Changes in Important Apoptosis-Related Molecules in the Endothelin-1–Induced Hypertrophied Cardiomyocytes: Effect of the Pretreatment with Eicosapentaenoic Acid

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Human heart failure is preceded by a process called cardiac remodeling, in which heart chambers progressively enlarge and contractile function deteriorates. Programmed cell death (apoptosis) of cardiac muscle cells has been identified as an essential process in the progression to heart failure. The execution of the apoptotic program entails complex interactions between and execution of multiple molecular subprograms. Endothelin (ET)-1, a potent vasoconstrictor peptide, is synthesized and secreted by cardiomyocytes and induces hypertrophy of cardiomyocytes. The cardiovascular benefit of fish oil containing eicosapentaenoic acid (EPA) in humans and experimental animals was reported. Recently, we found that ET-1–induced cardiomyocytic remodeling could be prevented by pretreatment with EPA. The aim of the present study is to investigate whether there would be any alteration in the expression of important apoptosis-related molecules in ET-1–administered hypertrophied cardiomyocytes. We also sought to determine, if there are alterations in apoptotic molecules, what type of role for EPA would then exist. Ventricular cardiomyocytes were isolated from 2-day-old Sprague-Dawley rats and were cultured for 3 days. At Day 4 of culture, the cardiomyocytes were divided into three groups: control, the ET-1 (0.1 n*M*)–treated group, and the ET-1 group pretreated with EPA (10 μ*M*). Twenty-four hours after the treatment, the gene expressions of three important molecules related to apoptosis (caspase-3, Bax, and Bcl-2) in three experimental groups were evaluated by real-time polymerase chain reaction. The present study could not demonstrate any significant or representative alteration in any of the above three apoptosis-related important markers in either ET-1–induced hypertrophied cardiomyocytes with or without EPA pretreatment. The present study would at least be able to exclude the involvement of some representative molecules related to apoptosis in ET-1–induced hypertrophied cardiomyocytes. In addition, the present study demonstrates that the antihypertrophic effect of EPA to ET-1–administered cardiomyocytes appears not to modulate the apoptosis signaling cascade.

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Introduction

Cardiac hypertrophy, which is characterized by an increase in cardiomyocyte size, is one of the most important predisposing risk factors for sudden death and the development of heart failure in human populations (1). Hypertrophy is initiated and maintained *in vitro* and *in vivo* by several factors, such as vasoactive peptides (i.e., endothelin [ET-1] and angiotensin [Ang] II), peptide growth factors, hormones and neurotransmitters, α_1-adrenergic agonists, fibroblast growth factors, insulin-like growth factor-1, and cardiotrophin-1 (2, 3). The hypertrophic factors stimulate intracellular signaling cascades, which initiate a hypertrophic, fetal-like gene program (1). Vasoconstrictor peptide ET-1 and its receptors ET_A and ET_B are on the cell surface of cardiomyocytes (4). Several lines of evidence indicate that ET-1 functions in a paracrine/autocrine manner in cardiac hypertrophy (4, 5). In cell cultures of cardiomyocytes, ET-1 induces hypertrophy, as assessed by cell size, increased myofibrillogenesis, and transcriptional changes associated

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with cardiac hypertrophy, such as expression of atrial natriuretic factor (4). Antagonists of the ET_A receptor reduce hypertrophic responses to other stimuli, including Ang II and aortic banding (5, 6). Thus, a wide range of studies concerning ET-1 extend our understanding about ET-1–mediated cardiac hypertrophy and, finally, apply to regulate it in human disease, which could have profound clinical implications.

