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Original Article

Analysis of Gene Expression Profiles in an Imatinib-Resistant Cell Line, KCL22/SR

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Key Words. *Imatinib mesylate · Drug resistance · KCL22/SR · Ras · MAPK*

ABSTRACT

The BCR/ABL tyrosine kinase inhibitor, imatinib, has shown substantial effects in blast crises of chronic myelogenous leukemia. However, most patients relapse after an initial clinical response, indicating that drug resistance is a major problem for patients being treated with imatinib. In this study, we generated a new imatinib-resistant BCR/ABL-positive cell line, KCL22/SR. The 50% inhibitory concentration of imatinib was 11-fold higher in KCL22/SR than in the imatinib-sensitive parental cell line, KCL22. However, KCL22/SR showed no mutations in the BCR/ABL gene and no increase in the levels of BCR/ABL protein and P-glycoprotein. Furthermore, the level of phosphorylated BCR/ABL protein was suppressed by imatinib treatment, suggesting that mechanisms independent of BCR/ABL signaling are involved in the imatinib resistance in KCL22/SR cells. DNA microarray analyses demonstrated that the signal transduction-related molecules, RAS p21 protein activator and RhoA, which could affect Ras signaling, and a surface tumor antigen, L6, were upregulated, while c-Myb and activin A receptor were downregulated in KCL22/SR cells. Furthermore, imatinib treatment significantly suppressed the level of phosphorylated p44/42 in KCL22 cells but not in KCL22/SR cells, even when BCR/ABL was inhibited by imatinib. These results suggest that various mechanisms, including disturbance of Ras-mitogen-activated protein kinase signaling, are involved in imatinib resistance. *Stem Cells 2003;21:315-321*

INTRODUCTION

Imatinib (imatinib mesylate; formally STI571), a specific ABL tyrosine kinase inhibitor, has been reported to have a significant clinical effect on chronic myelogenous leukemia (CML) in blast crisis as well as in the chronic phase [1-2]. However, many patients in blast crisis who are being treated with imatinib relapse at a relatively early time [2], suggesting that leukemia cells tend to acquire resistance to imatinib easily in blast crisis. Thus, drug resistance is a major problem even for CML patients in blast crisis who are being treated with imatinib.

Recently, there have been several studies on the mechanisms of imatinib resistance. These studies have shown that amplification of the BCR/ABL gene, increased expression of BCR/ABL protein, and upregulation of P-glycoprotein (P-gp) occurred in some imatinib-resistant BCR/ABL-positive cell lines [3-5]. P-gp belongs to the ATP-binding cassette (ABC) family and has been shown to expel drugs

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outside cells. In addition, BCR/ABL gene amplification or point mutations in the ATP-binding pocket of the gene have been observed in patients who had responded to imatinib treatment but finally relapsed [6-7]. This point mutation causes the replacement of a threonine residue with an isoleucine residue, resulting in inhibition of binding of imatinib to the ATP-binding pocket. On the other hand, *Passerini et al.*, who transplanted KU812 human CML cells into nude mice, found that the association between imatinib and .1 acid glycoprotein resulted in inactivation of imatinib [8]. These previous studies strongly suggest that various mechanisms are involved in the acquirement of resistance to imatinib.

In this work, we established a new imatinib-resistant cell line, KCL22/SR, and examined the differences in the gene expression profiles of imatinib-sensitive and imatinib-resistant cells by DNA microarray analyses. We found that BCR/ABL signaling-independent, continuous activation of Ras signaling occurred in KCL22/SR cells.

Materials and Methods

Cell Lines

KCL22 is a Philadelphia chromosome-positive cell line established from peripheral blood of a patient with CML in blast crisis [9]. The cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and split every 4 days. To generate imatinib-resistant clones, KCL22 cells were treated with step-wise increasing concentrations of imatinib (0.1-1.0 μM) and colonized on a medium containing methylcellulose; then individual colonies were selected. The clone KCL22/SR had the highest 50% inhibitory concentration (IC_{50}) value of imatinib and was used for further examinations.

Imatinib was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland; http://www.novartis.com). Cells were incubated with various concentrations of imatinib for 3 days, and then numbers of viable cells were counted by trypan blue staining. The fold resistance was calculated by dividing the IC_{50} of KCL22/SR cells by that of KCL22 cells.

Sequence Analysis of BCR/ABL Gene

Total RNA from KCL22 and KCL22/SR cells was isolated by the acid guanidium thiocyanate-phenol-chloroform method [10]. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using cDNA that had been prepared from total RNA by SuperScript II reverse transcriptase (Invitrogen Corp.; Carlsbad, CA; http://www.invitrogen.com). The primers used for this experiment were 5′-GCGCAA CAAGCCCACTGTCTATGG-3′ (forward) and 5′-GCCAG GCTCTCGGGTGCAGTCC-3′ (reverse). PCR products were cloned into the pT-Adv vector (Clontech; Palo Alto, CA; http://www.clontech.com). The sequences of both strands of 10 amplified cDNA clones were determined with the forward primer 5′-CACCATGAAGCACAAGCTGG-3′ and the reverse primer 5′-CAGCTACCTTCACCAAGTGG-3′ by an ABI prism 377 automated sequencer (Applied Biosystems; Foster City, CA; http://www.appliedbiosystems.com).

Western Blot Analysis

Nuclear extracts were prepared from 1×10^7 cells according to the method described previously [11]. Ten μg of nuclear extracts was separated electrophoretically using 10% polyacrylamide gel. Immunoblotting and detection by enhanced chemiluminescence were performed as described previously [11]. Anti-BCR rabbit polyclonal antibody and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; http://www.scbt.com) and Chemicon International (Temecula, CA; http://www.chemicon.com), respectively. Anti-p44/42 (extracellular signal-regulated kinase 1 and 2 [ERK1/2]) mitogen-activated protein (MAP) kinase and anti-phospho p44/42 (ERK1/2) MAP kinase rabbit polyconal antibodies were purchased from Cell Signaling Technology (Beverly, MA; http://www.cellsignal.com). Densitometric analysis was performed to determine the levels of ERK1 protein.

Flow Cytometry Analysis

Cells were incubated with a phycoerythrin (PE)-labeled anti-P-gp antibody (Immunotech; Marseille, France; http://www.immunotech.fr) at room temperature for 30 minutes and then washed with phosphate-buffered saline. PE-labeled mouse IgG1 (Becton Dickinson Immunocytometry Systems; Mountain View, CA; http://www.bd.com) was used as a control. The expression of P-gp was determined by flow cytometry.

DNA Microarray Analysis

Total RNA from KCL22 and KCL22/SR cells was prepared using the acid guanidium thiocyanate-phenol-chloroform method [10]. DNA microarray analyses were performed as described previously [12]. Briefly, biotin-labeled cRNA was synthesized and subjected to hybridization with HO2 and HO3 microarrays (Mergen; San Leandro, CA; http://www.mergen.com) and GeneChip HU95Avs2 microarrays (Affymetrix; Sana Clara, CA; http://www.affymetrix.com) representing a total of 2,304 and 12,625 known human genes, respectively. Hybridization signals were analyzed using a GMS418 Array Scanner (Affymetrix) and GeneSpring 3.2.2. software (Silicon Genetics; Redwood, CA; http://www.sigenetics.com).

RT-PCR and Real-Time PCR Analysis

cDNA was generated from total RNA extracted from KCL22 and KCL22/SR cells by SuperScript II reverse transcriptase. The primers used for RT-PCR and real-time PCR were as follows: Ras p21 protein activator (RASAP1): 5′-CCAACTAACCAGTGGTATCACGG-3′ (forward) and 5′-GCAGGGAAGTCTGGCAGTTATC-3′ (reverse); RhoA: 5′-TAACGATGTCCAACCCGTCTG-3′ (forward) and 5′-CTGACACACCAGGCGCTAATT-3′ (reverse); L6: 5′-GGAGTGCTTGGAGGCATATGTGGC-3′ (forward) and 5′-GTGGCTCTGTCCTGGGTTGGTTCT-3′ (reverse); c-Myb: 5′-CCTGGATTCCAAGGCCCTGGTGCCCTGAGC-3′ (forward) and 5′-CCACACCCCTGGTGAGTACCAGA CGCTGCC-3′ (reverse); and activin A receptor: 5′-GTG GATCAGCAGACCCCCACCATCCC-3′ (forward) and 5′-GAGCTAGGCCTGAGAGGACCGGGTCT-3′ (reverse). PCR products were electrophoresed on a 1.2% agarose-formaldehyde gel (RT-PCR) or analyzed using an ABI PRISM 7700 system (Applied Biosystems; Foster City, CA; http://home.appliedbiosystems.com) (real-time PCR). cDNA corresponding to the β-actin gene was used for the internal control of these real-time analyses.

Results

Development of an Imatinib-Resistant BCR/ABL-Positive Cell Line

We established a new imatinib-resistant cell line, KCL22/SR, from the human bcr/abl-positive cell line KCL22 by treatment with step-wise increasing concentrations of imatinib (0.1-1.0 μM). The IC_{50} value of imatinib to KCL22/SR was about 11.6-fold higher than that to KCL22, indicating that KCL22/SR has acquired significant resistance to imatinib.

Recent studies have suggested that several mechanisms, including amplification of the BCR/ABL gene, increased expression of BCR/ABL protein, point mutation in the ATP-binding pocket of the BCR/ABL gene, and overexpression of P-gp, are involved in the resistance to imatinib. However, there was no amplification of or point mutation in the BCR/ABL gene in KCL22/SR cells (data not shown). Immunoblot analysis using an anti-BCR antibody showed that there was no difference between the BCR/ABL protein levels in KCL22 and KCL22/SR cells (Fig.1A). Furthermore, the expression levels of P-gp in these two cell lines were almost the same (Fig. 1B). We therefore concluded that other unknown mechanisms are involved in the acquirement of resistance to imatinib in KCL22/SR cells.

