Biphasic Activation of the JAK/STAT Pathway by Angiotensin II in Rat Cardiomyocytes

Hiroaki Kodama, Keiichi Fukuda, Jing Pan, Shinji Makino, Motoaki Sano, Toshiyuki Takahashi, Shingo Hori, Satoshi Ogawa

Abstract—This study was designed to demonstrate the characteristic pattern of angiotensin II–induced JAK/STAT (indicating just another kinase/signal transducer and activator of transcription) activation in cultured rat cardiomyocytes by comparing it with leukemia inhibitory factor (LIF)-induced activation. Angiotensin II (10^{-7} mol/L) induced rapid phosphorylation of JAK2 and Tyk2, but not JAK1, and phosphorylated STAT1 and STAT2, but not STAT3, in the early stage up to 30 minutes. The time course of JAK/STAT activation by angiotensin II was apparently slower than that by LIF. Interestingly, angiotensin II phosphorylated STAT3 and phosphorylated STAT1 in the late stage at 120 minutes. We also found that angiotensin II induced the formation of interferon-stimulating gene factor (ISGF) complexes biphasically, in the early stage at 15 to 30 minutes and in the late stage at 120 minutes, and that angiotensin II induced delayed activation of the sI-1 inducing factor (SIF) complex at 120 minutes. Formation of ISGF and SIF complexes in response to angiotensin II paralleled the phosphorylation pattern of STAT1 and STAT3 and was quite different from those obtained in response to LIF. The phosphorylation of STAT1 was suppressed by pretreatment with the angiotensin II type-1 (AT_{1}) receptor antagonist CV11974, but the delayed addition of CV11974 failed to suppress phosphorylation of STAT3 at 120 minutes. In conclusion, angiotensin II–induced JAK/STAT activation in rat cardiomyocytes is biphasic and entirely different from LIF-induced activation. (Circ Res. 1998;82:244-250.)

Key Words: angiotensin II ▪ cardiomyocyte ▪ cardiac hypertrophy ▪ JAK/STAT pathway ▪ signal transduction

Angiotensin II has multiple physiological effects on the cardiovascular, endocrine, and nervous system that are initiated by binding to G-protein–coupled specific receptors located on the plasma membrane.1-3 Although both AT_{1} and AT_{2} receptor subtypes are equally distributed on cardiomyocytes,4-6 a number of previous studies have revealed that most of the biological response to Ang II is mediated by the AT_{1} receptor subtype.7-12 Ang II directly stimulates heart rate, cardiac contractility, and the growth of cardiac fibroblasts and cardiomyocytes.13-15 It also activates transcription of proto-oncogenes (such as c-fos, c-jun, c-myc, and Egr-1), genes encoding extracellular matrix proteins (such as collagen and fibronectin), and growth factors (such as platelet-derived growth factor and transforming growth factor-\(\beta\)).16-18 Induction of gene expression usually requires second messenger–mediated activation of transcription factors. AT_{1} receptors transactivate phospholipase C\(\beta\) via G protein and increase inositol triphosphate, which in turn increases Ca\(^{2+}\) release from endoplasmic reticulum, and diacylglycerol, which activates protein kinase C.19 Ang II is also known to activate tyrosine kinases, such as p125\(^{\text{FAK}}\), p46\(^{\text{SHC}}\), and p56\(^{\text{SHC}}\), and to induce serine/threonine kinases, such as mitogen-activated protein kinase (extracellular signal–regulated kinases 1 and 2).20,21 The AT_{1} receptor also stimulates phospholipase A\(_{2}\) and phospholipase D.22 The results of these studies indicate that Ang II can induce rapid changes in gene expression that may ultimately lead to increased cell growth.

