

Janus Kinase-Signal Transducer and Activator of Transcription Mediates Phosphatidic Acid-Induced Interleukin (IL)-1 β and IL-6 Production

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ABSTRACT

We have found previously that phosphatidic acid (PA) can induce inflammatory mediators such as cytokines, which implies that PA plays a role in inflammatory response. In the present study, we provide evidence of the PA-mediated activation of the Janus tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, which results in the production of interleukin (IL)-1 β and IL-6. PA elicited the rapid phosphorylations of JAK2 and STAT1/3, and the subsequent nuclear translocation. Macrophages that had been transiently transfected with a luciferase reporter construct containing eight consecutive γ -interferon activating sequence (GAS) elements, a known STAT binding site, exhibited enhanced reporter gene activity in response to PA stimulation, which further supports the involvement of JAK-STAT activation in the PA-induced signaling pathway. Of the inflammatory cytokines, IL-1 β , IL-6, and tumor necrosis factor (TNF)- α were detected in media from macrophages stimulated with PA.

Moreover, the JAK2 inhibitor α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide (AG-490) abolished PA-induced IL-1 β and IL-6 release but not TNF- α production, which is consistent with the notion that IL-1 β and IL-6 but not TNF- α contain a STAT binding element in their promoter region. The knockdown of JAK2 in macrophages by small interfering RNA significantly attenuated PA-induced IL-1 β and IL-6 production. In addition, JAK2 inhibitor suppressed PA-induced Akt phosphorylation, and the Akt inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) blocked GAS activation (GAS contains a promoter that responds to PA), suggesting that PA-mediated JAK2 activation leads to phosphatidylinositol 3-kinase/Akt phosphorylation and STAT activation, and the subsequent translocation of STAT to the nucleus. Together, our data demonstrate that PA-activated macrophages produce IL-1 β and IL-6 and that these processes require the activation of the JAK2-STAT1/3 or JAK2-Akt-STAT signaling pathways.

Phosphatidic acid (PA) is an important metabolite that is involved in phospholipid biosynthesis and membrane remodeling (Lim et al., 2003). PA can be generated by several cellular processes, such as the hydrolysis of phosphatidylcholine by phospholipase D, the phosphorylation of DAG by DAG

kinase, and the acylation of lyso-PA by lyso-PA acyltransferase (Koch et al., 2004; van Baal et al., 2005), and can be metabolized to other bioactive lipids, such as lyso-PA and DAG (Nanjundan and Possmayer, 2003).

It has been suggested that PA may play a crucial role in the regulation of various biological events. For example, PA is involved in the phosphorylations of many proteins (Chen et al., 2003; Avila-Flores et al., 2005), activation because of oxidative stress (de Jong et al., 2004), modulation of membrane trafficking (Kooijman et al., 2003), and regulation of inflammatory response (Lim et al., 2003; Tou and Gill, 2005).

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ABBREVIATIONS: PA, phosphatidic acid; DAG, diacylglycerol; mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase; JAK, Janus tyrosine kinase; STAT, signal transducers and activators of transcription; siRNA, small interfering RNA; IL, interleukin; PI3K, phosphatidylinositol 3-kinase; AG-490, α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PD98059, 2'-amino-3'-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, 1,9-pyrazolo anthrone anthra (1,9-cd) pyrazol-6(2*H*)-one; MAPK, mitogen-activated protein kinase; GAS, γ -interferon activating sequence; Ab, antibody; PAGE, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; TF, transcription factor; TTBS, Tris-buffered saline/0.05% Tween 20.

We found evidence that the addition of PA to macrophages induces a number of inflammatory responses, such as proinflammatory cytokine production, cyclooxygenase-2 expression, and the up-regulation of inducible nitric-oxide synthase (Lim et al., 2003). Other studies have also found that PA stimulates cardiac ATP-sensitive K⁺ channels with novel gating kinetics (Fan et al., 2003), which is also observed in the study of phosphatidylinositol, and promotes hair growth in epithelial cells (Anthony et al., 2004).

It is noteworthy that many of these processes are highly selective and specific; thus, it was suggested that like other lipid second messengers, PA seems to function via selected targets in specific cell membrane regions (Ktistakis et al., 2003). It was recently reported that PA causes the activation of the mammalian target of rapamycin (mTOR), and subsequently targets mTOR, S6 kinase, and 4E-binding protein 1 (Avila-Flores et al., 2005). Likewise, PA directly regulates protein kinase activities such as Fgr and protein kinase C- ζ , and also regulates the mitogen-activated protein kinase kinase/ERK cascade (Bollag et al., 2005). Moreover, Alderton et al. (2001), who reported PA-mediated ERK activation, speculated that ERK phosphorylation might occur via a specific PA receptor, although its existence was unproven. Another study demonstrated that PA binds to the orphan receptor GPR63 (Niedernberg et al., 2003; Kostenis, 2004), suggesting that PA acts through binding to cell membrane targets. However, the cellular target of PA has not been identified, and the mechanisms involved remain to be elucidated.

The JAK pathway may be triggered by diverse ligands, including cytokines and growth factors (Ihle, 1995; Yadav et al., 2005). Moreover, JAK-mediated signals trigger proliferation, migration, inflammatory responses, immune responses, and other cellular events (Hu et al., 2002; Niwa et al., 2005; Smith et al., 2005). The JAK family consists of four members, namely, JAK1, JAK2, JAK3, and TYK2, which are all ubiquitously expressed in cells, whereas in macrophages JAK2 is dominantly expressed (Natarajan et al., 2004; de Jonge et al., 2005). The stimulation of cells with suitable ligands, such as cytokines, induces receptor oligomerization and causes the local aggregation of associated JAKs, which results in their activation by transphosphorylation. JAKs are activated by tyrosine transphosphorylation, which allows them to phosphorylate their major downstream targets (e.g., STATs). When tyrosine is phosphorylated by JAKs, STATs are translocated to the nucleus and turn on target genes (Murray et al., 2005; Niwa et al., 2005).