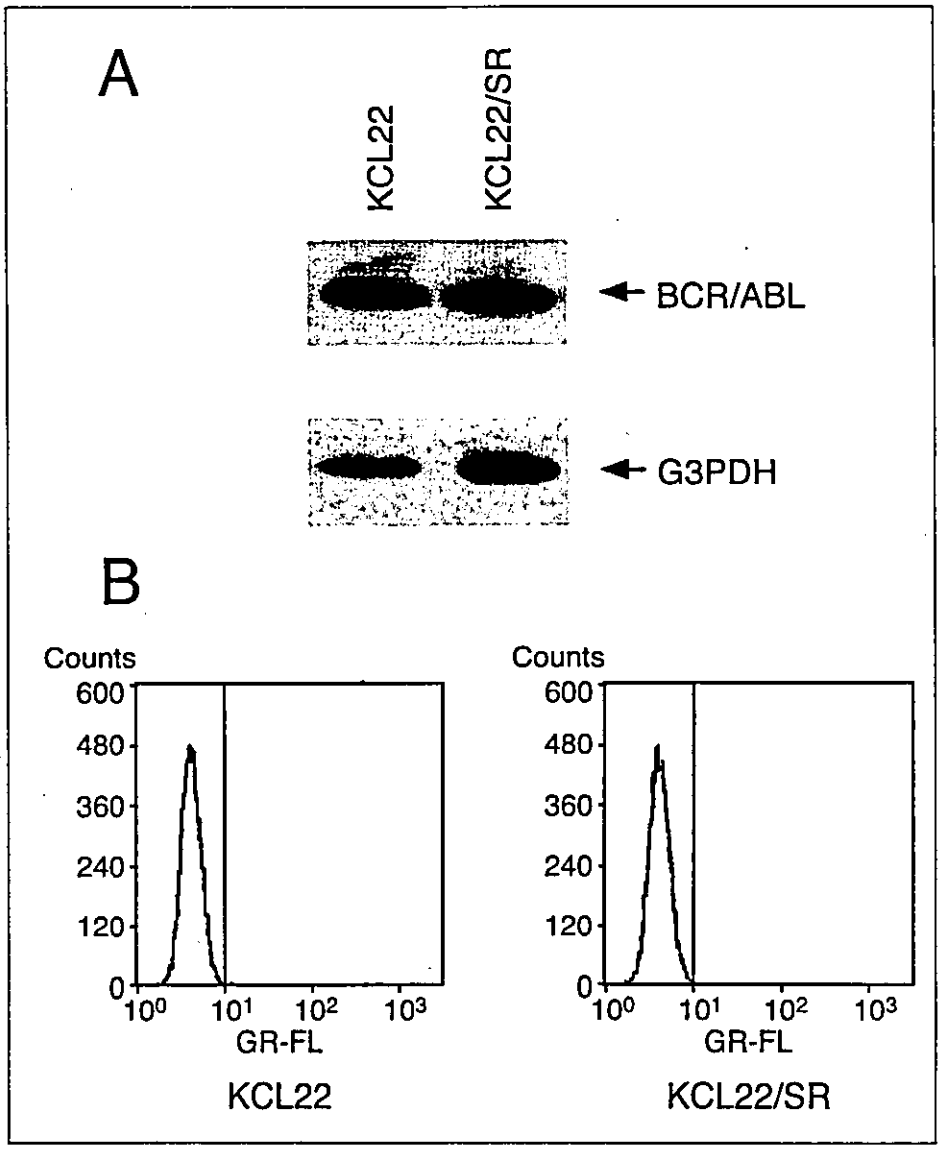

Figure 1. Levels of BCR/ABL and P-gp in KCL22 and KCL22/SR cells. A) The expression of BCR/ABL protein was determined by Western blot analysis using an anti-BCR antibody. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was demonstrated as an internal control. B) Expression of P-gp in KCL22 and KCL22/SR cells was determined by flow cytometry as described in Materials and Methods.

Upregulation of Signal Transduction-Related Molecules in KCL22/SR Cells

The level of autophosphorylation of BCR/ABL protein in KCL22/SR cells was immediately suppressed by imatinib treatment, similar to that in KCL22 cells (Fig. 2). This finding suggests that imatinib could inhibit BCR/ABL signaling even in KCL22/SR cells and that other mechanisms that were independent of BCR/ABL signaling were involved in the imatinib resistance. To understand the mechanisms involved in the imatinib resistance in KCL22/SR cells, we then examined the differences in gene expression profiles of KCL22 and KCL22/SR cells. DNA microarray analysis is an appropriate method for this purpose because KCL22 and KCL22/SR cells have common genetic backgrounds. Biotin-labeled cRNA was synthesized from KCL22 and KCL22/SR cells and was subjected to hybridization with an oligonucleotide chip, representing a total of 2,304 known human genes. While only three genes exhibited lower expression levels in KCL22/SR cells, 46 genes were initially demonstrated to be preferentially expressed in KCL22/SR cells. However, only two genes were confirmed to be expressed at higher levels in KCL22/SR cells by RT-PCR and real-time PCR methods. These were RASAP1 and RhoA, which are both signal transduction-related molecules (Table 1).

We next tried another DNA microarray analysis using microarrays (Affymetrix) to identify additional genes whose expressions were up- or downregulated in KCL22/SR cells. By this analysis, four genes that were differently expressed in KCL22 and KCL22/SR cells were identified (Table 2). The expression level of complement cytolysis inhibitor (CLI), which may be involved in tumor cell resistance to complement-mediated cytotoxicity [13], was shown to be higher in KCL22/SR cells by microarray analysis. However, this could not be confirmed by real-time PCR because of the failure of RT-PCR to detect an amplified product. On the other hand, real-time PCR clearly demonstrated that the expression of L6 was upregulated and that the expression of c-Myb and activin A receptor was downregulated in KCL22/SR cells (Table 2).

Ras-MAP Kinase Signaling in KCL22/SR Cells Is not Suppressed by Imatinib Treatment

BCR/ABL activates Ras-MAP kinase signaling, which is involved in cellular proliferation and differentiation. Since a Ras mediator, RASAP1, and RhoA, which has cross-talk with Ras signaling, were expressed at high levels in KCL22/SR cells, we speculated that disturbance of Ras-MAP kinase signaling might have occurred in KCL22/SR cells even when BCR/ABL signaling was inhibited by imatinib. To verify this hypothesis, we examined the level of ERK1/2, which belongs to the family of MAP kinases and plays an important role in many gene expressions, by Western blot analysis using anti-p44/42 MAP kinase and anti-phospho-p44/42 MAP kinase (active form) antibodies. The results showed that the level of phosphorylated ERK1/2 was significantly suppressed by imatinib treatment, with no remarkable change in the total amount of ERK1/2 in KCL22 cells (Fig. 3). In contrast, neither the level of phosphorylated ERK1/2 nor that of total ERK1/2 in KCL22/SR cells were changed (Fig. 3). These results indicated that continuous activation of Ras signaling occurred in KCL22/SR cells and that this activation was not under the control of BCR/ABL signaling.

Discussion

Recently, various new anticancer agents that target specific oncogenic molecules have been developed. Imatinib is one of the most promising reagents among them [14].

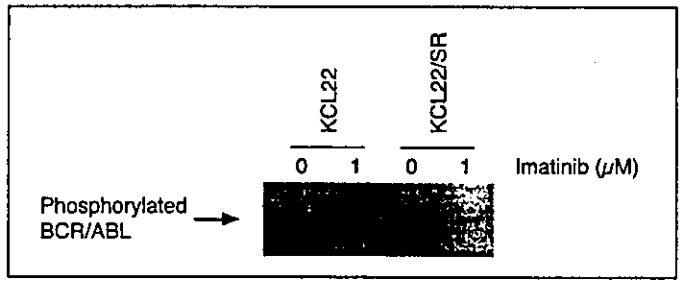

Figure 2. Autophosphorylation of BCR/ABL protein in KCL22 and KCL22/SR cells. KCL22/SR cells were cultured without imatinib for 3 days prior to treatment. Cells were treated with 1 or 5 μM imatinib for 6 hours. Immunoblot analysis using anti-phosphotyrosine antibody was performed as described in Materials and Methods. BCR/ABL autophosphorylation levels normalized on the basis of GAPDH are shown in the lower panel.

Table 1. Differently expressed genes identified by DNA microarray analysis using an oligonucleotide chip

	Gene	Description	Change*
Upregulation	RASAP	GTPase-activating protein	5.66
	RhoA	Small GTP-binding protein	2.07

*Changes in expressions of genes were evaluated by the real-time PCR method.

Table 2. Differently expressed genes identified by DNA microarray analysis using Affymetrix arrays

	Gene	Description	Change*
Upregulation	L6	Cell-surface tumor antigen	22.1
Downregulation	c-Myb	Transcription factor	-11.0
	activin A receptor	Receptor for the TGF-β superfamily	-10.3

*Changes in expressions of genes were evaluated by the real-time PCR method.

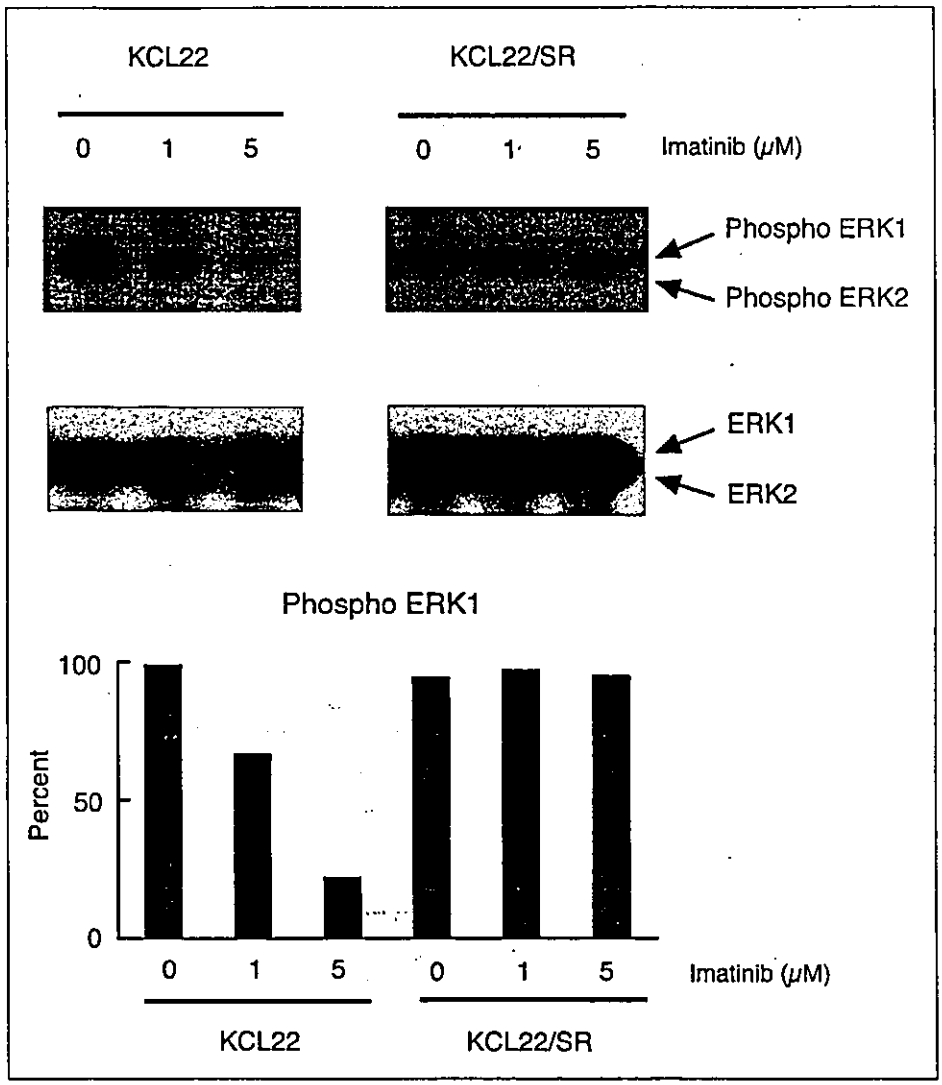

Figure 3. Changes in ERK1/2 phosphorylation caused by imatinib in KCL22 and KCL22/SR cells. KCL22/SR cells were cultured without imatinib for 3 days prior to treatment. Cells were treated with 1 or 5 μM imatinib for 6 hours. Immunoblot analysis using anti-phospho ERK1/2 (upper panel) and anti-ERK1/2 (lower panel) antibodies was performed as described in Materials and Methods. ERK1 protein levels normalized on the basis of GAPDH are shown in the figure.

