Molecular Pharmacology Fast Forward. Published on December 14, 2005 as doi: 10.1124/mol.105.018481
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Janus Kinase-Signal Transducer and Activator of Transcription Mediates Phosphatidic Acid-Induced IL-1β and IL-6 Production

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Molecular Pharmacology Fast Forward. Published on December 14, 2005 as DOI: 10.1124/mol.105.018481
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a) Running Title: JAK-dependent pathways involve in PA-induced cytokine release

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c) number of text pages (30),

number of figures (6),

number of references (40)

number of words in the abstract (250),

number of words of introduction (675)

number of words of discussion (1219)

d) Abbreviations used are: STAT, signal transducers and activators of transcription; GAS, γ-interferon activating sequence; phosphatidylinositol 3-kinase (PI3K); ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-jun amino-terminal kinase; mTOR, mammalian target of rapamycin; S6K, p70 S6 kinase

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Abstract

Previously, we found that phosphatidic acid (PA) can induce inflammatory mediators like 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 JAK-STAT signaling pathway, which results in the production of IL-1β 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-1β, IL-6, and TNF-α were detected in media from macrophages stimulated with PA. Moreover, the JAK2 inhibitor, AG490, abolished PA-induced IL-1β and IL-6 release, but not TNF–α production, which is consistent with the notion that IL-1β and IL-6, but not TNF–α, contain an STAT-binding element in their promoter region. The knock-down of JAK2 in macrophages by small interfering (si) RNA significantly attenuated PA-induced IL-1β and IL-6 production. In addition, JAK2 inhibitor suppressed PA-induced Akt phosphorylation and the Akt inhibitor, LY294002, blocked GAS activation (GAS contains a promoter that responds to PA), suggesting that PA-mediated JAK2 activation leads to PI3K/Akt phosphorylation and STAT activation, and the subsequent translocation of STAT to the nucleus. Taken together, our data demonstrate that PA-activated macrophages produce IL-1β and IL-6, and that these processes require the activation of the JAK2-STAT1/3 or JAK2-Akt-STAT signaling pathways.

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Introduction

Phosphatidic acid (PA) is an important metabolite which 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 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 (Avila-Flores et al., 2005; Chen et al., 2003), activation due to oxidative stress (de Jong et al., 2004), the modulation of membrane trafficking (Kooijman et al., 2003), and the regulation of inflammatory response (Lim et al., 2003; Tou and Gill, 2005). Previously, we found evidence that the addition of PA to macrophages induces a number of inflammatory responses, such as, pro-inflammatory cytokine production, cyclooxygenase-2 expression, and the upregulation 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).

Interestingly, many of these processes are highly selective and specific, and thus, it was

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suggested that like other lipid second messengers, PA appears to function via selected targets in specific cell membrane regions (Ktistakis et al., 2003). Recently, it was 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 MEK/ERK cascade (Bollag et al., 2005). Moreover, Alderton et al., who reported PA-mediated ERK activation, speculated that ERK phosphorylation might occur via a specific PA receptor, although its existence was unproven (Alderton et al., 2001). Another study demonstrated that PA binds to the orphan receptor GPR63 (Kostenis, 2004; Niedernberg et al., 2003), thus 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 (de Jonge et al., 2005; Natarajan et al., 2004). The stimulation of cells with suitable ligands, such as, cytokines induces receptor oligomerization and also brings about 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 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-1β 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 GAS element in response to PA, thus suggesting that STAT might be a downstream target of Akt in PA-induced signaling. Taken 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.

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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 Amoebocyte Lysate Kit (BioWhittaker, Walkersville, MD). AG490, LY294002, and several MAPK inhibitors, PD95065 and SB203580, and SP60025, were purchased from BioMol (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, Korea). For Western blot analysis, we used Abs against JAK1, JAK2, JAK3 (Chemicon, Temecula, CA), and TYK2 (Santa-Cruz Biotechnology, Santa-Cruz, CA), and against phosphorylated JAK2 (Tyr1007/1008), STAT1 (Tyr701), STAT3 (Tyr705), and Akt (Thr308) (Cell Signaling Technology, Beverly, MA). Peroxidase-conjugated anti-rabbit IgG, anti-goat IgG, or anti-mouse IgG (Santa-Cruz Biotechnology, Santa-Cruz, CA) were used as secondary antibodies.

