Coordinate regulation of endothelin and adrenomedullin secretion by oxidative stress in endothelial cells

TAKATOSHI SAITO, HIROSHI ITOH, TAE-HWA CHUN, YASUTOMO FUKUNAGA, JUN YAMASHITA, KENTARO DOI, TOKUJI TANAKA, MAYUMI INOUE, KEN MASATSUGU, NAOKI SAWADA, SATSUKI SAKAGUCHI, HIROSHI ARAI, MASASHI MUKOYAMA, KATSUYOSHI TOJO, TATSUO HOSOYA, and KAZUWA NAKAO

Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507; and Second Department of Internal Medicine, Jikei University School of Medicine, Tokyo 10-8461, Japan

Received 27 April 2000; accepted in final form 15 May 2001

Saito, Takatoshi, Hiroshi Itoh, Tae-Hwa Chun, Yasutomo Fukunaga, Jun Yamashita, Kentaro Doi, Tokuji Tanaka, Mayumi Inoue, Ken Masatsugu, Naoki Sawada, Satetsuki Sakaguchi, Hiroshi Arai, Masashi Mukoyama, Katsuyoshi Tojo, Tatsuo Hosoya, and Kazuwa Nakao. Coordinate regulation of endothelin and adrenomedullin secretion by oxidative stress in endothelial cells. Am J Physiol Heart Circ Physiol 281: H1364–H1371, 2001.—To elucidate the significance of oxidative stress in the modulation of endothelial functions, we examined the effects of H2O2 on the expression of two endothelium-derived vasoactive peptides, endothelin (ET) and adrenomedullin (Am), and their interaction. H2O2 dose dependently suppressed ET secretion and ET-1 mRNA expression in bovine carotid endothelial cells (ECs). Menadion sodium bisulfate, a redox cycling drug, also decreased ET secretion in a dose-dependent manner. Catalase, a H2O2 reductase, and dl-α-tocopherol (vitamin E) significantly inhibited H2O2-induced suppression of ET secretion. Downregulation of ET-1 mRNA under oxidative stress was regulated at the transcriptional level. In contrast, H2O2 increased Am secretion (and its mRNA expression) accompanied by the augmentation of cAMP production. Am, as well as 8-bromo-cAMP and forskolin decreased ET secretion in a dose-dependent fashion. Furthermore, an anti-Am monoclonal antibody that we developed abolished H2O2-induced suppression of ET secretion at 6–24 h after the addition of H2O2. H2O2 increased the intracellular Ca2+ concentration ([Ca2+]i). Moreover, treatment with ionomycin, a Ca2+ ionophore, and thapsigargin, an inhibitor of endoplasmic reticulum ATPase, decreased ET secretion dose dependently for 3 h. These results suggest that the production of ET was decreased via activation of the Am-cAMP pathway and by the elevation of [Ca2+]i, under oxidative stress. These findings elucidate the coordinate expression of two local vascular hormones, ET and Am, under oxidative stress, which may protect against vascular diseases.

intrinsic Ca2++; hydrogen peroxide; cAMP; nitric oxide; C-type natriuretic peptide

THE PHYSIOLOGICAL PRODUCTION OF reactive oxygen species (ROS) such as superoxide and H2O2 is necessary for maintenance of cell function and cell homeostasis. However, a surplus generation of ROS, termed oxidative stress, has been implicated in pathophysiological mechanisms. In the vasculature, oxidative stress has been recently found to play a role in the pathophysiology of hypertension and atherosclerosis. Recent studies have shown that an excess amount of oxidant induces profound vascular endothelial cell (EC) dysfunction such as vascular permeability aggravation via stimulation of platelet-activating factor production (27), abnormal coagulation due to inhibition of prostaglandin I2 synthesis (38), and cell lysis (40).

