Endothelin-1 stimulates expression of cyclin D1 and S-phase kinase–associated protein 2 by activating the transcription factor STAT3 in cultured rat astrocytes

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Yutaka Koyama, Satoshi Sumie, Yasutaka Nakano, Tomoya Nagao, Shiko Tokumaru, and Shotaro Michinaga

From the Laboratory of Pharmacology, Kobe Pharmaceutical University, 4-19-1 Motoyamakita, Higashinada, Kobe, 658-8558, Japan and the Laboratory of Pharmacology, Faculty of Pharmacy, Osaka Ohtani University, 3-11-1 Nishikiori-Kita, Tondabayashi, Osaka 584-8540, Japan

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Brain injury–mediated induction of reactive astrocytes often leads to gliarial scar formation in damaged brain regions. Activation of signal transducer and activator of transcription 3 (STAT3), a member of the STAT family of transcription factors, plays a pivotal role in inducing reactive astrocytes and gliarial scar formation. Endothelin-1 (ET-1) is a vasoconstrictor peptide, and its levels increase in brain disorders and promote astrocytic proliferation through ETB receptors. To clarify the mechanisms underlying ET-1–mediated astrocytic proliferation, here we examined its effects on STAT3 in cultured rat astrocytes. ET-1 treatment stimulated Ser-727 phosphorylation of STAT3 in the astrocytes, but Tyr-705 phosphorylation was unaffected, and ET-induced STAT3 Ser-727 phosphorylation was reduced by the ETB antagonist BQ788. ET-1 stimulated STAT3 binding to its consensus DNA-binding motifs. Monitoring G1/S phase cell cycle transition through bromodeoxyuridine (BrdU) incorporation, we found that ET-1 increases BrdU incorporation into the astrocytic nucleus, indicating cell cycle progression. Of note, STAT3 chemical inhibition (with static or 5,15-diphenyl-porphine (5,15-DPP)) or siRNA-mediated STAT3 silencing reduced ET-induced BrdU incorporation. Moreover, ET-1 increased astrocytic expression levels of cyclin D1 and S-phase kinase–associated protein 2 (SKP2), which were reduced by static, 5,15-DPP, and STAT3 siRNA. ChIP-based PCR analysis revealed that ET-1 promotes the binding of STAT3 to the 5’-flanking regions of rat cyclin D1 and SKP2 genes. Our results suggest that STAT3-mediated regulation of cyclin D1 and SKP2 expression underlies ET-induced astrocytic proliferation.

In brain disorders, phenotypic conversion of resting astrocytes to reactive ones is commonly observed and is characterized by the expression of glial fibrillary acidic protein (GFAP) and hypertrophy of the cell body. Reactive astrocytes produce and release a variety of bioactive substances that regulate the pathophysiological responses of injured nerve tissues (1, 2). In addition, reactive astrocytes show proliferative properties when nerve tissues are severely damaged. Hyperplasia of reactive astrocytes results in gliarial scar formation around injured nerve tissues. Gliarial scar formation largely affects the repair process of injured nerve tissue by regulating axonal elongation and inflammatory responses (3–5). Based on these observations, the management of reactive astrocyte and gliarial scar formation is suggested to be an effective therapeutic strategy to promote neuroprotection and repair of damaged nerve tissues (6, 7). STAT3, a member of the Stat family of transcription factors, plays a role in the transcription of genes regulating cell differentiation, proliferation, and phenotypic conversion. The transcription activity of STAT3 is modulated by phosphorylation of tyrosine and serine residues. Phosphorylation of STAT3 is stimulated by the activation of gp130-linked cytokine receptors and some G protein–coupled receptors, where tyrosine phosphorylation causes the dimerization and nuclear translocation of STAT3 (8). Meanwhile, the serine phosphorylation of STAT3 is reported to show stimulatory or inhibitory action on its transcriptional activity (9–12). In animal models of brain injury (13–15) and patients with neurological diseases (16, 17), increases in phosphorylated STAT3 were observed in reactive astrocytes. STAT3 stimulates the transcription of some genes coding proteins characterizing astrocytic activation, which includes GFAP and cell cycle regulatory proteins (18–22). Thus, STAT3 is considered to be a key molecule to induce the phenotypic conversion to reactive astrocytes (23). In support of this, the inhibition of STAT3 has been found to prevent the induction and proliferation of reactive astrocytes in animal models of nerve injury (4, 24–27). However, the signal substances regulating astrocytic STAT3 activation in brain disorders are not fully clarified.

Production of endothelin-1 (ET-1), a vasoconstrictor peptide, is increased owing to brain disorders and promotes many signal–regulated kinase; JAK, Janus kinase; MEM, minimal essential medium; ChIP, chromatin immunoprecipitation; PI, propidium iodide; TBI, traumatic brain injury; FPI, fluid percussion injury; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ANOVA, analysis of variance.

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This article contains Figs. S1–S3.
1 To whom correspondence should be addressed. Tel/Fax: 81-78-441-7572; E-mail: koyama-y@kobepharma-u.ac.jp.
2 The abbreviations used are: GFAP, glial fibrillary acidic protein; ET, endothelin; SKP2, S-phase kinase–associated protein 2; BrdU, bromodeoxyuridine; 5,15-DPP, 5,15-diphenyl-porphine; PKC, protein kinase C; ERK, extracellular signal–regulated kinase; JAK, Janus kinase; MEM, minimal essential medium; ChIP, chromatin immunoprecipitation; PI, propidium iodide; TBI, traumatic brain injury; FPI, fluid percussion injury; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ANOVA, analysis of variance.

