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Lipopolysaccharides stimulate adrenomedullin synthesis in intestinal epithelial cells; release kinetics and secretion polarity

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Running head: Adrenomedullin synthesis in intestinal epithelial cells

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ABSTRACT

Adrenomedullin (AM), a potent vasodilator peptide initially isolated from a human pheochromocytoma, functions as an antimicrobial peptide in host defense. In this study, we investigated changes in AM levels in intestinal epithelial cells and the mechanism of its secretion and cellular polarity after exposure to lipopolysaccharides (LPS). When a rat small intestinal cell line (IEC-18 cells) was exposed to LPS, enzyme-linked immunosorbent assay revealed a dose-dependent increase in AM together with an increase in AM mRNA expression, as determined by real-time polymerase chain reaction. Up-regulation of AM by LPS was dose-dependently inhibited by LY294002, PD98059, SP600125 and calphostin-C, suggesting the involvement of the phosphatidylinositol 3 kinase, extracellular signal-regulated kinase, c-Jun NH2-terminal kinase and protein kinase C pathways, respectively, in this process. When polarized IEC-18 cells in a Transwell chamber were stimulated with

LPS, AM secretion was directed primarily toward the side of LPS administration (either the apical or basolateral side). *In situ* hybridization revealed that AM mRNA was expressed in epithelial cells and in the connective tissue in the lamina propria of the jejunum after intraperitoneal or oral administration of LPS. Higher levels of AM mRNA expression were observed in rats treated with LPS via the intraperitoneal route, compared with those treated via the oral route. These findings suggest that intestinal AM plays an important role in mucosal defense in the case of intestinal luminal infection, as well as in the modulation of hemodynamics in endotoxemia.

Key words: adrenomedullin, small intestine, antimicrobial peptides, polarity, *in situ* hybridization

Abbreviations: AM, adrenomedullin; LPS, lipopolysaccharides; PI3K, phosphatidylinositol 3 kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; PKC, protein kinase C.

1. Introduction

Adrenomedullin (AM), a 52-amino-acid peptide originally isolated from a human pheochromocytoma and the adrenal medulla, belongs to the calcitonin family, which includes calcitonin gene-related peptides and amylin [16]. A potent vasodilator, AM relaxes vascular smooth muscle via cAMP and the release of nitric oxide from the endothelium [12]. It has also recently been implicated in a diverse range of physiological functions, including bronchodilation, antimicrobial activity, cell growth regulation, survival from apoptosis and induction of angiogenesis [2, 16]. IL-1β, TNF-α and lipopolysaccharide (LPS) potently stimulate AM production in vascular smooth muscle cells, endothelial cells, cardiac myocytes and fibroblasts [7, 11, 13, 19, 25]. Plasma AM levels are increased in various pathological conditions such as hypertension, chronic renal failure, congestive heart failure and particularly, sepsis, in which the highest levels of circulating AM have been observed [10, 14, 18, 21]. Zhou et al. previously reported that the AM level was 42% higher in the portal blood than in the systemic blood after cecal ligation and puncture suggesting that the small intestine is an important source of adrenomedullin release during polymicrobial sepsis [31]. Intestinal epithelial cells form the first line of defense against microorganisms in the intestinal lumen and actively participate in intestinal immune networks. The ability to prevent infection depends on both the innate and adaptive immune mechanisms. Increasing evidence suggests that antimicrobial peptides act as signaling molecules for communication between the innate and adaptive immune systems. Several investigators have recently reported that AM is expressed on various mucosal surfaces, where it functions as an important antimicrobial effector molecule in host defense [2, 4, 9, 20]. Some epithelial antimicrobial peptides are constitutively expressed, whereas others are induced by the presence of microorganisms, endogenous proinflammatory cytokines, hypoxic conditions and/or LPS [6, 19, 23, 27]. Although a variety of cytokines may be important in the initiation and propagation of intestinal injury, LPS, a component of Gram-negative bacteria, appears to be one of the earliest to act [5]. Whether intestinal epithelial cells release AM after exposure to LPS, however, remains to be determined.

