAW 264.7 cells compared with the control (Table I). In contrast, TNF-α and IL-1β, which augmented AM production in rat VSMCs and ECs (5, 6), showed no apparent effect on AM production in RAW 264.7 cells.

TPA, RA, LPS, and IFN-γ dose-dependently stimulated secretion of IR-AM from RAW 264.7 cells, and increased IR-AM concentration in the media to 164, 190, 600, and 460% of the control at concentrations of 10^{-7} and 10^{-5} M, 1000 ng/ml, and 100 units/ml, respectively (Fig. 3). Time-dependent effects of these substances in concentrations inducing almost maximal stimulation are shown in Fig. 1. AM production was augmented after 8 h stimulation in the cases of TPA, RA, and IFN-γ, whereas LPS was found to have already enhanced AM production before 4 h stimulation.

AM gene transcription was estimated by RNA blot analysis, and AM mRNA levels in RAW 264.7 cells stimulated with LPS (100 ng/ml) were measured after 0, 4, 8, and 14 h stimulation. LPS time-dependently increased AM mRNA level (Fig. 4, a and c), and this result was parallel to the peptide production level of AM in RAW 264.7 cells. Furthermore, AM mRNA level after 8 h stimulation was elevated according to the increase of LPS concentration and was also well correlated with the peptide production level of AM shown in Fig. 3c. AM mRNA levels were also increased by stimulation with RA, TPA, and IFN-γ to 116, 148, and 199% of the control in concentrations of 10^{-7} and 10^{-5} M and 100 units/ml after 8 h stimulation, respectively (data not shown).

Next, we examined effects of steroid hormones on IR-AM production in RAW 264.7 cells. Dexamethasone, hydrocortisone, and estradiol reduced IR-AM concentrations to 24, 49, and 67% of the control at the concentrations of 10^{-5}, 10^{-6}, and 10^{-7} M, respectively (Fig. 5, a, b, and c). We also investigated the effects of TGF-β that has been recognized as a potential inhibitor of macrophage activation (27). TGF-β suppressed AM production to 75% of the control in a concentration of 10 ng/ml (Fig. 5d).

To evaluate cooperative effects of stimulators and suppressors, IR-AM concentration in the cultured medium of RAW 264.7 cells was measured by co-administering substances that had significantly altered AM production. When 10^{-5} M of RA was administered with TPA (10^{-8} M), LPS (100 ng/ml), or IFN-γ (100 units/ml), IR-AM concentration in the medium was elevated to a level higher than that derived from summation of the effect of each substance (Fig. 6). Specifically, simultaneous
addition of LPS and RA increased IR-AM content in the medium to 10 times higher than that of control. Although IL-1β, TNF-α, and LPS showed additive effects on AM production in cultured rat VSMCs (5), combining these three substances did not induce any cooperative effect on AM production in RAW 264.7 cells (data not shown). Since combination of LPS and IFN-γ has been reported to induce synergistic effects on cytotoxic activity and nitric oxide (NO) production in RAW 264.7 cells (28), we examined the effect of co-administration of LPS and IFN-γ. In contrast, IFN-γ suppressed IR-AM secretion stimulated with 10 ng/ml LPS even at a concentration of 1 unit/ml (Fig. 7).

We also examined effects of glucocorticoids and TGF-β on AM production in RAW 264.7 cells stimulated with LPS, since these substances have been reported to suppress production of NO and cytokines, such as TNF-α, under stimulation with LPS (29, 30). When dexamethasone and hydrocortisone were each administered simultaneously with LPS, these substances elicited slight inhibitory effects on AM secretion from RAW 264.7 cells (data not shown). However, 1 h pretreatment of the cells with dexamethasone or hydrocortisone strongly inhibited IR-AM production stimulated with LPS (100 ng/ml) (Fig. 8). These results indicate that glucocorticoids are potent inhibitors of AM production in RAW 264.7 cells, overcoming the effect of LPS. TGF-β (10 ng/ml) also suppressed AM production to 60% that stimulated with LPS (100 ng/ml) (data not shown).

We examined the effect of the substances that elevate intracellular cAMP concentrations, such as forskolin, 8-Br-cAMP, and vasoactive intestinal polypeptide, on AM production in...
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RAW 264.7 cells, since we previously showed that these substances suppressed AM production in VSMCs (31). However, these substances elicited no significant effect on AM production in RAW 264.7 cells (Table I).

