EPA Effect on NOS Gene Expression and on NO Level in Endothelin-1–Induced Hypertrophied Cardiomyocytes

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Cardiomyocytes release (or metabolize) several diffusible agents (e.g., nitric oxide [NO], endothelin-1 [ET-1], and angiotensin II) that exert direct effects on myocyte function under various pathologic conditions. Although cardiac hypertrophy is a compensatory mechanism in response to different cardiovascular diseases, there can be a pathologic transition in which the myocardium becomes dysfunctional. Recently, NO has been found to be an important regulator of cardiac remodeling. Specifically, NO has been recognized as a potent antihypertrophic and proapoptotic mediator in cultured cardiomyocytes. We demonstrated that ET-1–induced hypertrophic remodeling in neonatal cardiomyocytes was arrested by pretreatment with eicosapentaenoic acid (EPA), a major component of fish oil. In some recent studies, EPA has demonstrated cardioprotective effects by modulating NO. This study investigated the changes in NO synthase (NOS) in ET-1–induced hypertrophied cardiomyocytes and in total levels of nitrates and nitrites. Ventricular cardiomyocytes were isolated from 2-day-old Sprague-Dawley rats and were cultured in D-MEM/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 three groups: control group, ET-1 (0.1 n*M*) group, and ET-1 pretreated with EPA (10 μ*M*) group. NOS gene expression was evaluated 24 hrs after treatment using real-time polymerase chain reaction. Endothelial NOS (eNOS) mRNA expression was decreased in the ET-1 group compared with controls and was unchanged by pretreatment with EPA. mRNA expression of inducible NOS (iNOS) was significantly increased in ET-1–treated cardiomyocytes and was suppressed by EPA pretreatment. Neuronal NOS gene expression and total NO level did not exhibit a statistically significant change in any of the groups. There may be some interaction between ET-1, eNOS, and iNOS in ET-1–induced and EPA-regressed hypertrophied cardiomyocytes that suppress iNOS expression without modulating total NO level or eNOS gene expression. Exp Biol Med 231:913–918, 2006

Key words: endothelin-1; eicosapentaenoic acid (EPA); neonatal cardiomyocytes; hypertrophy; nitric oxide and nitric oxide synthase

Introduction

Nitric oxide (NO) is generated by the NO synthase (NOS) family of enzymes, which catalyze the conversion of the amino acid L-arginine to L-citrulline (1). To date, three NOS isoforms have been identified, each encoded by a separate gene. The constitutively expressed Ca^{2+}-regulated isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS), initially were identified in nerve tissue and in endothelium, respectively (2). Inducible NOS (iNOS), which is not Ca^{2+}-regulated, was first identified in macrophages but can be expressed in almost any cell type on appropriate stimulation (2). All three NOS isoforms may be expressed in cardiomyocytes (2). nNOS expression occurs in the sarcoplasmic reticulum of cardiomyocytes (3), while iNOS can be expressed in cardiomyocytes, infiltrating inflammatory cells, coronary vascular smooth muscle, fibroblasts, and other areas, depending on the stimulus (4). Although eNOS is expressed predominantly in coronary vascular and endocardial endothelial cells (5), it is also expressed in cardiomyocytes at a much lower level (6, 7). eNOS expression is heterogeneous with respect to level of expression and subcellular localization (8).

Cardiac hypertrophy marked by cell enlargement without proliferation is a compensatory mechanism in response to a variety of cardiovascular diseases (9). Chronic

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increases in hemodynamic load often cause cardiac hypertrophy (10). Although hypertrophy can initially be viewed as and considered a salutary response, it may enter a phase of pathologic remodeling that ultimately leads to heart failure and premature death (10). NO is an important and potential regulator of cardiac remodeling (2). It has been recognized as a potent antihypertrophic and proapoptotic mediator in cultured cardiomyocytes (2). These findings have been extended to *in vivo* investigations in genetically engineered mice (11). Depending on NO level and NOS expression by which NO is predominantly produced, NO exerts differential roles in cardiac remodeling (2). Low levels and transient release of NO by eNOS exert beneficial effects on the remodeling process by reducing cardiomyocyte hypertrophy, cavity dilation, and mortality, while high levels and sustained production of NO by iNOS seem to be maladaptive by reducing ventricular contractile function and by increasing cardiomyocyte apoptosis and mortality (2). Extension of these mechanistic insights into the role of NO in cardiac remodeling would further the development of novel therapeutic strategies to treat cardiac remodeling and failure. Moreover, understanding the function and regulation of the antihypertrophic NO cyclic guanosine 3′,5′-monophosphate system offers the promise of new therapeutic strategies for treating cardiac hypertrophy and heart failure.