The goals of the present study were to investigate induction of AM expression and the

polarity of its secretion by exposing IEC-18 cells, a non-transformed rat small intestinal cell line, to LPS and clarify the underlying mechanisms of AM up-regulation by LPS. In addition, to determine the cellular localization of AM expression, we performed an *in situ* hybridization study in rat intestine using probes specific for AM peptide-coding regions.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum, penicillin, streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, U.S.A.). LY294002, PD98059, SP600125, SB203580 and calphostin-C were purchased from Calbiochem (San Diego, CA, U.S.A.). *Escherichia coli* O111:B4 LPS and dimethyl sulfoxide (DMSO) were purchased from Sigma (St Louis, MO, U.S.A.). Tissue Fixative and Probe Diluent were purchased from Genostaff (Tokyo, Japan). The adrenomedullin EIA kit was purchased from Phoenix Pharmaceuticals (Belmont, CA, U.S.A.). The RNeasy Kit was purchased from Qiagen (Chatswoth, SA, U.S.A). SYBER R ExScript TM RT-PCR Kit was obtained from Takara Bio. (Shiga, Japan). Transwell chambers were purchased from Corning (NY, U.S.A.). CellTiter® 96 AQueous One Solution was purchased from Promega (WI, U. S. A.). Sprague-Dawley rats were purchased from Charles River Laboratories Japan (Kanagawa, Japan).

2.2. Cell Culture

IEC-18 cells (derived from fetal rat ileum) were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan) and cultured as described previously [15].

2.3. IEC-18 cell monolayers on Transwell filters

For the polarity assay, IEC-18 cells were seeded at confluent density on the tops of collagen-coated microporous supports (0.4 μm pore size; 1.0 cm^2 growth area) in Transwell chambers. After culturing the IEC-18 cells for 14 days, the cells were stimulated for 24 h with 1 ng/mL of LPS in the upper compartment (apical side) or lower compartment (basolateral side), and the culture supernatants in both compartments were collected to measure AM concentrations. The impermeability of the monolayers to AM was verified by

addition of a relatively high dose of AM (3 ng/mL) to the basolateral compartment of non-stimulated monolayers.

2.4. AM Assays

The AM concentration was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) and a commercially available kit according to the manufacturer's instructions. The samples were read using an MTP-120 Microplate Reader (Corona Electric Co., Ibaragi, Japan) set at an absorbance of 450 nm. The cell proliferation assay was performed with the CellTiter® 96 AQueous One Solution kit according to the manufacturer's instructions, and the results for AM secretion were corrected by cell number.

2.5. Inhibition study

IEC-18 cells were pre-exposed for the indicated time period to either calphostin C (60 min, 200 nM, 20 nM; an inhibitor of PKC), SB203580 (60 min, 10 μM, 1 μM; an inhibitor of the enzymatic activity of p38 MAPK), PD98059 (30 min, 5 μM, 0.5 μM; an inhibitor of induced protein phosphorylation and the activation of ERK), SP600125 (30 min, 10 μM, 1 μM; an inhibitor of JNK), LY294002 (180 min, 10 μM, 1 μM; an inhibitor of PI3K) or the corresponding vehicle controls. LPS was then added for another 24 h and the supernatants were harvested as described above. Stock solutions of the inhibitors were prepared using DMSO. The final DMSO concentration in the medium for the reagents was kept at no more than 0.1% in all cell cultures, and no detectable effects on cell growth or AM secretion were noted.

2.6. RNA extraction and real-time polymerase chain reaction

Total cellular RNA was isolated using the RNeasy Kit (Qiagen, Chatswoth, SA) according to the manufacturer's instructions. RNA was quantitated by measuring OD260 before reverse transcription and PCR amplification. A 200-ng sample of total RNA was used to synthesize the first-strand of cDNA using the SYBER R ExScript TM RT-PCR Kit (Takara Bio, Japan) according to the manufacturer's instructions. The AM expression levels were determined using real-time PCR with the Smart Cycler version 2 (Takara Bio., Tokyo, Japan) and DNA binding dye SYBR Green I for the detection of the PCR products. The gene-specific PCR primers used for AM were as follows: sense, 5'-

GGGAACTACAAGCGTCCAGCA-3'; antisense, 5'- TCTGGCGGTAGCGTTT GACTC-3'; and for GAPDH: sense, 5'- GACAACTTTGGCATCGTGGA-3'; and antisense, 5'- ATGCAGGGATGATGTTCTGG-3'. Primers were chosen according to their cDNA sequences in the EMBL data library index, GeneBank accession no. **NM 012715** for AM and **NM 017008** for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression level of the AM gene was evaluated as the ratio of its mRNA level to the GAPDH mRNA level.