Recent studies have revealed that Ang II stimulates the JAK/STAT pathway, including JAK2, Tyk2, STAT1, and STAT2, via the AT_{1} receptor in rat aortic smooth muscle cells.23 The JAK/STAT pathway was initially discovered as a major signal transduction pathway of the cytokine superfamilies.24-27 The AT_{1} receptor28,29 is a seven-transmembrane–type G-protein–coupled receptor and is quite different from members of the cytokine receptor superfamilies. Because the configuration of the AT_{1} receptor is completely different from the receptors of other cytokines, the activation mechanism or modality of the JAK/STAT pathway may be different for Ang II and other cytokines. Cytokine receptors can directly bind to JAK family kinases. When ligands bind to the cytokine receptor, JAK kinases are immediately activated by tyrosine phosphorylation. Interestingly, Bhat and colleagues\(^{30,31}\) have reported that Ang II causes delayed activation of SIF in CHO-K1 cells expressing stably transfected AT_{1A} receptors and have suggested that there is a special relationship between the IL-6 family of cytokines and the Ang II signaling pathway. The
relation between activation of the JAK/STAT pathway and transactivation of the SIF complex by Ang II is not well understood to date.

We recently reported that LIF, a member of the IL-6 family of cytokines, induced cardiac hypertrophy, activated the JAK/STAT pathway, and caused a gel mobility bandshift of SIE in cultured rat cardiomyocytes. Because Ang II is known to be a critical factor that functions as an autocrine/paracrine hypertrophic growth factor for cardiomyocytes, the precise mechanism of the JAK/STAT signal transduction pathway by Ang II in cardiomyocytes should be clarified. In the present study, we report unique characteristic activation patterns of the JAK/STAT pathways and gel mobility shifts induced by Ang II, which were entirely different from those induced by LIF, in cardiomyocytes.

Materials and Methods

Cell Culture

Primary cultures of cardiomyocytes were prepared from the ventricles of 1-day-old Wistar rats (Clea Japan Inc, Tokyo, Japan) by enzymatic digestion in 0.03% trypsin, 0.03% collagenase, and 20 μg/mL DNase I. The cardiomyocytes were collected by differential adherence. Cells were seeded at a density of 1×10^5 cells/cm² on gelatin-coated dishes. The cells were grown in medium 199/DMEM supplemented with 10% fetal bovine serum and penicillin (50 μg/mL)/streptomycin (50 μg/mL) at 37°C in humid air with 5% CO₂. Twelve hours after plating, bromodeoxyuridine was added to the medium at a concentration of 10⁻⁶ M for 12 hours to deplete residual metabolite of TCV116. For the protein binding analysis, the same buffer without glycerol, SDS, and proteinase inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μmol/L dithiothreitol) supplemented with proteinase and phosphatase inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μmol/L leupeptin, 10 μmol/L aprotinin, 1 mmol/L NaF, and 1 mmol/L Na3VO4) incubated for 10 minutes on ice, and sedimented. The nuclei were then resuspended in 2 vol of the same buffer, homogenized (Dounce homogenizer), and sedimented at 1000g for 10 minutes, and the pellet (nuclei) was collected. The pellet nuclei were incubated for 30 minutes at 4°C in high-salt buffer (20 mmol/L HEPES [pH 7.9], 1 M NaCl, 0.5 M dithiothreitol) supplemented with proteinase and phosphatase inhibitors (see above). The nuclear extracts were dialyzed against low-salt buffer (same composition as the high-salt buffer except that the NaCl content was 50 mmol/L) overnight. The protein concentration was determined by Bradford assay.

Immunoprecipitation and Western Blot Analysis

Antibodies to JAK1, JAK2, Tyk2, STAT1, and STAT3 were purchased fromSanta Cruz Laboratory. Monoclonal antibody to STAT2 was purchased from Chemicon International Inc. Ang II was purchased from Sigma Chemical Co. Recombinant murine LIF was purchased from Genzyme.