However, one of the major problems in imatinib treatment, especially for patients in blast crisis, is acquirement of resistance. Many previous works have shown that several ABC family proteins such as P-gp, multidrug-resistant protein and lung cancer-resistant protein play important roles in multidrug resistance in leukemic cells [15-18]. It is still not clear, however, whether these proteins are involved in resistance to imatinib. Although overexpression and mutations of BCR/ABL have been suggested to be major causes of resistance to imatinib [6], neither overexpression nor mutations of BCR/ABL have been found in some imatinib-resistant cell lines and patients. We therefore tried to determine other mechanisms involved in resistance to imatinib.

For this purpose, we established a new imatinib-resistant cell line, KCL22/SR, in this work. KCL22/SR showed very strong resistance to imatinib, whereas no mutation in the BCR/ABL gene and no increase in BCR/ABL protein and P-gp levels were observed (Fig. 1A-B). To the best of our knowledge, two resistant cell lines in which these changes were not observed were previously reported [5], but the mechanisms involved in the resistance of those cells have not been elucidated. The level of autophosphorylation of BCR/ABL protein in KCL22/SR cells was decreased 6 hours after the addition of imatinib, as was also found in KCL22 cells (Fig. 2). These results strongly suggest that some mechanisms that are not under the control of BCR/ABL play important roles in the resistance to imatinib in KCL22/SR cells.

To identify imatinib resistance-related molecules, we performed DNA microarray analyses. First, using an oligonucleotide chip, we identified two genes that are expressed at higher levels in KCL22/SR cells than in KCL22 cells (Table 1). These were RASAP1 and RhoA, both of which play important roles in signal transduction pathways. RASAP1, which is one of the GTPase-activating proteins and can enhance the intrinsic GTPase activity of Ras proteins, is an effector of Ras protein action [19]. RhoA belongs to the Rho family of small G proteins, which are involved in remodeling of the actin cytoskeleton [20] and cellular proliferation [21]. Rho proteins have also been shown to have cross-talk with Ras signaling [22] and to participate in Ras-mediated induction of carcinogenesis [23, 24]. The upregulation of these molecules strongly suggests that intracellular signal transductions were disturbed in KCL22/SR cells. In fact, while the level of phosphorylated p44/42 was suppressed by imatinib treatment in accordance with the decrease in tyrosine autophosphorylation of BCR/ABL protein in KCL22 cells, it remained high in KCL22/SR cells even when BCR/ABL autophosphorylation was inhibited by

imatinib treatment (Fig. 3). Since there is no point mutation in the Ras genes in KCL22/SR cells (data not shown), it is possible that the continuous activation of Ras-MAP kinase signaling is caused by unusual expressions of molecules such as RASAP1 and RhoA, and that such disturbance of signal transduction pathways contributes to the resistance to imatinib in these cells.

DNA microarray analysis using Affymetrix microarrays demonstrated that the expressions of L6 and CLI were upregulated and that the expressions of c-Myb and activin A receptor were downregulated in KCL22/SR cells compared with the expressions in KCL22 cells (Table 2). L6, whose expression was upregulated 22-fold in KCL22/SR cells, is known to be a surface antigen and to be expressed at high levels in some tumors [25], though its function has not been clarified. CLI may be involved in tumor cell resistance to complement-mediated cytotoxicity [13], but its expression level could not be confirmed by real-time PCR because RT-PCR yields no products. On the other hand, the expression levels of c-Myb and activin A receptor were significantly decreased in KCL22/SR cells.

C-Myb is a transcription factor that is important for the proliferation of early hematopoietic progenitors [26]. A previous study showed that MAP kinase could suppress the transactivating activity of c-Myb through phosphorylation at serine 528 of the carboxy-terminal negative regulatory domain [27]. This finding together with the fact that c-Myb expression is downregulated in KCL22/SR cells suggests that c-Myb function may be suppressed in these cells. Activin A receptor, also called erythroid differentiation factor, is a cell-surface receptor for activin A, which belongs to the transforming growth factor-β superfamily [28]. Although it remains to be clarified how dysregulation of these molecules contributes to the acquirement of resistance, these results strongly suggest that various mechanisms are involved in the acquirement of resistance to imatinib. Determination of which mechanisms are involved in each case should enable the establishment of effective methods for overcoming the problem of resistance in patients.

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Cardiovascular Drugs and Therapy 17 231–236 2003

BASIC PHARMACOLOGY

Effects of Olmesartan, an Angiotensin II Receptor Blocker, on Mechanically-Modulated Genes in Cardiac Myocytes

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Summary. Background: **Angiotensin II plays an important role in cardiac hypertrophy or remodeling. Angiotensin II receptor blockers (ARB) are clinically useful for the treatment of hypertension and heart failure. However, the molecular effects of ARB in the mechanically-stressed myocardium have not been completely defined. We investigated the effects of ARB on mechanically-modulated genes in cardiac myocytes.**

Methods: **We used powerful DNA microarray technology to study the effects of the ARB, CS-886 (olmesartan), on genes modulated in neonatal rat cardiac myocytes using mechanical stimuli. Mechanical deformation was applied to a thin and transparent membrane on which neonatal rat cardiac myocytes were cultured in the presence or absence of RNH-6270, an active metabolite of CS-886. Expression profiles of 8000 rat genes using the Affymetrix GeneChip (Rat Genome U34A) were investigated with mRNA obtained from the samples above.**

Results: **Nine genes induced under 4% mechanical strain were significantly suppressed by RNH-6270 in rat cardiac myocytes: monoamine oxidase B, neuromedine B receptor, olfactory receptor, synaptotagmin XI, retinol-binding protein, and 4 expressed sequence tags (ESTs). In contrast, 21 genes suppressed under mechanical strain were significantly restored by RNH-6270: major acute phase alpha 1-protein, Sp-1, Bcl-Xalpha, JAK2, 2 genes encoding detoxification, few genes for receptor, structure, metabolism or ion channel, and 10 ESTs.**

Conclusions: **As some of these genes may be involved in promoting or modulating cardiac remodeling, these findings suggest that ARB may affect cardiovascular morbidity and mortality partially via these molecular alterations.**

Key Words. **angiotensin II, mechanical, stress, gene expression, cardiac, myocyte**

Introduction

The renin-angiotensin system plays an important role in the regulation of blood pressure and fluid-electrolyte balance. The previous placebo-controlled trials such as CONSENSUS and SOLVD showed a significant benefit of angiotensin-converting enzyme (ACE) inhibitors in terms of morbidity and mortality in congestive heart failure [1,2]. In addition, treatment with ACE inhibitors attenuates left ventricular remodeling and improves prognosis in patients with significant left ventricular dysfunction after acute myocardial infarction [3]. ACE inhibitors, generally given with diuretics and digoxin, are the standard treatments for patients with heart failure and systolic left ventricular dysfunction. However, ACE inhibitors are associated with a variety of adverse events, cough being the most common [4].

An alternative approach to blockade of the renin-angiotensin system is the use of an angiotensin II type 1 receptor blocker (ARB). Since a new class of medication, ARB, has been developed to better block the vasoconstrictor-growth-promoting effects of angiotensin II directly at the receptor level [5], these drugs should provide similar benefits to ACE inhibitors in blocking the harmful effects of angiotensin II with fewer side effects. Recently, the Valsartan Heart Failure Trial (Val-HeFT) demonstrated that treatment with valsartan resulted in significant improvements in the New York Heart Association class, ejection fraction, signs and symptoms of heart failure, and quality of life as compared with placebo [6]. CS-866, olmesartan, is a new ARB that was first identified during a systemic survey of the angiotensin II type 1 receptor binding properties of substituted imidazole-5-carboxylic acids [7]. *In vivo*, CS-866 is rapidly and completely de-esterified to an active metabolite, RNH-6270, that is a highly selective antagonist of angiotensin II binding to the angiotensin II type 1 receptor [8].

With the recent discovery of the complete sequence of the human genome, new high-throughput approaches to studying these complex pathways have been made possible. In addition to identifying large clusters of genes that respond to a given stimulus, DNA microarray technology may be used to identify a few genes that comprise highly specific molecular responses [9]. Already, some studies using microarray technology have yielded interesting results regarding the pathogenesis

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of cardiovascular diseases, such as myocardial infarction [10], cardiac hypertrophy [11], and human heart failure [12]. In the present study, using a DNA microarray and a mechanical deformation device that applies a highly uniform biaxial strain field to a culture substrate, we investigated the effects of ARB on genes modulated in neonatal rat cardiac myocytes with mechanical stimuli.

Methods

Materials

Fibronectin, bovine fetal calf serum (FCS) and Hanks' balanced salt solution (HBSS) were purchased from Life Technologies, Inc. (Rockville, MD). RNH-6270 was a gift from Sankyo Co., LTD. (Tokyo, Japan). All other chemicals used were of the highest grade commercially available.

Culture of neonatal rat ventricular myocytes (NRVM)

NRVM from 1-day old Sprague-Dawley rats were isolated by previously described methods [13]. The cells were cultured at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker, Walkersville, MD) containing 7% FCS, 50 U/mL penicillin and 50 μg/mL streptomycin (PS).