Cell Culture. Raw 264.7 cells were obtained from the American Type Culture Collection and were cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD) containing 10% FBS, 2mM 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 10ml of RPMI-1640. Cells from 5 mice were pooled, pelleted by centrifugation (200g, 10 min), and washed once with RPMI-1640.

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The cells were then seeded in a 100mm culture dish to remove any contaminating cells and to enrich adhering macrophages, and after 2 hours 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-1β, and IL-6 were determined by specific enzyme-linked immunosorbent assay (ELISA), according to the manufacturers' instructions (R&D Systems, Minneapolis).

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 50mM Tris-HCl, pH8.0, 5mM EDTA, 150mM NaCl, 1% Triton X-100, 50mM NaF, 1μM sodium orthovanadate, 1mM PMSF, and protease inhibitor cocktail. To determine JAK activities, 0.5mg of total protein was incubated with phosphotyrosine Ab (4G10) for 4h, and then with Protein A-agarose for 2h at 4°C. After being washed 7 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

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resuspended in 400µl of buffer A (10 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 10 mM KCl, 1mM $ZnCl_2$, 0.2 mM EGTA, 1mM Na_3VO_4, 10 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, and protease inhibitors). After cells had been incubated on ice for 10 min, and lysed by adding 50µl of 10% Nonidet P-40 (to a final concentration of 1.1%), nuclei were harvested by centrifugation. Nuclear pellets were then resuspended in 60µl of extraction buffer (10mM Hepes (pH 7.9), 5 mM $MgCl_2$, 300 mM NaCl, 1mM $ZnCl_2$, 0.2 mM EGTA, 25% glycerol, 1mM Na_3VO_4, 10mM NaF, 0.5 mM DTT, 0.5 mM PMSF, and protease inhibitors) and incubated on ice for 15 min. Nuclear debris was then removed by centrifugation (13,000 rpm x 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 oligonucleotides 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 µg of nuclear extract, labeled TF probe, 2 µg/ml poly[d(I-C)] in a buffer containing 20 mM Hepes (pH 7.9), 50 mM NaCl, 1mM dithiothreitol, 1mM EDTA, and 5% glycerol and these were incubated at room temperature for 30 min. Reaction mixtures were then separated on 6% Tris-glycine non-denaturing polyacrylamide gels in a 2X 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

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complexes.

***Transfection and promoter activity assays*.** A promoter-reporter construct containing 8 copies of the GAS element was trasnfected into RAW 264.7 cells using Lipofectamine 2000. 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 prior to PA stimulation. Cell lysates were assayed for luciferase activity using a luminometer (Promega) according to the manufacturer's instructions. For JAK2 silencing, Raw 264.7 cells were transfected with 100 nM of a pool of JAK2-specific siRNA (SMARTpool kit, Dharmacon, Chicago, IL) using lipofectamine 2000 (GIBCO BRL). As a control, non-specific 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 cold PBS, and lysed on ice using lysis solution (1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1mM PMSF, 1mM Na_3VO_4, and protease inhibitor cocktail). Protein concentrations were determined using Bio-Rad protein assays. Briefly, proteins from cell lysates (50 μg) 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 minutes at room temperature in Tris buffered saline-0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing a primary Ab for 4 h at room temperature. After 5x10min washes in TTBS, membranes were incubated with peroxidase-conjugated

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secondary Ab for 1 h. After 5x10min with TTBS, protein bands of interest were visualized using an enhanced chemiluminescence detection system (Amersham).

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Results

PA induces the phosphorylation and activation of JAK2 in RAW 264.7 cells.

Our previous work showed that PA induces the pro-inflammatory cytokines IL-1β, 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 demonstrates 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. Briefly, whole cell lysates from macrophages exposed to 50μM 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 1007/1008), 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.

