

# Endothelin-3 Induces Hypertrophy of Cardiomyocytes by the Endogenous Endothelin-1-mediated Mechanism

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## Abstract

We have recently reported that endothelin-1 (ET-1) mediates angiotensin II-induced hypertrophy of cardiomyocytes as an autocrine/paracrine factor. In the present study, we examined whether endothelin-3 (ET-3) induces hypertrophy of cultured neonatal rat cardiomyocytes and whether endogenous ET-1 mediates this effect. ET-3 ( $10^{-7}$  M) increased the cell surface area of cardiomyocytes after 48 h. ET-3 dose dependently ( $10^{-9}$ – $10^{-7}$  M) stimulated protein synthesis as evaluated by [ $^3$ H]leucine incorporation; the maximum response was 1.4-fold increase over the control at  $10^{-7}$  M. Since the response of cardiac hypertrophy is characterized by enhanced expression of fetal isoforms of muscle specific genes, the effect of ET-3 on steady state levels of mRNA for skeletal  $\alpha$ -actin was evaluated by Northern blot analysis. ET-3 ( $10^{-9}$ – $10^{-7}$  M) increased mRNA level for skeletal  $\alpha$ -actin with a maximum response after 6 h. ET-3-induced [ $^3$ H]leucine incorporation, skeletal  $\alpha$ -actin mRNA and cell surface area were inhibited by a synthetic ET<sub>B</sub> receptor antagonist (BQ788). Interestingly, ET-3-induced skeletal  $\alpha$ -actin gene expression and [ $^3$ H]leucine incorporation were inhibited by a synthetic ET<sub>A</sub> receptor antagonist (BQ123) as well as by antisense oligonucleotides against preproET-1 mRNA. ET-3 ( $10^{-7}$  M) transiently increased mRNA levels for ET-1 peaking at 30 min and stimulated the release of immunoreactive ET-1 from cardiomyocytes. These results suggest that endogenous ET-1 locally generated and secreted by cardiomyocytes may contribute to ET-3-induced cardiac hypertrophy as an autocrine/paracrine factor. (*J. Clin. Invest.* 1996; 97:366–372.) Key words: muscle-specific gene expression • leucine incorporation • ET-1-LI • BQ123

## Introduction

Endothelin (ET)<sup>1</sup> is a potent vasoconstrictor peptide with 21 amino acid residues, originally characterized from the supernatant of cultured porcine endothelial cells (1). Three ET

isopeptides (ET-1, ET-2, ET-3) have been identified so far in human (2), porcine (1), and rat (3). These three isopeptides have different pressor/vasoconstrictor activities; ET-1 is the strongest pressor/vasoconstrictor ever isolated, whereas ET-3 exerts the most potent initial depressor response among the three ET isopeptides (4). We and other investigators have previously demonstrated that ET-1 induces hypertrophy of cultured rat cardiomyocytes (5, 6). In the subsequent studies we have demonstrated that cultured rat cardiomyocytes express abundant ET-1 transcripts and release mature ET-1 into culture medium. Furthermore, the selective ET<sub>A</sub> receptor antagonist, BQ123, partially blocked cardiomyocyte hypertrophy stimulated by angiotensin II (Ang II) (7). These results have led us to speculate that endogenous ET-1 produced by cardiomyocytes may be a mediator for Ang II-induced hypertrophy of cardiomyocytes via an autocrine/paracrine mechanism.

Currently, two distinct ET receptors with differing affinities for ET-1 and ET-3 have been identified. ET-1 is more potent than ET-3 at the ET<sub>A</sub> receptor, whereas these two peptides have similar potencies at the ET<sub>B</sub> receptor. ET-2 has similar potency to ET-1 at both receptor subtypes. Both ET<sub>A</sub> and ET<sub>B</sub> receptors and their mRNAs are widely expressed in a variety of tissues, including the heart (8). We have recently reported that ET<sub>B</sub> receptor mRNA is up-regulated in hypertrophied rat cardiomyocytes (9), suggesting that ET<sub>B</sub> receptor in cardiomyocytes may partly contribute to the development of cardiac hypertrophy. However, a role of the ET<sub>B</sub> receptor and its ligand, ET-3, in the production and maintenance of cardiac hypertrophy remains unknown.

In the present study, we report that ET-3 induces hypertrophy of neonatal rat cardiomyocytes. Furthermore, using a synthetic ET<sub>A</sub> receptor antagonist and antisense oligonucleotides against ET-1, we show a possible autocrine/paracrine role of ET-1 in the ET-3-induced hypertrophy of rat cardiomyocytes.

## Methods

**Drugs and cDNAs.** The following drugs were used: synthetic ET-1, ET-3, (Peptide Institute, Osaka, Japan), cytosine arabinoside (Sigma Chemical Co., St. Louis, MO), BQ123 (10), BQ3020 (11), and BQ788 (12) were provided by Banyu Pharmaceutical Co. Ltd. (Tokyo, Japan). cDNA for rat preproendothelin-1 (ppET-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used for probes were generous gifts from Dr. T. Masaki (Kyoto University, Japan), and Dr. K. Webster (SRI International, Menlo Park, CA), respectively. Skeletal  $\alpha$ -actin cDNA was synthesized as previously described (13).