This investigation was performed according to the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Mechanical strain device and preparation of cells

Mechanical deformation was applied to a thin and transparent membrane on which cells were cultured, an approach which produces controlled cellular strain as well as visualization of cells [14]. For the preparation of NRVM to be subjected to mechanical strain, autoclaved membrane dishes were coated with 2 μg/mL of fibronectin in 13 mL of HBSS for 12 h at 4°C and then washed twice with 10 mL of PBS. NRVM were plated on the coated membrane dish at a density of 2,000,000 cells/dish in 13 mL of DMEM containing 7% FCS and incubated for 48 h. NRVM were then made quiescent by washing with 10 mL of HBSS twice and incubating with 10 mL of DMEM containing 1% insulin, transferrin, selenium media supplement (ITS; Sigma, St. Louis, MO) and PS. All experiments were performed on NRVM that had been serum-starved for 24 h.

Transcriptional profiling

NRVM cultured on fibronectin-coated membranes were harvested immediately after 6 h of cyclic deformation (1 Hz) or no deformation, and total RNA was extracted using RNeasy kit (QIAGEN K.K., Tokyo, Japan), and the purity was checked by spectrophotometry and agarose gel electrophoresis. Total RNA (20 μg) was converted to double-stranded cDNA using an oligo dT primer containing the T7 promoter (Gibco BRL Superscript® Choice System; Life Technologies, Inc.), and the template for an *in vitro* transcription reaction was used to synthesize biotin-labeled antisense cRNA (BioArray™ High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY). The biotinylated cRNA was fragmented and hybridized for 16 h at 45°C to GeneChip Test2 arrays (Affymetrix, Inc., Santa Clara, CA) to assess sample quality, and then to Rat Genome arrays (U34A, Affymetrix, Inc.). The arrays were washed, and then stained with streptavidin-phycoerythrin. The arrays were scanned with the GeneArray scanner (Agilent Technologies, Palo Alto, CA) and analyzed using the GeneSpring software package (Silicon Genetics, Redwood City, CA). Rat Genome U34A chip can detect 7,000 well-characterized genes with putative functions and 1,000 expressed sequence tags (ESTs).

Detailed protocols for data analysis of Affymetrix oligonucleotide microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described [15]. Raw data from array scans were averaged across all gene probes for each array.

Real-time reverse transcription (RT)-PCR analysis

For RT, RNA was reverse transcribed using T7-dT primer (5′-TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG TTT TTT TTT TTT TTT TTT TTT-3′) and Superscript II reverse transcriptase (Life Technologies, Inc.). Real-time quantitative PCR was performed in optical tubes in a 96-well microtiter plate (Perkin-Elmer/Applied Biosystems, Foster City, CA) with an ABI PRISM 7700 Sequence Detector Systems (Perkin-Elmer/Applied Biosystems) according to the manufacturer's instructions. By using the SYBR Green PCR Core Reagents Kit (Perkin-Elmer/Applied Biosystems, P/N 4304886), fluorescence signals were generated during each PCR cycle via the 5′- to 3′-endonuclease activity of Taq Gold [16] to provide real-time quantitative PCR information. The primer set used for real-time PCR analysis contained the 5′-CATTGTGGCCTTCTTCTCCTT-3′ sense and 5′-TCCCGTAGAGATCCACAAAAGT-3′ antisense oligonucleotides for Bcl-Xalpha, 5′-GCCGTATGGAAGTTTACGAGAC-3′ sense and 5′-AGATCCCGGTGGATATACCTTT-3′ antisense oligonucleotides for JAK2 and 5′-TTCCAGTATGACTCTACCCACG-3′ sense and 5′-AGACTCCACGACATACTCAGCA-3′ for glyceraldehyde-3 phosphate dehydrogenase (GAPDH). No template controls as well as the samples were added in a total volume of 50 μL/reaction. Potential PCR product contamination was digested by uracil-N-glycosylase, because dTTP is substituted by dUTP [16]. All PCR experiments were performed with the hot start method. In the reaction system,

Table 1. Suppression of mechanically-induced genes by RNH-6270

GeneBank #	Description	Fold expression relative to control	
		Mechanical strain	Mechanical strain + RNH-6270
Metabolism			
M23601	Monoamine oxidase B	1.43 ± 0.04	1.15 ± 0.07
M10934	Retinol-binding protein	271.60 ± 19.51	140.92 ± 18.62
Receptors			
AF091575	Olfactory receptor	2.83 ± 0.07	1.99 ± 0.46
U37058	Neuromedin B receptor	24.77 ± 0.82	13.97 ± 4.19
Other			
AF000423	Synaptotagmin XI	3.38 ± 0.34	2.27 ± 0.28
ESTs			
AI639318		2.33 ± 0.08	1.00 ± 0.42
AI639100		3.94 ± 0.33	1.86 ± 0.88
AI639307		8.56 ± 1.61	2.94 ± 1.20
AA945585		9.25 ± 0.93	0.47 ± 0.20

Note: Values are mean ± SEM ($n = 3$). Nine mechanically-induced genes were significantly suppressed by RNH-6270 ($p < 0.05$).

uracil-N-glycosylase and Taq Gold (Perkin-Elmer/Applied Biosystems) were applied according to the manufacturer's instructions [16,17]. Denaturing and annealing reactions were performed 40 times at 95°C for 15 s, and at 60°C for 1 min, respectively. The increase in the fluorescence signal is proportional to the amount of specific product [18]. The intensity of emission signals in each sample was normalized to that of GAPDH as an internal control.

Statistical analysis

Data are expressed as the mean ± SEM. The data were analyzed by the nonparametric Kruskal-Wallis method to avoid assumptions about the distribution of the measured variables. Subsequent pairwise-comparisons were made with the Mann-Whitney U test; values of $p < 0.05$ were considered statistically significant.

Results

DNA microarray analysis of mechanically-modulated genes in cardiac myocytes

4% cyclic mechanical strain at 1 Hz in cultured rat cardiac myocytes significantly induced 45 genes (>2.0 fold, $p < 0.05$), including genes for heat shock protein 70, heme oxygenase, c-fos and adenosine A3 receptor, and 17 ESTs (available in an online only Data Supplement at http://www.kluweronline.com/issn/0920-3206). In contrast, 4% cyclic mechanical strain at 1 Hz in cultured rat cardiac myocytes significantly suppressed 94 genes (<0.5 fold, $p < 0.05$), including genes for cytochrome P450f, major acute phase alpha-1-protein and arylamine N-acetyltransferase, and 43 ESTs (available in an online only Data Supplement at http://www.kluweronline.com/issn/0920-3206).

DNA microarray analysis of suppression of mechanically-induced genes by RNH-6270 (Table 1)

Although the microarray hybridizations were performed three times, the results of these hybridizations regarding modulated genes were nearly identical. Of genes that were significantly induced under 4% cyclic mechanical strain at 1 Hz in cultured rat cardiac myocytes, we identified 9 genes that were significantly suppressed by RNH-6270 (0.1 μmol/L, $n = 3$, $p < 0.05$): monoamine oxidase B, retinol-binding protein, olfactory receptor, neuromedine B receptor, synaptotagmin XI, and 4 ESTs. There was suppression of genes encoding metabolism, monoamine oxidase B and retinol-binding protein, and receptors, olfactory receptor and neuromedine B receptor. It may be preferable that RNH-6270 significantly inhibited the mechanically-induced genes for monoamine oxidase B and neuromedine B receptor in rat cardiac myocytes, because monoamine oxidase B and neuromedine B are profoundly involved in catechol metabolism and mitosis or growth, respectively.

DNA microarray analysis of restoration of mechanically-suppressed genes by RNH-6270 (Table 2)

Next, we investigated whether mechanically-suppressed genes were restored by ARB. Of genes that were significantly suppressed under 4% cyclic mechanical strain in rat cardiac myocytes, we identified 21 genes that were significantly restored by RNH-6270 (0.1 μmol/L, $n = 3$, $p < 0.05$): Sp-1, Bcl-Xalpha, JAK2, neuritin, major acute phase alpha 1-protein, 2 genes encoding detoxification, few genes for receptor, structure, metabolism or ion channel, and 10 ESTs. Interestingly, RNH-6270, one of ARBs, restored the mechanically-suppressed genes for detoxification, cytochrome P-450f and arylamine N-acetyltransferase. In addition,

Table 2. Restoration of mechanically-suppressed genes by RNH-6270

GeneBank #	Description	Fold expression relative to control	
		Mechanical strain	Mechanical strain + RNH-6270
Transcription factor			
D12768	Sp-1	0.30 ± 0.18	0.90 ± 0.35
Detoxification			
M31031	CytochromeP-450f	0.15 ± 0.03	0.99 ± 0.21
U01344	Arylamine N-acetyltransferase	0.45 ± 0.16	0.88 ± 0.07
Humoral factor			
K02814	Major acute phase alpha 1-protein	0.35 ± 0.07	0.94 ± 0.31
Receptor			
M15682	Nicotinic acetylcholine receptor alpha subunit	0.10 ± 0.02	0.86 ± 0.30
Structure			
D26495	Dynein-like protein 4	0.10 ± 0.02	0.60 ± 0.20
Metabolism			
D26073	Phosphoribosylpyrophosphate synthetase-associated protein	0.38 ± 0.01	0.79 ± 0.03
Aitiapoptosis			
U72350	Bcl-Xalpha	0.33 ± 0.01	0.62 ± 0.10
Cell signaling			
U13396	JAK2	0.13 ± 0.06	0.55 ± 0.05
U88958	Neuritin	0.22 ± 0.08	0.51 ± 0.08
Channel			
M88751	Calcium channel beta subunit-III	0.11 ± 0.02	0.48 ± 0.02
ESTs			
AI178267		0.08 ± 0.02	0.77 ± 0.53
AI639401		0.10 ± 0.02	0.98 ± 0.36
AA893193		0.10 ± 0.03	0.33 ± 0.13
AA892754		0.14 ± 0.03	0.35 ± 0.06
AA894337		0.19 ± 0.08	0.35 ± 0.19
AI639146		0.20 ± 0.03	0.69 ± 0.24
AA891739		0.20 ± 0.02	0.94 ± 0.35
AI639162		0.28 ± 0.07	0.79 ± 0.04
AA799464		0.44 ± 0.03	0.91 ± 0.18
AA944973		0.54 ± 0.10	0.94 ± 0.12

Note: Values are mean ± SEM ($n = 3$). Twenty-one mechanically-suppressed genes were significantly restored by RNH-6270 ($p < 0.05$).

RNH-6270 restored the mechanically-suppressed gene expression for major acute phase alpha 1-protein of the rat that contains the sequence for bradykinin [19]. These findings suggest that ARB may provide much benefit in cardiovascular diseases, such as heart failure.

