Hirosaki Med. J. 65 : 218—226, 2014

ORIGINAL ARTICLE

INVOLVEMENT OF ENDOTHELIN-1 IN ADRENAL CATECHOLAMINE REGULATION

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Abstract *Aims:* The purpose of the current study was to evaluate the significance of the endothelin system in catecholamine release and synthesis in the adrenal gland.
Main Methods: Primary cultures of isolated bovine adrenal medullary cells were prepared. Gene expression, intracellular calcium changes, epinephrine release, and other factors were investigated.
Key findings: Expression of endothelin receptors in the rat adrenal gland was confirmed. Intravenous infusion of endothelin-1 (1.0 nmol) increased blood pressure (systolic and diastolic). Endothelin-1 stimulated intracellular calcium changes, resulting in increased nuclear factor of T cell (NFAT) activity and epinephrine release from cultured adrenal medullary cells. Furthermore, endothelin-1 increased catecholamine synthesis and caused hypertrophic changes in the cell size.
Significance: Our findings indicate involvement of the endothelin system in the sympathetic regulation of the adrenal medulla.

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Key words: adrenal gland; calcium; catecholamine; blood pressure; endothelin.

Introduction

Endothelin-1, a 21-amino acid residue peptide, was first isolated as a potent vasoconstrictor peptide from cultured porcine vascular endothelial cells[1)]. Two additional endothelin isopeptides (endothelin-2 and -3) have pharmacological profiles similar to that of endothelin-1[2)]. In addition to its cardiovascular vasoconstrictive effects, endothelin-1 has a stimulatory effect on norepinephrine release in rat mesenteric artery[3)] and acetylcholine release from parasympathetic neurons in guinea pig ileum[4)]. Furthermore, endothelin-1 potentiated adrenal catecholamine secretion in response to exogenous acetylcholine *in vitro*[5)], suggesting pre- and postsynaptic implications of cholinergic mechanisms in the endothelin-l-induced catecholamine release. In addition, endothelin-1 induces cardiac hypertrophy via an autocrine/paracrine fashion[6)]. These previous observations indicate the involvement of endothelin-1 in catecholamine release. Therefore, we speculated that endothelin-1 might regulate catecholamine synthesis or cell proliferation in the adrenal gland.

In the current study, we analyzed the effect of endothlin-1 in catecholamine release and synthesis. Endothelin-1 stimulated catecholamine secretion. Furthermore, endothelin-1 increased the phosphorylation of tyrosine hydroxylase, which catalyzes the rate-determining step

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Received for publication, December 27, 2013
Accepted for publication, February 26, 2014

in catecholamine synthesis, suggesting the involvement of the endothelin system in adrenal catecholamine regulation.

Materials and Methods

Materials

Endothelin-1 was purchased from Peptide Institute (Osaka, Japan). Bosentan was kindly supplied from Actelion Pharmaceuticals (Allschwil, Switzerland).

Primary culture of adrenal medullary cells

Isolated bovine adrenal medullary cells were cultured (4×10^6 per dish, 35-mm diameter) in Eagle's minimum essential medium containing 10% calf serum in a 5% CO_2/95% air atmosphere at 37° C[7].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was isolated from cells using the TRIzol® reagent (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was purified using Oligotex-dT30 (Takara, Shiga, Japan). The reverse-transcription reaction was performed with a first-strand cDNA synthesis kit (SuperScript® II Reverse Transcriptase, Invitrogen). PCR amplification was performed using GoTaq® Green Master Mix (Promega, Madison, WI).

