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Arachidonic Acid Induces Endothelin-1 Gene Expression in Vascular Endothelial Cells

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Abstract Endothelin-1 (ET-1) is a potent vasoconstrictor peptide synthesized and secreted by vascular endothelial cells. Regulation of ET-1 production occurs at the level of gene transcription. We previously demonstrated a role for arachidonic acid as an intracellular mediator in the regulation of gene expression. This study investigated the role of arachidonic acid in induction of ET-1 production in endothelial cells. Challenge of bovine aortic endothelial cells (BAECs) with arachidonic acid induced a dose- and time-dependent increase in the amount of immunoreactive ET-1 in the supernatant. The maximum effect was observed at concentrations of 20 μM. Release of ET-1 by arachidonic acid was preceded by induction of ET-1 gene expression. Arachidonic acid increased ET-1 gene expression by increasing transcription of the ET-1 gene. The effect of arachidonic acid was mimicked by other polyunsaturated fatty acids, whereas saturated fatty acids had no effect. Moreover, inhibitors of the lipoxygenase pathway blocked arachidonic acid-induced release of ET-1. These results suggest that arachidonic acid stimulated the production of ET-1 in BAECs by inducing ET-1 gene transcription. Arachidonic acid-induced production of ET-1 is dependent on lipoxygenase products of arachidonate metabolism. J. Cell. Biochem. 75:724–733, 1999.

Key words: arachidonic acid; endothelial cells; endothelin-1 gene

Several lines of evidence have demonstrated that the vascular endothelium participates in the control of vascular tone and function through the release of vasoactive mediators [Arai et al., 1990; Vanhoutte et al., 1988]. Endothelin-1 (ET-1), a peptide of 21 amino acid residues originally isolated from porcine aortic endothelial cells, is one of the most potent known vasoconstrictor [Yanagisawa et al., 1988]. There are at least three different isoforms of endothelins (ET-1, ET-2, and ET-3) each regulated by separate genes [Inoue et al., 1989]. Although these isoforms share a high degree of structural homology, their production is regulated differently ,as only ET-1 is produced by endothelial cells [Nakamura et al., 1990]. Endothelial cells do not contain secretory granules for the storage of ET-1 and the release of ET-1 after cell stimulation results from induction of ET-1 gene expression [Block et al., 1989]. Different regulatory elements, including an AP-1 motif, have been identified in the promoter region of the ET-1 gene as potential sites for control and regulation of ET-1 gene expression [Lee et al., 1995]. ET-1 is initially synthesized as preproendothelin, a precursor of 202-amino acid, which is subsequently cleaved into big endothelin, a biologically inactive precursor of 38 amino acid residues [Kimura et al., 1989]. Big endothelin is subsequently subjected to proteolytic cleavage into the final biologically active peptide by the endothelin-converting enzyme (ECE-1) a membrane-bound metalloprotease [Opgenorth et al., 1992]. The release of ET-1 occurs upon cell stimulation by a wide array of stimuli, including cytokines, shear stress, thrombin, vasopressin, and angiotensin II [Golden et al., 1995; Marsden et al., 1992; Yoshizumi et al., 1989; Emori et al., 1989]. ET-1 possesses a wide range of biological activities both in vivo and in vitro, including mitogenesis, muscle contraction, release of vasoactive substances, and vasoconstriction [Weissberg et al., 1990; Sumner et al., 1992; Yang et al., 1990]. The effects of ET-1 on cell function are mediated by its binding to two distinct subtypes of G proteins-coupled receptors, ET-A and ET-B, which are expressed

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differently in a wide variety of tissue types [Arai et al., 1990].

Arachidonic acid, a biologically active essential fatty acid, is produced in response to agonist stimulation by a variety of cells, including endothelial cells [Gryglewski et al., 1988]. Several studies have shown that arachidonic acid metabolism plays a pivotal role in vascular physiology [Whatley et al., 1990]. Thus, arachidonic acid metabolites are involved in regulation of endothelial cell proliferation and in the control of vascular tone [Harder et al., 1995]. Cyclooxygenase metabolites of arachidonic acid, including prostacyclin (PGI_2) and prostaglandins, have been shown to be potent vasodilators [Sumner et al., 1992; Furchgott et al., 1989]. By contrast, thromboxane A_2, prostaglandins $F_{2\alpha}$, several leukotrienes, or metabolites of the cytochrome-P-450 pathway are implicated in the induction of vasoconstriction [Harder et al., 1988]. The rate-limiting step in the biosynthesis of these products is the availability of arachidonic acid. Once released upon cell stimulation, arachidonic acid is rapidly transformed in eicosanoids, which in turn regulate the vascular tone [Mayer et al., 1993; Marsden et al., 1991]. Although there is substantial evidence implicating arachidonic acid metabolites in regulation of vascular tone, little information is available with regard to the cell signaling mechanisms by which arachidonic acid or its metabolites exert their actions. Previous investigations by our group as well as other workers, have defined a role for arachidonic acid as mediator of cytokine-induced expression of genes containing an AP-1 motif in their promoter [Rao et al., 1996; Rizzo et al., 1995]. Moreover, we demonstrated activation of c-jun by arachidonic acid in stromal cells [Rizzo et al., 1996, 1999]. In the present study, we have explored the hypothesis that arachidonic acid induces ET-1 gene expression, leading to an increased production of ET-1 by vascular endothelial cells. We present evidence that arachidonic acid stimulates production of ET-1 from bovine aortic endothelial cells (BAECs) by increasing the transcription of the ET-1 gene. The signaling pathway of arachidonic acid-induced ET-1 release appears to be dependent on lipoxygenase products of arachidonic acid metabolism. The control of ET-1 production by arachidonic acid may represent a mechanism by which arachidonate metabolites participate in regulation of endothelial cell function and the control of vascular tone.