**Cell culture.** The primary culture of neonatal rat cardiomyocytes was prepared using the method originally described by Simpson and Savion (14) with minor modifications (15). Briefly, the hearts from 1- or 2-d-old Wistar rats (Japan Laboratory Animals, Tokyo, Japan) were minced and dissociated with 0.1% tyrosine. After the dispersed cells were incubated on 100-mm culture dishes (Falcon Labware, Becton Dickinson Co., Oxnard, CA) for 60 min at 37°C in 5% CO<sub>2</sub>, nonattached viable cells were collected and seeded onto 60-mm dishes ( $2 \times 10^6$  cells/dish) or 12-well plates ( $4 \times 10^5$  cells/well). Cardio-

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Received for publication 31 May 1995 and accepted in revised form 30 October 1995.

1. Abbreviations used in this paper: Ang II, angiotensin II; ET, endothelin; ET-1-LI, ET-1-like immunoreactivity; ppET-1; preproET-1.

J. Clin. Invest.

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0021-9738/96/01/366/07 \$2.00

Volume 97, Number 2, January 1996, 366–372

myocytes were incubated in MEM supplemented with 5% calf serum plus  $10^{-5}$  M cytosine arabinoside for 48 h, and then replaced with serum-free MEM without cytosine arabinoside for 48 h. In this stage, the cultures contained 5–7% of nonmyocytes, but no endothelial cells were identified by immunocytochemical studies using antibody for Factor VIII (Ito, H., unpublished data). All experiments were performed in serum-free MEM.

**Binding experiments.** Confluent cardiomyocytes ( $4 \times 10^5$  cells in 12-well plates) were pretreated with BQ3020 or BQ123 for 60 min and then incubated with 6 pM  $^{125}$ I-labeled ET-1 in the presence or absence of various doses of competitors at 37°C for 60 min in HBSS containing 0.1% BSA, as reported previously (16). After completion, cells were extensively washed with PBS and solubilized in 0.5 N NaOH, and the cell-bound radioactivity was determined. Specific binding was obtained by subtracting nonspecific binding in the presence of an excess ( $10^{-6}$  M) of unlabeled ET-1 from total binding and expressed as a percentage of total binding without competitor.

**Measurements of cell surface area and cell number.** Cardiomyocytes were fixed with 10% buffered formalin after treatment with or without ET-3 for 48 h. Phase contrast pictures (original magnification 200) were taken on 35-mm film (Kodak Japan Inc., Tokyo, Japan) and the images were digitized by an image scanner (Model GT-8000; Epson Inc., Tokyo, Japan). The measurement software program (Image, version 1.41; Wetness@helix.nih.gov) controlled by an Apple computer (Apple Computer, Inc., Cupertino, CA) was used to measure the surface area of cardiomyocytes. The surface area was determined by the mean value of 50 cells.

**Northern blot analysis.** Total RNA from neonatal rat cardiomyocytes was isolated by the guanidium thiocyanate-phenol-chloroform method (17) using ISOGEN (Nippon Gene, Tokyo, Japan). RNA (10  $\mu$ g) was size fractionated through 1.4% agarose gel in 0.7 M formaldehyde and 20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA. Northern blot hybridization was performed with hybridization buffer containing 50% formamide,  $5 \times$  Denhardt's solution, 100 mg/ml salmon sperm DNA, and  $5 \times$  SSPE (0.75 M NaCl, 0.05 M  $\text{NaH}_2\text{PO}_4$ , and 0.005 M EDTA).  $^{32}$ P-labeled cDNA probes were prepared by the random primer method (18). The membranes (Managrap nylon; Micron Separations Inc., Westborough, MA) were washed twice with  $5 \times$  SSPE/10% SDS at room temperature, once with  $2 \times$  SSPE/10% SDS at 37°C, and once with  $0.2 \times$  SSPE/10% SDS at 37°C for 15 min each. Autoradiography was performed on an RX film (Fuji Film Corp., Tokyo, Japan) with an intensifying screen at  $-80^\circ\text{C}$ . Radioactivities were quantitated by BAS 2000 (Fuji Film Corp.). Results were normalized to GAPDH expression.

**Incorporation of [ $^3\text{H}$ ]leucine.** Protein synthesis by cardiomyocytes was evaluated by incorporation of [ $^3\text{H}$ ]leucine into cells. Cardiomyocytes plated on 12-well plates ( $2 \times 10^5$  cells/well) were incubated with or without test compounds in serum-free MEM for 24 h. 4 h before the end of the incubation period, 0.5  $\mu\text{Ci}$  [ $^3\text{H}$ ]leucine was added in 1 ml of culture medium. Cultures were then rinsed three times with ice-cold PBS, and incubated with 5% TCA on ice for 20 min. After the cells were washed twice with ice-cold 5% TCA, they were solubilized in 0.5 N NaOH. An aliquot of TCA-insoluble materials was neutralized and radioactivity was determined by a liquid scintillation counter (Model 460CD, Packard Instrument Co., Inc., Meriden, CO).

