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α_1-Adrenergic Receptor Stimulates Interleukin-6 Expression and Secretion through Both mRNA Stability and Transcriptional Regulation: Involvement of p38 Mitogen-Activated Protein Kinase and Nuclear Factor-κB

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ABSTRACT

Our previous studies have demonstrated that activation of α_1-adrenergic receptors (ARs) increased interleukin-6 (IL-6) mRNA expression and protein secretion, which is probably an important yet unknown mechanism contributing to the regulation of cardiac function. Using Rat-1 fibroblasts stably transfected with the α_{1A}-AR subtype and primary mouse neonatal cardiomyocytes, we elucidated the basic molecular mechanisms responsible for the α_1-AR modulation of IL-6 expression. IL-6 mRNA production mediated by α_1-AR peaked at 1 to 2 h. Studies of the mRNA decay rate indicated that α_1-AR activation enhanced IL-6 mRNA stability. Analysis of IL-6 promoter activity using a series of deletion constructs indicated that α_1-ARs enhanced IL-6 transcription through several transcriptional elements, including nuclear factor κB (NF-κB). Inhibition of α_1-AR mediated IL-6 production and secretion by actinomycin D and the increase of intracellular IL-6 levels by α_1-AR activation suggest that α_1-AR mediated IL-6 secretion through de novo synthesis. Both IL-6 transcription and protein secretion mediated by α_1-ARs were significantly reduced by chemical inhibitors for p38 mitogen-activated protein kinase (MAPK) and NF-κB and by a dominant-negative construct of p38 MAPK. Serum level of IL-6 was elevated in transgenic mice expressing a constitutively active mutant of the α_{1A}-AR subtype but not in a similar mouse model expressing the α_{1B}-AR subtype. Our results indicate that α_1-ARs stimulated IL-6 expression and secretion through regulating both mRNA transcription and stability, involving p38 MAPK and NF-κB pathways.

Adrenergic receptors (ARs), belonging to the superfamily of G-protein coupled receptors, have been classified into α_1-ARs, α_2-ARs, and β-ARs. Three subtypes of α_1-ARs (α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR) have been cloned and characterized pharmacologically (Perez et al., 1991, 1994; Ramarao et al., 1992). Expressed in various tissues and cell types including cardiomyocytes (CMs), α_1-ARs mediate the action of endogenous catecholamines and induce a variety of physiological effects such as the regulation of blood pressure, cardiac functions, and the development of cardiac hypertrophy. Hypertrophy due to AR activation in the heart can initially be adaptive in terms of muscular economy by normalizing wall stress and preserving cardiac contractile function. However, prolonged AR activation can become maladaptive and even pathological by exerting deleterious effects and promoting the apoptosis of CMs (Selvetella et al., 2004).

Interleukin-6 (IL-6) is a multifunctional cytokine and has a broad range of activities regulating not only host immune responses but also cell growth and differentiation (Sehgal et al., 1989). Evidence indicates that IL-6 is involved in cardiovascular diseases, such as cardiac hypertrophy, myocardial infarction, and heart failure (Hirota et al., 1995; Kanda et al., 2000). Overexpression of signal transducer and activator of transcription 3, a major component in IL-6 signaling, was

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ABBREVIATIONS: AR, adrenergic receptor; ActD, actinomycin D; MAPK, mitogen-activated protein kinase; AP-1, activated protein 1; Bay, Bay 11-7085 [(2*E*)-3-[[4-(1,1-dimethylethyl)phenyl]sulfonyl]-2-propene nitrile]; CAM, constitutively active mutant; CH, chelerythrine; CM, cardiomyocyte; CRE, cAMP response element; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; Epi, epinephrine; ERK, extracellular signal-regulated kinase; Go, Go 6983 [3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione]; IL, interleukin; NF-κB, nuclear factor-κB; PD, PD 98059 [2′-amino-3′-methoxyflavone]; SB, SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole]; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; WT, wild type; DN, dominant-negative; PBS, phosphate-buffered saline; ARE, adenine- and uridine-rich element.

sufficient to induce hypertrophy of CMs (Kunisada et al., 1998). On the other hand, IL-6 has also been shown to be protective in the ischemic heart by inhibiting CM apoptosis (Matsushita et al., 2005). Thus, whether the net effect of IL-6 in cardiac functions is protective or mainly harmful is still an open question.

It has been previously shown that norepinephrine mediates significant up-regulation of IL-6 expression in rat CMs, fibroblasts (Bürger et al., 2001; Briest et al., 2003), and in mouse heart (Song et al., 1999), but the AR subtype(s) and mechanisms were not known. In addition, stress and injections of epinephrine (Epi) have been shown to increase plasma levels of IL-6 in rats (Takaki et al., 1994). These data indicate a potential neurohormone-cytokine cross-talk between the ARs and IL-6, which could possibly regulate cardiac functions. Activation of β-ARs can lead to increased IL-6 mRNA expression in several cell types, including cardiac fibroblasts (Bürger et al., 2001), but nothing is known about the mechanism. Furthermore, virtually nothing has been published about α_1-AR regulation of IL-6 expression until our recent studies (Gonzalez-Cabrera et al., 2003). Using Rat-1 fibroblasts stably transfected with individual α_1-AR subtypes, we demonstrated that activation of any α_1-AR subtype can greatly enhance IL-6 mRNA expression and protein secretion. Cross-talk between α_1-AR and IL-6 signaling potentially represents an important yet unknown mechanism contributing to the regulation of cardiac function and hypertrophy. Thus, further studies including the use of primary CMs as a cell model to understand the molecular mechanisms involved in this cross-talk are highly significant.

The purpose of the present study is to explore the basic mechanisms underlying the modulation of α_1-AR activation on IL-6 expression and secretion and to identify potential signaling pathways involved that might be used for the treatment and management of cardiac malfunctions. Using Rat-1 fibroblasts and neonatal CMs as cell systems, we report our findings that α_1-ARs modulate IL-6 expression and secretion through regulating both mRNA transcription and stability, involving p38 MAPK and NF-κB pathways.