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**Activation of astrocytic STAT3 by ET-1**

ET receptors, especially ETA receptors, are highly expressed in astrocytes (30–33). The roles of ET receptors in induction of reactive astrocytes have been examined. Administration of an ET-A agonist into rat brain increased the number of reactive astrocytes (34), whereas inhibition of ET-B receptor reduced the reactive astrocytes in several brain disorders (33, 35–39). Based on these observations, ET-1 is proposed to be one of the factors that induce phenotypic conversion and proliferation of reactive astrocytes (40). In cultured astrocytes, ET-1 stimulated astrocytic proliferation through altered expression of cell cycle regulatory proteins (41–43). The present study examines the role of STAT3 in ET-induced expression of astrocytic cyclin D1 and S-phase kinase-associated protein 2 (SKP2), which are cell cycle regulatory proteins highly expressed in reactive astrocytes (44–48).

**Results**

**ET-1–induced Ser phosphorylation and activation of astrocytic STAT3**

The transcriptional activity of STAT3 is regulated by phosphorylation of Tyr-705 and Ser-727 residues. Treatment with 100 nM ET-1 for 10–480 min did not largely affect either total or Tyr-705–phosphorylated STAT3 protein levels (Fig. 1A). Ser-727–phosphorylated STAT3 was increased 3–4-fold by treatment with ET-1 for 20–60 min, and the increased levels of Ser-727–phosphorylated STAT3 were maintained for 240 min (Fig. 1A). The effects of ET-1 on Ser-727 phosphorylation of STAT3 were dose-dependent, where statistically significant increases were observed at higher concentrations than 10 nM (Fig. 1B). Ala1,3,11,15-ET-1, an ET-B-selective agonist, increased Ser-727–phosphorylated STAT3 in cultured astrocytes (Fig. 2A). Increases in Ser-phosphorylated STAT3 were reduced by BQ788, an ET-B antagonist, whereas FR139317, an ET-A antagonist, had no effect (Fig. 2B). Immunocytochemical observations of nontreated astrocytes showed that large populations of cells had Tyr-705–phosphorylated STAT3 in the nucleolus (Fig. 3A). Treatment with ET-1 did not increase the numbers of Tyr-705–phosphorylated STAT3–positive astrocytes. In contrast, cultured astrocytes showing Ser-727–phosphorylated STAT3 were partly reduced by Go6983 (1 μM; extracellular signal–regulated kinase (ERK) inhibitor) and FR180204 (5 μM; protein kinase C (PKC) inhibitor) and FR139317, an ET-B antagonist, whereas ruxolitinib (500 nM; Janus

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**Figure 1. Effects of ET-1 on Tyr and Ser phosphorylation of STAT3 in cultured rat astrocytes.** A, time course. Serum-starved astrocytes were treated with 100 nM ET-1 for the time indicated. Tyr-705– and Ser-727–phosphorylated STAT3 were detected by immunoblotting. After detection of phosphorylated proteins, the same blots were reprobed with an anti-STAT3 antibody to detect levels of total STAT3. The protein bands in X-ray films were subjected to densitometry analyses. Results are means ± S.D. (error bars) of four experiments. Individual data points are indicated to the right of the error bars. STAT3 phosphorylation values are presented as ratios of phosphorylated/total STAT3. ●, phospho-Tyr STAT3; ○, phospho-Ser STAT3. Expression levels of total STAT3 are presented as a ratio to β-actin protein. *, *p < 0.05; **, *p < 0.01 versus zero time by one-way ANOVA followed by Dunnett’s test. B, dose-response. Astrocytes were treated with the indicated concentrations of ET-1 for 20 min. Results are the means ± S.D. of 4–8 experiments and presented as ratios of Ser-phosphorylated/total STAT3. Individual data points are indicated by dots on the error bars. **, *p < 0.01 versus none by one-way ANOVA followed by Dunnett’s test.
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kinase (JAK) inhibitor) and PP-1 (1 μM; Src kinase inhibitor) had no effect (Fig. 4). The basal level of STAT3 Ser phosphorylation was not affected by these kinase inhibitors at the concentrations used (Fig. S1A). Go6983, FR180204, and PP-1 did not affect the basal level of STAT3 Tyr phosphorylation, whereas ruxolitinib reduced it (Fig. S1A).

ETb antagonism reduced traumatic brain injury (TBI)-induced phosphorylation of STAT3

In animal models of brain injury, increases in phosphorylation of astrocytic STAT3 were observed (13–15). To examine an involvement of ETb receptors in the brain injury–induced phosphorylation of STAT3, effects of ETb antagonist were examined in a mouse model of TBI. Lateral fluid percussion injury (FPI) to the mouse cerebrum increased protein levels of Tyr-705– and Ser-727–phosphorylated STAT3 5 days after the initial insult (Fig. 5), whereas total STAT3 protein levels were not altered. Repeated intracerebroventricular administration of BQ788 (15 nmol/day) from 2 days after FPI reduced the increases in Tyr-705– and Ser-727–phosphorylated STAT3 proteins (Fig. 5).

Prevention of ET-induced astrocytic G1/S phase transition by the inhibition of STAT3

The role of STAT3 in ET-induced cell cycle progression of cultured astrocytes was examined. Treatment with 100 nM ET-1 increased the numbers of cultured astrocytes taking up bromodeoxyuridine (BrdU) in the nucleus, which indicates the promotion of G1/S phase transition (Fig. 6, A and B). In the presence of Stat inhibitors (i.e. stattic (5 μM) and 5,15-diphenyl-porphine (5,15-DPP; 5 μM)), increases in BrdU-positive astrocytes by ET-1 were not observed. Transfection of STAT3 siRNA decreased STAT3 proteins in cultured astrocytes (Fig. 8, A and B). Stattic (5 μM) and 5,15-DPP (5 μM) reduced mRNA expression of astrocytic cyclin D1 and SKP2 by ET-1 (Fig. 8A). ET-induced increases in BrdU-positive astrocytes were reduced by STAT3 siRNA (Fig. 6, D and E).

