Induction of JAB/SOCS-1/SSI-1 and CIS3/SOCS-3/SSI-3 Is Involved in gp130 Resistance in Cardiovascular System in Rat Treated With Cardiotrophin-1 In Vivo

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Abstract—CIS (cytokine-inducible SH2 protein), SOCS (suppressor of cytokine signaling), or SSI (signal transducers and activators of transcription [STAT]-induced STAT inhibitor) proteins are a family of cytokine-inducible negative regulators of cytokine signaling via Janus kinase (JAK)-STAT pathways. Given the evidence that the JAK-STAT pathway plays a critical role in the cardiovascular system, the primary objective of this study was to assess the effects of the CIS family on JAK-STAT signaling in the cardiovascular system in rats treated with cardiotrophin-1 (CT-1), an interleukin-6 family of cytokines. Intravenous injection of 20 μg/kg body weight of CT-1 induced a transient, marked increase in STAT3 activation in various tissues, including heart and lung, and subsequent upregulation of 2 members of the CIS family, JAK-binding protein (JAB)/SOCS-1/SSI-1 and CIS3/SOCS-3/SSI-3, in the same tissues. It was also observed that CIS3 was directly associated with JAK2 in vivo. Pretreatment with the same dose of CT-1 60 minutes before significantly attenuated the STAT3 activation induced by a second injection of CT-1. We previously reported that intravenous injection of CT-1 results in the nitric oxide (NO)-dependent hypotension accompanied by the induction of inducible NO synthase mRNA. In rats pretreated with CT-1, the induction of inducible NO synthase mRNA or hypotension by subsequent CT-1 injection was not observed. Forced expression of JAB or CIS3, but not other CISs, directly blocked CT-1–induced STAT3 activation in 293 cells. These results suggest that JAB and CIS3 serve as endogenous inhibitors of CT-1–mediated JAK-STAT signaling in the cardiovascular system in vivo. (Circ Res. 2001;88:727-732.)

Key Words: CIS family • JAB/SOCS-1/SSI-1 • cardiotrophin-1 • JAK-STAT signaling

The Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway is activated in a rapid, transient manner and has been shown to be critical in many biological responses to cytokines.1,2 Recently, a family of proteins that functions in a negative feedback loop to regulate signaling by cytokine receptors via JAK-STAT pathway has been identified by several groups and is referred to as the CIS (cytokine-inducible SH2-containing protein),3 SOCS (suppressor of cytokine signaling),4 or SSI (STAT-induced STAT inhibitor)5 family. Presently, the CIS/SOCS/SSI family consists of at least 7 members of proteins and shares 2 homologous domains, an SH2 domain and a C-terminal–conserved domain called CIS homology domain.6,7 Most CISs seem to be induced by several cytokines, and at least 3 CIS family members, CIS, JAB/SOCS-1/SSI-1, and CIS3/SOCS-3/SSI-3, are proved to block JAK-STAT signaling by binding cytokine receptors or JAKs.6 JAB and CIS3 directly bind to the kinase domain of JAKs and thereby inhibit interferon (IFN)-mediated STAT1 signaling and leukemia inhibitory factor (LIF)-mediated or interleukin (IL)-6–mediated STAT3 activation in mouse myeloid leukemia M1 cells.5 CIS3 is also found to bind to gp130 and regulate the signaling.9–11 Thus, evidence was accumulated that JAB or CIS3 exerts negative regulators of JAK-STAT signaling in hematopoietic and immune systems.8,12 However, the significance of the negative regulatory mechanism of JAK-STAT pathway in cardiovascular system is little understood.

Cardiotrophin-1 (CT-1), a member of the IL-6 superfamily, is substantially expressed in the heart, and its expression is upregulated in hypertrophied ventricles of genetically hypertensive rats.13 CT-1 binds to the gp130-LIF receptor complex in myocytes, activating the JAK-STAT pathway and inducing hypertrophic responses.14–18 Other investigators19 and we20 recently reported additionally that the intravenous injection of...

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CT-1 induces nitric oxide (NO)-dependent hypotension dose-dependently in conscious rats. Taken together, these findings strongly suggest that CT-1 acts as a cytokine with a variety of important actions in the cardiovascular system.

