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# Interleukin-6 inhibition of peroxisome proliferator-activated receptor alpha expression is mediated by JAK2- and PI3K-induced STAT1/3 in HepG2 hepatocyte cells

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Received: 6 September 2013 / Accepted: 5 November 2013


**Abstract** Interleukin-6 (IL-6) is the major activator of the acute phase response (APR). One important regulator of IL-6-activated APR is peroxisome proliferator-activated receptor alpha (PPARα). Currently, there is a growing interest in determining the role of PPARα in regulating APR; however, studies on the molecular mechanisms and signaling pathways implicated in mediating the effects of IL-6 on the expression of PPARα are limited. We previously revealed that IL-6 inhibits PPARα gene expression through CAAT/enhancer-binding protein transcription factors in hepatocytes. In this study, we determined that STAT1/3 was the direct downstream molecules that mediated the Janus kinase 2 (JAK2) and phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathways in IL-6-induced repression of PPARα. Treatment of cells with pharmacological inhibitors of JAK2, PI3K, AKT, and mTOR attenuated the inhibitory effect of IL-6 on PPARα protein in a dose-dependent manner. These inhibitors also decreased the IL-6-induced repression of PPARα mRNA expression and promoter activity. Overexpression of STAT1 and STAT3 in HepG2 cells cotransfected with a reporter vector containing this PPARα promoter region revealed that both the expression plasmids inhibited the IL-6-induced repression of PPARα promoter activity. In the presence of inhibitors of JAK2 and mTOR (AG490 and rapamycin, respectively), IL-6-regulated protein expression and DNA binding of STAT1 and STAT3 were either completely or partially inhibited simultaneously, and the IL-6-induced repression of PPARα protein and mRNA was also inhibited. This study has unraveled novel pathways by which IL-6 inhibits PPARα gene transcription, involving the modulation of JAK2/STAT1–3 and PI3K/AKT/mTOR by inducing the binding of STAT1 and STAT3 to STAT-binding sites on the PPARα promoter. Together, these findings represent a new model of IL-6-induced suppression of PPARα expression by inducing STAT1 and STAT3 phosphorylation and subsequent down-regulation of PPARα mRNA expression.

**Keywords** Acute phase response · Interleukin-6 · Peroxisome proliferator alpha · Cell signaling pathways · Human HepG2 hepatocyte cells

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## Introduction

Organisms respond toward changes in local or systemic disturbances in homeostasis caused by infection, injury, and trauma by triggering a highly complicated but precise gene regulation network. Acute phase response (APR) is one of the major reactions initiated in human liver to restore disturbed homeostasis to normal physiological levels [1–3]. APR mediates changes in the concentration of plasma proteins known as acute phase proteins (APPs) which act as effectors of APR [4–6]. APPs are modulated positively or negatively by cytokines especially interleukin-6 (IL-6) mainly by altering the rate of their synthesis in the liver [7–9]. The onset of APR triggered by IL-6 may



Journal : Large 11010 | Dispatch : 13-11-2013 | Pages : 13
Article No. : 1896 | ☐ LE | ☐ TYPESET
MS Code : | ☑ CP | ☑ DISK

lead to local inflammation in cancer, atherosclerosis, and infection of hepatic origin [10–13].

IL-6 belongs to a family which includes IL-11, IL-27, ciliary neurotropic factor, oncostatin M, leukemia inhibitory factor, cardiotrophin-1, cardiotrophin-like cytokine, and neurotrophin. IL-6 is a typical pleiotropic cytokine that plays a pivotal role in the immune and the hematopoietic system, as well as various physiological events such as cell proliferation and differentiation [14, 15]. IL-6 was first demonstrated to be the major inducer of APPs in primary cultures of human hepatocytes where it induced the synthesis of C-reactive protein (CRP) and serum amyloid A (SAA) [16]. Subsequently, studies using human liver cell lines such as HepG2 showed that the gene expression of other APPs including fibrinogen, $\alpha_1$-antichymotrypsin, ceruloplasmin, haptoglobin, $\alpha_1$-acid glycoprotein (gp), and $\alpha_1$-antitrypsin is also modulated by IL-6 [17–19].

Many studies have reported the role of transcription factors in mediating the action of cytokines in modulating gene expression of APPs such as members of CAAT/enhancer-binding proteins (C/EBPs) and hepatocyte nuclear factors HNF-1, HNF-3, HNF-4, and HNF-6 [20–22]. There is also evidence indicating the transcriptional regulation of APPs by a member of ligand-activated nuclear hormone receptors, peroxisome proliferator activator receptor alpha (PPARα). PPARα which is highly expressed in hepatocytes, skeletal muscle, cardiomyocytes, and brown adipose tissues, regulates cholesterol homeostasis and fatty acid catabolism [23–25]. In mice treated with the PPARα agonist, fenofibrate, the actions of IL-6 on modulation of gene expression on APPs are suppressed. Furthermore, this proved that the effect of PPARα prevents the IL-6-induced expression of the APR genes via PPARα-dependent mechanisms, directly at the transcriptional level [26]. Studies also demonstrated that regulation of PPARα expression is of crucial importance not only in the development of the APR in liver, but also in inflammation and atherosclerosis [25–29]. The signal transduction cascades represent a complex regulatory network in processes of diseases and may provide a common target for therapeutic intervention. At present, the IL-6 signaling pathway in the regulation of PPARα during APR is poorly understood. Although the expression of PPARα has been found to be down-regulated by cytokines during physiological and pathophysiological changes [30–33], studies to determine the action of IL-6 on PPARα gene expression in liver cells are limited. Recently, we reported that IL-6 decreased PPARα mRNA expression, and this was mediated by C/EBP members [20]. However, the mechanisms of IL-6 and IL-6 receptor-mediated signaling in liver cells on PPARα expression and APR have not been fully explored.

In this study, we demonstrated that Janus kinase 2 (JAK2)–STAT1–3 and phosphatidylinositol-3 kinase (PI3K) pathways were involved in IL-6-induced suppression of PPARα expression. Furthermore, our results also suggest that IL-6-induced STAT1 and STAT3 proteins were essential for mediating the IL-6-inhibitory action on PPARα gene expression in human hepatocarcinoma HepG2 cells.