Real-time RT-PCR analysis

We confirmed expressions of two genes by real-time quantitative RT-PCR analysis. In real-time RT-PCR analysis, Bcl-Xalpha and JAK2 mRNA expressions were suppressed under 4% cyclic mechanical strain in rat cardiac myocytes (0.21 ± 0.01 fold and 0.21 ± 0.04 fold versus control, respectively, $n = 3$). These were significantly restored by RNH-6270 (0.38 ± 0.07 fold and 0.41 ± 0.06 fold versus control, respectively, $p < 0.05$).

Discussion

The present study using oligonucleotide microarray analysis demonstrates the changes in gene expression in stretch versus control neonatal rat cardiomyocytes and that nine genes induced under 4% mechanical strain, such as monoamine oxidase B and neuromedine B receptor were suppressed by RNH-6270 and that 21 genes suppressed under mechanical strain, such as genes for detoxification, cytochrome P-450f and arylamine N-acetyltransferase, and major acute phase alpha 1-protein were restored by RNH-6270. These molecular alterations might lead to the effects of ARB and a significant reduction in cardiovascular morbidity and mortality.

In the present study, nine genes induced under 4% mechanical strain were significantly suppressed by RNH-6270 in rat cardiac myocytes. Two of these genes encode the enzyme monoamine oxidase B and neuromedin B receptor. Human monoamine oxidase B plays a major role in the degradation of biogenic and dietary amines such as dopamine, thus increased myocardial degradation of dopamine may in part explain the deterioration of heart failure. In addition, the expression of neuromedin B receptor was also normalized by

RNH-6270. Neuromedin B, a member of the bombesin family of peptides [20], is a potent mitogen and growth factor for normal and neoplastic lung [21]. Moody et al. [22] reported that nonpeptide neuromedin B receptor antagonists inhibited the proliferation of C6 cells. Therefore, ARB may prevent the progression of cardiac hypertrophy or heart failure partially via the modulation of monoamine oxidase B and neuromedin B receptor expression.

Twenty-one mechanically-suppressed genes were significantly restored by RNH-6270. Genes for detoxification, cytochrome P-450f and arylamine N-acetyltransferase, which were suppressed under mechanical strain, were restored by RNH-6270. Cytochrome P450s are one of the major phase I-type classes of detoxification enzymes [23]. Arylamine N-acetyltransferases catalyse the biotransformation of many primary arylamines, hydrazines and their N-hydroxylated metabolites, thereby playing an important role in the detoxification [24]. In addition, major acute phase alpha 1-protein of the rat carries the sequence for bradykinin, a potent cardioprotective hormone [19]. These molecular alternations might provide the clinical advantage of ARB. In the present study, genes for Sp-1 and JAK2 were suppressed under mechanical strain. However, the previous studies demonstrated that mechanical strain induced Sp-1 mRNA expressions in vascular smooth muscle cells [25] and rapid phosphorylation of JAK2 in rat cardiomyocytes [26]. Further studies will be required to clarify these discrepancies. Rat Bcl-Xalpha corresponds to the human Bcl-X_L, as designated by Boise et al. [27]. Shiraiwa et al. [28] reported that Bcl-Xalpha mRNA is expressed in the heart, and that overexpression of Bcl-Xalpha delayed apoptosis induced by withdrawing IL-3 in the promyeloid cells. Therefore, RNH-6270 may prevent apoptosis of cardiomyocytes from mechanical stress.

Recent randomized trials of ARB in the treatment of patients with congestive heart failure have demonstrated clinical benefits of ARB as well as ACE inhibitors [6,29]. Evaluation of Losartan in the Elderly (ELITE) II study showed equivalent effect on mortality and morbidity between losartan and captopril and less adverse events in losartan [29]. Val-HeFT demonstrated the additional benefits of varsartan to the standard treatment with ACE inhibitors, diuretics and digitalis in patients with heart failure [6]. Although the roles of other genes including ESTs in the heart except for genes described above remain still unknown, the genes screened in this study may provide insights into the beneficial effects of ARB, olmesartan, on the cardiovascular system, because DNA microarray is a highly effective method for screening genes.

Study Limitations

The dosage of RNH-6270, olmesartan, used in the present study was 0.1 μmol/L and at the range of treatment, because the maximum observed plasma concentration of RNH-6270 following oral administration of 20 mg CS-866 in healthy subjects was 0.9 μmol/L [30] and RNH-6270 inhibited [^{125}I]angiotensin II binding to bovine adrenal cortical membranes (angiotensin II type 1 receptors) with an IC50 of 7.7 nmol/L [8]. The time course and dosage of the drugs are very important factors in evaluation of gene expression in cells stimulated with mechanical or chemical stimuli. Therefore, further studies should be performed at several time points and several dosages of olmesartan. In addition, there is a difference in the fold change in expression between the microarray and RT-PCR experiments in the present study. The results of microarray experiments should be confirmed by the RT-PCR experiments or other methods, although DNA microarray is a powerful method for screening genes.

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Antiapoptotic Effect of Endothelin-1 in Rat Cardiomyocytes In Vitro

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Abstract—Apoptosis of cardiac myocytes is thought to be a feature of many pathological disorders, including congestive heart failure (CHF) and ischemic heart disease (IHD). Because recent investigations indicate that endothelin-1 (ET-1) plays an important role in CHF and IHD, we investigated the effect of ET-1 on cardiomyocyte apoptosis. The presence of apoptosis in rat cardiomyocytes (H9c2 and neonatal) was evaluated by morphological criteria, electrophoresis of DNA fragments, 4′,6′-diamidine-2′-phenylindole staining, and TUNEL analysis. ET-1, but not angiotensin II, prevented apoptosis induced by serum deprivation via ET_A receptors in a dose-dependent manner (1 to 100 nmol/L). ET-1 also prevented cytochrome *c* release from mitochondria to the cytosol. The use of specific pharmacological inhibitors demonstrated that the antiapoptotic effect of ET-1 was mediated through a tyrosine kinase pathway (genistein and AG490) but not through protein kinase C (PKC; calphostin C), mitogen-activated protein kinases (PD98059 and SB203580), or PKA (KT5270) pathways. Adenovirus-mediated gene transfer of kinase-inactive (KI) c-Src reversed the antiapoptotic effect of ET-1. We further investigated whether Bcl-x_L, an antiapoptotic molecule, would be upregulated by using a luciferase-based reporter system. ET-1 upregulated Bcl-x_L, and this upregulation was inhibited by genistein or AG490 but not by calphostin C. The experiments with KI mutants for various tyrosine kinases revealed that c-Src and Pyk2 (but not JAK1, Jak2, Syk, and Tec) are involved in ET-1–induced upregulation of Bcl-x_L expression. These findings suggest that ET-1 prevents apoptosis in cardiac myocytes through the ET_A receptor and the subsequent c-Src/Bcl-x_L–dependent pathway. (***Hypertension.* 2003;41:1156-1163.**)

Key Words: signal transduction ■ kinase ■ endothelin ■ apoptosis ■ myocardium

Cardiac myocyte cell death by apoptosis accompanies several heart diseases.[1,2] It has been demonstrated in the myocardium from failing human hearts,[3] in patients with dilated cardiomyopathy and arrhythmogenic right ventricular dysplasia,[4,5] and in association with myocardial infarction.[6] Apoptosis causes loss of contractile cells, compensatory hypertrophy of myocardial cells, and reparative fibrosis.[7] Because a reduction of contractile material is a prominent feature in heart failure, modification of apoptosis in the myocardium might provide a new therapeutic target for cardiovascular diseases.

A number of stimuli induce a hypertrophic response in cardiac myocytes, including α-adrenergic agents, heparin-binding epidermal growth factor–like growth factor, insulin-like growth factor-1, leukemia inhibitory factor, neuregulin, cardiotropin-1, angiotensin II (AII), and interleukin-1β.[8–12] Several of these factors have also been shown to be proapoptotic, whereas others have an antiapoptotic role in cardiac myocytes.[13,14]

Endothelin-1 (ET-1), a family of 21–amino acid peptides, is 1 of the most potent hypertrophic stimuli for cardiac myocytes.[15,16] Furthermore, a number of clinical and experimental investigations have demonstrated that ET-1 might play an important role in the pathophysiology of cardiovascular diseases, including congestive heart failure (CHF) and ischemic heart disease.[17] The plasma and myocardial tissue levels of ET-1 increase in patients with CHF.[18,19] In patients with acute myocardial infarction, plasma ET-1 levels are elevated[20,21] and are correlated with 1-year mortality.[21] We therefore hypothesized that ET-1 regulates apoptosis in the myocardium.

The effect of ET-1 on apoptosis is controversial. ET-1 has been reported to be an antiapoptotic factor in endothelial cells.[22] On the other hand, there are studies of smooth muscle cells in which ET-1 causes apoptosis.[23] In cardiac myocytes, ET-1 prevents oxidative stress– and β-adrenergic agonist–induced apoptosis.[24,25] In the present study, we demonstrate that ET-1 prevents apoptosis induced by serum deprivation in cultured cardiac myocytes and investigate the signaling pathways that mediate the antiapoptotic effect of ET-1.

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Methods

Cell Culture and Materials

The embryonic rat heart–derived myogenic cell line H9c2 was obtained from American Type Culture Collection (Rockville, Md). Rat neonatal cardiomyocytes were prepared from ventricles of 1-day-old Sprague-Dawley rats as described previously.[26] The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin solution. The investigation was performed in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (Her Majesty's Stationary Office, London, UK).

The cDNA for a kinase domain–deleted Tec was subcloned into an expression vector, pSRa.[27] The kinase-deleted forms of porcine Syk (amino acids 1 to 504), human c-Src (amino acids 1 to 253), and mouse Jak1 (amino acids 1 to 879) were amplified by the polymerase chain reaction and inserted individually into the same vector. The expression plasmid for the kinase-deleted Jak2 was constructed as described previously.[27] Expression plasmids for a kinase-inactive (KI) form of Pyk2 were described previously.[28] Adenovirus containing either the β-galactosidase cDNA (Ad.LacZ) or a cDNA encoding chicken KI–c-Src was prepared, amplified, and purified as described previously.[29] Human ET-1 was purchased from the Peptide Institute Inc. Antibodies against Bcl-x_L, Bcl-2, c-Src (clone GD11, ED10), and an activated form of c-Src ([pY418] phosphospecific antibody) were purchased from Santa Cruz Inc, Upstate Biotechnology Inc, and Biosource International, respectively. Antibodies against phosphosignal transducer and activator of transcription 3 (Stat3 [Tyr705]) and Stat3 were purchased from Cell Signaling Technology Inc. BQ123, BQ788, genistein, AG490, calphostin C, KT5270, PD98059, SB203580, and PP2 were purchased from Calbiochem. The remaining reagents including AII were obtained from Sigma unless otherwise indicated.

