Cytochrome P-450 metabolites in endothelin-stimulated cardiac hormone secretion

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The mammalian heart synthesizes and secretes a family of peptides that have potent diuretic, natriuretic, and vascular smooth muscle relaxant effects as well as complex interactions with the hormonal and nervous systems (18, 23). Atrial natriuretic peptide (ANP) is secreted by the heart in response to increased atrial stretch (2, 4) such as would typically be produced by an increase in blood volume. ANP is initially stored in granules in the heart as a 126-amino acid prohormone (pro-ANP). During the secretion process (4, 3, 10), the pro-ANP molecule is cleaved to form ANP, the 28-amino acid COOH-terminus of pro-ANP, and several NH2-terminal prohormone peptides, pro-ANP-(1–30), -(31–67), and -(79–98). Our recent data suggest that pro-ANP-(1–30) also increases sodium excretion and plays a role in blood volume and blood pressure regulation (5).

Numerous factors have been shown to modulate the secretion of ANPs, including endothelin-1 (ET-1), a potent vasoconstrictor synthesized and secreted by endothelial cells (8). ET-1 has also been shown to have potent hypertrophy-promoting effects in a variety of mammalian cells, including vascular smooth muscle cells, cardiomyocytes (13), and fibroblasts (25). Various models of cardiac hypertrophy have demonstrated increased ET expression and secretion in the heart (13). ET-1 has been shown to be a potent stimulus for the hypertrophy of neonatal rat ventricular myocytes (NRVM) in vitro (24). In addition, ET-1 increases cell contraction frequency (14) and protein synthesis and modulates stretch-induced ANP release (21, 20) and gene expression (8, 9, 12, 13, 19). Skvorak et al. (22) reported that the cyclooxygenase inhibitor indomethacin abolished ET-1-stimulated ANP secretion in anoxic conditioned atria, suggesting a role for prostaglandins in the ANP secretion stimulated by ET-1. Subsequently, it has been shown that the cytochrome P-450 (CYP450)-arachidonic acid (AA) metabolites modulate the intracellular free Ca2+ signal via cardiac L-type Ca2+ channels as well as cardiac contraction via changes in intracellular cAMP (28).

In the present study, we investigated the role of CYP450-AA metabolites on cardiac hormone secretion stimulated by ET-1. In addition, we studied the mechanisms by which CYP450-AA metabolites regulate pro-ANP secretion at the cellular level.

MATERIALS AND METHODS

Materials

ET-1, 17-ODYA, and 20-hydroxyeicosatetraenoic acid (20-HETE) were purchased from Sigma (St. Louis, MO). Staurosporine and H-89 were obtained from Calbiochem-Novabiochem (La Jolla, CA). All chemical products for the cell isolation buffer were also purchased from Sigma. Collagenase CLS 2 was obtained from Worthington Biochemical (Freehold, NJ). DMEM, medium-199 (M199), Dulbecco’s PBS, antibiotic solution (penicillin-streptomycin; PS), and BSA were purchased from Gibco-BRL Life Technologies (Baltimore, MD). All products needed for the RIAs of ANP and pro-ANP-(1–30) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Adult and neonatal Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN).

Buffers and Media for Cell Isolation and Culture

ADS buffer. One liter of ADS buffer contained 4.36 g HEPES (free acid, pH 7.35), 6.80 g NaCl, 1.00 g d-glucose, 0.40 g KCl, 0.14 g NaH2PO4·H2O, and 0.10 g MgSO4 (anhydrous).

Myocyte maintenance medium. Myocyte maintenance medium consisted of 70% (vol/vol) DMEM with 20% M199 with PS, and 10% BSA.

Challenger medium. Challenge medium was composed of 80% (vol/vol) DMEM with 20% M199 with PS containing 0.2% BSA.