2.7. In situ hybridization

Experiments were performed on 6-week-old male Sprague-Dawley rats weighing 200 -250 g each according to a procedure approved by the Committee on the Use of Living Animals for Research and Teaching of Tokyo Dental College. Rats were fasted overnight, anesthetized with intraperitoneal injection of 50 mg/kg sodium pentobarbital, treated with 10 mg/kg LPS via either an intraperitoneal or oral route and decapitated after 3 h. Control animals were treated with 0.9% sodium chloride intraperitoneally or orally. *In situ* hybridization was carried out, as described previously, under a contract with Genostaff (Tokyo, Japan) [8]. The jejunum was dissected, fixed with Tissue Fixative, embedded in paraffin and sectioned. Tissue sections were deparaffinized, fixed in 4% paraformaldehyde in PBS. A 411-base pair DNA fragment corresponding to nucleotide positions 5-415 of rat AM cDNA (GeneBank accession no. **NM 012715**) was subcloned and used for the generation of sense and antisense RNA probes. RNA probes were prepared using a digoxigenin RNA labeling mixture (Roche Applied Science, Mannheim, Germany). Hybridization was performed using the probes at concentrations of 100 ng/mL in Probe Diluent at 60°C for 16 h. The probes were visualized using the Digoxigenin Detection Kit (Roche Diagnostics). The sections were counterstained with Kernechtrot stain solution (Mutoh, Tokyo Japan), dehydrated and mounted with Crystal/Mount (Biomeda, Plovdiv, Bulgaria).

2.8. Statistical analysis

Data are expressed as means ± S.E. The statistical significance of differences in the results was evaluated using an unpaired ANOVA, and *P* values were calculated by Tukey's method. $P<0.05$ was accepted as statistically significant.

3. Results

3.1. AM release by LPS-exposed intestinal epithelial cells

The AM concentrations in conditioned medium containing LPS-stimulated IEC-18 cells were determined using ELISA. Conditioned media containing IEC-18 cells exposed to LPS at concentrations of 10^{-1} ~ 10 ng/mL contained significantly higher concentrations of AM than the controls (Fig. 1A). Figure 1B shows the time course of changes in AM release induced by 1 ng/mL LPS. Stimulation of AM release by IEC-18 cells was evident within 24 h of LPS exposure.

3.2. Real-time PCR analysis of AM mRNA in IEC-18 cells

We then determined whether LPS stimulation increased AM mRNA concentration in IEC-18 cells. The time-course changes in LPS-induced AM mRNA synthesis are shown in Fig. 2A. AM mRNA transcripts were first noted at 1 h after stimulation with LPS. AM expression peaked at 3 h after stimulation, decreasing thereafter. The dose-response relationship between LPS and AM mRNA is shown in Fig. 2B. Although exposure of cells to LPS at concentrations of 0.1 ng/mL and 1.0 ng/mL resulted in a dose-dependent increase in AM mRNA synthesis, exposure to a higher concentration (10 ng/mL) had a suppressive effect on AM mRNA synthesis.

3.3. Polarized secretion of AM by IEC-18 cells after stimulation with LPS

As intestinal epithelial cells are functionally polarized, we cultured IEC-18 cells to determine the polarity of AM secretion in LPS (1 ng/mL)-stimulated intestinal epithelial cells. After 24 h incubation, the culture medium was harvested from both compartments and the AM concentrations were determined using an ELISA. As shown in Fig. 3, AM was constitutively secreted predominantly to the apical medium in the control cells. When LPS was added to the apical compartment, secretion of AM into the medium was significantly enhanced, predominantly into the apical compartment. In contrast, when LPS was administered basolaterally, AM was secreted predominantly to the basolateral medium.

3.4. Inhibition study

To elucidate the molecular mechanisms of AM secretion, we preincubated the IEC-18 cells with a panel of specific inhibitors of signaling. Treatment of the IEC-18 cells with LY294002 (a PI3K inhibitor), PD98059 (an ERK inhibitor), SP600125 (a JNK inhibitor) and calphostin-C (a PKC inhibitor) dose-dependently decreased LPS-mediated AM secretion. However, SB203580 (a p38 MAKP inhibitor) showed no effect on LPS-stimulated AM secretion (Fig. 4).

3.5. Detection and localization of AM mRNA in rat jejunum using in situ hybridization

Sections of the jejunum were subjected to *in situ* hybridization 3 h after administration of LPS via either an intraperitoneal or an oral route. At 3 h after intraperitoneal injection of LPS (10 mg/kg), AM-coding mRNA was exclusively localized in epithelial cells in the crypt area or villus area, in the connective tissue in the lamina propria and in tunica muscularis (Fig. 5A (a and c)). In the jejunum of rats treated with LPS via the oral route, AM mRNA expression was found in the epithelial cells in the crypt area and in the connective tissue in the lamina propria, although the signals were smaller than those in the jejunum of rats treated via the intraperitoneal route (Fig. 5B (a and c)). The absence of staining with the sense-strand riboprobe demonstrated the specificity of the technique (Fig. 5A (b and d), 5B (b and d)). In each experiment, *in situ* hybridization from small intestinal sections from the control rats, which received an intraperitoneal saline injection or oral saline administration, revealed no positive signals with the antisense probes (data not shown).