Specific sequences of endothelin-1 were amplified by PCR using the following primer pairs: ET1s (5'-tctggacatcatctgggtca-3') and ET1as (5'-cttggcaaaaattccagcat -3'), which correspond to the sequences of the bos taurus endothelin neuropeptide endothelin-1 H_{68}LDIIWV$_{74}$ and K_{119}CWNFCQ$_{125}$ (173 bp); ET2s (5'-ccaagacgctgtgagtgcta-3') and ET2as (5'-tgctgttgcctagcaaagtg-3'), which correspond to the sequences of the bos taurus ET2 P_{92}RRCECY$_{98}$ and I_{154}HFARQQ$_{160}$ (209 bp); ET3s (5'-gggacaccaaggagactgtg-3') and ET3as (5'-gttcaggggtgttgatccag-3'), which correspond to the sequences of the bos taurus ET3 G_{44}DTKETV$_{50}$ and I_{108}WINTPE$_{114}$ (213 bp); ETAs (5'-agtcgtgttcagggaattgg-3'), and ETAas (5'-aggggcatgcagaagtagaa-3'), which correspond to the sequences of the bos taurus ETA S_{191}RVQGIG$_{197}$ and G_{261}FYFCMP$_{267}$ (233 bp); ETBs (5'-ggtggtgtcctgcctagtgt-3') and ETBas (5'-acacatctcaaccccaaagg-3'), which correspond to the sequences of the bos taurus ETB T_{104}VVSCLV$_{110}$ and P_{167}FGVEMC$_{173}$ (208 bp). As a control, β-actin cDNA was amplified for 25 cycles using the primers β-actin S (5'-ctcttccagccttccttcct -3') and β-actin AS (5'-gggcagtgatctctttctgc -3') to produce a 178-bp fragment.

Blood pressure measurement

Male SD rats weighing ca. 400 g were anaesthetized with isoflurane (2%) and the carotid artery and vein were cannulated for blood pressure recording and compound administration, respectively. Endothelin-1 (1.0 nmol kg^{-1}) was administered intravenously, and the blood pressure was monitored. Blood pressure responses were recorded using a transducer (TP-400T; Nihon Kohden Ltd., Tokyo, Japan) connected to an amplifier (AP-601G; Nihon Koden Ltd.). Data were A/D converted using PowerLab 4/20 (AD Instruments Ltd., Castle Hill, NSW, Australia) and recorded by a computer.

Intracellular calcium changes

To measure intracellular calcium changes, adrenal medullary cells were incubated with Fluo-4-AM (10 μM for 1 h, at 37° C). Fluo-4-loaded cells were perfused with Krebs-Ringer phosphate (KRP) buffer (mM; 154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4, and 5 glucose, pH 7.4) at a flow rate of 5 ml/min. The fluorescence of Fluo-4-AM (excitation wavelength 488 nm, emission

wavelength 509 nm) was measured using a fluorescent microscope (DMIRE2; Leica Microsystems, Wetzlar, Germany). The Ca^{2+} image required 1.0 sec to scan a full frame (512 × 512 pixels). Images were recorded at 5-s intervals.

Plasmid transfection into adrenal chromaffin cells

The nuclear factor of T cell (NFAT) reporter plasmid (NFAT-EGFP), with its enhanced green fluorescent protein, enables the evaluation of NFAT activity using its EGFP fluorescence[8]. The NFAT-EGFP plasmid was transfected using the HilyMax transfection reagent (Dojindo, Kumamoto, Japan). NFAT activity in response to endothelin-1 (10 nM) was evaluated by EGFP (excitation wavelength, 488 nm; emission wavelength, 509 nm) using a Leica DMIRE2 fluorescence microscope.

Catecholamine secretion

Cells were washed with ice-cold Krebs-Ringer phosphate buffer, and incubated in KRP buffer with or without endothelin-1 at 37° C for 30 min. The catecholamine (epinephrine) concentration was measured by high-performance liquid chromatography (HPLC).

Western blotting analysis of Ser^{40}-phosphorylated tyrosine hydroxylase and tyrosine hydroxylase

Identical quantities of afterprotein (ca. 7.0 μg per lane) were separated by 12% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Hybond-P; GE, London, UK). The membrane was incubated overnight at 4° C in Can Get Signal Solution-1 (Toyobo, Osaka, Japan) with antibody (1:2000) against Ser^{40}-phosphorylated tyrosine hydroxylase (Cell Signaling Technology, Beverly, MA), or tyrosine hydroxylase (Chemicon, Temecula, CA). The immunoreactive bands were reacted with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody, and then visualized using the ECL Plus enhanced chemiluminescent detection system (GE, London, UK). Each Western blotting assay was repeated at least six times for statistical evaluation.