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Preparation of NRVM in Culture

Primary cultures of ventricular myocytes were isolated from 1- to 2-day-old neonatal Sprague-Dawley rats. Ventricular myocytes were used rather than atrial myocytes to enhance the myocyte yield. Briefly, the thoracic cavity was opened to expose the heart. The atria were isolated and discarded. The ventricles were excised and then placed in sterile ADS buffer. Once all the tissue was collected, the ventricles were minced and enzymatically digested using ADS buffer containing collagenase (CLS2, 155 U/ml; Worthington) for 15 min on a rotating platform inside an incubation chamber at 37°C. The supernatant was collected after the first digestion and discarded. Fresh enzyme solution was added to the tissue and digested for another 15 min. This step was repeated three to four more times until the tissue was completely digested. The supernatant from each digestion was rendered inactive by adding FBS to the cell-enzyme mixture, centrifuging, and resuspending the cell pellet in 100% FBS. After processing the supernatant from each digestion step, all the dissociated cells from each step were pooled, centrifuged, and resuspended in ADS buffer.

To segregate myocytes from nonmyocytes, the cell suspension was layered on discontinuous Percoll density gradients consisting of the following two phases: 4 ml of 40.5% Percoll and 3 ml of 58.5% Percoll in ADS buffer. The volume of the cell suspension layered on each gradient did not exceed 1.5 ml and was followed by centrifugation at 1,200 g for 30 min to yield two distinct layers of cells. The upper layer consisted of a mixed population of nonmyocyte types, and the lower layer consisted entirely of cardiac myocytes. The myocyte-enriched fraction was removed, centrifuged, and resuspended in ADS buffer. This washing procedure was performed two times to remove all traces of Percoll. The myocytes were resuspended in the myocyte maintenance medium and tested for cell viability by trypan blue exclusion. Cells were plated at a density of 1.2 × 10^5 cells/well on 12-well culture plates precoated with 1% gelatin. The media were changed 16 h after seeding and replaced with fresh maintenance medium. The final cell preparations routinely contained >95% myocytes, as judged by morphology and spontaneous contraction. Cells were allowed to adhere to the plates for 48 h, after which the media were changed to the challenge medium.

Measurement of Pro-ANP-(1–30) Secretion from Cultured NRVM

Secretion rates calculated from the pro-ANP-(1–30) RIA (3–5) were used in all NRVM experiments as an index of cardiac hormone secretion. This assay measures the NH2-terminus of the pro-ANP molecule, and we have shown that in both plasma and in our atrial tissue perfusates the peptide measured by this NH2-terminal assay is consistent with pro-ANP-(1–30) (3, 10). In preliminary experiments in NRVM, we found that the pro-ANP assay provided more reproducible results than the ANP [pro-ANP-(99–126)] assay. We attribute this to the high degree of adhesion of the COOH-terminal molecule [pro-ANP-(99–126)] with the plastic culture dishes and the coating employed. We have observed a similar phenomenon earlier using peptide separation with high-pressure gel permeation chromatography (3, 10).

Experiments were conducted after 12 h incubation in the challenge medium. For pro-ANP-(1–30) release, 12-well tissue culture plates containing confluent, spontaneously beating ventricular myocyte monolayers were washed two times with 1 ml serum-free media. After the supernatant was replaced with 500 μl of fresh challenge medium, the cells were incubated at 37°C in a 5% CO2–95% air atmosphere incubator for the indicated times in the presence of various pharmacological agents. 17-ODYA (10^-7 M) was prepared in DMSO. The final percentage of DMSO in the incubation media was <0.1%. Control wells were treated with the same concentration of DMSO.

Cultured cells were pretreated with the agonists/antagonists for 5 min before exposure to ET-1. Medium was collected at defined incubation times, centrifuged, and assayed for ANP and pro-ANP-(1–30) by RIA using methods previously described by our laboratory (4, 5). The data are expressed as picograms of ANP or pro-ANP-(1–30) per tissue culture well or as ANP secretion rate in picograms per minute for atrial perfusates.