In the present study, we show that pretreatment with CT-1 60 minutes before inhibits STAT3 activation, induction of inducible NO synthase (iNOS) mRNA, and resultant hypotensive effects induced by subsequent injection of CT-1 in vivo and that forced expression of JAB and CIS3 significantly attenuates CT-1–induced STAT3 activation in 293 cells. Thus, pretreatment of CT-1 induces resistance to subsequent cytokine stimulation in vivo.

Materials and Methods

Animals
Male Wistar rats (250 to 270 g, Shimizu Exp, Tokyo) were housed in a light- and temperature-controlled room, where they received rat chow and water ad libitum. Rats were maintained in accordance with guidelines of the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Administration of CT-1
Rat recombinant CT-1 was prepared as previously reported.4 Rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital (Abbott Labs), and then a catheter (PE-50) filled with heparin and saline (200 U/mL) was implanted in the right external jugular vein. The animals were allowed to recover consciousness, and at least 12 hours after the operation, 20 μg/kg body weight recombinant rat CT-1 in 100 μL saline was injected via the catheter. Control rats received an equal volume of saline alone.

RNA Extraction and Northern Blot Analysis
Rats were anesthetized with 50 mg/kg pentobarbital and then killed at selected times after CT-1 administration. The lung, heart (left ventricle), liver, spleen, kidney, muscle, and brain were removed, immediately frozen, and stored at −80°C until use. Tissues were homogenized in TRIZol Reagent (GIBCO/BRL, Life Technologies, Inc), and total RNA was isolated according to the manufacturer’s instructions. Northern blot analysis was performed using 50 μg of total RNA from each sample as previously described.

Protein Extraction and Western Blot Analysis
Protein extraction and Western blotting were performed as previously reported.22 For the detection of STAT3 and phosphorylated-STAT3 (p-STAT3), membranes were incubated with antibodies (Abs) raised against STAT3 and its phosphorylated form (New England Biolabs, Inc) followed by incubation with peroxidase-conjugated goat anti-rabbit IgG Ab and chemiluminescence detection. For the detection of CIS3, rabbit polyclonal anti-CIS3 Ab (Immuno-Biological Laboratories) was used.

Measurement of Systemic Arterial Pressure
Arterial blood pressure (BP) was subsequently measured using a pressure transducer (Fukuda Denshi) connected to the arterial cannula implanted in the internal carotid artery, as previously reported.

Luciferase Assay
Luciferase reporter gene assay was performed as previously reported.6 Expression vector (pcDNA3) containing cDNA of CIS family and luciferase reporter gene driven by the STAT3-responsive element acute phase reactive element (APRE) was cotransfected into 293 cells by the calcium-phosphate method as previously reported.6 Plasmid construction and culture conditions were described previously.6 The transfected cells were cultured for 48 hours and then prepared, and luciferase activity was measured as described previously.

Detection of STAT3 Phosphorylation in 293 Cells
The 293 cells were transfected with pcDNA3-CISs and pcDNA-Myc-STAT3. For the detection of STAT3 and p-STAT3, cell lysates were immunoprecipitated with anti-cMyc Ab (Santa Cruz), and then Western blotting was performed.

Statistical Analysis
All values are presented as mean±SD. Comparisons between 2 groups were made using unpaired Student’s t test. ANOVA with post hoc Fischer’s tests was used to determine significant differences among 3 or 4 groups. A value of P<0.05 was considered significant.

Results

CT-1 Activates STAT3 Followed by Upregulation of CIS Family Expression In Vivo
First, to determine whether or not STAT3 is activated by CT-1 in vivo, Western blot analyses were carried out using tissue samples from heart, lung, and skeletal muscle probed with anti-STAT3 and anti–p-STAT3 Abs. In each case, CT-1 injection resulted in the transient appearance of p-STAT3, the level of which peaked within 15 minutes (Figure 1). In the second, CT-1–induced in vivo expression of CIS family gene was assessed by Northern blot analysis. Before CT-1 treatment, low levels of JAB and CIS3 mRNA were detected in heart, lung, liver, spleen, kidney, muscle, and brain (Figure 2). Injection of 20 μg/kg body weight CT-1 induced dramatic increases in the expression of JAB and CIS3 mRNA, which peaked within 15 to 60 minutes and then returned to baseline levels within 120 minutes in all tissues examined. CIS2 mRNA was slightly increased by CT-1 injection in heart and lung but was not significantly increased in other tissues. Neither CIS1 nor CIS4 mRNA was elevated in the tissues examined. Upregulation of CIS3 protein was also confirmed by Western blotting in heart and lung (Figure 3). The level of CIS3 protein was increased just after the CIS3 mRNA upregulation, peaked 60 minutes after the CT-1 injection, and returned to the basal level within 120 minutes. Because of lack of high affinity anti-JAB Ab suitable for Western blotting, upregulation of JAB protein could not be examined.