Hypertrophic effect of endothelin-1

Isolated bovine adrenal medullary cells were cultured (4×10^6 per dish, 35-mm diameter) in Eagle's minimum essential medium containing 10% calf serum in a 5% CO_2/95% air atmosphere at 37° C[7]. Three days (60–62 h) later, the cells were treated with or without endothelin-1 (10 nM) for 24 h. Hypertrophic changes in response to endothelin-1 were evaluated by measuring mean diameter ((major + minor diameter)/2) of each cell.

Statistical analysis

All experiments were repeated at least three times. Data are presented as the means ± SEM. At first, Shapiro-Wilk normality test was examined for further analysis. Significance ($P < 0.05$) was determined by a one- or two-way analysis of variance (ANOVA) with *post hoc* mean comparisons using the Newman-Keuls multiple-range test. Student's *t*-tests were used for comparison of the means of two groups.

Results

RT-PCR detection of endothelin and its receptors in bovine adrenal medullary cells

To examine the expression of the endothelin system in the adrenal medulla, endothelin and its receptor mRNAs were evaluated using RT-PCR. Amplification of bovine adrenal medullary mRNA with endothelin-1-specific primers resulted in PCR amplification of a single band (Fig. 1A). On the other hand, specific primers against endothelin-2 resulted in no DNA amplification. Specific PCR products for endothelin-3 and endothelin receptors (ET-A