Endothelin-1 (ET-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 (12). The critical balance of NO and ET-1 is vital in normal physiology and is disrupted in pathologic conditions (2, 13). There has been little research about NO system changes in cardiomyocyte hypertrophy induced by ET-1.

The cardiovascular benefits of fish oil enriched in the n-3 long-chain polyunsaturated fatty acids, including eicosapentaenoic acid (EPA), have been demonstrated in humans and in experimental animals (14, 15). In diabetic rats, long-term oral administration of EPA may stimulate NO production, and increased NO likely inhibits enhanced cardiac sympathetic activity (16). The n-3 fatty acids promote the synthesis of beneficial NO in the endothelium (17). In rabbit heart, EPA reduces myocardial infarct size, mainly by inducing calcium channel–mediated and partially NO-mediated mechanisms (18). Therefore, the beneficial effects of EPA by modulating NO have been noted. We recently demonstrated that ET-1 induced significant hypertrophic changes in neonatal cultured ventricular cardiomyocytes and that these changes could be prevented by pretreatment with EPA. More recently, we found that ET-1 caused the dose-dependent upregulation of vascular endothelial growth factor and its receptors in ET-1–treated hypertrophied cardiomyocytes. The present study investigated the NO system changes (NO level and NOS expression) in ET-1–induced hypertrophied cardiomyocytes. As a therapeutic option, we also investigated whether EPA-mediated regression of ET-1–induced cardiomyocyte hypertrophy would involve NO signaling.

Materials and Methods

Ventricular Cardiomyocyte Culture. Ventricular cardiomyocytes were isolated from 2-day-old Sprague-Dawley rats and were cultured in D-MEM/Ham F12 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 a charge-coupled device (CCD) camera (Olympus, Tokyo, Japan). At Day 4 of culture, the cardiomyocytes were divided into three groups: control group, ET-1 (0.1 n*M*) group, and ET-1 pretreated with EPA (10 μ*M*) group. Cardiomyocytes in the 3 groups were evaluated 24 hrs after treatment. In preliminary experiments, we used EPA at different doses and found that the 10 μ*M* dose of EPA is effective in regressing the ET-1–induced cardiomyocyte hypertrophy. In endothelial cells, NO production was upregulated by EPA administration at doses of 30 to 300 μ*M* (19). In an earlier study, when 9 g/day of fish oil (containing 1.6 g of EPA) was orally administered to German patients with hyperlipoproteinemia, the EPA levels as total lipids in plasma increased from a mean ± SD of 119 ± 70 μ*M* to 619 ± 172 μ*M*, and the EPA levels as free fatty acids in plasma increased from 7 ± 3 μ*M* to 40 ± 10 μ*M* (20).

Morphologic Evaluation. Cell images captured by a CCD camera were traced and analyzed using free image software from the National Institutes of Health (Bethesda, MD). The area was then doubled to account for the surface portion in contact with the dish. All cells from randomly selected fields in 2 or 3 dishes were examined for each condition. We measured more than 100 cells in each condition.

Quantitative Real-Time Polymerase Chain Reaction (RT-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. 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.

mRNA expression levels of eNOS, iNOS, and nNOS in ventricular myocytes were analyzed by quantitative RT-PCR with TaqMan probe using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA) as previously described (21). The gene-specific primers and TaqMan probes were designed using Primer Express v. 1.5 software (Perkin-Elmer Applied Biosystems) specific for eNOS, iNOS, nNOS, and glycer-

aldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The sequences of the oligonucleotides were as follows:

eNOS forward: 5′-GATCCTAACTTGCCTTGCATCCT-3′
eNOS reverse: 5′-TGTAATCGGTCTTGCCAGAATCC-3′
eNOS probe: 5′-CTGGTATTGCACCCTTCC-3′
iNOS forward: 5′-GTGGGTGGCCTCGAGTTC-3′
iNOS reverse: 5′-CCAATCTCGGTGCCCATGTAC-3′
iNOS probe: 5′-CTGCCCCTTCAATGGTT-3′
nNOS forward:
5′-GGAGTCCTTCATCAAACACATGGA-3′
nNOS reverse: 5′-GACATGGGAGGCACAATCCA-3′
nNOS probe: 5′-CTCTGCAGCGGTATTC-3′
GAPDH forward:
5′-GTGCCAAAAGGGTCATCATCTC-3′
GAPDH reverse:
5′-GGTTCACACCCATCACAAACATG-3′
GAPDH probe: 5′-TTCCGCTGATGCCCC-3′

GAPDH mRNA expression was used as an internal control. Each PCR amplification was performed in triplicate, using the following profile: 1 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 quantitative RT-PCR, serial dilutions of rat ventricular myocyte cDNA were performed within the range of various concentrations (1×, 2×, 4×, 8×, and 16×). A no-template (water) reaction mixture was prepared as a negative control.

