Nitric Oxide Inhibits Endothelin-1-Induced Cardiomyocyte Hypertrophy through cGMP-mediated Suppression of Extracellular-Signal Regulated Kinase Phosphorylation


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ABSTRACT
Cardiac hypertrophy is a compensatory mechanism in response to a variety of cardiovascular diseases. Recently, reactive oxygen species and nitro-oxide (NO) have been demonstrated to be involved in the pathogenesis of atherosclerosis; however, the role of these free radicals in the development of cardiac hypertrophy remains unclear. In this study, we investigate NO modulation of cellular signaling in endothelin-1 (ET-1)-induced cardiomyocyte hypertrophy in culture. ET-1 treatment of cardiomyocytes increased constitutive NO synthase activity and induced NO production via the stimulation of ET-receptor subtype ET(A). Using Northern blot analysis and chloramphenicol acetyltransferase assay, we found that NO suppressed the ET-1-induced increase in c-fos mRNA level and promoter activity. In contrast, ET-1 stimulation of c-fos expression was augmented by depletion of endogenous NO generation with the addition of NO scavenger PTIO into cardiomyocytes. Cells cotransfected with the dominant negative and positive mutants of signaling molecules revealed that the Ras/Raf/extracellular signal regulated kinase (ERK) signaling pathway is involved in ET-induced c-fos gene expression. Furthermore, NO directly inhibited ET-1-induced ERK phosphorylation and activation in a cGMP-dependent manner, indicating that NO modulates ET-1-induced c-fos expression via its inhibitory effect on ERK signaling pathway. The ET-1-stimulated activator protein-1 (AP-1) mRNA binding activity and AP-1-mediated reporter activity were attenuated by NO. In addition, NO also significantly inhibited ET-1-stimulated promoter activity of hypertrophic marker gene β-myosin heavy chain and the enhanced protein synthesis. Taken together, our findings provide the molecular basis of NO as a negative regulator in ET-1-induced cardiac hypertrophy.

Cardiac hypertrophy marked by cell enlargement without proliferation is a compensatory mechanism in response to a variety of cardiovascular diseases (Bueno and Molkentin, 2002). Although the initial hypertrophic response may be beneficial, sustained hypertrophy often evolves into heart failure (Bueno and Molkentin, 2002). The extra stimuli that induce hypertrophy include mechanical overload and neurohumoral factors such as endothelin-1 (ET-1). These stimuli bind to heterotrimeric G protein-coupled receptors and rapidly activate immediate-early genes (c-fos, c-jun, egr-1, etc.)

ABBREVIATIONS: PKC, protein kinase C; MAPK, mitogen-activated protein kinase; β-MHC, β-myosin heavy chain; ROS, reactive oxygen species; mERK, mutant of ERK2; SNAP, S-nitroso-N-acetylpenicillamine; SIN-1, 3-morpholinosydnonimine; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; NOS, nitric-oxide synthase; CAT, chloramphenicol acetyltransferase; PMSF, phenylmethylsulfonyl fluoride; MBP, myelin basic protein; ET-1, endothelin-1; ET(A), endothelin-1 receptor type A; ET(B), endothelin-1 receptor type B; BQ485, (hexahydro-1H-azepinyl)carbonyl-Leu-D-Trp-D-OH; BO778, cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxybenzyltryptophan-D-norleucine; RLI1820, Suc-[Glu³, Ala¹¹,¹⁹] endothelin-1 (8-21); PD98059, 2′-amino-3′-methylxavone; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated protein kinase; DEA-NO, diethylnitroso-NONOate; 8-Br-cGMP, 8-bromo-cGMP; PKG, cGMP-dependent protein kinase; AP-1, activator protein-1; L-NAME, N⁰-nitro-L-arginine methyl ester; KT5823, C₂₉H₂₅N₃O₅; 6-nitro-L-arginine, etc.)

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that encode transcription factors via protein kinase C (PKC) pathway and mitogen-activated protein kinase (MAPK) phosphorylation cascade (Bueno and Molkentin, 2002). Subsequently, late target genes such as atrial natriuretic factor, β-myosin heavy chain (β-MHC), skeletal α-actin, and myosin light chain 2, etc. (normally expressed in the embryonic ventricle), are induced, ultimately leading to cardiomyocyte hypertrophy (Chen et al., 2001). Recently, reactive oxygen species (ROS) and nitric oxide (NO) have been demonstrated to be involved in the pathogenesis of atherosclerosis (Alexander and Dzau, 2000). However, the role of these radicals in the development of cardiac hypertrophy remains unclear. Using ET-1-induced cultured cardiomyocyte hypertrophy as a model, we have previously demonstrated that ET-1 treatment to cardiomyocytes increases intracellular ROS generation, which subsequently modulates c-fos gene expression via Ras pathway (Cheng et al., 1999). It has been shown that NO antagonizes the action of ET-1 in vascular smooth muscle cells (Rizvi and Myers, 1997). Calderone et al. (1998) reported that NO inhibited the growth-promoting effects of norepinephrine in cardiomyocytes. It remains to be determined whether ET-1 induces NO production in cardiomyocytes and whether intracellular NO could modulate ET-1-induced cardiomyocyte hypertrophic response, including the induction of early response gene and re-expression of fetal gene program. Therefore, the purpose of this study was to examine the effect of ET-1 on NO production and elucidate how NO is involved in ET-1-induced cardiomyocyte hypertrophy. Herein, we demonstrate that cardiomyocytes exposed to ET-1 increase NO production via ETB receptor. The released NO consequently attenuated ET-1-induced c-fos gene expression through cGMP-mediated suppression of the ERK signaling pathway.