Finally, effects of lipoproteins, such as LDL, ac-LDL, and α-LDL, on AM production in RAW 264.7 cells were estimated, since macrophages were well known to have an important role in cholesterol deposition in atherosclerosis (32). LDL, ac-LDL, and α-LDL significantly increased AM production in RAW 264.7 cells to 141, 123, and 181% of the control, respectively (Table I).

AM Modulates Secretion and Gene Transcription of TNF-α and IL-6 in RAW 264.7 Cells—To elucidate physiological functions of AM secreted from macrophages and their cell lines, we examined the effects of AM on production of inflammatory cytokine, TNF-α, IL-1β, and IL-6, in RAW 264.7 cells, since these cytokines were known to play important roles in the pathophysiology of sepsis. AM increased TNF-α concentration in the culture medium of RAW 264.7 cells to about 160% in the absence of LPS stimulation (Fig. 9a). LPS (1 ng/ml) markedly enhanced TNF-α production about 120-fold in RAW 264.7 cells.

Next, TNF-α mRNA level in RAW 264.7 cells was evaluated by real time quantitative PCR. TNF-α mRNA level was time-dependently increased up to 8 h by stimulation with LPS (100 ng/ml), and AM (10^{-6} M) suppressed TNF-α mRNA levels maximally to 77% at 4 h after LPS stimulation (Fig. 10b). In addition, LPS dose-dependently increased TNF-α mRNA levels, and AM (10^{-6} M) suppressed TNF-α mRNA levels maximally to 66% at a dose of 10 ng/ml after 4 h of stimulation (Fig. 10c).

Production of IR-AM in Murine Peritoneal Macrophages—Based on data obtained from macrophage cell line, RAW 264.7,
we next assessed whether AM was produced and secreted from primary mouse macrophages. Mouse primary macrophages secreted IR-AM without any stimulation at a rate (0.2 ± 0.04 fmol/10⁶ cells/24 h) about 15% that of RAW 264.7 cells (Table II). Peritoneal exudate macrophages were treated with TPA (10⁻² M), RA (10⁻⁶ M), LPS (100 ng/ml), and IFN-γ (100 units/ml) for 24 h. Among them, LPS increased IR-AM content in the culture medium about 7-fold compared with that of control, but TPA and RA slightly increased IR-AM secretion (Table II). We found that IFN-γ (10⁻³ M) inhibited IR-AM secretion stimulated with LPS in a manner similar to that of RAW 264.7 cells. However, TPA inhibited IR-AM secretion stimulated with LPS in contrast to the results obtained in RAW 264.7 cells. Based on these results, mouse primary macrophages were shown to have the ability to produce and secrete AM. Regulation of AM production in primary macrophage was deduced to be similar to that of RAW 264.7 cells, as observed in the case of LPS, but was not completely identical to that of the established cell line.

Intracellular cAMP Production in RAW 264.7 Cells and Mouse Peritoneal Macrophages—Since AM was shown to modulate production of cytokines in RAW 264.7 cells, we examined the expression and properties of AM receptors on this macrophage cell line and mouse peritoneal macrophages. AM (10⁻⁶ M) did not increase cAMP production in RAW 264.7 cells (data not shown). On the other hand, AM (10⁻⁶ M) augmented the intracellular cAMP level in mouse peritoneal macrophages 6.7-fold compared with the basal level. Rat CGRP dose-dependently elevated the intracellular cAMP level with an ED₅₀ value of 7.6 × 10⁻¹⁰ M, and AM antagonist, human AM-(22-52) did not affect the ED₅₀ value. In the presence of 10⁻⁶ M CGRP antagonist, human CGRP-(8–37), the dose-response curve of
the cAMP production was markedly shifted to the high concentration side, and the ED_{50} value was 145 times higher than that without CGRP antagonist. In contrast, the ED_{50} value of rat AM in the cAMP production assay was 4.3 × 10^{-7} M, being 570 times higher than that of rat CGRP. Furthermore, the dose-response curve of AM was not shifted with human AM (22–52) but with human CGRP (8–37). Based on these results, the receptors expressed on mouse peritoneal macrophages are found to be specific for CGRP, and AM is not deduced to significantly stimulate the cAMP production under the physiological conditions.