CIS3 Directly Associates to JAK2 In Vivo
Considering the earlier evidence that JAB and CIS3 bind to JAK2-JH1 and inhibit JAK-STAT signaling in vitro,6,8 we investigated the direct interaction between CIS3 and JAK2 in vivo. Anti-JAK2 immunoprecipitates in lung tissues from rats injected by CT-1 were separated on SDS-PAGE and subjected to Western blotting analysis with anti-CIS3 Ab. As shown in Figure 4, CIS3 was coprecipitated with JAK2, and the coprecipitated band was increased and decreased in

Figure 1. Representative Western blots showing the time course of STAT3 phosphorylation in heart and lung after intravenous injection of 20 μg/kg body weight of CT-1. At each time point, 50 μg of cell lysate was probed using anti–p-STAT3 and anti-STAT3 Abs.
in accordance with the upregulation and downregulation of CIS3 protein after CT-1 injection (Figures 3 and 4).

CT-1 Pretreatment Attenuates STAT3 Activation Induced by a Second Injection of CT-1 In Vivo

To assess whether CT-1–induced expression of JAB and CIS3 would inhibit later activation of STAT3 in vivo, groups of rats were pretreated by the intravenous bolus injection of either CT-1 (20 μg/kg body weight) or saline. After 60 minutes, when JAB and CIS3 were upregulated by the first injection of CT-1, members of each group received a second injection containing CT-1 or saline, and the ratios of p-STAT3 to STAT3 were analyzed by Western blot 15 minutes after a second injection in heart (Figure 5A) and lung (Figure 5B). In animals pretreated with saline, ratios of p-STAT3 to STAT3 were both markedly increased 15 minutes after subsequent injection of CT-1. On the other hand, the pretreatment with CT-1 significantly diminished STAT3 activation elicited by a second CT-1 injection. Attenuation of STAT3 activation by the pretreatment with CT-1 was also confirmed by electrophoretic mobility shift assay using lung nuclear extracts from rats with or without CT-1 pretreatment (data not shown).

Pretreatment With CT-1 Inhibits the Hypotension Induced by a Second Injection of CT-1 In Vivo

We previously showed that injection of CT-1 elicits NO-dependent hypotension in a dose-dependent manner. We therefore tested whether in addition to attenuating STAT3 activation, pretreatment with CT-1 would also inhibit an integrated biological response, such as decline in BP. Figure 6A shows that whereas the first injection of CT-1 (20 μg/kg body weight) elicited a clear decline in BP, a second injection 60 minutes later had a significantly smaller effect. Mean values of BP before and after CT-1 injection in rats from each group are summarized in online Table 1 available in the data supplement at http://www.circresaha.org. To rule out the possibility that the attenuated hypotensive effect was attributable to suppressed reactivity to NO, in some experiments the NO donor S-nitroso-N-acetylpenicillamine (SNAP) was injected instead of CT-1. As shown in Figure 6B, injection of 4 μmol (0.88 mg)/kg body weight SNAP elicited a sharp decline in BP, clearly demonstrating that there was no suppression of reactivity to NO. Next, because our earlier work showed that CT-1–induced hypotension was blocked by the treatment with aminoguanidine, an iNOS inhibitor, we examined expression of iNOS mRNA after injection of either CT-1 or saline using the same 2-injection protocol described above. After CT-1 pretreatment, a second injection of CT-1 failed to induce iNOS mRNA expression, although a significant response was elicited by CT-1 in rats pretreated with saline (Figure 7). Thus, pretreatment of CT-1 attenuated hypotensive effects of the subsequent CT-1 injection probably by reducing iNOS mRNA induction resulting from JAB and CIS3 upregulation.
JAB and CIS3 Directly Inhibit CT-1–Induced STAT3 Activation In Vitro