Materials and Methods

Materials. The catalytic inactive mutant of ERK2 (mERK) was a gift from Dr R. J. Davis (University of Massachusetts Medical School, Worcester, MA). RasN17, RasL61, and Raf310 were described previously (Cheng et al., 2003). S-Nitroso-N-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), and diethylamine NONOate were purchased from Calbiochem (San Diego, CA). The plasmid AP-1-Luc containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 binding element was obtained from Stratagene (La Jolla, CA). All other chemicals were of reagent grade and were obtained from Sigma (St. Louis, MO).

Cell Culture. Primary cultures of neonatal rat ventricular myocytes were prepared as described previously (Cheng et al., 1999). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). In brief, ventricles from 1- to 2-day-old neonatal Sprague-Dawley rats were cut into pieces of approximately 1-mm3 and subjected to trypsin (0.125%; Invitrogen, Carlsbad, CA) digestion in phosphate-buffered saline (PBS). Trypsin-digested cells were collected by centrifugation at 1200 rpm for 5 min. The cell pellet was re-suspended in a medium containing 80% F-10 nutrient mixture (Invitrogen), 20% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and plated into a Petri dish. The nonattached myocytes in the medium were collected and plated on a culture dish (10-cm diameter) with a cell density of 1 × 106 cells/dish. After 2 days in culture, culture medium was replaced with medium containing 90% DMEM nutrient mixture, 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Myocyte cultures thus obtained were >90% pure as revealed by immunofluorescence microscopy and counting of all nuclei (stained by 4,6-diamidino-2-phenyindole diacetate (Sigma)) and of cells that stain positive for α-actinin (Sigma). The culture medium was replaced after 24 h with serum-free medium consisting of DMEM, 10 μg/ml transferrin, 10 μg/ml insulin, and 0.1 mM BrdU. Serum-containing medium from these cultured myocytes was replaced with serum-free medium and exposed to agents as indicated.

Measurement of Nitrate/Nitrite Levels. The culture medium was stored at −70°C until use. After the medium had been thawed, the sample was deproteinated with 2 volumes of 4°C 99% ethanol and centrifuged (3000g for 10 min). These medium samples (100 μl) were injected into a collection chamber containing 5% VCl3. This strong reducing environment converts both nitrate and nitrite to NO. A constant stream of helium gas carried NO into a NO analyzer (270B NOA; Seivers Instruments Inc., Boulder, CO), where the NO reacted with ozone, resulting in the emission of light. Light emission is proportional to the NO formed; standard amounts of nitrate were used for calibration.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from cardiomyocytes by the guanidine isothiocyanate/phenol/chloroform method. The RNA (10 μg/lane) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran; Whatman Schleicher and Schuell, Keene, NH) by a vacuum blotting system (VacuGene XL; GE Healthcare, Little Chalfont, Buckinghamshire, UK). After hybridization with the32P-labeled cDNA probes, the membrane was washed with standard saline citrate containing 1% SDS at 42°C for 30 min and then exposed to X-ray film at −70°C. Autoradiography results were analyzed by using a densitometer (Computing Densitometer 300S; GE Healthcare).

Transfections. For the transient transfections, cardiac cells were transfected with different expression vectors by the calcium phosphate method (Cheng et al., 1999). DNA concentration for all samples was adjusted to equal amount with empty vector pSRo in each experiment. In brief, cardiomyocytes (1 × 106 cells/100-mm diameter dish) were maintained in culture for 48 h before transfection. The indicated expression vectors were mixed with calcium phosphate and immediately added to the cardiomyocyte cell culture. After incubation for 5 h, cells were then washed three times with PBS and incubated with 10% serum DMEM. After 24 h, cells were washed with serum-free medium and incubated in the same medium for an additional 24 h. Cells were then treated with different agents. To correct for transfection efficiency, 5 μg of pSV-f-galactosidase plasmid, which contains a β-galactosidase gene driven by the simian virus 40 promoter and enhancer, was cotransfected into cells. The c-fosCAT was 5′ flanking regions of a 2.25-kilobase EcoRI-Nael fragment containing upstream sequences and the promoter of the c-fos gene. In some experiments, mERK, RasN17, RasL61, or Raf310 were transfected in the presence of c-fosCAT. The empty vector pSRo was
transfected and used as a control. The β-MHC CAT was 5’ flanking regions of a 1306-base pair HindIII-PstI fragment containing upstream sequences and the promoter of the β-myosin heavy chain gene as described previously (Shih et al., 2001). Except for routine β-galactosidase activity, cells were constantly collected for measuring chloramphenicol acetyltransferase (CAT) activities as described below.

Chloramphenicol Acetyltransferase Assays and β-Galactosidase Assays. The chloramphenicol acetyltransferase and β-galactosidase assays were performed. The relative chloramphenicol acetyltransferase activity was corrected by normalizing the respective CAT value to that of β-galactosidase activity. Cotransfected β-galactosidase activity varied by <10% within a given experiment and was not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) were included in every assay.

cGMP Concentration. Cardiomyocytes were stimulated with different agents and then disintegrated with ethyl alcohol (100%). The lysate was centrifuged (12,000 rpm, 2 min). The supernatant was used for cGMP determination and the pellet for analysis of protein content. cGMP was determined by radioimmunoassay using the cGMP [H] assay system (TRK500; GE Healthcare) according to the manufacturer’s protocol. The cGMP concentration was normalized to cellular protein content.