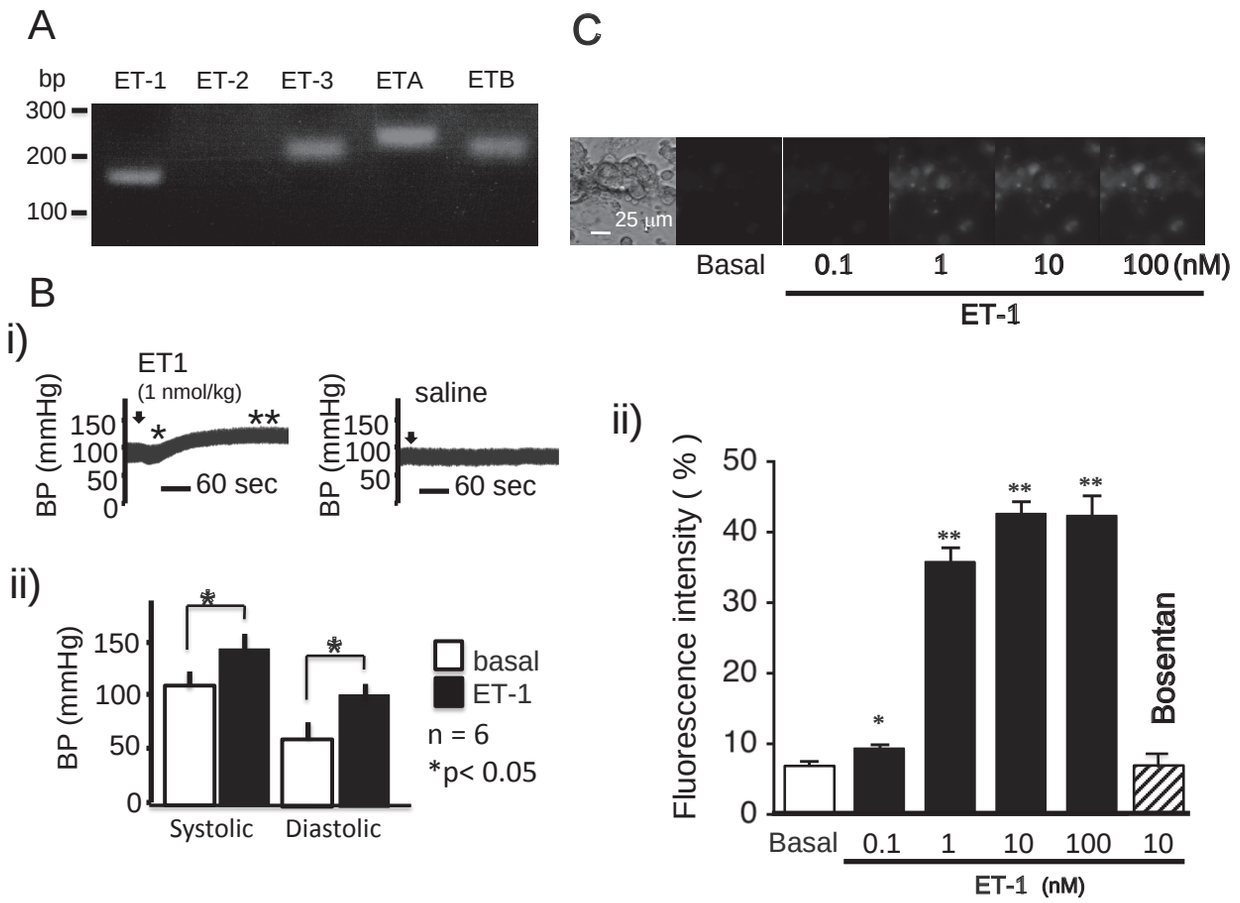

Figure 1. Expression of endothelins and their receptors.
A) RT-PCR analysis of bovine adrenal medullary cells.
Identification of endothelins and their receptors (ET-A and ET-B) in the bovine adrenal gland. Expression of ET-1, ET-3, ET-A and ET-B were confirmed. The primer sets used for each PCR amplification are indicated.
B) ET-1 induced an increase in blood pressure.
i) Representative blood pressure changes in response to ET-1 (1.0 nmol/kg). Control (saline infusion) showed no changes in blood pressure. Systolic and diastolic blood pressure increased.
ii) Statistical analysis of blood pressure changes to ET-1 (1.0 nmol/kg). *P < 0.05: significantly different versus basal (Student's t-test).
C) Changes in intracellular calcium concentrations.
i) Representative fura-2 related $[Ca^{2+}]i$ images of adrenal medullary cells in response to endothelin-1.
ii) Statistical analysis revealed dose-dependent changes in $[Ca^{2+}]i$. An endothelin antagonist (bosentan, 10 μM; hatched bar) prevented the significant increase. Corresponding DIC image is shown (left). *P < 0.05, **P< 0.01: significantly different versus control (ANOVA).

and ET-B) were also detected.

Increased blood pressure in response to endothelin-1

To confirm the vasoconstrictive effect of endothelin-1, we evaluated blood pressure changes in response to endothelin-1 treatment. Intravenous injection of endothelin-1 (1.0 nmol/kg) resulted in increases in systolic and diastolic blood pressure (Fig.1Bi left panel). As reported previously[9], intravenous infusion of endothelin-1 showed slight decreases in systolic and diastolic blood pressure (Fig. 1Bi, asterisk), followed by increases in systolic and diastolic blood pressure (asterisks). No blood pressure changes were observed after saline injection (Fig. 1Bi right panel). Statistical analysis revealed significant increase in systolic and diastolic blood pressure after 5 min of endothelin-1 (1.0 nmol/kg)