Since the JAK/STAT pathway is the main effector of signaling by many bioactive

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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 Tyr 701) 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 (Tyr 705) under the same conditions. More, the pattern of STAT3 phosphorylation appeared similar to that of STAT1 phosphorylation. Next, we examined whether phosphorylated STAT1 and STAT3 translocate to the nucleus. Accordingly, nuclear extracts from PA-treated macrophages were subjected to Western blot analysis for STAT1 and STAT3. Fig. 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 STAT 1 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 8 copies of the interferon activating sequence (GAS) element, which is a STAT-binding site, and we found that PA stimulated 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 were 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

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specific binding of these factors to STAT1 or STAT3 consensus sequence.

JAK2-STAT pathway activation is required for PA-induced cytokine release.

Previously, we showed that PA induced the expressions of IL-1β 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-1β and IL-6, and TNF-α in conditioned media were determined by ELISA. AG490 strongly reduced PA-induced IL-1β and IL-6 production, but not that of TNF-α (Fig. 4A, B, C), indicating that the PA-stimulated productions of IL-1β 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-1β and IL-6 production, since IL-1β and IL-6 contain a STAT-binding element in their promoter region. The involvement of JAK2 in PA-stimulated IL-1β and IL-6 production was confirmed by treating cells with JAK2 specific small interfering

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(si) RNA. 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-1β 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. Taken 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, since 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. Interestingly, Akt phosphorylation by PA was inhibited by AG490 (Fig. 6A), but not by 4-(4′-Hydroxyphenyl) amino-6,7-dimethoxyquinazoline WHI-P131, 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, thus confirming the functional activation of

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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.

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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 (Avila-Flores et al., 2005; de Jong et al., 2004). Previously, we reported that PA regulates the expression of pro-inflammatory 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-1β 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 the phosphorylations of JAK2 and STAT1/3, the translocation of STATs to the nucleus, and the 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 show 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 productions of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, are of particular interest in terms of toxicity, as they are the most stringently regulated pro-inflammatory 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,

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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 (Chen et al., 2005; Watanabe et al., 2004). Consistent with these findings, others and we have found that various kinases, including Akt and MAPKs can be activated by PA stimulation (Chen et al., 2003; Li and Malik, 2005; Lim et al., 2003). 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-1β and IL-6 release were found to sensitive to AG490 treatment, thus implying JAK2 involvement in PA-induced signal transduction. However, AG490 had no effect on TNF-α release in response to PA, thus indicating the existence of a regulatory mechanism not requiring JAK2 activation for TNF-α production by PA. Since 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 expressions of IL-1β 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 hetero-dimerization and translocate to the nucleus, where they bind γ-interferon activating sequence (GAS) element, a consensus DNA-binding sequence present in the promoter regions of genes that is 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 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 were also found to be elevated by gel mobility shift assay. We then transfected macrophages with a luciferase reporter plasmid containing 8 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 appears 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 following 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 suggested to contribute to its biological ability in cells (Kostenis, 2004). Fang et al. showed that PA is required for the activation of mTOR downstream effectors, and that it directly interacts with the domain in mTOR (Fang et al., 2003). 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., KSR, isoforms of cAMP-specific phosphodiesterase, PDE4A1 and PDE4D3 (Grange et al., 2000), and tyrosine protein phosphatase SHP-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

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before it is distributed in the cytosol (data not shown). These findings correspond with reports by Fukami and Takenawa and Pillai et al. (Fukami and Takenawa, 1992; 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, since 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 which indicates that PA-activated macrophages produce IL-1β and IL-6 via the transcriptional activities of STAT 1, 3 and that this process is regulated by two different pathways that involve JAK2 activation or JAK2 activation followed by Akt activation.

Footnotes

This work was supported by the Basic Research Program (Grant No. R01-2004-000-10023-0 (2004)) and the Aging-Associated Vascular Disease Research Center at Yeungnam University of Korea Science and Engineering Foundation (Grant No. R13-2005-005-01003-0 (2005)).