4. Discussion

AM is expressed in the mucosal surfaces of the digestive, respiratory and reproductive systems, and plays a key role in mucosal defense, possibly as an antimicrobial agent [1, 2, 4, 20]. On the other hand, AM plays an important role in regulating systemic hemodynamics under various pathophysiological conditions, especially in sepsis [18, 21, 30]. The AM released by the gut is postulated to be of biological significance in two ways: first, it contributes to mucosal defense by its antimicrobial activity, and secondly, it increases AM to the required levels under fatal conditions such as sepsis

The results of our study clearly show that LPS-stimulated secretion of AM by cultured intestinal epithelial cells. The time course for this change was similar to that observed in portal blood in a sepsis model, suggesting the possibility that the small intestine is an important source of AM during sepsis [21].

Few reports have referred to the polarity of AM secretion in intestinal epithelial cells. In our study, the secretion of AM was primarily toward the side of LPS stimulation. Luminal secretion of AM will predominate when infection occurs at the apical surface of the mucosa, and may contribute to the mucosal defense system by blocking bacterial invasion. In contrast, basolaterally secreted AM would counter bacteria or endotoxins that have penetrated as far as the subepithelium by bacterial translocation or where disturbed mucosal integrity has resulted in enhanced permeability [15]. Moreover, in the presence of systemic inflammation, such as in sepsis, AM secretion from the basolateral cell surface could explain why the small intestine is a source of AM release. The fact that the polarity of AM secretion is directed toward the side of inflammation seems to be relevant to explaining the biological significance of AM, both as an antimicrobial peptide and a vasodilator peptide, in systemic circulation.

Ishimitsu et al. reported that both NF-IL6 and AP-2 sites were necessary for the regulation of AM production [11]. However, the intracellular signal transduction pathways involved in the regulation of AM production are, as yet, largely unexplored. Recently, the significance of the MAPK pathways including ERK, p38 and JNK pathway, in response to LPS have been reported [3, 24]. The classical ERK pathway can be activated by Ras-GTP; however, new mechanisms of ERK activation have been suggested by the PI3K and PKC pathways [22, 24]. On the other hand, in several receptors, AP-2, which is also a key transduction pathway in the regulation of AM, was activated by PKC [28]. Therefore, we chose ERK, p38, JNK, PI3K and PKC pathway inhibitors to elucidate the mechanism of AM secretion. To the authors' knowledge, the present study is the first to demonstrate that the PI3K, ERK, PKC and JNK pathways are involved in LPS-induced AM.

The localization of AM expression in the intestine is controversial [2, 4, 9, 17, 31]. Cameron and Fleming reported that AM mRNA was strongly expressed in the microvilli of

the plicae circulares, as well as in the mucosa and submucosa [4]. In our *in situ* hybridization study AM-coding mRNAs were localized exclusively in simple columnar epithelial cells, goblet cells, intestinal crypt cells and connective tissue in submucosa after intraperitoneal administration of LPS. The lack of positive AM mRNA signals in the control rat group in our study indicates that AM mRNA is expressed relatively weakly, as suggested by Zhou et al., so that positive signals cannot be detected, even when actually present [31]. The other purpose of this study was to investigate how the distribution and intensity of the AM mRNA signals are modified by the intraperitoneal and oral routes of LPS administration. As described in previous reports, the plasma endotoxin level is not affected by acute oral LPS treatment [26]. Therefore, oral administration of LPS is a model of luminal bacterial infection in the intestine, whereas intraperitoneal administration of LPS is a model of sepsis. In the orally treated rats in this study, a lower proportion of cells showed positive signals than in the intraperitoneal group. One possibility is that only a small dose of LPS actually reaches the small intestine as a result of the detoxification of LPS, including LPB-binding protein [29]. Another possibility is that intraperitoneal LPS administration, an experimental model of sepsis, stimulates AM more strongly than oral LPS administration. Together with the results of our polarity study, the predominant secretion of AM in intestinal epithelial cells by subepithelial LPS stimulation may explain why the intestine plays an important role in increasing the levels of circulating AM during sepsis.

