Effect of Eicosapentaenoic Acid on the Different Endothelin System Components in Endothelin-1–Induced Hypertrophied Cardiomyocytes

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The cardiovascular benefit of fish oil, including eicosapentaenoic acid (EPA), in humans and experimental animals has been reported. The role of endothelin-1 (ET-1) in cardiac hypertrophy is well known. Endothelin-1 stimulates prepro–ET-1 mRNA expression in cardiomyocytes, and the autocrine/paracrine system of ET-1 is important for cardiomyocyte hypertrophy. Although many studies link EPA to cardiac protection, the effect of EPA on cardiac hypertrophy has yet to be clarified. Recently, we demonstrated that ET-1–induced cardiomyocytic change could be prevented by pretreatment with EPA. The present study investigated the changes of different components of the ET system at the mRNA level in ET-1–administered cardiomyocytes, and examined the effect of EPA pretreatment. Ventricular cardiomyocytes were isolated from 2-day-old Sprague-Dawley rats, cultured in Dulbecco's modified Eagle's medium and Ham F12 supplemented with 0.1% fatty acid–free bovine serum albumin for 3 days. At Day 4 of culture, the cardiomyocytes were divided into 3 groups: control group, ET-1–treated (0.1 n*M*) group, and ET-1–treated group pretreated with EPA (10 μ*M*). Twenty-four hours after treatment, the gene expressions of different components of the endothelin system in three experimental groups were evaluated by real-time polymerase chain reaction. Prepro–ET-1 mRNA expression was 53% upregulated in ET-1–induced hypertrophied cardiomyocytes and suppressed in the EPA-pretreated group. Endothelin-converting enzyme-1 (ECE-1) was also increased in ET-1–administered cardiomyocytes by 42% compared with the control group and was reversed in the EPA-pretreated group. The two receptors of ET system, ET_A and ET_B, tended to be increased in the ET-1–treated group, but no statistical significance was seen among study groups. Endothelin-1 increased prepro–ET-1 and ECE-1 mRNA expression in hypertrophied-neonatal cardiomyocytes, and this was reversed with EPA pretreatment. Thus, EPA may play a crucial role in the regression of ET-1–induced cardiomyocyte hypertrophy, partly through the suppression of ET-1 and ECE-1 expression. Exp Biol Med 231:888–892, 2006

Key words: endothelin-1; eicosapentaenoic acid (EPA); neonatal cardiomyocytes; hypertrophy; endothelin system

Introduction

Cardiomyocyte hypertrophy is one of the central features of many cardiac diseases and is characterized by an increase in myocyte size, accumulation of contractile proteins in the cells, upregulation of embryonic gene marker expression, and lack of a concomitant effect on muscle cell proliferation (1). Cardiomyocyte hypertrophy is an adaptive response to excessive workload on the myocardium or myocardial injury. This mechanism is, at least initially, favorable and adaptive, but subsequently a pathological transition can occur in which the myocardium becomes dysfunctional (2).

The endothelin (ET) system is composed of three active ETs (3, 4). Endothelin-1 (ET-1) is the main isoform, which is synthesized in endothelial cells, the muscular coat of the arterial wall, and in the heart, kidney, and central nervous system (4). The actions of ET are mediated through interactions with two classes of cell surface receptors. The ET_A receptor has been associated with vasoconstriction and cell growth, whereas the ET_B receptor has been linked to

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endothelial-cell–mediated vasodilation and release of other neurohormones, such as aldosterone. Endothelin-converting enzyme-1 (ECE-1) converts big ET-1 to ET-1 (3).

Endothelin-1, a potent vasoconstrictor peptide identified from the conditioned medium of vascular endothelial cells, is also synthesized and secreted by cardiomyocytes, and induces hypertrophy of cardiomyocytes (2). Detailed signal transduction of ET-1–induced cardiomyocyte hypertrophy is currently under investigation, and different therapeutic approaches toward the regression of ET-1–induced cardiomyocyte hypertrophy have also gained recent interest. Thus, different approaches that suppress ET-1 levels and ET-1 signal transduction molecules are of potential importance. Among the different types of endothelins, ET-1 plays a predominant role in the pathogenesis of a wide variety of cardiovascular complications (3).