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Molecular Pharmacology Fast Forward. Published on December 14, 2005 as DOI: 10.1124/mol.105.018481
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Figure legends

FIGURE 1. Effect of PA on the tyrosine phosphorylation of JAK2. A, RAW 264.7 cells were treated with synthetic PA (50 μM) 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 on the lower panel. A representative result of three independent experiments is shown. *B,* Cells were incubated with 50 μM of PA for the indicated times. To detect JAK2 phosphorylation, equals amount of cell extracts were analyzed by Western blotting using phospho-specific JAK2 antibody. As a loading control, same blots were re-probed with anti-JAK2 antibody. *C,* Peritoneal macrophage cells were prepared and stimulated with 50 μM of PA for 10 min. Whole cell extracts were used for immunoblot analysis to detect phosphor-JAK2 using specific antibody. As a loading control, same blots were re-probed with anti-JAK2 antibody.

FIGURE 2. Effect of PA on STAT1 and STAT3 phosphorylation and nuclear translocation. RAW 264.7 cells were stimulated with PA (50 μM) for the indicated times. *A,* Whole cell lysates were prepared and immunoblotted with phosphotyrosine-specific STAT1 or STAT3 antibodies. Blots were then stripped and re-probed with anti-STAT1 and STAT3 antibodies. *B,* Nuclear extracts were prepared as described in "Materials and Methods". Nuclear extracts were resolved by SDS-PAGE and immunoblotted with anti-STAT1 and STAT3 antibodies.

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FIGURE 3. Effects of PA on STAT1 and STAT3 phosphorylation and nuclear translocation

A, RAW 264.7 cells were transfected with 5μg 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, STAT3.

FIGURE 4. Effect of JAK2 inhibitor on PA-induced cytokine release and GAS-dependent luciferase activity. *A,B,C,* RAW 264.7 cells were pretreated with the indicated concentrations of AG490 for 1h and then treated with 50 μM PA for 18h. The levels of IL-1β and IL-6, and TNF-α in conditioned media were determined by ELISA. *D,* Cells were transfected with 5 μg 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.

FIGURE 5. Knock-down of JAK2 with siRNA suppressed the PA-induced productions of IL-1β and IL-6. *A,* RAW 264.7 cells were transfected with 5 μg of JAK2 or control siRNA using Lipofectamine 2000. Whole cell lysates from transfected cells were

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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 μM PA for 18 h and IL-1β, IL-6, and TNF-α levels were determined by ELISA.

FIGURE 6. JAK2 activation is required for PA-stimulated Akt phosphorylation.

A, RAW 264.7 cells were treated with PA (50 μM) with/without AG490 (20μM) for 30 min, and cell extracts were then prepared. Equal amounts of protein (50μg) from non-treated controls and from each treatment were analyzed by Western blotting for Akt phosphorylation using a specific antibody. As a loading control, blots were re-probed with anti-Akt antibody. *B,* Cells were transfected with 5 μg of GAS-luciferase construct. And the following day, transfected cells were collected, divided into 12 well plates, preincubated for 1 h with/without LY294002 (25 μM), and then stimulated with PA. Eight hours later, luciferase assays were performed.

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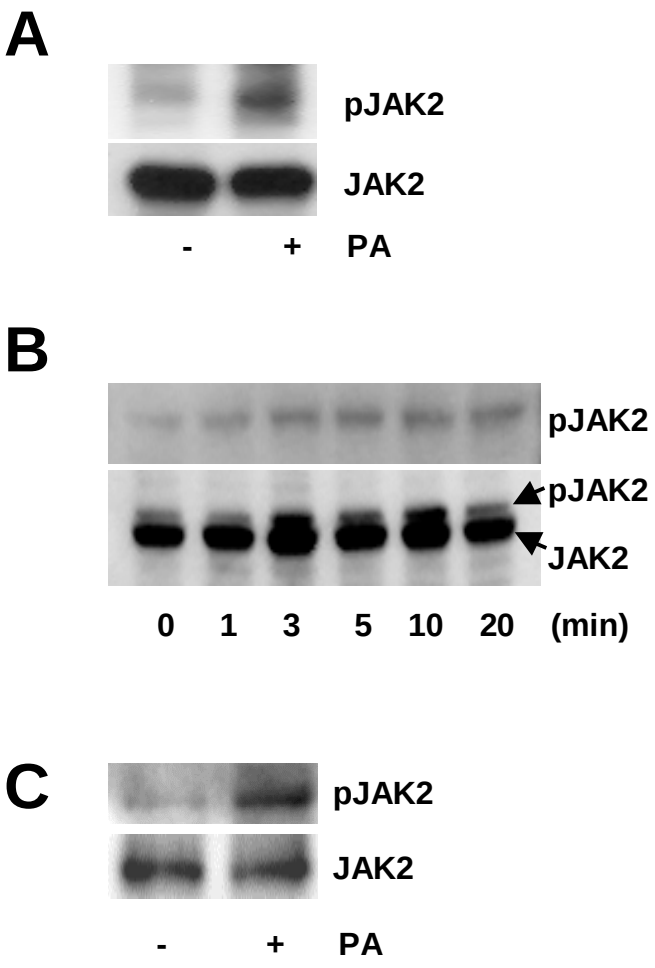