In conclusion, we have presented evidence that intestinal epithelial cells secrete significant amounts of AM after exposure to LPS and that the AM secretion is directed primarily toward the side of LPS administration. *In situ* hybridization revealed that higher levels of AM mRNA signals were observed in rat jejunum after intraperitoneal administration of LPS, compared with oral administration. These findings suggest a role for AM both in mucosal defense and in regulating systemic hemodynamics during systemic inflammation.

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FIGURE LEGENDS

Figure 1. (A) Kinetics of AM release by rat intestinal epithelial cells stimulated with LPS. IEC-18 cells were exposed to LPS at concentrations ranging from 10^{-1} to 10 ng/mL. After 24 h, conditioned medium was collected and AM levels measured using an enzyme-linked immunosorbent assay (ELISA). Conditioned medium from untreated IEC-18 cells served as a control. Results are expressed as means ± SE. * $P < 0.001$, ** $P < 0.01$ compared with control.

Fig. 1. (B) Time course of AM release by LPS-exposed IEC-18 cells. Cells were exposed to LPS at 1.0 ng/mL, and conditioned medium collected 6, 12, 24 and 48 h later. AM levels were measured using an ELISA. Results are expressed as means ± SE. * $P < 0.001$, compared with unstimulated control cells.

Fig. 2. (A) Kinetics of time-course changes in amounts of AM mRNA in response to LPS. Confluent IEC-18 monolayers were incubated with LPS for period indicated. For quantification of AM mRNA, total RNA was reverse-transcribed and analyzed using real-time PCR. Results are expressed as means ± SE. * $P < 0.01$, ** $P < 0.05$ compared with control.

Fig. 2. (B) Dose-dependent increase in AM mRNA after LPS stimulation. IEC-18 cells were exposed to concentrations of LPS ranging from 10^{-1} to 10 ng/mL. Results are expressed as means ± SE. * $P < 0.001$ compared with control.

Fig. 3. Polarized secretion of AM induced by LPS. Monolayers of IEC-18 cells in Transwell were stimulated with LPS (1.0 ng/mL) on apical and basolateral sides, and supernatants obtained from upper and lower chambers. AM levels were measured using an ELISA. Results are expressed as means ± SE. * $P < 0.01$, ** $P < 0.05$ compared with the same side of control.

Fig. 4. Effect of intracellular kinase inhibitors on AM secretion in IEC-18 cells. IEC-18 cells were preincubated with vehicle control, calphostin-C, LY294002, PD98059, SB203580 or SP600125 for indicated time period and at indicated concentration, as described in Section 2, and then stimulated with LPS for 24 h. AM released into culture supernatants was measured using an ELISA. Results are expressed as means ± SE. * $P <$

0.001 ** P <0.01 *** P <0.05 compared with LPS alone.

Fig. 5. (A) Photomicrograph of results of *in situ* hybridization performed with antisense (a and c) or sense (b and d) AM riboprobes in rat ileum after 3 h intraperitoneal LPS treatment. Expression of AM mRNA was evident in enterocytes (small arrows, a), goblet cells (small arrowheads, a and c), and connective tissue in lamina propria (large arrows, a and c). Signals were also seen in crypt epithelium (asterisks, c), and tunica muscularis (large arrowhead, c). No hybridization signals were detected using sense riboprobe (b and d). Bars, 100 μm.

(B) Photomicrograph of results of *in situ* hybridization performed with antisense (a, c) or sense (b, d) AM riboprobes in rat ileum after 3 h oral LPS treatment. Expression of AM mRNA was evident in enterocytes (small arrow, a), goblet cells (small arrowhead, a), connective tissue in lamina propria (large arrows, a and c), and crypt epithelium (asterisk, c). No hybridization signals were detected using sense riboprobe (b and d). Bars, 100 μm.

Fig.1(A)

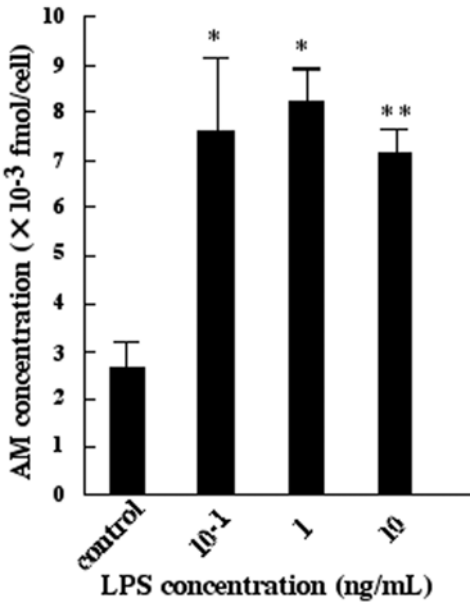
AM concentration (×10-3 fmol/cell)
0
1
2
3
4
5
6
7
8
9
10
*
*
**
control
10-1
1
10
LPS concentration (ng/mL)