The balance between cell death and cell survival is a tightly controlled process, especially in terminally differentiated cells, such as the cardiomyocyte (7). Accumulating data support a role for cardiomyocyte apoptosis in the development of several cardiac diseases, including the transition from hypertensive compensatory hypertrophy to heart failure (7). It has been proposed that ventricular dilatation and neurohumoral activation during heart failure lead to upregulation of transcription factors and prepare the cell for reentry into the cell cycle, which fails and induces apoptosis (1, 8). Although the functional relevance of apoptosis is still obscure, recently significant progress has been made in demonstrating the role of apoptosis in various heart diseases and in elucidating the molecular mechanisms of cardiac apoptosis (1, 8). In addition, the effects of various hypertrophic signaling molecules on apoptosis have been characterized (9). Ang II type-1 receptor pathways, nitric oxide, and natriuretic peptides are involved in the induction of apoptosis in these cells, while alpha1- and beta2-adrenergic receptors, ET_A receptor pathways, and gp130-activating cytokines are antiapoptotic (10). In addition to being a well-known cardiac hypertrophy-inducible factor, ET-1 represents a protective factor against myocardial cell apoptosis in heart failure, and this effect is mediated mainly through the ET_A receptor-dependent pathway involving multiple downstream signalings in cardiac myocytes (11, 12).

Contrary to these reports, Oie *et al*. (13) demonstrated that a mixed ET_A/ET_B receptor antagonist may attenuate peri-postinfarcted cardiomyocyte cell loss through apoptosis in the area at risk after induction of ischemia in rat. Thus, conflicting results exist concerning the role of ET-1 on cardiomyocyte apoptosis.

Epidemiologic evidence has established that ingestion of long-chain polyunsaturated omega-3 fatty acids, abundant in fish oils, has profound effects on many human disorders and diseases, including cardiovascular disease (14). Recently we found that ET-1 administration caused a significant hypertrophy in neonatal ventricular cardiomyocyte and that this hypertrophy could be prevented by the pretreatment of eicosapentaenoic acid (EPA). Despite the numerous reports linking EPA to cardiac protection (15), the effect of EPA on ET-1–induced cardiac remodeling has not been investigated in detail. We have already found that EPA prevents ET-1–induced cardiomyocyte hypertrophy from interfering with several hypertrophic signaling molecules, such as transforming growth factor–beta, JNK, and Ang II (unpublished data). We also understand that the nitric oxide system is not involved in the EPA-mediated prevention of ET-1–induced cardiomyocyte hypertrophy (unpublished data). The present study is an extension and continuation of the previous findings, in which we gain an even greater understanding of the several intracellular signaling systems after ET-1 administration in cardiomyocytes as well as how much protective action EPA may offer to ET-1–induced hypertrophied cardiomyocytes. In the present study, we intend to investigate whether there is any involvement or alteration of apoptosis-related molecules in cardiomyocytes after ET-1 administration. Studies with cancer cells indicate that docosahexaenoic acid, another important long-chain polyunsaturated omega-3 fatty acid, induces cell cycle arrest and apoptosis by activating protein phosphatases, and protein phosphatases are involved with the protein Bcl-2, which regulates the release of cytochrome *c* from mitochondria and, eventually, activation of the apoptotic enzyme caspase-3 (16). Finally, we are interested in exploring the role and effect of EPA on the expression of several apoptosis-related genes in cardiomyocytes after ET-1 administration. In the current investigation, we examined the gene expression of three important molecules related to apoptosis (Caspase-3, Bax, and Bcl-2) in ET-1–induced hypertrophied cardiomyocytes with and without pretreatment with EPA.

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/Ham's F12 medium supplemented with 0.1% fatty acid–free bovine serum albumin for 3 days. This preparation yielded a 95% pure population of cardiomyocytes on cell images captured by CCD Camera (Olympus, Tokyo, Japan). At Day 4 of culture, the cardiomyocytes were divided into three groups: the control group, the ET-1 (0.1 n*M*)–treated group, and the ET-1 with EPA (10 μ*M*)–pretreated group. Twenty-four hours after the treatment, cardiomyocytes of the three groups were evaluated.

Morphologic Evaluation. Cell images captured by CCD Camera (Olympus) were traced and analyzed with National Institutes of Health (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 examined 60 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 Ltd., Toyama, Japan), according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically at 260 nm.

Total cellular RNA was primed with 0.05 μg of oligo $d(pT)_{12–18}$ and was reverse transcribed by omniscript

reverse transcriptase using a first-strand cDNA synthesis kit (Qiagen GmbH, Hilden, Germany). The reaction was performed at 37°C for 60 mins.