Fig. 1A,B,C

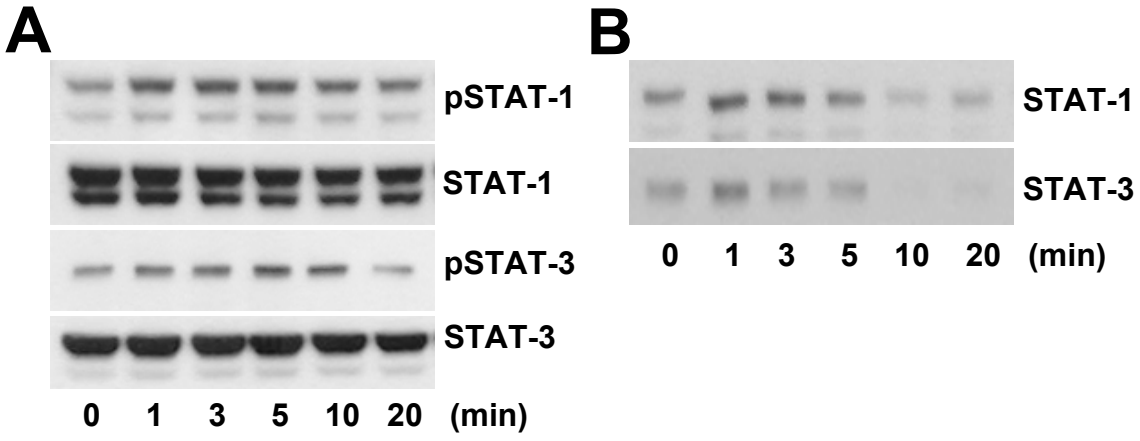
A
pSTAT-1
STAT-1
pSTAT-3
STAT-3
0 1 3 5 10 20 (min)
B
STAT-1
STAT-3
0 1 3 5 10 20 (min)