### DISCUSSION

In this study, we demonstrated that the macrophage cell line RAW 264.7 produced and secreted IR-AM that was identified as native AM by chromatographic procedures (Fig. 2). Although the production level of AM in RAW 264.7 cells was lower than that of cultured rat ECs and VSMCs (4, 5), we found substances that stimulated differentiation and activation of monocyte/macrophage potently augmented AM production (Table I). As shown in Fig. 3, the secretion rate of AM from RAW 264.7 cells stimulated with LPS or IFN-γ was comparable to that of cultured VSMCs, indicating that monocyte/macrophage system was another candidate for secreting AM into the bloodstream.

TPA and IFN-γ increased IR-AM concentration in the culture medium of RAW 264.7 cells (Figs. 1 and 3). TPA, a potent activator of protein kinase C, induces the differentiation of this cell line into macrophage and stimulates production of IL-1 activator of protein kinase C, induces the differentiation of this culture medium of RAW 264.7 cells (Figs. 1 and 3). TPA, a potent inducer of differentiation of monocyte/macrophage and to enhance production of NO, TNF-α, and IL-1β (28, 34, 35). RA increased AM production in RAW 264.7 cells (Figs. 1 and 3) and stimulated AM production synergistically when administered with TPA, IFN-γ, or LPS (Fig. 6). RA has been reported to activate macrophage and potentiate phagocytosis and IL-1 activity in RAW 264.7 cells (36). This result suggests that RA activates macrophages to conditions where they can respond more potently to any stimulation and produce AM at higher rates. AM production is thought to be increased by stimulators that induce differentiation or activation of macrophages. On the other hand, mouse peritoneal macrophages failed to increase AM production by stimulation with TPA, RA, and IFN-γ. RAW 264.7 cells were established from peritoneal macrophages, but the cells have been transformed and immortalized by infection of Abelson leukemia virus (16). Although peritoneal macrophages and RAW 264.7 cells share many biological features, these cells are reported to elicit different responses in NO synthesis when stimulated with phorbol ester (37). We also observed in this study that AM increased cAMP production in peritoneal macrophages but not in RAW 264.7 cells. These findings lead us to deduce that the differences in results are due to different properties of established cell lines and primary culture.

Glucocorticoids have been shown to enhance AM production, and estradiol increased it in rat EC and VSMC EC (6, 38), but these reagents were found to be potent suppressors of AM production in RAW 264.7 cells (Fig. 5). Glucocorticoids have been shown to antagonize differentiation of macrophage (39) and are potent anti-inflammatory agents that inhibit production and release of NO and cytokines, such as IL-1, IL-6, and TNF-α, from macrophages (29, 40–42). These data indicate that gene transcription and production of AM in the macrophage cell line are regulated in a manner similar to NO synthase and inflammatory cytokines but distinct from AM in EC and VSMC (4–6, 38). In the vascular wall, glucocorticoids are known to increase vascular tone, but AM secreted from EC and VSMC contributes to reduction of vascular tone (43, 44). In the monocyte/macrophage system, AM is shown to be produced and secreted from RAW 264.7 cells and to participate in the regulation of TNF-α and IL-6 production. Taken together, we deduce that the intracellular signal integration mechanism regulating transcription of AM gene must be different in each cell line in order to exert its divergent effects after secretion from the cells. TGF-β is recognized as a potential inhibitor for activation of macrophage as well as for production of NO and TNF-α (27, 30) and also suppresses AM production in RAW 264.7 cells. Based on these results, AM production in RAW 264.7 cells is thought to be increased or decreased mainly by the substances that stimulate or suppress differentiation and activation of macrophages.

We have reported that production and secretion of AM from VSMC and EC are augmented by LPS stimulation (5, 6) and that intravenous administration of LPS into rats increases plasma AM concentration 20-fold and augments AM gene transcription in almost all tissues including blood vessels (10). Furthermore, an average concentration of plasma AM in patients with septic shock has recently been reported to be 10–45 times higher than that of healthy volunteers (11, 12). Based on these data, we have attributed high plasma AM concentrations in LPS-injected rats and in patients with septic shock to the elevated production of AM in ECs and VSMCs. As shown in the present study, LPS is shown to be the most potent stimulator of AM production and gene transcription in RAW 264.7 cells (Figs. 1, 3, 4, and 11). Even in primary mouse macrophages, LPS in a concentration of 1000 ng/ml also increases AM production about 9-fold compared with that of control. We also found in our recent studies that human leukemia cell line and peripheral blood monocytes produce AM according to their differentiation into macrophages and that LPS stimulation augments AM production in monocyte-derived macrophages (45). Regulation of AM production in RAW 264.7 cells is found to be similar to that of inflammatory cytokines, such as IL-1, IL-6, and TNF-α, which contribute to the pathophysiology of sepsis (7–9). These data indicate that macrophage is another candidate for secreting circulatory AM in the case of sepsis.