Different doses of ET-1 exert different systemic and local effects. The effective dose of ET-1 used to induce hypertrophic change in cardiomyocytes is much higher than that found in plasma ET-1 levels in patients with cardiovascular diseases (5). We recently found that in a streptozotocin-induced diabetic rat model, the plasma ET-1 level was unchanged whereas significant upregulation of ET-1 was found in different risk-prone tissues such as the heart, eye, and kidney.[1] Moreover, ET antagonism may have significant reversal effects on tissue and plasma ET-1 alterations in diabetic rats. Thus, one might reasonably speculate that locally produced ET-1, which may act as an autocrine and/or paracrine factor, is also vital in exerting the effects of ET-1 locally together with circulating ET-1, and locally produced ET-1 might have a greater potential to exert its hypertrophic effect in cardiomyocytes than systemic ET-1 (5).

The cardiovascular benefit of fish oil enriched with n-3 long-chain polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA), was reported in both humans and experimental animals (6, 7). Despite the numerous reports linking EPA to cardiac protection, the effect of EPA on cardiac hypertrophy has not been investigated in detail. We hypothesized that ET-1–induced cardiomyocyte hypertrophy may be regressed by EPA pretreatment, and that there may be alterations in the different components of ET system in ventricular cardiomyocytes after ET-1 administration when the hypertrophic process is ongoing. Finally, we investigated whether there is significant alteration in the gene expressions of different components of ET system in ET-1–treated hypertrophied cardiomyocytes, and if EPA pretreatment normalizes these changes.

Materials and Methods

Ventricular Cardiomyocyte Culture. Ventricular cardiac myocytes were isolated from 2-day-old Sprague-Dawley rats, cultured in Dulbecco's modified Eagle's medium and Ham F12 supplemented with 0.1% fatty acid–free bovine serum albumin (Sigma, St. Louis, MO) for 3 days. At Day 4 of culture, the cardiomyocytes were divided into three groups: a control group, an ET-1–treated (0.1 n*M*) group, and an ET-1–treated group pretreated with EPA (10 μ*M*). Twenty-four hours after treatment, cardiomyocytes were evaluated.

Morphological Evaluation. Cell images captured by CCD Camera (Olympus, Tokyo, Japan) were traced and analyzed with NIH image. The area was then doubled to account for the surface portion in contact with the dish. All cells from randomly selected fields in two or three dishes were examined for each condition. We measured over 100 cells in each condition.

Quantitative Real-Time Polymerase Chain Reaction (PCR). Total RNA from ventricular myocytes was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene, Toyama, Japan). Briefly, the accumulated cardiomyocytes of different groups were homogenized in Isogen (100 mg tissue to 1 ml Isogen) with a Polytron tissue homogenizer (model PT10SK/35, Kinematica, Lucerne, Switzerland). The precipitated RNA was then extracted with chloroform, precipitated with isopropanol, and washed with 75% (vol/vol) ethanol. The resulting RNA was resolved in diethyl pyrocarbonate–treated water, treated with DNase I (Takara, Shiga, Japan), and extracted again with Isogen (Nippon Gene) to eliminate the genomic DNA. The RNA concentration was determined spectrophotometrically at 260 nm.

Total cellular RNA was primed with 0.05 μg oligo d $(pT)_{12-18}$ and reverse transcribed by omniscript reverse transcriptase using a first-strand cDNA synthesis kit (Qiagen, Tokyo, Japan). The reaction was performed at 37°C for 60 mins.

The mRNA expression levels of prepro–ET-1, ECE-1, and ET_A and ET_B receptors in the ventricular myocytes were analyzed by quantitative real-time PCR with TaqMan probe using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster, CA) as previously described (8). The gene-specific primers and TaqMan probes were synthesized from Primer Express v.1.5 software (Perkin-Elmer) according to the published cDNA sequences for each of the following: prepro–ET-1 (9), ECE-1 (10), ET_A receptor (11), ET_B receptor (12), and glyceraldehyde phosphate dehydrogenase (GAPDH) (13) mRNA. The sequences of the oligonucleotides were as follows: prepro–ET-1 forward: 5′-TCTACTTCTGCCACCTGGACAT-3′; prepro–ET-1 reverse: 5′-GAAGGGCTTCCTAGTCCATACG-3′; prepro–ET-1 probe: 5′-CATCTGGGTCAACACTCC-3′; ECE-1 forward: 5′-TCAGACAAGTCTCCACACTCATCA-3′; ECE-1 reverse: 5′-CCAGGTTCCACATCATGTAGTTGTT-3′; ECE-1 probe: 5′-ACAGCACCGACAAATG-3′, ET_A receptor forward: 5′-GAATCTCTGCGCTCTCAGTGT-3′; ET_A receptor reverse: 5′-GAGACAATTTCAATGGCGGTAATCA-

[1] Jesmin S, Zaedi S, Shimojo N, Maeda S, Miyauchi T. 2005. Unpublished observations.