Materials and Methods

Isolation of Primary Cultures of CMs. Neonatal CMs were isolated using kits from Cellutron following the procedures provided by the manufacturer (Cellutron Life Technology, Baltimore, MD). The procedures have been used successfully before (Saucerman et al., 2006). In brief, hearts from 2- to 3-day neonatal mouse pups were aseptically excised, the atria were removed, and the ventricles were digested at 37°C for 15 min in buffered solution containing collagenase type II. The liquid portion of the digestion mixture was centrifuged, and the resulting pellet containing CMs was resuspended in Dulbecco's modified Eagle's medium (DMEM-F12)-based solution from the company. The remaining ventricle tissue was subjected up to a total of six more rounds of digestion, and the resulting pellet from each digestion was pooled, centrifuged, and resuspended in DMEM-F12 medium containing 10% fetal bovine serum. The suspension containing CMs was preplated in a sterile tissue-culture flask at 37°C in the presence of 5% CO_2 for 1 h to reduce fibroblast contamination. The CM-enriched cell suspension after preplating was transferred to six-well plates precoated for 2 h with the coating solution provided by the company and incubated for 48 h before experimental treatments. Yields typically generated >97% purity for CMs.

Fibroblast Cell Culture and Treatments. Rat-1 fibroblasts stably transfected with human α_{1A}-AR cDNA was a gift from GlaxoSmithKline (Uxbridge, Middlesex, UK). The expression level of α_1-AR in this cell line is approximately 1 pmol/mg membrane protein as previously assessed through ligand binding (Gonzalez-Cabrera et al., 2003). Rat-1 fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum, 500 μg/ml G418 (Invitrogen, Carlsbad, CA), 10 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C. Confluent cell monolayers in culture plates were detached by trypsinization and subcultured at a ratio of 1:3. When cells are at 80% confluence, the β-AR blocker propranolol and the α_2-AR blocker rauwolscine (both from Sigma, St. Louis, MO) were added into the cell culture medium at final concentrations of 1 and 0.1 μM, respectively, and incubated for 30 min before adding other agents. For α_1-AR agonist-treated cells, Epi (Sigma) was added into the culture medium at a final concentration of 10 μM, and incubation continued for the designated time periods as described in the figure legends. For treatment with inhibitors or α_1-AR antagonist, cells were preincubated with α_2-AR and β-AR blockers along with each individual inhibitor or α_1-AR antagonist prazosin (1 μM) for 30 min. Different final concentrations were tested based on the literature or the IC_{50} value of each chemical inhibitor. Cytotoxicity assays were performed using the XTT cell viability assay kit (Biotium Inc., Hayward, CA) following the manufacturer's procedures to decide the optimum final concentration(s) of the inhibitors to be used as follows: PKC inhibitor chelerythrine (CH) (Sigma) at 2 μM; NF-κB inhibitor Bay 11-7085 (Bay) (Calbiochem, San Diego, CA) at 1, 2, and 5 μM; PKC inhibitor Go 6983 (Go) (Calbiochem) at 1 μM; Erk inhibitor PD 98059 (PD) (Calbiochem) at 10 μM; p38 MAPK inhibitor SB 203580 (SB) (Calbiochem) at 2, 5, 10 μM; prazosin (Sigma) at 1 μM; and the transcriptional inhibitor actinomycin D (ActD) (Sigma) was at a final concentration of 5 μg/ml.

RNA Extraction and Real-Time RT-PCR. Media were removed from cells by aspiration, and total RNAs were immediately isolated using TRIzol reagent (Invitrogen) following the manufacturer's procedures. cDNAs were made by reverse transcription of total RNA using oligo$(dT)_{20}$ and SuperScript III reverse transcriptase (Invitrogen) following the company's protocol. Real-time RT-PCR amplification was performed using cDNA preparations as templates and SYBR Green Supermix from Bio-Rad (containing real-time polymerase chain reaction buffer, iTag DNA polymerase, dNTPs, SYBR Green I, fluorescein, and stabilizers) using the iCycler System following the company's protocol (Bio-Rad, Hercules, CA). Polymerase chain reaction amplification was performed with an initial 3-min step at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. The fluorescent signal from SYBR Green was detected immediately after the extension step and the threshold cycle (Ct) was recorded. The Ct value from α-tubulin served as an internal control for normalization.

Plasmids and Transfection. A series of rat IL-6 promoter deletion constructs that have been described previously (Franchimont et al., 1997) was kindly provided by Professor Ernesto Canalis (University of Connecticut School of Medicine, Farmington, CT) with the permission of Dr. G. Fey (Friedrich-Alexander Universitat, Erlangen, Germany), who originally made the constructs. These constructs cover different portions of the IL-6 promoter region ranging from 34 base pairs to approximately 3 kilobases upstream of the transcription start site. All constructs were linked to a luciferase reporter gene to detect the promoter activity. A wild-type (WT) construct of p38 MAPK (p38-Wt) and a dominant-negative construct of p38 MAPK (p38-DN) were described previously (Whitmarsh et al., 1995) and kindly provided by Dr. Philip Howe (The Cleveland Clinic Foundation, Cleveland, OH). A construct containing the β-galactosidase gene driven by the cytomegalovirus promoter was used for the normalization of transfection efficiency. All plasmid constructs were transiently transfected into Rat-1 fibroblasts at 80% confluence using Lipofectamine 2000 (Invitrogen) following the manufacturer's

instructions. Cells were incubated for 24 to 48 h after transfection, followed by chemical treatments as described in the figure legends.

Immunoblotting. For total and phosphorylated IκBα determination, proteins were extracted using SDS-based lysis buffer (50 mM Tris, 100 mM dithiothreitol, 2% SDS, and 10% glycerol). Fresh solutions of proteinase inhibitors [final concentrations in lysis buffer: 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 0.15 μM aprotinin, 0.5 mM EDTA, and 1 μM leupeptin] and phosphatase inhibitors (final concentrations in lysis buffer: 10 mM sodium fluoride, 2 mM β-glycerophosphate, 2 mM sodium pyrophosphate decahydrate, and 1 mM sodium orthovanadate) were added to the lysis buffer immediately before use. Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was immunoblotted with antibodies against total IκBα or phosphorylated IκBα (Cell Signaling Technology, Danvers, MA) overnight at 4°C. After removal of blotting solution containing primary antibody, the blot was incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h, and the signal was detected by chemiluminescence (Pierce, Rockford, IL).