ECs participate in the regulation of vascular tone and remodeling by modulating the secretion of vasoactive substances. Itoh et al. (18, 19) reported that vaso-active substances regulate not only vascular tone but also vascular growth; i.e., angiotensin II, a potent vasoconstrictor, promotes vascular growth. Furthermore, we elucidated that C-type natriuretic peptide (CNP) is secreted from ECs (24, 37) and regulates vascular remodeling (6, 23, 35, 36).

Endothelin (ET) is a 21-amino acid peptide isolated from the supernatant of porcine aortic ECs (39). ET exerts potent cardiovascular actions, the most prominent of which is vascular constriction. Increased ET-1 expression has been observed after treatment of cultured ECs with growth factors and cytokines such as thrombin (7), transforming growth factor-β (25), tumor necrosis factor-α (26), or insulin (32). Vasoactive substances, such as angiotensin II (5, 15) and anti-diuretic hormone (15), also increase ET-1 mRNA expression in ECs. On the other hand, cGMP-elevating vasodilators, such as nitric oxide (NO) (14) and atrial natriuretic peptide (13), have been known to decrease endothelial ET secretion. Recently, Michael et al. (29) reported that oxidative stress elicited by glucose oxidase decreased the expression of ET in cultured rat pulmonary arterial ECs; however, the mechanisms by which oxidants regulate ET production remain unclear.

Address for reprint requests and other correspondence: H. Itoh, Dept. of Medicine and Clinical Science, Kyoto Univ. Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan (E-mail address: hiito@kuhp.kyoto-u.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Adrenomedullin (Am), which was first isolated from human pheochromocytoma, is a potent vasorelaxant peptide that is secreted from vascular ECs (21). Am exerts a hypotensive effect and an antiproliferative effect through a cAMP-dependent process (20). Chini et al. (1) reported that Am acts as a cytoprotective autacoid to suppress oxidative stress through the cAMP signaling pathway. In vascular smooth muscle cells (VSMCs), exogenously administered Am has been reported (22) to suppress ET-1 production.

The intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) is an important significant signal transduction in the cardiovascular system. It was reported that oxidants increased [Ca\textsuperscript{2+}]i in ECs (9). Furthermore, treatment with Ca\textsuperscript{2+} ionophores inhibited ET production in ECs (31). These findings suggest that oxidative stress suppresses ET production by modulating [Ca\textsuperscript{2+}]i. The underlying regulation mechanisms between ET production and [Ca\textsuperscript{2+}]i during oxidative stress are not fully understood.

In the present study, to elucidate the significance of oxidative stress in the modulation of endothelial functions, we examined the effects of H\textsubscript{2}O\textsubscript{2} on the expression of two endothelium-derived vasoactive peptides, ET and Am, and their interaction. Moreover, we investigated whether alteration of [Ca\textsuperscript{2+}]i due to oxidative stress contributes to ET production or not.