Determination of cGMP and cAMP Levels in Cultured NRVM

Cultured NRVM were plated on 12-well plates and grown to confluence. Cells were incubated with the challenge medium for 10–12 h. Before the experiments, cells were washed with 1 ml BSA-omitted challenge medium and pretreated with IBMX (1 mM for cGMP determination and 0.5 mM for cAMP) for 5 min in BSA-omitted challenge medium, followed by various pharmacological agents for another 15 min. The reaction was stopped by adding 6% TCA and incubated at 4°C for 10 min. All supernatants were collected, extracted four times with water-saturated diethyl ether, and dried using a Speed-Vac evaporator. The samples were reconstituted in cGMP or cAMP assay buffer, respectively. The cGMP and cAMP secreted from cultured NRVM were determined by an RIA kit (Amersham Pharmacia). Data are expressed as femtomoles of cGMP or cAMP per culture well.

Protocol for Isolated Perfused Atria

Male Sprague-Dawley rats (300–375 g) were anesthetized with pentobarbital sodium (65 mg/kg). The chest was opened; the left atrium was removed and placed in cold, oxygenated Krebs buffer. The atria were carefully dissected and secured to a perfusion cannula with 4–0 silk. The tissues were then placed in a reservoir tube containing 4 ml modified Krebs buffer perfusate warmed to 38°C and gassed with 95% O2–5% CO2. An infusion pump continuously circulated fluid from the reservoir through the atria and back to the reservoir at a rate of 0.25 ml/min. All atria were paced at 200 beats/min. Atrial pressure, used as an index of atrial stretch, could be adjusted by changing the height of the atrial outflow cannula. Atrial pressure was maintained at ~2 mmHg (baseline level) for three 10-min equilibration periods, during which time the fluid in the reservoir was changed but discarded. The experiment consisted of three 10-min periods where atrial pressure was maintained at a 2-mmHg baseline level, then the atrial pressure was increased to 8–10 mmHg for three additional 10-min periods (distension), and ET-1 was added in a concentration of 50 nM in both the control and experimental groups. In one group of experiments, the standard Krebs buffer perfusate was used throughout the experiment. In a second group, 17-ODYA was added to the Krebs buffer perfusate at a concentration of 10 μM. Reservoir perfusate was removed at the end of each 10-min period and frozen at −20°C for later analysis of ANP by the method that we have used for many years (4).

The ANP assay (4) employs an antibody from Peninsula Laboratories (now Bachem Bioscience, King of Prussia, PA). The antibody cross-reacts 100% with both the rat and human sequences of ANP but does not cross-react significantly with brain natriuretic peptide (BNP) or C-type natriuretic peptide (CNP). The pro-ANP-(1–30) antibody (Bachem Bioscience) cross-reacts with both the human and rat forms of the NH2-terminal portion of the ANP prohormone (3). It does not cross-react with ANP or any of the other COOH-terminal peptides.

Statistics

The results are expressed as means ± SE. Statistical significance for experimental data from the ventricular myocytes was determined using one-way ANOVA followed by the Newman-Keuls multiple-comparison test. Student’s t-test was used for all between-group comparisons to analyze the data obtained from the isolated perfused atria. In all cases, statistical significance was defined as P < 0.05.

RESULTS

In preliminary experiments in the isolated perfused rat atria, we found that the CYP450-AA inhibitor 17-ODYA (10^-5 M)
suppressed ET-1 (10^{-8} M)-stimulated ANP secretion at both low and high levels of atrial stretch (Fig. 1). At 30 min, ANP secretion from atria at low atrial pressure (2 mmHg) was 162 ± 17 pg/min in the control group vs. 60 ± 13 pg/min in the 17-ODYA-treated group (Fig. 1, P < 0.05, t-test), and at 60 min with atrial pressure set at 8–10 mmHg, ANP secretion in the control group was nearly double the secretion rate of the 17-ODYA-treated group (393 ± 58 vs. 214 ± 19 pg/min, P < 0.05). Also, it should be pointed out that increased stretch in both groups more than doubled ANP secretion compared with the low stretch period in their respective group (P < 0.05, repeated-measures ANOVA). These results suggested that CYP450-AA metabolites might provide a stimulatory input to the ET-1-augmented ANP secretion pathway. However, 17-ODYA also reduced stroke volume in the isolated atria, a factor that can alter ANP release by changing the rate of extracellular translocation of ANP (1). Therefore, we performed several additional experiments in cultured myocytes to examine this phenomenon where ANP secretion rate can be directly observed without the complicating effects of hemodynamic factors.