NO Assay. NO was indirectly detected in cardiomyocyte extracts as nitrite using an NO assay kit (R&D Systems, Minneapolis, MN). In this method, the nitrate present in the sample was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (GADPD) in the presence of the enzyme nitrate reductase. The nitrite formed reacted with sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye. Based on its absorbance within the visible range, the diazo dye was measured at 550 nm.

Statistical Analysis. Values are means ± SEM. Statistical assessment was performed using analysis of variance, with multiple comparisons using the Fisher protected least significant difference test. Nonparametric data were analyzed using the Mann-Whitney *U* test or the Wilcoxon signed rank test. $P < 0.05$ was considered significant.

Results

Cell Surface Area of Cardiomyocytes. As shown in Figure 1, ET-1 caused a significant increase in the cell surface area of cardiomyocytes (1.97-fold, $P < 0.001$ vs. the control group). This morphologic change in cardiomyocytes was ameliorated by pretreatment with EPA ($P < 0.001$ vs. ET-1).

NOS Gene Expression and NO Level in Ventricular Myocytes. mRNA expression of eNOS in neonatal cardiomyocytes was significantly decreased in the ET-1 group compared with the control group, and there was no significant improvement in eNOS gene expression by pretreatment with EPA (Fig. 2A). iNOS gene expression was significantly increased in hypertrophied cardiomyocytes induced by ET-1, and EPA pretreatment suppressed this upregulation (Fig. 2B). mRNA expression of nNOS and NO level were unchanged in the experimental study groups irrespective of ET-1 or EPA administration (Fig. 2C and Fig. 3, respectively).

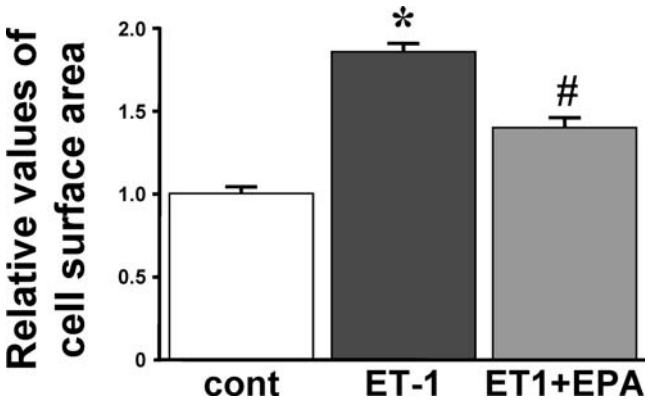

Figure 1. Quantitation of cell surface area of cardiomyocytes in the control group (cont), ET-1 group (ET-1), and ET-1 pretreated with EPA group (ET1 + EPA). Cell surface areas are expressed as relative values when the control group is normalized to 1. Data are means ± SEM (60 cells per group). *$P < 0.001$ vs. the control group. #$P < 0.001$ vs. the ET-1 group.

Discussion

The present study showed that eNOS mRNA was significantly decreased in ET-1–induced hypertrophied cardiomyocytes, but pretreatment with EPA did not reverse this downregulation. Despite eNOS gene downregulation, mRNA expression of iNOS was increased significantly after ET-1 administration, and EPA had a reversal effect on it. There was no effect of ET-1 or EPA on nNOS expression in cardiomyocytes. Compared with the control group, NO concentration was unchanged in cardiomyocytes after ET-1 administration, although iNOS expression was upregulated and EPA had no additive beneficial or detrimental effect on NO concentration. The present study presents an important overview of NOS expression and NO level in ET-1–induced hypertrophied cardiomyocytes. EPA, a major component of fish oil that is useful in cardioprotection, may arrest the hypertrophic progression caused by ET-1 in cardiomyocytes, suppressing iNOS gene expression without affecting NO level or eNOS gene expression in cardiomyocytes.