Involvement of STAT3 in ET-induced cyclin D1 and SKP2 expression

Cyclin D1 and SKP2 are cell cycle regulatory proteins that promote G1/S phase transition. Studies in animal models of brain injury showed that expression of cyclin D1 and SKP2 was increased in reactive astrocytes (44–48). Treatment with 100 nM ET-1 for 6–12 h increased expression of cyclin D1 mRNA in serum-starved astrocytes (Fig. 7A). Protein levels of cyclin D1 were increased by treatment with ET-1 (Fig. 7B). ET-1 increased mRNA expression and protein levels of SKP2 in cultured astrocytes (Fig. 7, A and B). Stattic (5 μM) and 5,15-DPP (5 μM) reduced mRNA expression of astrocytic cyclin D1 and SKP2 by ET-1 (Fig. 8A). ET-induced increases in cyclin D1 and SKP2 proteins were also reduced by stattic and 5,15-DPP (Fig. 8B). p27, a cell cycle regulatory protein suppressing G1/S phase transition, is ubiquitinated by SKP2 complex and degraded through a proteasome-mediated mechanism. mRNA and protein levels of p27 in cultured astrocytes were decreased by treatment with ET-1 (Fig. 7, A and B). Stattic and 5,15-DPP did not prevent the effects of ET-1 on astrocytic p27 mRNA (Fig. 8A).
To confirm the involvement of STAT3, the effects of STAT3 siRNA on ET-induced cyclin D1 and SKP2 expression were examined. ET-1 did not increase cyclin D1 or SKP2 mRNAs in cultured astrocytes transfected with STAT3 siRNA (Fig. 9A).

ET-induced increases in cyclin D1 and SKP2 proteins in cultured astrocytes were inhibited by transfection with STAT3 siRNA (Fig. 9B). STAT3 siRNA did not affect the reduction of p27 mRNA by ET-1 (Fig. 9A).

Stimulation of STAT3 binding to 5′-flanking regions of cyclin D1 and SKP2 genes by ET-1

The effects of ET-1 on STAT3 binding to the 5′-flanking regions of cyclin D1 and SKP2 were examined by ChIP-PCR analysis. PCR of the input astrogial nuclear extract by a primer pair detecting rat cyclin D1 or SKP2 promoter amplified DNA fragments with predicted length (Fig. 10A). PCR of immunoprecipitate using anti-STAT3 antibody amplified DNA fragments of the same size as those obtained with the input nuclear extract. Quantitative PCR of STAT3 immunoprecipitate showed that treatment with 100 nM ET-1 for 3 h increased the binding of STAT3 to 5′-flanking regions of the cyclin D1 gene (Fig. 10B). ET-1 also increased the binding of STAT3 to 5′-flanking regions of the SKP2 gene.

Figure 3. A and B, immunocytochemical observations of phosphorylated STAT3 in cultured astrocytes. Serum-starved astrocytes were treated with 100 nM ET-1 for 30 min. After fixation, cells were labeled with anti-phospho-Tyr (A) or anti-phospho-Ser (B) STAT3 antibody. For counterstaining, PI was included in the secondary incubations with an FITC-conjugated secondary antibody. Typical micrographs of phospho-STAT3-positive astrocytes treated with ET-1 are shown. Phospho-STAT3 immunoreactivity was observed in the nucleus (arrowheads). Bar, 50 μm.

C, increases in DNA binding activities of STAT3 to consensus oligonucleotides by ET-1. Cultured astrocytes were treated with 100 nM ET-1 for 30 and 60 min. After nucleus extracts were obtained from cultured astrocytes, the binding activity to STAT3 consensus DNA sequence was measured using an ELISA-based kit. Results are expressed as the mean ± S.D. (error bars) of 7–8 experiments. Individual data points are indicated by dots on the error bars. **, p < 0.01 versus none by one-way ANOVA followed by Dunnet’s test.

Figure 4. Effects of inhibitors for PKC, ERK, JAK, and Src on the ET-induced Ser phosphorylation of STAT3. Astrocytes were treated with 100 nM ET-1 for 20 min. Go6983 (1 μM), FR180204 (5 μM), ruxolitinib (500 nM), or PP-1 (1 μM) was added to the medium 30 min before treatment with ET-1. Results are means ± S.D. (error bars) of 7–8 experiments and are presented as ratios of Ser-phosphorylated/total STAT3. Individual data points are indicated by dots on the error bars. **, p < 0.01 versus none; #, p < 0.05 versus ET-1 by one-way ANOVA followed by Tukey’s test.