p38 Kinase Activity Assay. The Nonradioactive p38 MAP Kinase Assay Kit from Cell Signaling Technology was used to determine p38 MAP kinase activity by measuring phosphorylated ATF-2. In brief, cells were chilled on ice, washed once with ice-cold PBS, and lysed with cold cell lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin) included in the kit for 5 min on ice. Cells were scraped and transferred to cold tubes, sonicated on ice four times for 5 s each, and centrifuged at 14,000*g* for 10 min at 4°C. The supernatants were transferred to fresh tubes, and protein concentrations were determined using protein assay dye reagent from Bio-Rad following the company's instructions. Levels of total p38 MAPK in the cell lysates were determined as input. Approximately 200 μl of cell lysate containing 200 μg of total protein was immunoprecipitated with 20 μl of immobilized phosphor-p38 MAPK antibody overnight at 4°C with gentle rocking. Cell lysate/immobilized antibody was centrifuged at 14,000*g* for 10 min at 4°C, and the resulting pellet was washed twice with 500 μl of cell lysis buffer and kinase buffer (25 mM Tris, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$), respectively. The kinase assay was performed by incubating the immunoprecipitates in 50 μl of kinase buffer supplemented with 200 μM ATP and 1 μg of ATF-2 fusion protein for 30 min at 30°C. The reaction was terminated by adding 30 μl of 3× SDS sample buffer and centrifuged at 14,000*g* for 30 s. The supernatant was loaded onto 10% SDS-polyacrylamide gel electrophoresis gel for immunoblotting. Levels of phosphorylated ATF-2 and phosphorylated p38 MAPK were determined using antibodies that specifically recognize phospho-ATF-2 (Thr71) or phospho-p38 MAPK.

Luciferase Assay. Luciferase activity was determined using The Luciferase Assay System, and β-galactosidase activity was determined using the β-Galactosidase Enzyme Assay System. Both systems were purchased from Promega (Madison, WI). Luciferase assays were performed using flat-bottomed 96-well Microlite plates (Thermo Fisher Scientific, Waltham, MA) using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). The β-galactosidase assay was performed using transparent 96-well plates (Sarstedt Inc., Newton, NC) using a Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) following the manufacturer's instructions.

IL-6 Protein Determination. Protein levels of IL-6 either in cell culture medium or serum was determined by ELISA (sensitivity, 1.6 pg/ml) using the Quantikine kit from R&D Systems (Minneapolis, MN) following the manufacturer's instructions.

Immunocytochemistry. Rat-1 α_{1A}-AR fibroblasts in DMEM were seeded (1×10^5 cells) in 12-well plates containing a sterile circular coverslip coated with poly(L-lysine). Cells were grown to 80% confluence. For the experimental group, α_2-AR and β-AR blockers with or without prazosin (1 μM) were added to each well, and the plate was incubated for 30 min. Epinephrine (10 μM) was then added to the wells, and the incubation continued for an additional 18 h. For the control group, only blockers were added to each well. After incubation, the coverslips were washed three times with PBS, and the cells were fixed overnight in 4% paraformaldehyde at 4°C. After fixation, the paraformaldehyde was removed by washing the coverslip twice with PBS. The coverslips were first incubated with blocking buffer (6% bovine serum albumin and 0.3% Triton X-100) for at least 1 h at room temperature with shaking. Primary rabbit antibody against IL-6 (R&D Systems) was then added to the blocking solution at 5 μg/ml, and incubation continued for 1 day at 4°C. The coverslips were then washed three times with PBS and incubated with goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) at 1:3000 for 1 h at room temperature followed by washes in PBS. Coverslips were mounted in VectaShield medium with 4,6-diamidino-2-phe-nylindole (Vector Laboratories, Burlingame, CA) and sealed with nail polish. Sections were analyzed on a confocal laser-scanning microscope (Leica Microsystems, Inc., Deerfield, IL) representing optical sections of 2- to 3-μm axial resolution and an average of three line scans.

Mouse Serum Collection. Mice were injected with 0.2 ml of sodium pentobarbital solution (50 mg/ml; Lundbeck Inc., Deerfield, IL), and blood samples were collected through the tail vein and set at room temperature for 2 h. Samples were then centrifuged at 5000*g* for 20 min. After centrifugation, serum samples were transferred to a fresh tube, and IL-6 levels were determined immediately or stored at −70°C.

Statistics. One way analysis of variance with Bonferroni's post test was performed using Prism (version 3.0; GraphPad Software Inc., San Diego, CA). *P* values less than 0.05 were considered significant.

Results

α_1-ARs Stimulated IL-6 mRNA Expression in Both Rat-1 Fibroblasts and Neonatal CMs. Our previous studies used microarray gene expression profiling and real-time RT-PCR and indicated that α_1-AR activation stimulated dramatic IL-6 mRNA up-regulation in Rat-1 fibroblasts (Gonzalez-Cabrera et al., 2003; Shi et al., 2006). To determine whether our previous observations from Rat-1 fibroblasts were physiologically relevant, we compared our results from Rat-1 fibroblasts with those from primary neonatal CMs throughout this article.

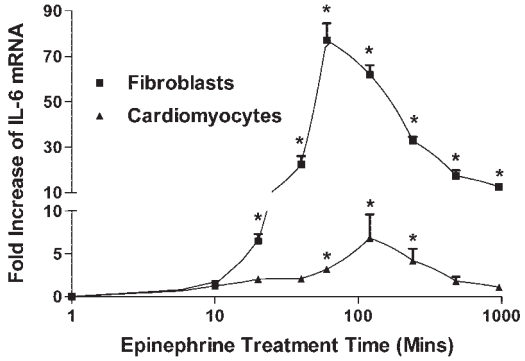

Fig. 1. Time course of IL-6 mRNA expression induced by α_1-AR in Rat-1 fibroblasts or in neonatal mouse cardiomyocytes. Cells were pretreated with the β-AR blocker propranolol (1 μM) and the α_2-AR blocker rauwolscine (0.1 μM) for 30 min and then treated with epinephrine (10 μM) for 0, 10, 20, 40, 60, 120, 240, 480, and 960 min. -Fold increase of mRNA level was assessed by real-time RT-PCR after being normalized by the mRNA level of α-tubulin. Each data point is the mean ± S.E.M. *, significant mRNA increase compared with mRNA levels from cells that receive no epinephrine treatment ($P < 0.05$).