**Concentration-dependent Response of ET-1 in Cultured NRVM**

Before determining the effect of CYP450-AA metabolites or inhibitors on pro-ANP-(1–30) secretion by ET-1, we first examined the effect of ET-1 itself on pro-ANP-(1–30) secretion in cultured NRVM (Fig. 2). The cells were incubated for 1 h in the presence of ET-1 in concentrations of 10^{-9}, 10^{-8}, and 10^{-7} M. ET-1 increased pro-ANP-(1–30) secretion in cultured NRVM in a concentration-dependent manner (Fig. 2). For all ET-1 concentrations employed, pro-ANP-(1–30) secretion was significantly greater than the vehicle (0 dose)-treated group (all P < 0.05). The responses to the 10^{-8} and 10^{-7} M concentrations were also significantly greater (P < 0.05) than the response to 10^{-9} M ET-1 (1-way ANOVA).

**Effects of 20-HETE and 17-ODYA on Pro-ANP-(1–30) Secretion in NRVM**

Figure 3 shows the effect of the CYP450-AA metabolite 20-HETE (3 × 10^{-10} M) and its inhibitor 17-ODYA (10^{-6} and 10^{-5} M) on pro-ANP-(1–30) secretion in NRVM. 20-HETE (3 × 10^{-10} M) tended to increase pro-ANP-(1–30) secretion compared with control (127 ± 12 vs. 145.9 ± 10 pg), but this was not statistically significant (Fig. 3). 17-ODYA (both 10^{-6} and 10^{-5} M) suppressed pro-ANP-(1–30) secretion by 20-HETE in a dose-dependent fashion (Fig. 3).

**Effects of CYP450-AA metabolites on cGMP and cAMP Production in NRVM**

We examined both cGMP and cAMP production from NRVM to determine if the effects of CYP450-AA metabolites on ET-1-induced natriuretic peptide secretion are induced via cyclic nucleotides. ET-1 alone tended to decrease cGMP production from ventricular myocytes compared with vehicle, but the difference was not significant. 17-ODYA in a dose of 10^{-5} M had no significant effect on cGMP production (Fig. 4A). On
the other hand, cAMP production was stimulated by the combination of 17-ODYA \((10^{-5}\text{ M})\) and ET-1 \((P < 0.05, \text{Fig. 4B})\).

Effects of Protein Kinase Inhibitors on 17-ODYA Inhibition of Pro-ANP-(1–30) Secretion in NRVM

To explore mechanism by which CYP450-AA metabolites inhibit ET-1-induced cardiac hormone secretion, we treated the cells with protein kinase inhibitors (Figs. 5 and 6). Both staurosporine \((10^{-6}\text{ M})\) and H-89 \((5 \times 10^{-5}\text{ M})\) in combination with ET-1 inhibited ANP and pro-ANP-(1–30) secretion by ET-1 compared with ET-1 alone. Staurosporine also blocked the effects of 17-ODYA (Fig. 6) on ET-1-stimulated pro-ANP-(1–30) secretion. On the other hand, in the presence of H-89, 17-ODYA \((10^{-5}\text{ M})\) still showed a significant inhibitory effect on ET-1-stimulated pro-ANP-(1–30) secretion. This suggests that the inhibitory effect of 17-ODYA on pro-ANP secretion is not mediated through protein kinase A (Fig. 5).