Here, we demonstrate that the JAK2-STAT1/3 pathway has critical and distinctive role in PA-induced cytokine release. Our data show that PA elicits JAK2 activation and phosphorylation and the nuclear translocation of STAT1/3 in Raw 264.7 cells (a murine macrophage cell line). A JAK2 inhibitor or JAK2 siRNA suppressed PA-induced IL-1 β and IL-6 production, indicating the involvement of the JAK2 pathway in PA-activated macrophages. We also found that Akt is activated by PA and that an Akt inhibitor abolished promoter activity containing γ -interferon activating sequence (GAS) element in response to PA, suggesting that STAT might be a downstream target of Akt in PA-induced signaling. Together, our data demonstrate that novel signal transduction pathways are required for cytokine release by PA and that these include the JAK2-STAT1/3 or the JAK2-PI3K/Akt-STAT pathway in PA-activated macrophages.

Materials and Methods

Reagents and Antibodies. Dioctanoyl PA (>99%) was obtained from Sigma-Aldrich (St. Louis, MO), and its endotoxin content was determined using a chromogenic *Limulus polyphemus* ameocyte lysate kit (BioWhittaker, Walkersville, MD). AG-490, LY294002, and several MAPK inhibitors—PD98059, SB203580, and SP600125—were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA), and JAK3 inhibitor was from Calbiochem (San Diego, CA). The GAS-luciferase reporter construct was generously provided by Dr. M. H. Song (Chungnam University, Daejeon, Korea). For Western blot analysis, we used Abs against JAK1, JAK2, JAK3 (Chemicon International, Temecula, CA), and TYK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and against phosphorylated JAK2 (Tyr1007/1008), STAT1 (Tyr701), STAT3 (Tyr705), and Akt (Thr308) (Cell Signaling Technology, Inc., Beverly, MA). Peroxidase-conjugated anti-rabbit IgG, anti-goat IgG, or anti-mouse IgG (Santa Cruz Biotechnology, Inc.) were used as secondary antibodies.

Cell Culture. Raw 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum, 2 mM L-glutamine, 10 U/ml penicillin, and 10g/ml streptomycin at 37°C in 5% CO₂ in a water-saturated atmosphere. Cells were treated with synthetic PA for the indicated times.

Isolation of Murine Peritoneal Macrophages. Resident peritoneal macrophages were obtained by peritoneal cavity lavage using 10 ml of RPMI 1640 medium. Cells from five mice were pooled, pelleted by centrifugation (200g; 10 min), and washed once with RPMI 1640 medium. The cells were then seeded in a 100-mm culture dish to remove any contaminating cells and to enrich adhering macrophages, and after 2 h of incubation, adherent cells were collected and used in experiments.

Cytokine Measurements. The conditioned media from RAW 264.7 cells stimulated with PA amounts of TNF- α , IL-1 β , and IL-6 were determined by specific enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Kinase Activity Measurement. Raw 264.7 cells were seeded in 35-mm dishes, cultured overnight, and treated with PA for the indicated times. Whole cell lysates were prepared in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 μ M sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail. To determine JAK activities, 0.5 mg of total protein was incubated with phosphotyrosine Ab (4G10) for 4 h and then with protein A-agarose for 2 h at 4°C. After being washed seven times with lysis buffer, immunocomplexes were resolved by SDS-PAGE and transferred to nitrocellulose for subsequent immunoblot analysis using JAK2 Ab. For kinase phosphorylation, immunoblotting was performed using Abs specific for the phosphorylated, activated forms of proteins.

Nuclear Extracts. Raw 264.7 cells were incubated with PA as indicated. Cells were harvested in PBS containing 2% serum, washed twice with ice-cold PBS, and resuspended in 400 μ l of buffer A (10 mM HEPES, pH 7.9, 5 mM MgCl₂, 10 mM KCl, 1 mM ZnCl₂, 0.2 mM EGTA, 1 mM Na₃VO₄, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM PMSF, and protease inhibitors). After cells had been incubated on ice for 10 min and lysed by adding 50 μ l of 10% Nonidet P-40 (to a final concentration of 1.1%), nuclei were harvested by centrifugation. Nuclear pellets were then resuspended in 60 μ l of extraction buffer (10 mM HEPES, pH 7.9, 5 mM MgCl₂, 300 mM NaCl, 1 mM ZnCl₂, 0.2 mM EGTA, 25% glycerol, 1 mM Na₃VO₄, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM PMSF, and protease inhibitors) and incubated on ice for 15 min. Nuclear debris was then removed by centrifugation (13,000 rpm \times 10 min), and nuclear protein extracts were subjected to gel shift analysis. Protein concentrations were determined using the Bradford method.

Electrophoretic Mobility Shift Assays. Mobility shift assays were performed with nuclear extracts and biotin-labeled oligonucle-

otides containing the consensus sequences of transcription factor (TF) STAT1 or STAT3 using gel shift kits (Panomics, Redwood City, CA). Typical binding reaction mixtures consisted of 5 μ g of nuclear extract, labeled TF probe, 2 μ g/ml poly[d(I-C)] in a buffer containing 20 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol, and these were incubated at room temperature for 30 min. Reaction mixtures were then separated on 6% Tris-glycine nondenaturing polyacrylamide gels in a 2 \times Tris-glycine buffer system and transferred to nitrocellulose membranes. The shifted bands corresponding to protein/DNA complexes were visualized using a chemiluminescence system. Unlabeled TF probes were used as a competitor to confirm the identities of the protein/DNA complexes.