**RIA.** Cardiomyocytes plated on 35-mm dishes ( $4 \times 10^5$  cells/dish) were incubated with or without ET-3 in serum-free MEM for 6 and 24 h. ET-1-like immunoreactivity (ET-1-LI) in the medium was determined by specific RIA for ET-1 as previously reported (19). The polyclonal antibody used cross-reacted fully with ET-1, 2% with big ET-1, and  $< 0.1\%$  with ET-2 and ET-3. The number of cells was counted using a hemocytometer (Kayagaki Irika Kogyo Co. LTD., Tokyo, Japan), and the level of ET-1-LI was expressed in picograms per  $10^5$  cells.

**Antisense oligonucleotides.** Antisense oligonucleotides (oligonucleoside phosphorothioates) against the first 15 nucleotides of the coding region of rat ppET-1 including AUG codon (3'TACCTA

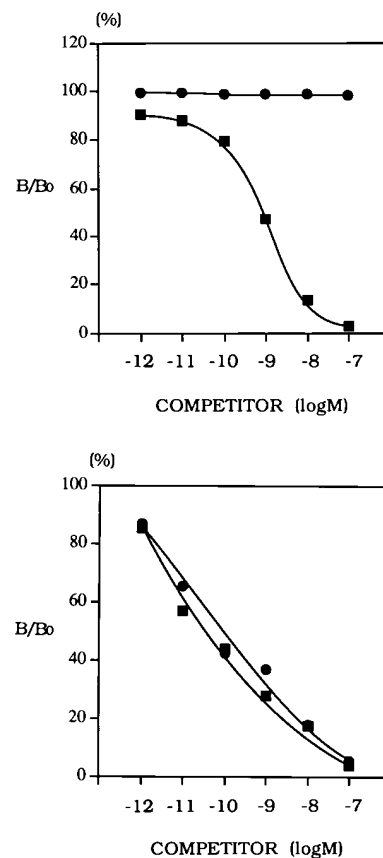
ATAAAAGGG5') were synthesized by a DNA synthesizer (Model 8909, PerSeptive Biosystems, Cambridge, MA) and purified by a HPLC. Mismatch oligonucleotides at four nucleosides (3'TACCTA GTACAATGG5') were similarly synthesized. Cells were incubated with antisense or mismatch oligonucleotides ( $10^{-5}$  M) for 18 h before treatment with ET-3. Oligonucleotides were further added at the start of ET-3 treatment.

**Statistical analyses.** Either the Student's *t* test or one-way ANOVA with multiple comparison methods by Scheffe was used for statistical analyses. A value of  $P < 0.05$  was considered significant.

## Results

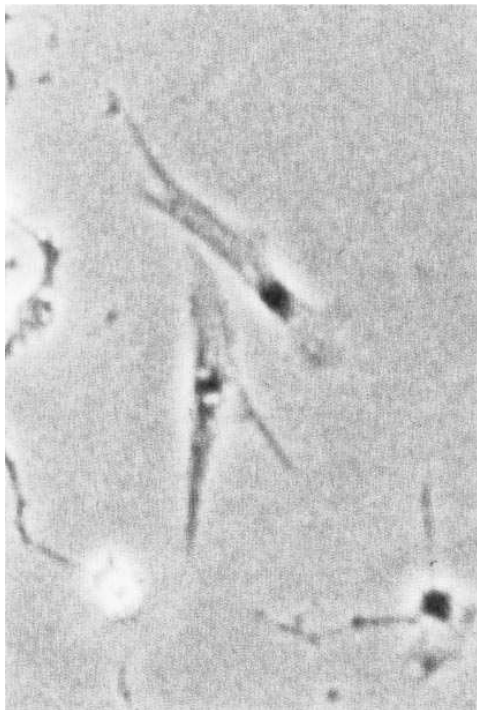
**ET-3 selectively binds  $\text{ET}_B$  receptor of cardiomyocytes.** We performed competitive binding studies using neonatal rat cardiomyocytes in the presence of an excess amount of either an  $\text{ET}_B$  receptor agonist (BQ3020) or an  $\text{ET}_A$  receptor antagonist (BQ123) to fully block  $\text{ET}_B$  receptor or  $\text{ET}_A$  receptor, respectively. ET-1 competitively inhibited the binding of [ $^{125}\text{I}$ ]ET-1 in the presence of  $10^{-7}$  M BQ3020 with apparent  $\text{IC}_{50}$  of  $5 \times 10^{-10}$  M, whereas ET-3 showed a minimal inhibition up to the dose of  $10^{-7}$  M (Fig. 1 A). In contrast, both ET-1 and ET-3 almost equally inhibited the binding of  $^{125}\text{I}$ -labeled ET-1 in the presence of  $10^{-6}$  M BQ123 (Fig. 1 B).

