Inverse regulation of preproendothelin-1 and endothelin-converting enzyme-1β genes in cardiac cells by mechanical load

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EMERGING EVIDENCE HAS INDICATED that a complex pattern of humoral and autocrine/paracrine factors, including endothelin-1 (ET-1) and ANG II, participates in the pathophysiology of cardiovascular diseases (15). Plasma levels of ET-1 precursor peptide, big ET-1, have been shown to increase in patients with cardiac hypertrophy, and the levels correlate with the clinical severity of the heart failure (27). Besides the potential role as a humoral factor, more than 80% of ET-1 is secreted to the basolateral compartment by endothelial cells (42), and ET-1 may act as an important local regulator of vascular tone as well as cardiac growth and function.

Pressure overload increases the levels of preproendothelin-1 (ppET-1) mRNA in rat hypertrophied myocardium (10). Moreover, both in rats with chronic heart failure due to myocardial infarction (12) and in dogs with tachycardia-induced heart failure (23), cardiac ET-1 peptide and ppET-1 mRNA levels increase. ET-1 treatment of cultured cardiac myocytes increases myocyte size accompanied by sarcomere organization and activates protein synthesis and transcription of hypertrophy-associated genes (29, 39). In the nonmyocyte pool of cardiac cells, ET-1 stimulates the proliferation of cardiac fibroblasts (7). In the deoxytocorticosterone acetate salt-fed rats with hypertension and an activated ET-1-system, inhibition of the ET-1 system reduces development of cardiac fibrosis (2). Similarly, chronic treatment with endothelin receptor antagonist bosentan reduces myocardial fibrosis in rats with heart failure due to myocardial infarction (24). Collectively, ET-1 may regulate both myocyte growth and proliferation of fibroblasts in the development of cardiac hypertrophy.

The mechanisms that control the ET-1 system are of fundamental interest in the pathophysiology of heart failure. To directly examine the effect of mechanical loading on ET-1 system, we used an in vitro model of mechanical load. We studied which cardiac cells respond to load by increasing ET-1 production. Our results suggest that the effect of mechanical stretch on the ET-1 system is cell type specific: ET-1 generation is activated in myocytes and decreased in fibroblasts. Interestingly, mechanical stretch induces opposite changes in mRNA levels of endothelin-converting enzyme-1β (ECE-1β) and ppET-1. Our results further demonstrate that activation of ppET-1 gene transcription in stretched ventricular myocytes is mediated via extracellular signal-regulated kinase (ERK).

MATERIALS AND METHODS

Plasmids. Plasmid for Rous sarcoma virus promoter linked to the β-galactosidase gene (RSV-β-gal) was purchased from Clontech (Palo Alto, CA). The pGL3 basic vector driven by a 1.3-kb fragment of rat ppET-1 promoter (ppET-1-luc) was described previously (28). Plasmid encoding wild-type mitogen-activated ERK-activating kinase-1 (pCMV-MEK1) was a kind gift from Dr. K. L. Guan (University of Michigan, Ann Arbor, MI), and pMT2 plasmid was
generously provided by Dr. D. B. Wilson (Boston Children’s Hospital, Boston, MA).