Figure 10. Effects of inhibitors for PKC, ERK, JAK, and Src on the ET-induced Ser phosphorylation of STAT3. Astrocytes were treated with 100 nM ET-1 for 20 min. Go6983 (1 μM), FR180204 (5 μM), ruxolitinib (500 nM), or PP-1 (1 μM) was added to the medium 30 min before treatment with ET-1. Results are means ± S.D. (error bars) of 7–8 experiments and are presented as ratios of Ser-phosphorylated/total STAT3. Individual data points are indicated by dots on the error bars. **, p < 0.01 versus none by one-way ANOVA followed by Tukey’s test.
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![Figure 5. Effects of BQ788 administration on FPI-induced STAT3 phosphorylation in the mouse cerebrum. BQ788 (15 nmol/day) or vehicle was repeatedly administered into the mouse brain from 2 days after FPI. Expression levels of phosphorylated and total STAT3 proteins in the injured areas of the cerebrum were measured 5 days after FPI. Typical immunoblots are indicated in the top left of the quantitative results. Expression levels of phosphorylated STAT3 were normalized to that of total STAT3. Expression levels of total STAT3 protein were normalized to that of β-actin. Results are mean ± S.D. (error bars) for four mice expressed as percentages of sham/vehicle. Individual data points are indicated by dots on the error bars. *p < 0.05 versus sham/vehicle; #, p < 0.05 versus sham/vehicle; **, p < 0.01 versus sham/vehicle by one-way ANOVA with Tukey’s test.](image)

**Figure 5. Effects of BQ788 administration on FPI-induced STAT3 phosphorylation in the mouse cerebrum. BQ788 (15 nmol/day) or vehicle was repeatedly administered into the mouse brain from 2 days after FPI. Expression levels of phosphorylated and total STAT3 proteins in the injured areas of the cerebrum were measured 5 days after FPI. Typical immunoblots are indicated in the top left of the quantitative results. Expression levels of phosphorylated STAT3 were normalized to that of total STAT3. Expression levels of total STAT3 protein were normalized to that of β-actin. Results are mean ± S.D. (error bars) for four mice expressed as percentages of sham/vehicle. Individual data points are indicated by dots on the error bars. *p < 0.05 versus sham/vehicle; #, p < 0.05 versus sham/vehicle; ***, p < 0.01 versus sham/vehicle by one-way ANOVA with Tukey’s test.**

**Discussion**

**ET-1 stimulates Ser-727 phosphorylation and activation of astrocytic STAT3**

In response to various extracellular signals, STAT3 is phosphorylated in Tyr-705 and Ser-727 residues. Phosphorylation of Tyr-705 increases the transcriptional activity of STAT3 by stimulating dimerization and nuclear translocation (8). Meanwhile, phosphorylation of Ser-727 is shown to cause positive or negative action on STAT3 activity depending on cell types or target genes (9–12). In this study, treatment with ET-1 increased Ser-727–phosphorylated STAT3 in cultured astrocytes (Fig. 1). Immunocytochemical observation showed that Ser phosphorylated STAT3 was located in the astrocytic nucleus (Fig. 3B), accompanied by increases in the binding of STAT3 to its recognition DNA sequence (Fig. 3C). These findings indicate that ET-1 stimulates astrocytic STAT3 activity through phosphorylation of Ser-727 residue. The effects of ET receptor agonist and antagonists (Fig. 2) showed that ET-induced Ser-727 phosphorylation of STAT3 was mainly mediated by ETβ receptors. ETβ receptors are linked to Gq protein and activate PKC and ERK signals in astrocytes. PKC and ERK directly phosphorylate Ser-727 residues of STAT3 (49–52). ET-induced Ser phosphorylation of STAT3 was partially reduced by Go6983 and FR180204 (Fig. 4). These results suggest the involvement of PKC/ERK signals in ET-induced Ser-727 phosphorylation of astrocytic STAT3 under an ETβ receptor signal. Washburn and Neary (53) reported that activation of the purinergic P2y receptor, which is also linked to Gq protein, stimulated STAT3 Tyr-705 phosphorylation in cultured astrocytes. In contrast with P2y agonists, activation of ETβ receptors did not greatly affect STAT3 Tyr-705 phosphorylation (Fig. 1). Therefore, we examined the effects of ET-1 on JAK, which is a pivotal kinase that induces Tyr-705 phosphorylation of STAT3. However, unexpectedly, ET-1 stimulated activation of JAK2 in cultured astrocytes (Fig. S1B). Although it is still unclear why ETβ receptor-mediated activation of astrocytic JAK2 does not induce STAT3 Tyr-705 phosphorylation, a negative regulation of the JAK/STAT3 pathway may also be activated with astrocytic ETβ receptors. In cardiomyocytes, ET-1 is shown to attenuate cytokine-induced STAT3 Tyr-705 phosphorylation (54).

**Activation of ETβ receptors induced STAT3 phosphorylation in a TBI model**

STAT3 is activated in response to brain injury, where reactive astrocytes have high levels of activated STAT3 (13–16). Studies in animal models of brain injury showed that the inhibition of STAT3 prevented the induction and proliferation of reactive astrocytes (4, 24–27). Thus, STAT3 is considered to be a key molecule to induce the phenotypic conversion of reactive astrocytes (23). We previously showed that administration of BQ788 reduced FPI-induced induction of reactive astrocytes in a mouse TBI model using FPI (38). In this study, effects of BQ788 in FPI-induced phosphorylation of STAT3 were examined in the same experimental conditions where induction of reactive astrocyte was reduced (Fig. 5). These results showed that BQ788 reduced FPI-induced Ser-727 phosphorylation of STAT3, indicating an involvement of astrocytic ETβ receptors in STAT3 activation by TBI. We also found that BQ788 reduced FPI-induced Tyr-705 phosphorylation of STAT3 in the mouse cerebrum (Fig. 5), which indicates that activation of ETβ receptors stimulates Tyr-705 phosphorylation of STAT3 in TBI. However, ET-1 did not stimulate Tyr-705 phosphorylation of STAT3 in cultured astrocytes (Fig. 1A). The different involvement of ETβ receptors between the in vivo and the in vitro experiments can be explained by indirect actions of ETβ receptor–mediated signals on STAT3 in damaged brain. In other words, Tyr-705 phosphorylation of STAT3 is stimulated by the activation of gp130-linked cytokine receptors (8). Activation of brain ETβ receptors stimulates production of cytokines that activate gp130-linked receptors, such as IL-6 (55, 56). ETβ receptor–mediated cytokine productions may cause Tyr-705 phosphorylation of STAT3 in the TBI model.