Selected Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
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<td>AT₁</td>
<td>Ang II type-1</td>
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<td>GAS/ISRE</td>
<td>interferon gamma activation</td>
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<td>IL-6</td>
<td>interferon-stimulating response element</td>
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<td>ISGF</td>
<td>interferon-stimulating gene factor</td>
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<td>LIF</td>
<td>leukemia inhibitory factor</td>
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<td>SIE</td>
<td>ſ-u-inducing element</td>
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<tr>
<td>SIF</td>
<td>ſ-u-inducing factor</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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Antibodies to JAK1, JAK2, Tyk2, STAT1, and STAT3 were purified from mouse hybridomas by protein A-Sepharose and G-Sepharose columns. The purity of the antibodies was >98% as determined by immunofluorescence staining with cardiomyocytes. The antibodies were used at 1:1000 dilution for Western blotting and at 1:100 dilution for immunoprecipitation. The antibodies showed no cross-reaction with other JAK family members or other STAT family members.

Preparation of Nuclear Extracts

After Ang II stimulation, cultured neonatal cardiomyocytes were rinsed with PBS at 0°C and scraped into the same buffer. Nuclear extracts were prepared according to standard methods. Briefly, harvested cells were resuspended in 5 vol of hypotonic buffer (10 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 1.5 mmol/L MgCl₂, and 0.5 mmol/L dithiothreitol) supplemented with proteinase and phosphatase inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μmol/L leupeptin, 10 μmol/L aprotinin, 1 mmol/L NaF, and 1 mmol/L Na3VO4) incubated for 10 minutes on ice, and sedimented. The nuclei were then resuspended in 2 vol of the same buffer, homogenized (Dounce homogenizer), and sedimented at 1000g for 10 minutes, and the pellet (nuclei) was collected. The pellet nuclei were incubated for 30 minutes at 4°C in high-salt buffer (20 mmol/L HEPES [pH 7.9], 1 M NaCl, and 1 mmol/L EDTA) supplemented with proteinase and phosphatase inhibitors (see above). The nuclear extracts were dialyzed against low-salt buffer (same composition as the high-salt buffer except that the NaCl content was 50 mmol/L) overnight. The protein concentration was determined by Bradford assay.

Gel Mobility Shift Assay

Gel mobility shift assays were performed as described previously, with minor modifications. Nuclear extracts (5 μg) were incubated with 1 μg of poly(dI-dC)–poly(dI-dC) (Pharmacia Biotech), with or without competitor oligonucleotide in 20 μL of 10 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 1 mmol/L EDTA, and 10% glycerol, for 20 minutes, at 25°C. The samples were then incubated with 1 or 2 finol of radiolabeled probes (~5000 cpm) for 10 minutes at 25°C. The probes used in the present study were purchased from Santa Cruz Biotechnology, and their sequences have been described (SIE-DNA, 5′-CAGTTCCCGTCAATC-3′; mutant SIE-DNA, 5′-CAGCCACCCGTAATAC-3′; mutant SIE-DNA, 5′-CAGCCACCCGTAATAC-3′; and mutant-GAS/ISRE-DNA, 5′-AATCTTTCAGTGGTCTATTACTCTA-3′). Binding reactions were resolved by electrophoresis on a 4% native polyacrylamide gel containing 0.5× TBE buffer (25 mmol/L Tris, 25 mmol/L boric acid, and 0.5 mmol/L EDTA). Gels were run at 150 V in a cold room (4°C).
for 2 to 3 hours in 0.5X TBE buffer and dried, and x-ray film was exposed to them for 12 to 24 hours.

**Results**

**Effect of Ang II on Phosphorylation of JAK Family Kinases**

As the first step in elucidating the characteristics of Ang II–induced JAK/STAT activation in cardiomyocytes, we compared Ang II–induced and LIF–induced tyrosine phosphorylation of JAK family kinases. The results are shown in Fig 1a. LIF induced strong phosphorylation of JAK1 and JAK2 and weak phosphorylation of Tyk2 as early as 2 minutes, peaking at 5 minutes. In contrast, although tyrosine phosphorylation of both JAK2 and Tyk2 increased when the cells were stimulated with Ang II, the time course was different from that after LIF stimulation. Phosphorylation of JAK2 and Tyk2 increased by 5 minutes, peaked at 15 minutes, and continued up to 30 minutes. Phosphorylation of JAK1 was not observed. The differences between the time course of activation of JAK kinases by Ang II and LIF are summarized in Fig 1b. Phosphorylation of JAK2 was weaker and more slowly induced by Ang II than by LIF. The phosphorylation of Tyk2, on the other hand, was more intense in response to Ang II than to LIF. Similar results were obtained in five separate experiments.