Transfection and Promoter Activity Assays. A promoter-reporter construct containing eight copies of the GAS element was transfected into RAW 264.7 cells using LipofectAMINE 2000 (Gibco BRL). After transfection, cells were incubated in complete media for 24 h at 37°C and stimulated with PA for 8 h at 37°C. In some experiments, cells were preincubated with specific inhibitor for 1 h at 37°C before PA stimulation. Cell lysates were assayed for luciferase activity using a luminometer (Promega, Madison, WI) according to the manufacturer's instructions. For JAK2 silencing, Raw 264.7 cells were transfected with a 100 nM concentration of a pool of JAK2-specific siRNA (SMARTpool kit; Dharmacon, Chicago, IL) using LipofectAMINE2000. As a control, nonspecific siRNA duplexes were transfected in parallel into cells.

Protein Extraction and Western Blot Analysis. Raw 264.7 cells were stimulated with PA, washed twice in ice-cold PBS, and lysed on ice using lysis solution (1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM PMSF, 1 mM Na₃VO₄, and protease inhibitor cocktail). Protein concentrations were determined using Bio-Rad protein assays. In brief, proteins from cell lysates (50 μ g) were boiled at 95°C in Laemmli SDS loading buffer, separated on 8% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Membranes were blocked for 30 min at room temperature in Tris-buffered saline/0.05% Tween 20 (TTBS) containing 5% nonfat dry milk and then incubated with TTBS containing a primary Ab for 4 h at room temperature. After five 10-min washes in TTBS, membranes were incubated with peroxidase-conjugated secondary Ab for 1 h. After 5 \times 10 min with TTBS, protein bands of interest were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences Inc., Little Chalfont, Buckinghamshire, UK).

Results

PA Induces the Phosphorylation and Activation of JAK2 in RAW 264.7 Cells. Our previous work showed that PA induces the proinflammatory cytokines IL-1 β , IL-6, and TNF- α in macrophages (Lim et al., 2003). Although activation of JAK/STAT signaling has been associated with inflammation-related stimulation by many cytokines, no published data demonstrate a direct link between this pathway and PA stimulation in macrophages. Thus, we examined whether PA activates the JAK/STAT pathway in RAW 264.7 cells. Thus, the tyrosine phosphorylations of different JAKs were examined in PA-stimulated macrophages. In brief, whole cell lysates from macrophages exposed to 50 μ M PA were immunoprecipitated with anti-phosphotyrosine antibody (4G10) and subjected to Western blot analysis with antibodies against JAKs. A strong phospho-JAK2 band was detected (Fig. 1A), but the phosphorylations of other members of the JAK family—JAK1, JAK3, and TYK2—were not observed (data not shown), indicating that JAK2 is the only member of the JAK family activated by PA in macrophages. PA-induced JAK2 phosphorylation was also confirmed by the Western blotting of PA-stimulated cell lysates using phospho-specific

anti-JAK2 antibody (Tyr 007/1008) and was detected as early as 5 min after stimulation (Fig. 1B). We next examined PA-induced JAK2 phosphorylation in primary macrophages. Peritoneal macrophages from 10-week-old mice were stimulated with PA, and we found PA-induced JAK2 phosphorylation (Fig. 1C).

PA Promotes STAT1/3 Phosphorylation and Nuclear Translocation. Because the JAK/STAT pathway is the main effector of signaling by many bioactive ligands (Niwa et al., 2005), we examined whether STATs are involved in JAK2 pathway induction by PA. As shown in Fig. 2A, STAT1 was phosphorylated (at Tyr701) in response to PA, and this phosphorylation reached a maximum level at 1 min and began to decline at 10 min. Total STAT1 protein levels were unchanged during this phosphorylation. In addition to STAT1 phosphorylation, we detected the PA-induced phosphorylation of STAT3 (Tyr705) under the same conditions. Moreover, the pattern of STAT3 phosphorylation seemed similar to that of STAT1 phosphorylation. Next, we examined whether phosphorylated STAT1 and STAT3 translocate to the nucleus. Therefore, nuclear extracts from PA-treated macrophages were subjected to Western blot analysis for STAT1 and STAT3. Figure 2B shows that both STAT1 and STAT3 were rapidly translocated to the nucleus after being activated by PA, suggesting that PA caused JAK2 activation, which resulted in the phosphorylation of STAT1 and STAT3 and the subsequent nuclear translocation. To confirm that PA-induced STAT activation is associated with transcriptional activation, macrophage cells were transfected with a luciferase construct containing eight copies of the GAS element, which is a STAT binding site, and we found that PA stimulated

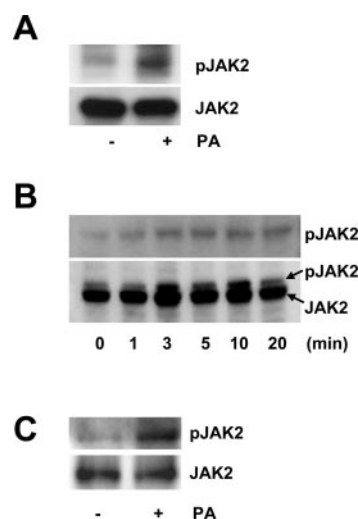


Fig. 1. Effect of PA on the tyrosine phosphorylation of JAK2. A, RAW 264.7 cells were treated with synthetic 50 μ M PA for the indicated times. Whole cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine (4G10) monoclonal antibody. After immunoprecipitation, phosphorylated JAK2 was detected using a specific antibody. The total amount of JAK2 protein in lysates is shown (bottom). A representative result of three independent experiments is shown. B, cells were incubated with 50 μ M PA for the indicated times. To detect JAK2 phosphorylation, equal amounts of cell extracts were analyzed by Western blotting using phospho-specific JAK2 antibody. As a loading control, the same blots were reprobed with anti-JAK2 antibody. C, peritoneal macrophage cells were prepared and stimulated with 50 μ M PA for 10 min. Whole cell extracts were used for immunoblot analysis to detect phospho-JAK2 using specific antibody. As a loading control, the same blots were reprobed with anti-JAK2 antibody.