3′; ET_A receptor probe: 5′-CAGGAAGCCACTGCTCT-3′; ET_B receptor forward: 5′-GCTGGTGCCCTTCATACA-GA-3′; ET_B receptor reverse: 5′-CTTAGAGCACATA-GACTCAACACTGT-3′; ET_B receptor probe: 5′-ATCCCCACAGAAGCCT-3′; GAPDH forward: 5′-GTGCCAAAAGGGTCATCATCTC-3′; GAPDH reverse: 5′-GGTTCACACCCATCACAAACATG-3′; GAPDH probe: 5′-TTCCGCTGATGCCCC-3′.

The expression of GAPDH mRNA was used as an internal control. The PCR mixture (25 μl total volume) consisted of 450 n*M* of both forward and reverse primers for prepro–ET-1, ECE-1, ET_A receptor, ET_B receptor, and GAPDH (Perkin-Elmer), 200 n*M* FAM-labeled primer probes (Perkin-Elmer), and TaqMan Universal PCR Master Mix (Perkin-Elmer). Each PCR amplification was performed in triplicate, using the following profile: one cycle of 95°C 10 mins, and 40 cycles of 94°C for 15 secs and 60°C for 1 min. For the standard curve in the real-time quantitative PCR, serial dilutions of a rat ventricular myocyte cDNA were performed within the range of various concentrations (1×, 2×, 4×, 8×, and 16×). No template (water) reaction mixture was prepared as negative control.

Staistical Analysis. Values are shown as means ± SEM. Statistical analysis was performed by ANOVA with multiple comparisons by Fisher protected least-significant difference *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Cell-Surface Area of Cardiomyocytes. The cardiomyocyte cell surface area calculated by NIH image significantly increased to 2375 ± 66 pixels after ET-1 administration compared with the control value of 1272 ± 53 pixels ($P < 0.001$ vs. control group). This increase in cell surface area was remarkably ameliorated by EPA pretreatment and reversed to 1789 ± 70 pixels ($P < 0.001$ vs. ET-1–treated group).

Gene Expression of ET System in Ventricular Myocytes. Prepro–ET-1 mRNA expression was 53% upregulated in ET-1–induced hypertrophied cardiomyocytes compared with control cells, and this upregulation was suppressed in the EPA-pretreated group (Fig. 1A). Endothelin-converting enzyme-1 was also increased by 42% in ET-1–treated cardiomyocytes compared with controls and was reversed in EPA-pretreated cells (Fig. 1B). Expression of ET_A and ET_B receptors tended to be increased in ET-1–treated cardiomyocyte, but no statistical significance was seen among the study groups (Fig. 2).