MATERIALS AND METHODS

Chemicals and Other Reagents

CS-C medium for endothelial cell growth, endothelial cell growth factor (ECGF) supplement, arachidonic acid, oleic acid, palmitic acid, linoleic acid, linolenic acid, indomethacin, caffeic acid, nordihydroguaiaretic acid (NDGA), actinomycin D, cycloheximide, and the lactate dehydrogenase (LDH) kit were purchased from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). The endothelial cell attachment factor was purchased from Cell Systems (Kirkland, WA). The endothelin assay kit was purchased from R&D Systems (Minneapolis, MN). Trizol was purchased from Gibco-BRL Life Technology (Gaithersburg, MD). RNA molecular markers were purchased from Boehringer-Mannheim (Indianapolis, IN). [α-^{32}P] dCTP, Hyperfilms-MP, and nitrocellulose membranes were obtained from Amersham Life Science (Arlington Heights, IL), and [α-^{32}P] dUTP was obtained from New England Nuclear (Boston, MA).

Endothelial Cell Culture

BAECs were purchased from Cell Systems. Cells were received in the second passage and propagated in CS-C medium containing 10% FBS and 1% ECGF supplement (containing porcine heparin and HBGF-1) at 37°C in a humidified 5% CO_2 atmosphere. After initial plating, medium was changed every 48 h. Confluent cells exhibited a cobblestone appearance. The cells were used when confluent and at passages 4–10. The cells did not show changes in morphology or response to stimuli over this range of passages.

Arachidonic Acid Supplementation

Stock solutions of arachidonic acid were prepared by dissolving sodium arachidonate in ethanol (100%). Before use, arachidonic acid was complexed to fatty acid-free bovine serum albumin (BSA) in a 1:4 molar ratio. The same procedure was used to prepare the other fatty acids. Trypan blue dye exclusion and measurement of LDH activity in culture cell supernatant confirmed that treatment with arachidonic acid at the concentrations used in the present study did not produce significant cytotoxicity.

Isolation of Cytosolic RNA

Total cellular RNA was isolated using the Trizol reagent following the manufacturer's instructions. The RNA was precipitated overnight at −70°C in isopropanol and washed in 70% ethanol; the concentration was determined by spectrophotometer analysis as previously described [Rizzo et al., 1994].

Northern Blot Analysis

For Northern blot analysis, samples of 30 µg of RNA were denatured in a mixture of formaldehyde and formamide for 5 min at 65°C. RNA was size fractionated by electrophoresis through denaturing 1% agarose gel, transferred onto nitrocellulose membranes, and hybridized with ^{32}P-labeled cDNA probes as previously described [Rizzo et al., 1994]. A 1.2-kb *Eco*RI insert isolated by preparative agarose gel electrophoresis from a full-length human ET-1 cDNA (obtained from ATCC, Rockville, MD), was prepared and labeled with the Klenow fragment using 25 µCi of [α-^{32}P] dCTP after priming with random hexamers, as previously described [Rizzo et al., 1994]. Blots were exposed to x-ray and ^{32}P-labeled ET-1 was visualized by autoradiography. A 1.2-kb fragment of ^{32}P-labeled CHO-B was used to normalize the amount of RNA loaded in each lane. The relative intensities of the bands on the autoradiographs were quantified using the Un-Scan-It software program (Silk Scientific, Orem, UT).

Nuclear Run-on Assay

Confluent cultures of BAECs were stimulated in serum-free conditions as indicated. Cells were washed with ice-cold phosphate-buffered saline (PBS), and nuclei were isolated by cell lysis with 0.5% Nonidet P-40 (NP-40) [Rizzo et al., 1995]. Isolated nuclei were stored at −70°C in a buffer containing 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol until further use. The transcription reaction and isolation of labeled RNA were performed as previously described [Rizzo et al., 1995]. Briefly, nuclei (10^7) were incubated for 30 min at 26°C in a reaction buffer containing 100 µCi of α-^{32}P (3,000 Ci/mmol)[Oriji and Kaiser, 1997]. Labeled RNA was isolated and hybridized to nitrocellulose paper containing 5 µg each of cDNA probes for ET-1, CHO-B, and pGEM. Filters were washed, dried, and exposed to x-ray films as previously described [Rizzo et al., 1995].