DISCUSSION

The present results clearly show that the CYP450 inhibitor 17-ODYA decreases ET-1-augmented ANP secretion in both the isolated perfused rat atria and in cultured rat ventricular myocytes (Figs. 1 and 3). In addition, the data suggest that 17-ODYA inhibits ANP secretion by enhancing cAMP production (Fig. 4) through activation of protein kinase C, since the protein kinase C inhibitor staurosporine blocked the response to 17-ODYA (Fig. 6).

ET-1 has been shown to promote ANP secretion under a variety of experimental conditions, but the mechanism for this stimulation has only been partially elucidated \((8, 20–22)\). In a previous study, our laboratory clearly showed that ET-1 stimulated ANP secretion in the isolated perfused rat atria and augmented the ANP response to atrial stretch \((21)\). Also, it was shown that a specific ET-1 receptor blocker, BQ-123, attenuated the ANP secretory response to atrial stretch in the isolated atria, even in the absence of exogenous ET-1. The results demonstrated that endogenous endothelin acts as an essential paracrine role in the regulation of ANP secretion \((8)\). Subsequently, we found that the prostaglandin inhibitor indomethacin attenuated the ANP release to both ET-1 and atrial stretch in the isolated perfused rat atria \((22)\). In addition, indomethacin attenuated the ANP release to ischemia in the perfused atria, a response that we could clearly show was mediated by ET-1 \((22)\). Prostaglandins are known to play a significant role in the ANP secretion produced by ET-1, although prostaglandin production itself is not essential for the increased contractility induced by ET-1 \((22)\). The CYP450-AA metabolites are converted to prostaglandins by cyclooxygenase. Therefore, we hypothesized that CYP450-AA metabolites could have an important regulatory role in cardiac hormone secretion through the ET-1-stimulated pathway.

![Fig. 4](http://ajpregu.physiology.org/)  
Fig. 4. Effects of 17-ODYA on cGMP (A) and cAMP (B) production by ET-1 in NRVM. Cultured cells were preincubated with 1 mM IBMX for 5 min and then incubated for another 15 min in the presence of experimental drugs (cGMP, \(n = 4\); cAMP, \(n = 9\)). Values are means ± SE. Although ET-1 had no detectable effect on either cGMP or cAMP secretion, 17-ODYA, a CYP450-AA metabolite inhibitor, significantly increased cAMP secretion. \(*P < 0.05, \text{significant differences from ET-1 (10^{-8}\text{ M}) alone-treated group (1-way ANOVA)}.\)

![Fig. 5](http://ajpregu.physiology.org/)  
Fig. 5. Effects of H-89, a selective inhibitor of protein kinase A, on pro-ANP-(1–30) secretion in response to 17-ODYA in combination with ET-1 in NRVM. Cultured cells were preincubated with H-89 \((5 \times 10^{-5}\text{ M})\) for 5 min, followed by an additional incubation period of 30 min with the experimental drugs \((n = 6)\). Values are means ± SE. \(*P < 0.05, \text{significant differences from H-89 (5 \times 10^{-5}\text{ M}) and ET-1 (10^{-8}\text{ M})-treated group (1-way ANOVA)}.\)

![Fig. 6](http://ajpregu.physiology.org/)  
Fig. 6. Effects of staurosporine, a selective inhibitor of protein kinase C, on pro-ANP-(1–30) secretion in response to 17-ODYA in combination with ET-1 in NRVM. Cultured cells were preincubated with staurosporine \((10^{-6}\text{ M})\) for 5 min, followed by an additional incubation period of 30 min with the experimental drugs \((n = 6)\). Values are means ± SE.
Several lines of evidence suggest an important role for CYP450-AA metabolites in cell metabolism and cardiovascular regulation (28). Recently, it has been shown that the CYP450-AA metabolites modulate the intracellular free Ca\(^{2+}\) signal via changes in intracellular cAMP (28). During salt loading or hypertension, CYP450 expression is increased (7) and renal production of 20-HETE is elevated. Inhibitors of 20-HETE decrease arterial pressure in DOCA-salt hypertensive rats, and Lyon hypertensive rats (17).