**ET-3 induces hypertrophy of cardiomyocytes via the  $\text{ET}_B$  receptor.** Neonatal rat cardiomyocytes were apparently hypertrophied after incubation with ET-3 ( $10^{-7}$  M) for 48 h as compared to the cells cultured in serum-free MEM for same period (Fig. 2). The hypertrophy-promoting effects of ET-3 were also confirmed by morphometrical evaluation using an image analyzer system. The cell surface area of cardiomyocytes treated with ET-3 ( $10^{-7}$  M) ( $692 \pm 32 \mu\text{m}^2$ ,  $n = 50$ ) was significantly



**Figure 1.** Competitive binding of  $^{125}\text{I}$ -labeled ET-1 to the neonatal rat cardiomyocytes in the presence of excess  $\text{ET}_B$  receptor agonist or  $\text{ET}_A$  receptor antagonist. To select out effects on the other receptor subtype, either  $10^{-7}$  M BQ3020 (top) or  $10^{-6}$  M BQ123 (bottom) was included in the medium, then confluent rat cardiomyocytes ( $4 \times 10^5$  cells/well) were incubated with  $^{125}\text{I}$ -labeled ET-1 in the presence of various concentrations of ET-1 (■) or ET-3 (●). Results are expressed as the percentage of specific binding in the absence of peptides ( $B_0$ ); each point is the means of three samples.

# CON

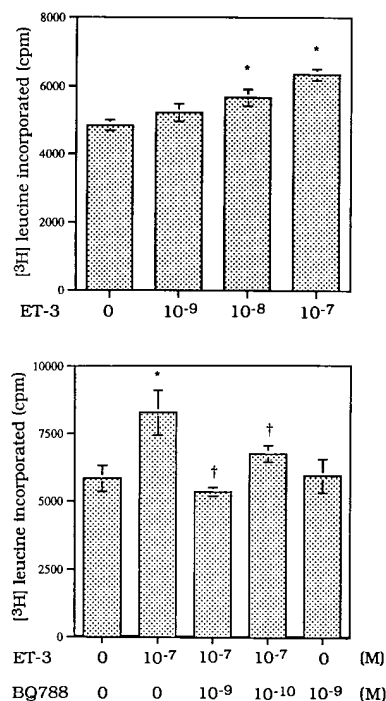


# ET-3



**Figure 2.** Phase contrast microscopic pictures of hypertrophied cardiomyocytes by ET-3. Neonatal rat cardiomyocytes cultured in serum-free MEM were treated with or without ET-3 ( $10^{-7}$  M) for 48 h. Note the hypertrophied cardiomyocytes with ET-3 (*right*) as compared with the control cells (*left*).

( $P < 0.05$ ) larger than that of the control cells ( $305 \pm 14 \mu\text{m}^2$ ,  $n = 50$ ). Furthermore, ET-3 significantly increased [ $^3\text{H}$ ]leucine incorporation into cardiomyocytes in a dose-dependent manner ( $10^{-9}$ – $10^{-7}$  M); the maximum response was up to 1.4-fold over the control value at the dose of  $10^{-7}$  M (Fig. 3, *top*). ET-3-stimulated [ $^3\text{H}$ ]leucine incorporation into cardiomyocytes was blocked completely by the  $\text{ET}_B$  receptor antagonist, BQ788 ( $10^{-9}$  M) (Fig. 3, *bottom*). The steady state mRNA level

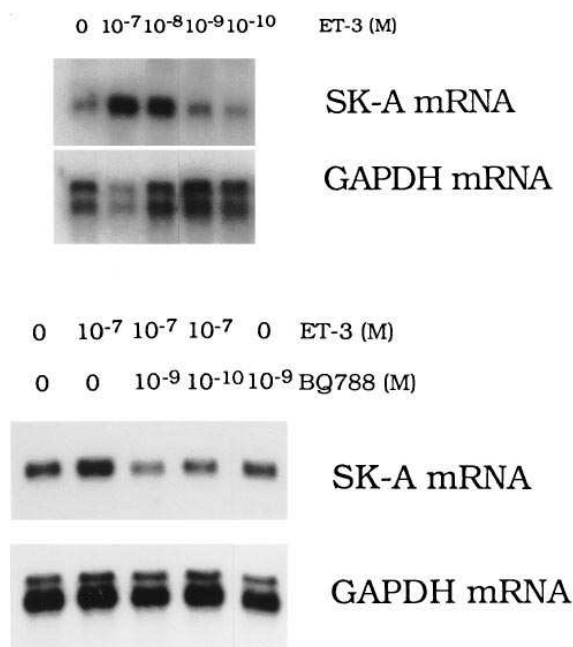


**Figure 3.** Effect of ET-3 and BQ788 on protein synthesis of cultured rat cardiomyocytes. Rat cardiomyocytes were treated with indicated dose of ET-3 (*top*), and in the presence or absence of BQ788 ( $10^{-9}$  M– $10^{-10}$  M) for 24 h (*bottom*). Protein synthesis was assessed by incorporation of [ $^3\text{H}$ ]leucine into the cells. Each column represents mean of four samples; error bars show SEM. Statistically significant: \*difference from the cells without ET-3, and † difference from the cells with ET-3 only ( $10^{-7}$  M) ( $P < 0.05$ ).

of skeletal  $\alpha$ -actin, a genetic marker for cardiomyocyte hypertrophy, increased in a dose-dependent manner after 6-h incubation with ET-3 (Fig. 4, *top*). ET-3-stimulated skeletal  $\alpha$ -actin gene expression was blocked completely by BQ788 ( $10^{-9}$  M) while BQ788 ( $10^{-9}$  M) alone had no effect (Fig. 4, *bottom*).  $\text{ET}_B$  receptor agonist (BQ3020) ( $10^{-6}$  M) mimicked ET-3-induced mRNA level for skeletal  $\alpha$ -actin (data not shown).

