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Angiotensin II Interferes with Leukemia Inhibitory Factor-Induced STAT3 Activation in Cardiac Myocytes

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Recently, we reported that leukemia inhibitory factor (LIF), a member of the interleukin (IL)-6 cytokine family, transduced hypertrophic and cytoprotective signals via Janus Kinase-signal transducer and activator of transcription (JAK-STAT) pathway in cardiac myocytes. Angiotensin II (AII) is also known to activate STATs and reported to induced apoptosis in adult rat ventricular myocytes. In the present study, we investigated potential interactions between gp130 dependent and AII signaling pathways, by examining AII regulation of LIF-induced anti-apoptotic effect and STAT3 activation in cardiac myocytes. Although LIF attenuated the DNA fragmentation induced by serum depletion, AII augmented the DNA fragmentation in cultured neonatal rat cardiac myocytes. Furthermore, LIF-mediated cytoprotective effect was inhibited by AII pretreatment. LIF rapidly and transiently tyrosine phosphorylated STAT3 in cardiac myocytes which was not observed by AII. AII pretreatment inhibited LIF-induced phosphorylation of STAT3 in a dose dependent manner. This inhibitory effect of AII on STAT3 activation was blocked by the AII type I (AT1) receptor antagonist CV11974. These results demonstrate that negative crosstalk between gp130 and AT1 receptor dependent signaling exists in cardiac myocytes. This crosstalk may contribute to the modulation of pathophysiological process in myocardial disease.

We and others have reported that interleukin (IL)-6 related cytokines, such as cardiotrophin (CT)-1 and leukemia inhibitory factor (LIF), transduce hypertrophic signals through gp130 in cardiac myocytes (1–4). The signaling pathway downstream of gp130 is reported to consist of two distinct pathways, one a Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway and the other a mitogen-activated protein kinase (MAPK) pathway (5). More recently, Kunisada et al. transfected wild-type and mutated-type STAT3 to cardiac myocytes to show that the JAK-STAT pathway, especially the STAT3-mediated pathway, played a critical role in gp130-dependent myocardial hypertrophy (6). On the other hand, Fujio et al. reported the ability of gp130 signaling to exert cytoprotective effect in cardiac myocytes, resulting from upregulation of bcl-x gene expression via STAT1-binding *cis*-element (7).

Angiotensin II (AII) is known to have a variety of effects on the cardiovascular system, including promotion of myocardial hypertrophy (8, 9). Recent reports provide evidence that AII activates not only the MAPK but also the JAK-STAT pathways directly through AII type 1 (AT1) receptors in smooth muscle cells and in cardiac myocytes (10, 11). Another report using $AT1_A$ receptor expressing cells (CHO-K1 cells) demonstrated the ability of AII to transiently inhibit IL-6-induced STAT3 tyrosine phosphorylation (12). In addition, AII is reported to induce apoptosis of adult rat ventricular myocytes and human endothelial cells (13, 14).

Although much progress has been made in elucidating the intracellular signal transduction pathways for inducing hypertrophy or protection against apoptosis in cardiac myocytes, much less is known about the crosstalk between distinct signaling induced by two separate stimuli. In the present study, we investigated potential interactions between gp130 dependent and AII signaling pathways in cardiac myocytes and demonstrated negative regulation of LIF-induced STAT3 tyrosine phosphorylation by AII via the AT1 receptor.