Quantitative Determination of ET-1 by Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

Measurement of the released ET-1 peptide in the conditioned medium of cells stimulated with arachidonic acid was performed by ELISA following manufacturer's instructions. Briefly, BAECs grown in 24-well/plates were washed and stimulated under serum-free conditions with either vehicle or various concentrations of arachidonic acid for the indicated times at 37°C and 5% CO_2. Supernatants were removed and centrifuged at 800*g* to remove debris. Supernatants were either used immediately for ET-1 quantification or kept at −20°C until further use. Preliminary experiments showed that storage of the supernatants at −20°C did not affect subsequent measurement of ET-1. For quantification of ET-1, supernatants were incubated for 1 h at room temperature in a 96-well microplate coated with rat antibody to ET-1 in the presence of a second ET-1 antibody bound to horseradish peroxidase (HRP) to allow any ET-1 present in the supernatant to form a bridge between the two antibodies. Unbound material was removed, each well was washed several times before the addition of a substrate specific for HPR and incubation was continued for 30 min at room temperature. Reactions were terminated by the addition of HCl, and the optical density of each well was determined using a microtiter plate reader set at 450 nm. The amount of ET-1 present in the supernatants was calculated by comparison of the optical density readings to that of a standard curve generated with known amount of ET-1. Each assay included an ET-1-positive control. Sensitivity of the assay was 0.25 pg/ml. This assay was specific for ET-1. Cross-reactivity with big ET-1, safarotoxin, ET-2, and ET-3 was $<$1%, $<$2%, 45%, and 14%, respectively.

Protein Determination

Protein concentration was quantified by the method of Bradford, using BSA as the standard [Bradford et al., 1976].

Statistical Analysis

Unless otherwise indicated, data are expressed as means ±SEM of the mean obtained from at least three independent experiments.

All statistical analyses were performed with the Sigma Stat (St. Louis, MO) program using an IBM-compatible personal computer. Comparisons between two means were made with the Student's *t*-test. *P* values of <0.05 were considered statistically significant.

RESULTS

Arachidonic Acid Induces Endothelin-1 Secretion

The amount of ET-1 released from BAECs into the conditioned medium upon stimulation with arachidonic acid was measured. Stimulation with various concentrations of arachidonic acid under serum-free conditions induced a significant increase of ET-1 released from BAECs in a dose-dependent manner. Arachidonic acid (10 and 20 μM) induced 1.3-fold ($P < 0.0002$) and 2-fold ($P < 0.00009$) increase in the amount of ET-1 released from endothelial cells compared with cells stimulated with vehicle only (Fig. 1A). Levels of immunoreactive ET-1 increased from 134 7.7 pg/mg protein in unstimulated cells to 150 ± 21, 306 ± 1.7, and 404 ± 15.3 pg/mg protein in cells stimulated for 4 h with 5, 10, and 20 μM arachidonic acid, respectively (Fig. 1A). At these concentrations, arachidonic acid was not cytotoxic, as determined by measurement of the release of LDH into the supernatant and by Trypan blue exclusion (data not shown). Arachidonic acid induced the release of ET-1 from BAECs in a time-dependent manner (Fig. 1B). After 2-h stimulation, arachidonic acid (20 μM) induced a 3.9-fold increase ($P < 0.0035$) over unstimulated cells of ET-1 released in the conditioned medium of BAECs. The amount of ET-1 recovered in the supernatant of arachidonic acid-stimulated BAECs increased in a linear fashion over a period of 6 h (Fig. 1B). A time-dependent increase in the release of ET-1 was also observed in cells stimulated with vehicle only (Fig. 1B). These findings are consistent with previous reports showing constitutive secretion of ET-1 from endothelial cells in culture [Marsden et al., 1992, Vogel et al., 1997].

We also examined cell-associated ET-1 under basal conditions or after stimulation with arachidonic acid. Cells were stimulated with various concentrations of arachidonic acid (5–20 μM) for 4 h. Supernatants were removed, cells were recovered and lysed in the presence of 1% Triton X-100, and the ET-1 content of cell lysates was measured. Stimulation with arachidonic acid increased the amount of cell-associated ET-1 from 28 pg ± 6.4/mg/protein in unstimulated cells to 49 ± 14, 55 ± 15, and 106 ± 10 pg/mg/protein at concentrations of 5, 10, and 20 μM, respectively (Fig. 2).