**MATERIALS AND METHODS**

**Materials.** Human Am was obtained from the Peptide Institute (Osaka, Japan). Menadion sodium bisulfite (MSB), N\textsuperscript{6}-nitro-L-arginine methyl ester (L-NAME), catalase, 8-bromo-cAMP (8-Br-cAMP), 8-Br-cGMP, actinomycin D, forskolin, fura 2-acetoxymethyl ester (AM), ionomycin, A-23187, thapsigargin (TSG), EGTA, 1,2-bis(2-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid (BAPTA), and cromophor EL were purchased from Sigma (St. Louis, MO). H\textsubscript{2}O\textsubscript{2} was purchased from Santoku (Tokyo, Japan). We purchased dl-\alpha-tocopherol from Nacalai Tesque (Kyoto, Japan). Cell culture. Bovine artery endothelial cells (BAECs) were isolated from the adult bovine carotid artery by digestion with collagenase and elastase (37). The BAECs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hazleton Biologics; Richmond, CA) (30). The neutralizing effects of KY-CNP-1 purified with an Affi-Gel Protein A MAPS II kit (Bio-Rad; Richmond, CA) (30). After incubation for 24 h with or without H\textsubscript{2}O\textsubscript{2}, the supernatants were collected, succinylated, and mixed and transferred to a nitrocellulose membrane. The samples were then hybridized using rat ET-1 full-length cDNA or bovine Am partial-length cDNA, prepared by polymerase chain reaction by using the sense (408–428 bases; 5'-GCAGCCCCGATCCGATCAAG-3') and antisense (602–622 bases; 5'-GTAATAGTCCGACCCACGC-3') primers as probes. The blots were probed by random priming with [\alpha\textsuperscript{32P}]-deoxyctydine 5'-triphosphate. The blot was washed finally with 0.1x saline-sodium citrate buffer at 65°C. Blots were exposed to an imaging plate (Fuji Film, Japan) for 24 h. The signals on the imaging plate were quantified with an image analyzer (BAS2000, Fuji Film) and expressed by the intensity of photo-stimulated luminescence (PSL). The background PSL values were subtracted. PSL is proportional to the adsorbed radiation energy on the imaging plate. The main signals, with a size of 2.3 Kb, were quantified. The density of 18S rRNA stained with ethidium bromide was used to monitor the amount of total RNA in each sample.

**Half-life study of ET-1 mRNA.** BAECs were incubated in the presence of 10 µg/ml actinomycin D for specific periods, at which time total RNA was extracted. In parallel, the cells were reincubated with actinomycin D and 0.5 mM H\textsubscript{2}O\textsubscript{2} for the same period. cAMP measurement. Basal and H\textsubscript{2}O\textsubscript{2}-stimulated cAMP production in the medium were measured by RIA as described (30). After incubation for 24 h with or without H\textsubscript{2}O\textsubscript{2}, the supernatants were collected, succinylated, and mixed with assay buffer. The treated samples were measured with the use of a commercial kit (Yamasa; Tokyo, Japan).

**Effects of monoclonal anti-CNP and anti-Am antibodies.** To examine the action of endogenous CNP and Am, we utilized a monoclonal anti-CNP antibody (KY-CNP-1) and a monoclonal anti-Am antibody (KY-Am-1), which we previously developed (12, 30). These antibodies in ascites were purified with an Affi-Gel Protein A MAPS II kit (Bio-Rad; Richmond, CA) (30). The neutralizing effects of KY-CNP-1 (10 µg/ml) and KY-Am-1 (50 µg/ml) were confirmed by blockade of CNP-stimulated cGMP production in cultured VSMCs and the Am-induced cAMP elevation in BAECs, respectively.

**Radioimmunoassay for ET-1 and Am.** After incubation, the concentrations of ET and Am in the media were measured by specific radioimmunoassays (RIAs) developed by us (30, 33). In the RIA for ET-1, the cross-reactivities with ET-2, ET-3, and Big ET-1 were 100%, 60%, and 100% on a molar basis, respectively. A concentration giving half-maximal response (EC\textsubscript{50}) was achieved at a concentration of 2.4 fmol/tube and minimal inhibition was detected at 0.2 fmol/tube. H\textsubscript{2}O\textsubscript{2} had no effect on ET-like immunoreactivity during the assay. We measured synthetic ET (22 pg/tube) and the same amount of ET mixed with 5 µM H\textsubscript{2}O\textsubscript{2} by RIA and compared the ET-like immunoreactivity. The ET-like immunoreactivity in the tube containing synthetic ET only was 22.4 ± 0.4 pg/tube (n = 4) and that containing synthetic ET and 5 µM H\textsubscript{2}O\textsubscript{2} was 22.9 ± 0.3 (n = 4). In the RIA for Am, the cross-reactivity with calcitonin gene-related peptide-1, which has structural homology with Am, was <0.01%. EC\textsubscript{50} was achieved at a concentration of 8.0 fmol/tube and minimal inhibition was detected at 1.0 fmol/tube.