## Materials and methods

### Materials

The recombinant human IL-6 was obtained from National Institute for Biological Standards and Control, UK. Human HepG2 cell line was purchased from American Type Culture Collection. All cell culture reagents were purchased from Gibco/BRL. Rabbit polyclonal antibodies directed against the STAT1, STAT3, PI3K, AKT, mammalian target of rapamycin (mTOR), PPARα, β-actin; and the phosphorylated forms $Tyr^{701}$ STAT1, $Tyr^{705}$ STAT3, $Ser^{727}$ STAT1, $Ser^{727}$ STAT3, $Tyr^{458/199}$ PI3K, $Tyr^{30}$ AKT, and $Ser^{2448}$ mTOR were purchased from Cell Signaling Technology (Beverly, MA, USA). Pharmacological inhibitors, AG490, were purchased from Tocris Bioscience (St. Louis, MO, USA); wortmannin and AKT were obtained from Calbiochem (Pacific Center Court, San Diego, CA, USA); and rapamycin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Cell culture and IL-6 treatment

HepG2 cell lines were cultured in MEM supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (HI-FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5 % (v/v) $CO_2$. Before treatment with 1,000 U/ml of IL-6 or various inhibitors against components of signal transduction pathways, the cells were pre-incubated in reduced concentration of HI-FBS (0.5 %) for 4 h [34].

### Real-time RT-PCR

Total cellular RNA was isolated from HepG2 cell line using Tri-Reagent LS (Molecular Research Center) according to the manufacturer's instructions. Real-time RT-PCR was performed using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) [20]. In brief, the reaction contained 600 ng of DNase-treated RNA isolated from HepG2 cells, 15 μl of 2× SYBR Green RT-PCR Reaction Mix, 1 μl of each forward and reverse primers (10 μl), and 1 μl of iScript Reverse Transcriptase in a final volume of 30 μl. Real-time PCR was performed in a iCycler iQ

Multicolor Real-Time PCR Detection System (Bio-Rad). The program consisted of 1 cycle each of 20 min at 50 °C and 5 min at 95 °C, followed by 30 cycles of denaturing at 95 °C for 15 s; annealing at 60 °C for 20 s; and primer extension at 72 °C for 30 s. The quantity of PPARα mRNA was normalized against the β-actin housekeeping gene as previously described [20].

### SDS-PAGE and western blot analysis

Total cellular protein was isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce) following the manufacturer's instructions. The cytoplasmic extracts were prepared from control and treated cells. 100 and 30 μg of proteins were used in SDS-PAGE to determine the levels of protein content for phosphorylated $Tyr^{701}$ STAT1, $Tyr^{705}$ STAT3, $Ser^{727}$ STAT1, $Ser^{727}$ STAT3, $Tyr^{458/199}$ PI3K, $Tyr^{30}$ AKT, and $Ser^{2448}$ mTOR; and for PPARα and β-actin, respectively. Subsequently, proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with blocking solution [1× PBS containing 5 % (w/v) skimmed milk powder and 0.1 % (v/v) Tween-20] for 1 h at room temperature by shaking. The membrane was washed three times for 10 min each in washing solution [1× PBS and 0.1 % (v/v) Tween-20] and incubated with primary antibodies (rabbit anti-human $Tyr^{701}$ STAT1, $Tyr^{705}$ STAT3, $Ser^{727}$ STAT1, $Ser^{727}$ STAT3, $Tyr^{458/199}$ PI3K, $Tyr^{30}$ AKT, $Ser^{2448}$ mTOR, PPARα, and β-actin), which was diluted 1/1,000 in 1× PBS containing 1 % (w/v) skimmed milk powder and 0.1 % (v/v) Tween-20, for 1 h at room temperature. The membrane was then washed and immersed in secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) diluted 1/2,000 in 1× PBS containing 1 % (w/v) skimmed milk powder and 0.1 % (v/v) Tween-20. Detection of membrane-bound antigen–antibody complexes was carried out as per the instructions provided with the ECL kit (GE Healthcare) and Fuji Medical X-ray film. Westview™ Western Size Marker (Mbiotech, Inc., Songpagu, Seoul) and immunoreactive proteins were visualized on X-ray film.

### Electrophoretic mobility shift assay (EMSA) and antibody supershift assay

Nuclear extracts were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce). The sequences of the oligonucleotides containing the STAT-binding site (−612 to −604; [20]) were 5′-AGAATTCTTCCTCCAAGAGTTA-3′ and 5′-TAACTCTTGGAGGAAGAATTCT-3′. The oligonucleotides were labeled using biotin 3′-End DNA Labeling kit (Pierce) according to manufacturer's instructions. For binding reactions, 20 μg of nuclear extracts were incubated with labeled oligonucleotides using LightShift® Chemiluminescent EMSA kit (Pierce) according to manufacturer's instructions. DNA–protein complexes were then resolved by electrophoresis using 6 % (w/v) nondenaturing polyacrylamide gels (29:1, acrylamide:bisacrylamide). DNA–protein complexes were transferred to nylon membranes (Hybond N+), and subsequently, detection of the biotin-labeled DNA was carried out using a Fuji Medical X-ray film. For antibody supershift (SS) assays, 1 μl of STAT1 or STAT3 antibody (Cell Signaling) was mixed with the binding reactions, and the mixture was incubated on ice for 20 min followed by 20 min at room temperature before the labeled oligonucleotides were added.

### PPARα promoter–reporter plasmids

The construction of human PPARα promoter −765/+34 in pGL3-Basic Vector was described previously [20]. The PPARα promoter −765/+34 was mutated at positions −607, −608, and −610 within the STAT recognition element (nucleotides −612 to −604) using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). The sample reaction consisted of 1× reaction buffer, 50 ng double-stranded DNA template, and 125 ng of complimentary oligonucleotides [5′-CAAGAATT CTTGCCACAAGAGTTACAG-GTCAGT-3′ and 5′-ACT GACCTGTAACTCTTGTGGcAAGAATTCTTG-3′] of the human PPARα promoter. The mutated construct was designated as STATmut PPARα promoter. The change in sequence was confirmed by DNA sequencing.