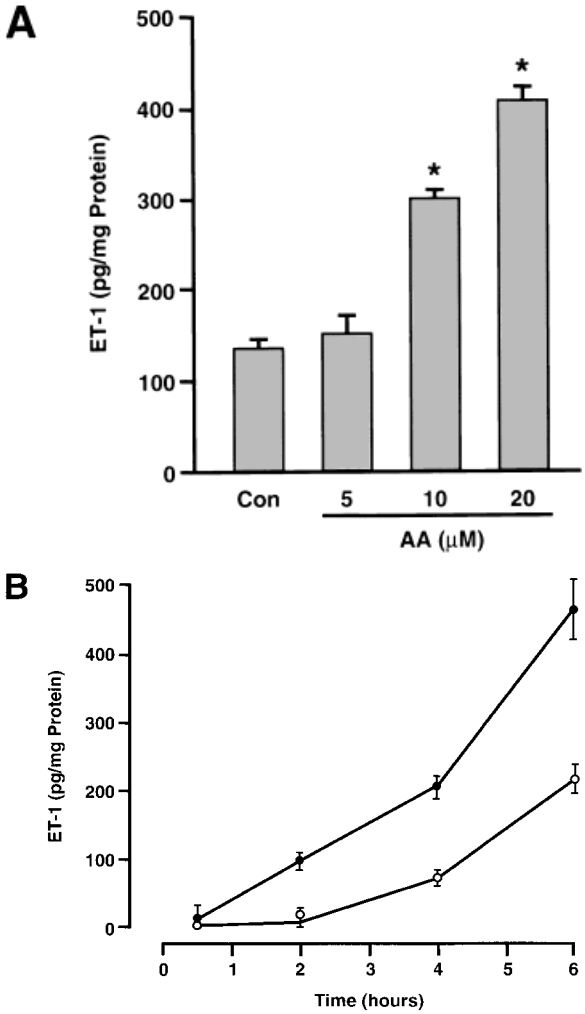

Fig. 1. Arachidonic acid-induced release of endothelin-1 (ET-1) from bovine aortic endothelial cells. **A:** Effect of various concentrations of arachidonic acid. Confluent cells growing on 12-well/plates were washed and stimulated in serum-free conditions with various concentrations of arachidonic acid (AA) for 4 h. Vehicle-treated cells are indicated as control (Con). Supernatants were collected and ET-1 measured by enzyme-linked immunosorbent assay (ELISA). Results are expressed as pg ET-1 released in the supernatant and normalized for mg protein/well. Results represent mean ±SEM of five independent experiments. *Statistical significant ($P < 0.05$). **B:** Kinetics of arachidonic acid-stimulated ET-1 release. Bovine aortic endothelial cells were stimulated with arachidonic acid (AA 20 μM) or with vehicle (Con) for the indicated time intervals. Supernatants were collected and ET-1 measured by ELISA. Results are expressed as pg/mg protein/well. Results are mean ± SEM of four independent experiments, each performed in duplicate. *Statistical significant ($P < 0.05$)

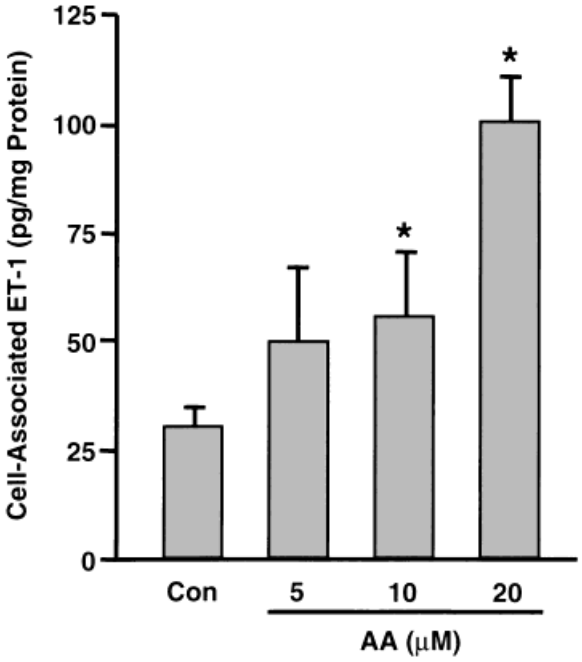

Fig. 2. Effect of arachidonic acid (AA) on cell-associated endothelin-1 (ET-1). Bovine aortic endothelial cells were stimulated with various concentrations of arachidonic acid (AA 5–20 μM) or with vehicle (Con) for 2 h. Cells were collected and lysed in 1% Triton X-100. ET-1 was measured from the cell lysates by enzyme-linked immunosorbent assay (ELISA). Results are expressed as pg of cell-associated ET-1 per mg protein. Results are mean ±SEM of three independent experiments. *Statistically significant ($P < 0.05$).

Effect of Arachidonic Acid on Endothelin-1 Gene Expression

Experiments were then undertaken to determine whether increased synthesis and release of ET-1 upon cell stimulation with arachidonic acid was associated with induction of ET-1 gene expression. BAECs were stimulated under serum-free conditions with various concentrations of arachidonic acid (5–20 μM), RNA was extracted, resolved and hybridized with a radiolabeled ET-1 cDNA probe. As shown in Figure 3A, arachidonic acid induced a dose-dependent increase of ET-1 gene expression at 1 h of stimulation. Densitometric analysis showed a 1.75- and 3.7-fold increase of ET-1 gene expression upon stimulation of BAECs with arachidonic acid at concentrations of 10 and 20 μM, respectively (Fig. 3A). Lower concentrations (5 μM) of arachidonic acid were ineffective (Fig. 3A). Arachidonic acid induced ET-1 gene expression in a time-dependent manner (Fig. 3B). The effect of arachidonic acid (20 μM) on ET-1 gene expression was detected after 30 min of stimulation