GAS-luciferase activity in a dose-dependent manner (Fig. 3A). Consistent with this result, gel shift assays using biotin-labeled oligonucleotides containing a STAT1 or STAT3 consensus sequence and nuclear extracts from PA-treated cells demonstrated STAT1- or STAT3-probe complexes. Major gel shift complexes were also found in nuclear extracts from unstimulated cells, but increment of the same complexes was evident in nuclear extracts from PA-treated cells (Fig. 3B). In addition, we also found competition of unlabeled probe in the formation of STAT1- or STAT3-probe complex, confirming the specific binding of these factors to STAT1 or STAT3 consensus sequence.

JAK2-STAT Pathway Activation Is Required for PA-Induced Cytokine Release. We have shown previously that PA induced the expressions of IL-1 β and IL-6, and TNF- α and that PI3K/Akt pathway activation is involved in these processes (Lim et al., 2003). Thus, we examined whether PI3K/Akt is engaged in the JAK2-STAT pathway initiated by PA-mediated signaling. In the presence of the JAK2 inhibitor AG490, macrophages were treated with PA for 18 h, and cytokine levels of IL-1 β and IL-6, and TNF- α in conditioned media were determined by ELISA. AG490 strongly reduced PA-induced IL-1 β and IL-6 production but not that of TNF- α (Fig. 4, A–C), indicating that the PA-stimulated productions of IL-1 β and IL-6 are dependent on JAK2. To exclude possible inhibition of JAK3 and subsequent PI3K activation by AG490, which was observed in T cells (Dadi et al., 1994; Sharfe et al., 1995), cells were treated with JAK3 inhibitor. However, it had no effect on PA-stimulated IL-6 and TNF- α production (data not shown), indicating that JAK2-STAT pathway activation is involved in the PA-induced cytokine release.

We next examined the effect of AG490 on PA-induced nuclear factor binding to the GAS element. PA-induced GAS-luciferase activity was significantly suppressed by AG490 (Fig. 4D), implying that PA stimulates JAK2-STAT pathway activation, resulting in IL-1 β and IL-6 production, because IL-1 β and IL-6 contain a STAT binding element in their promoter region. The involvement of JAK2 in PA-stimulated IL-1 β and IL-6 production was confirmed by treating cells with JAK2-specific siRNA. Cells were transfected with a commercially available JAK2 siRNA pool, and Western blot analysis showed significantly, but not completely, reduced JAK2 levels in siRNA-containing cell lysates (Fig. 5A). Moreover, this reduced JAK2 expression was found to be associated with reduced amounts of IL-1 β and IL-6 in response to

PA treatment (Fig. 5B). TNF- α production was not affected by JAK2 expression, which is consistent with a result of Fig. 4C, namely, that AG490 had no effect on PA-induced TNF- α production. Together, these data suggest that the activation of the JAK2-STAT pathway by PA is not required for TNF- α production.

Akt, a Downstream Target of JAK2, Is Involved in PA-Induced Cytokine Release. In this study, we investigated the potential engagement of the Akt pathway in PA-activated signaling, because we previously showed that Akt is an important signaling molecule in PA-mediated cytokine production (Lim et al., 2003), and because it has been reported that JAK2 plays an important role in STAT binding to the GAS element of via the Akt pathway (Krasilnikov et al., 2003). Thus, we tested the effect of AG490 on PA-induced Akt phosphorylation. It is noteworthy that Akt phosphorylation by PA was inhibited by AG490 (Fig. 6A) but not by 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131), a JAK3 inhibitor (data not shown).

Furthermore, reporter gene activity in RAW 264.7 cells transfected with luciferase reporter plasmid containing the STAT binding element was significantly blocked when cells were preincubated with LY294002, confirming the functional activation of the JAK2-Akt-STAT pathway (Fig. 6B). These results suggest a requirement for JAKs, particularly JAK2, in the activation of the Akt pathway and of its downstream target STAT by PA in RAW 264.7 cells.

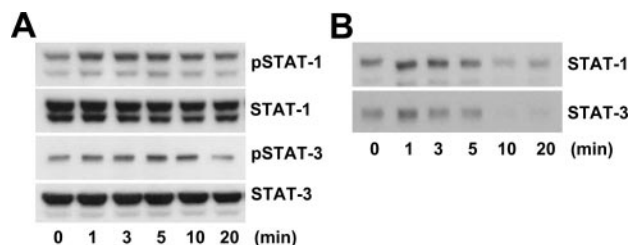


Fig. 2. Effect of PA on STAT1 and STAT3 phosphorylation and nuclear translocation. RAW 264.7 cells were stimulated with 50 μ M PA for the indicated times. A, whole cell lysates were prepared and immunoblotted with phosphotyrosine-specific STAT1 or STAT3 antibodies. Blots were then stripped and reprobed with anti-STAT1 and STAT3 antibodies. B, nuclear extracts were prepared as described under *Materials and Methods*. Nuclear extracts were resolved by SDS-PAGE and immunoblotted with anti-STAT1 and STAT3 antibodies.