To confirm that JAB and CIS3 directly inhibit STAT3 signaling, we examined effects of forced expressed CISs on luciferase reporter gene activity driven by the STAT3-responsive element APRE in 293 cells. In the control group, in which empty vector was cotransfected with reporter gene, stimulation with CT-1 increased relative luciferase activity 6-fold (Figure 8A). In contrast, when pcDNA-JAB or pcDNA-CIS3 was cotransfected, STAT3-responsive luciferase activity was dose-dependently attenuated until the highest dose, at which activity was completely blocked (Figures 8B and 8C). Forced expression of CIS1, CIS2, and CIS4 did not have marked effect on luciferase activity (Figure 8A). Furthermore, we performed Western blotting using cell lysates from 293 cells in which both pcDNA-Myc-STAT3 and pcDNA-JAB or pcDNA-CIS3 were cotransfected. Transfection of JAB or CIS3 in 293 cells inhibited CT-1–induced STAT3 phosphorylation (online Figure 1 available in the data supplement at http://www.circresaha.org).

Discussion

We have shown for the first time to our knowledge that pretreatment with CT-1 in vivo confers resistance to subsequent CT-1 administration when measuring STAT3 activation, induction of iNOS mRNA, and effects on BP. Administration of CT-1 evoked activation of STAT3 in heart and lung, accompanied by the induction of JAB and CIS3 and by the direct interaction of JAK2 and CIS3 in vivo. Furthermore, forced expression of JAB and CIS3 strongly attenuated CT-1–induced activation of STAT3 in a cell-based system. Taken together, we consider that JAB and CIS3 may serve as endogenous inhibitors of CT-1–mediated JAK-STAT signaling in the cardiovascular system in vivo.

Previous studies showed that CIS, JAB, CIS2, and CIS3 are induced within 15 to 30 minutes by a wide variety of cytokines and hormones in vitro and in vivo.24–26 However, the induction profile of CIS genes by certain cytokines often varies with respect to tissues.4,27 The present study demonstrates that CT-1, a member of IL-6 family of cytokines, markedly induced JAB and CIS3 but not CIS, CIS2, and CIS4 in all tissues examined in vivo, suggesting a strong interaction between CT-1 and JAB or CIS3. This expression profile is similar to that induced by IL-6 or LIF.3–5

In the present study, we also investigated the significance of JAB and CIS3 in CT-1–induced resistance to subsequent CT-1 administration, in other words, CT-1 induced CT-1 resistance. We proved that forced expression of JAB or CIS3 inhibited STAT3 activation induced by CT-1 in accordance with expression levels of JAB or CIS3 in 293 cells. In the next step, to examine roles of endogenous JAB or CIS3 in the mechanism, we examined the effects of pretreatment with CT-1 on STAT3 activation by subsequent CT-1 stimulation in wild 293 cells that were not transfected with JAB or CIS3. However, after pretreatment with CT-1, JAB and CIS3 were not upregulated besides STAT3 phosphorylation in 293 cells, in which pretreatment with CT-1 failed to inhibit STAT3 phosphorylation by subsequent CT-1 stimulation (data not shown), suggesting that CT-1–induced CT-1 resistance depends on the induction of JAB and CIS3. These data clearly suggest that JAB and CIS3 inhibit STAT3 signaling and their downstream biological phenotype in vivo.

In addition to analyses of intracellular signaling, the present study showed that CT-1 pretreatment attenuates biological actions by a second injection of CT-1 in vivo. Jin et al19 and we20 recently reported that intravenous injection of CT-1 caused systemic hypotension in conscious rats, which was blocked by L-arginine methyl ester (L-NAME), an NOS inhibitor, and by aminoguanidine, an iNOS-specific inhibitor. Given the evidence that CT-1 induces iNOS mRNA
in vivo and that activation of JAK-STAT pathways stimulates iNOS induction,\textsuperscript{28} we consider the induced expression of iNOS mRNA and the resultant decrease in BP as potential markers of the biological actions of CT-1. Consistent with the proposed suppressive effect of JAB and CIS3, pretreatment with CT-1 attenuated subsequent induction of iNOS expression and systemic hypotension. These results together with in vitro data clearly indicate that CT-1 resistance observed after CT-1 pretreatment is attributable to increased JAB or CIS3 protein expression in vivo.