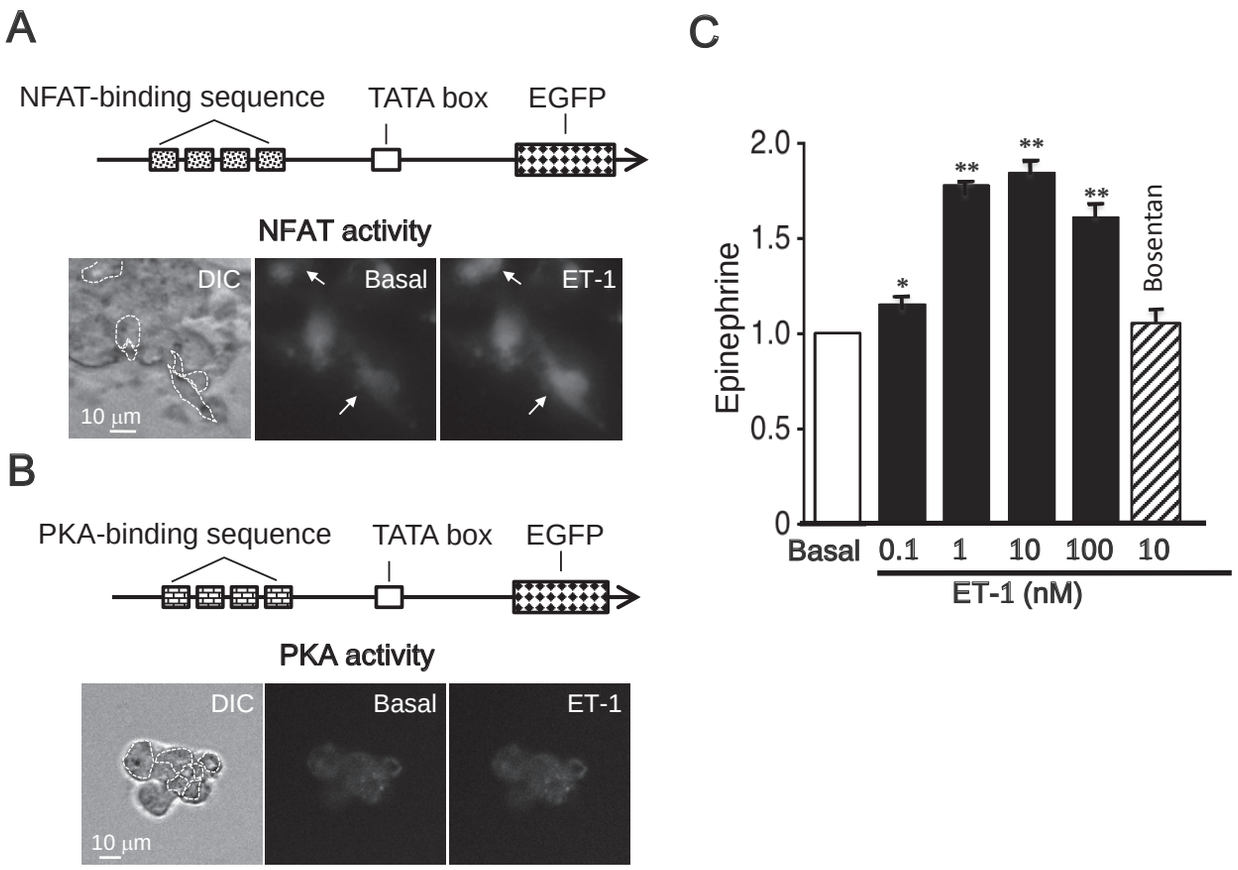

Figure 2. A) Fluorescence imaging of NFAT promoter activity with EGFP.
Structure of the NFAT promoter activity (EGFP) reporter plasmid (upper panel). Representative results for EGFP fluorescence and the corresponding phase-contrast images are shown (lower panel). Endothelin-1 (10 nM) significantly increased EGFP fluorescence (ET-1, arrows). The corresponding DIC image is indicated (left).
B) Fluorescence imaging of PKA-binding promoter activity with EGFP.
Structure of the PKA-binding promoter activity (CREB-EGFP) reporter plasmid (upper panel). Representative results for EGFP fluorescence and the corresponding phase-contrast images are shown (lower panel). The corresponding DIC image is shown (left).
C) Endothelin-1-related epinephrine release from bovine adrenal medullary cells.
Endothelin-1 stimulated the release of epinephrine from bovine adrenal medullary cells in a dose-dependent fashion. Incubations were 30 min. An endothelin antagonist (bosentan, 10 μM; hatched bar) prevented the significant increase. All data are expressed as means ± SEM, n = 8–10. *P < 0.05, **P< 0.01: significantly different versus the control (ANOVA).

injection (Fig. 1Bii, *p< 0.05 vs. basal, Student's t-test).