Cell culture and transfection. Cell cultures of neonatal rat ventricular myocytes and fibroblasts and of human umbilical vein endothelial cells (HUVEC) were prepared and transfected as previously described (14, 29). The Ethical Committee and The Animal Use and Care Committee of the University of Oulu approved the experimental design. Briefly, after digestion of ventricular tissue with collagenase (2 mg/ml), cell suspension was prepared for two passages (divided at a ratio of 1:5 per passage) in the presence of 10% fetal bovine serum (FBS) to ensure proliferation of fibroblasts over other cell types. The nonattached cells or myocyte-enriched cells (MC) were plated at a density of 2 × 10^5 cells/cm^2 on cell culture plates (Falcon) or on flexible-bottomed collagen I-coated elastomere plates (Bioflex; Flexcell International) for stretch experiments and then cultured overnight with DMEM/F-12 medium containing 10% FBS and thereafter in complete serum-free medium (CSFM). Fibroblasts were propagated in 10% FBS containing DMEM/F-12 medium until 24 h before the experiment, when medium was replaced with CSFM. HUVEC culture was prepared as previously described (14). To examine the presence of endothelial cells, we stained cells for an endothelial cell marker (vWF) (19). The second passage of fibroblasts and primary MC cultures stained negative for vWF, whereas >95% of HUVEC stained positive (data not shown). Transfection of cells (if designated) was performed on the second day in culture. Cells were exposed to 1.5 μl of FuGENE 6 and 0.75 μg of DNA (0.5 μg of luciferase, 0.25 μg of β-galactosidase plasmids) per milliliter of CSFM for 6 h and cultured thereafter in CSFM. For cotransfection experiments (30), cells were grown on 24-well plates and transfected with 0.5 μg of ppET-1-luc plasmid and 0.025 μg of pCMV-MEK1 or empty control plasmid (pMT2) with 0.25 μg of RSV-β-gal and 1.5 μg of FuGENE 6 per milliliter. Reporter gene activities were measured using luciferase and β-galactosidase (to correct transfection efficiency) assays (Promega) with a luminometer (29). Cells on Bioflex plates were exposed to cyclic mechanical stretch. Frequency of cyclic stretch was 0.5 Hz with a pulsation of 10-25% elongation of cells from 1 min to 24 h. Cells were stretched by applying a cyclic vacuum suction under Bioflex plates with computer-controlled equipment (FX-3000; Flexcell International). This in vitro model of mechanical load leads to a hypertrophic phenotype of cardiac myocytes within 24-48 h (activation of hypertrophy-associated genes, reorganization of sarcomeric proteins) (31).

mRNA analysis and radioimmunoassay. RNA was isolated from cells using the guanidine thiocyanate-CsCl method (41). Northern blots with fibroblast RNA were hybridized with specific cDNA probes for rat ET-1 and rat ribosomal 18S labeled with [32P]dCTP (Amerham Pharmacia) with a T7 Quick Prime kit (Amersham Pharmacia). ET-1 levels of culture medium were measured by radioimmunoassay. For HUVEC, bars represent means ± SE of 4–5 (3 h), 8–12 (6 h), 35 (24 h, control), and 17 (24 h, stretch) experiments. For F and MC, bars represent means ± SE of 24 and 12 experiments, respectively. *P < 0.05; **P < 0.01 vs. control.

ERK activity assay. Activity of ERK was measured as described previously (11). Briefly, cells were sonicated and supernatant was collected after centrifugation. Protein extract (15 μl) was incubated at 30°C for 15 min with 10 μl of substrate buffer containing specific ERK substrate peptide in the presence of [γ-32P]ATP. The terminated reaction was blotted on peptide-binding paper disks, which were washed repeatedly with 75 mM orthophosphoric acid. Incorporated radioactivity was measured with a scintillation counter (Rack-beta II; LKB Wallac). Fibroblasts were lysed, and samples (6.8 μg protein/lane) were subsequently subjected to Western blot analysis measurement of site-specific phosphorylation of ERK [phospho-p44/p42 MAPK (Thr202/Tyr204) antibody; New England Biolabs] and total protein/lane) were subsequently subjected to Western blot analysis measurement of site-specific phosphorylation of ERK [phospho-p44/42 MAPK (Thr202/Tyr204) antibody; New England Biolabs] and total levels of ERK (p44/42 MAPK antibody; New England Biolabs) as described in detail previously (11).

Statistical analysis. Results are expressed as means ± SE. To determine the statistical significance between two groups and for analysis of multiple groups, we used Student’s t-test and one-way ANOVA followed by Bonferroni’s post hoc test, respectively. Differences at the 95% level were considered statistically significant.