Fig.1(B)

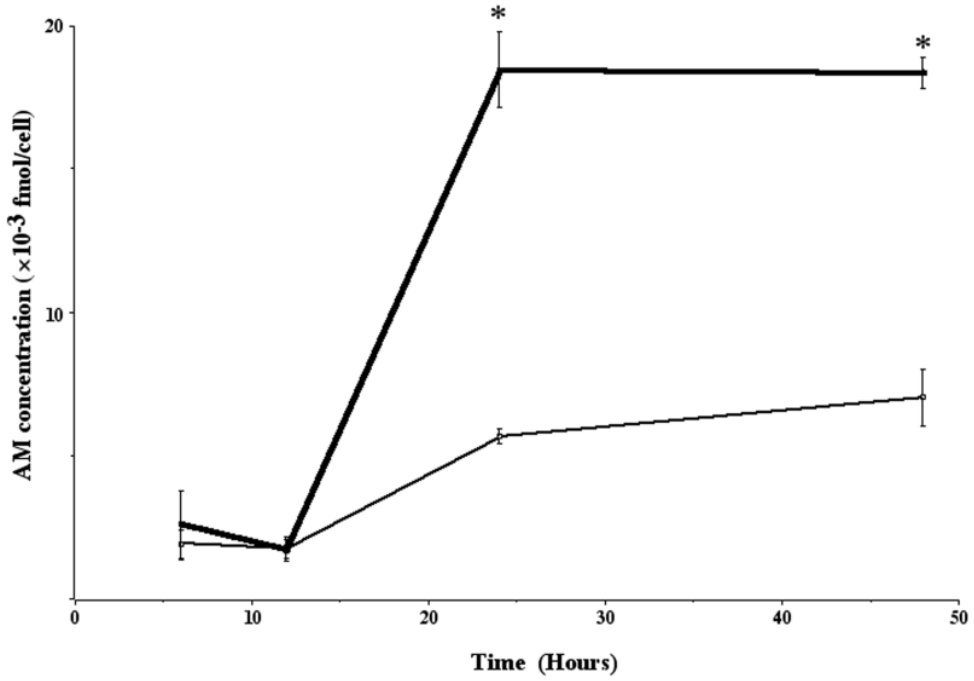
AM concentration (×10-3 fmol/cell)
10
20
*
*
0
10
20
30
40
50
Time (Hours)

Fig.2(A)

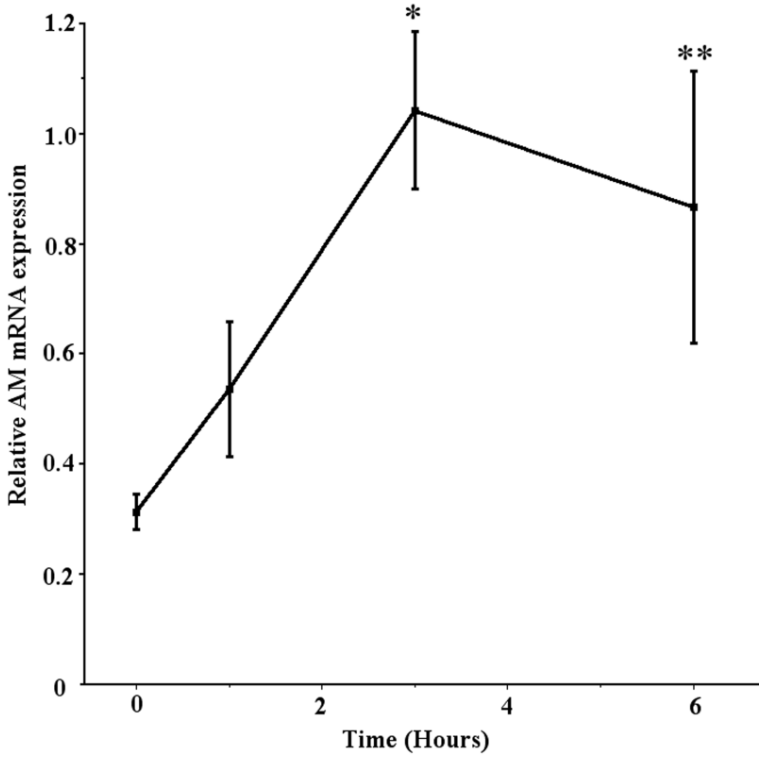
Relative AM mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
Time (Hours)
*
**