### Transient transfection assays

HepG2 cells were transfected with plasmids using lipofectin transfection reagent (Invitrogen® Life Technologies) according to manufacturer's instruction with slight modification as previously described [20, 34]. Cells were replated 12 h before transfection at a density of $9 \times 10^5$ cells in 2 ml of fresh culture medium in a six-well plastic dish. The cells were transfected with 2 μg of wild-type (wt) PPARα promoter luciferase construct or the STATmut PPARα promoter luciferase construct and 0.5 μg thymidine kinase/Renilla (pRL-TK) vector as a control for transfection efficiency. For the over expression studies, HepG2 cells were cotransfected with 2 μg of PPARα promoter luciferase construct, 0.5 μg pRL-TK plasmid, and either 2 μg of STAT1 or STAT3 expression plasmids or 2 μg of empty vector. Following transfection, cells were treated and then harvested for analysis for firefly luciferase and Renilla luciferase activities using the Dual Luciferase Reporter Assay System (Promega). Relative light units were determined by dividing firefly luciferase activity by Renilla luciferase activity. Results are given as an average of three independent assays ± SD.

### Assay of luciferase activity

Luciferase activity was measured by means of the luciferase assay system. Transfected cells grown in six-well plastic dishes were washed with PBS and lysed, each with 500 µl of passive lysis buffer per well. Luciferase activity was determined as instructed by the manufacturer (Promega). In brief, 20 µl of cell extracts were mixed at room temperature with 100 µl of Luciferase Assay Buffer II, LARII (Promega), and measurements were read in a luminometer (TD-20/20 Turner Designs, CA, USA). *Renilla* luciferase was used as an internal control for transfection efficiency.

### Statistical analysis

Statistical analysis was determined using one-way analysis of variation (ANOVA) or paired *t* test available in GraphPad Prism® Version 3.02 software. $P < 0.05$ was considered to be statistically significant, $P < 0.01$ very significant and $P < 0.001$ highly significant.

## Results

### IL-6 activates the components of JAK/STAT and PI3K signal transduction pathways in HepG2 cells

IL-6 has been shown to regulate target genes via the activation of the JAK/STAT and PI3K pathways in hepatocytes [35, 36]. However, studies investigating the action of IL-6 phosphorylation of the components of JAK–STAT and PI3K pathways in HepG2 cells are limited. Western blot analysis showed that the treatment of HepG2 cells with IL-6 over a period of 60 min produced a marked increase in the level of phosphorylation of STAT1 $Tyr^{701}$ and STAT3 $Tyr^{705}$ within 15–30 min but not the total STAT1 and STAT3 (Fig. 1a). The levels of phosphorylated STAT1 $Ser^{727}$ and STAT3 $Ser^{727}$ were also increased after 15–30 min treatment with IL-6 albeit at lower levels.

For the components of the PI3K signaling pathway, treatment of HepG2 cells with IL-6 over a period of 240 min produced a dose-dependent response on the levels of phosphorylated PI3K $Tyr^{458/199}$, AKT $Tyr^{30}$, and mTOR $Ser^{2448}$ (Fig. 1a). In all cases, the levels of phosphorylated proteins were increased after 15 min, sustained at 30 min, and gradually decreased thereafter. The total protein levels of PI3K, AKT, and mTOR remained unchanged.

In order to corroborate that JAK/STAT and PI3K pathways were activated in response to IL-6 treatment, various concentrations of pharmacological inhibitors against the identified signaling proteins were used to preincubate the cells for 2 h, followed by IL-6 treatment for requisite time. AG490, a JAK inhibitor, was used to identify the JAK–STAT pathway [37–40], and the wortmannin, AKTIV, and rapamycin inhibitors were used, respectively, to identify the PI3K, AKT, and mTOR components in the PI3K pathway [41, 42]. AG490 inhibited the IL-6-induced phosphorylation of STAT1 ($Tyr^{705}$) and STAT3 ($Tyr^{705}$) in a dose-dependent manner (Fig. 1b).

In delineating the PI3K pathway, we discovered that wortmannin was able to attenuate the phosphorylation of AKT, a downstream component of PI3K pathway. In addition, AKTIV inhibitor at a concentration of 0.5 µM abolished the IL-6-induced phosphorylation of mTOR, another downstream component of PI3K pathway (Fig. 1b).

In order to delineate the existence of crosstalk between JAK–STAT and PI3K pathways in IL-6-treated HepG2 cells, rapamycin and AG490 were used to pretreat cells followed by incubation using IL-6 for requisite time. Subsequently, the levels of phosphorylated PI3K, STAT1, and STAT3 were determined, respectively. IL-6-mediated induction of the phosphorylated STAT1 and STAT3 was slightly inhibited by rapamycin (Fig. 1c). Similarly, the attenuated effect of AG490 was also observed on the IL-6-induced phosphorylation of PI3K, albeit at relatively lower levels than the effect of AG490 on STAT phosphorylation. Based on these results, PI3K/AKT/mTOR pathway may play a minor role in the activation of JAK–STAT pathway, and vice versa.

### IL-6-inhibitory effects on PPARα mRNA expression are mediated by JAK–STAT and PI3K signal transduction pathways

Previously, we showed that IL-6 down-regulated the expression of PPARα at the levels of mRNA, protein content, and binding activity [20]. Having established that IL-6 mediated its cellular effect on HepG2 via JAK–STAT and PI3K/AKT/mTOR pathways (Fig. 1), we next examined whether these pathways were involved in mediating the inhibitory action of IL-6 on PPARα gene expression. We examined the effects of JAK–STAT and PI3K inhibitors on IL-6-inhibitory action on PPARα protein content. The inhibitors dose dependently inhibited the IL-6-suppressed PPARα protein expression (Fig. 2a). AG490 produced a statistically significant inhibition on the action of IL-6 with the highest level at 500 µM, and its levels of protein content were increased by 38 % compared with IL-6-treated HepG2 cells that were not preincubated with the inhibitor. Similarly, the inhibitory effect of IL-6 on PPARα protein content was significantly attenuated by wortmannin, AKTIV, and rapamycin with the highest levels being achieved at 4 mM (28 %), 1 mM (32 %), and 2 mM (38 %), respectively.