Intracellular calcium changes in response to endothelin

Because endothelin and its receptors are known to be coupled with the Gq protein pathway and increased IP3 concentrations[10], we evaluated intracellular calcium changes in adrenal medullary cells. A typical recording of changes in $[Ca^{2+}]i$ in adrenal medullary cells is presented in Fig 1Ci. Endothelin-1 (10 nM, 42.9 ± 2.9* % increase in fluorescence intensity, mean ± SEM, n = 12, *p< 0.05, or **p< 0.01 vs. basal, ANOVA) increased intracellular calcium concentrations in a dose-dependent manner (Fig. 1Cii). Because the endothelin-induced calcium increase was inhibited by bosentan (10 μM),

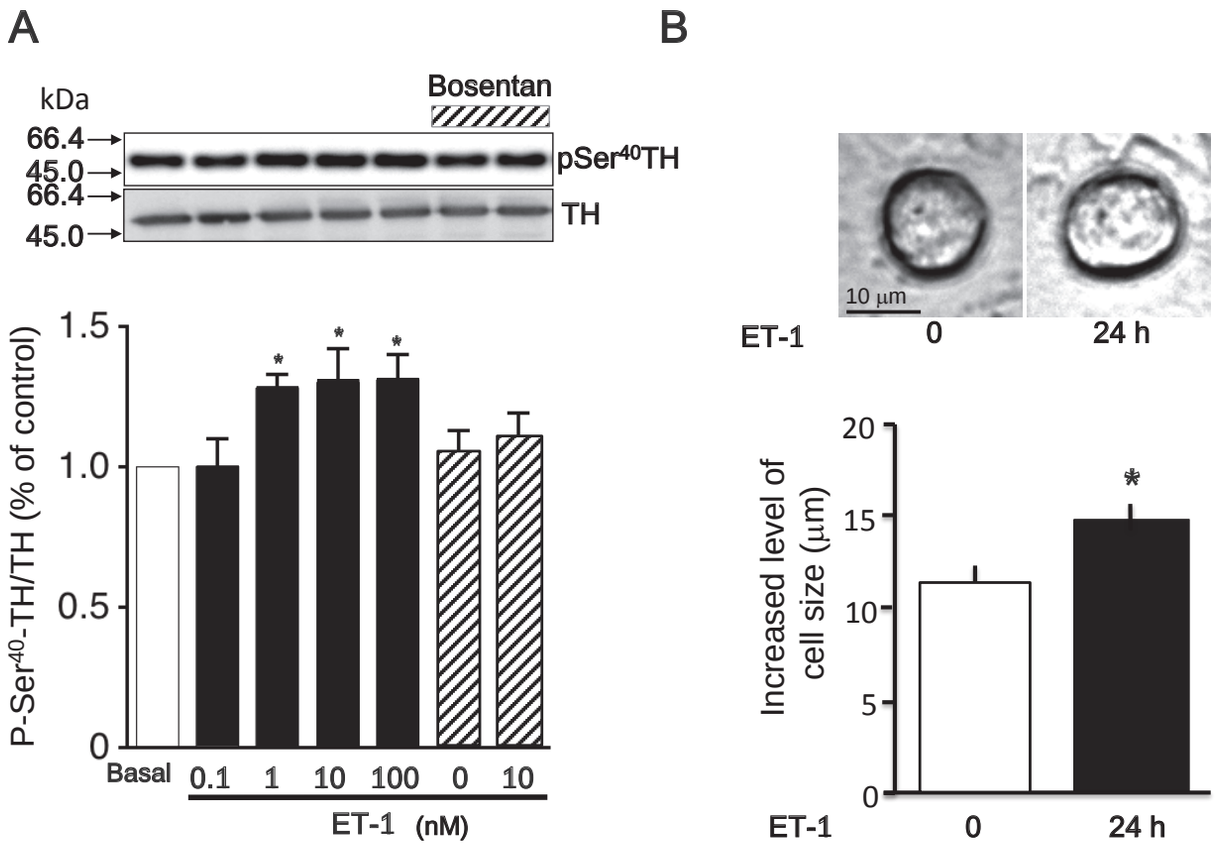

Figure 3. A) Increased catecholamine synthesis in response to endothelin-1.
An antibody specific for phospho-tyrosine hydroxylase, which catalyzes the rate-determining step in catecholamine synthesis, showed increased phosphorylation of tyrosine hydroxylase in response to endothelin-1.
Representative Western blots are shown in the upper panels. Endothelin-1 treatment increased the amount of phospholylated tyrosine hydroxylase (p-Ser^{40}-TH), while the total amount of tyrosine hydroxylase (TH) did not change. Statistical analysis revealed a dose-dependent increase in phospho-tyrosine hydroxylase levels in response to endothelin-1. An endothelin antagonist (bosentan, 10 μM; hatched bar) prevented this increase. All data are expressed as means ± SEM, n = 6–10. *P < 0.05: significantly different versus the control (ANOVA).
B) Hypertrophic effect of endothelin-1.
Representative change of adrenal medullary cells in response to endothelin-1 (10 nM, upper panels). Treatment with endothelin-1 (24 h) caused an increase in cell size. Statistical analysis revealed significant changes in cell size (lower panel). All data are expressed as means ± SEM, n = 6–10. *P < 0.05: significantly different versus the control (Student's t-test).

an endothelin receptor antagonist, we consider the intracellular calcium changes in response to endothelin to be specific.

Increased NFAT activity