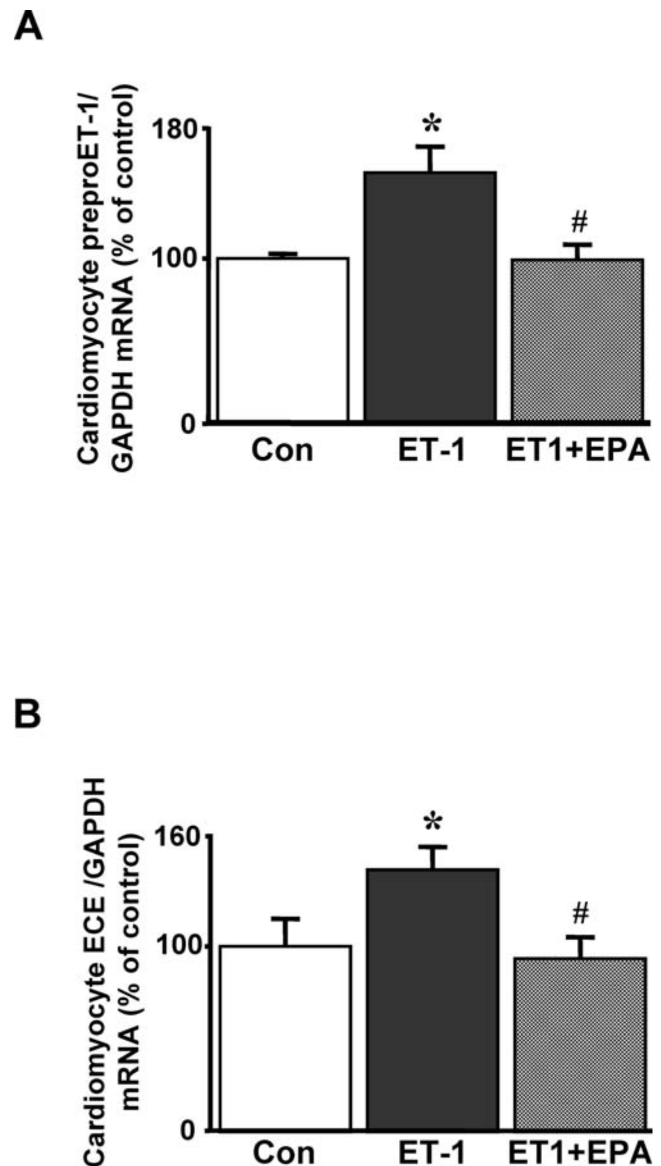

Figure 1. The gene expressions of prepro–ET-1 (A) and ECE-1 (B) in the neonatal ventricular myocytes of the control group (Con), ET-1–treated group (ET-1), and the EPA-pretreated ET-1 group (ET-1+EPA). Expression of mRNA was determined by real-time PCR. Data are shown as mean ± SEM of seven separate experiments. *$P < 0.01$ vs. Con; $^{\#}P < 0.01$ vs. ET-1 (prepro–ET-1); *$P < 0.05$ vs. Con; $^{\#}P < 0.05$ vs. ET-1 (ECE-1).

Discussion

The present study demonstrates that the mRNA expression of ET-1 and ECE-1 are upregulated in ET-1–treated hypertrophied cardiomyocytes, and that these upregulations are reversed by EPA pretreatment. It should be noted that EPA concomitantly suppresses cardiomyocyte hypertrophy, which is induced by ET-1. In the present study, there was no significant change in ET receptor expression, although an increasing tendency of mRNA expression of these receptors was seen after ET-1 administration in ventricular myocytes.

The role of ET-1 in cardiac hypertrophy has been demonstrated both *in vivo* and *in vitro* (5). Our previous reports show that the production of ET-1 is markedly increased both in the hypertrophied heart and in the failing heart (14, 15), and that prepro–ET-1 mRNA is overexpressed in ET-1–induced hypertrophied cardiomyocytes compared with control cells (1). The potential role of