*ET-3 stimulates ET-1 mRNA and ET-1 production in cardiomyocytes.* As previously reported (7), abundant amounts of ppET-1 mRNA was detectable in cultured neonatal rat cardiomyocytes. ET-3 ( $10^{-7}$  M) up-regulated ppET-1 mRNA by ~ fourfold over the control level as early as 30 min. ppET-1 gene expression then returned to basal level after 2 h and remained constant during 12 h. Up-regulation of ppET-1 mRNA by ET-3 was followed by the induction of skeletal  $\alpha$ -actin mRNA, which was observed after 6 h (Fig. 5, *A* and *B*). ET-1-LI was released from cardiomyocytes incubated in serum-free MEM as early as 6 h, and ET-3 ( $10^{-7}$  M) almost doubled the release of ET-1-LI into the medium at 6 and 24 h (Fig. 6).

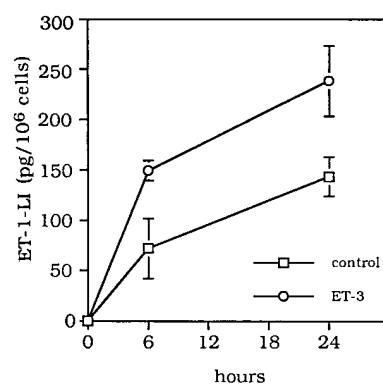
*ET-3-induced hypertrophy of cardiomyocytes is inhibited by an  $\text{ET}_A$  receptor antagonist and antisense oligonucleotides against ET-1 mRNA.* We next examined the effects of the  $\text{ET}_A$  receptor antagonist, BQ123, on ET-3-induced hypertrophy. The increase in the cell surface area of cardiomyocytes induced by ET-3 ( $10^{-7}$  M for 48 h) was significantly inhibited by BQ123 (Fig. 7). The [ $^3\text{H}$ ]leucine incorporation stimulated by ET-3, as well as by BQ3020, was also inhibited by treatment with BQ123 ( $10^{-8}$ – $10^{-6}$  M) in a dose-responsive manner, while BQ123 ( $10^{-6}$  M) alone had no effect (Fig. 8). Furthermore, ET-3-stimulated skeletal  $\alpha$ -actin gene expression was significantly ( $P < 0.05$ ) inhibited by BQ123 (Fig. 9, *top*); quantitative analysis of the data from four independent experiments revealed that the level of skeletal  $\alpha$ -actin mRNA in cardiomyo-



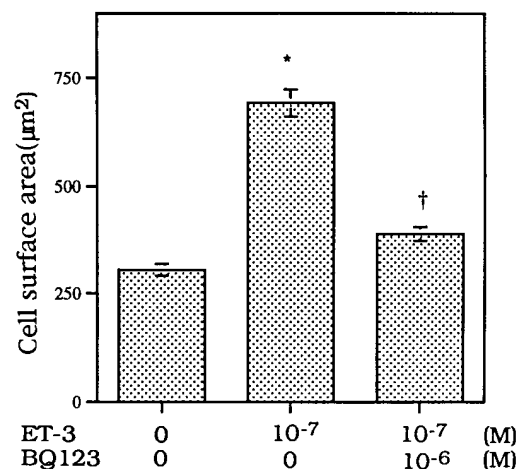
**Figure 4.** Induction of skeletal  $\alpha$ -actin mRNA by ET-3 with or without BQ788. Rat cardiomyocytes were treated with indicated dose of ET-3 (*top*), and in the presence or absence of BQ788 ( $10^{-9}$  M– $10^{-10}$  M) for 6 h (*bottom*). Northern blot analysis (10  $\mu$ g of total RNA/each lane) was performed using  $^{32}$ P-labeled rat skeletal  $\alpha$ -actin and GAPDH cDNA probes as described in the text. Exposure time: 72 h for skeletal  $\alpha$ -actin and 24 h for GAPDH. SK-A, skeletal  $\alpha$ -actin.

cytes treated with ET-3 ( $10^{-7}$  M) increased by  $383 \pm 20\%$  (mean  $\pm$  SE,  $n = 4$ ) over control, whereas that in cotreated with ET-3 and BQ123 ( $10^{-6}$  M) ( $103 \pm 7\%$ ) was almost equal to that of control cells ( $103 \pm 7\%$ ) (mean  $\pm$  SE,  $n = 4$ ). Similarly, BQ123 blocked mRNA level of skeletal  $\alpha$ -actin induced by BQ3020 to the control level (Fig. 9, *bottom*).