Fig.2(B)

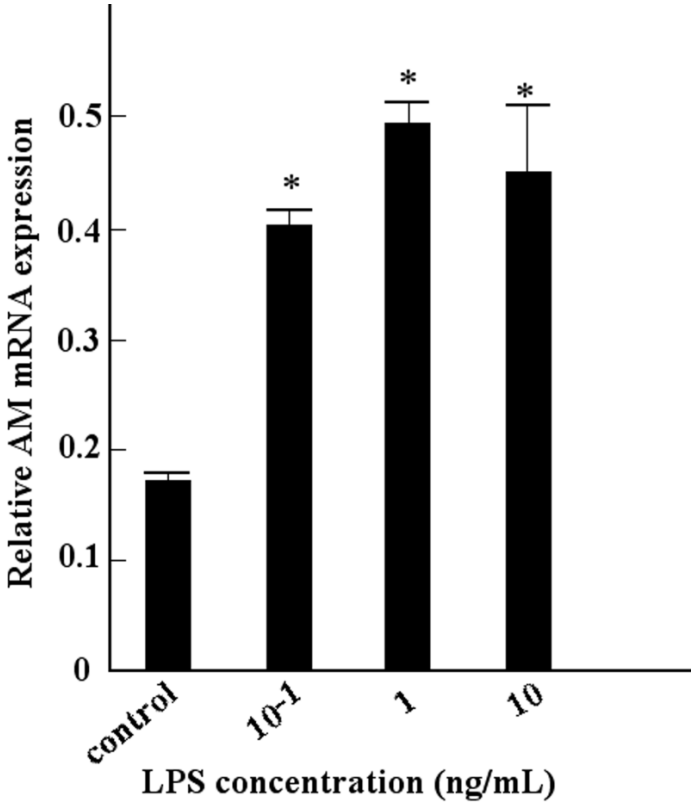
Relative AM mRNA expression
0.5
0.4
0.3
0.2
0.1
0
control
10-1
1
10
LPS concentration (ng/mL)
*
*
*

Fig.3

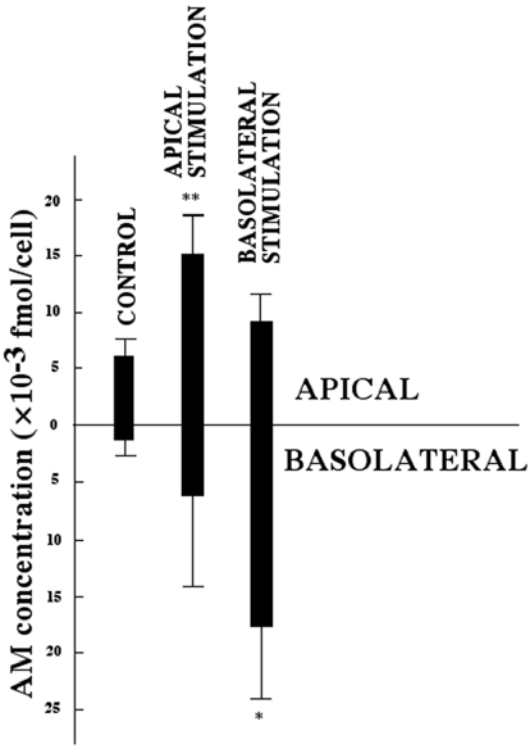
AM concentration (×10-3 fmol/cell)
CONTROL
APICAL STIMULATION
BASOLATERAL STIMULATION
APICAL
BASOLATERAL
**
*