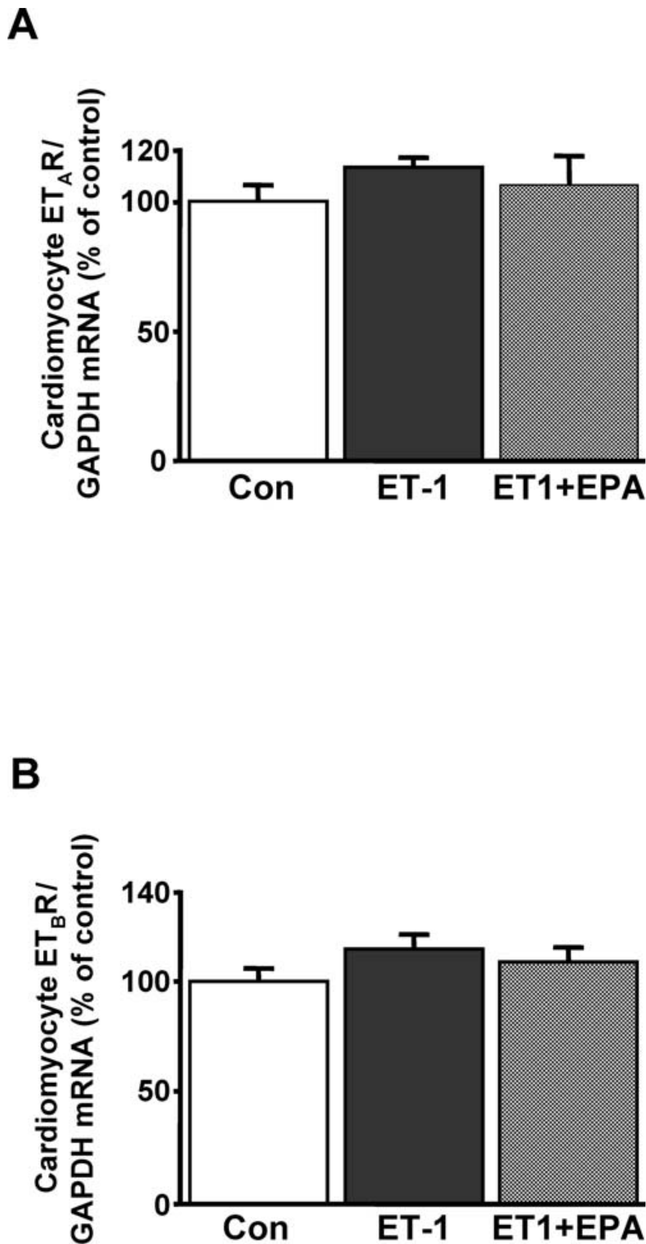

Figure 2. The gene expression of ET_A receptor (A) and ET_B receptor (B) in the neonatal ventricular myocytes of the control group (Con), ET-1–treated group (ET-1), and the EPA-pretreated ET-1 group (ET-1+EPA). Expression of mRNA was determined by real-time PCR. Data are shown as mean ± SEM of seven separate experiments.

locally produced endogenous ET-1 in cardiomyocyte hypertrophy *via* an autocrine/paracrine manner is well documented (16). Thus, the local production of ET-1 after ET-1 administration might be an important mechanism in the development of cardiomyocyte hypertrophy. In the present study, the ET-1 mRNA levels were significantly increased after ET-1 administration, suggesting that administered ET-1 regulates the expression of prepro–ET-1 mRNA in ventricular cardiomyocytes. Moreover, the suppression of this upregulated prepro–ET-1 mRNA in hypertrophied cardiomyocytes by EPA warrants further studies to further understand the specific mechanisms concerning this suppressive effect. In the current investigation, the mRNA expression of ECE-1, the vital enzyme converting big ET-1 to ET-1 peptide (3), has the parallel correlation with changes in cardiomyocyte prepro–ET-1 mRNA levels after ET-1 administration. The parallel alterations in-between prepro–ET-1 and ECE-1 further suggest that locally produced cardiomyocyte ET-1 plays an important role in the development and progression of cardiomyocyte hypertrophy. This parallel correlation may also help us to extend our knowledge regarding the expression of signal transduction molecules, growth factors, and several cytokines altered by locally produced ET-1 and their role in the hypertrophic process in cardiomyocytes. Kaburagi *et al.* (17) showed that the conversion of big ET-1 to ET-1 was accelerated in phenylephrine (PE)-induced hypertrophied cardiomyocytes, and that this accelerated level of ET-1 production was associated with the marked increase in ECE-1 mRNA levels after PE stimulation. A specific ECE-1 antagonist, FR901533, inhibited the PE-stimulated increase in protein synthesis by 45% in hypertrophied cardiomyocytes (17). To further dissect the role of locally produced-ET-1 and their upstream genes in ET-1–induced cardiomyocyte hypertrophy, a specific ECE-1 antagonist should also be used in the current experimental setting. To gain more mechanistic insight into the suppressive effect of EPA on upregulated ECE-1 mRNA expression, one can investigate the results of both the EPA and ECE antagonist pretreatment before the ET-1 administration in cardiomyocytes.

It is well established that most of ET-1's action is mediated by either of its receptor types (3, 5). Accordingly, it would be reasonable to expect an upregulation of any or both of the receptor types in cardiomyocytes after ET-1 administration. Surprisingly, in the present study, neither ET-1 nor EPA altered the gene expressions of ET receptors in cardiomyocytes at statistically significant levels. However, a tendency toward upregulation of both receptor types was seen after ET-1 administration in cardiomyocytes, and in the EPA-pretreated group there was a reversal of this trend of statistically insignificant upregulation. How ET-1 mediates its hypertrophic action on cardiomyocytes is unclear. Lee *et al.* (18) report that ET_B receptor causes cardiac hypertrophy without any change in ET_B receptor mRNA expression levels in the prehypertensive stage of spontaneously hypertensive rats compared with Wistar-Kyoto rats; in their study, the expression of ET_A receptor was not altered. But at *in vitro* level, Kanno *et al.* (19) demonstrated the upregulation of ET_B receptor mRNA expression in hypertrophying neonatal cardiomyocytes. The present study cannot make any specific comment regarding the involvement of ET receptors in ET-1–induced cardiomyocyte hypertrophy. Endothelin receptors should be investigated at the protein level, and a specific ET receptor antagonist should be applied in the present model before a concrete conclusion can be

made concerning the involvement of the ET receptor subtypes in the current experimental setting.

In conclusion, we demonstrate that the overexpression of prepro–ET-1 and ECE-1 mRNA in ET-1–induced cardiomyocyte hypertrophy is arrested by EPA pretreatment without any alteration in ET receptor mRNA expression. These findings partly explain the reversal mechanism of EPA in cardiomyocyte hypertrophy *in vitro*.

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