DNA Laddering

To evaluate DNA fragmentation, cellular fragmented DNA was extracted by the Triton X-100 lysis method, which efficiently eliminates intact chromatin. Floating and/or adherent cells were collected, and DNA fragments were extracted, fractionated by 1.8% agarose gel electrophoresis, and stained with ethidium bromide.[30]

DAPI Staining

Cells were fixed in 3% paraformaldehyde in phosphate-buffered saline for 20 minutes and stained with a solution of 4′,6-diamidino-2-phenylindole (DAPI; 10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 100 mmol/L NaCl, 500 ng/mL DAPI) for 10 minutes at room temperature. The apoptotic cells were evaluated under a fluorescent microscope.[31]

TUNEL Analysis

Cells were fixed and then labeled using terminal deoxyribonucleotidyl transferase according to the manufacturer's instructions (in situ apoptosis detection kit, Wako).

Detection of Cytochrome *C* Release

Cell lysates were prepared for the detection of cytochrome *c* in cytosolic and mitochondrial fractions, and detection of cytochrome *c* release was performed by Western blot analysis with an anti–cytochrome *c* antibody according to the manufacturer's instructions (cytochrome *c* releasing apoptosis assay kit, Biovision).

Western Blot Analysis

Expression levels of Bcl-x_L, Bcl-2, c-Src, the activated form of c-Src, Stat3, and the phosphorylated form of Stat3 were analyzed by Western blot analysis. In brief, cells were lysed in a modified radioimmunoprecipitation assay buffer (10 mmol/L HEPES, pH 7.4, 5 mmol/L EDTA, 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 mmol/L NaCl, 100 μmol/L Na_3VO_4, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, and fresh 0.5 mmol/L PMSF and 10 μg/mL leupeptin). Cell lysates were prepared by scraping, sonication, and centrifugation for 20 minutes at 14 000 rpm in a microfuge at 4°C. Cell lysates were subjected to 5% to 20% SDS-polyacrylamide gradient gel electrophoresis. The separated proteins were electrophoretically transferred onto nitrocellulose membranes, and the resultant blots were incubated with the first antibody for 2 hours, followed by incubation for 1 to 2 hours with the secondary antibody (horseradish peroxidase conjugated). Immunoreactive bands were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech UK Ltd).

Transfection and Luciferase Assay

Transfections were performed with the Tfx-50 lipofectin reagent (Promega). In brief, cells were plated at 10^5 cells/well in DMEM supplemented with 10% fetal calf serum (FCS) in 6-well plates and allowed to attach overnight. Transfections were performed 1 day after seeding by using a combination of 1.5 μg of expression plasmids, 1.5 μg Bcl-x_L-luc, 0.3 μg pRL-TK (Promega), and 9.9 μL Tfx-50. Cells were deprived of serum for 16 hours and then treated with 100 nmol/L ET-1 for 5 hours. Cell lysates were prepared, and the activity of *Photinus pyralis* luciferase was measured with the dual-luciferase reporter assay system (Promega) and normalized by the activity of *Runilla reniformis* luciferase.

Statistical Analysis

Data are expressed as the mean±SD. For comparisons between multiple groups, we determined the significance of differences between group means by ANOVA with the least significant difference for multiple comparisons. $P<0.05$ was considered statistically significant.

Results

Effect of ET-1 on Serum Deprivation–Induced Apoptosis

We first examined the effects of ET-1 on apoptosis in cultured cardiomyocytes. Electrophoresis of DNA fragments showed that 100 nmol/L ET-1 prevented formation of the characteristic apoptosis ladder induced by serum deprivation in both H9c2 cells and rat neonatal cardiomyocytes (Figure 1A). In contrast, AII, which is a vasoconstrictive peptide similar to ET-1, had no effect on apoptosis in H9c2 cells, although these cells express the angiotensin II type 1 (AT_1) receptor.[32] The antiapoptotic effect of ET-1 was dose dependent over the range used (1 to 100 nmol/L; Figure 1B). We confirmed that ET-1 prevented serum deprivation–induced apoptosis by TUNEL analysis and DNA-binding dye (DAPI) staining (Figures 1C through 1E). Serum deprivation reduced cell viability, as measured by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (data not shown). Furthermore, ET-1 prevented serum deprivation–induced cytochrome *c* release from mitochondria to the cytosol (Figure 2).

The effects of ET-1 are initiated by their binding to G protein–coupled heptahelical receptors, ET_A and ET_B, expressed in a wide variety of tissues and cells.[33] To identify which ET receptor (ET_A or ET_B) is responsible for the antiapoptotic effect of ET-1 in H9c2 cells, we used selective ET_A and ET_B receptor antagonists. The antiapoptotic effect of ET-1 was almost completely inhibited by treatment with the selective ET_A receptor antagonist BQ123, but not by the ET_B receptor antagonist BQ788 (Figures 3A and 3B). These observations indicate that ET-1 participates in the survival of cardiac myocytes by preventing apoptosis by way of the ET_A receptor in myocytes.

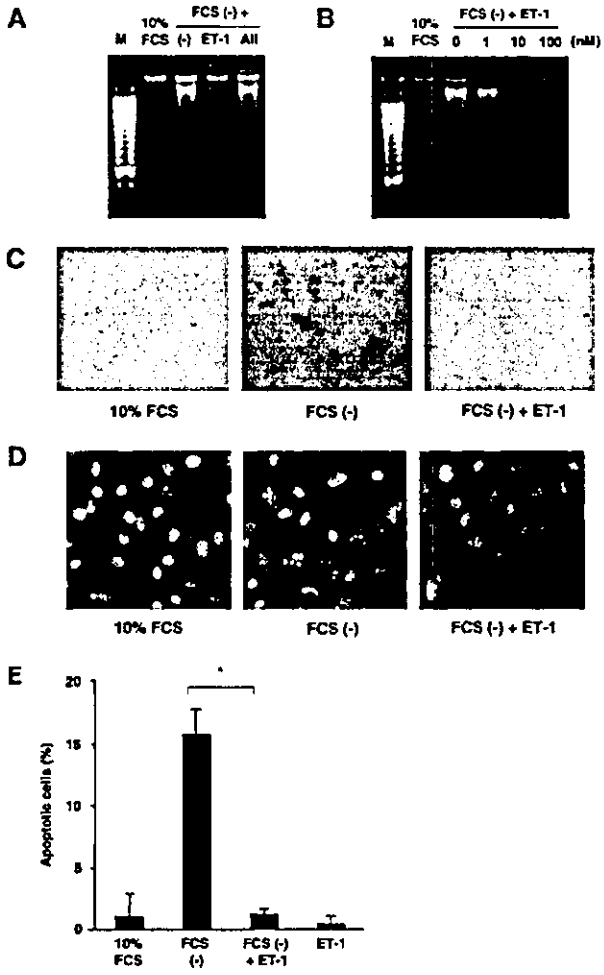

Figure 1. Effect of ET-1 on serum (FCS) deprivation-induced apoptosis. A, H9c2 or rat neonatal cardiomyocytes (CM) were deprived of serum for 24 hours in the presence or absence of 100 nmol/L ET-1 or 100 nmol/L AII. B, H9c2 cells were deprived of serum for 24 hours in the presence or absence of indicated concentrations of ET-1. Genomic DNA was extracted by the Triton X-100 lysis method. DNA (10 μg/lane) was loaded onto 1.8% agarose gel containing ethidium bromide. After electrophoresis, DNA bands were visualized under ultraviolet light. M indicates DNA size markers. Results are representative of 3 independent experiments. C to E, H9c2 cells were deprived of serum for 24 hours in the presence or absence of 100 nmol/L ET-1. TUNEL analysis (C) and DAPI staining (D) were performed as described in Methods. Results are representative of 3 independent experiments. E, Bar graphs show mean±SD of 1200 to 1600 cells of 3 independent experiments (DAPI staining). *$P<0.01$ vs control.

Effects of Various Signaling Inhibitors on ET-1–Prevented Apoptosis

Because the intracellular protein kinases mediate the prevention of apoptosis in cardiac myocytes,[1,2,34] we next examined whether protein kinases were involved in the antiapoptotic effect of ET-1 in H9c2 cells by using tyrosine kinase inhibitors (genistein and AG490), protein kinase C inhibitors (calphostin C), mitogen-activated protein (MAP) kinase inhibitors (PD98059 for extracellular-regulated kinase and SB20380 for p38-MAP kinase), and a cAMP-dependent kinase inhibitor (KT5270). Genistein and AG490 inhibited

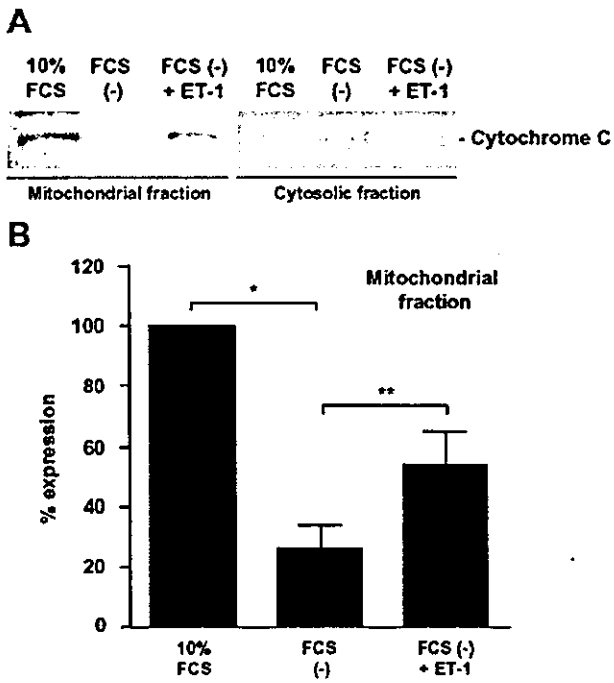

Figure 2. Prevention of cytochrome *c* release by ET-1. H9c2 cells were deprived of serum for 24 hours in the presence or absence of 100 nmol/L ET-1 and prepared for detection of cytochrome *c* in mitochondrial (Mito.fr.) and cytosolic (Cyto.fr.) fractions, as described in Methods. Cytochrome *c* release was measured by Western blotting (A and B). Relative expression of cytochrome *c* was quantified by densitometry (n=3). Bar graphs show mean±SD of 3 independent experiments. *$P<0.01$ and **$P<0.05$.

the effect of ET-1, whereas calphostin C, PD98059, SB20380, and KT5270 failed to show any effect (Figures 4A and 4B). In addition, treatment with the phosphatidyl inositol-3 kinase inhibitor wortmannin showed no effect on ET-1–prevented apoptosis (data not shown). These results suggest that ET-1 prevents serum deprivation–induced apoptosis in cardiac myocytes through a tyrosine kinase–dependent mechanism.

c-Src Involved in the Antiapoptotic Effect of ET-1

Recent evidence suggests that the c-Src family of protein tyrosine kinases is involved in apoptotic cell death in certain types of cells.[35–37] We therefore hypothesized that c-Src might participate in the antiapoptotic effect of ET-1. To determine whether c-Src activity was regulated by ET-1, cells were stimulated by 100 nmol/L ET-1 for varying amounts of time, and c-Src activity was analyzed by Western blotting by using an antibody that selectively recognizes the activated form of c-Src. The activity of c-Src clearly increased by 4.5-fold within 30 seconds in response to ET-1 stimulation and then declined (Figure 5, top). We confirmed that there were no significant changes in c-Src protein levels of the same amount of cell lysates (Figure 5, bottom).