RESULTS

Regulation of ET-1 secretion by mechanical stretch. First, we evaluated our stretch model by introducing cyclic mechanical stretch on HUVEC culture and measured secretion of ET-1 with RIA (Fig. 1A). Compared with static cultures, at 3 h of cyclic stretch the levels of immunoreactive ET-1 (ir-ET-1) in culture medium had already increased 41% (P < 0.05), and ir-ET-1 levels remained 81% (P < 0.05) and 38% elevated

Table 1. Oligonucleotide sequences in RT-PCR analysis using TaqMan chemistry

<table>
<thead>
<tr>
<th>Sense Primer (Forward)</th>
<th>Antisense Primer (Reverse)</th>
<th>Fluorogenic Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppET-1</td>
<td>ATGGACGAGCCGAGGCTTCTG</td>
<td>GGCAGGCCGCGCTGC</td>
</tr>
<tr>
<td>ECE-1β</td>
<td>GCATGCAGCCGCGCAGAT</td>
<td>GAGGTCGATGATGGCCGAG</td>
</tr>
<tr>
<td>ETa</td>
<td>GGAATGCTGGCCTGCGG</td>
<td>TTTGCCACCTGTCGGCCG</td>
</tr>
<tr>
<td>c-Fos</td>
<td>GGGAGGTCCACGCGGCA</td>
<td>TGTTCGCGGATCTAGCGG</td>
</tr>
<tr>
<td>18S</td>
<td>TGGTTGCAAAAGCTGAAACTTAAAG</td>
<td>AGCTGAAATTAGCGGGAGG</td>
</tr>
</tbody>
</table>

ppET-1, preproendothelin-1; ECE-1β, endothelin-converting enzyme-1β; ETa, endothelin receptor type A.

Fig. 1. Effect of mechanical stretch on ET-1 secretion by cultured human umbilical vein endothelial cells (HUVEC; A) and neonatal rat ventricular fibroblasts (F) and myocytes (MC) (B). Immunoreactive ET-1 (ir-ET-1) levels in cell culture media were measured by radioimmunoassay. For HUVEC, bars represent means ± SE of 4–5 (3 h), 8–12 (6 h), 35 (24 h, control), and 17 (24 h, stretch) experiments. For F and MC, bars represent means ± SE of 24 and 12 experiments, respectively. *P < 0.05; **P < 0.01 vs. control.
(P < 0.001) at 6 and 24 h, respectively. Thereafter, we applied mechanical stretch on rat fibroblast and MC cultures for 24 h (Fig. 1B). Interestingly, ir-ET-1 mRNA levels decreased 53% (P < 0.001) in fibroblasts and increased 202% (P < 0.01) in MC, respectively. The levels of ir-ET-1 in confluent culture media of HUVEC, fibroblasts, and MC were 120, 60, and 1 pM after 24-h incubation without mechanical stretch, respectively. Together, these data show that cyclic mechanical stretch augments release of ET-1 by endothelial cells and cardiac myocytes but inhibits secretion of ET-1 by cardiac fibroblasts.

**Regulation of ET-1 synthesis in stretched fibroblasts.** The initial gene product of 212 amino acids (ppET-1) is cleaved into a 38-amino acid big ET-1, which is subsequently cleaved into a 13-amino acid ET-1. The ppET-1 mRNA levels transiently 62% (P < 0.05) and 106% (P < 0.05) at 3 and 6 h, respectively (Fig. 2B). Using TaqMan chemistry with RT-PCR, we found that ETA mRNA levels were below detection in both control and stretched fibroblasts (data not shown).

![Fig. 2. Effect of mechanical stretch on ET-1 (A), endothelin-converting enzyme-1β (ECE-1β; B) and c-Fos mRNA levels (C) of neonatal rat ventricular fibroblasts. mRNA levels were related to the 18S RNA level of each sample. mRNAs and 18S RNA were measured with Northern blot analysis (A) and real-time quantitative RT-PCR analysis with the use of TaqMan chemistry on an ABI Prism 7700 sequence detection system (B and C). Bars represent means ± SE of 3 (1 h), 5 (4–6 h), 8–10 (12 h), and 11–14 (24 h) separate experiments, respectively. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.](http://ajpregu.physiology.org/)