**ET-induced activation of STAT3 promotes astrocytic proliferation through cyclin D1 and SKP2**

Several studies suggest an involvement of STAT3-mediated signals in astrocytic proliferation (4, 24, 25, 26, 27). In this study, ET-induced BrdU incorporation into cultured astrocytes was reduced with STAT3 inhibitors (Fig. 6B) and STAT3 knockdown by siRNA (Fig. 6E), which indicates that activation of STAT3 mediated the astrocytic G/S phase of cell cycle transition by ET-1. In response to mitogenic stimuli, the expression of cyclin D1 and SKP2 increased in the late G1 phase to promote...
Transition to S phase. Increases in cyclin D1 expression cause activation of CDK4/CDK6, whereas increases in SKP2 expression cause a reduction in p27 protein, a negative regulator of G1/S phase transition, through ubiquitination-dependent proteolysis. In animal models of brain injury, conversion to reactive astrocytes was accompanied by increased expression of cyclin D1 and SKP2 (44–47), suggesting an involvement of these proteins in the proliferation of reactive astrocytes. ET-1 increased...
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(A) Cyclin D1

(B) Cyclin D1, Skp2, and p27

Figure 6. Effects of ET-1 on expression of cyclin D1, SKP2, and p27 in cultured astrocytes. A, serum-starved astrocytes were cultured with 100 nM ET-1 for the times indicated. The expression of cyclin D1, SKP2, and p27 mRNAs was normalized to that of G3PDH. Results are expressed as means ± S.D. (error bars) of 10–12 experiments. Individual data points are indicated by the right of the error bars. *p < 0.05; **p < 0.01; ***p < 0.001 versus zero time by one-way ANOVA followed by Tukey’s test. B, astrocytes were treated with 100 nM ET-1 for the time indicated. Cyclin D1, SKP2, and p27 proteins were detected by immunoblotting. After detection of phosphorylated proteins, the same blots were reprobed with anti-β-actin antibody. The protein bands in X-ray films were subjected to densitometry analyses and normalized to those of β-actin. Results are expressed as means ± S.D. of 5–8 experiments. Individual data points are indicated by the right of the error bars. *p < 0.05; **p < 0.01; ***p < 0.001 versus zero time by one-way ANOVA followed by Dunnett’s test.

Figure 7. Effects of ET-1 on expression of cyclin D1, SKP2, and p27 in cultured astrocytes. A, serum-starved astrocytes were cultured with 100 nM ET-1 and 100 μM BrdU for 48 h. After fixation, cells were labeled with anti-BrdU mouse antibody. For counterstaining, PI was included in the secondary incubations with an antibody. The numbers of BrdU-positive cells observed in each experiment. Individual data points are indicated by the right of the error bars. *p < 0.05; **p < 0.01; ***p < 0.001 versus zero time by one-way ANOVA followed by Dunnett’s test. B, the numbers of BrdU-positive cells under each condition are expressed as percentages of the total number of PI-positive cells observed. Results are expressed as means ± S.D. of six experiments, with more than 400 PI-positive cells observed in each experiment. Individual data points are indicated by dots on the error bars. *p < 0.05; **p < 0.01 versus none without STAT3 inhibitors; ##p < 0.01 versus ET-1 without STAT3 inhibitors by one-way ANOVA followed by Tukey’s test.

Based on these findings, increases in STAT3-mediated transcription of cyclin D1 and SKP2 underlie ET-induced astrocytic G1/S phase transition.

We also found that ET-1 decreased both mRNA and protein levels of astrocytic p27 (Fig. 7, A and B). However, inhibition of STAT3 did not affect p27 mRNA levels (Fig. 8A). Thus, it is likely that ET-1 decreased p27 proteins through both STAT3/activation of astrocytic cyclin D1 and SKP2 mRNAs as well as their proteins (Fig. 7). These effects of ET-1 on cyclin D1 and SKP2 expression were reduced by inhibition of STAT3 (Figs. 8 and 9). 5′-Flanking regions of rat cyclin D1 and SKP2 have STAT3 recognition sequences (18, 57). The present ChIP-PCR analysis showed that ET-1 stimulated the binding of STAT3 to the 5′-promotor sequence of cyclin D1 and SKP2 genes (Fig. 10B).
SKP2-mediated protein degradation and STAT3-independent transcriptional regulation.

In addition to cyclin D1 and SKP2, 5′-regions of GFAP and ETB receptor genes have STAT3 recognition sequences (20, 33) and are thought to be target genes of astrocytic STAT3. We found that transfection of STAT3 siRNA decreased basal levels of astrocytic GFAP and ETB receptor mRNAs (Fig. S2A), indicating the involvement of STAT3 in their transcription. However, treatment with ET-1 did not increase GFAP and ETB receptor mRNAs in cultured astrocytes (Fig. S2B). The implication of STAT3 Ser-727 phosphorylation in GFAP and ETB receptor transcription may differ from that in cyclin D1 and SKP2, although the present study does not clarify the mechanisms. The regulation of STAT3-mediated gene transcription by Ser-727 phosphorylation differs among target genes or cell types (9–12).