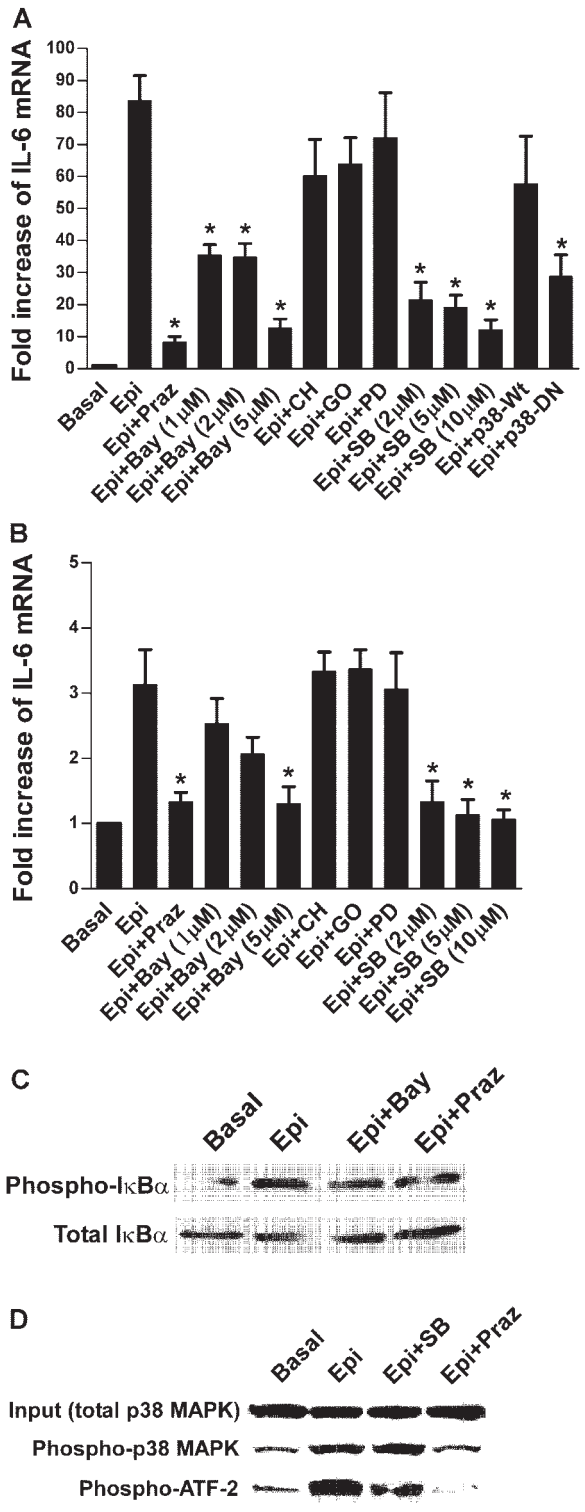

Fig. 2. Signaling pathways involved in α_1-AR-stimulated IL-6 mRNA expression. A, Rat-1 fibroblasts were pretreated with propranolol (1 μM) and rauwolscine (0.1 μM) alone (basal) or along with individual protein or kinase inhibitors or α_1 antagonist (Bay at 1, 2, and 5 μM; CH at 2 μM; Go at 1 μM; PD at 10 μM; SB at 2, 5, and 10 μM; and prazosin at 1 μM) for 30 min and then treated with Epi (10 μM) for 1 h. Alternatively, cells were transfected with a dominant-negative or WT construct of p38 MAPK for 48 h and then pretreated with propranolol and rauwolscine for 30 min, followed by Epi treatment for 1 h. mRNA levels were assessed by real-time RT-PCR after being normalized by the mRNA level of α-tubulin. Data are the mean ± S.E.M. of the -fold increase of mRNA level in each experimental group compared with basal. *, significant difference compared with Epi treated sample ($P < 0.05$). B, neonatal cardiomyocytes were subjected to the same chemical treatments, mRNA level assessment, and data analysis as Rat-1 fibroblasts described in A. C, immunoblots assessing the levels of phospho-IκBα in Rat-1 fibroblasts under the same treatment conditions as described in A. Treatment concentration for Bay 11-7085 is 5 μM. D, phospho-p38 MAPK levels and p38 MAPK kinase activity assessment in Rat-1 fibroblasts under the same treatment conditions as described in A. Treatment concentration for SB 203580 is 10 μM. Praz, prazosin (α_1-AR antagonist).

We first investigated whether α_1-AR activation stimulated IL-6 mRNA expression in primary neonatal CMs. Therefore, we characterized the α_1-AR-mediated regulation of IL-6 mRNA expression in time-course studies. Cells were stimulated with Epi (10 μM), an AR agonist, in the presence of α_2- and β-AR blockers, for various times, and IL-6 mRNA levels were determined via real-time RT-PCR. The results indicated that IL-6 mRNA levels in Rat-1 fibroblasts increased significantly as early as 20 min and peaked near 1 h. Exponential increases in IL-6 mRNA levels were observed up to 1 h (Fig. 1). The increase of IL-6 mRNA in neonatal CMs was slower and not as robust, but it was significant and peaked near 2 h (Fig. 1). For both cell systems, the observed increases in IL-6 mRNA levels were significantly blocked by α_1-AR antagonist prazosin (Fig. 2), confirming that the increase was mediated by α_1-AR activation. We also found that α_1-AR activation increased IL-6 mRNA in H9C2, which is a cell line derived from rat heart muscle tissue (data not shown).

p38 MAPK and NF-κB Mediate IL-6 mRNA Expression Induced by α_1-AR. To investigate the signaling pathways involved in the α_1-AR-stimulated IL-6 mRNA expression, we used specific kinase or protein inhibitors of common α_1-AR-mediated biochemical pathways. Whereas the inhibition of PKC and ERK had no significant effect, the inhibition of NF-κB and p38 MAPK significantly blocked the α_1-AR-induced increase of IL-6 mRNA in either Rat-1 fibroblasts (Fig. 2A) or CMs (Fig. 2B) in a dose-response. The involvement of p38 MAPK was supported by the results using a dominant-negative construct of p38. These results provide evidence for the involvement of NF-κB activity and the p38 MAPK cascades in α_1-AR stimulated IL-6 mRNA expression. Because phosphorylation of IκBα is a key event before NF-κB activation, the specificity of Bay 11-7085 was verified by showing that Bay 11-7085 reduced the phosphorylation of IκBα (Fig. 2C). SB 202580, a specific p38 MAPK activity inhibitor, did not block p38 MAPK phosphorylation (Fig. 2D, middle) under our experimental conditions. Subsequent immunoprecipitation and p38 MAPK kinase assay indicate that SB 203580 suppressed the kinase activity of p38 MAPK (Fig. 2D, bottom). This is typically seen for kinase inhibitors, which compete with ATP for the substrate of the kinase (Davies et al., 2000) rather than blocking phosphorylation and has been previously verified for SB 203580 (Kumar S et al., 1999).