NO is an important regulator of cardiac function, and multiple interactions between ET and NO signaling are suggested. There is a close interaction between ET-1 and NO: ET-1 generally stimulates NO release by activation of eNOS via the ETB receptor, while NO inhibits ET-1 release by blocking ET-1 gene expression. In the present study, ET-1 administration caused a decrease of eNOS mRNA

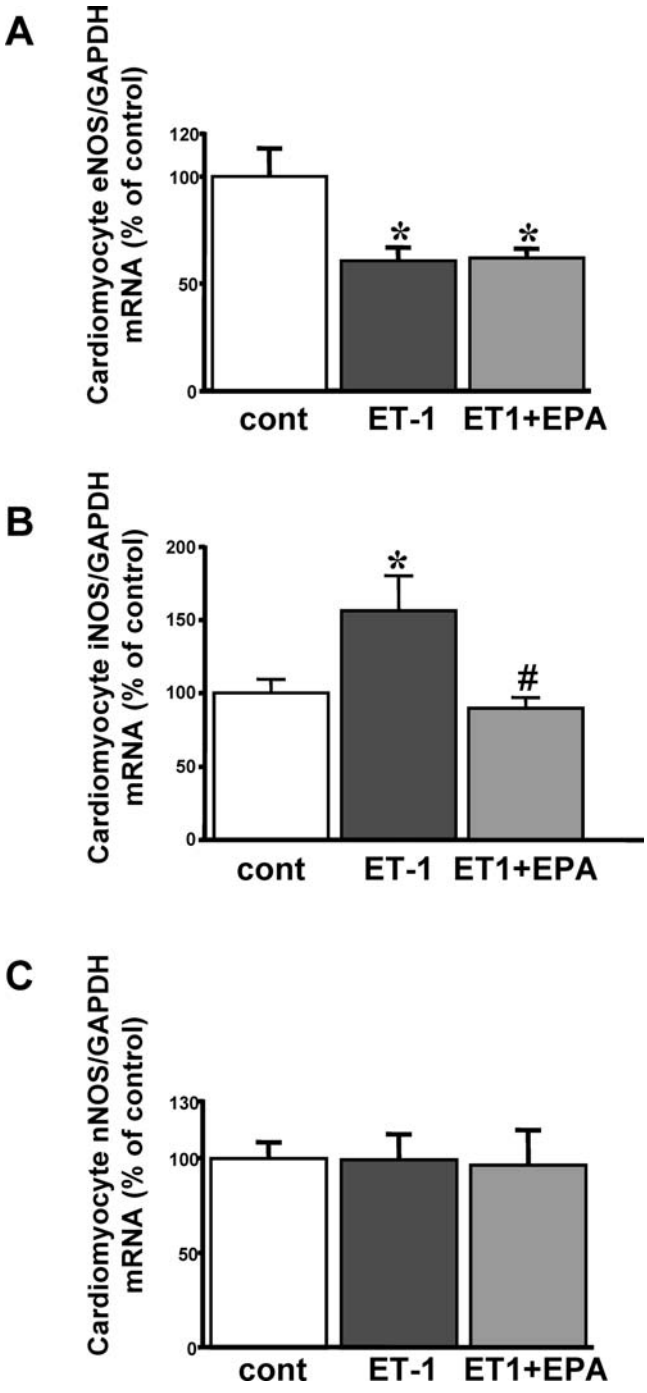

Figure 2. Gene expression of eNOS (A), iNOS (B), and nNOS (C) in neonatal ventricular myocytes of the control group (cont), ET-1 group (ET-1), and ET-1 pretreated with EPA group (ET-1 + EPA). mRNA expression was determined using RT-PCR. Data are means ± SEM. *$P < .01$ vs. the control group (in case of eNOS). *$P < 0.05$ vs. the control group. #$P < 0.05$ vs. the ET-1 group (in case of iNOS).