Mechanical stretch of fibroblasts induced transient increase of c-Fos mRNA levels at 1 and 4 h (Fig. 2C). We exposed fibroblasts to 12-O-tetradecanoylphorbol-13-acetate (TPA; a positive control), which effectively increases ET-1 synthesis in HUVEC (14). In fibroblast culture, TPA (100 nM, 24 h) increased the release of ir-ET-1 447% (P < 0.05) (Fig. 3A) and activated rat ppET-1 promoter 71% (P < 0.05) (Fig. 3B). However, mechanical stretch of fibroblasts for 24 h inhibited ppET-1 promoter activity 59% (P < 0.001) (Fig. 3B). Together, these data show that ppET-1 mRNA levels and gene transcriptional activity decreased in stretched fibroblasts, while simultaneously, mRNA levels of ECE-1β transiently increased.

**Regulation of ET-1 synthesis in stretched myocytes.** Mechanical stretch augmented ET-1 synthesis in MC culture: ET-1 mRNA levels increased transiently 87% (P < 0.01) at 1 h (Fig. 4A). In contrast, mechanical stretch of MC decreased ECE-1β and ETA mRNA levels 35% (P < 0.05) and 38% (P < 0.01) at 12 h and 37% (P < 0.01) and 48% (P < 0.01) at 24 h, respectively (Fig. 4, B and C). In stretched MC, ppET-1 promoter activity increased 78% (P < 0.001) and 39% (P < 0.05) at 6 and 24 h, respectively (Fig. 4D). Therefore, ppET-1 mRNA levels and ppET-1 promoter activity increase in stretched ventricular myocytes, but mRNA levels of its receptor (ETA) and processing enzyme (ECE-1β) decline.

**Regulation of ET-1 transcription by mechanical stretch via ERK.** Recently, it was shown that ERK is involved in activation of ppET-1 gene transcription in cardiac myocytes by α-adrenergic stimulation (22). To examine whether the regulation of ERK activity is upregulated by mechanical stretch in cultured MC, we applied an ERK-specific kinase assay (11). Mechanical stretch had elevated ERK activity already at 1 min, and activity peaked at 10 min (Fig. 5A). PD-98059, an inhibitor of the upstream kinase of ERK (MEK1), decreased the activation of ERK by mechanical stretch (Fig. 5B). When PD-98059
was added to incubation medium, activation of ppET-1 promoter activity was abolished (Fig. 5C). An inhibitor of p38 MAPK (SB-203580) (11) had no effect on ppET-1 promoter induction by mechanical stretch (Fig. 5C). To evaluate whether ERK activation was sufficient to induce ppET-1 promoter, we cotransfected ppET-1-luc and MEK1 with RSV-β-gal control plasmid. Activation of ERK pathway by MEK1 overexpression induced ppET-1 promoter activity 78% (P < 0.001) (Fig. 5D). In stretched fibroblasts, activation of ERK was detected by Western blot analysis of site-specific phosphorylation of ERK (Fig. 6A). In separate experiments conducted for analysis of the effect of PD-98059, we observed a 55% decrease (P < 0.01) in ET-1 mRNA levels in stretched fibroblasts at 24 h but only a 35% decrease in the presence of PD-98059 (P < 0.05 vs. static or stretched cells without PD-98059).

**DISCUSSION**

In the present study, we have shown that mechanical loading increases the release of ET-1 by endothelial cells and ventricular myocytes but not by ventricular fibroblasts. In agreement with our results, it has been reported that cyclic stretch of vascular endothelial cells rapidly augments release of ET-1 (18). Elevated ppET-1 mRNA levels have been detected in stretched cardiac myocytes (16). However, previous studies have suggested that cardiac fibroblasts increase the release of ET-1 in response to mechanical stretch or growth factors (8, 9). We found that transcription of ppET-1 gene, as well as mRNA and ir-ET-1 levels, was significantly attenuated in stretched ventricular fibroblasts. It is notable that basal ppET-1 gene