α_1-AR Activation Enhanced IL-6 mRNA Stability. Cytokine mRNA can be regulated through stabilization mechanisms. To study whether the rapid increase in IL-6 mRNA expression by α_1-AR activation involved the regulation of mRNA stability, we performed IL-6 mRNA decay experiments using actinomycin D, a transcription-arresting agent. In Rat-1 fibroblasts, IL-6 mRNA degraded quite rapidly with a half-life of 30 min. When cells were treated with Epi (10 μM), IL-6 mRNA was significantly stabilized (Fig. 3A). This

result demonstrates that the observed α_1-AR-mediated increase in IL-6 mRNA is at least partially due to enhanced IL-6 mRNA stability. To determine what signaling pathways regulated the mRNA stability, we inhibited p38 MAPK, ERK, and PKC kinases and evaluated their effects on IL-6 mRNA stability. The results indicate that none of the kinases tested had significant effect on α_1-AR-mediated IL-6 mRNA stability (Fig. 3A). In case of neonatal CMs, the stabilizing effect of α_1-AR activation was less than fibroblasts but significant, and none of the pathways studied showed effects on IL-6 mRNA stability (Fig. 3B).

α_1-AR Activation Enhances IL-6 Transcription. We determined that the half-life of IL-6 mRNA was approximately 30 min (Fig. 3A), but beyond this time point, an exponential increase in IL-6 mRNA levels in stimulated Rat-1 fibroblasts continued and reached very high levels in 1 h (80-fold). This fact suggested that enhanced mRNA stability was unlikely to account fully for the observed increase in IL-6 mRNA. To study whether α_1-AR activation can stimulate transcription of IL-6, we transiently transfected a series of IL-6 promoter constructs into Rat-1 fibroblasts. We found that α_1-AR activation did enhance IL-6 transcription (Fig. 4). The first increase of transcriptional activity was observed at −105, which includes the promoter elements AP-1 and NF-κB. The second increase of transcriptional activity occurred from −257 to −276, which included the promoter elements simian virus 40 promoter factor 1, nuclear factor for IL-6, CRE, and multiple response element. An even further increase of transcriptional activity was facilitated by promoter elements from −276 to −2906 (Fig. 4). These results suggested that α_1-AR mediated IL-6 transcription through several distinct promoter elements.

p38 MAPK and NF-κB Mediate α_1-AR Transcriptional Regulation of IL-6. We investigated which signaling pathways are involved in α_1-AR-mediated transcriptional regulation of IL-6 expression. For cells transfected with the construct carrying the IL-6 minimal promoter (−34), a basal level of luciferase activity was detected. Basal activity in cells transfected with the construct containing the largest promoter region (−2906) was also increased. But promoter activity was dramatically increased when α_1-ARs were stimulated (Fig. 5). Inhibition of NF-κB by Bay 11-7085 or inhibition of p38 MAPK activity by either SB 203580 or a dominant-negative construct of p38 MAPK significantly blocked the transcription activity induced by α_1-AR activation, whereas PKC inhibition had no effect (Fig. 5).

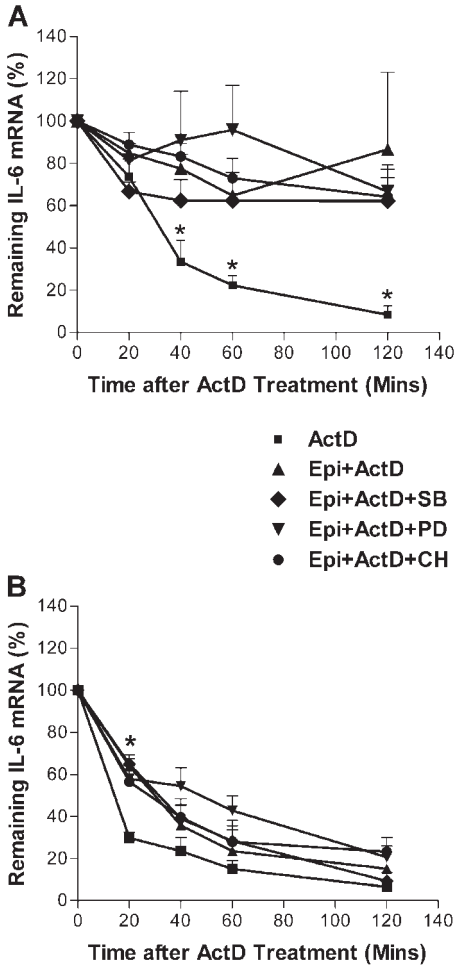

Fig. 3. Stabilization of IL-6 mRNA by α_1-AR activation in Rat-1 fibroblasts (A) or neonatal cardiomyocytes (B). Cells were pretreated with propranolol (1 μM) and rauwolscine (0.1 μM) or along with individual protein or kinase inhibitors (CH at 2 μM, PD at 10 μM, and SB at 2 μM) for 30 min. Cells were then treated with Epi (10 μM) for 1 h followed by ActD treatment (5 μg/ml) for various time periods. The IL-6 mRNA levels after 0, 20, 40, 60, and 120 min of ActD treatment were measured by real-time RT-PCR and normalized with α-tubulin mRNA levels. Each data point is the mean ± S.E.M. ∗, significant difference between ActD treatment alone and Epi treatment followed by ActD treatment at that time point, $P < 0.05$.

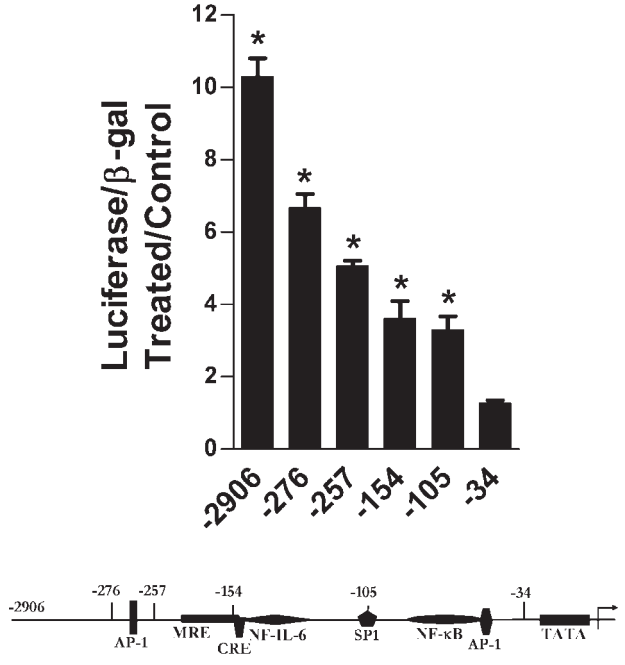

Fig. 4. Effects of α_1-AR activation on IL-6 promoter activity. Rat-1 fibroblasts were transiently transfected with a series of rat IL-6 promoter deletion constructs ranging from 34 to 2906 base pairs upstream of the transcription start site. All constructs were linked to a luciferase reporter gene. A construct containing CMV-β-galactosidase (β-gal) was cotransfected for normalization of transfection efficiency. After 48 h, cells were pretreated with propranolol (1 μM) and rauwolscine (0.1 μM) for 30 min and then treated with epinephrine (10 μM) for 1 h. The cells were harvested and analyzed for luciferase and β-gal activity. Data shown are the ratio expressed as mean ± S.E.M. between each normalized luciferase activity from each deletion construct and the control group transfected with a vector containing the IL-6 minimal promoter (−34). ∗, significant difference compared with the activity from minimal promoter (−34), $P < 0.05$. Location of the known regulatory elements on the rat IL-6 promoter is supplied under the graph.