To further investigate the role of c-Src in the antiapoptotic effect of ET-1, adenoviruses were used to overexpress either β-galactosidase (Ad.LacZ) or a KI–c-Src. Transfection of H9c2 cells with a KI–c-Src, but not with Ad.LacZ, increased c-Src in

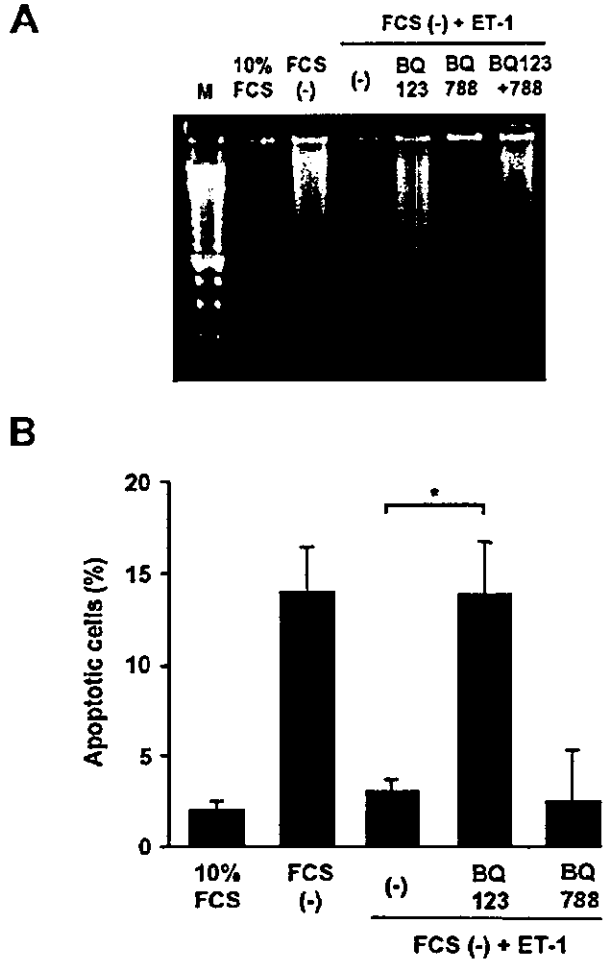

Figure 3. Antiapoptotic effect of ET-1 is mediated via ET_A receptor. H9c2 cells were deprived of serum (FCS) for 24 hours with 100 nmol/L ET-1 in the presence or absence of 100 nmol/L BQ123, BQ788, or BQ123 plus BQ788. DNA fragmentation (A) and DAPI staining (B) were performed as described in Methods. Genomic DNA was extracted from cells as described in the legend to Figure 1. M indicates DNA size markers. Bar graphs show mean±SD of 1200 to 1600 cells of 3 independent experiments (DAPI staining). *$P<0.01$ vs control.

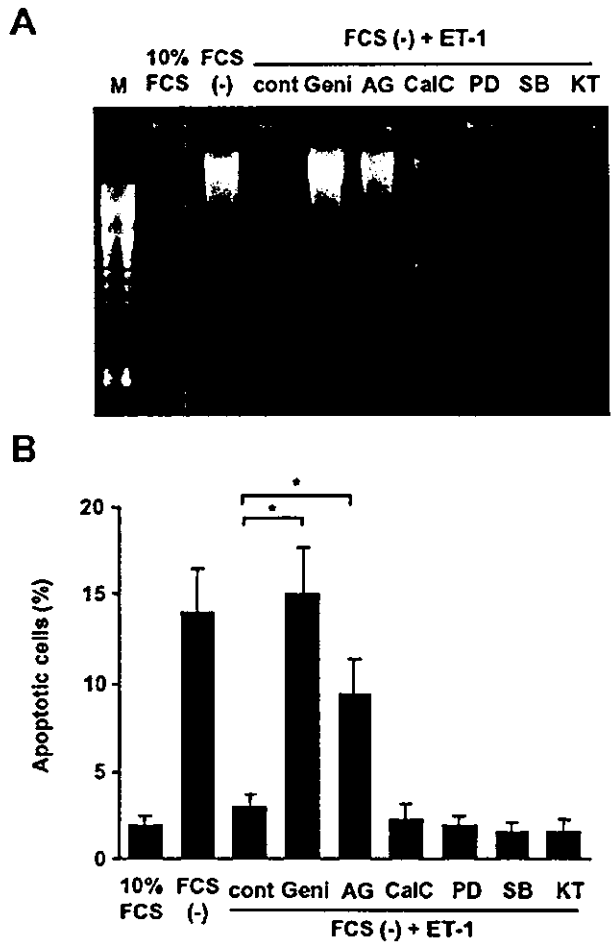

Figure 4. Effects of various inhibitors on ET1-prevented apoptosis. After H9c2 cells were pretreated with 0.1% dimethyl sulfoxide (cont), 10 μmol/L genistein (Geni), 100 μmol/L AG490 (AG), 1 μmol/L calphostin C (CalC), 1 μmol/L KT 5720 (KT), 10 μmol/L PD98059 (PD), or 10 μmol/L SB203580 (SB) for 1 hour, cells were deprived of serum (FCS) for 24 hours in the presence of ET-1. DNA fragmentation (A) and DAPI staining (B) were performed as described in Methods. Genomic DNA was extracted from cells as described in the legend to Figure 1. M indicates DNA size markers. Bar graphs show mean±SD of 1200 to 1600 cells of 3 independent experiments (DAPI staining). *$P<0.01$ vs control.

a concentration-dependent manner (Figure 6A). Expression of KI–c-Src significantly inhibited the antiapoptotic effect of ET-1, whereas expression of β-galactosidase with Ad.LacZ had no effect (Figures 6B and 6C). These results indicate that c-Src is required for the antiapoptotic effect of ET-1.

Effects of KI Mutants for Various Tyrosine Kinases on Bcl-x_L Expression

Because c-Src has been shown to negatively regulate apoptosis via Bcl-x_L, an antiapoptotic molecule, in several cell types,[35–37] we investigated whether Bcl-x_L was involved by using a luciferase-based reporter system. Western blot analysis showed that ET-1 stimulated Bcl-x_L expression (Figures 7A and 7B). ET-1 upregulated Bcl-x_L gene expression, which was inhibited by treatment with genistein or AG490, but not by calphostin C (Figure 7C). The experiments with KI mutants for various tyrosine kinases showed that KI–c-Src completely inhibited ET-1–induced Bcl-x_L gene expression (Figure 7D). In addition, KI-Pyk2 partially inhibited its expression, whereas KI-JAK1, KI-Jak2, KI-Syk, or KI-Tec showed no effect. These findings suggest that c-Src and Pyk2 are involved in Bcl-x_L expression induced by ET-1.

ET-1 Stimulates STAT3 Phosphorylation

Because it has been reported that STAT3 regulates Bcl-x_L expression in cardiac myocytes,[38] finally we examined whether ET-1 stimulates STAT3 phosphorylation. ET-1 clearly stimulated STAT3 phosphorylation in a time-dependent manner, and this STAT3 phosphorylation was inhibited by treatment with a specific c-Src inhibitor, PP2 (Figure 8).

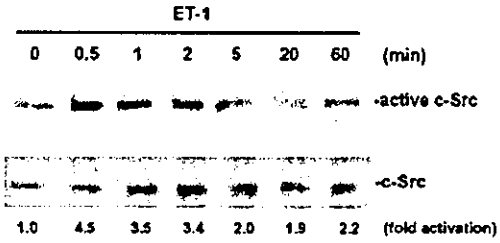

Figure 5. ET-1 stimulates c-Src activation. After H9c2 cells were deprived of serum in the presence or absence of 100 nmol/L ET-1 for the indicated periods, cell lysates were prepared. Anti-phosphospecific-c-Src (top, active c-Src) and c-Src antibody (bottom, c-Src) were used to quantify these proteins by Western blotting of whole lysates that were obtained in the same experiments. Relative c-Src kinase activity was quantified by densitometry. Results are representative of 2 independent experiments.

Discussion

The major findings of this study are that ET-1 prevents apoptosis induced by serum deprivation in a dose-dependent manner via an ET_A receptor in H9c2 cardiomyocytes and that the antiapoptotic effect of ET-1 is mediated through a c-Src/Bcl-x_L pathway. Evidence for this proposal includes the following: (1) ET-1, but not AII, prevented mitochondrial cytochrome *c* release and apoptosis induced by serum deprivation in a dose-dependent manner, and this antiapoptotic effect was inhibited by an ET_A receptor antagonist (BQ123) but not by an ET_B receptor antagonist (BQ788); (2) the inhibitory effects of ET-1 on apoptosis were inhibited by tyrosine kinase inhibitors and adenovirus-mediated overexpression of KI-c-Src; (3) ET-1 stimulated c-Src activation; and (4) ET-1 upregulated an antiapoptotic molecule, Bcl-x_L, and this upregulation was inhibited by tyrosine kinase inhibitors or cotransfection with KI-c-Src.

Recent evidence suggests that apoptosis of cardiac myocytes is a feature in cardiovascular diseases, including CHF and myocardial infarction.[1,2,39] The levels of plasma and myocardial ET-1 increase in patients with CHF and myocardial infarction,[18–21] suggesting the critical role of ET-1 in these cardiovascular disease states. Therefore, we investigated whether ET-1 affects myocardial apoptosis in this study. We showed here that ET-1 prevents serum deprivation–induced mitochondrial cytochrome *c* release and apoptosis, suggesting that the antiapoptotic effect of ET-1 is mediated through a mitochondrial apoptotic pathway. We further demonstrated that ET-1 prevents apoptosis in a dose-dependent manner via the ET_A receptor. The effects of ET were mediated through 2 distinct receptor subtypes of G protein–coupled receptors, termed ET_A and ET_B, expressed in a wide variety of cells and tissues.[40,41] In myocardium, ET_A receptors are mainly expressed, and small amounts of ET_B receptors are expressed.[33] Consistent with the expression levels in the myocardium, ET_A receptors act as a major pathway for several effects of ET-1, such as myocardial contraction and hypertrophy.[17] Similar to these effects of ET-1, our findings indicate that the antiapoptotic effect of ET-1 in cardiac myocytes is also mediated via the ET_A receptor.