The mRNA expression levels of caspase-3, Bax, and Bcl-2 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 City, CA), as previously described (17). The gene-specific primers and TaqMan probes were designed from Primer Express v.1.5 software (Perkin-Elmer) specific for caspase-3, Bax, Bcl-2, and GAPDH mRNA. The sequences of the oligonucleotides were as follows:

caspase-3 forward: 5′- GCAGCTAACCTCAGAGA-GACATTC-3′; caspase-3 reverse: 5′-ACGAGTAAGGT-CATTTTTATTCCTGACTT-3′; caspase-3 probe: 5′-ATGGCCCTGAAATAC-3′; Bax forward: 5′-CAA-GAAGCTGAGCGAGTGTCT-3′; Bax reverse: 5′-CAAT-CATCCTCTGCAGCTCCATATT-3′; Bax probe: 5′-CCAGTTCATCTCCAATTCG-3′; Bcl-2 forward: 5′-TGCGCTCAGCCCTGTG-3′; Bcl-2 reverse: 5′-GGTAGC-GACGAGAGAAGTCATC-3′; Bcl-2 probe: 5′-CCACCTGTGGTCCACCTG-3′.

The expression of GAPDH mRNA was used as an internal control. Each PCR amplification was performed in triplicate, using the following profile: 1 cycle of 95°C for 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 rat cardiomyocyte cDNA performed within the range of various concentrations (1×, 2×, 4×, 8×, and 16×). No-template (water) reaction mixture was prepared as a negative control.

Statistical Analysis. Values are shown as means ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance with multiple comparisons by Fisher's protected least-significant difference *t* test. Nonparametric data were analyzed by the Mann-Whitney's *U* test or the Wilcoxon signed-rank test. A *P* value of <0.05 was considered to be statistically significant.

Results

Cell-Surface Area of Cardiomyocytes. The quantitation of cell surface area of cardiomyocytes was 90% increased after ET-1 administration, and this increase in cell surface area was remarkably blocked by the EPA pretreatment (Fig. 1).

Gene Expression of Apoptosis-Related Molecules in Ventricular Myocytes. As demonstrated in Figure 2A, the gene expression of the important initiator for apoptotic cascade, caspase-3, was not significantly altered in cardiomyocytes after ET-1 administration, regardless of EPA pretreatment. In the apoptotic process, the relative ratio of Bax and Bcl-2 is important, and in the present study, none of them was significantly altered after ET-1 administration in cardiomyocytes with or without pretreatment with EPA (Fig. 2B and C).

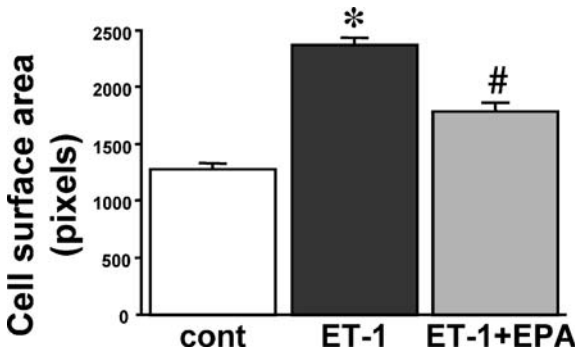

Figure 1. The quantitation of cell surface area of cardiomyocytes. This graph shows the cell surface area of control group (cont), ET-1–administered group (ET-1), and EPA-pretreated ET-1 group (ET1+EPA), respectively. All cells ($n = 60$, each group) randomly selected from two or three dishes were examined for each condition. Data are shown as means ± SEM. *, $P < 0.0001$ versus cont; #, $P < 0.0001$ versus ET-1.

Discussion

In the present study, the gene expressions of three important apoptosis-related molecules, caspase-3, Bax, and Bcl-2, were not altered in neonatal cardiomyocyte after ET-1 administration. Moreover, the present study did not demonstrate any effect of EPA on apoptotic markers in ET-1–administered cardiomyocytes.