**RNA extraction and Northern blot analysis.** BAECs grown to confluence in 10-cm dishes were incubated with several agents for specific periods. Total cellular RNA was extracted by a method previously reported (36). RNA samples (25 µg) were applied to 1.6% formamide-agarose gels and transferred to a nitrocellulose membrane. The samples were then hybridized using rat ET-1 full-length cDNA or bovine Am partial-length cDNA, prepared by polymerase chain reaction by using the sense (408–428 bases; 5'-GCAGCCCCGATCCGATCAAG-3') and antisense (602–622 bases; 5'-GTAATAGTCCGACCCACGC-3') primers as probes. The blots were probed by random priming with [\alpha\textsuperscript{32P}]-deoxyctydine 5'-triphosphate. The blot was washed finally with 0.1x saline-sodium citrate buffer at 65°C. Blots were exposed to an imaging plate (Fuji Film, Japan) for 24 h. The signals on the imaging plate were quantified with an image analyzer (BAS2000, Fuji Film) and expressed by the intensity of photo-stimulated luminescence (PSL). The background PSL values were subtracted. PSL is proportional to the adsorbed radiation energy on the imaging plate. The main signals, with a size of 2.3 Kb, were quantified. The density of 18S rRNA stained with ethidium bromide was used to monitor the amount of total RNA in each sample.

**Effects of monoclonal anti-CNP and anti-Am antibodies.** To examine the action of endogenous CNP and Am, we utilized a monoclonal anti-CNP antibody (KY-CNP-1) and a monoclonal anti-Am antibody (KY-Am-1), which we previously developed (12, 30). These antibodies in ascites were purified with an Affi-Gel Protein A MAPS II kit (Bio-Rad; Richmond, CA) (30). The neutralizing effects of KY-CNP-1 (10 µg/ml) and KY-Am-1 (50 µg/ml) were confirmed by blockade of CNP-stimulated cGMP production in cultured VSMCs and the Am-induced cAMP elevation in BAECs, respectively.
The same concentration of mouse immunoglobulin (50 μg/ml, Sigma) was used as a control.

Measurement of \([\text{Ca}^{2+}]_i\). \([\text{Ca}^{2+}]_i\) was measured as described elsewhere (9). Briefly, the BAECs seeded and grown to confluence on the coverslips were loaded with 2 μM fura 2-AM and 0.01% cremophor EL for 30 min at room temperature in modified Hanks’ balanced salt solution composed of (in mM) 137 NaCl, 3.5 KCl, 0.44 KH2PO4, 25 NaHCO3, 0.33 Na2HPO4, and 0.5 CaCl2 with a pH of 7.4. The cells were then washed three times with fresh Hanks’ balanced salt solution and incubated for 20 min at 37°C. The coverslips were mounted on the stage of a microscope (Diaphot, Nikon; Tokyo, Japan) that was fitted with a charge-coupled device imaging system (Hamamatsu Photonics; Hamamatsu, Japan). The cells were continuously superfused at 3 ml/min with Hanks’ balanced salt solution, to which H2O2 was added.

Statistics. Data are presented as means ± SE. The significance of differences between groups was evaluated by repeated-measure analysis of variance, followed by Fisher’s protected least-significant difference test. A value of \(P < 0.05\) was considered significant.

RESULTS

H2O2 decreased ET secretion and suppressed ET-1 mRNA expression. Figure 1A illustrates the effect of H2O2 on ET secretion from BAECs. A significant decrease in ET secretion was first detectable 3 h after exposure to 0.5 mM H2O2 and was maintained for up to 24 h (\(P < 0.01\) vs. basal secretion, \(n = 6\)). At each time point, ~50% suppression compared with control was observed in response to treatment with H2O2. Three hours after the administration of H2O2 (0.25–0.75 mM), ET secretion was suppressed in a dose-dependent manner, and EC50 was ~0.5 mM, as shown in Fig. 1C (\(P < 0.01\) vs. basal secretion, \(n = 6\)). Twenty-four hours of incubation with 0.5 mM of H2O2 did not significantly affect the cell number.