Cardiomyocyte cultures obtained by preplating and bromodeoxyuridine treatment contain ~10% nonmyocytes, and cardiac nonmyocytes are known to possess Ang II receptors. In order to confirm that the signals obtained in Fig 1a were produced by cardiomyocytes, we used highly purified cardiomyocytes obtained by Percoll gradient for immunoprecipitation/Western blot of the JAK kinase in some experiments. Cardiac nonmyocytes were also examined. Percoll-purified cardiomyocytes revealed JAK2 phosphorylation having the same time course as in Fig 1a. Cardiac nonmyocytes also exhibited JAK2 phosphorylation, and the time course of the phosphorylation was also similar to that of cardiomyocytes (Fig 1c). These findings indicate that although cardiac nonmyocytes may have affected the data somewhat, the main signals in the present study were produced by cardiomyocytes.

The above findings indicate that JAK2 and Tyk2 are involved in the Ang II–induced signaling pathway in cardiomyocytes and that not only the type of JAK kinase but also the time course of activation differed for LIF compared with Ang II.

**Effect of Ang II on Tyrosine Phosphorylation of STATs**

In order to further investigate the differences in STAT activation between LIF and Ang II in cardiomyocytes, we analyzed tyrosine phosphorylation of the STAT family by immunoprecipitation/Western blot analysis. The results are shown in Fig 2a. All STATs, 1, 2, and 3, were almost completely unphosphorylated in unstimulated cardiomyocytes. LIF induced immediate and intense phosphorylation of STAT1 at 5 minutes and of STAT3 as early as 2 minutes, which peaked at 5 to 15 minutes and decreased at 30 minutes. LIF did not induce phosphorylation of STAT2. In contrast, STAT1 and STAT2 were phosphorylated after stimulation with Ang II, and their phosphorylation increased gradually, peaking at ~15 minutes. Tyrosine phosphorylation of STAT1 was clearly more slowly activated by Ang II than by LIF. The time course of phosphorylation of STAT2 by Ang II was also slow and was almost the same as the time course for STAT1. STAT3 was not phosphorylated as of 30 minutes. Five separate
experiments yielded similar results. Comparisons between phosphorylation of the STATs by LIF and Ang II, assessed by densitometry, are summarized in Fig 2b. The results indicated that STAT1 and STAT2, but not STAT3, mediate Ang II signaling in cardiomyocytes in the early stage for up to 30 minutes. Since Bhat and colleagues reported that Ang II induces delayed activation of SIE in stably AT1A receptor–transfected CHO-K1 cells, we tested whether Ang II induces phosphorylation of STAT1 and STAT3 as early as 2 minutes, whereas phosphorylation by Ang II was weak and observed at 120 minutes. The results of densitometric analysis of tyrosine phosphorylation of STAT1 and STAT3 are shown in Fig 6.

Figure 2. Effect of Ang II on tyrosine phosphorylation of STATs. 
a, Tyrosine phosphorylation of STATs by Ang II and LIF in cardiomyocytes was demonstrated. Ang II induced tyrosine phosphorylation of STAT1 and STAT2, but not STAT3, in the early stage up to 30 minutes, whereas LIF strongly phosphorylated STAT1 and STAT3 as early as 2 minutes. Similar results were obtained in four separate experiments. IP indicates immunoprecipitation; anti-pY, anti-phosphotyrosine. b, The time courses of the phosphorylation of STATs by Ang II and LIF were demonstrated by densitometric analysis. Ang II–induced phosphorylation of STATs was slower than LIF-induced phosphorylation. 