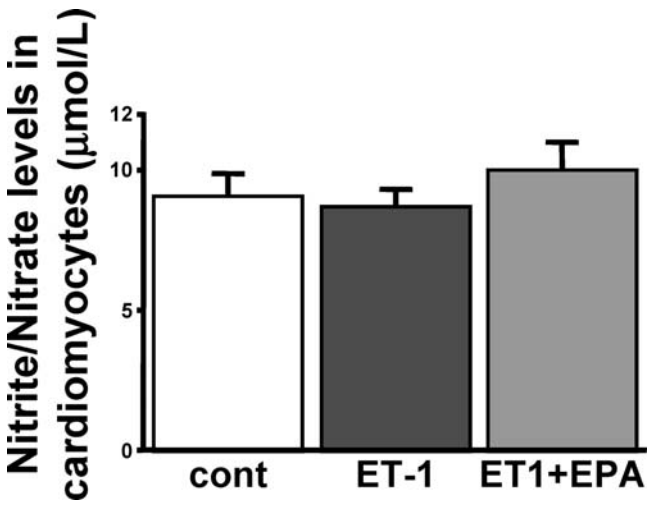

Figure 3. Levels of nitrates and nitrites (NO) in neonatal ventricular myocytes of the control group (cont), ET-1 group (ET-1), and ET-1 pretreated with EPA group (ET-1 + EPA). NO level was determined by nitrate reduction assay. Data are means ± SEM.

expression in cardiomyocytes, which is not surprising. ET-1 increased reactive oxygen species (ROS) in fetal pulmonary endothelial cells, and the ROS increase caused a decrease in eNOS gene expression (22). ET-1 treatment of cardiomyocytes induced ROS via an ETA receptor pathway, and the ROS increase is involved in increased c-fos expression (23). As shown in the present study, ET-1 may induce the formation of ROS in hypertrophied cardiomyocytes, which may in turn downregulate eNOS gene expression. ROS measurement in a scenario similar to that of the present study should clarify the involvement of ROS in ET-1–mediated eNOS downregulation in hypertrophied cardiomyocytes. ET-1 has recently been shown to mediate oxygen production through uncoupled NOS in an endothelium-dependent manner, and endothelium-independent oxygen production stimulated by ET-1 is not mediated via activation of uncoupled NOS (24). Therefore, ET-1 also mediates oxygen production from activated NADPH oxidase, providing "kindling" radicals to uncouple NOS and facilitate endothelial dysfunction. Pretreatment with EPA arrested the development and progression of hypertrophic changes in cardiomyocytes after ET-1 administration but was independent of eNOS expression. Saralasin acetate inhibited angiotensin II–induced cardiomyocyte hypertrophy through the upregulation of eNOS gene expression in cardiomyocytes; a decrease in eNOS activity and gene expression was observed in angiotensin II–induced hypertrophied cardiomyocytes (25). Before making a conclusive statement about EPA and the recovery of eNOS in ET-1–treated cardiomyocytes based on the findings of our study, the activity and protein level of eNOS should be examined.

Nevertheless, iNOS expression in the present study was upregulated after ET-1 was administered to cardiomyocytes, and the upregulation was reversed by pretreatment with EPA. Indeed, a recent study showed ET-1–induced iNOS upregulation in human osteoarthritic chondrocytes (26). BigET-1, which increases the substrate availability for ET-1, significantly enhanced expression of myocardial iNOS at the time of sepsis (27). EPA suppressed increased iNOS

mRNA expression induced by lipopolysaccharide in mouse fibroblasts (28). Moreover, EPA caused significant inhibition of arachidonic acid–mediated iNOS gene expression in human osteoblastic cells (29). EPA protection of angiotensin II–induced renal damage involves inhibition of activator protein-1 and the nuclear factor-κB–dependent pathway (30). iNOS inhibition did not provide renoprotection similar to that of EPA relative to the angiotensin II–induced renal damage (30). Based on our findings, the antihypertrophic action of EPA may be linked to the alteration of iNOS in ET-1–induced cardiomyocyte hypertrophy, despite unchanged NO level. The functional effects of myocardial iNOS expression are controversial, and there appear to be intimate feedback mechanisms between iNOS expression and NO level in different pathologic conditions.

Cardiomyocyte-restricted nNOS overexpression was recently shown to limit left ventricular dysfunction and remodeling after myocardial infarction, in part by decreasing myocyte hypertrophy in noninfarcted myocardium (31). The role of nNOS in cardiomyocyte protection is just beginning to be understood. In the present study, nNOS expression was unchanged in the experimental groups. Either the induction of cardiomyocyte hypertrophy of by ET-1 or the reversal of ET-1–induced cardiomyocyte hypertrophy by EPA does not involve nNOS gene expression.

In conclusion, the present study showed that expression of eNOS mRNA was significantly downregulated in ET-1–induced cardiomyocyte hypertrophy**,** without significant alteration of nNOS expression. EPA was effective in reversing cardiomyocyte hypertrophy morphologically but failed to recover downregulated eNOS gene expression. Moreover, iNOS gene expression was significantly upregulated in ET-1–induced hypertrophied cardiomyocytes, and this upregulation was reversed by EPA pretreatment.

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