A number of proapoptotic and antiapoptotic signaling pathways in cardiac myocytes have been demonstrated.[1,2] To investigate the molecular mechanisms of the antiapoptotic effect of

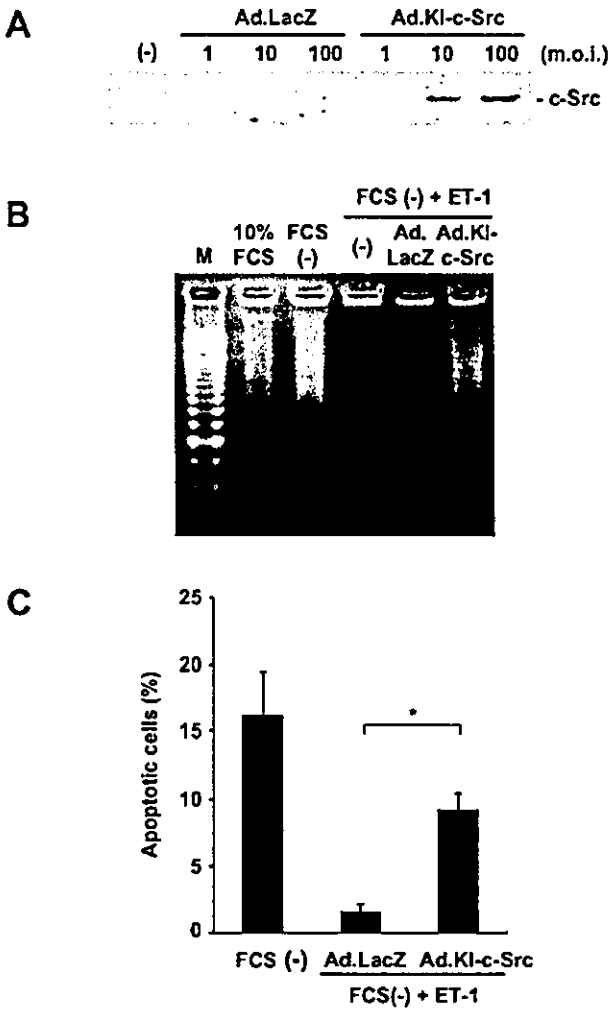

Figure 6. Overexpression of Ad.KI-c-Src inhibits antiapoptotic effect of ET-1. A, H9c2 cells were infected with either Ad.LacZ (10 and 100 m.o.i.) or Ad.Klc-Src (1, 10 and 100 m.o.i.) for 1 hour at 37°C and then incubated with DMEM supplemented with 10% FCS for 48 hours. Cell lysates were prepared and analyzed by Western blotting with anti-c-Src antibody. B, Cells were infected with 100 m.o.i. of either Ad.LacZ or Ad.KI-c-Src for 1 hour at 37°C, incubated with DMEM supplemented with 10% FCS for 48 hours, and then deprived of serum (FCS) in the presence of 100 nmol/L ET-1 for 24 hours. Fragmented DNA was extracted from cells as described in the legend to Figure 1. M indicates DNA size markers. Results are representative of 3 independent experiments. C, Bar graphs show mean±SD of 1200 to 1600 cells of 3 independent experiments (DAPI staining). *$P<0.01$ vs control.

ET-1 in cardiac myocytes, we used an adenovirus-based vector system that allows for highly efficient DNA transfection in many cell types. The efficiency of expression examined with Ad.LacZ in H9c2 cells infected by adenovirus was found to be almost 100% (data not shown). Because tyrosine kinase inhibitors, such as genistein and AG490, reversed the antiapoptotic effect of ET-1, we examined whether protein tyrosine kinase c-Src is involved in the antiapoptotic effect of ET-1 by using the KI-c-Src–expressing adenovirus. We demonstrated that overexpression of KI-c-Src reversed the antiapoptotic effect of ET-1, suggesting that c-Src plays a critical role in the ET-1–mediated antiapoptotic pathway in cardiac myocytes.

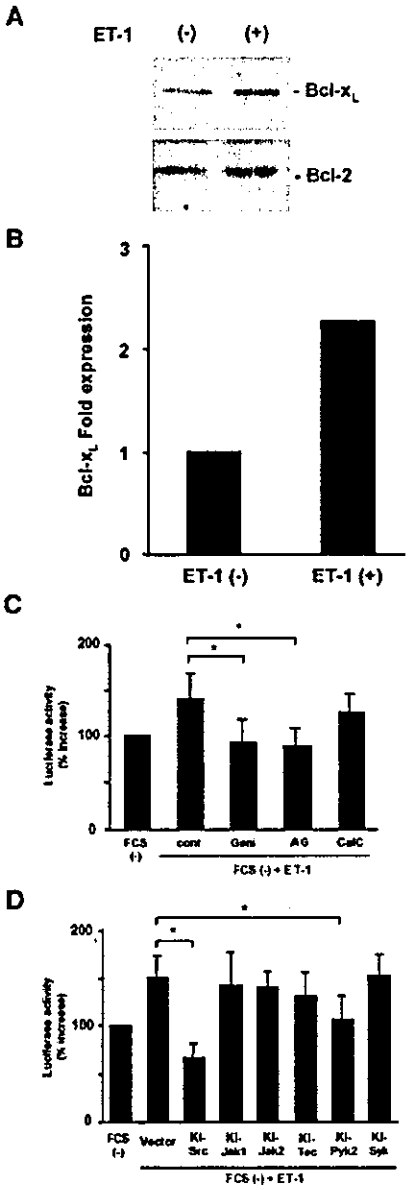

Figure 7. Effects of KI mutants for various tyrosine kinases on Bcl-x_L expression. A, H9c2 cells were deprived of serum for 24 hours in the presence or absence of ET-1. Cell lysates were prepared, and expression of Bcl-x_L and Bcl-2 was analyzed by Western blotting, as described in Methods. B, Relative Bcl-x_L expression was quantified by densitometry. Results are representative of 2 independent experiments. C, After pBcl-x_L/luc (1.5 μg) and pRL-TK (0.3 μg) were introduced into cells, they were deprived of serum for 16 hours and pretreated with 0.1% dimethyl sulfoxide (cont), 10 μmol/L genistein (Geni), 100 μmol/L AG490 (AG), or 1 μmol/L calphostin C (CalC) for 1 hour; then cells were treated with 100 nmol/L ET-1 for 5 hours. Cell extracts were subjected to luciferase assay as described in Methods. The activity of *Photinus pyralis* luciferase was normalized by activity of *Runilla reniformis* luciferase. Bar graphs show mean±SD (n=4). *P<0.01 vs control. D, After pBcl-x_L/luc (1.5 μg) and pRL-TK (0.3 μg) were introduced into cells together with 1.5 μg each of blank vector (Vector), expression plasmid for control vector (vector), or the KI form of each protein tyrosine kinases (Src, Jak1, Jak2, Tec, Pyk2, Syk), cells were deprived of serum (FCS) for 16 hours, and then treated with 100 nmol/L ET-1 for 5 hours. Cell extracts were analyzed as described above. Bar graphs show mean±SD (n=6). *P<0.01 vs control.

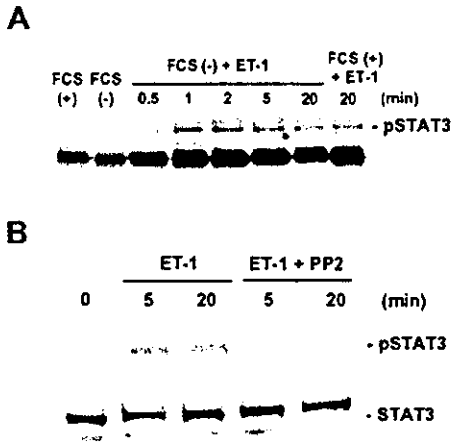

Figure 8. ET-1 stimulates STAT3 phosphorylation. A, After H9c2 cells were deprived of serum (FCS) in the presence or absence of 100 nmol/L ET-1 for indicated periods, cell lysates were prepared. B, After cells were pretreated with 10 μmol/L PP2 for 1 hour, they were stimulated with ET-1, and then cell lysates were prepared. Anti-phospho-STAT3 (pSTAT3) and STAT3 antibody (STAT3) were used to quantify these proteins by Western blotting. Results are representative of 2 independent experiments.

Bcl-x_L plays a critical role in the antiapoptotic signaling pathway in a variety of cells, including cardiac myocytes.[42] In addition, recent investigations have suggested that c-Src regulates Bcl-x_L in several cell types.[35–37] Therefore, we next focused on Bcl-x_L expression in the antiapoptotic pathway by ET-1. We demonstrated that ET-1 upregulated Bcl-x_L expression, and this upregulation was completely inhibited by both KI–c-Src and tyrosine kinase inhibitors, which inhibited the antiapoptotic effect of ET-1. These findings suggest that c-Src is an upstream molecule for Bcl-x_L expression in cardiac myocytes. c-Src itself has also induced the activation of several signaling molecules, including MAP kinase and STAT3,[43] and both can stimulate Bcl-x_L expression.[36,38] Regarding this, Araki et al[25] recently reported that ET-1 prevents apoptosis induced by β-adrenergic agonists, and this effect is inhibited by treatment with the MAP kinase inhibitor PD98059. This difference might be due to apoptosis-inducing stimuli, because the β-adrenergic agonist itself stimulates MAP kinase activity in cardiac myocytes.[44] Downstream from c-Src, STAT3 is another molecule that might stimulate Bcl-x_L expression. Karni et al[37] reported that c-Src positively regulates Bcl-x_L expression via STAT3 activation. In addition, Negoro et al[45] recently reported that pretreatment with AG490 significantly inhibited STAT3 phosphorylation and increased apoptosis in rat hearts after infarction. In our study, AG490 was found to inhibit the antiapoptotic effect of ET-1. In addition, ET-1 stimulated STAT3 phosphorylation through a c-Src–dependent mechanism. Thus, STAT3 is a possible molecule that participates in a c-Src/Bcl-x_L pathway. Another signaling molecule responsible for ET-1–prevented apoptosis is Pyk2, because KI-Pyk2 significantly inhibited ET-1–induced Bcl-x_L expression in part. At present, however, the role of Pyk2 in