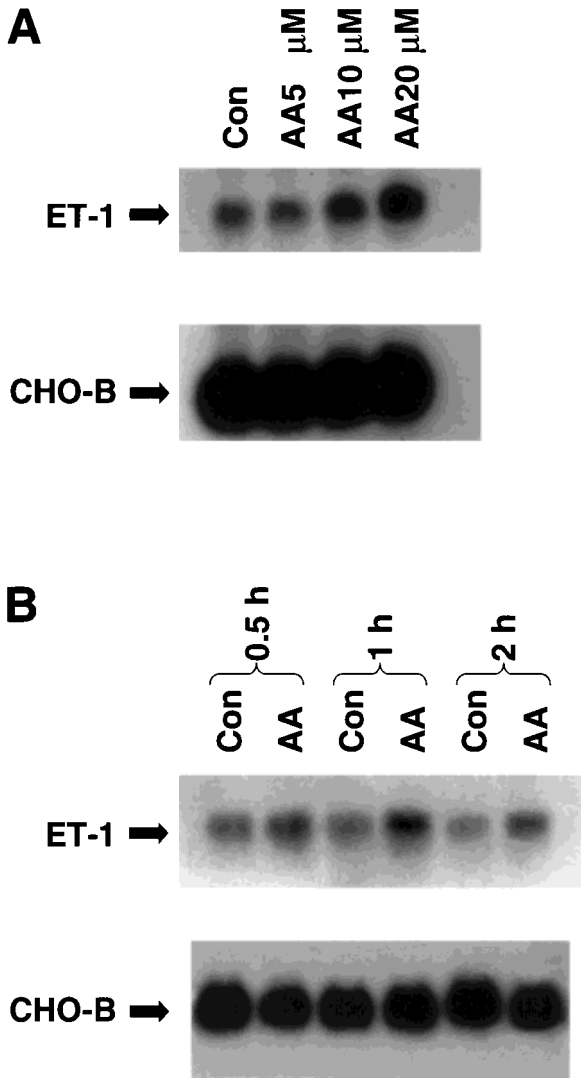

Fig. 3. Induction of endothelin-1 (ET-1) gene expression by arachidonic acid. **A:** Effect of various concentrations of arachidonic acid. Confluent monolayers of bovine aortic endothelial cells were stimulated in serum-free conditions with arachidonic acid (AA) or with vehicle (Con). Total RNA (30 μg /lane) was sequentially hybridized with ^{32}P-labeled ET-1 probe (1.2-kb) (upper) and with CHO-B (1.2 kb) used as loading control (lower). **B:** Kinetics of ET-1-induced gene expression. Cells were stimulated with arachidonic acid (AA, 20 μM) or with vehicle (Con) from 30 min to 2 h. Total RNA (10 μg /lane) was hybridized with ^{32}P-labeled ET-1 and CHO-B probes. Shown are representative autoradiographs.

and increased over a period of 2 h (Fig. 3B). Longer incubations with arachidonic acid did not further increase the expression of the ET-1 (data not shown). In initial Northern blot analyses β-actin was used as a housekeeping gene to check for loading. However, these experiments showed that arachidonic acid increased the ex-

pression of β-actin gene in a time- and dose-dependent manner (M. T. Rizzo and W.-M. Yu, unpublished observations). Therefore, blots were sequentially hybridized with the constitutive active mouse gene CHO-B to monitor for loading.

To clarify whether induction of ET-1 gene expression induced by arachidonic acid was caused by an increase in ET-1 mRNA half-life, we performed actinomycin D pulse-chase experiments. Cells were stimulated with arachidonic acid (20 μM, 30 min); actinomycin D (20 μg /ml) was then added to arrest RNA transcription and permit quantification of the rate of disappearance of ET-1 mRNA. Cells were harvested at 30 min, 1 h, and 2 h after addition of actinomycin D. RNA was isolated and analyzed for ET-1 gene expression. The addition of actinomycin D did not increase ET-1 gene expression induced by arachidonic acid, suggesting that the effect of the fatty acid on ET-1 gene expression is not attributable to increased stabilization of the ET-1 mRNA (Fig. 4). To determine whether the increased ET-1 gene expression upon exposure of BAECs to arachidonic acid was the result of increased of gene transcription, nuclei run on analyses were performed. Cells were stimulated with arachidonic acid (20 μM for 30 min) or with vehicle, nuclei were prepared and the transcription reaction was performed as previously described [Rizzo et al., 1995]. Challenge of BAECs with arachidonic acid induced a 3.7-fold increase in the transcription rate of the ET-1 gene (Fig. 5). No effect of arachidonic acid was observed on the transcription rate of the housekeeping gene CHO-B or of the empty vector pGEM (Fig. 5). Taken together, these results suggested that arachidonic acid induced ET-1 gene expression by stimulating ET-1 gene transcription.

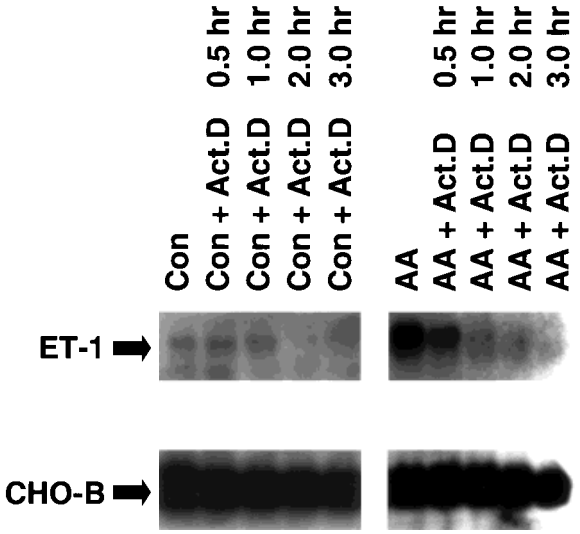

Fig. 4. Effect of arachidonic acid on ET-1 mRNA stability. Confluent cultures of bovine aortic endothelial cells were stimulated with either 20 μM arachidonic acid (AA) or vehicle for 60 min. Actinomycin D (10 μg /ml) was then added to cells for various time intervals. Total RNA was extracted at subsequent time points and analyzed for ET-1 and CHO-B gene expression. A representative autoradiograph from an experiment performed twice is shown.