Pathophysiological significance of ET-induced astrocytic STAT3 activation

In this study, ET-1 stimulated the G1/S cell cycle transition of cultured astrocytes through activation of STAT3 mediated by Ser-727 phosphorylation. In brain disorders, brain ET-1 increases and causes several pathophysiological responses in damaged nerve tissue (28, 29, 40). Activation of astrocytic ETB receptors promoted phenotypic conversion to reactive astrocytes (33, 35–37, 39). Moreover, the expression of astrocytic

Figure 8. Effects of STAT3 inhibitors on expression of cyclin D1, SKP2, and p27 in cultured astrocytes. A, serum-starved astrocytes were treated with 100 nM ET-1 for 6 h. Stattic (5 μM) and 5,15-DPP (5 μM) were included in the medium at 30 min before the addition of ET-1. The expression of cyclin D1, SKP2, and p27 mRNAs was normalized to that of G3PDH. Results are expressed as means ± S.D. (error bars) of 8–13 experiments. Individual data points are indicated by dots on the error bars. *, p < 0.05; **, p < 0.01 versus none without STAT3 inhibitors; #, p < 0.05; ##, p < 0.01 versus ET-1 without STAT3 inhibitors by one-way ANOVA followed by Tukey’s test. B, astrocytes were treated with 100 nM ET-1 for 16 h. Stattic (5 μM) and 5,15-DPP (5 μM) were included in the medium at 30 min before the addition of ET-1. The expression of cyclin D1 and SKP2 proteins was normalized to that of β-actin. Results are expressed as means ± S.D. of four experiments. Individual data points are indicated by dots on the error bars. **, p < 0.01 versus none without STAT3 inhibitors; #, p < 0.05; ##, p < 0.01 versus ET-1 without STAT3 inhibitors by one-way ANOVA followed by Tukey’s test.
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ETβ receptors is increased, accompanied by the conversion to reactive astrocytes (33, 36, 38), suggesting a positive feedback loop of reactive astrocytes through ETβ receptors. In a mouse TBI model, antagonism of ETβ receptors decreased the number of reactive astrocytes (38). As well as reduction of reactive astrocytes, the present examination showed that antagonism of ETβ receptors also reduced activation of STAT3 in the mouse TBI model (Fig. 5). Considering these observations, ET-induced STAT3 activation is thought to be a mechanism underlying the hyperplasia of reactive astrocytes. In damaged nerve tissue, reactive astrocytes produce and release various bioactive substances that affect neural viability, neurogenesis, and axonal regeneration. In addition, hyperplasia of reactive astrocytes leads to glial scar formation. Studies have shown that glial scar formation has both beneficial and detrimental effects during the repair of damaged nerve tissues (4, 24, 58). Thus, it has been proposed that the control of astrocytic activation and glial scar formation is a promising therapeutic strategy for treating several brain disorders (6, 7). Accordingly, astrocytic ETβ/STAT3 signals may have significance as effective targets to control astrocytic activation and glial scar formation.

**Experimental procedures**

**Preparation of primary cultured astrocytes from rat brain**

The experimental protocols conformed to the Guiding Principles for the Care and Use of Animals by the Japanese Pharmacological Society and were approved by the Animal Experiment Committee of Kobe Pharmaceutical University. Astrocytes were prepared from the cerebra of 1–2-day-old Wistar rats, as described previously (42). The isolated cells were seeded at 1 × 10⁴ cells/cm² in 75-cm² culture flasks and grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum. To remove small process-bearing cells (mainly oligodendrocyte progenitors and microglia from the protoplasmic cell layer), the culture flasks were shaken at 250 rpm overnight, 10–14 days after seeding. The monolayer cells were trypsinized and seeded on 6-well culture plates or 15-mm glass coverslips.

Figure 9. Effects of STAT3 siRNA on expression of cyclin D1, Skp2, and p27 in cultured astrocytes. A, after transfection of STAT3 siRNA, 100 nm ET-1 was included in serum-free MEM, and cultured astrocytes were incubated for 3 h (Skp2) or 6 h (cyclin D1 and p27). The expression of cyclin D1, Skp2, and p27 mRNAs was normalized to that of G3PDH. Results are expressed as means ± S.D. (error bars) of 8–12 experiments. Individual data points are indicated by dots on the error bars. *, p < 0.05; **, p < 0.01 versus no ET-1. #, p < 0.05 versus control siRNA by one-way ANOVA followed by Tukey’s test. B, after transfection of STAT3 siRNA, astrocytes were treated with 100 nm ET-1 for 16 h. Then cell lysate was prepared. The expression of cyclin D1 and Skp2 proteins was normalized to that of β-actin. Results are expressed as means ± S.D. of 4–6 experiments. Individual data points are indicated by dots on the error bars. *, p < 0.05; ***, p < 0.001 versus no ET-1. #, p < 0.05; ###, p < 0.001 versus control siRNA by one-way ANOVA followed by Tukey’s test.
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Cultured astrocytes in 6-well culture plates were lysed in 100 μl of ice-cold homogenization buffer (20 mM Tris/HCl, pH 7.4, 1% SDS, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin) at 4 °C. To prepare lysate of the mouse brain, coronal brain sections (between 0 and 5 mm posterior to the bregma) were made, and the cerebrum was dissected from the brain sections. The cerebral tissue from each animal was homogenized in 200 μl of the lysis buffer. The lysates were centrifuged at 15,000 x g for 10 min, and the protein contents of the supernatants were measured. The cell lysates were applied to SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes. For detection of Tyr-705- and Ser-727-phosphorylated STAT3, the membranes were first probed with anti-phospho-Tyr-705 STAT3 rabbit antibody (1:2,000 dilution, catalog no. 9131, Cell Signaling Technology, Danvers, MA) or anti-phospho-Ser-727 STAT3 rabbit antibody (1:1,000 dilution, catalog no. sc8001R, Santa Cruz Biotechnology, Inc.), diluted with sterilized saline. For the vehicle, saline containing 1% DMSO was prepared. The weight of mice was within a defined range of 30~36 g before antagonist administration.