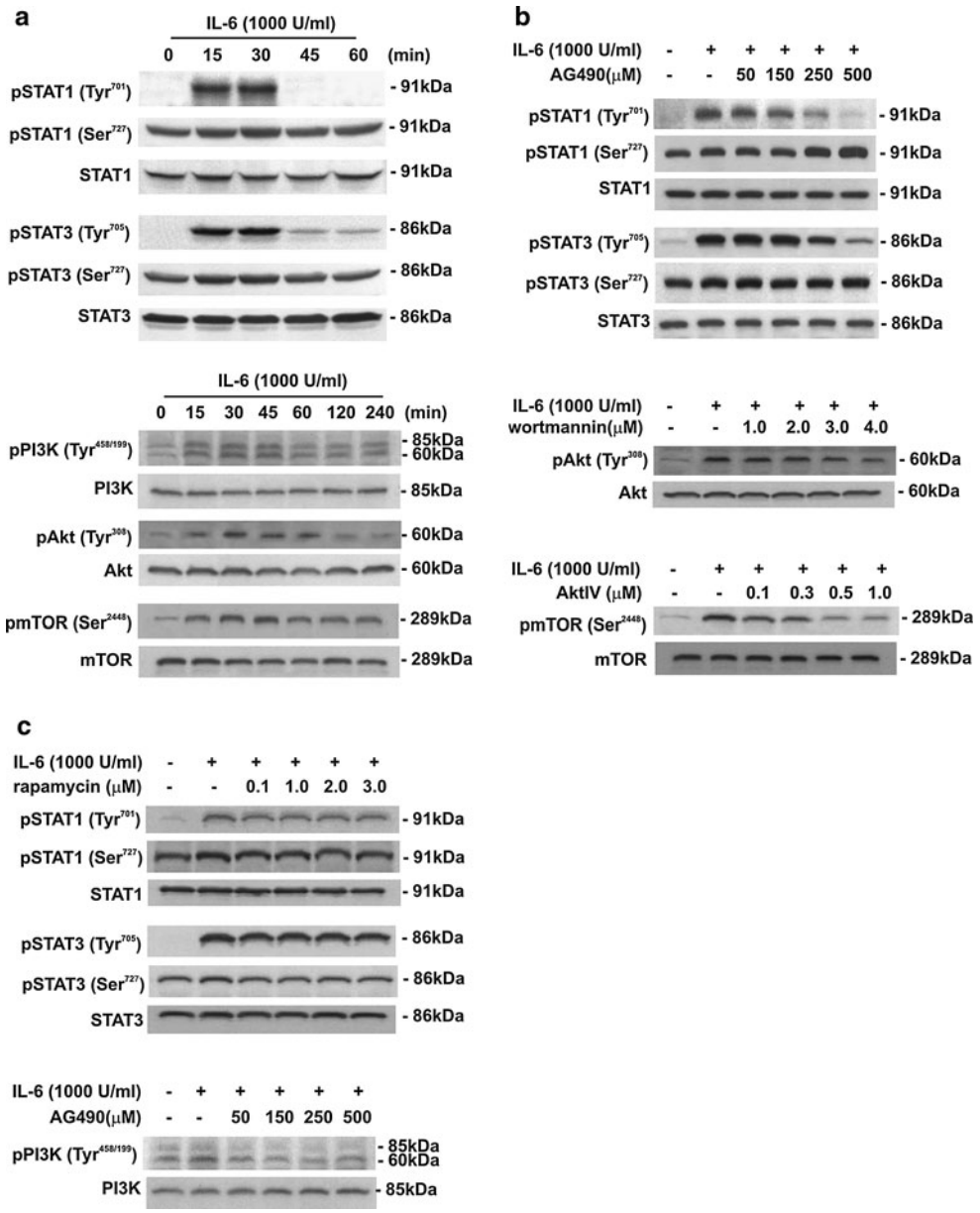


**Fig. 1** IL-6 activated the components of JAK/STAT and PI3K signal transduction pathways in HepG2 cells. **a** Time-dependent effect of IL-6 on STAT1 and STAT3, PI3K, AKT, and mTOR activation. Western blotting was used to detect for phosphorylated form of STAT1 and STAT3, PI3K, AKT, mTOR and their total STAT1 and STAT3, PI3K, AKT, and mTOR proteins, respectively. **b** Concentration-dependent inhibition of pharmacological inhibitors on their target proteins at the upstream to delineate downstream proteins. Cells were pretreated with various concentrations of inhibitors for STAT1 and STAT3, PI3K, AKT, and mTOR for 2 h, followed by stimulation with 1,000 U/ml for 15 min for STAT1 and STAT3, 30 min for AKT and 45 min for mTOR, respectively. Western blot was used to detect for STAT1 and phosphorylated form of STAT1, STAT3 and phosphorylated form of STAT3, AKT and phosphorylated form of AKT and mTOR and phosphorylated form of mTOR. **c** Rapamycin and AG490 were used to pretreat the cells followed by incubation with IL-6 for requisite time to determine the levels of phosphorylated STAT1 and STAT3, and phosphorylated form of PI3K, respectively, in delineating the crosstalk between JAK–STAT and PI3K pathways

To determine if the same inhibitors also attenuated the inhibitory action of IL-6 on PPARα mRNA levels, real-time PCR was performed on RNA isolated from IL-6-treated HepG2 cells preincubated with the inhibitors against JAK–STAT and PI3K/AKT/mTOR pathways. Messenger RNA expression of PPARα was decreased to approximately 50 % of that of the control in the presence of 1,000 U/ml of IL-6 (Fig. 2b), which is consistent with our previous study [20]. Treatment of HepG2 cells with 1,000 U/ml IL-6 was significantly attenuated by the inhibitors in suppressing PPARα mRNA levels which also correspond with the protein content. AG490, wortmannin,