Specificity of Arachidonic Acid-Induced Release of ET-1

The effect of arachidonic acid on the production of ET-1 from BAECs was compared with the effect of other fatty acids with various degree of saturation, to address the question of structural specificity. Cells were stimulated in serum-free conditions with equimolar concentrations (20 μM) of different unsaturated fatty acids, including oleic acid (C18:1, n-9), linoleic acid (C18:2, n-6), and linolenic acid (C18:3, n-3), and with the saturated palmitic acid (C16:0). Palmitic acid had no effect on the release of ET-1 (Fig. 6). Among the unsaturated fatty acids, linolenic acid and linoleic acid had a modest effect, whereas oleic acid was the most potent in inducing an increase of released ET-1 from BAECs (Fig. 6).

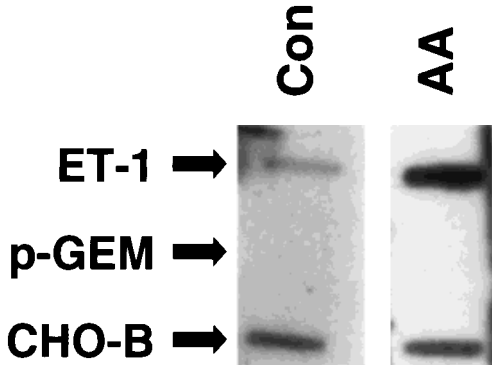

Fig. 5. Effect of arachidonic acid on ET-1 gene transcription. Nuclei were isolated from bovine aortic endothelial cells stimulated with 20 μM arachidonic acid for 30 min or with vehicle and subsequently used in transcription run-on experiments as described under Material and Methods. ^{32}P-labeled RNA transcripts were hybridized to 5 μg of ET-1, CHO-B and pGEM cDNA probes immobilized on nitrocellulose filters. After autoradiography for 96 h, the relative levels of gene transcription were quantitated by densitometry. A representative autoradiograph from an experiment performed twice with independent nuclei preparation is shown. Each experiment yielded similar results.

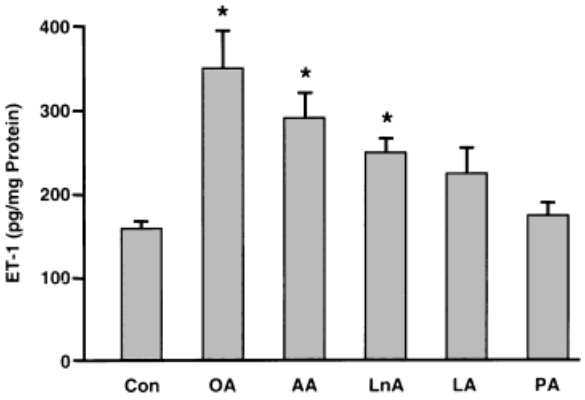

Fig. 6. Effect of fatty acids on ET-1 released from bovine aortic endothelial cells. Confluent monolayers of bovine aortic endothelial cells growing on 12-well/plates were washed and stimulated under serum-free conditions with 20 μM arachidonic acid (AA), oleic acid (OA), linolenic acid (LnA), linoleic acid (LA), and palmitic acid (PA) for 2 h. The amount of immunoreactive ET-1 was measured by enzyme-linked immunosorbent assay (ELISA). Results represent mean ± SEM of experiments performed in triplicate. *Statistically significant ($P < 0.05$).

Effect of Inhibitors of Arachidonic Acid Metabolism on ET-1 Release From Endothelial Cells

We investigated whether the effect of arachidonic acid on ET-1 release was mediated by its conversion into eicosanoids. Preincubation of BAECs with the cyclooxygenase inhibitor indomethacin (10 μM for 30 min) before stimulation with arachidonic acid (20 μM for 2 h) had no significant effect on arachidonate-induced release of ET-1 (Fig. 7). By contrast, preincubation with the lipoxygenase inhibitor caffeic acid (10 μM for 30 min) induced a significant ($P < 0.05$) decrease in immunoreactive ET-1 released from arachidonic acid-stimulated BAECs (Fig. 7). Similarly, preincubation with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) also inhibited ET-1 release from arachidonic acid-stimulated cells ($P < 0.009$) (Fig. 7). Pretreatment of BAECs with ketoconazole, an inhibitor of the P-450 cytochrome system, had no effect on arachidonic acid-stimulated release of ET-1 (data not shown). The decrease of arachidonic acid-stimulated ET-1 release in the presence of the inhibitors was not a consequence of cell toxicity, as cell morphology, attachment, and viability were not affected by these pharmacological treatments.