Electrophoretic Mobility Shift Assay. To prepare nuclear protein extracts, cardiomyocytes were washed with ice-cold PBS and then immediately removed by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in ice-cold buffer A (10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF) for 15 min. The cells were lysed by adding 10% Nonidet P-40 and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in ice-cold buffer B (20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF, and 0.4 M NaCl), vigorously agitated, and then centrifuged. The supernatant containing the nuclear proteins was used for the electrophoretic mobility shift assay or stored at −70°C until used. Double-stranded oligonucleotides (30 bp) containing the AP-1 binding site were prepared. The oligonucleotides were end-labeled with [32P]ATP. Extracted nuclear proteins (10 μg) were incubated with 0.1 ng of 32P-labeled DNA for 15 min at room temperature in 25 μl of binding buffer containing 1 μg of poly(dI-dC). In the antibody supershift assay, anti-c-fos and anti-c-jun antibodies (1 μg each; Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the mixtures for 30 min at room temperature followed by the addition of the labeled probe. The mixtures were electrophoresed at 5% non-denaturing polyacrylamide gels. Gels were dried and imaged by autoradiography.

Luciferase Assay. Cardiomyocytes plated on 3-cm diameter culture dishes were transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) (Stratagene). After incubation for 24 h in serum-free DMEM, myocytes were cultured under different treatments as indicated for 48 h. Myocytes were assayed for luciferase activity with a luciferase reporter assay kit (Stratagene). The firefly luciferase activities as AP-1 transcriptional activities were normalized for transfection efficiency to its respective β-galactosidase activity and expressed as relative activity to control.

Assay of ERK Activity and Phosphorylation. ERK activity was assayed. In brief, cardiomyocytes were lysed with buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). Cells were disrupted by repeated aspiration through a 21-gauge needle. The same amount of protein from each sample was incubated with anti-ERK1/ERK2 antibody (Santa Cruz Biotechnology) for 2 h at 4°C with gentle shaking. The immune complex was then incubated with protein A/G agarose for 1 h. This agarose-bound immune complex was then incubated with kinase reaction buffer containing myelin basic protein (MBP). The kinase reaction was carried out for 20 min at 30°C in buffer containing 0.3 g/L MBP, 50 μM ATP, and 1 μCi of [32P]ATP. The reaction was stopped by adding an equal volume of sample buffer containing SDS and boiling for 3 min. The samples were electrophoresed on a 15% polyacrylamide gel. After drying, the gel was exposed to X-ray film. For detection of ERK phosphorylation in cardiac myocytes, the cell lysates were collected and boiled. Total cell lysates (100 μg of protein) were separated by SDS-PAGE (12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P; 0.45-μm pore size). The membrane was then incubated with anti-active ERK1/ERK2 antibody (Promega, Madison, WI). Immunodetection was performed by using the Western-Light chemiluminescent detection system (Tropix, Inc., Bedford, MA).

Protein Synthesis Measurement ([3H]Leucine Incorporation). To measure synthesis of new protein, cardiomyocytes cultured in six-well plates were incubated with 1.0 μCi/ml [3H]leucine in serum-free medium. After addition of the test agent, cells were harvested by incubation at 4°C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH, and radioactivity determined by scintillation counting.

Statistical Analysis. Results are expressed as mean ± S.E.M. Statistical analysis was performed using Student’s t test and analysis of variance (ANOVA) followed by a Dunnett multiple comparison test using Prism (GraphPad Software, San Diego, CA). A value of p < 0.05 was considered statistically significant.

Results

Effects of ET-1 on NO Synthesis and NOS Activity. The effects of ET-1 on NO generation and NOS activity in cardiomyocytes were analyzed. Exposure of cardiomyocytes to ET-1 (10 nM) induced a 2-fold increase of NO generation at 30 min after stimulation (Fig. 1A). To determine the ET-1 receptor subtype responsible for the generation of NO in cardiomyocytes, cells were pretreated with ET-1 receptor type A (ET A) antagonist (BQ485) or type B (ET B) antagonist (BQ788) before the ET-1 exposure. As shown in Fig. 1B, in contrast to BQ485 treatment, BQ788 significantly inhibited the ET-1-induced NO generation. Cardiomyocytes treated with ET B agonist IRL1620 (100 nM) consistently induced NO generation. These results indicate that induction of NO generation upon ET-1 treatment is mediated via the ET B receptor. All three NOS isoforms characterized so far depend upon calmodulin activation but are distinguished from each other by their calcium sensitivity and enzymatic activity. A constitutive NO synthase activity was detected and quantified in whole extracts of cardiomyocytes, and this activity was dependent upon the presence of calcium (Fig. 1C). Exposure of cardiomyocytes to ET-1 increased NOS activity by 2-fold at 30 min after stimulation (Fig. 1A). To determine the ET-1 receptor subtype that is responsible for the increase of NOS activity in cardiomyocytes, they were pretreated with ET A receptor antagonist (BQ485) or ET B receptor antagonist (BQ788) before the ET-1 exposure. As shown in Fig. 1D, BQ788 (100 nM) significantly inhibited the ET-1-induced NOS activity. In contrast, BQ485 (100 nM) had no significant effect on NOS activity. Cardiomyocytes treated with ET B receptor agonist IRL1620 (100 nM) alone also increased NOS activity.