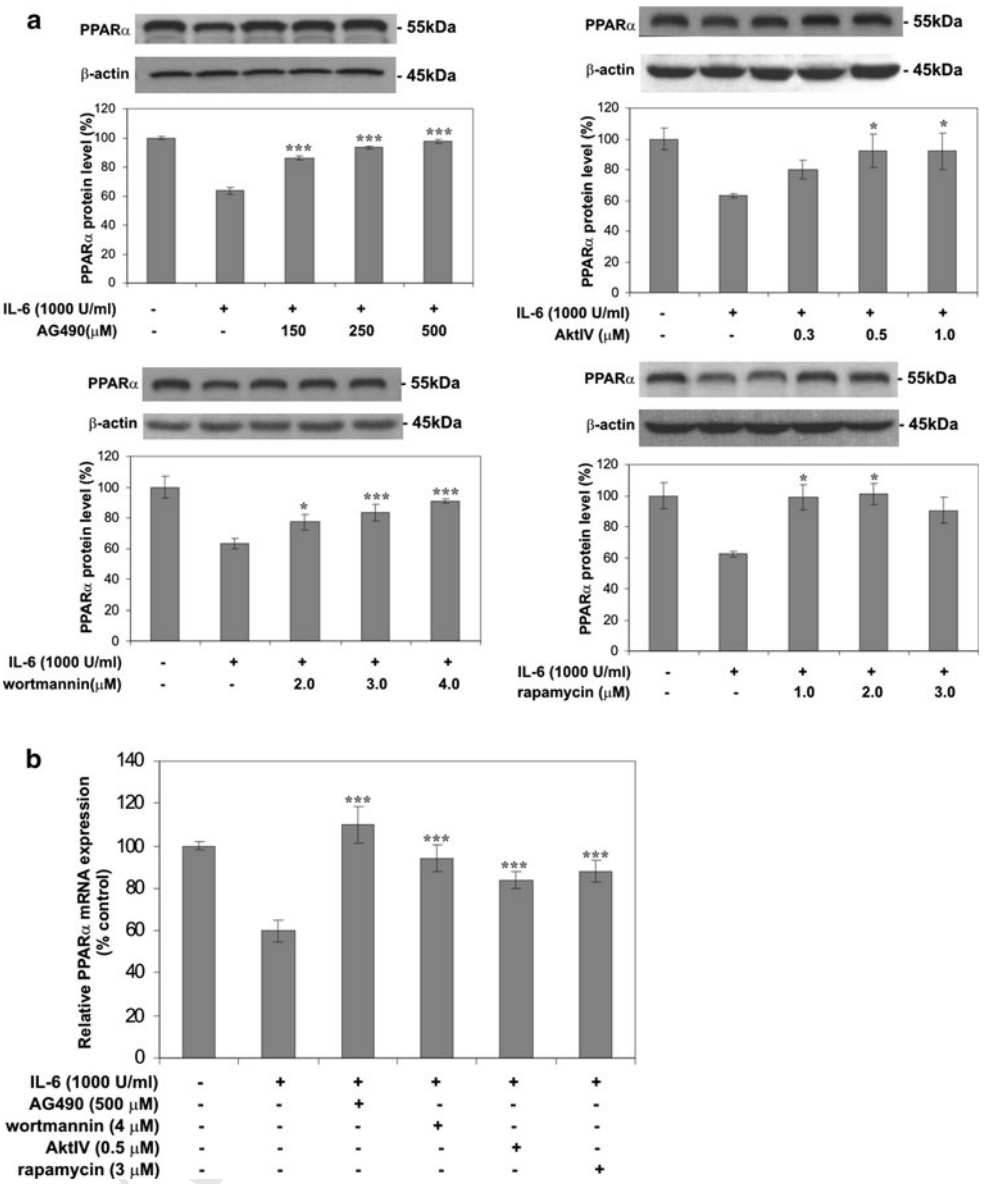


**Fig. 2** IL-6-inhibitory effects on PPARα mRNA and protein expression are mediated by JAK–STAT and PI3K pathways. **a** Dose-dependent inhibition of AG490, wortmannin, AKTIV, and rapamycin on IL-6-induced repression of PPARα protein. **b** The effects of AG490, wortmannin, AKTIV, and rapamycin on IL-6-mediated suppression of PPARα mRNA. Total cellular RNA was isolated and subjected to real-time PCR, assigning the signals for PPARα/β-actin ratio in unstimulated cells as 100 %, the expression of PPARα for each dose response treatment was relative to this control value. Effect of inhibitors on the IL-6-treated PPARα mRNA suppression was statistically analyzed by one-way analysis of variation (ANOVA). Statistical significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) is indicated *above bar*

AKTIV, and rapamycin increased the levels of PPARα mRNA by 50, 35, 22, and 30 %, respectively, compared with IL-6-treated cells in the absence of inhibitors (Fig. 2b).

These results showed that the inhibitors abolished the inhibitory action of IL-6 on PPARα gene expression both at the mRNA and protein level. Taken together, the results suggest that JAK2–STAT1/3 and PI3K/AKT/mTOR pathways are involved in IL-6-induced PPARα suppression in HepG2 cells.

## JAK/STAT and PI3K pathways are involved in IL-6-induced suppression on PPARα promoter activity

Previously, we demonstrated that the human PPARα promoter construct region from −765 to +34 displayed an approximately 50 % decrease in luciferase activity in HepG2 cells treated with IL-6, indicating that the IL-6 down-regulated the PPARα mRNA expression at the levels of transcription [20]. Since IL-6 decreased PPARα mRNA and protein via JAK2–STAT1/3 and PI3K/AKT/mTOR pathways, we investigated the role of similar pathways in modulating the PPARα promoter activity by means of pharmacological inhibitors against the identified components of signaling pathways.

In order to confirm the JAK2–STAT1/3 inhibition effects on the action of IL-6 in down-regulating PPARα transcriptional activity, HepG2 cells were preincubated with 500 μM AG490 for 2 h followed immediately by IL-6 treatment for 24 h on PPARα promoter transiently transfected cells. In agreement with our previous study [20], the activity from wt PPARα promoter was reduced by 50 %

when the cells were treated with IL-6. Interestingly, pretreatment with AG490 at 500 μM almost abolished the suppression caused by IL-6 by increasing the transcriptional activity of the PPARα promoter by 25 %, suggesting the involvement of the JAK2–STAT1/3 pathway in mediating the suppression of IL-6-mediated PPARα gene transcription (Fig. 3). The inhibitors used to elucidate the PI3K/AKT/mTOR pathway increased the PPARα promoter activities to 20, 10, and 6 % when IL-6-treated cells were preincubated with wortmannin, AKTIV, and rapamycin, respectively. However, it should be noted that only wortmannin (20 % induction) attained significance. The inhibition of IL-6-induced suppression of PPARα promoter activity by the inhibitors wortmannin, AKTIV, and rapamycin strongly suggests that the PI3K–AKT–mTOR pathway may be responsible in mediating the inhibitory effects of IL-6 on PPARα promoter activity. This pattern of attenuation also indicates that the wortmannin inhibitor at the upstream cascade blocked the IL-6 suppression on the PPARα promoter activity more effectively than the inhibitor used at the downstream cascade of PI3K pathway.