After the administration of Ang II, the phosphorylation of STAT3 by LIF and Ang II was completely different. Phosphorylation of STAT3 by LIF was intense and observed as early as 2 minutes, whereas phosphorylation by Ang II was weak and observed at 120 minutes. The results of densitometric analysis of tyrosine phosphorylation of STAT1 and STAT3 are shown in Fig 6.

Gel Mobility Shift Assay of GAS/ISRE and SIE in Ang II–Induced Cardiac Hypertrophy

Our previous study revealed that LIF does not affect ISGF (heterodimer of STAT1 and STAT2)–like activity in cardiomyocytes. In order to investigate whether Ang II induces ISGF–like activity in rat cardiomyocytes, nuclear extracts were prepared at various time intervals after Ang II stimulation and incubated with 32P-labeled GAS/ISRE. The DNA-protein complexes were analyzed by gel mobility shift assay, and the results are shown in Fig 4. The DNA-protein complexes with GAS/ISRE seemed to show an immediate increase after Ang II stimulation in the early stage up to 30 minutes. Moreover, GAS/ISRE was reactivated in the late stage at 120 minutes, paralleling the delayed rephosphorylation of STAT1.

To investigate whether Ang II induces SIF (homodimer and/or heterodimer of STAT1 and STAT3)–like activity in cardiomyocytes, we performed a gel mobility shift assay for SIE and compared the results with those of LIF-induced activation of SIE (Fig 5). LIF immediately and strongly induced SIF–like activity as early as 5 minutes. In contrast, no gel mobility shift of SIE was observed for the first 60 minutes after Ang II stimulation. Interestingly, we found that the DNA-protein complex with SIE was observed at 120 minutes after Ang II stimulation.

Figure 3. Delayed phosphorylation of STAT1 and STAT3 by Ang II. Immunoprecipitation (IP)/Western blot analysis revealed that STAT1 was rephosphorylated at 120 minutes, whereas STAT3 was phosphorylated in the late stage at 120 minutes. The results were confirmed in four separate experiments. IP indicates immunoprecipitation; anti-pY, anti-phosphotyrosine.

Figure 4. Gel mobility shift assay of GAS/ISRE. Ang II induced formation of the ISGF complex, which interacted with GAS/ISRE in the early stage up to 30 minutes. Note that Ang II also induced a gel mobility shift of both SIE and GAS/ISRE in the late stage that was maximal at 120 minutes. The results were reproduced in four separate experiments.

Figure 4. Gel mobility shift assay of GAS/ISRE. Ang II induced formation of the ISGF complex, which interacted with GAS/ISRE in the early stage up to 30 minutes. Note that Ang II also induced a gel mobility shift of both SIE and GAS/ISRE in the late stage that was maximal at 120 minutes. The results were reproduced in four separate experiments.
stimulation. This parallels the delayed phosphorylation of STAT1 and STAT3. The time course of STAT phosphorylation and the gel shift of GAS/ISRE and SIE by Ang II stimulation are summarized in Fig 6.

Effect of AT1 Receptor Blocker on STAT1 Phosphorylation

To determine which receptor subtypes are involved in the JAK/STAT signaling pathway in response to Ang II stimulation, we performed a similar experiment with and without the AT1 receptor–specific antagonist CV11974. The results, shown in Fig 7, revealed that phosphorylation of STAT1 was significantly suppressed by pretreatment with CV11974. This finding indicated that Ang II–induced activation of STAT1 is mediated by the AT1 receptor.

We examined whether delayed addition of an AT1 blocker would affect the delayed activation of STAT3 by adding CV11974 at a concentration of $10^{-5}$ mol/L at 30 and 60 minutes after Ang II stimulation. Delayed addition of the AT1 blocker at both times failed to inhibit the delayed activation of STAT3 at 120 minutes, indicating that delayed Ang II stimulation does not affect STAT3 phosphorylation.