DISCUSSION

Vascular endothelial cells elaborate a variety of vasoactive substances including vasodilators and vasoconstrictors, which modulate intrinsic vascular tone [Davies et al., 1993; Vanhoutte et al., 1988; Marsden et al., 1991]. The vascular endothelium is a primary target of arachidonic acid metabolism, as suggested by evidence that eicosanoids are actively involved in the control of vascular tone [Gryglewski et al., 1988; Furchgott et al., 1989]. However, the signaling pathways involved in mediating these responses are largely unknown. The present study provides evidence of the involvement of arachidonic acid in the production and release of ET-1, a potent vasoconstrictor peptide, from vascular endothelial cells, suggesting a potential mechanism by which arachidonic acid metabolism regulates vascular tone. Several studies have been focused primarily on the signaling pathways by which ET-1 influences cellular functions or the variety of external stimuli, which control ET-1 production [Levin et al., 1995; Golden et al., 1995; Marsden et al., 1992; Yoshizumi et al., 1989; Emori et al., 1989]. However, the mechanisms of production of ET-1 in endothelial cells remain unclear. Furthermore, the potential role of lipid second messengers in the production and release of ET-1 has not yet been investigated.

Previous investigations by our group and others have shown that arachidonic acid is a potent activator of gene expression [Rao et al., 1996; Rizzo et al., 1994, 1995; Sellmayer et al., 1997]. Among the genes activated by arachidonic acid are those containing an AP-1 motif in their promoter [Rao et al., 1996]. The transcription factor c-jun, which participates in the formation of the AP-1 complex, is also activated by arachidonic acid in various different cell types [Rizzo et al., 1995, 1999]. The endothelin-1 gene contains in its 5′ flanking region, the consensus sequence that binds c-jun/AP-1 complex [Lee et al., 1989]. These findings prompted us to explore the possibility that arachidonic acid is involved in activation of ET-1 gene expression. The results of this study are consistent with this hypothesis. Thus, stimulation of BAECs with arachidonic acid induced a dose- and time-dependent increase of ET-1 gene expression. The increased ET-1 gene expression detected upon stimulation with arachidonic acid is due to an increase of ET-1 transcription rather to an increase of ET-1 mRNA half-life as shown by nuclear run on assays and actinomycin D pulse-chase experiments. Stimulation of BAECs with arachidonic acid induced c-jun gene expression

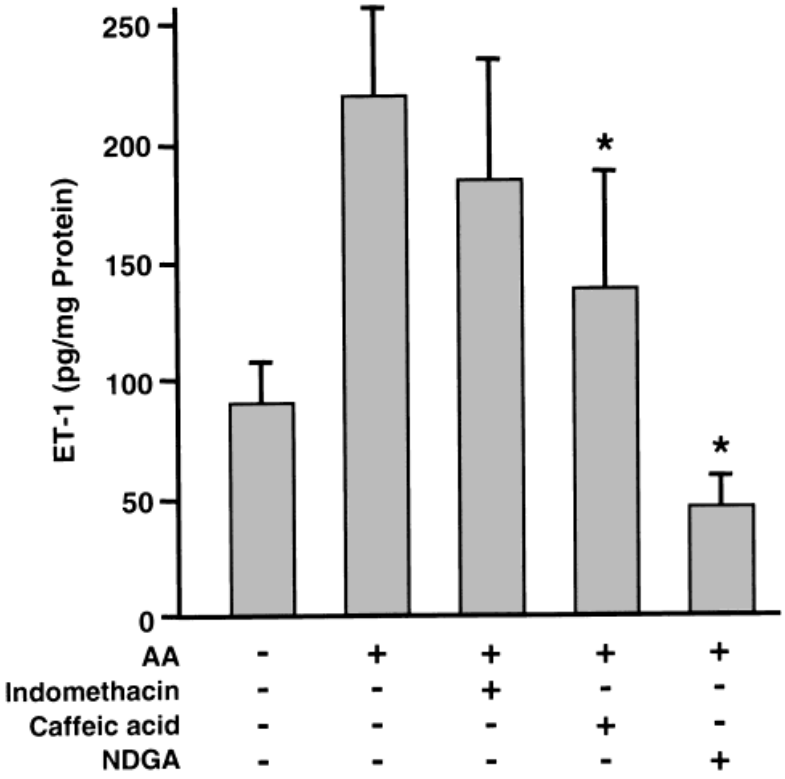

Fig. 7. Effect of inhibitors of arachidonic acid metabolism on the release of endothelin-1 (ET-1) from endothelial cells. Confluent bovine aortic endothelial cells (BAECs) were preincubated with the indicated inhibitors for 30 min before stimulation with arachidonic acid (20 μM, 2 h). Vehicle-treated cells are indicated as control (Con). Supernatants were collected after 2 h of incubation at 37°C, and the amount of immunoreactive ET-1 was measured by enzyme-linked immunosorbent assay (ELISA). Results represent mean ±SEM of three independent experiments. *Statistically significant ($P < 0.05$).

and activation of the c-jun N-terminal kinase (M.T. Rizzo, unpublished observations). Thus, is possible that the effect of arachidonic acid on ET-1 transcription is mediated by the AP-1 motif present in the 5′ flanking region of the ET-1 gene. Further studies are required to clarify this possibility.