MATERIALS AND METHODS

Reagents. Murine recombinant LIF (10^6 U/ml) from AMRAD Biotech (Boronia, Australia) and human Angiotensin II (Sigma Chemical Co.) were used in this study. MTT [3- (4,5-Dimethyl-2-thiazolyl) -2, 5-diphenyltetrazolium Bromide] was purchased from NACALAI TESQUE, Inc. (Kyoto, Japan). Rabbit anti-STAT3 antibody was purchased from Santa Cruz Biotechnology Inc. and mouse anti-

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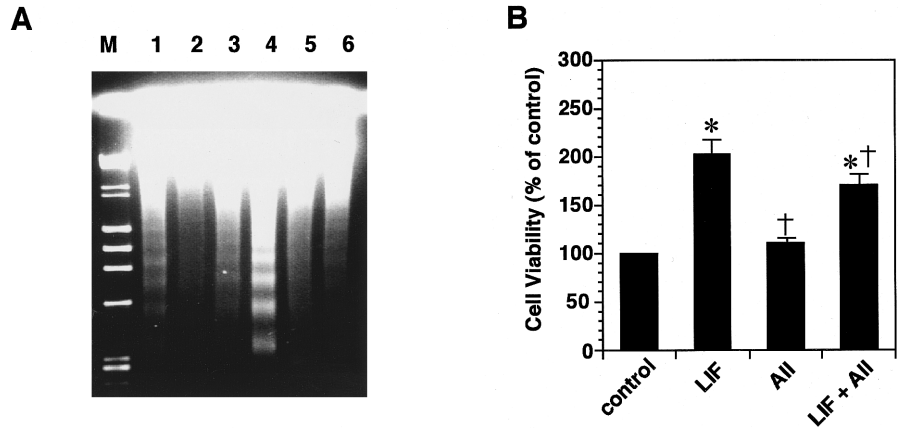

FIG. 1. Effect of AII on LIF-mediated cytoprotection. (A) Electrophoretic pattern of low molecular weight DNA fragments extracted from cultured neonatal rat cardiac myocytes incubated with either medium-199 alone (lane 1), or medium-199 supplemented with 10 % newborn calf serum (lane 2), combination with 10 % newborn calf serum and 1 μM AII (lane 3), 1 μM AII (lane 4), 1×10^3 U/ml LIF (lane 5), or combination with 1×10^3 U/ml LIF and 1 μM AII (lane 6). Two μg of DNA sample is electrophoresed in each lane. Representative result is presented from four independent experiments. M: molecular weight markers of ϕ X174/*Hae* III digest. (B) Cell viability measured by MTT assay. Cardiac myocytes were incubated with either medium-199 (control), 1×10^3 U/ml LIF, 1 μM AII, or combination of both 1×10^3 U/ml LIF and 1 μM AII for 24 hours. The cells were lysed and MTT assay was performed as described in the Methods. Results are expressed as percent of control. Data are mean ± SD from six samples. *$P<.05$ vs control; †$P<.05$ vs LIF.

phosphotyrosine monoclonal antibody (4G10) from Upstate Biotechnology Inc.. The AT1 receptor antagonist CV11974 was kindly provided by Takeda Co. Ltd. (Osaka, Japan).

Cell culture. Primary cultures of neonatal rat cardiac myocytes were prepared from the ventricles of 1-day-old Sprague-Dawley rats obtained from Nippon Dobutsu (Osaka, Japan) as described previously (1). Cardiac myocytes were suspended in medium-199 supplemented with 10 % newborn calf serum and 0.1 mM bromodeoxyuridine, plated onto 35- or 60-mm plastic culture dishes at a concentration of 5×10^2 cells/mm^2 and cultured in 95 % air/5 % CO_2 at 37°C for 48 hours. Before the experiments, cardiac myocytes were cultured in serum-free medium for 24 hours.

DNA fragmentation and MTT assay. Cardiac myocytes were washed twice with ice-cold phosphate buffered saline (PBS), and lysed in 0.6 % sodium lauryl sulfate (SDS), 0.1 % ethylenediamine-tetraacetic acid (EDTA), pH 8.0; and 0.3 mM NaCl over 8 hours at 4 °C, followed by the centrifugation (15,000 rpm for 15 minutes at 4 °C). The supernatants were incubated with 1 mg/ml RNase A for 90 minutes at 45 °C, addition of 200 μg/ml proteinase K for another 60 minutes, and extracted with phenol and chlorofolm. Two μg of DNA was electrophoresed on a 2 % agarose gel and visualized by staining with ethidium bromide. MTT assay was performed as described previously (15). Briefly, cardiac myocytes cultured in 96- well plates (2×10^4 cells/well) were incubated with 0.5 mg/ml MTT for 5 hours. The cell culture medium was removed, cells were lysed in 100 μl of dimethyl sulfoxide (DMSO), and the amount of MTT was photometrically determined.