α_1-AR Stimulated IL-6 Secretion in Both Rat-1 Fibroblasts and Neonatal CMs. Because IL-6 is a secreted protein, we characterized the time course of IL-6 protein secretion into the culture medium via ELISA. For nonstimulated Rat-1 fibroblasts, IL-6 levels in the media were barely detectable. After α_1-AR stimulation, IL-6 levels were first detectable after 4 h and increased significantly with continued stimulation (Fig. 6A). For neonatal CMs, we also observed that α_1-AR activation stimulated IL-6 secretion, but nonstimulated CMs had higher basal levels of secretion than fibroblasts (Fig. 6B).

α_1-AR Induced IL-6 Secretion through Regulating De Novo Protein Synthesis. To investigate whether α_1-AR-induced IL-6 secretion is through regulating new protein synthesis or through the release of IL-6 from storage granules, we performed immunofluorescence cytochemistry to evaluate intracellular IL-6 protein levels. The results indicated that intracellular IL-6 protein levels increased when α_1-ARs were activated, suggesting that IL-6 was not prestored (Fig. 7, A and B). This effect was blocked by prazosin, an α_1-AR antagonist (Fig. 7C).

IL-6 Secretion Induced by α_1-ARs Is Mediated by p38 MAPK and NF-κB. Consistent with signaling pathways regulating the IL-6 transcription, both p38 MAPK and NF-κB inhibition decreased α_1-AR mediated IL-6 secretion (Fig. 8A). Transfection of a dominant-negative construct of p38 MAPK resulted in similar inhibition. Blocking the transcription of IL-6 using actinomycin D dramatically reduced IL-6 secretion, a result consistent with those from immunocytochemistry. This result again suggested that the secreted IL-6 is mainly derived from de novo protein synthesis that is stimulated by α_1-AR activation. Likewise, IL-6 secreted from CMs also seemed to be derived from de novo synthesis because it was inhibited by actinomycin D, and NF-κB and p38 MAPK were the major signals involved (Fig. 8B).

Serum IL-6 Levels Are Increased in Transgenic α_{1A}-AR Mice. To determine whether IL-6 secretion is regulated by α_1-ARs in vivo, we determined serum levels of IL-6 in transgenic mice that systemically overexpress the α_{1A}-AR or α_{1B}-AR subtypes. These mice express constitutively active mutants (CAMs) of the receptors under the control of their endogenous mouse promoter to increase basal subtype-specific signaling in tissues that naturally express that subtype (Zuscik et al., 2000). We found that only the CAM α_{1A} mice had increased IL-6 serum levels, approximately 4-fold more than the levels measured in normal mice (Fig. 9).

Discussion

We have previously demonstrated that activation of α_1-ARs stimulated IL-6 mRNA expression and protein secretion in Rat-1 fibroblasts stably transfected with any of the three α_1-AR subtypes (Gonzalez-Cabrera et al., 2003; Shi et al., 2006). In the present study, we have shown for the first time that α_1-ARs can stimulate IL-6 mRNA expression and protein secretion by regulation of transcription through p38 and NF-κB signaling and mRNA stability through yet unknown

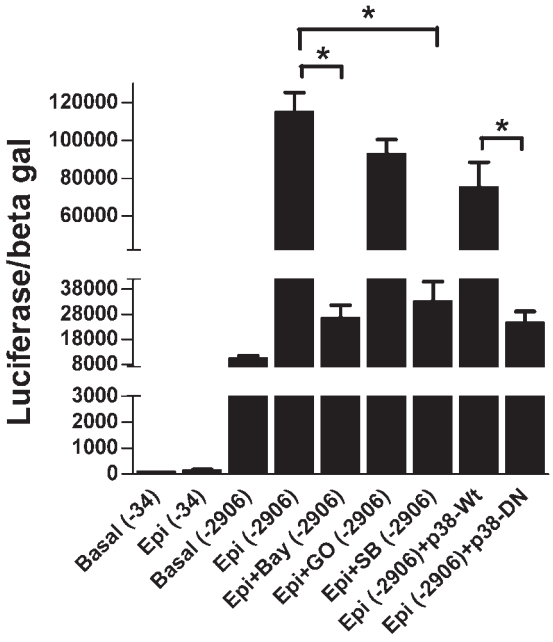

Fig. 5. Signaling pathways involved in α_1-AR-stimulated IL-6 promoter activity. Rat-1 fibroblasts were transiently transfected with a vector containing the IL-6 minimal promoter (−34), the full-length promoter construct (−2906), or full-length promoter construct (−2906) plus either p38-Wt construct or p38-DN construct, in addition to a construct containing CMV-β-gal. After 48 h, cells were pretreated with propranolol (1 μM) and rauwolscine (0.1 μM) alone or along with individual protein or kinase inhibitors (Bay at 5 μM, Go at 1 μM, and SB at 2 μM) for 30 min. Cells were then treated with Epi (10 μM) for 1 h, harvested, and analyzed for luciferase and β-gal activity. Data are the mean ± S.E.M. of normalized luciferase activities. *, significant difference between the indicated comparisons ($P < 0.05$).