Production and release of the ET-1 peptide by arachidonic acid into the supernatant of endothelial cells followed induction of ET-1 gene expression. Release of ET-1 peaked at 4–6 h of stimulation, whereas induction of the ET-1 gene was detected as early as 30 min of stimulation. The release of ET-1 was attenuated by pretreatment with actinomycin D, suggesting that the release of ET-1 was dependent on transcriptional activation of the ET-1 gene. Furthermore, upon treatment of BAECs with arachidonic acid there was an increase of cell-associated ET-1 as compared with unstimulated cells. This finding was consistent with the possibility that increased release of ET-1 was attributable to increased synthesis of the peptide as a result of activation of the ET-1 gene.

Pharmacological inhibition of the lipoxygenase pathway by caffeic acid or NDGA markedly reduced arachidonate-induced release of ET-1 from BAECs. This finding implies that conversion of arachidonic acid into one or more lipoxygenase products was required for its effect on ET-1 production. NDGA was more potent that caffeic acid in reducing arachidonate-induced release of ET-1. NDGA inhibits the 5-, 12-, and 15-lipoxygenases, whereas caffeic acid selectively inhibits the 5-lipoxygenase only [Koshihara et al., 1984]. It has been reported that NDGA also inhibits the cytochrome P-450 pathway [Kozak et al., 1998]. However, our data suggest that the effect of NDGA on arachidonate-induced ET-1 release is due to inhibition of the lipoxygenase system, as ketoconazole, an inhibitor of the cytochrome P-450 pathway, did not affect ET-1 released upon challenge of BAECs with arachidonic acid. The inhibitory effect of NDGA on arachidonic acid-induced ET-1 production may also be partially attributed to its antioxidant properties [Keogh et al., 1998]. Consistent with this possibility, NAC (N-acetylcysteine), a structurally distinct antioxidant, partially blocked arachidonate-induced release of ET-1 (data not shown).

The effect of arachidonic acid was compared with that of other fatty acids. The unsaturated fatty acids tested oleic acid and linolenic acid

induced a significant increase of ET-1 release compared with untreated cells, whereas palmitic acid, a saturated fatty acid, had no effect. These findings may be relevant to the physiological activity of certain fatty acids, as oleic acid is the major extracellular free fatty acid found circulating in the blood, whereas arachidonic acid is the major intracellular free fatty acid [Blobe et al., 1995]. Oleic acid is not metabolized into the bioactive eicosanoids, whereas the evidence presented in this study suggested that effect of arachidonic acid on ET-1 release was mediated by metabolites of the lipoxygenase pathway. Thus, it may be possible that arachidonic acid and oleic acid exert their effects on the release of ET-1 by different signaling mechanisms and under different pathophysiological conditions.

The signaling mechanism by which arachidonic acid induces the production of ET-1 from endothelial cells was not investigated in the current study. Arachidonic acid and other polyunsaturated fatty acids have been reported to activate protein kinase C PKC in a cell-free system [McPhail et al., 1984], and certain effects of arachidonic acid are dependent on PKC activation [Blobe et al., 1990]. However, we have shown that arachidonic acid activates c-jun by a PKC-independent pathway [Rizzo et al., 1995]. Activation of PKC is involved in the production of ET-1 by different stimuli, including erythropoietin, angiotensin, and mechanical strain [Vogel et al., 1997; Oriji and Keiser, 1997; Wang et al., 1995]. By contrast, activation of PKC by oncostatin M induced downregulation of ET-1 production from endothelial cells [Saijonmaa et al., 1998]. The potential involvement of PKC or other kinases in the signaling pathway of arachidonic acid-induced ET-1 production remains to be determined.

We previously reported that arachidonic acid mediated interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) induction of granulocyte-macrophage colony stimulating factor (GM-CSF) and c-jun gene expression in stromal cells [Rizzo et al., 1994, 1995]. Furthermore, we demonstrated that cytokine activation of JNK was mediated by arachidonic acid [Rizzo et al., 1996]. In the present study, we investigated the potential involvement of arachidonic acid in IL-1 and TNF-α-induced release of ET-1. However, pretreatment of cells with inhibitors of different PLA_2s failed to demonstrate any effect on ET-1 released by BAECs upon stimulation with IL-1 and TNF-α (data not shown). Thus, arachidonic acid metabolism, under the experimental conditions used in this study, is not involved in transducing the effect of IL-1 and TNF-α on ET-1 release from endothelial cells. Current studies are being carried out to identify agonists upstream to the release of arachidonic acid in the pathway leading to the production of ET-1 in endothelial cells.

In conclusion, the results reported in this study are the first to demonstrate induction of ET-1 gene expression by arachidonic acid in vascular endothelial cells. Although the specific endothelial cell function regulated by arachidonic acid through the production of ET-1 remains to be identified, the ET-1 signaling pathway described herein may represent a novel mode of arachidonic acid action in the physiological control of vascular tone.

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