Fig. 2A,B

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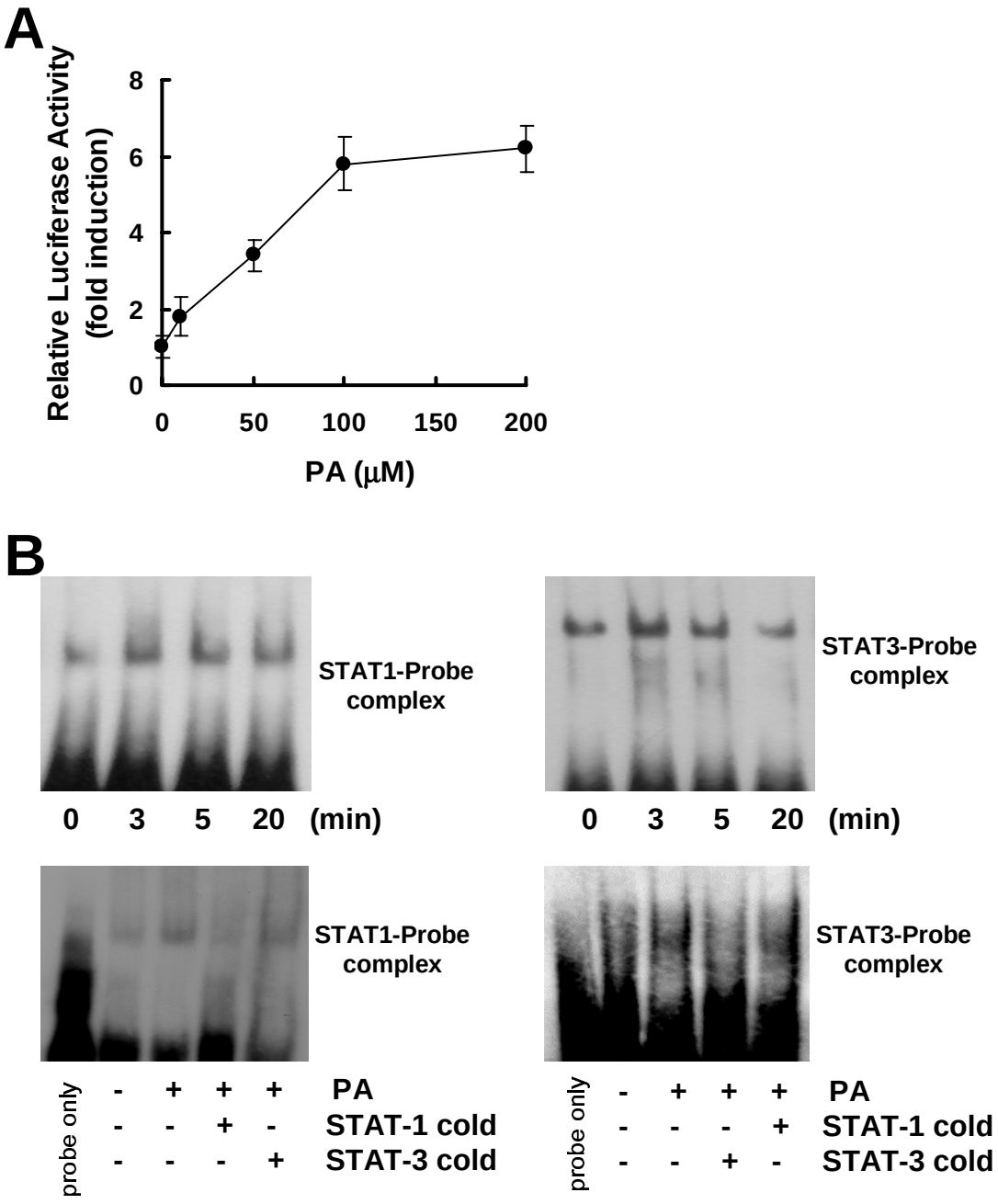