Measurement of protein levels by immunoblotting

Cultured astrocytes were treated with 100 nM ET-1 for 3 h, nuclear extract for ChIP-PCR was prepared. After ChIP using an anti-STAT3 antibody, DNA fragments were extracted from the STAT3 immunoprecipitants and amplified by PCR using primer pairs to analyze STAT3 binding to 5′-flanking regions of rat cyclin D1 and SKP2 genes. DNA fragments were extracted from the STAT3 immunoprecipitants before ChIP (input DNA) or H2O (blank) as negative and positive controls, respectively. DNA fragments were extracted from nuclear preparation before ChIP (input DNA) or H2O (blank) as negative and positive controls, respectively. DNA fragments were extracted from the STAT3 immunoprecipitants and amplified by PCR using primer pairs to analyze STAT3 binding to 5′-flanking regions of rat cyclin D1 and SKP2 genes, as determined by ChIP-PCR assay.
Activation of astrocytic STAT3 by ET-1

Dallas, TX). Then membranes were incubated with peroxidase-conjugated secondary antibodies. The exposed X-ray films were scanned, and the densities of the protein bands were measured using Image J version 1.45. After detection of the phosphorylated STAT3, the membranes were reprobed with anti-STAT3 rabbit antibody (1:2,000 dilution, C-20, Santa Cruz Biotechnology), and total STAT3 protein levels were measured as described above. Levels of protein phosphorylation were indicated as a ratio of phosphorylated protein to total protein. For the determination of cyclin D1, SKP2, and p27 protein levels, blotted polyvinylidene difluoride membranes were first probed with anti-cyclin D1 mouse antibody (1:2,000 dilution; 72-13-G, Santa Cruz Biotechnology), anti-SKP2 rabbit antibody (1:2,000 dilution; H-432, Santa Cruz Biotechnology), and anti-p27 rabbit antibody (1:1,000 dilution; C-17, Santa Cruz Biotechnology), respectively. After determination of total STAT3, cyclin D1, SKP2, and p27 proteins, the membranes were reprobed with mouse anti-β-actin primary antibody (1:4,000 dilution; Chemicon, Temecula, CA). Expression levels of total STAT3, cyclin D1, SKP2, and p27 were indicated as a ratio to β-actin protein.

Immunochemistry for phospho-STAT3

After astrocytes on 15-mm glass coverslips were incubated in serum-free MEM for 48 h, ET-1 (100 nM) was applied in serum-free MEM. ET-1–treated and nontreated cells were fixed with 3% (w/v) paraformaldehyde and then incubated with phospho-Tyr-705 STAT3 rabbit antibody (1:200 dilution; Cell Signaling Technology) or phospho-Ser-727 STAT3 rabbit antibody (1:200; Santa Cruz Biotechnology). To identify astrocytes, anti-GFAP mouse mAb (1:400; Sigma) was included in the incubation with primary antibodies. After the incubation with primary antibodies, cells were labeled by FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG. Labeled cells were observed using an epifluorescence microscope.

Measurement of astrocytic proliferation

Proliferation of cultured astrocytes was determined by BrdU incorporation as described previously (43). After being cultured in serum-free MEM for 48 h, astrocytes on 15-mm glass coverslips (60–70% confluent) were treated with ET-1 and 100 µM BrdU in serum-free medium. Forty-eight hours later, cells were fixed with 3% paraformaldehyde and then stained with anti-BrdU mouse antibody (1:100 dilution; BU33, Sigma) and 5 µg/ml propidium iodide (PI). Labeled cells were observed using an epifluorescence microscope. Astrocytes with high BrdU immunoreactivity in their nuclei were scored as cells entering into the S phase through the G1 phase of the cell cycle. The numbers of BrdU-positive cells were expressed as percentages of total PI-positive cells observed.

DNA-binding assay of STAT3

Nuclear extraction from cultured astrocytes was performed using a nuclear extract kit (Active Motif (Carlsbad, CA), catalog no. 40010). After cultured astrocytes were collected from 6-well plates, nuclear extraction was performed according to the manufacturer’s protocol. Binding of STAT3 protein to the DNA consensus sequence was measured using an ELISA-based assay kit (TransAM® STAT3 transription factor assay kit, Active Motif, catalog no. 45196). After nuclear extraction from astrocytes, the binding assay was performed according to the manufacturer’s protocol.