To further determine the possible involvement of endogenous ET-1 in ET-3-induced hypertrophy, we introduced antisense oligonucleotides against coding region of ppET-1 mRNA in rat cardiomyocytes, and examined the skeletal  $\alpha$ -actin mRNA expression and protein synthesis stimulated by ET-3. The antisense sequence ( $10^{-5}$  M) blocked skeletal  $\alpha$ -actin

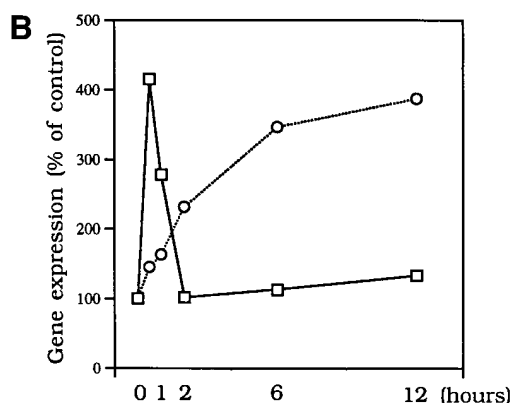
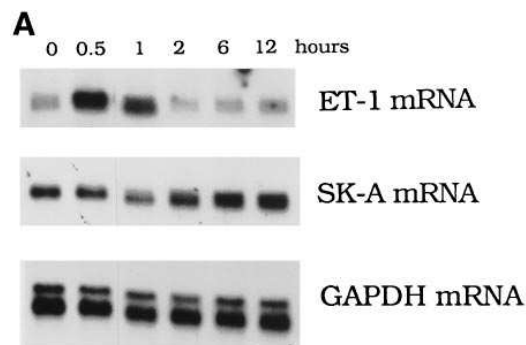


**Figure 6.** Effect of ET-3 on release of immunoreactive ET-1 (ET-1-LI) from cultured rat cardiomyocytes. Cells were treated with (○) or without (□) ET-3 ( $10^{-7}$  M) for 6 or 24 h. ET-1-LI was measured by RIA as described in the text. Each point represents mean of six samples; bars show SEM.

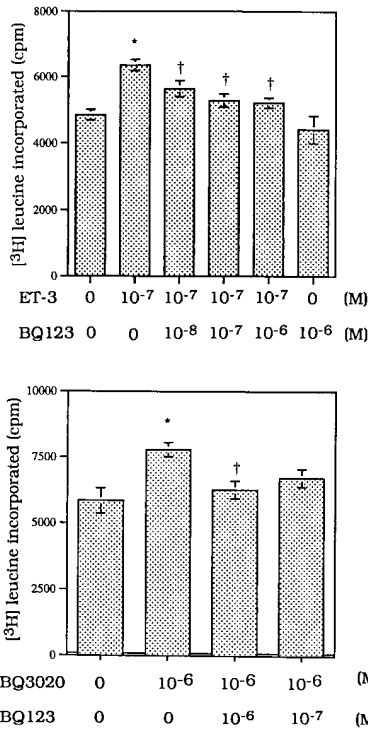


**Figure 7.** Inhibition of ET-3-induced increases in surface area of rat cardiomyocytes by BQ123. Surface area of cardiomyocytes treated with or without ET-3 ( $10^{-7}$  M) in the presence or absence of BQ123 ( $10^{-6}$  M) was assessed by an image analyzer system as described in the text. Each column represents mean value of 50 cells; error bars show SEM. Statistically significant; \*difference from the cells without ET-3, and † difference from the cells with ET-3 only ( $P < 0.05$ ).

mRNA (Fig. 10 A) and inhibited the ET-3-induced [ $^3$ H]leucine incorporation (Fig. 10 B). The mismatch sequences have no effect on either ET-3-induced skeletal  $\alpha$ -actin mRNA or protein synthesis.



**Figure 5.** Time course of ppET-1 and skeletal  $\alpha$ -actin mRNA induced by ET-3. Neonatal rat cardiomyocytes were exposed to ET-3 ( $10^{-7}$  M) for indicated times. Northern blot hybridization (10  $\mu$ g of total RNA/each lane) was performed as in Fig. 4. (A) Autoradiograms. Exposure time: 72 h for ppET-1 and skeletal  $\alpha$ -actin (SK-A) and 24 h for GAPDH. (B) Quantitative results of gene expression of ppET-1 (□) and skeletal  $\alpha$ -actin (○) mRNA normalized for GAPDH expression.