Northern blot analysis detected ET-1 mRNA with a size of 2.3 kb in BAECs. ET-1 mRNA levels were downregulated 3 h after exposure to 0.5 mM of H2O2 and this effect persisted for the 24-h experimental period (Fig. 1B). Exposure to 0.25–0.75 mM of H2O2 for 3 h markedly suppressed the expression of ET-1 mRNA, as shown in Fig. 1D. H2O2 (0.5 mM) suppressed ET-1 mRNA level to 43 ± 7% of the control (\(n = 4, P < 0.05\)).

To elucidate whether the observed decrease in ET-1 mRNA expression was a result of destabilization of ET-1 mRNA or of the transcriptional suppression of ET-1 mRNA, the BAECs were incubated in the presence of 10 μg/ml actinomycin D with or without 0.5 mM H2O2. In the absence of H2O2, ET-1 mRNA half-life was ~20 min, and the blot completely disappeared 2 h after exposure to this inhibitor of transcription. The...
presence of H2O2 had no significant effect on the stability of ET-1 mRNA.

**MSB decreased ET secretion.** Treatment with MSB mimicked the suppressive effect of H2O2 on ET secretion. Administration of 0.25–1.0 mM MSB for 3 h remarkably suppressed ET secretion from BAECs in a concentration-dependent manner (Fig. 2, P < 0.01 vs. basal secretion, n = 3). MSB (0.25 mM) suppressed ET secretion by 40% compared with the control.

**Antioxidants prevented H2O2-induced decrease of ET secretion.** Pretreatment with 300 U/ml of catalase completely abrogated the H2O2-induced decrease in ET secretion (Fig. 3, P < 0.01 vs. H2O2 treatment, n = 6). Catalase alone slightly augmented the basal secretion of ET (P < 0.05 vs. basal secretion, n = 6). Pretreatment with 50 μg/ml dl-α-tocopherol also significantly attenuated the H2O2-induced decrease of ET secretion (Fig. 3, P < 0.05 vs. H2O2 treatment, n = 4).

**H2O2 increased Am secretion and Am mRNA expression with augmentation of cAMP production.** Basal Am secretion from BAECs incubated for 24 h without any treatment was 8.1 ± 0.2 × 10² fmol/10⁵ cells (n = 4).

Fig. 2. Ir-ET secretion from BAECs (n = 3) incubated for 3 h with increasing doses of menadion sodium bisulfate (MSB, 0.25–1.0 mM). *P < 0.01 vs. control.

Fig. 3. Ir-ET secretion from BAECs (n = 6) pretreated for 30 min with 300 μg/ml catalase (CAT) or 50 μg/ml dl-α-tocopherol (VitE), followed by a 3-h incubation with or without H2O2 (0.5 mM). *P < 0.05, **P < 0.01 vs. control; †P < 0.05, ††P < 0.01 vs. H2O2 alone.

Fig. 4. A: adrenomedullin (ir-Am) secretion from BAECs (n = 3) incubated for 24 h with or without H2O2 (0.25–0.5 mM). *P < 0.05 vs. control. B: Am mRNA expression in BAECs incubated for 24 h with or without H2O2 (0.5 mM). *P < 0.05, **P < 0.01 vs. control. C: ir-cAMP secretion from BAECs incubated for 24 h with or without H2O2 (0.25–0.75 mM). *P < 0.05, **P < 0.01 vs. control.