Leptin is an adipocyte-derived satiety factor that, when peripherally administered, acts at the hypothalamus via JAK-STAT--signaling pathways to reduce food intake.\textsuperscript{29} Recent evidence indicates that plasma leptin levels are elevated in obese humans and in animal models of obesity, and a concept of leptin resistance has been accepted to explain such high levels of plasma leptin in some obese individuals. Although the mechanism of this resistance has not yet been identified, peripheral administration of leptin rapidly induces hypothalamus.\textsuperscript{30} In the present study, we proposed that pretreatment of JAB and CIS3 at least partly play a role in CT-1 resistance in vivo.

In the present study, we proposed that pretreatment of CT-1 confers resistance to subsequent administration of the same cytokine. However, evidence has been accumulated that the upregulation of JAB, CIS1, CIS2, and CIS3 results in the attenuation of signaling by many different cytokines and hormones in vitro.\textsuperscript{32} It is therefore likely that pretreatment of CT-1 inhibits biological actions mediated by other groups of cytokines or substances producing cytokines, such as lipopolysaccharide. In fact, Pulide et al.\textsuperscript{33} reported that CT-1 attenuates bacterial lipopolysaccharide--induced neutrophil accumulation and edema in lung. Thus, application of CT-1--induced cytokine resistance to some pathological states may be plausible, such as sepsis, myocarditis, and cardiomyopathy, in which cytokines play roles in their pathogenesis.\textsuperscript{35,36}

References


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Induction of JAB/SOCS-1/SSI-1 and CIS3/SOCS-3/SSI-3 Is Involved in gp130 Resistance in Cardiovascular System in Rat Treated with Cardiotrophin-1 (CT-1) in vivo.

Ichiro Hamanaka¹, Yoshihiko Saito¹, Hideo Yasukawa², Ichiro Kishimoto¹, Koichiro Kuwahara¹, Yoshihiro Miyamoto¹, Masaki Harada¹, Emiko Ogawa¹, Noboru Kajiyama¹, Nobuki Takahashi¹, Takehiko Izumi¹, Rika Kawakami¹, Izuru Masuda¹, Akihiko Yoshimura² and Kazuwa Nakao¹.
Online Figure Legend

online Figure 1.
(A) pcDNA-JAB, pcDNA-CIS3 and control vector at a dose of 0.25 µg and 50ng/well of Myc tagged STAT3 were co-transfected in 293 cell and stimulated with 10ng/ml CT-1 for 60minutes. Cell lysates were immunoprecipitated with anti-Myc Ab, then immunoblotted with anti pSTAT3 Ab (upper lane) and STAT3 Ab (lower lane). (B) Bar graph depicts relative quantities of phosphorylation of STAT3 (pSTAT3/STAT3), which was normalized to the STAT3 phosphorylation in immunoprecipitate (with anti-Myc Ab) from CT-1-stimulated 293 cells, which were transfected with empty vector and was arbitrarily assigned a value of 1.0. (n=3 each) *P<0.05 vs. empty vector.

online Figure 2.
Western blotting illustrating the time course of LIF receptor of the heart (left panel) and of the lung (right panel) in CT-1 treated rats. Fifty microgram of cell lysate was loaded on each lane. This is a representative blotting out of three independent experiments.
(A) CT-1 plasmid control (+) control (+) JAB (+) CIS3

- p-STAT3
- STAT3
- IP: c-Myc

(B) (AU)

- no treat
- EMP
- JAB
- CIS3

* indicates significant difference.
Table 1 Hypotensive effect of cardiotrophin-1 (CT-1) in rats with or without CT-1-pretreatment

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<th>CT-1 pretreatment(-) (n=4)</th>
<th>CT-1 pretreatment(+) (n=4)</th>
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Values are indicated as mean±S.D., * P <0.05 compared with no pretreatment
† Post BP were evaluated 15min after CT-1 injection.