Discussion

In the present study, we demonstrated involvement of the JAK/STAT pathway in Ang II–induced signal transduction in rat cardiomyocytes. The following findings were obtained: (1) Ang II induced the phosphorylation of JAK2 and Tyk2, but not JAK1. Ang II–induced phosphorylation was slower than LIF–induced phosphorylation. (2) Ang II phosphorylated STAT1 and STAT2, but not STAT3, in the early stage up to 30 minutes. Ang II–induced phosphorylation of the STAT family was slower than LIF–induced phosphorylation. (3) Ang II phosphorylated STAT3 and rephosphorylated STAT1 in the late stage at 120 minutes. (4) The phosphorylation of STAT1 was significantly suppressed by pretreatment with the AT1 receptor antagonist CV11974. (5) Late addition of CV11974 did not inhibit delayed phosphorylation of STAT3 at 120 minutes. (6) Ang II induced biphasic formation of ISGF complexes in the early stage at 15 to 30 minutes and the late stage at 120 minutes. (7) Ang II induced delayed activation of the SIF complex at 120 minutes.

It is well known that Ang II plays a critical physiological and molecular biological role in cardiomyocytes as well as in smooth muscle cells. Accumulating evidence indicates that Ang II mediates cardiac hypertrophy and that it exerts its effect through G-protein–coupled receptors. Marrero et al reported that stimulation of smooth muscle cells by Ang II activates JAK2, Tyk2, STAT1, and STAT2. The JAK kinases and STAT family members activated by Ang II were found to be the same in cardiomyocytes in the early stage as in smooth muscle cells. The principle findings in the present study are that (1) not only does the JAK/STAT family differ, but the time courses of activation of the pathway by Ang II and LIF are different, and (2) Ang II induced delayed phosphorylation of STAT1 and STAT3, which parallels the delayed activation of SIE and GAS/ISRE.

We and others have recently found that LIF, a member of the IL–6 cytokine family, causes cardiac hypertrophy and that its...
The mechanism of activation of the JAK/STAT pathway has been well investigated in cytokine receptors. 52–60 JAK kinases bind directly to cytokine receptors. When ligands bind to cytokine receptors, conformational changes in the receptors occur, and this induces autophosphorylation of the JAK kinases. On the other hand, the AT1 receptor is a seven-transmembrane–type G–protein–coupled receptor. Signal transduction of the AT1 receptor has been extensively studied in various cell types, 61 but the mechanism of activation of the JAK/STAT pathway is still unknown. Investigation of the mechanism, eg, at sites upstream from the JAK/STAT pathway, or the protein–protein interaction between the AT1 receptor and JAK kinases in the future may explain the different time course of these two factors.

The present study also revealed that Ang II induces late phosphorylation of STAT1 and STAT3 and stimulates late ISGF complex formation. a, The effect of pretreatment with the AT1 receptor specific antagonist CV11974 on STAT1 phosphorylation was tested. Tyrosine phosphorylation of STAT1 was significantly suppressed by CV11974. b, The effect of delayed addition of CV11974 on late phosphorylation of STAT3 was tested. CV11974 was added to the culture media at 30 and 60 minutes after Ang II stimulation. c, A summary of the densitometric analysis of the five separate experiments is shown. *P<.01 vs control.

Figure 7. Effect of AT1 receptor blocker on STAT phosphorylation. a, The effect of pretreatment with the AT1 receptor specific antagonist CV11974 on STAT1 phosphorylation was tested. Tyrosine phosphorylation of STAT1 was significantly suppressed by CV11974. b, The effect of delayed addition of CV11974 on late phosphorylation of STAT3 was tested. CV11974 was added to the culture media at 30 and 60 minutes after Ang II stimulation. c, A summary of the densitometric analysis of the five separate experiments is shown. *P<.01 vs control.
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