The IP3 pathway should increase intracellular calcium concentrations, resulting in increased NFAT activity; thus, we next conducted NFAT-EGFP assays to evaluate NFAT activities[8]. Because endothelin increased the intracellular calcium concentration, we expected an increase in NFAT activity. Indeed, endothelin-1 (10 nM, 19.4 ± 3.7* %, mean ± SEM, n = 10, *p< 0.05 vs. basal, Student's t-test) elevated NFAT activity in adrenal medullary cells (Fig. 2A), confirming that endothelin increased NFAT activity, likely by activating phospholipase-C, which was in good agreement with the previous

experiments[8]. We also examined transfection of the pCRE-hrGFP plasmid to evaluate PKA activities (Agilent Technologies, La Jolla, CA); this resulted in no significant changes, suggesting no contribution of cAMP-related pathways to endothelin- signal transduction (Fig. 2B).

Effect of endothelin on catecholamine secretion

The effect of endothelin-1 on catecholamine secretion from adrenal medullary cells was examined. Endothelin-1 (10 nM, 81.1 ± 5.2* %, mean ± SEM, n = 10, *p< 0.05, or **p< 0.01 vs. basal, ANOVA) significantly enhanced epinephrine release from bovine adrenal medullary cells (Fig. 2C) in a dose-dependent manner. Following pretreatment with bosentan (10 μM), an endothelin receptor antagonist, endothelin-1 (10 nM) showed no significant effects, suggesting the specific effect of endothelin-1 and involvement of the endothelin system in catecholamine release in the adrenal gland.

Increased catecholamine synthesis due to endothelin

To further investigate the effects of endothelin, we evaluated changes in the phosphorylation of tyrosine hydroxylase, which catalyzes the rate-determining step in catecholamine synthesis (Fig. 3A). Endothelin-1 (10 nM, 32.3 ± 6.7%, mean ± SEM, *p< 0.05 vs. basal, ANOVA) stimulated the phosphorylation of tyrosine hydroxylase in a dose-dependent manner. Bosentan (10 μM) prevented this increase in catecholamine synthesis.

Hypertrophic changes in response to endothelin-1

Because expression of the endothelin system was confirmed in the adrenal gland (Fig. 1), and the application of endothelin-1 resulted in an increased release of catecholamines (Fig. 2C), we next analyzed hypertrophic changes in response to endothelin-1 (Fig. 3B). Treatment with endothelin-1 increased the size of cultured adrenal medullary cells (*p< 0.05 vs. basal, Student's t-test). These results suggest that endogenous endothelin-l locally generated and secreted by adrenal medullary cells may contribute to hypertrophy via an autocrine/paracrine fashion.