## The STAT-binding site in PPARα promoter (from −612 to −604) is responsible for IL-6 suppression of PPARα activity

The role of JAK2–STAT1/3 pathway may indicate a pivotal role for STAT in modulating the activity of PPARα promoter by IL-6. In addition, scanning the PPARα promoter using MatInspector [43] revealed the presence of a potential binding site for STAT transcription factor located between nucleotides −612 and −604 (Fig. 4a). In order to determine the importance of the STAT site, we transiently transfected HepG2 cells with wt PPARα promoter (−765/+34) or with STATmut PPARα promoter which had three nucleotides altered within the predicted STAT-binding site (Fig. 4a). The STAT site was mutated according to Yu et al. [44], which abolished unwanted binding sites for the other transcription factors and completely abrogates STAT-binding activity. The mutations (muts) of STAT-binding site almost abolished the suppression induced by IL-6 on the PPARα promoter activity (Fig. 4b). In addition, cotransfection with STAT1 and STAT3 expression plasmids in untreated HepG2 cells significantly reduced the transcriptional activities of the PPARα promoter and the control to 82 and 90 %, respectively (Fig. 4c). Thus, these results clearly demonstrate the role of STAT-binding site present in the PPARα promoter in mediating the IL-6 inhibition of PPARα gene expression.

In order to determine that down-regulation of PPARα by IL-6 was indeed mediated by the bindings of STAT1 and STAT3 transcription factors to the STAT-binding site

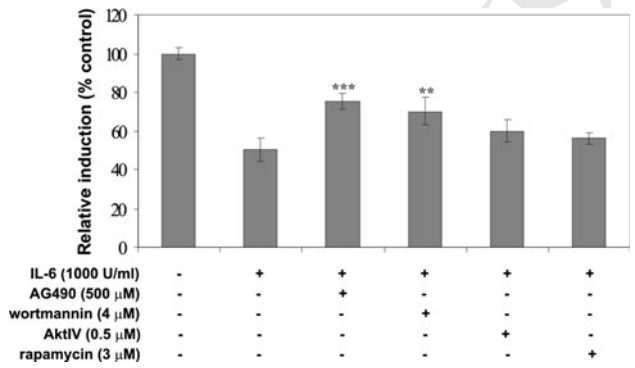


**Fig. 3** Effects of AG490, wortmannin, AKTIV, and rapamycin on IL-6-mediated suppression of PPARα promoter activity. HepG2 cells were transfected with luciferase reporter construct containing the promoter of PPARα gene (−765/+34) in the absence and the presence of IL-6. The transient-transfected cells were then pretreated with AG490, wortmannin, AKTIV, and rapamycin for 2 h before treatment with 1,000 U/ml of IL-6 for 24 h. Relative induction (%) of the promoter constructs activities in IL-6-treated cells inhibitors and without treatment of individual inhibitors, compared with untreated cells (control) which has been assigned as 100 %

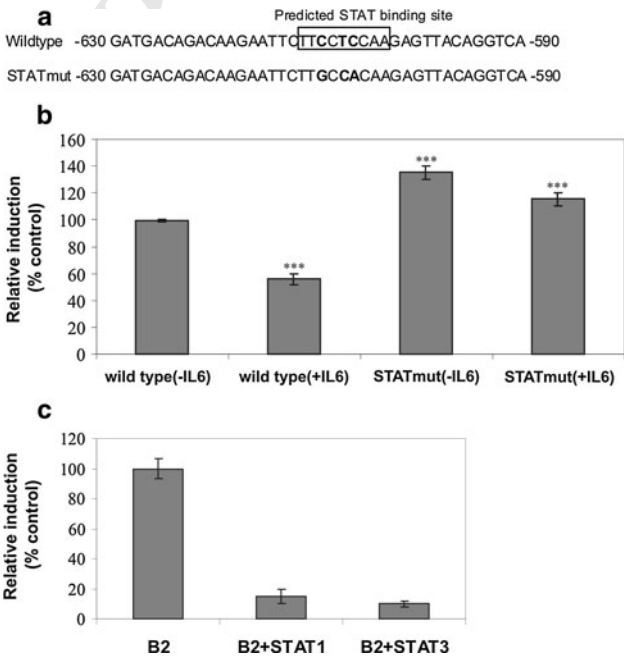


**Fig. 4** STAT-binding site in PPARα promoter played a significant role in IL-6 suppression of PPARα activity. **a** Identification of the *cis*-acting element responsible for IL-6 response on PPARα gene expression. The predicted STAT-binding site on PPARα promoter (−765/+34) is shown in *box* and the mutated nucleotides within STAT-binding site is indicated in *bold*. **b** Relative induction (%) of the effect of mutations in the STAT-binding sites on IL-6-regulated PPARα promoter activities. **c** The influence of STAT1 and STAT3 expression plasmids on PPARα promoter. HepG2 cells were cotransfected with the (B2) PPARα promoter luciferase constructs (−765/+34), along with 2 μg STAT1 and STAT3 expression plasmids. The fragments without IL-6 treatment have been assigned as 100 % and the values of their respective fragments in cells treated with IL-6 being shown in relative to the control value

(−612 to −604), we examined the interaction of nuclear extracts, prepared from HepG2 cells treated with IL-6 over the period of 120 min. EMSA showed three major DNA–protein complexes designated as C1–C3 that were present in the untreated cells, with IL-6-treated nuclear extracts producing a significant induction in the DNA–protein complexes C2 and C3 at 15 and 30 min, respectively (Fig. 5a). The specificities of complexes C2 and C3 were confirmed when these complexes were competed out using 400× of unlabeled STAT oligonucleotides (Fig. 5b). Complex C1 may represent a nonspecific interaction as the complex was not competed out by unlabeled oligonucleotides. Unlabeled STAT wild-type oligonucleotides (STATwt) completely abolished the formation of complexes C2 and C3 (Fig. 5c) and is consistent with Fig. 5b. Cold competition using the unlabeled STAT containing mutations (STATmut) binding site showed no effect on the formation of complexes C2 and C3, which strongly indicated that C2 and C3 represented specific interactions of proteins to STAT binding (Fig. 5c). We then confirmed that the complexes C2 and C3 contained STAT proteins by performing SS analysis using antibodies for STAT1 and STAT3 in EMSA. The incubation with antibodies against STAT1 and STAT3 produced an inhibition in the mobility of complexes C2 and C3, resulting in a formation of new supershifted complexes, SS1 and SS2 (Fig. 6a). Specifically, both antibodies partially reduced the formation of complex C3 and completely abolished the presence of complex C2. In contrast, complex C1 was not affected by antibodies against STAT1 and STAT3. These results suggest that STAT1 and STAT3 participated in the DNA–protein interactions which mediated the IL-6-inhibition of the PPARα promoter transcriptional activity. Interestingly, these results may also suggest that STAT1 and STAT3 proteins may form homo- and heterodimer between themselves.