Fig. 3A,B

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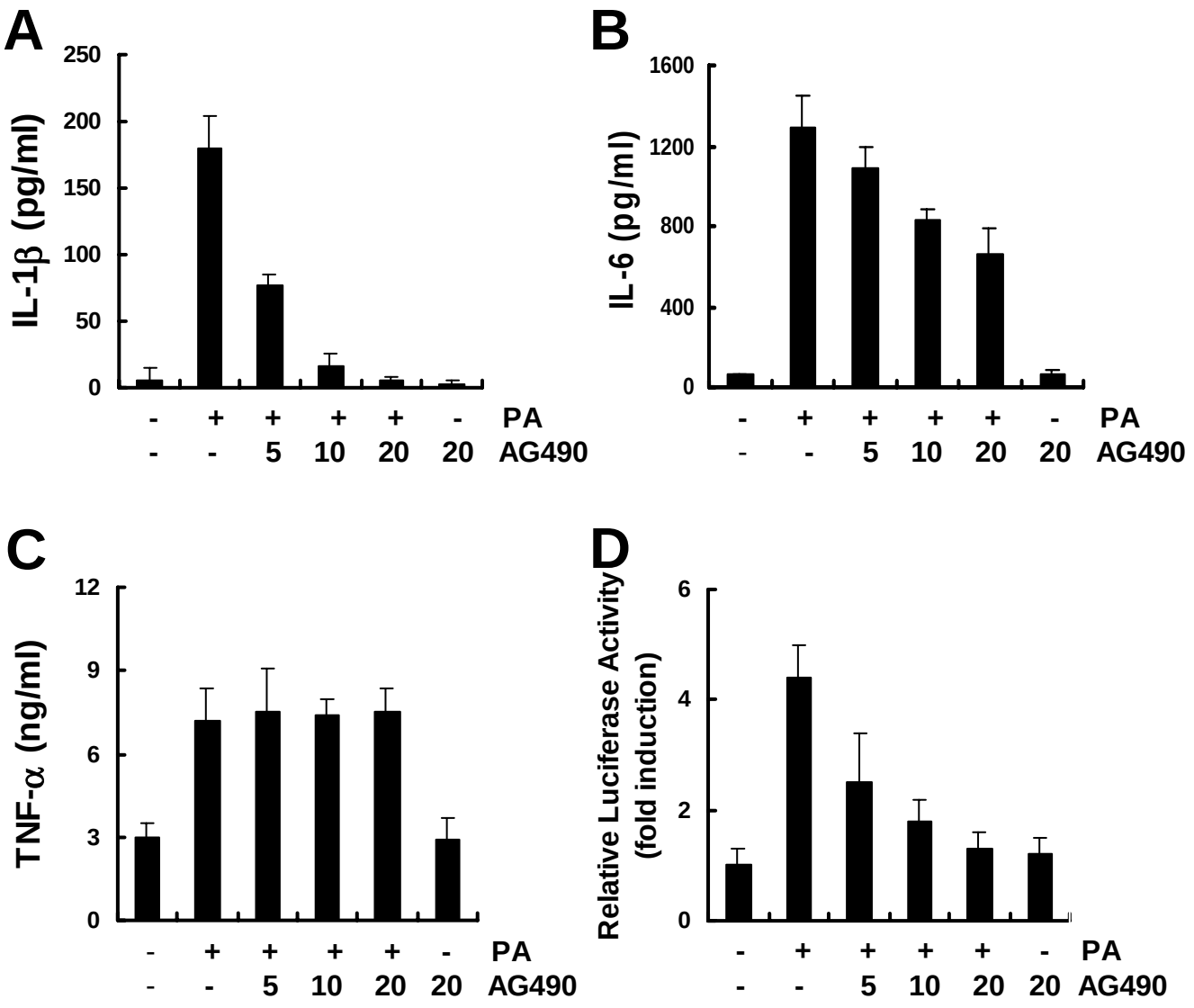

Fig. 4A,B,C,D

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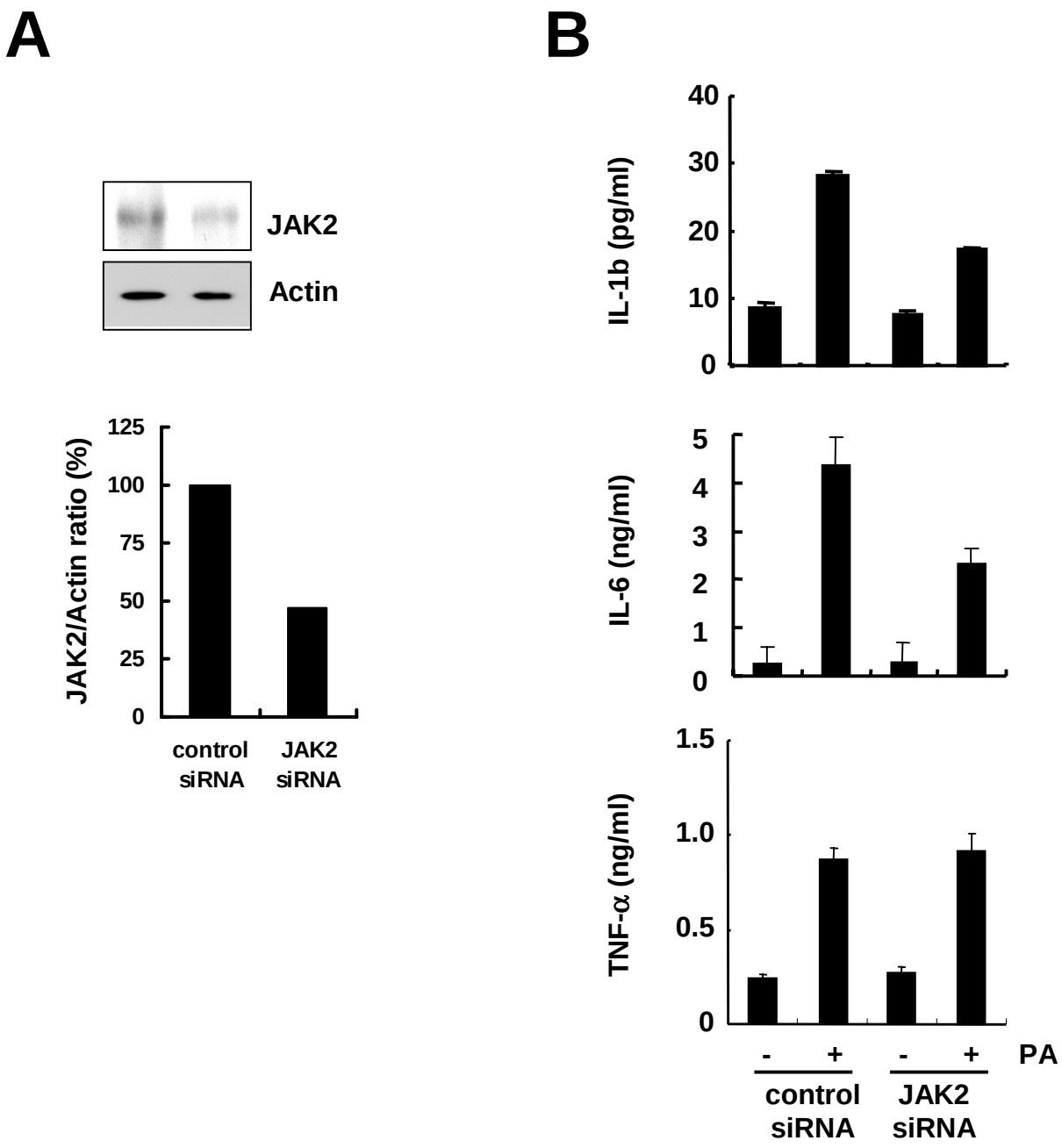