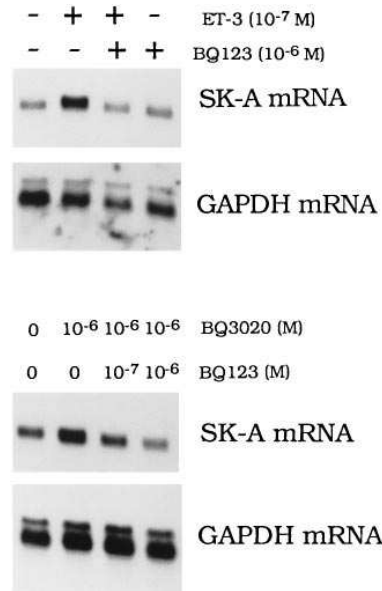


**Figure 8.** Inhibition of ET-3-induced protein synthesis of rat cardiomyocytes by BQ123. Protein synthesis of cardiomyocytes treated with or without ET-3 ( $10^{-7}$  M) (*top*), or BQ3020 ( $10^{-6}$  M) (*bottom*) in the presence or absence of indicated doses of BQ123 was assessed by incorporation of [ $^3$ H]leucine into the cells. Each column represents mean value of four samples; error bars show SEM. Statistically significant; \*difference from the cells without ET-3, and †difference from the cells with ET-3 only ( $P < 0.05$ ).

## Discussion

In the present study, we reported that ET-3 has a hypertrophic effect on rat cardiomyocytes associated with increases in the transcripts for skeletal  $\alpha$ -actin. Furthermore, we demonstrated that endogenous ET-1 locally generated by cardiomyocytes may contribute to ET-3-induced cardiac hypertrophy as an autocrine/paracrine factor.

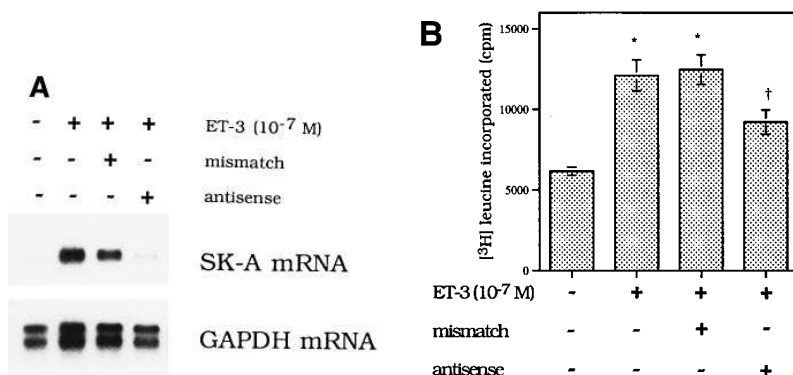
During the development of cardiomyocyte hypertrophy induced by neurohumoral factors, transcripts of several muscle-specific genes such as myosin light chain 2 (20), troponin I (5), and the skeletal  $\alpha$ -actin isoform (21) are induced in vitro. Among those muscle-specific genes, induction of mRNA for skeletal  $\alpha$ -actin, an embryonic or fetal isoform of sarcomeric actin, was clearly demonstrated in vivo in pressure-overloaded rat hearts (22). Therefore, skeletal  $\alpha$ -actin mRNA is one of the useful genetic markers for cardiomyocyte hypertrophy both in vitro and in vivo. The present results with respect to ET-3-induced expression of skeletal  $\alpha$ -actin, in addition to the in-



**Figure 9.** Inhibition of ET-3-induced expression of skeletal  $\alpha$ -actin mRNA by BQ123. Cells were exposed to ET-3 ( $10^{-7}$  M) (*top*), or BQ3020 ( $10^{-7}$  M) (*bottom*) in the presence or absence of BQ123 ( $10^{-6}$  M) for 6 h. Northern blot hybridization (10  $\mu$ g of total RNA/each lane) was performed as in Fig. 4. Exposure time: 72 h for skeletal  $\alpha$ -actin (SK-A) and 24 h for GAPDH.

creases of cell surface area and protein synthesis in rat cardiomyocytes, may be supportive evidence that ET-3 is capable of inducing cardiomyocyte hypertrophy.

ET receptors are distributed widely, not only in vascular, but also in nonvascular tissues. In the myocardium, the presence of two receptor subtypes, ET<sub>A</sub> and ET<sub>B</sub>, have been reported (8). Recently several receptor antagonists and agonists specific for each ET receptor subtype, such as the ET<sub>A</sub> antagonist, BQ123, the ET<sub>B</sub> antagonist, BQ788, and the ET<sub>B</sub> agonist, BQ3020, have been developed. In the present binding experiments, in the presence of an excess amount of an ET<sub>B</sub> receptor agonist, ET-3 showed far less potent inhibition than ET-1 in cardiomyocytes. In contrast, in the presence of an excess of an ET<sub>A</sub> receptor antagonist, ET-1 and ET-3 almost equally inhibited the binding of [ $^{125}$ I]-labeled ET-1. These data suggest that ET-3 has high affinity with ET<sub>B</sub> receptors, whereas very low affinity with ET<sub>A</sub> receptors in cardiomyocytes. In this study, ET-3-induced expression of skeletal  $\alpha$ -actin mRNA, as well as [ $^3$ H]leucine incorporation, was inhibited completely by BQ788 at the dose  $10^{-9}$  M, which antagonist is ET<sub>B</sub> subtype specific (12). Taken together, our data indicate that the hypertrophy-promoting action of ET-3 is mediated via the ET<sub>B</sub> receptor. Furthermore, our results showing that the ET<sub>B</sub>-specific agonist