Measurement of cyclin D1, SKP2, and p27 mRNA levels using quantitative RT-PCR

Before treatment with ETs, astrocytes in 6-well culture plates were incubated in serum-free MEM for 48 h. After treatment with ETs, total RNA in cultured astrocytes was extracted using a total RNA extraction kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). First-strand cDNA was synthesized from total RNA (1 µg) using Maloney murine leukemia virus reverse transcriptase (200 units; Invitrogen), random hexanucleotides (0.2 µg; Invitrogen), and an RNase inhibitor (20 units; Takara, Tokyo, Japan) in 10 µl of a buffer supplied by the enzyme manufacturer. The mRNA levels of cyclin D1, SKP2, and p27 in each sample were determined by quantitative PCR using SYBR Green fluorescent probes. Each reverse transcription product was added to SYBR Green Master Mix (Toyobo, Tokyo, Japan) along with the primer pairs, and the mixture was placed in a thermal cycler (Opticon 2; MJ Research, Waltham, MA). The following primer pairs were used: rat cyclin D1, 5′-TGCTGGGGAATGTGTGTTGG-3′ and 5′-AATGCGCACTACGGTCCTAC-3′; SKP2, 5′-AGGAGGTGACATGAAAT-3′ and 5′-CTCTCTGCAAACCTCCAGAGACT-3′; p27, 5′-GCTTGGATGTCAAGCGGA-3′ and 5′-TCAGAGTTTCGCTGACCCA-3′; and G3PDH, 5′-CTCATGACACGTCCATGC-3′ and 5′-TACATTGGGGTACGACAC-3′. As a standard for the copy numbers of PCR products, serial dilutions of each amplification product were amplified in the same manner. The amount of cDNA was calculated as the copy number of each reverse transcription product equivalent to 1 µg of total RNA and then normalized to the value for G3PDH.

Knockdown of astrocytic STAT3 by siRNA

Knockdown of astrocytic STAT3 was attained by transient transfection of STAT3 siRNA. Rat STAT3 siRNA was obtained from Santa Cruz Biotechnology (catalog no. sc-270027) and was composed of a mixture of three different types of siRNA targeting rat STAT3 mRNA. Transient transfection of STAT3 siRNA to cultured astrocyte was carried out using Lipofectamine® RNAiMAX (Thermo Fisher Scientific), according to the supplier’s protocol. Briefly, a stock solution of STAT3 siRNA and a negative control siRNA (Santa Cruz Biotechnology, sc-37007) were diluted to 200 nM in Opti-MEM (Thermo Fisher Scientific). Meanwhile, Lipofectamine® RNAiMAX was diluted to 2% in Opti-MEM. Then diluted siRNA and transfection reagent solutions were mixed and left for 10 min to form an siRNA–transfection reagent complex. To examine the effects of STAT3 knockdown on mRNA and protein expression, astrocytes were seeded in 6-well plates. To examine the effects on BrdU incorporation, cells were seeded on 15-mm glass coverslips inserted in 24-well plates. When cultured astrocytes reached 60–70% siRNA and transfection reagent was included in serum-free MEM, final concentrations of siRNA and trans-
fection reagent were 50 nM and 0.5%, respectively. Forty-eight hours later, siRNA-containing medium was replaced by fresh serum-free MEM, and cultured astrocytes were subjected to each experimental condition.

**ChiP-PCR analysis of STAT3 binding to 5'-flanking regions of cyclin D1 and SKP2 genes**

After incubation with 100 nM ET-1 for 1 and 3 h, cultured astrocytes in 10-cm dishes were treated with parafomaldehyde (1.1% final concentration) to cross-link nuclear proteins and DNA. Cells were collected by centrifugation, and the pellet was washed in ice-cold PBS twice. Nuclear extract of cultured astrocytes was prepared using a ChiP assay kit (SimpleChiP® Plus Enzymatic Chromatin IP Kit, Cell Signaling Technology, catalog no. 9004) according to the supplier’s protocol. Astrocytic nuclear fractions were digested by micrococcal nuclease (Cell Signaling Technology, catalog no. 10011) at 37 °C for 20 min and collected by centrifugation. The resulting pellet was suspended in 1X ChiP buffer and dispersed using a tip sonicator (Ultrasones SonifierTM S-250D, Branson) to fragment genomic DNA. After centrifugation, the resultant supernatant was used for STAT3 immunoprecipitation as an astrocytic nuclear extract. The astrocytic nuclear extract (40 μg of protein) was incubated with 1 μg of anti-STAT3 rabbit IgG (C-20, Santa Cruz Biotechnology) in 200 μl of 1X ChiP assay buffer at 4 °C overnight. For negative or positive control, an equal amount of nonimmune rabbit IgG (Cell Signaling Technology, catalog no. 2729) or anti-histone H3 rabbit antibodies (Cell Signaling Technology, catalog no. 4620) was included in the ChiP mixture instead of anti-STAT3 antibody. Then protein G–agarose was included, and the ChiP mixture was further incubated for 2 h. After being collected by centrifugation, the immunoprecipitants were digested by proteinase K (40 μg; Cell Signaling Technology, catalog no. 10012) at 65 °C for 5 h. DNA fragments in the immunoprecipitants were purified using a DNA spin column. The amount of DNA fragment containing STAT3-binding sequence was measured by quantitative PCR as described above. For detection of the cyclin D1 promoter fragment, a primer pair was designed using the 5'-flanking region of the rat cyclin D1 gene containing the STAT3-binding sequence (18): forward, 5’-AAACACACCCCAACCGAACGCT-3’; reverse, 5’-CTCCACTGCCGGAAGAAAT-3’ (Fig. S3). For detection of the SKP2 promoter fragment, the STAT3-binding sequence in the 5'-flanking region of the rat SKP2 gene (57) was analyzed using TFBIND, a software for searching transcription factor–binding sites (59). A primer pair was designed to amplify DNA fragments containing the predicted STAT3 binding sequences: forward, 5’-CGAAGGCTGTGGGCTC-3’; reverse, 5’-CATCCCCGCTCCACGACAG-3’ (Fig. S3).

**Statistical analysis**

Experimental data are presented as mean ± S.D. Results from more than three groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s test or Tukey’s test. Results from two groups were analyzed by Student’s t test. p values were calculated using Ekuseru-Toukei2015 (Social Survey Research Information Co., Ltd., Tokyo, Japan). p values < 0.05 were considered statistically significant.

### References


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