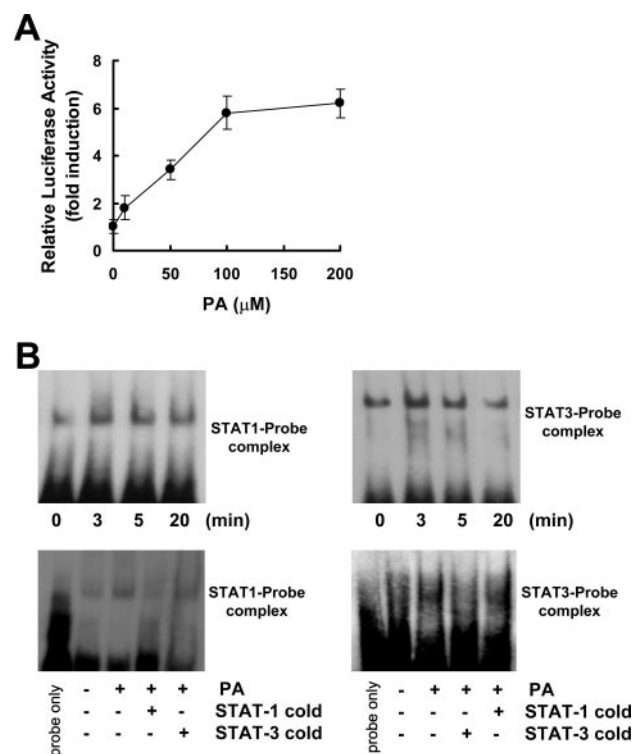


Fig. 3. Effects of PA on STAT1 and STAT3 phosphorylation and nuclear translocation. A, RAW 264.7 cells were transfected with 5 μ g of GAS-luciferase reporter construct using LipofectAMINE 2000. The transfected cells were then divided into 12-well plates. The following day, cells were stimulated with the indicated concentrations of PA for 8 h and then luciferase activities were measured. B, nuclear extracts from PA-stimulated cells were prepared and subjected to EMSA using unlabeled and biotin-labeled STAT1 and STAT3.

Discussion

PA has been implicated as an important phospholipid biosynthesis metabolite during membrane remodeling and has been further suggested to be a crucial second messenger in various cellular signaling events, for example, in inflammatory response (de Jong et al., 2004; Avila-Flores et al., 2005). We reported previously that PA regulates the expression of proinflammatory cytokines, nitric oxide, and prostaglandin E_2 by modulating the Akt-mTOR-S6K pathway (Lim et al., 2003). In this study, we describe a novel mechanism for the up-regulations of the productions of IL-1 β and IL-6 by PA and a novel biological role for JAK2 in the regulation of the release of both of these cytokines. We found that PA induced phosphorylations of JAK2 and STAT1/3, translocation of STATs to the nucleus, and activation of GAS-luciferase activity. We also found that JAK2 is essential for PA-induced cytokine release by using a pharmacological inhibitor of JAK2 and an siRNA of JAK2, and we showed that Akt is involved in the transcriptional activation of STAT, a downstream target of JAK2.

Macrophages are professional phagocytic cells that function predominantly in nonspecific host defense, and as one of the most active secretory cells in the body, they release hundreds of mediators that can regulate cytotoxicity, inflammation, and proliferation. The production of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 are of particular interest in terms of toxicity, because they are the most stringently regulated proinflammatory cytokines identified to date. Resting macrophages produce low basal levels of these cytokines, but various factors can stimulate these cells to induce huge amounts. Because of the potent biological actions of these cytokines, considerable attention has been focused on identifying the mechanisms that activate and limit cytokine gene expression. Only recently was it recognized that PA may signal through novel mechanisms,

including the MAPK signaling network, which might be linked to transcription factor in the nucleus (Watanabe et al., 2004; Chen et al., 2005). Consistent with these findings, we and others have found that various kinases, including Akt and MAPKs can be activated by PA stimulation (Chen et al., 2003; Lim et al., 2003; Li and Malik, 2005). Therefore, it is conceivable that PA activates cells through an as yet unidentified molecule belonging to the JAK family. PA-induced effects in macrophages, such as IL-1 β and IL-6 release, were found to sensitive to AG490 treatment, implying JAK2 involvement in PA-induced signal transduction. However, AG490 had no effect on TNF- α release in response to PA, indicating the existence of a regulatory mechanism not requiring JAK2 activation for TNF- α production by PA. Because AG490 has been reported to also block JAK3 activity in T cells (Dadi et al., 1994; Sharfe et al., 1995), we examined its effects and found that PA-induced IL-6 and TNF- α production were not affected by JAK3 inhibitor, confirming JAK2 activation of PA.

The activity of JAK2 is crucial for the induction of numerous genes, including cytokines and inflammatory mediators (Hu et al., 2002). Although other mechanisms have also been reported to be involved in the activation of these transcription factors, it is known that as downstream targets of JAKs, STATs are phosphorylated by JAKs at tyrosine residues and thus activated. To further understand the signaling events underlying the PA-induced expression of IL-1 β and IL-6, we next examined the JAK-STAT pathway and found that PA induced the tyrosine phosphorylations of STAT1 and STAT3 in a time-dependent manner. This is the first report that PA activates the JAK-STAT signaling pathway.

Upon tyrosine phosphorylation, STATs undergo either homo- or heterodimerization and translocate to the nucleus, where they bind GAS element, a consensus DNA binding sequence present in the promoter regions of genes that is