As shown in Fig. 8, glucocorticoids strongly suppressed AM production in RAW 264.7 cells even in the presence of LPS. In the in vivo experiments, glucocorticoids have been reported to induce tolerance to LPS and reduce mortality in animals with experimentally induced septic shock (46). Glucocorticoids suppress production of inflammatory cytokines and NO, which results in reduction of vasodilation (40–42). This suppressive...
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effect on production of inflammatory cytokines and NO is thought to be one of the reasons why glucocorticoid induces tolerance to LPS and prevents septic shock. Thus, our results raise the possibility that AM produced in macrophage is an additional modulator in the pathogenesis of sepsis. Pretreatment of macrophages with TGF-β has been reported to attenuate their ability to produce TNF-α, NO, and prostaglandin E2 in response to LPS (30, 47). AM production in RAW 264.7 cells was also suppressed by TGF-β (Fig. 5d). In this study, macrophage and macrophage cell line were shown to actively produce AM after LPS stimulation, whereas AM production was suppressed by glucocorticoids and TGF-β. These results suggest that AM plays a significant role in the pathogenesis of sepsis and inflammation in addition to its function as a vasodilator.

Our previous study has shown that IFN-γ suppresses AM production in rat VSMCs (31). However, IFN-γ stimulates AM secretion in RAW 264.7 cells when administered alone (Fig. 3). AM gene has many IFN-γ-responsive elements in the promoter region (48). McDowell et al. (49) reported that RAW 264.7 cells, but not less mature WEHI-3 cells, were able to utilize trans-activated IFN-γ-responsive elements and induce rapid transcription by IFN-γ stimulation. These facts indicate that the IFN-γ-responsive element is utilized differently depending on the cells and that in RAW 264.7 cells IFN-γ induces gene transcription of AM via IFN-γ-responsive elements. On the contrary, IFN-γ strongly inhibits AM secretion from RAW 264.7 cells and mouse peritoneal macrophages stimulated with LPS (Fig. 7 and Table II). In the case of RAW 264.7 cells stimulated with LPS, IFN-γ was reported to induce hyporesponsiveness to LPS and reduce the production of TNF-α (50). Another report also showed that IFN-γ strongly suppressed the expression of IL-1β gene stimulated with LPS (51). These data suggest that IFN-γ acts as inhibitory cytokine under the chronically inflammatory conditions stimulated with LPS. Since the effects of IFN-γ are different in each cell type and dependent on the physiological condition of the cells, discordant responses of IFN-γ are likely to be derived from differences in transcriptional regulation between VSMCs and RAW 264.7 cells as well as between the presence and absence of LPS.

In order to identify physiological functions of AM secreted from macrophages, we examined whether AM modulates secretion of TNF-α, IL-1β, and IL-6, the cytokines mainly produced in macrophages and RAW 264.7 cells (7–9). As shown in Fig. 9, AM increased a basal secretion level of TNF-α from RAW 264.7 cells, whereas AM significantly suppressed TNF-α secretion when the cells were stimulated with LPS. AM was also shown to suppress secretion of IL-6 stimulated with LPS in this study. RNA blot analysis and real time quantitative PCR analysis showed that AM lowered TNF-α and IL-6 mRNA levels in RAW 264.7 cells stimulated with LPS (Fig. 10), suggesting that AM inhibits cytokine production at the step of gene transcription. Kamoi et al. (52) reported that AM inhibited secretion of cytokine-induced neutrophil chemoattractant, belonging to IL-8 superfamily, in rat alveolar macrophages stimulated with LPS. These results indicate that AM secreted from macrophages not only dilates blood vessels but also modulates production of inflammatory cytokines, TNF-α, IL-6, and cytokine-induced neutrophil chemoattractant.

To assess the properties of AM receptors expressed on RAW 264.7 cells and mouse peritoneal macrophages, we measured intracellular cAMP concentrations in these cells. However, AM did not significantly increase the intracellular cAMP concentration in RAW 264.7 cells. In the case of mouse peritoneal macrophages, only 10−6 M AM increased intracellular cAMP concentration, and receptors expressed on primary macrophages were found to be specific for CGRP. These results sug-

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