NO Modulates ET-1–Induced c-fos Gene Expression in Cardiomyocytes. To explore whether NO modulates ET-1–induced c-fos gene expression, cardiomyocytes were preincubated with an NO donor (i.e., SNAP or SIN-1) for 30 min...
followed by ET-1 treatment. As shown in Fig. 2A, cardiomyocytes treated with SNAP (100 μM) or SIN-1 (100 μM) significantly suppressed ET-1-induced c-fos mRNA expression. In contrast, when cardiomyocytes were pretreated with PTIO (100 μM), a NO scavenger, for 1 h and followed by ET-1 treatment, ET-1-induced c-fos mRNA level was significantly augmented (Fig. 2A). These results suggest that ET-1-induced c-fos expression in cardiomyocytes is modulated by NO.

To further determine whether the modulation of NO in ET-1-induced c-fos expression is a transcriptional event, a c-fos promoter construct containing the c-fos promoter region (−2.25 kb) and the reporter gene CAT were transiently transfected into cardiomyocytes. Cardiomyocytes exposed to 24 h of ET-1 significantly increased c-fos promoter activity by 2.4-fold compared with untreated cells (Fig. 2B). The addition of either SNAP or SIN-1 to cardiomyocytes completely abolished the increased c-fos promoter activity. Conversely, treatment of cardiomyocytes with PTIO enhanced this promoter activity. These results suggest that the modulation of NO on c-fos induction by ET-1 is a transcriptional event.

**ET-1-Induced c-fos Expression Is Mediated via the Ras/Raf/ERK1/2 Pathway.** To identify the signaling pathway involved in ET-1-induced c-fos expression, we transiently transfected cardiomyocytes with various dominant-negative mutants, Ras (RasN17) or Raf-1 (Raf301) or a catalytically inactive mutant of ERK2 (mERK), all of which are associated with the Ras/Raf/ERK pathway. Cardiomyocytes cotransfected with the empty vector PSR, with or without the empty vector PSRs, revealed no effect on ET-1-induced c-fos promoter activity (Fig. 3). However, cells cotransfected with RasN17, Raf301, or mERK resulted in a significant inhibition of ET-1-induced c-fos promoter activity. Cardiomyocytes treated with a specific inhibitor to mitogen-activated protein kinase kinase (MEK) (i.e., PD98059) consistently attenuated ET-1-induced c-fos promoter activity. In contrast, cardiomyocytes cotransfected with a dominant-positive mutant of Ras (RasL61) or MEK1 greatly increased c-fos promoter activity either with or without ET-1-stimulation. These results suggest that the Ras/Raf/ERK signaling pathway is involved in ET-1-induced c-fos gene expression in cardiomyocytes.

**ERK Signaling Pathway Is Involved in the Inhibitory Effect of NO on ET-1-Induced c-fos Gene Expression.** To explore the role of ERK signaling pathway in the attenuating effect of NO on ET-1-induced c-fos expression,

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**Fig. 1.** ET-1 increases NO production and NO synthase activity in cardiomyocytes. A, time course of ET-1-induced NO production in cardiomyocytes. Cardiomyocytes were in control condition, treated with ET-1 (10 nM) for 10, 30, 60, or 120 min. Results are shown as mean ± S.E.M. (n = 5). *, P < 0.05 versus control. B, control cardiomyocytes or those treated with ET-1 in the absence (column 2) or presence of BQ485 (100 nM, column 3) or BQ788 (100 nM, column 4) for 30 min. Cells treated with the ET_{β} receptor agonist IRL1620 (100 nM, column 5) are shown as positive controls. Results are shown as mean ± S.E.M. (n = 6). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone. C, time course of ET-1-induced NOS activity in cardiomyocytes. Cardiomyocytes were treated with ET-1 for 0, 10, or 30 min. NOS activities were measured by citrulline formation from cell lysate incubated with (black bars) or without Ca^{2+} (white bars). NOS activity in the absence of Ca^{2+} indicates inducible NOS activity, whereas its activity in the presence of Ca^{2+} represents constitutive NOS activity. Results are shown as mean ± S.E.M. (n = 5). *, P < 0.05 versus control. D, control cardiomyocytes or those treated with ET-1 in the absence or presence of BQ485 or BQ788 for 30 min. IRL1620-treated cells are shown as positive controls. Results are shown as mean ± S.E.M. (n = 6). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone.

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**Fig. 2.** NO modulates ET-1-induced c-fos gene expression in cardiomyocytes. A, NO modulates ET-1-induced c-fos mRNA expression in cardiomyocytes. Cardiomyocytes were untreated or pretreated with PTIO, SNAP, or SIN-1 for 30 min at a concentration of 100 μM and then treated with or without ET-1 (10 nM) for 30 min. Total RNA was extracted and Northern hybridization was performed with 32P-labeled rat c-fos as probe. 18S RNA was used to normalize the RNA applied in each lane. Data were presented as a percentage change of experimental groups compared with untreated controls. Results are shown as mean ± S.E.M. (n = 6). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone. B, NO modulating ET-1-induced c-fos expression is a transcriptional event. Cardiomyocytes were transfected with 15 μg of c-fosCAT chimeric gene for 24 h. Some cells were pretreated with SNAP, SIN-1, or PTIO for 30 min. Cardiomyocytes were then treated with ET-1 (10 nM) for 24 h. CAT2 and CAT3 are shown as positive and negative controls of a CAT assay system. CAT activities are represented as -fold induction relative to control groups. Results are shown as mean ± S.E.M. (n = 6). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone.
we pretreated cells with PD98059, which specifically blocks the activation of the MAPK pathway. Cardiomyocytes treated with PD98059 showed no ET-1-induced increase in c-fos mRNA expression. ET-1-induced c-fos expression enhanced by the NO scavenger PTIO was also consistently inhibited by pretreating cells with PD98059 (Fig. 4A). To further confirm that the ERK signaling pathway is involved, the dominant inhibitory mERK or wild-type MEK1 was transiently transfected into cells. Cardiomyocytes cotransfected with mERK showed neither ET-1-induced c-fos promoter activity nor PTIO-enhanced ET-1-stimulated c-fos promoter activity (Fig. 4B). Cardiomyocytes cotransfected with wild-type MEK1 failed to further increase ET-1-stimulated c-fos promoter activity. The addition of SNAP to cardiomyocytes significantly inhibited the increased c-fos promoter activity by ET-1 alone but not ET-1 with MEK1 overexpression. These results indicate that the attenuating effect of NO on ET-1-induced c-fos promoter activity is at least partially mediated via MAPK/ERK pathway.