**Figure 10.** Inhibition of ET-3-stimulated skeletal  $\alpha$ -actin mRNA and protein synthesis by antisense oligonucleotides against ppET-1 mRNA in cultured rat cardiomyocytes. (A) Cells were pretreated with antisense oligonucleotides ( $10^{-5}$  M) or oligonucleotides of mismatch sequence ( $10^{-5}$  M) for 18 h, followed by exposure to ET-3 ( $10^{-7}$  M) for 6 h in the presence of each oligonucleotide. Northern blot hybridization (10  $\mu$ g of total RNA/each lane) was performed as in Fig. 4. Exposure time: 72 h for skeletal  $\alpha$ -actin (SK-A) and 24 h for GAPDH. (B) Cells were pretreated with antisense oligonucleotides ( $10^{-5}$  M) or oligonucleotides of mismatch sequence ( $10^{-5}$  M) for 18 h, followed by exposure to ET-3 ( $10^{-7}$  M) for 24 h in the presence of each oligonucleotide. Synthesis of protein was assessed by

incorporation of [ $^3$ H]leucine into cells. Each column represents mean value of four samples; error bars show SEM. Statistically significant; \*difference from the cells without ET-3, and †difference from the cells with ET-3 only ( $P < 0.05$ ).

(BQ3020) also induces skeletal  $\alpha$ -actin mRNA expression support the contribution of ET<sub>B</sub> receptor to cardiac hypertrophy. We have previously reported that ET<sub>B</sub> receptor mRNA is up-regulated during the course of Ang II-induced cardiomyocyte hypertrophy, while ET<sub>A</sub> receptor mRNA remained unchanged (9). This also suggested a role for the ET<sub>B</sub> receptor in the development of cardiac hypertrophy. The present data that ET-3, as well as BQ3020, induced hypertrophy of rat cardiomyocytes suggest that the ET<sub>B</sub> receptor contributes to induction of cardiac hypertrophy.

In our previous study, we have demonstrated that ppET-1 mRNA is abundantly expressed in neonatal rat cardiomyocytes and ET-1-LI is released into media (7). In this study, ET-3 also stimulated ppET-1 transcripts in cardiomyocytes and ET-1-LI release from them. These results are compatible with the previous report by Yokokawa and co-workers (23) that ET-3 caused an increase in ET-1 production in human umbilical vein endothelial cells. In our previous study, ppET-1 mRNA is rapidly up-regulated in rat cardiomyocytes by the treatment with a protein kinase C activator, 12-*O*-tetradecanoyl-phorbol-13-acetate (7). These data suggest that ppET-1 gene expression is up-regulated by an intracellular signaling pathway coupled to phosphoinositide breakdown in cardiomyocytes. On the other hand, stimulation of ET<sub>B</sub> receptor by BQ3020 induced formation of inositol-1,4,5-triphosphate and increased intracellular Ca<sup>2+</sup> concentrations in vascular endothelial cells (24). Taken together, it is suggested that intracellular phosphoinositide breakdown contributes, at least in part, to the up-regulation of ppET-1 mRNA induced by ET-3 in cardiomyocytes.

In the present study, increases in cell surface area, [<sup>3</sup>H]leucine incorporation, and skeletal  $\alpha$ -actin gene expression in rat cardiomyocytes were significantly and almost completely inhibited by the selective ET<sub>A</sub> receptor antagonist, BQ123. Furthermore, the increases of [<sup>3</sup>H]leucine incorporation as well as skeletal  $\alpha$ -actin gene expression in rat cardiomyocytes by ET-3 were blocked by the antisense oligonucleotides against ppET-1 mRNA. Our previous data indicated that this kind of antisense oligonucleotides specifically and almost completely blocked the increase of ppET-1 mRNA (7). Taken together, we speculate that endogenous ET-1 locally produced by cardiomyocytes contributes, at least in part, to the mechanism of ET-3-induced hypertrophy. This notion is consistent with our previous report that BQ123 as well as antisense oligonucleotides, inhibited Ang II-induced [<sup>3</sup>H]leucine incorporation and skeletal  $\alpha$ -actin gene expression in rat cardiomyocytes (7). The inhibitory action of BQ123 on cardiac hypertrophy in vivo has also been reported in the pressure overloaded left ventricle of rats with aortic banding (25) and the right ventricle of rats with monocrotaline-induced pulmonary hypertension (26). On the basis of these experimental results, it may be proposed that endogenous ET-1 plays a potential role in the development of cardiac hypertrophy as an autocrine/paracrine factor.

In conclusion, the results of our present studies indicate that ET-3 induces hypertrophy of cardiomyocytes and suggest a possible autocrine/paracrine role of ET-1 in ET-3-induced hypertrophy of cultured rat cardiomyocytes.

## Acknowledgments

We thank Ms. K. Hayashi for her excellent technical support.

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, and

the Ministry of Health and Welfare, Japan, and by a grant from the Study Group of Molecular Cardiology, Tokyo.

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