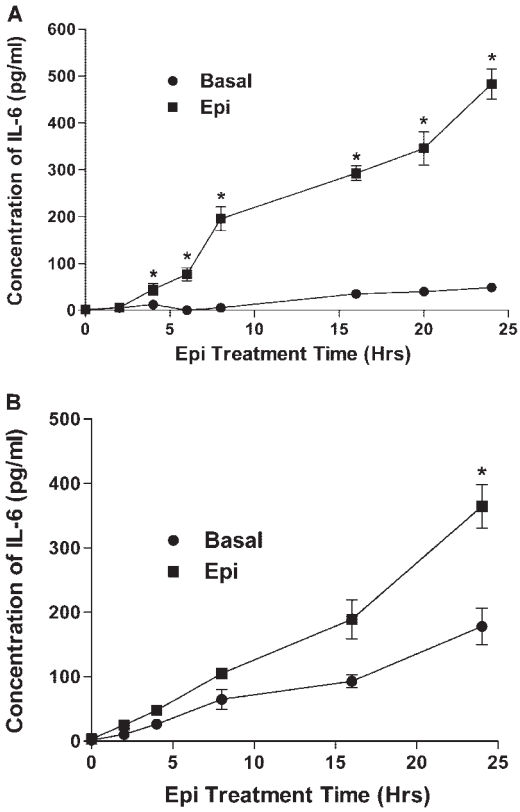

Fig. 6. Effects of α_1-AR activation on IL-6 secretion in Rat-1 fibroblasts (A) or neonatal cardiomyocytes (B). Cells were pretreated with propranolol (1 μM) and rauwolscine (0.1 μM) for 30 min (basal) and then treated with Epi (10 μM) for 0, 1, 2, 3, 4, 5, 6, 7, 8, 16, 20, and 24 h. The IL-6 concentration in the media was measured by ELISA. Data are the mean ± S.E.M. *, significant IL-6 increase compared with the corresponding basal IL-6 level ($P < 0.05$).

signals (Figs. 1–3, 5, and 8). Although previous work stimulating β-ARs in CMs demonstrated the increase of IL-6 mRNA, the effect was purely transcriptional through a cAMP response (Rohrbach et al., 2007). The differences between fibroblasts and CMs in the ability of α_1-ARs to stimulate IL-6 expression and secretion are most likely due to the differences in receptor expression levels (~1 pmol/mg in rat fibroblasts versus 0.05 pmol/mg in primary CMs). α_1-AR modulation of IL-6 expression is probably a common phenomenon in many cell types that express α_1-ARs.

Regulation of mRNA stability can be a major control point in gene expression, especially for transiently expressed genes that require tightly controlled mRNA levels. Studies have revealed that many cytokine mRNAs, which are often short-lived, contain adenine- and uridine-rich sequences known as AU-rich elements (AREs) that confer instability to otherwise stable mRNA (Chen and Shyu, 1995). Although many studies have focused on transcriptional initiation and promoter functions of the IL-6 gene, relatively few studies have addressed post-transcriptional regulation of IL-6 mRNA, and nothing is known about AR regulation of IL-6 mRNA stability. In the present study, we demonstrated for the first time that enhancing IL-6 mRNA stability is part of the mechanism that determines how ARs increase IL-6 mRNA expression (Fig. 3) and that subsequently leads to enhanced IL-6 protein production and secretion.

Evidence suggests that stimulus-induced stabilization of ARE-containing mRNAs is regulated via signaling pathways involving various phosphoproteins (Winzen et al., 1999; Ming et al., 2001). These signals could include the common α_1-AR mediators PKC, MAPKs, and their downstream effectors such as MK-2 (Chen and Shyu, 1995; Winzen et al., 1999; Kontoyiannis et al., 2001) that control mRNA stabilization by regulating interactions between sequence elements and RNA binding proteins. However, our results did not identify any of these pathways in regulating α1-AR mediated IL-6 mRNA stability (Fig. 3), even though p38 regulated IL-6 transcription (Fig. 5). Our result is in contrast with a recent report

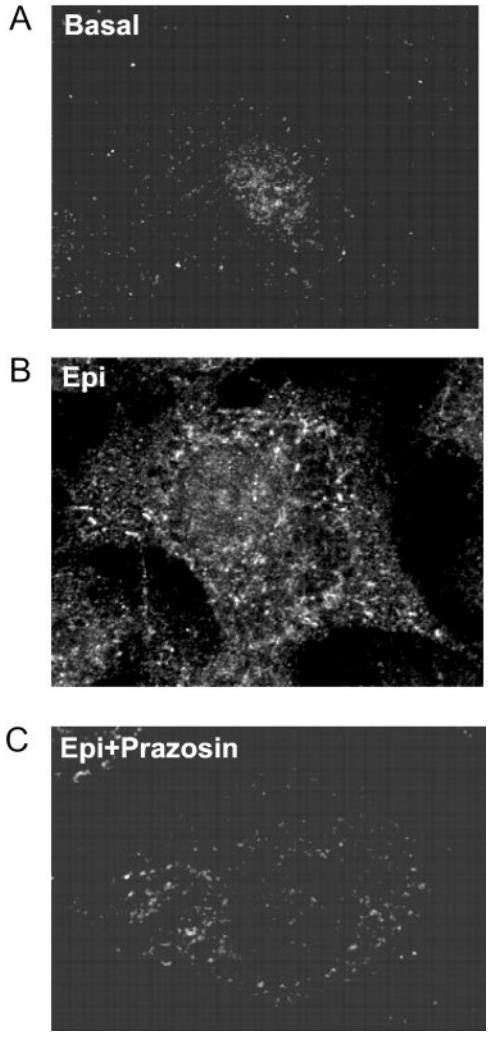

Fig. 7. Effects of α_1-AR activation on intracellular IL-6 levels. Rat-1 fibroblasts were treated with propranolol (1 μM) and rauwolscine (0.1 μM) for 18 h (A, basal), pretreated with β- and α_2-AR blockers for 30 min followed by Epi (10 μM) treatment for 18 h (B, Epi), or pretreated by β- and α_2-AR blockers and prazosin (1 μM) for 30 min followed by Epi (10 μM) treatment for 18 h (C, Epi + Prazosin). Cells were then fixed and incubated with anti-rabbit IL-6 followed by a goat anti-rabbit antibody conjugated with Fluor 488. Cells were analyzed on a confocal laser-scanning microscope representing optical sections of 2 to 3 μm axial resolution and an average of three line scans.