**NO Regulates ET-1-Induced ERK1/2 Phosphorylation and Activity in Cardiomyocytes.** Given that the ERK signaling pathway is involved in ET-1-induced c-fos expression and that NO modulates ET-1-induced c-fos expression, we further investigated whether NO modulates ERK pathway in ET-1-treated cardiomyocytes. We first examined the phosphorylation of ERK1/2 in cardiomyocytes exposed to ET-1 in the presence of the NO donor or NO scavenger. As shown in Fig. 5A, cardiomyocyte exposure to ET-1 for 15 min rapidly induced phosphorylation of ERK1/2 by 4.7-fold. However, cardiomyocytes pretreated with an NO donor (SNAP) or a specific MEK1 inhibitor (PD98059) significantly inhibited ET-1-induced ERK1/2 phosphorylation by 80%. Cardiomyocytes pretreated with PTIO consistently augmented ET-1-induced ERK1/2 phosphorylation by 40% above that of ET-1-treated cells (Fig. 5B). NO modulation of ERK1/2 signaling pathway was further elucidated by its inhibitory effect on ET-1-induced ERK1/2 activity. Cardiomyocytes exposed to ET-1 rapidly induced ERK1/2 activity by 3.4-fold as indicated by an increase of 32P-labeled phosphorylation of MBP by ERK1/2 (Fig. 5C). Treatment of cardiomyocytes with SNAP or SIN-1 significantly attenuated ERK1/2 activation by 90%. In contrast, PTIO treatment of cardiomyocytes enhanced ERK activation by 50% (Fig. 5D). Taken together, these findings imply that NO attenuation of ET-1-induced c-fos expression is mediated via the ERK1/2 signaling pathway in cardiomyocytes.

**NO Modulates ET-1-Induced c-fos Gene Expression and Suppresses ERK Phosphorylation via cGMP/PKG Pathway.** We next tested whether ET-1 stimulates cGMP accumulation by activating ETB receptors. ET-1 alone (10 nM; 30 min) significantly increased intracellular cGMP concentration. In cells pretreated with BQ485 (100 nM; 30 min), ET-1 still significantly increased intracellular cGMP concentration. In contrast, cells treated with BQ788 (100 nM) or Nω-nitro-L-arginine methyl ester (l-NAME; 100 μM), ET-1 had no significant effect on intracellular cGMP concentration (Fig. 6A). BQ485, BQ788, or l-NAME alone had no significant effect on basal levels of intracellular cGMP. IRL1620 (100 nM) or SNAP (100 μM) also significantly increased intracellular cGMP concentration. These data suggest that ET-1 increases intracellular cGMP concentration by activating the ETB receptors in cardiomyocytes. To examine whether NO donor antagonizes ET-1-induced c-fos gene ex-

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Fig. 3. ET-1-induced c-fos gene expression is mediated via Ras/Raf/ERK signaling pathway. Cardiomyocytes were transfected with either pSRα-empty vector (5 μg) or an expression plasmid encoding the dominant-negative mutant mERK, Raf301, or RasN17 (5 μg) was cotransfected with 15 μg of c-fosCAT. Cardiomyocytes transfected with c-fosCAT were pretreated with PD98059 (30 μM) for 30 min before treatment with ET-1. Cardiomyocytes cotransfected with an expression plasmid encoding MEK1 (5 μg) or RasL61 (5 μg) were used as positive controls. Results are shown as mean ± S.E.M. (*P < 0.05 versus pSRα-transfected control; #, P < 0.05 versus ET-1 alone).

Fig. 4. NO modulates ET-1-induced c-fos gene via extracellular signal-regulated kinase pathway in cardiomyocytes. A, NO scavenger augments ET-1-induced c-fos mRNA via extracellular signal-regulated kinase pathway in cardiomyocytes. PTIO pretreatment augments ET-1-induced c-fos mRNA. Cardiomyocytes were untreated or pretreated with PD98059 in the absence or presence of PTIO for 30 min and then treated with or without ET-1 (10 nM) for 30 min. Data were presented as a percentage change of experimental groups compared with untreated controls. Results are shown as mean ± S.E.M. (*P < 0.05 versus control; #, P < 0.05 versus ET-1 alone). B, NO modulating ET-1-induced c-fos promoter activity is mediated via ERK signaling pathway in cardiomyocytes. Cardiomyocytes were transfected with either pSRα-empty vector (5 μg) or an expression plasmid encoding the dominant-negative mutant mERK or an expression plasmid encoding MEK1 (5 μg), was cotransfected with 15 μg of c-fosCAT. Cardiomyocytes transfected with c-fosCAT were pretreated with PTIO or SNAP for 30 min before being treated with ET-1. Results are shown as mean ± S.E.M. (*P < 0.05 versus ET-1 alone).
pression through cGMP and cGMP-dependent protein kinase (PKG) pathways, we used KT5823 (1 μM), a specific inhibitor of PKG, to determine the involvement of GMP/PKG pathway in NO modulation of ET-1-induced c-fos gene expression. Cardiomyocytes exposed to ET-1 in the presence of the NO donor SNAP or cGMP analogs, 8-bromo-cGMP (8-Br-cGMP), which activates PKG, showed significant inhibition of ET-1-stimulated ERK phosphorylation (Fig. 6B). In contrast, in the presence of KT5823 (1 μM), the inhibitory effect of SNAP or 8-Br-cGMP on ET-1-induced ERK phosphorylation was reversed. As shown in Fig. 6, C and D, KT5823 significantly reversed the inhibitory effect of SNAP (100 μM) on ET-1-induced c-fos gene expression. These results indicate the involvement of cGMP/PKG pathway in NO modulation of c-fos gene expression.