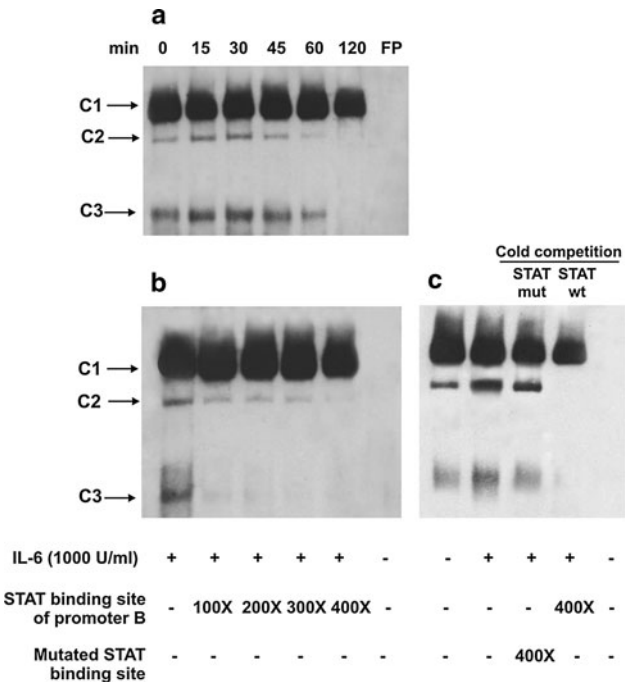


**Fig. 5** Analysis of the DNA-binding proteins to the STAT-binding site of PPARα promoter. **a** Time course of DNA-binding proteins to the STAT-binding site of PPARα promoter in response to IL-6 treatment. HepG2 cells were either untreated or treated with 1,000 U/ml of IL-6 for 15, 30, 45, 60, and 120 min. Nuclear extracts were then isolated, and 20 μg of nuclear extract was subjected to EMSA using biotin-labeled STAT (−612/−604) oligonucleotides. C represents patterns of DNA–protein complexes (C1–C3) detected, indicated by *labeled arrows*. **b** Analysis of the binding specificity of DNA–protein interactions in EMSA. Competition EMSA was carried out using nuclear extracts (20 μg) from IL-6-treated HepG2 cells, in the presence of 100×, 200×, 300×, and 400× molar excesses of unlabeled STAT-binding site oligonucleotides containing corresponding sequence of human STAT-binding site. **c** Competition experiments using mutated STAT oligonucleotides. Cold competition experiments were performed with labeled STAT-binding site oligonucleotides against unlabeled wild-type (STATwt) and labeled STAT-binding-site oligonucleotides against unlabeled STAT containing mutations (STATmut) at positions −607, −608, and −610 within the STAT recognition element (nucleotides from −612 to −604)

## STAT1 and STAT3 are responsible in mediating the IL-6-induced suppression of PPARα expression through JAK–STAT and PI3K pathways

To substantiate that an increase in the formation of complexes C2 and C3 was mediated through JAK2–STAT1/3 pathway, EMSA was performed using nuclear extracts from cells pretreated with 500 μM of AG490. As expected, the formation of complexes C2 and C3 was induced upon IL-6 stimulation compared with the untreated nuclear extract cells (Fig. 6b). The formation of these complexes was almost abolished when cells were pretreated with AG490 (Fig. 6b), corresponding to the inhibition of the phosphorylation of tyrosine residue of STAT1 and STAT3 and attenuated effect on the inhibitory action of IL-6 on PPARα expression levels. These results suggest that the binding of STAT1 and STAT3 is essential for mediating the IL-6-induced suppression effect on PPARα expression via JAK2–STAT1/3 pathway in HepG2 cells. We also investigated the connection of STAT-binding activity in the downstream PI3K pathway when induced by IL-6 using rapamycin to block the STAT activation. Interestingly, rapamycin was found to inhibit the binding activity of STAT proteins to the labeled STAT oligonucleotides, thereby reducing the formation of complexes C2 and C3 (Fig. 6c). These results strongly indicate that the formation of complexes C2 and C3 was also regulated by PI3K activation, confirming the role of PI3K in IL-6-induced STAT phosphorylation and STAT-binding activity.