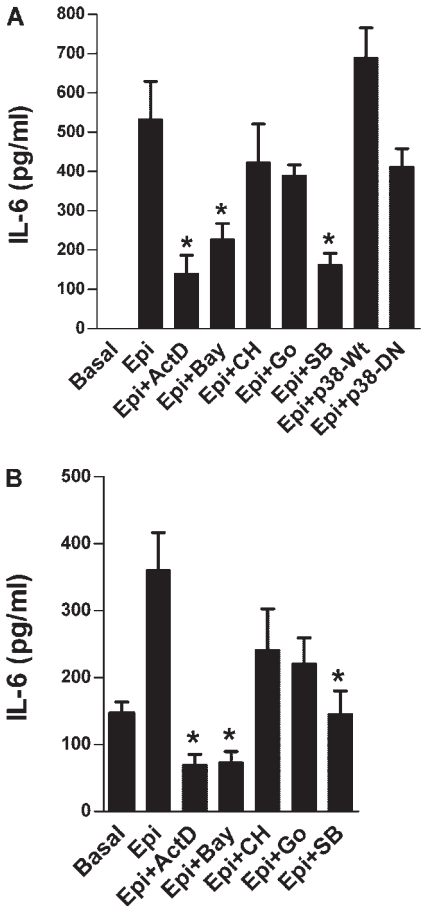

Fig. 8. Signaling pathways involved in α_1-AR stimulated IL-6 secretion in Rat-1 fibroblasts (A) or neonatal cardiomyocytes (B). Cells were pretreated with propranolol (1 μM) and rauwolscine (0.1 μM) alone or along with individual protein or kinase inhibitors (Bay at 5 μM, CH at 2 μM, Go at 1 μM, and SB at 2 μM) or ActD at 5 μg/ml for 30 min and then treated with Epi (10 μM) for 24 h. Alternatively, cells were transfected with a dominant-negative or a WT construct of p38 MAPK for 48 h and then treated with β- and α_2-blockers for 30 min followed by Epi treatment (10 μM) for 24 h. IL-6 levels in the medium were determined by ELISA. Data are mean ± S.E.M. *, significant difference compared with Epi treated sample ($P < 0.05$).

indicating that p38α significantly stabilized IL-6 mRNA in mouse embryonic fibroblasts under IL-1β stimulation (Zhao et al., 2008), suggesting that ARs use a different signaling mechanism than cytokines to regulate IL-6 mRNA.

In addition to IL-6 mRNA stability, transcriptional regulation was another contributing factor to the increased IL-6 mRNA expression and protein secretion mediated by α_1-ARs. We focused on NF-κB because the IL-6 promoter contains binding sites for the NF-κB protein (Sehgal et al., 1989), which has been previously linked to IL-6 regulation (Sehgal et al., 1989; Craig et al., 2000; Kobayashi et al., 2003). Studies also implicated that NF-κB can be regulated by α_1-ARs (Meldrum et al., 1997; Zhong et al., 2001). Our data suggest that besides NF-κB, other transcriptional elements such as AP-1 and CRE may contribute to α_1-AR mediated regulation of IL-6 transcription (Figs. 4 and 5). A recent study indicated that activation of β-ARs induced IL-6 expression in neonatal CMs through NF-κB, AP-1, and CRE (Rohrbach et al., 2007). Our results indicate that besides these transcription factors, p38 MAPK is also critical for α_1-AR-mediated IL-6 expression (Figs. 2, 5, and 8). Exactly how p38 and NF-κB regulate IL-6 transcription under our study system and conditions is unclear. It has been previously shown that p38 MAPK can regulate tumor necrosis factor-induced IL-6 gene expression by modulating a direct repressive effect on the transactivation potential of the p65 κB subunit (Vanden Berghe et al., 1998).

Cytokines can be stored in secretory granules and released quickly; however, α_1-AR-mediated secretion of IL-6 was detectable only after long stimulation (>4 h, Fig. 6). In addition, intracellular IL-6 was found by immunofluorescence to only increase when stimulated (Fig. 7), and secretion was reduced when transcription was arrested with actinomycin D (Fig. 8). These results support the conclusion that α_1-AR-mediated IL-6 secretion is derived mainly from de novo synthesis instead of a quick-release mechanism from granules (Tanda et al., 1998; Mahmudi-Azer et al., 2000).

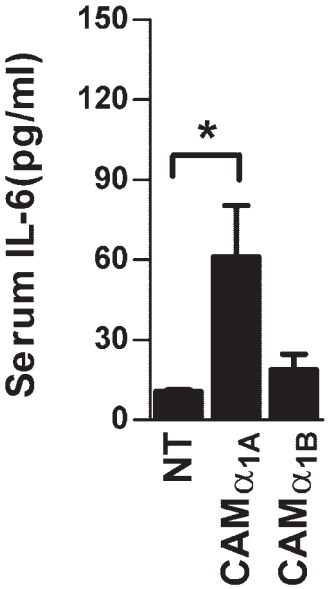

Fig. 9. Serum IL-6 levels in transgenic mice expressing CAMs of the α_1-adrenergic receptor subtype α_{1A} (CAMα_{1A}) or subtype α_{1B} (CAMα_{1B}). The expressions of CAMs of α_1-adrenergic receptors are under the control of endogenous mouse promoter. Blood samples were collected from 3- to 4-month-old mice and stored at room temperature for 2 h, centrifuged, and serum IL-6 levels were determined by ELISA. Data are the mean $\pm$ S.E.M. $*$, significant difference between the indicated comparisons ($P < 0.05$). NT, nontransgenic control.

Although IL-6 can be proinflammatory and maladaptive to cardiac functions in stressed myocardium when sustained at high concentrations (Mann 1996), low concentrations of IL-6 as typically induced by catecholamines (Rohrbach et al., 2007) can regulate antiapoptotic survival pathways in CMs (Matsushita et al., 2005) and prevent the onset of heart failure (Hirota at al., 1999). Although most cytokines are usually associated with inflammation, the level of circulating IL-6 increases in response to exercise and declines in the period after exercise (Pedersen and Hoffman-Goetz, 2000). The fact that the classic proinflammatory cytokines, tumor necrosis factor-α and IL-1β, do not increase with exercise indicates that the IL-6 cytokine cascade induced by exercise, and perhaps by α_{1A}-ARs, markedly differs from the cytokine cascade induced by infections. For instance, IL-6 can induce a phosphatidylinositol-3-kinase and NO-dependent protection of cardiomyocytes (Smart et al., 2006). It seems that only CAM α_{1A}-AR mice, which show cardioprotective phenotypes (Huang et al., 2007), have increased serum IL-6 levels. Although we do not know all of the sources contributing to the elevated serum IL-6, the CAM α_{1A}-AR mice show no signs of inflammation. We speculate that the long-term and moderate release of IL-6 into the serum of CAM α_{1A} mice (Fig. 9) may be adaptive and beneficial to cardiac functions, because CAM α_{1A}-AR mice were protected against myocardial ischemia and regulated an antiapoptotic survival signal in CMs (Rorabaugh et al., 2005). Therefore, the α_{1A}-AR regulation of IL-6 presented here may represent an adaptive mechanism during cardiac pathophysiology.

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