Immunoprecipitation and western blotting. Cardiac myocytes were washed with ice-cold TBS buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; and 1 mM sodium orthovanadate), homogenized, lysed in 20 mM Tris-HCl, pH 7.4; 1 % NP-40, 0.1 % SDS, 150 mM NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 1 mM sodium orthovanadate and 1 mM PMSF, and centrifuged as described previously (1). The supernatants were immunoprecipitated with anti-STAT3 antibody and the immunoprecipitants were stored at 80°C until assay. Western blot analyses were performed as described previously (1). The immune complexes were visualized with Kodak X-OMAT-AR film using the enhanced chemiluminescence detection system (Amersham International PLC).

Statistical analysis. Statistical analyses were performed by use of Student's *t* test. Values of $P<.05$ were considered to be statistically significant.

RESULTS

Effect of AII on LIF-mediated Cytoprotection

Since an internucleosomal DNA fragmentation pattern is a biochemical marker of apoptosis, a DNA agarose gel electrophoresis of low molecular weight DNA fragments was evaluated. Although DNA fragmentation was not observed in cardiac myocytes incubated in the medium supplemented with serum (Fig. 1A, lane 2), distinctive ladder was observed when the cells were incubated without serum for 24 hours (Fig. 1A, lane 1). DNA fragmentation induced by serum depletion was attenuated by LIF (Fig. 1A, lane 5), however, AII treatment significantly augmented DNA fragmentation both in serum-supplemented and -free medium (Fig. 1A, lanes 3 and 4, respectively). In addition, LIF-mediated attenuation of DNA fragmentation was deteriorated by combination with AII (Fig. 1A, lane 6).

As shown in Fig. 1B, we further examined cell viability using MTT assay for the quantitative analysis of cardiac myocytes apoptosis. LIF significantly promoted but AII did not affect the cardiac myocytes viability that was evaluated in serum-depleted medium. Treatment with AII significantly inhibited LIF-induced cytoprotective effect ($P<.05$).

Dose-Dependent Inhibition of LIF-Induced STAT3 Tyrosine Phosphorylation by AII

The evidence that interference of LIF-induced cytoprotective effect by AII suggests the existence of crosstalk between gp130 dependent and AII signaling pathways in cardiac myocytes. Fig. 2A shows the time course of LIF-induced tyrosine phosphorylation of STAT3 in cardiac