Treatment with 0.5 mM of H2O2 augmented Am secretion to 14.2 ± 0.5 × 10² fmol/10⁵ cells, as shown in Fig. 4A (P < 0.05 vs. basal secretion, n = 4). Am mRNA expression was also augmented by ~30% with 24-h treatment of 0.5 mM H2O2 compared with the control (Fig. 4B). Figure 4C showed that the addition of 0.25–0.75 mM of H2O2 resulted in dose-dependent increases of cAMP production for 24 h. H2O2 (0.5 mM) significantly augmented the cAMP level in the medium by ~30% compared with the control.
Exogenously administered Am and cAMP suppressed ET secretion. We then investigated the influence of Am and cAMP on ET secretion. Three hours of treatment with human Am and forskolin, which are well known to increase the intracellular cAMP levels, attenuated ET production from BAECs in a dose-dependent manner (Fig. 5, A and B; *P < 0.01 vs. basal secretion, n = 6) and this effect continued for up to 24 h (data not shown). As shown in Fig. 5C, addition of 8-Br-cAMP resulted in a dose-dependent decrease of ET secretion from BAECs (*P < 0.01 vs. basal secretion, n = 6).

Neutralizing Am monoclonal antibody attenuated \( \text{H}_2\text{O}_2 \)-induced suppression of ET secretion. To examine the significance of Am secreted from BAECs on ET secretion, we performed neutralization experiments with KY-Am-1 on \( \text{H}_2\text{O}_2 \)-induced suppression of ET secretion. Treatment with 50 \( \mu \text{g/ml} \) KY-Am-1 for 12 and 24 h, respectively, completely inhibited the suppressive effect of \( \text{H}_2\text{O}_2 \), whereas control mouse immunoglobulin did not affect ET levels (Fig. 6; \( P < 0.01 \) vs. basal secretion, \( n = 12 \)). KY-Am-1 alone significantly increased ET secretion compared with the control.

Role of cGMP pathway in ET secretion under oxidative stress. A few studies (13) suggested that NO inhibited ET secretion by elevating cGMP production in VSMCs. We therefore investigated the influence of NO and cGMP on ET secretion in the presence of \( \text{H}_2\text{O}_2 \). Administration of 10 \( \mu \text{M} \) 8-Br-cGMP for 3 and 24 h, respectively, decreased ET secretion from BAECs to approximately one-half of that seen with the control (3 h, control, 2.41 \( \pm \) 0.07, 1.57 \( \pm \) 0.17; and 24 h, control, 10.79 \( \pm \) 0.78; 5.82 \( \pm \) 0.51, \( \times 10^2 \) fmol/10\(^5 \) cells, \( P < 0.01 \) vs. basal secretion, \( n = 3 \)). On the other hand, pretreatment with 10 and 100 \( \mu \text{M} \) L-NAME did not affect basal ET secretion or \( \text{H}_2\text{O}_2 \)-induced suppression of ET secretion (Fig. 7).

Chun et al. (3) showed that CNP was mainly secreted from ECs and the secretion was augmented by addition of \( \text{H}_2\text{O}_2 \). We accordingly addressed the effect of KY-CNP-1 on \( \text{H}_2\text{O}_2 \)-reduced ET secretion. Treatment with KY-CNP-1 for 24 h had no significant effect on 0.5 mM of \( \text{H}_2\text{O}_2 \)-induced alteration of ET secretion (Fig. 7). KY-CNP-1 alone also had no effect on basal ET secretion.

\( \text{H}_2\text{O}_2 \) transiently increased \([\text{Ca}^{2+}]_i\), and ionomycin and TSG suppressed ET production in BAEC. Treatment with Am monoclonal antibody abrogated the \( \text{H}_2\text{O}_2 \)-induced alteration of ET secretion for 12 or 24 h; however, it had no effect on ET secretion at 3 h (data not shown). To examine whether an increase in \([\text{Ca}^{2+}]_i\),
affects ET production, we measured \([\text{Ca}^{2+}]_i\) under oxidative stress. As shown in Fig. 8A, treatment with 1 mM of \(\text{H}_2\text{O}_2\) markedly increased \([\text{Ca}^{2+}]_i\). Furthermore, ionomycin, a \(\text{Ca}^{2+}\) ionophore, and TSG, an inhibitor of endoplasmic reticulum ATPase, suppressed ET secretion dose dependently (Fig. 8, B and C). On the other hand, EGTA and BAPTA had no effect on \(\text{H}_2\text{O}_2\)-induced suppression of ET secretion and ET-1 mRNA expression (data not shown).