NO Inhibits ET-1-Induced AP-1 Transcriptional Activity. The ET-1-induced c-fos gene expression was concordant with an increase in activator protein-1 (AP-1) activity. Using electrophoretic mobility shift assay, AP-1 binding activity to consensus AP-1 binding sequence was assayed in cells treated with ET-1 for 6 h (Fig. 7A). Pretreating cells with NO donor, SNAP, or SIN-1 attenuated this ET-1-stimulated AP-1 binding activity. In contrast, cells pretreated with the NO scavenger PTIO enhanced AP-1 binding activity, indicating that intracellular NO level is involved in the modulation of ET-1-stimulated AP-1 activity. This binding seems to be specific to AP-1, because it was abolished by coinubation of nuclear proteins with c-Fos and c-Jun antibodies. The effects of NO donors on ET-1-induced AP-1 functional activity were also assessed in a reporter gene assay. ET-1 significantly increased AP-1-luciferase activities (Fig. 7B). SNAP or SIN-1 significantly attenuated ET-1-induced AP-1 reporter activation. In contrast, cells pretreated with the NO scavenger PTIO enhanced AP-1 reporter activation (Fig. 7B). These results clearly indicate that NO inhibits the transcriptional activity of AP-1 induced by ET-1.

The Activation of β-MHC and Cardiomyocyte Hypertrophy by ET-1 Is Modulated by NO. ET-1 is known to induce cardiac hypertrophy (Shohet et al., 2004; Xu et al., 2004). The hypertrophy responses in cardiac myocytes are characterized by the induction of a fetal gene program (Stewart et al., 1998). To investigate further the attenuation effects of NO on ET-1-induced cardiac responses, we examined whether NO modulates ET-1-induced expression of cardiac β-MHC. ET-1 (10 nM, 48 h) increased β-MHC promoter activity by 2.8-fold compared with control level (Fig. 8A). When cardiomyocytes were preincubated with NO donors, SNAP (100 μM), or DEA-NO (100 μM) for 60 min, ET-1-induced activation of β-MHC promoter activity was significantly suppressed by DEA-NO. Using [3H]leucine incorporation, SNAP (100 μM) or DEA-NO (100 μM) also attenuated the enhanced protein synthesis in cardiomyocytes induced by ET-1 (Fig. 8B). Conversely, treatment of cardiomyocytes with PTIO enhanced β-MHC promoter activity and protein synthesis induced by ET-1 (Fig. 8, A and B). These results further support the hypothesis that NO plays an important role as a negative regulator in ET-1-induced cardiomyocyte hypertrophy.

Discussion

ET-1, a 21-amino acid peptide with a terminal tryptophan residue, was originally characterized from supernatant of cultured endothelial cells (Goraca, 2002). It is a potent and long-lasting vasoconstrictor, exerts inotropic and chronotropic effects on cardiomyocytes, and induces cardiomyocyte hypertrophy (Goraca, 2002). Two mammalian ET-1 receptor subtypes termed ET$_A$ and ET$_B$ have been characterized. The
ET\(_A\) receptor is specific for ET-1, whereas the ET\(_B\) receptor shows no specificity for ET isoforms (Goraca, 2002); ET-1 binds to both receptor subtypes. ET-1 treatment of cardiomyocytes increased NOS activity and induced NO production (Fig. 1, A and C). The free radical gas NO is synthesized from l-arginine and O\(_2\) by NOS, which requires NADPH and tetrahydrobiopterin as cofactors. These NOS isoforms have been cloned and characterized. At least two of the three isoenzymes (inducible, Ca\(^{2+}\)-insensitive NOS and constitutive, Ca\(^{2+}\)-sensitive endothelial NOS) are now known to be present in cardiomyocytes and play an important role in the regulation of cardiac contractile function (Buchwalow et al., 2001; Petroff et al., 2001). The inducible isoform of NOS is not normally expressed in the myocardium but is synthesized de novo in response to inflammatory cytokines (Stein et al., 1996). The constitutive endothelial NOS is distinguished from inducible iNOS by their calcium sensitivity for the binding of activator calmodulin, and enzymatic activity within the physiological range of intracellular calcium (Ebadi and Sharma, 2003). These results indicate the activation of a constitutive Ca\(^{2+}\)-dependent NOS that generates NO formation. In this study, we demonstrated that NO formation upon ET-1 treatment is mediated via ET\(_B\) receptor stimulation (Fig. 1B). In agreement with our results, several studies showed a relationship between ET\(_B\) receptor activation and NO formation (Yamada et al., 1999; Liu et al., 2003). Interestingly, we have reported previously that ROS generation induced by ET-1 is via ET\(_B\) receptor activation in cardiomyocytes (Cheng et al., 1999). To our knowledge, this is the first report documenting the fact that ET-induced stimulation of NOS activity via the ET\(_B\) receptor subtype in a cardiomyocyte system (Fig. 1D). However, considering that the cardiac ET\(_B\) level was relatively lower than cardiac ET\(_A\) level in cardiomyocytes (data not shown) and that the ET\(_B\)-mediated effect observed in the present study was modest, we should be more cautious in speculating about the potential relevance of the finding observed in the present study to clinical situations.