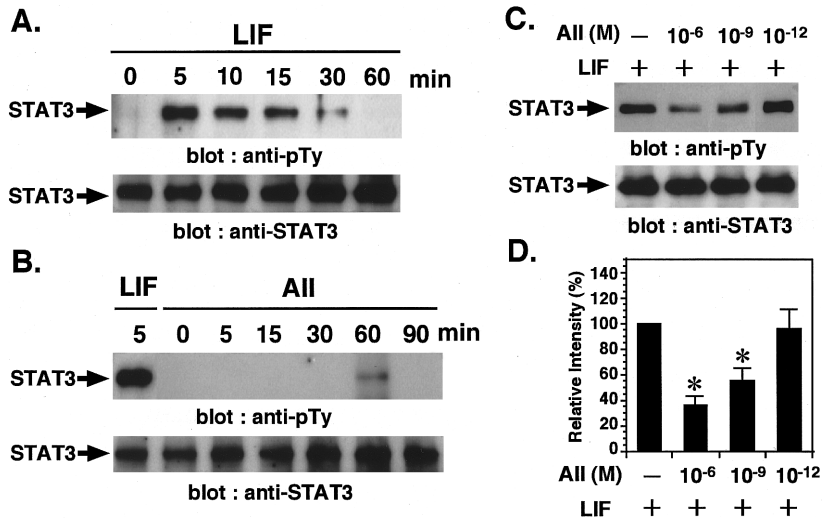

FIG. 2. Dose-dependent inhibition of LIF-induced STAT3 tyrosine phosphorylation by AII. Cultured neonatal rat cardiac myocytes were stimulated with 1×10^3 U/ml LIF (A) or 1 μM AII (B) for the time indicated. Cardiac myocytes were pretreated with indicated concentrations of AII for 15 minutes and then stimulated with LIF for 5 minutes (C). The cell lysates were immunoprecipitated with anti-STAT3 antibody and immunoblotted with anti-phosphotyrosine antibody (top). The blot was reprobed with anti-STAT3 antibody (bottom). Representative blot is presented from six independent experiments. Phosphorylated STAT3 relative to immonoprecipitated STAT3 were quantified by densitometric analyses corresponding Fig. 2C (D). Results are expressed as percent of LIF (+). Data are mean ± SD from six samples. *$P<.05$ vs LIF (+).

myocytes. STAT3 was phosphorylated within 5 minutes after LIF (1×10^3 U/ml) stimulation, gradually dephosphorylated by 30 minutes and returned to the baseline after 60 minutes (Fig. 2A, top). We next examined whether STAT3 was tyrosine phosphorylated in the cardiac myocytes stimulated with AII. As shown in Fig. 2B, AII (1 μM) stimulation did not phosphorylate STAT3 in the early stages from 5 to 30 minutes. However, weak transient phosphorylation was observed at 60 minutes after AII stimulation (Fig. 2B, top).

To investigate the potential interactions between gp130 dependent and AII signaling pathways, cardiac myocytes were pretreated with different concentrations of AII for 15 minutes and then stimulated with LIF for 5 minutes. Pretreatment of cardiac myocytes with AII significantly inhibited LIF-induced phosphorylation of STAT3 in a dose-dependent manner (Figs. 2C and 2D). The amounts of immunoprecipitated STAT3 were the same for each lane (Figs. 2A, 2B and 2C, bottom).

CV11974 Blocks the AII-mediated Inhibition of LIF-Induced STAT3 Tyrosine Phosphorylation

To clarify the molecular mechanisms of the interference of LIF-induced STAT3 activation by AII, we examined the effects of CV11974, an AT1 receptor antagonist, on the AII-mediated inhibition of LIF-induced STAT3 tyrosine phosphorylation. Cardiac myocytes were preincubated with 1 μM CV11974 for 40 minutes before the addition of AII and LIF. Although treatment of cardiac myocytes with 1 μM AII for 15 minutes resulted in a 74% inhibition of STAT3 phosphorylation (Fig. 3, lane 2; $P<.05$ vs lane 1), preincubation with CV11974 completely blocked the inhibitory effects of AII (Fig. 3, lane 3; $P<.05$ vs lane 2). The amounts of immunoprecipitated STAT3 were the same for each lane (Fig. 3, middle panel).

DISCUSSION

Although both gp130 dependent and AII signaling pathways are well known to phosphorylate STATs and play important roles in myocardial hypertrophy, distinct evidence has been found with respect to apoptosis. In the present study, DNA fragmentation, as a marker of apoptosis, induced by serum depletion was attenuated with LIF treatment but augmented with AII treatment. LIF treatment significantly improved the cell viability which was measured by MTT assay. However, this effect was interfered with AII treatment; i.e., 15 % reduction in cell viability ($P<.05$ vs LIF). The reduction in LIF-promoted cell viability by AII pretreatment suggests the negative modulation of LIF-mediated cytoprotective effect by AII.