**DISCUSSION**

ROS have been suggested to act as intracellular second messengers affecting gene regulation in various cells (11). In the present study, we demonstrated that ET production was markedly downregulated by oxidative stress. \(\text{H}_2\text{O}_2\) suppressed ET production both dose and time dependently. The suppressive effect on ET secretion by MSB is consistent with the effect of \(\text{H}_2\text{O}_2\). These results indicate that oxidative stress is a negative regulator of ET secretion. Treatment with catalase, the specific reductase of \(\text{H}_2\text{O}_2\), not only inhibited \(\text{H}_2\text{O}_2\)-induced suppression of ET secretion but also significantly augmented basal ET secretion, implying that ECs are constantly exposed to oxidative stress at a certain level.

ECs are continuously subjected to flow-induced shear stress. Some studies (2, 4) recently reported that shear stress caused a sustained activation of prooxidant processes, resulting in redox-sensitive gene expression in human ECs, and that this activation was significantly attenuated by pretreatment with antioxidants. We have already shown that shear stress at a physiological level suppressed the production of ET and mRNA expression of ET-converting enzyme-1 (28). From our preliminary results, shear stress-induced downregulation of ET and ET-converting enzyme-1 suppression is blocked on pretreatment with \(\text{N}-\text{acetylcyestein}\), an antioxidant. These findings and our results indicate that ET production may be modulated via a redox-sensitive mechanism in the physiological state in vivo.

In the present study, we assessed ET-1 mRNA stability under oxidative stress by using actinomycin D. Inoue et al. (16) reported that the AUUUA motif, considered to be an RNA-destabilizing element, was found in the 3’ untranslated region of ET-1 mRNA. In the current study, the ET-1 mRNA half-life was \(\sim20\) min in both the presence and the absence of 0.5-mM \(\text{H}_2\text{O}_2\). This indicates that \(\text{H}_2\text{O}_2\) did not significantly alter the

---

**Fig. 7.** Ir-ET secretion from BAECs \((n = 12)\) pretreated with or without \(\text{N}^\text{G}\)-nitro-\(\text{L}\)-arginine methyl ester (L-NAME) \((100 \mu\text{M})\) or an anti-\(\text{C}\)-type natriuretic peptide monoclonal antibody (A-CNP-Ab) \((10 \mu\text{g/ml KY-CNP-1})\) and incubated for 24 h with or without \(\text{H}_2\text{O}_2\) \((0.5 \mu\text{M})\). *\(P < 0.01\) vs. control.

**Fig. 8.** Intracellular \(\text{Ca}^{2+}\) concentrations \(([\text{Ca}^{2+}]_i)\) in BAECs after treatment with 1.0 mM \(\text{H}_2\text{O}_2\) \((A)\). Ir-ET secretion from BAECs incubated for 3 h with or without ionomycin \((10–100 \mu\text{M}; B)\), and thapsigargin \((\text{TSG}, 0.3–3.0 \mu\text{M}; C)\). *\(P < 0.05\) and **\(P < 0.01\) vs. control.
ET-1 mRNA half-life, suggesting that the downregulation of ET-1 mRNA under oxidative stress was modulated at the transcriptional level.

In previous studies (17), administration of interleukin-β, tumor necrosis factor-α, phorbol ester, oxidized low-density lipoprotein, and lysophosphatidyl choline, which are well recognized to generate intracellular ROS, elevated Am production in cultured rat ECs. In the current study, we obtained direct evidence that Am secretion and Am mRNA expression were augmented by oxidative stress. Am exerts several actions through augmentation of cAMP production. In the present study, H2O2 increased cAMP production in a dose-dependent fashion. In addition, both 8-Br-cAMP and forskolin, as well as Am, suppressed ET secretion from BAECs dose dependently. We further demonstrated that treatment with a neutralizing monoclonal antibody against Am inhibited the suppressive effect of H2O2 on ET secretion for 24 h. These results indicate that Am secretion enhanced by oxidative stress inhibited ET secretion via a cAMP-dependent process in BAECs.