The immediate proto-oncogene c-fos is normally expressed at a minimal level in the mammalian heart. Increased proto-oncogene expression has been implicated in the development of cardiac hypertrophy (Zimmer, 1997). Induction of c-fos, a transcriptional factor that interacts with cis-regulatory elements (such as AP-1) in many genes, has important conse-

![Fig. 6](image-url). NO antagonizes ET-1 in a cGMP/PKG-dependent manner. A, ET-1 increases cGMP concentration in either cardiomyocytes control or those treated with BQ485 (100 nM), BQ788 (100 nM), or l-NAME (100 \(\mu\)M) in the absence or presence of ET-1 (10 nM) for 30 min. ET\(_B\) receptor agonist IRL1620- (100 nM) or SNAP- (100 \(\mu\)M) treated cells are shown as positive controls. Results are shown as mean ± S.E.M. (n = 4). * P < 0.05 versus control; #, P < 0.05 versus ET-1 alone. B, before ET-1 treatment, cardiomyocytes were preincubated with SNAP or 8-Br-cGMP (100 \(\mu\)M) for 30 min in the absence or presence of KT5823 (1 \(\mu\)M). After ET-1 (10 nM; 15 min) treatment, cardiomyocytes were lysed, and the phosphorylation of ERK was determined. White or black columns denote the degree of ERK1 or ERK2 phosphorylation, respectively. Data are represented as -fold induction relative to control groups. Results are shown as mean ± S.E.M. (n = 5). * P < 0.05 versus control; #, P < 0.05 versus ET-1 alone. C, KT5823 reverses the inhibitory effect of SNAP on ET-1-induced c-fos mRNA in cardiomyocytes. Cardiomyocytes were treated with ET-1 (10 nM) for 30 min in the absence or presence of SNAP or KT5823. Data were presented as a percentage change of experimental groups compared with untreated controls. Results are shown as mean ± S.E.M. (n = 6). * P < 0.05 versus control; #, P < 0.05 versus ET-1 alone. D, KT5823 reverses the inhibitory effect of SNAP on ET-1-increased c-fos promoter activity in cardiomyocytes. Cardiomyocytes transfected with c-fosCAT were treated with ET-1 (10 nM, 24 h) in the absence or presence of SNAP, or SNAP plus KT5823. Results are shown as mean ± S.E.M. (n = 6). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone.
quences for downstream gene expression that may determine the phenotypic response to ET-1. We demonstrated previously that ET-1 induced c-fos gene expression (Cheng et al., 1999). In this study, we further demonstrated that ET-1-induced c-fos expression is modulated by NO (Fig. 2). These findings reveal that ET-1 treatment of cardiomyocytes simultaneously generates both free radicals, ROS and NO, via ET-receptor subtype ETA and ETB stimulation, respectively. ROS stimulates c-fos expression, whereas NO exerts a partially inhibitory effect on c-fos induction. The positive net effect on c-fos gene induction modulated by interaction between endogenous ROS and NO formation induced by ET-1 is consistent with the overwhelming distribution of ETA receptors on cardiomyocytes. Therefore, ET-1 stimulation of c-fos expression is enhanced by depletion of endogenous NO generation with the administration of NO scavenger PTIO into cardiomyocytes. Thus, the cells possess an endogenous counterbalance mechanism for the ET-1-induced c-fos gene expression.

To investigate how NO modulates intracellular signal transduction in ET-1-induced c-fos expression, we identified the signaling pathway in regulating c-fos induction by ET-1. We clearly demonstrated that the Ras/Raf/ERK signaling pathway is involved in ET-1-induced c-fos gene expression in cardiomyocytes (Fig. 3). Previous studies have shown that activation of any of the three parallel MAPK pathways, which consist of the ERK, stress-activated protein kinase/c-Jun NH2-terminal kinase, and p38 subfamilies can lead to a hypertrophic response in cardiomyocytes (Molkentin, 2004). All three MAPK subfamilies are activated by the ET-1 and other hypertrophic G protein-coupled receptor agonists (Dorn and Brown, 1999). It is unlikely that any one pathway could be responsible for all features of the hypertrophic response induced by the diverse array of extracellular stimuli. In addition, there has been disagreement over which of the pathways is necessary for expression of various components of the hypertrophic response induced by specific stimulus. For instance, Choukroun et al. (1998) reported that stress-activated protein kinases are necessary for the development of

![Fig. 7.](image1)

**Fig. 7.** NO modulates ET-1-increased AP-1 activity in cardiomyocytes. A, cardiomyocytes were in control condition, treated with ET-1 (10 nM) for 6 h, pretreated with PTIO, SNAP, or SIN-1 for 30 min at a concentration of 100 μM, and then treated with ET-1 (10 nM) for 6 h or treated with phorbol 12-myristate 13-acetate (100 μg/liter) for 1 h. Total nuclear extracts were prepared and analyzed by electrophoretic mobility shift assay using a 32P-labeled oligonucleotide probe containing AP-1 binding sites. The specificity of the retarded complexes (AP-1) was assessed by preincubating the nuclear extracts with anti-c-Fos and anti-c-Jun antibodies (1 μg each). Results are representative of triplicate experiments with similar results. B, cardiomyocytes, transfected with AP-1-Luc, were incubated for 48 h with either no drug, PTIO, SNAP, or SIN-1 at a concentration of 100 μM in the absence or presence of ET-1 (10 nM) for 48 h. Data are represented as -fold induction relative to control groups. Results are shown as mean ± S.E.M. (n = 4). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone.