Two major apoptotic pathways (the mitochondrial pathway and the death-receptor pathway) are active in mammalian cells, including the cardiac muscle cell (1). The mitochondrial death pathway is used extensively in response to extracellular cues and internal insults such as DNA damages (1). In these different pathways, signaling results in the activation of a family of caspases, which act in a proteolytic cascade to dismantle and remove the dying cell, and, finally, caspase-3, downstream in the caspase cascade, behaves as an important trigger of apoptosis (18). Ang II increases the ratio of caspase-3 to procaspase-3 in ventricular myocytes from rats both in the adult and the neonate (19–21). In fact, caspase-3 exists as zymogen that must first be proteolytically cleaved to become activated protease (22). This ratio has been used as an index of caspase-3 activation. In the present study, we speculated on the activation of caspase-3 in cardiomyocytes after ET-1 administration. The discrepancy regarding the activation of caspase-3 in ventricular myocyte by ET-1 and Ang II cannot be answered based on the results of the present study. For further understanding, one should administer Ang II to the neonatal ventricular myocytes used in the present study. On the other hand, ET-1 should be administered to the adult cardiomyocytes, and then the expression of caspase-3 should be examined.

The cardiomyocytes isolated from the left ventricle of spontaneously hypertensive rat (SHR) exhibit enhanced

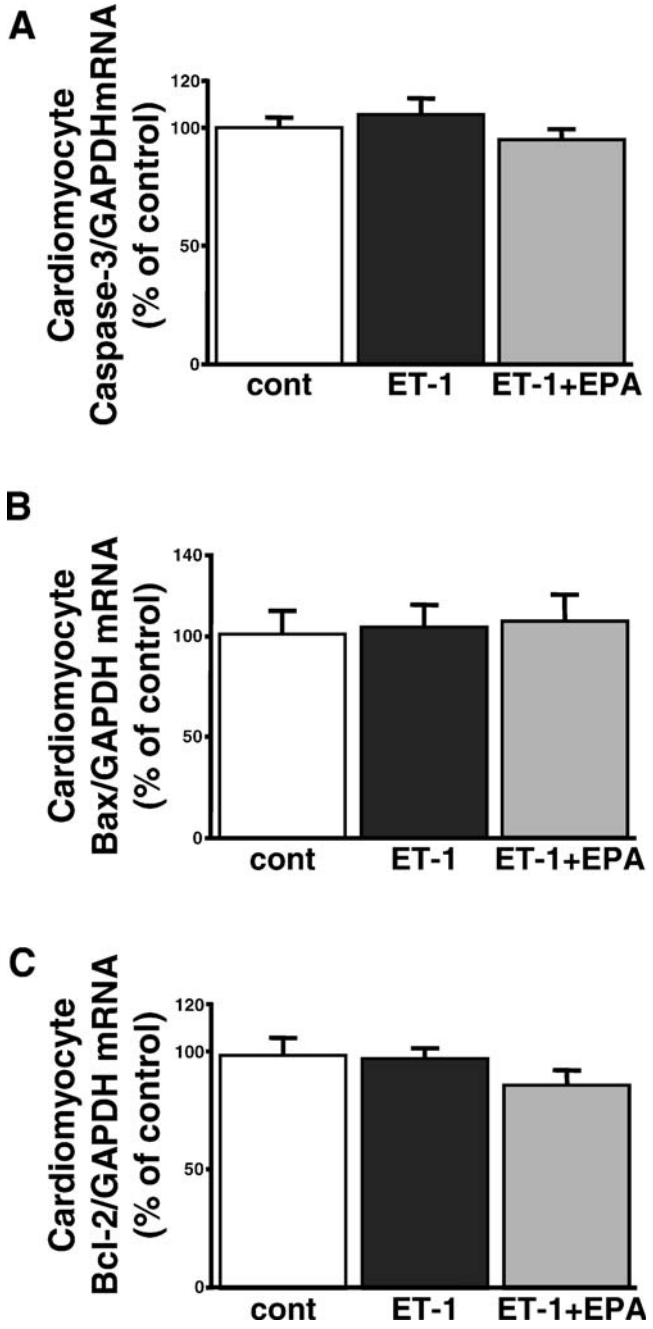

Figure 2. The mRNA expression of caspase-3 (A), Bax (B), and Bcl-2 (C) in the neonatal ventricular myocytes of control group (cont), ET-1–administered group (ET-1), and EPA-pretreated ET-1 group (ET1+EPA). The mRNA expressions were determined by real-time PCR. Data are shown as means ± SEM of five separate experiments.