However, the neutralizing monoclonal antibody against Am failed to prevent H2O2-induced suppression of ET secretion at 3 h. Because some reports demonstrated the suppressive effect of increase in [Ca2+]i on ET production in ECs, we investigated the role of intracellular Ca2+ in ET secretion. As shown in Fig. 8, H2O2 markedly increased [Ca2+]i. Furthermore, ionomycin and TSG significantly suppressed ET secretion dose dependently. This effect seems to be dependent on intracellular Ca2+ fluxes because EGTA and BAPTA had no effect on ET production with or without H2O2. These results suggest that H2O2 suppressed ET secretion also via an increase in [Ca2+]i in BAECs.

NO is known to produce cGMP in ECs through activation of soluble guanylate cyclase. It is reported that elevation of the cGMP levels in ECs inhibits ET secretion. Moreover, it is well established that an increase in [Ca2+]i stimulates NO synthesis in ECs. A previous report (34) indicated that treatment with 1.0 mM H2O2 increased [Ca2+]i and enhanced NO synthase (NOS) activity in ECs, leading to cell injury. Another group demonstrated that treatment with 1.0 and 10.0 mM H2O2 increased endothelial NO (eNOS) tyrosine-phosphorylation and its interaction with caveolin-1, the coat protein of caveolae (8). On the other hand, Gupta et al. (10) demonstrated that in intact ECs, 0.1–0.5 mM of H2O2 had little effect on NOS activity. To determine the possible involvement of the NO-cGMP pathway in the suppression of ET secretion by H2O2, we examined the effect of L-NAME. There was no difference in ET secretion between control and L-NAME-treated BAECs. In our previous study (3), inducible NOS and eNOS expression were not significantly altered by the treatment with 0.25–0.75 mM of H2O2. These findings suggest that, whereas higher concentrations of H2O2 induce NOS activation by extracellular Ca2+ influx, activation of NOS at a level high enough to cause suppression of ET secretion may not occur in ECs in response to lower concentrations of H2O2 as used in the present study.

We have already shown that CNP induces growth inhibition and redifferentiation of proliferating and dedifferentiated VSMCs (6). We also demonstrated that CNP has the ability to potently augment intracellular cGMP accumulation by binding to the atrial natriuretic peptide-B receptor, which has been shown (23) to be selectively activated by CNP in VSMCs. Therefore, we examined the effect of the CNP neutralizing monoclonal antibody to investigate the possible involvement of the CNP-cGMP pathway in H2O2-suppressed inhibition of ET production. The addition of anti-CNP monoclonal antibody KY-CNP-1, however, failed to prevent H2O2-induced suppression of ET secretion. It could be speculated that atrial natriuretic peptide-B receptor expression is very scarce in ECs, which we previously reported (35).

Our findings suggest that ECs possess the coordinate gene regulation mechanism to reduce the vasocontracting peptide (ET) secretion and to increase the vasorelaxant peptide (Am) production in response to oxidative stress. By this mechanism, ECs function to protect against oxidative stress by increasing blood flow via VSMC relaxation and growth inhibition.

We thank A. Sone, A. Nonoguchi, and Y. Takada for excellent secretarial work.

This work was supported in part by research grants from the Japanese Ministry of Education, Science and Culture, Japanese Ministry of Health, Japan Smoking Foundation, and Japanese Society for the Promotion of Science “Research for the Future” Program Grants JSPS-RPTP 96100204 and JSPS-RPTP 98L00801.

REFERENCES