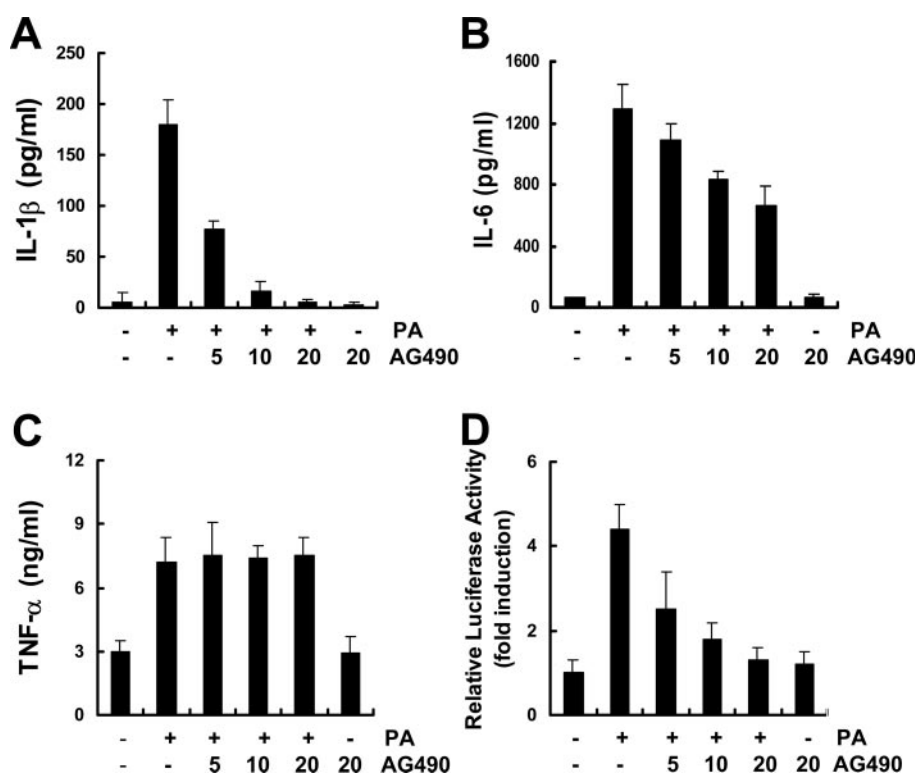


Fig. 4. Effect of JAK2 inhibitor on PA-induced cytokine release and GAS-dependent luciferase activity. A to C, RAW 264.7 cells were pretreated with the indicated concentrations of AG490 for 1 h and then treated with 50 μ M PA for 18 h. The levels of IL-1 β and IL-6, and TNF- α in conditioned media were determined by ELISA. D, cells were transfected with 5 μ g of GAS-luciferase construct. The following day, transfected cells were collected and divided into 12-well plates. And then cells were preincubated for 1 h with the indicated concentrations of AG490 (a JAK2 inhibitor) before adding PA, and 8 h later luciferase assays were performed.

required for transcription (Bach et al., 1997). To test nuclear translocations of STAT1/3 after PA stimulation, quiescent cells were treated with PA, and cytoplasmic and nuclear

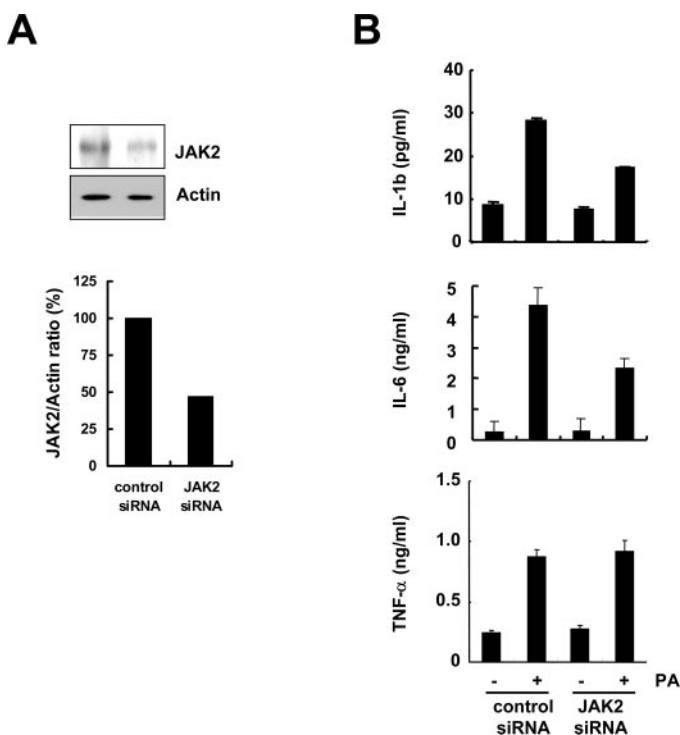


Fig. 5. Knockdown of JAK2 with siRNA suppressed the PA-induced productions of IL-1 β and IL-6. **A**, RAW 264.7 cells were transfected with 5 μ g of JAK2 or control siRNA using LipofectAMINE 2000. Whole-cell lysates from transfected cells were prepared and immunoblotted with phosphotyrosine-specific JAK2. **B**, cells transfected with JAK2 or control siRNA were collected and divided into 24-well plates. The next day, cells were stimulated with 50 μ M PA for 18 h, and IL-1 β , IL-6, and TNF- α levels were determined by ELISA.

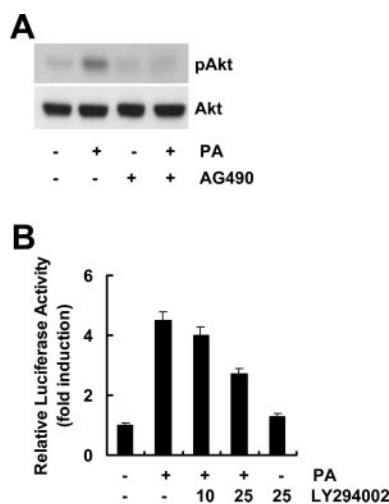


Fig. 6. JAK2 activation is required for PA-stimulated Akt phosphorylation. **A**, RAW 264.7 cells were treated with 50 μ M PA with or without 20 μ M AG490 for 30 min, and cell extracts were then prepared. Equal amounts of protein (50 μ g) from nontreated controls and from each treatment were analyzed by Western blotting for Akt phosphorylation using a specific antibody. As a loading control, blots were reprobed with anti-Akt antibody. **B**, cells were transfected with 5 μ g of GAS-luciferase construct. The following day, transfected cells were collected, divided into 12-well plates, preincubated for 1 h with or without 25 μ M LY294002, and then stimulated with PA. Eight hours later, luciferase assays were performed.

fractions were prepared. Both STAT1 and STAT3 levels in the nuclear fractions of PA-stimulated cells were increased, and STAT1/3 binding activity to their consensus sequences was also found to be elevated by gel mobility shift assay. We then transfected macrophages with a luciferase reporter plasmid containing eight copies of the GAS element and found that PA induced GAS-dependent luciferase activity by 4-fold and that AG490 substantially inhibited this response, confirming that enhanced STAT1/3 binding activity leads to an increase in transactivation activity.