The STATs family is known to be activated rapidly after the ligand occupation of a specific receptor. Although rapid STAT3 tyrosine phosphorylation was observed after LIF stimulation, AII-induced STAT3 phosphorylation occurred not in the early stages but in the late stage at 60 minutes in cardiac myocytes. This observation suggests that AII does not directly activate STAT3. The present study described the ability of AII to inhibit LIF-induced STAT3 tyrosine phosphorylation in cardiac myocytes via AT1 receptors for the first time. Epidermal growth factor mediated serine phos-

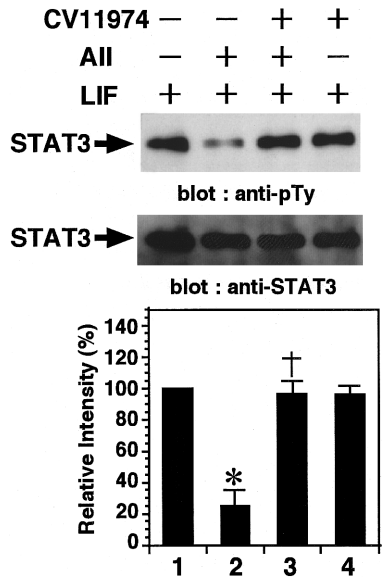

FIG. 3. CV11974 blocks the AII-mediated inhibition of LIF-induced STAT3 tyrosine phosphorylation. Cultured cardiac myocytes were stimulated with LIF (1×10^3 U/ml) for 5 minutes (lane 1), first treated with AII (1 μM) for 15 minutes and then stimulated with LIF for 5 minutes (lane 2), pretreated with CV11974 (1 μM) for 40 minutes before the treatment with AII (15 minutes) and LIF (5 minutes) (lane 3), or pretreated with CV11974 (1 μM) for 40 minutes before the stimulation with LIF (lane 4). The cell lysates were immunoprecipitated with anti-STAT3 antibody and immunoblotted with anti-phosphotyrosine antibody (top). The blot was reprobed with anti-STAT3 antibody (middle). Representative blot is presented from six independent experiments. Phosphorylated STAT3 relative to immonoprecipitated STAT3 were quantified by densitometric analyses (bottom). Results are expressed as percent of LIF (+). Data are mean ± SD from six samples. *$P<.05$ vs LIF (+); †$P<.05$ vs LIF (+) AII (+).

phorylation of STAT3 through extra-cellular signal regulated kinases is known to negatively modulate STAT3 tyrosine phosphorylation (16). Recently, AII has been reported to stimulate serine phosphorylation of STAT3 through a MAPK kinase dependent pathway in rat cardiac fibroblasts and CHO-K1 cells (17). AII may also stimulate serine phosphorylation of STAT3 in cardiac myocytes and this may result in the inhibition of LIF-induced STAT3 tyrosine phosphorylation.

In addition, IL-6-induced STAT3 tyrosine phosphorylation was found to be inhibited by the calcium ionophore ionomycin, increasing the association of JAK1 with protein tyrosine phosphatase 1D (18). AII elevates intracellular Ca^{2+} and may increase the association of JAK family kinases with protein tyrosine phosphatase. We examined the effects of intracellular calcium mobilization on LIF-induced STAT3 tyrosine phosphorylation. Pretreatment with calcium cheleter augmented LIF-induced STAT3 tyrosine phosphorylation, while ionomycin attenuated (data not shown). Interestingly, Kajstura et al. recently reported that AII induces apoptosis of adult rat ventricular myocytes by a mechanism involving protein kinase C-mediated increases in cytosolic calcium via AT1 receptor (13).

Although further study is necessary to clarify detail molecular mechanisms of AII-mediated inhibition of LIF-induced STAT3 tyrosine phosphorylation, evidence of modulatory crosstalk between these pathways would provide novel relevance in pathophysiological conditions such as myocardial hypertrophy or infarction. Present study demonstrated, for the first time, the existence of negative crosstalk between gp130 and AT1 receptor dependent signaling in cardiac myocytes.

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