We therefore performed preliminary experiments in the isolated perfused rat atria to examine the effects of the CYP450-AA inhibitor 17-ODYA on ANP secretion at both low and high levels of atrial stretch (Fig. 1). These results suggested that CYP450-AA metabolites might provide a stimulatory input to the ET-1-augmented ANP secretion pathway. However, 17-ODYA also reduced contractility and stroke volume in the perfused atria, a factor that has been suggested to alter ANP release by changing the rate of extracellular translocation of ANP (1). We have previously shown that ET-1 increases contractility in the isolated atria by augmenting Ca\(^{2+}\)-entry. Therefore, several additional experiments were performed in NRVM to examine the role of CYP450 on ANP secretion, where secretion rate can be directly observed without the complicating changes in hemodynamics.

In the cultured myocytes, ET-1 clearly stimulated pro-ANP-(1–30) secretion in a dose-dependent manner (Fig. 2). The stimulatory effect of ET-1 on cardiac hormone secretion was inhibited by 17-ODYA, a known inhibitor of CYP450-AA metabolism (Fig. 3). Conversely, 20-HETE, a CYP450-AA metabolite, slightly augmented pro-ANP-(1–30) secretion (Fig. 3), but this level of secretion was significant only at 17-ODYA to a level below that of baseline secretion (Fig. 3). The action of 17-ODYA on ET-1-stimulated pro-ANP-(1–30) secretion was completely blocked by an inhibitor of protein kinase C (staurosporine, 10\(^{-6}\) M; Fig. 6). However, an inhibitor of protein kinase A had no significant effect on the response to 17-ODYA (Fig. 5), suggesting that the protein kinase C pathway is important in CYP450-mediated natriuretic peptide secretion.

Recently, it has been reported that inhibition of 20-HETE mediates ET-1-induced vasoconstriction in the kidney (11). Moreover, 20-HETE shows increased production when the level of ET-1 in tissue or plasma is elevated (6). However, 20-HETE has not been shown to be increased in cardiac tissue. Our results with 20-HETE show a small increase in cardiac hormone secretion in normal atrial tissue and cultured NRVM, suggesting that 20-HETE could be a potential mediator of the ET-1-induced response. In addition to 20-HETE, epoxycyclo-satenoic acids (EETs), which are metabolites of the CYP450 epoxygenase pathway, have important physiological effects, such as regulation of Ca\(^{2+}\) flux and vasorelaxation and modulation of ion channels in cardiac myocytes (27, 28). In preliminary experiments in cultured NRVM, we found that 20-HETE increased cardiac hormone secretion while EETs showed the opposite effect, a decrease in secretion.

Intracellular receptors for CYP450 products have not been clearly demonstrated in the heart. However, Widstrom et al. (27) reported that oxidized fatty acids bind to fatty acid-binding proteins (FABPs) such as heart-FABPs (H-FABPs), and these H-FABPs have a significant binding affinity for AA, 11,12-EET, 5,6-EET, and 20-HETE, implying the possible role of 20-HETE and EETs in heart (27). Nitric oxide modulates 20-HETE formation by binding to CYP450 4A (7). Moreover, it has been suggested that NO-mediated inhibition of 20-HETE formation accounts for the cGMP-independent relaxant effects and the natriuretic and diuretic actions of NO in the kidney (15). This is in agreement with our results showing that ET-1 inhibited cGMP production, and this response was blocked by 17-ODYA. 17-ODYA has an inhibitory effect on the production of both 20-HETE and EETs with a similar potency (26).

In summary, the present results suggest that ET-1 stimulation of cardiac natriuretic hormone secretion is mediated in part by CYP450-AA metabolites and a protein kinase C-dependent pathway. Because 17-ODYA inhibits both 20-HETE and EET formation (26), additional experiments using specific inhibitors of each metabolite will be necessary to narrow the involvement of these important regulators in ANP secretion.

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