susceptibility to Ang II–induced apoptosis compared with cells isolated from the left ventricle of normotensive Wistar-Kyoto (WKY) control rats (21). Stimulation of cardiac apoptosis in the left ventricle of SHR has been shown to be related, in a temporal way, to an increase of local Ang-converting enzyme (ACE) activity and not to the elevation of blood pressure values (23). Thus, there might be several differences in between the contribution of Ang II and ET-1 on the apoptosis cascade in cardiomyocytes. In our other experiment, we found an upregulation of preproET-1 and ET-converting enzyme (ECE) expression in ET-1–administered cardiomyocytes, but in those myocytes there was no change in apoptosis signaling. But Ang II could induce apoptosis in WKY rat cells, which are normal cardiomyocytes. While ACE is an important factor in Ang II–induced apoptosis changes in cardiomyocytes, the contribution of ECE in the regulation of apoptotic signaling in cardiomyocytes should be reviewed as well. The modulation of ECE-1 activity would affect phosphorylation of p38-mitogen–activated protein kinase (p38-MAPK) and would potentiate apoptosis in adult rat ventricular myocytes during sepsis (24). In the future, before coming to a conclusion on the role of ET-1 in cardiomyocytes *in vitro,* one should treat the cells from SHR with ET-1 and then examine the changes in apoptotic markers in cardiomyocytes. Different biochemical stress, such as chronic hypertension and mechanical load, activates multiple parallel and converting signals for cardiac hypertrophy and apoptosis, which represent two distinct outcomes in individual cardiomyocytes.

Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis, the cell suicide program critical for development, tissue homeostasis, and protection against pathogens. Bcl-2 on mitochondrial membrane promotes cell survival by inhibiting the adapters (Bax, Bad, Bim, and Bid) needed for activation of caspases that dismantle the cell (25).

However, the effect of ET-1 on apoptosis is still controversial. There are studies of smooth muscle cells in which ET-1 causes apoptosis (26, 27). Oie *et al.* (13) demonstrated the activation of pro-apoptotic genes and evidence of cardiomyocyte apoptosis in the viable peri-infarct area and in the infarcted regions after myocardial infarction in rats, and they also demonstrated that bosentan, a mixed ET_A/ET_B receptor antagonist, has been shown to attenuate cardiomyocyte cell loss through apoptosis in the area at risk after induction of ischemia. On the other hand, ET-1 has been reported to be an antiapoptotic factor in endothelial cells (28). Many reports demonstrate that ET-1 represents a protective factor against myocardial cell apoptosis in heart failure and that this effect is mediated mainly through an ET_A receptor–dependent pathway involving multiple downstream signaling in cardiomyocytes (10–12). In cardiac myocytes, ET-1 prevents oxidative stress– and β-adrenergic agonist–induced apoptosis (11, 29). ET-1 prevents serum deprivation-induced apoptosis in cardiac myocytes *via* the ET_A receptor pathway (30). c-Src is activated by ET-1, upregulates Bcl-x_L expression, and shows an antiapoptotic effect in cardiac myocytes, and these findings indicate a potentially important role for the c-Src/Bcl-x_L pathway in the antiapoptotic effect of ET-1 (30). In fact, Bcl-2 gene product is a 25-kDa membrane protein that functions to prevent apoptosis by various stimuli (9).

Prevention of apoptosis by increased Bcl-2 expression has also been shown in adult cardiac myocytes (29).

Recent evidence indicates that apoptosis of cardiac myocytes is a feature in cardiovascular diseases, including chronic heart failure (CHF) and myocardial infarction. The levels of plasma and myocardial ET-1 increase in patients with CHF and myocardial infarction, indicating the critical role of ET-1 in these cardiovascular disease states (31). But, as is the case with Ang II, extensive studies concerning the role of ET-1 in cardiomyocytes both *in vivo* and *in vitro* have not yet been performed. More studies should be conducted on the role of ET-1 apoptosis in normal cardiomyocytes and also in the cardiomyocytes from different cardiac disease models. As EPA was effective in the prevention of ET-1–induced cardiomyocyte hypertrophy, the present study used EPA as a pharmacologic tool.

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