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**R1642** CIRCULAR STRETCH DISTINCTLY REGULATES ENDOTHELIN SYSTEM

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**Fig. 4. Effect of mechanical stretch of MC on ET-1 (A), ECE-1β (B), and endothelin-receptor type A (ETA) mRNA levels (C) and on ppET-1 promoter activity (D). A–C: mRNA levels were related to the 18S RNA level of each sample. mRNAs and 18S RNA were measured with real-time quantitative RT-PCR analysis by using TaqMan chemistry on an ABI Prism 7700 sequence detection system. D: MC were transfected with plasmids of −1.3-kb ppET-1-luc and RSV-β-gal by using cationic liposome delivery. The next day, cells were exposed to mechanical stretching for 6 or 24 h. After the experiment, cells were lysed and lysates were assayed for luciferase and β-galactosidase activity. In A–C, bars represent means ± SE of 17–18 (6 h) and 3 (24 h) separate experiments. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.**

**Fig. 5. Role of ERK in the mechanical stretch-induced ET-1 synthesis of MC.** A: MC were mechanically stretched for 0–20 min or for 10 min followed by 3-min recovery without stretch. Subsequently, cells were lysed and assayed for ERK activity. Bars represent means ± SE of 6 separate experiments. **P < 0.01; ***P < 0.001 vs. static cells. #P < 0.01 vs. MC stretched for 10 min. B: vehicle or PD-98059 (10 or 50 μM) was added to culture medium 2 h before start of stretching. After stretching for 10 min, cells were lysed and assayed for ERK activity. Bars represent means ± SE of 4 separate experiments. *P < 0.05; **P < 0.01; ***P < 0.001 vs. static cells. **P < 0.01 vs. MC stretched for 10 min without inhibitors. C: MC were transfected with plasmids of −1.3-kb ppET-1-luc and RSV-β-gal by using cationic liposome delivery. The next day, vehicle, PD-98059 (20 μM), or SB-203580 (20 μM) was added to cell culture medium, followed by initiation of mechanical stretch after 2 h. Cells were stretched for 6 h and then lysed for lucerase and β-galactosidase activity assays. Bars represent means ± SE of 4–6 separate experiments. **P < 0.01 vs. static cells. #P < 0.05 vs. stretched vehicle- or SB-203580-treated cells. D: MC were transfected simultaneously with a CMV promoter-driven MEK1 expression plasmid or empty control plasmid and with −1.3-kb ppET-1-luc and RSV-β-gal reporter plasmids by using cationic liposome delivery. Bars represent means ± SE of 4–6 separate experiments. **P < 0.001 vs. control.**
expression may further diverge between the cell types in adult heart. Earlier studies using semiquantitative RT-PCR measurements have demonstrated that ET-1 mRNA levels of unstimulated adult myocytes are unmeasurably low compared with highly expressing endothelial cells (both isolated from perfused adult rat heart) (20,33). Our results suggest that endothelial cells and, to a lesser extent, cardiac myocytes are the origin of increased ET-1 production in mechanically overloaded hearts.

In vivo after myocardial infarction, the surviving myocardium is exposed to increased mechanical load and neurohumoral stimulation. According to an immunohistochemical analysis of infarcted rat heart (12), ET-1 levels increase significantly in cardiac myocytes of the noninfarcted area and even more strongly in surviving myocytes of the marginal zone of infarcted area. However, fibrotic tissues, including fibroblasts in the marginal zone of infarcted area stained negative for ET-1 (12). Similarly to cardiac fibroblasts in the present study, cyclic mechanical stretch has been reported to decrease ET-1 generation by vascular smooth muscle cells (4), suggesting that the effect of mechanical stretch on ET-1 synthesis is cell type specific. In the heart, stretched cardiac myocytes rapidly release natriuretic peptides, which may further inhibit ET-1 generation by cardiac fibroblasts (6). Cultured myocytes secrete relatively low levels of ET-1 compared with fibroblasts or endothelial cells, but myocytes constitute a substantially greater proportion of ventricular mass, and ET-1 secretion by myocytes themselves results in higher local concentrations in the immediate vicinity of myocyte ET receptors (autocrine regulation). In support of the important role of myocyte ET-1 production, a recent study (38) demonstrated that mice with cardiac myocyte-specific inactivation of the ppET-1 gene are resistant to hyperthyroid cardiac hypertrophy. Moreover, conditional overexpression of human ET-1 in endothelium or in cardiac myocytes causes endothelial dysfunction and vascular remodeling or cardiomyopathy (see below), respectively, in transgenic mice (1,38). It is tempting to speculate that stretched myocytes may increase collagen synthesis and proliferation of fibroblasts via ET-1 (7) and hence augment fibrosis in the heart.