Several recent reports have demonstrated that JAK stimulation alone does not fully account for the functional activations of STAT-induced genes. Thus, it seems that the activation of STAT-dependent transcription requires the involvement(s) of other kinase(s), and PI3K and its downstream target Akt have been suggested to mediate this event (Krasilnikov et al., 2003; Xu et al., 2005). These findings caused us to consider the possibility that the Akt pathway might be involved in the regulation of cytokine production in response to PA. Our group previously reported that Akt is phosphorylated in macrophages after PA stimulation and that it is required for cytokine production induced by PA. To test the role of JAK2 in PA-induced Akt phosphorylation, cells were treated with PA in the presence or absence of AG490 or JAK3 inhibitor, and then Akt phosphorylation was measured. It was found that AG490 but not JAK3 inhibitor strongly suppressed PA-induced Akt phosphorylation. In addition, pan-JAK inhibitor also has a strong effect on PA-induced Akt phosphorylation and cytokine release (data not shown), suggesting that JAK2 participates in Akt and STAT phosphorylation in response to PA. A linkage between JAK2 and Akt was also confirmed by the finding that STAT transcriptional activation was attenuated by AG490 or LY294002, a JAK2 and an Akt inhibitor, respectively.

Orphan receptor GPR63 has been shown to bind dioleoyl PA and has been suggested to contribute to its biological ability in cells (Kostenis, 2004). Fang et al. (2003) showed that PA is required for the activation of mTOR downstream effectors and that it directly interacts with the domain in mTOR. Previous reports have demonstrated the existence of PA binding sites within the PX domain of p47phox (Stahelin et al., 2003) and the CR3 domain of cRaf-1 (Andresen et al., 2002) and direct interactions between PA and a small group of proteins [e.g., kinase suppressor of Ras; isoforms of cAMP-specific phosphodiesterase, PDE4A1 and PDE4D3 (Grange et al., 2000); and tyrosine protein phosphatase Src homology phosphatase-1 (Houslay and Adams, 2003)]. However, our preliminary data suggest other events occur in macrophages. Flow cytometry and confocal microscopy analyses suggest that PA penetrates the cell membrane, where it is rapidly incorporated before it is distributed in the cytosol (data not shown). These findings correspond with reports by Fukami and Takenawa (1992) and Pillai et al. (1998), which showed that exogenous PA added to cell culture media incorporates rapidly into cellular membranes and subsequently participates in cellular functions (Gascard et al., 1991). Moreover, the permeability of cell membranes to PA in macrophages might be physiologically and pathologically important, because the ability of PA to rapidly incorporate into cell membranes hints at its direct association with intracellular signaling molecules. However, any such mechanism remains speculative.

In summary, the present study presents a body of evidence that indicates that PA-activated macrophages produce IL-1 β and IL-6 via the transcriptional activities of STAT1 and -3 and that this process is regulated by two different pathways that involve JAK2 activation or JAK2 activation followed by Akt activation.