Discussion

We have demonstrated the expression of endothelins (ET-1 and ET-3) and their receptors (ETA and ETB) in the bovine adrenal gland. Endothelin-1 stimulated intracellular calcium changes, resulting in increased NFAT activity and epinephrine release from cultured bovine adrenal medullary cells. Endothelin-1 increased the size of adrenal chromaffin cells and TH phosphorylation, suggesting a hypertrophic effect and increased catecholamine synthesis.

Endothelins (ETs), a family of potent vasoactive and growth-regulatory peptides, are involved in the physiological and/or pathophysiological control of adrenocortical functions[11]. Furthermore, ETs mediate the paracrine control of vascular tone and secretion of catecholamines in the adrenal gland through two ET receptor subtypes, (ETA and ETB)[12, 13]. The enhancement of catecholamine release in response to ETs is mediated by both the ETA and ETB subtypes[14, 15]. Ikeda et al. reported binding of ^{125}I-labeled ET-1 in the rat adrenal gland[16]; they also reported the gene expression of ETA and ETB receptors, ET-1, ET-3 and ECE-1 using relative multiplex reverse transcription/PCR. Interestingly, endothelin-1 has also been found to be a growth factor in mammalian cells including vascular smooth muscle cells[17]. Ito et al. demonstrated that ET-1 induces hypertrophy of cardiomyocytes *in vitro*, associated with the induction of muscle-specific genes (myosin light chain 2, α-actin, and troponin I) and a proto-oncogene (c-fos) [6].

Therefore, it is tempting to speculate that ET-1 plays an important role in the pathogenesis of *hypothalamic-pituitary-adrenal* (PHA)-axis, and may cause hypertrophy in adrenal chromaffin cells, which is possibly associated with various cardiovascular diseases. Therefore, endothelins are thought to play a significant role in the autonomic adrenal gland, although their role in catecholamine synthesis and hypertrophic effect have not been reported.

In the current study, we confirmed the expression of endothelin-1 by RT-PCR (Fig.1a). In addition, our findings show that endothelin-1 induced a significant effect on catecholamine release (Fig. 2c). The endothelin receptor antagonist, bosentan, inhibited catecholamine release. Combined with the elevated intracellular calcium (Figs. 1c,d) and NFAT activity (Fig. 2a), our results strongly suggest that endothelin-1 induces catecholamine release by binding and activating its own receptor cascades. In addition, our data revealed that endothelin-1 induced catecholamine synthesis and had a hypertrophic effect on adrenal chromaffin cells. Considering the expression of two receptors (ETA and ETB), the endothelin system appears to play a significant role in maintaining the sympathetic tonus at the peripheral adrenal gland medulla in an auto- or paracrine manner. Endothelin receptors are G-protein coupled receptors (GPCR), which are thought to couple mainly with Gq protein following increases in IP3, intracellular calcium concentration, and NFAT activity[10]. In the current study, endothelin-1 showed similar effects, therefore endothelin's effect might be related to its Gq protein and following IP3 systems. However, further experiments are needed to clarify the mechanism of their effect on adrenal chromaffin cells, including increased catecholamine synthesis and hypertrophic effects.

Conclusions

Our data confirmed the expression of endothelins and two endothelin receptors in the adrenal gland. Endothelin-1 stimulated intracellular calcium increase and subsequent NFAT activity, resulting in increased epinephrine release from adrenal medullary cells. Endothelin-1 induced catecholamine synthesis and hypertrophic changes. These data suggest the involvement of the endothelin system in catecholamine release and synthesis in adrenal medullary cells.

Acknowledgements

We thank Dr. Masashi Yanagisawa. We also thank Ms. Izumi Miki for her assistance. This research was sponsored by Grants-in-Aid for Scientific Research from the JSPS, KAKENHI, Strategic Research Program and Joint Animal Research Project of Miyazaki University.

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