![Fig. 8.](image2)

**Fig. 8.** NO modulates ET-1-increased β-MHC promoter activity and protein synthesis. A, NO modulates ET-1-increased β-MHC promoter activity. Cardiomyocytes were transfected with 15 μg of β-MHC CAT chimeric gene. Cells were pretreated with PTIO, SNAP, or DEA-NO at a concentration of 100 μM in the absence or presence of ET-1 (10 nM) for 48 h. Data are represented as -fold induction relative to control groups. Results are shown as mean ± S.E.M. (n = 4). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone. B, cardiomyocytes treated with either no drug, PTIO, SNAP, or DEA-NO at a concentration of 100 μM in the absence or presence of ET-1 (10 nM) are then assayed for protein synthesis. Results are shown as mean ± S.E.M. (n = 6). *, P < 0.05 versus control; #, P < 0.05 versus ET-1 alone.
ET-1-induced cardiomyocyte hypertrophy (Choukroun et al., 1998), whereas we demonstrated that ET-1 certainly induces up-regulation of c-fos via Ras/Raf/ERK signaling pathway. ET-1 activates the small G protein Ras through a PKC-dependent transactivation mechanism. Ras in turn activates ERK cascade, which consists of three-member protein kinase “modules”, Raf/MEK/ERK. When activated, the ERKs translocate to the nucleus to activate the immediate downstream target, transcription factor Elk-1 (ternary complex factor), which subsequently transactivates the c-fos serum response element and induces c-fos expression.

Given that Ras/Raf/ERK signaling pathway is involved in ET-1-induced c-fos gene expression and NO regulates ET-1-induced c-fos expression in cardiomyocytes, we further demonstrated that the inhibitory effect of NO on ET-1-induced c-fos expression is at least partially mediated via MAPK/ERK pathway (Fig. 4). To identify the molecule(s) that NO modulates in the MAPK signaling pathway, we showed that NO modulates the phosphorylation and activation of ERK molecules (Fig. 5). ERKs are key molecules in intracellular signal transduction and play essential roles in cellular proliferation and differentiation in many cell types. In cardiomyocytes, activation of ERKs was reported to be necessary for α1-adrenoceptor agonist phenylephrine-induced transactivation of immediate early genes such as c-fos (Bueno and Molkentin, 2002). Our findings clearly demonstrate that NO modulates ET-1-induced c-fos gene expression via its inhibitory effect on the ERK signaling pathway in cardiomyocytes. It is well known that NO activates guanylyl cyclase with a resulting increase in cGMP accumulation that subsequently activates the receptor proteinPKG (Fiedler and Wollert, 2004). In agreement with our present findings, endothelins are reported to stimulate the NO-dependent cGMP formation in different tissues (Tack et al., 1997). This NO/cGMP signaling pathway plays an important role in regulating contractile properties of cardiac muscle in vitro and in vivo (Paulus and Bronzwaer, 2004). In this study, we further demonstrated that ET-1-induced c-fos gene expression is inhibited by NO via the suppression of the ERK signaling through NO/cGMP/PKG pathway (Fig. 6). Because the ET-1-induced c-fos gene expression is coincident with an increase in the activity of AP-1, which is composed of a heterodimer of c-fos and c-jun, we further test whether NO modulates ET-1-induced AP-1 transcriptional activity. The ET-1-induced AP-1 binding activity assay that the electrophoretic mobility shift assay and AP-1-luciferase activity both were attenuated by the NO scavenger PTIO (Fig. 7). Previous studies reveal that AP-1 and ERK seem to be activated by ROS, which is involved in the regulation of ET-1 gene expression (Cheng et al., 2003). Our findings further indicate that NO suppresses the ET-1-stimulated transcriptional activity of AP-1 via its inhibitory effect on ERK activation and c-fos expression.

At the gene level, the most characteristic finding in cardiac hypertrophy is re-expression of a group of muscle-specific fetal genes. Among these, only myosin heavy chain gene has physiological significance, because the ratio of its isoforms in myocardium is directly associated with contractile performance of the heart. We have previously reported that ET-1 stimulates α- and β-MHC mRNA level and promoter activity in cultured cardiomyocytes (Wang et al., 1992; Cheng et al., 2005). Because NO regulates ET-1-induced c-fos expression and AP-1 transcriptional activity, we further examine whether NO modulates the ET-1-stimulated promoter activity of hypertrophic marker gene, β-MHC. Our results also indicate that NO inhibits the ET-1-induced hypertrophic response (i.e., the induction of immediate-early gene, re-expression of fetal gene, and increased protein synthesis) (Fig. 8). These findings further support the concept that NO plays an important role as a negative regulator in ET-1-induced cardiomyocyte hypertrophy. In summary, ET-1 increases NO activity and NO formation via ET-receptor subtype ET_{1A} stimulation in cardiomyocytes. The expression of ET-1-induced immediate early gene c-fos is modulated by NO via the NO signaling pathway in a cGMP-dependent manner. NO regulates hypertrophic marker gene β-MHC, at least in part via modulation of c-fos expression and AP-1 transcriptional activity. Thus, our findings provide the molecular mechanisms of NO as a negative regulator in ET-1-induced hypertrophic response of cardiomyocytes and may allow the development of new strategies for the treatment of cardiac hypertrophy and heart failure.

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