References

- Alderton F, Darroch P, Sami B, McKie A, Ahmed IS, Pyne N, and Pyne S (2001) G-protein-coupled receptor stimulation of the p42/p44 mitogen-activated protein kinase pathway is attenuated by lipid phosphate phosphatases 1, 1a and 2 in human embryonic kidney 293 cells. *J Biol Chem* **276**:13452–13460.
- Andresen BT, Rizzo MA, Shome K, and Romero G (2002) The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade. *FEBS Lett* **531**:65–68.
- Anthony RG, Henriques R, Helfer A, Meszaros T, Rios G, Testerink C, Munnik T, Deak M, Koncz C, and Bogre L (2004) A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*. *EMBO (Eur Mol Biol Organ) J* **23**:572–581.
- Avila-Flores A, Santos T, Rincon E, and Merida I (2005) Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. *J Biol Chem* **280**:10091–10099.
- Bach EA, Aguet M, and Schreiber RD (1997) The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* **15**:563–591.
- Bollag WB, Zhong X, Dodd ME, Hardy DM, Zheng X, and Allred WT (2005) Phospholipase D signaling and extracellular signal-regulated kinase-1 and -2 phosphorylation (activation) are required for maximal phorbol ester-induced transglutaminase activity, a marker of keratinocyte differentiation. *J Pharmacol Exp Ther* **312**:1223–1231.
- Chen YC, Chang MF, Chen Y, and Wang SM (2005) Signaling pathways of magnolol-induced adrenal steroidogenesis. *FEBS Lett* **579**:4337–4343.
- Chen Y, Zheng Y, and Foster DA (2003) Phospholipase D confers rapamycin resistance in human breast cancer cells. *Oncogene* **22**:3937–3942.
- Dadi H, Ke S, and Roifman CM (1994) Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor is dependent on protein tyrosine kinase activity. *Blood* **84**:1579–1586.
- de Jong CF, Laxalt AM, Bargmann BO, de Wit PJ, Joosten MH, and Munnik T (2004) Phosphatidic acid accumulation is an early response in the CF-4/Avr4 interaction. *Plant J* **39**:1–12.
- de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, Berthoud HR, Uematsu S, Akira S, van den Wijngaard RM, et al. (2005) Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol* **6**:844–851.
- Fang Y, Park IH, Wu AL, Du G, Huang P, Frohman MA, Walker SJ, Brown HA, and Chen J (2003) PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. *Curr Biol* **13**:2037–2044.
- Fan Z, Gao L, and Wang W (2003) Phosphatidic acid stimulates cardiac KATP channels like phosphatidylinositols, but with novel gating kinetics. *Am J Physiol* **284**:C94–C102.
- Fukami K and Takenawa T (1992) Phosphatidic acid that accumulates in platelet-derived growth factor-stimulated Balb/c 3T3 cells is a potential mitogenic signal. *J Biol Chem* **267**:10988–10993.
- Gascard P, Tran D, Sauvage M, Sulpice JC, Fukami K, Takenawa T, Claret M, and Giraud F (1991) Asymmetric distribution of phosphoinositides and phosphatidic acid in the human erythrocyte membrane. *Biochim Biophys Acta* **1069**:27–36.
- Grange M, Sette C, Cuomo M, Conti M, Lagarde M, Prigent AF, and Nemoz G (2000) The cAMP-specific phosphodiesterase PDE4D3 is regulated by phosphatidic acid binding. Consequences for cAMP signaling pathway and characterization of a phosphatidic acid binding site. *J Biol Chem* **275**:33379–33387.
- Houslay MD and Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* **370**:1–18.
- Hu X, Herrero C, Li WP, Antoniv TT, Falck-Pedersen E, Koch AE, Woods JM, Haines GK, and Ivashkiv LB (2002) Sensitization of IFN-gamma Jak-STAT signaling during macrophage activation. *Nat Immunol* **3**:859–866.
- Ihle JN (1995) Cytokine receptor signalling. *Nature (Lond)* **377**:591–594.
- Koch T, Brandenburg LO, Liang Y, Schulz S, Beyer A, Schroder H, and Holt V (2004) Phospholipase D2 modulates agonist-induced mu-opioid receptor desensitization and resensitization. *J Neurochem* **88**:680–688.
- Kooijman EE, Chupin V, de Kruijff B, and Burger KN (2003) Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic* **4**:162–174.
- Kostenis E (2004) Novel clusters of receptors for sphingosine-1-phosphate, sphingosylphosphorylcholine and (lyso)-phosphatidic acid: new receptors for “old” ligands. *J Cell Biochem* **92**:923–936.
- Krasilnikov M, Ivanov VN, Dong J, and Ronai Z (2003) ERK and PI3K negatively regulate STAT-transcriptional activities in human melanoma cells: implications towards sensitization to apoptosis. *Oncogene* **22**:4092–4101.
- Ktistakis NT, Delon C, Manifava M, Wood E, Ganley I, and Sugars JM (2003) Phospholipase D1 and potential targets of its hydrolysis product, phosphatidic acid. *Biochem Soc Trans* **31**:94–97.
- Li F and Malik KU (2005) Angiotensin II-induced Akt activation through the epidermal growth factor receptor in vascular smooth muscle cells is mediated by phospholipid metabolites derived by activation of phospholipase D. *J Pharmacol Exp Ther* **312**:1043–1054.
- Lim HK, Choi YA, Park W, Lee T, Ryu SH, Kim SY, Kim JR, Kim JH, and Baek SH (2003) Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. *J Biol Chem* **278**:45117–45127.
- Murray M, Butler AM, Fiala-Beer E, and Su GM (2005) Phospho-STAT5 accumulation in nuclear fractions from vitamin A-deficient rat liver. *FEBS Lett* **579**:3669–3673.
- Nanjundan M and Possmayer F (2003) Pulmonary phosphatidic acid phosphatase and lipid phosphate phosphohydrolase. *Am J Physiol* **284**:L1–L23.
- Natarajan C, Sriram S, Muthian G, and Bright JJ (2004) Signaling through JAK2-STAT5 pathway is essential for IL-3-induced activation of microglia. *Glia* **45**:188–196.
- Niedernberg A, Tunaru S, Blaukat A, Ardati A, and Kostenis E (2003) Sphingosine 1-phosphate and dioleoylphosphatidic acid are low affinity agonists for the orphan receptor GPR63. *Cell Signal* **15**:435–446.
- Niwa Y, Kanda H, Shikachi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, and Yoshikawa H (2005) Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. *Oncogene* **24**:6406–6417.
- Pillai MG, Certik M, Nakahara T, and Kamisaka Y (1998) Characterization of triacylglycerol biosynthesis in subcellular fractions of an oleaginous fungus, *Mortierella ramanniana* var. *angulisporea*. *Biochim Biophys Acta* **1393**:128–136.
- Sharfe N, Dadi HK, and Roifman CM (1995) JAK3 protein tyrosine kinase mediates interleukin-7-induced activation of phosphatidylinositol-3' kinase. *Blood* **86**:2077–2085.
- Smith PL, Lombardi G, and Foster GR (2005) Type I interferons and the innate immune response—more than just antiviral cytokines. *Mol Immunol* **42**:869–877.
- Stahelin RV, Burian A, Bruzik KS, Murray D, and Cho W (2003) Membrane binding mechanisms of the PX domains of NADPH oxidase p40phox and p47phox. *J Biol Chem* **278**:14469–14479.
- Tou JS and Gill JS (2005) Lysophosphatidic acid increases phosphatidic acid formation, phospholipase D activity and degranulation by human neutrophils. *Cell Signal* **17**:77–82.
- van Baal J, de Widt J, Divecha N, and van Blitterswijk WJ (2005) Translocation of diacylglycerol kinase theta from cytosol to plasma membrane in response to activation of G protein-coupled receptors and protein kinase C. *J Biol Chem* **280**:9870–9878.
- Watanabe H, Yokozeki T, Yamazaki M, Miyazaki H, Sasaki T, Maehama T, Itoh K, Frohman MA, and Kanaho Y (2004) Essential role for phospholipase D2 activation downstream of ERK MAP kinase in nerve growth factor-stimulated neurite outgrowth from PC12 cells. *J Biol Chem* **279**:37870–37877.
- Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, et al. (2005) Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *Oncogene* **24**:5552–5560.
- Yadav A, Kalita A, Dhillon S, and Banerjee K (2005) JAK/STAT3 pathway is involved in survival of neurons in response to insulin-like growth factor and negatively regulated by suppressor of cytokine signaling-3. *J Biol Chem* **280**:31830–31840.

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