ECE-1 is the major ET-1-forming enzyme in the cardiovascular system. ECE-1β isoform mRNA (also called ECE-1α) is detected in both fetal and adult rat heart (26), and its expression is significantly induced in myocytes of failing heart (5). We did not measure ECE activity in this study, but previously it was shown that increased ECE-1β mRNA synthesis results in increased ECE activity of cardiac myocytes (5). We found that mechanical stretch increased ECE-1β mRNA levels in cardiac fibroblasts but decreased levels in cardiac myocytes. The alterations of ECE-1β mRNA levels were the opposite of changes in ET-1 production in both stretched cardiac myocytes and fibroblasts. ET-1 has been shown to inhibit ECE-1 gene expression in cultured rat pulmonary endothelial cells (25). Therefore, it is possible that alterations in ET-1 levels also may regulate ECE-1β gene expression in stretched cardiac cells (negative feedback loop). However, a concomitant increase in ET-1 and ECE-1 mRNA levels has been detected in rat hypertrophied myocardium at 12 wk after initiation of pressure overload (36), underscoring additional regulatory mechanisms in vivo. In the context of chronic heart failure, the cardiac levels of ECE-1 and ppET-1 mRNAs have been reported to remain unchanged in patients with idiopathic dilated cardiomyopathy (37,44) but to increase in patients with ischemic cardiomyopathy (21,37).

MAPKs are important signaling machinery between external stimuli and the nucleus, and their role in cardiac signal transduction has been studied extensively (40). In agreement with previous studies (17,35), we detected rapid activation of ERK by mechanical stretch of myocytes, and the activation was inhibited by the specific MEK1 inhibitor PD-98059. We found that ERK pathway is sufficient and necessary for activation of ppET-1 gene transcription in ventricular myocytes. In addition, we detected a transient activation of ERK in stretched fibroblasts. In contrast to myocytes, PD-98059 partially blocked the attenuation of ppET-1 mRNA levels by mechanical stretch. Therefore, our results suggest that ERK cascade may participate in regulation of ppET-1 gene expression in both cell types. Hypothetically, ERK signaling may target different effectors in cardiac myocytes and fibroblasts, and in myocytes this would include GATA-4 transcription factor. GATA-4 is activated by β-adrenergic agonist stimulation to ppET-1 gene transcription in neonatal rat cardiac myocytes (22,31). However, the exact cell type-specific mechanisms in ppET-1 gene expression remain to be addressed in further studies. Transgenic mice overexpressing kinases of ERK pathway have been shown to develop compensated cardiac hypertrophy (3). This is of particular interest, because activation of ERK augments ET-1 synthesis in cardiac myocytes, and the upregulated ET-1 system may contribute to preservation of the systolic performance in rat hearts with the compensated phase of cardiac hypertrophy (32). However, sustained and robust activation of myocyte ET-1 production may lead eventually to a fatal inflammatory cardiomyopathy (43), which was demonstrated by using mice with conditional myocyte-specific over-
expression of human ET-1 gene (43). Notably, these mice had an ~10-fold increase in cardiac ET-1 concentration, whereas circulating ET-1 levels were not elevated (43).

In summary, we found that mechanical stretching increased autocrine ET-1 production via ERK in cardiac myocytes, whereas stretching of cardiac fibroblasts induced their ET-1 generation. We detected cell type-specific alterations at the level of the ET-1-generating enzyme ECE-1β mRNA, which was induced in stretched fibroblasts but inhibited in stretched myocytes. Given the fundamental and potent effects of ET-1 on cardiac function and growth, we suggest that inhibition of the ET-1 system may effect cardiac mechanotransduction and therefore provides an approach to treatment of load-induced cardiac pathology.

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