Fig. 5A,B

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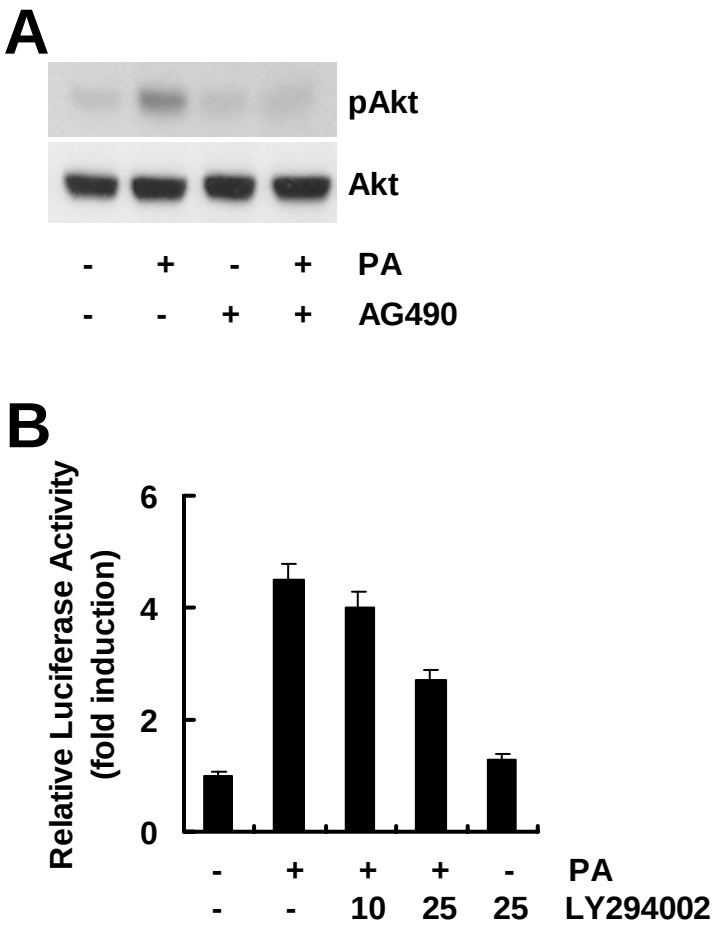

Fig. 6A,B