Fig.4

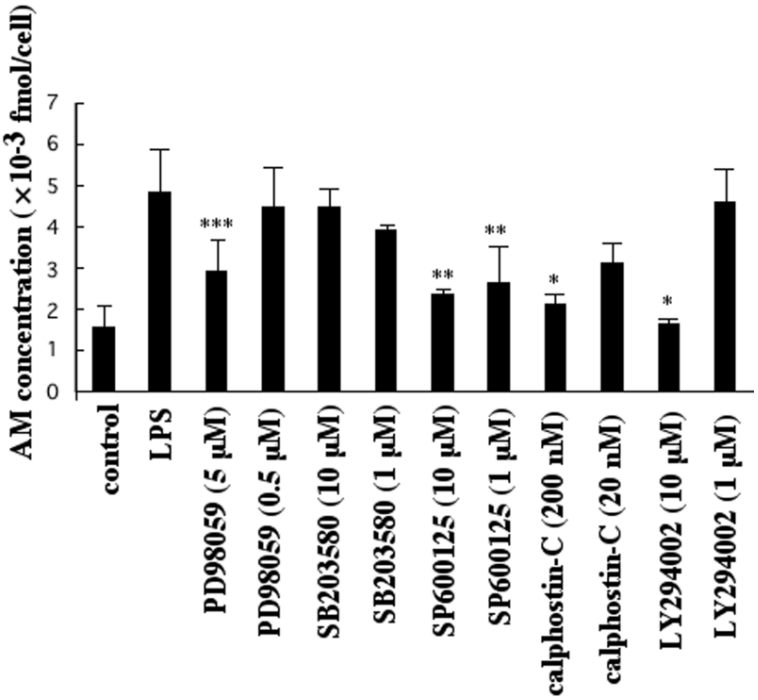
AM concentration (×10-3 fmol/cell)
control
LPS
PD98059 (5 μM)
PD98059 (0.5 μM)
SB203580 (10 μM)
SB203580 (1 μM)
SP600125 (10 μM)
SP600125 (1 μM)
calphostin-C (200 nM)
calphostin-C (20 nM)
LY294002 (10 μM)
LY294002 (1 μM)
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Fig.5(A)

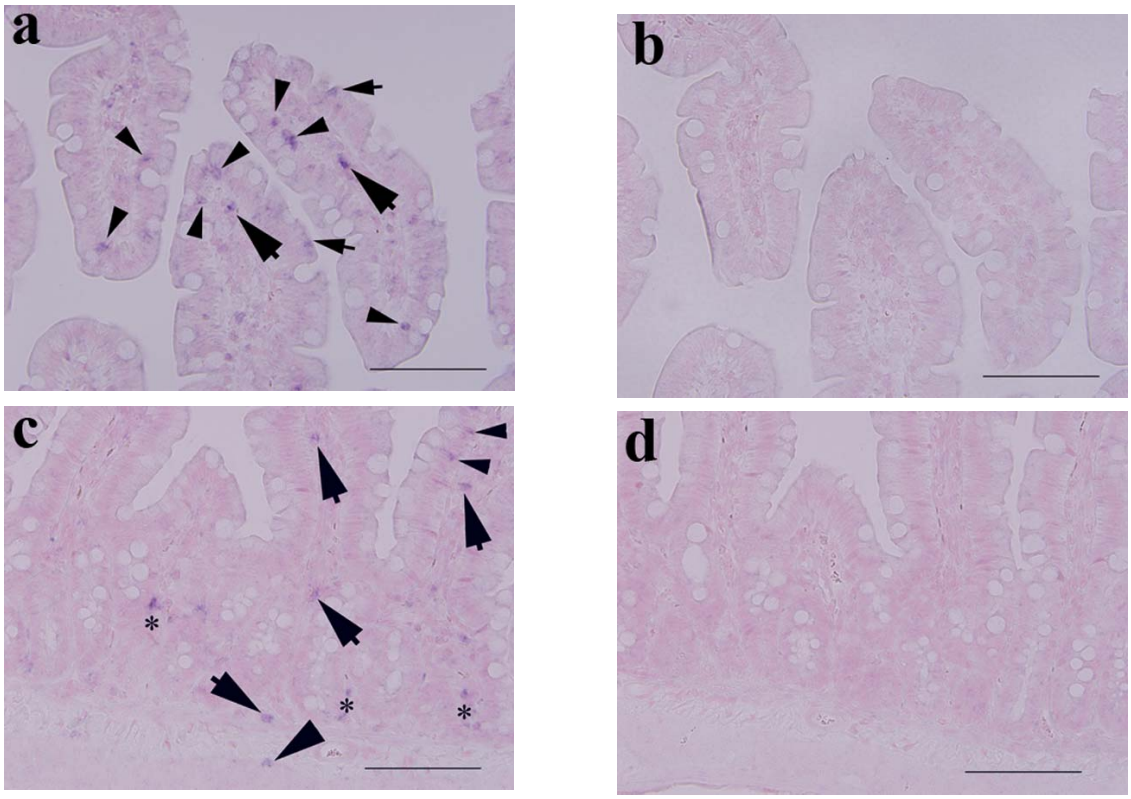

Fig.5(B)