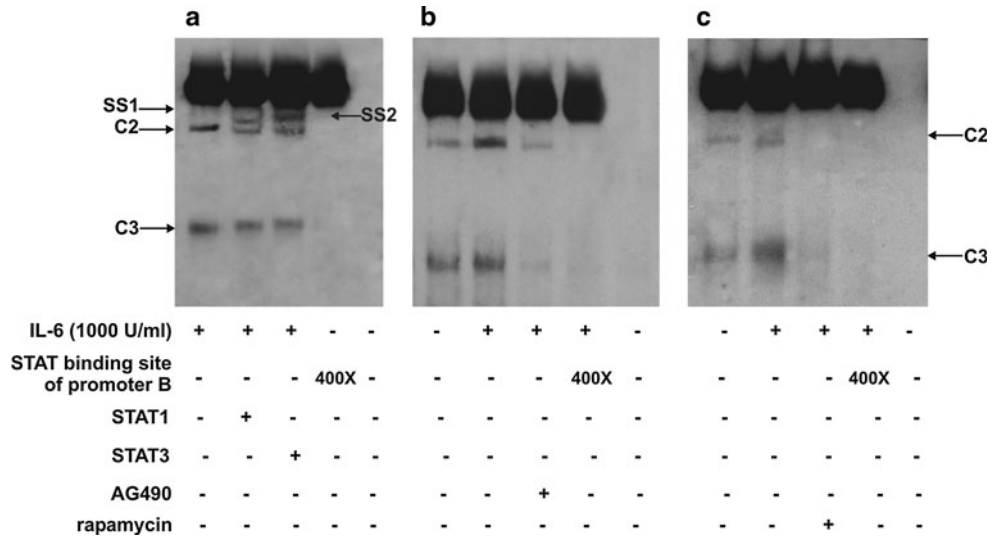


**Fig. 6** STAT1 and STAT3 are responsible for IL-6 suppression of PPARα activity. **a** IL-6-induced direct STAT1 and STAT3 DNA-binding protein of PPARα promoter to regulate the IL-6 suppression of PPARα gene expression. EMSA SS analysis was used to determine the presence of STAT1 and STAT3 proteins' complexes. IL-6-treated nuclear extracts (20 μg) from HepG2 cells were used for EMSA in the absence of antibody (IL-6) or in the presence of anti-STAT antibodies (STAT1 and STAT3). **b** The effects of AG490 on the IL-6-induced STAT-binding activity. HepG2 cells were either untreated or pretreated with AG490 for 2 h before treatment with 1,000 U/ml of IL-6 for 30 min. Nuclear extracts were then isolated and subjected to EMSA using biotin-labeled STAT oligonucleotides. **c** The effects of rapamycin on the IL-6-induced STAT-binding activity. HepG2 cells were either untreated or pretreated with rapamycin for 2 h before treatment with 1,000 U/ml of IL-6 for 30 min. Nuclear extracts were then isolated and subjected to EMSA using biotin-labeled STAT oligonucleotides. The DNA–protein complexes are shown with *labeled arrows* (C2 and C3), and the antibody *SS* band is as indicated. The result is representative of three independent experimental series

## Discussion

APR is a short-term response which is important in maintaining normal physiological homeostasis to counteract tissue injuries, infection, and trauma [45]. It is characterized by drastic changes in the concentration of APPs in the liver in response to activators [26], in which the activator IL-6 plays a major role [26, 28, 46]. IL-6 cytokine exerts its effect by binding to its receptor which is composed of two subunits, an 80-kDa IL-6 binding protein of mammalian cell surface and a 130-kDa transmembrane signal transducing component (gp130; [47]). IL-6 drives intracellular signaling pathways like JAK–STAT and MAPKs and activates a wide variety of APP genes including CRP, SAA, haptoglobin, fibrinogen, $\alpha_1$-acid gp, $\alpha_1$-antichymotrypsin, and albumin [20, 26].

IL-6 acts via activating the regulators that directly modulate the expression levels of APPs. Increasing evidence suggests that PPARα plays an important role in the development of APR, particularly in regulating the expression levels of APPs [26]. However, detailed analyses on the molecular mechanisms and pathways triggered by IL-6 in regulating the expression of PPARα are limited.

The present study demonstrated that JAK–STAT and PI3K pathways are involved in the STAT-mediated IL-6-inhibition of PPARα expression in HepG2 cells treated with IL-6. Four pieces of evidence from this study support this notion. First, specific JAK–STAT and PI3K inhibitors attenuated the mRNA and protein suppression of PPARα by IL-6. Second, this study proposed that the PI3K–AKT–mTOR pathway mediated the IL-6-inhibition of PPARα expression and was associated with JAK2–STAT1/3 activation. Third, the inhibitors of JAK–STAT and PI3K individually attenuated the IL-6-induced suppression on PPARα promoter activity in transient transfection assay. Finally, IL-6-inhibition of PPARα expression at the transcriptional level was regulated via the STAT-binding site of PPARα promoter.

We demonstrated for the first time that the signal transduction pathways of JAK2–STAT1/3 and PI3K/AKT/mTOR were responsible for mediating the inhibitory effect of IL-6 on PPARα gene expression in HepG2 cells. These results correspond with the previous studies reporting that the signaling routes triggered by IL-6 to exert its effects in liver mainly involved JAK–STAT [35, 48, 49] and PI3K pathways [36, 50, 51]. Our study also revealed that the activation of JAK2–STAT1/3 pathway by IL-6 depends largely on the phosphorylation of tyrosine residues. Since the JAK–STAT inhibitor, AG490, effectively inhibited the IL-6-induced repression of PPARα mRNA, protein, and promoter activity, it is likely that the IL-6-induced PPARα suppression was mainly activated by $Tyr^{701}$ STAT1 and $Tyr^{705}$ STAT3. Actually, accumulating evidence revealed that phosphorylation of tyrosine kinase residue is a

prerequisite for STAT activation by IL-6 [51, 52]. In addition, APPs are regulated by IL-6 via the phosphorylation of tyrosine residues of STAT1 and STAT3, SHP2, and gp130 [53].

It is well documented in IL-6-activated PI3K pathway studies that PI3K protein is the first component activated, which then responds to activate AKT, and subsequently phosphorylates and regulates the activity of mTOR [54–56]. In this study, we demonstrated that PI3K pathway inhibited the IL-6-induced suppression of PPARα mRNA, PPARα protein, and PPARα promoter activity with a PI3K inhibitor and produced a higher attenuated effect followed by the inhibitors for AKT and mTOR. The role of IL-6-activated PI3K pathway in regulating the APPs is not well established. However, there are studies demonstrating the involvement of this pathway in the prosurvival, antiapoptotic effect of IL-6 in protecting and regenerating the liver cells [57–59].

Our finding also suggested a potential crosstalk between JAK2–STAT1–3 and PI3K/AKT/mTOR in the activation of STAT at the downstream level of the PI3K pathway. We showed that AG490 and rapamycin abolished the STAT–DNA-binding activity represented by complexes C2 and C3 (Fig. 5b, c). In support of this discovery, these inhibitors were also found to inhibit the quantity of activated STAT isoforms present in STAT–DNA-binding activity assays in other cell types [60, 61]. Therefore, it is tempting to speculate that IL-6-induced suppression of PPARα gene expression may also be mediated through the crosstalk between PI3K/AKT/mTOR and JAK/STAT pathways by inhibiting tyrosine phosphorylation of STAT1 and STAT3 and blocking STAT dimer formation to reduce STAT–DNA-binding activity. Indeed, Tron et al. [48] reported that the inhibition of PI3K pathway completely obliterated the functional activity of STAT in inducing the heme oxygenase-1 gene by IL-6. In addition, a novel model has been strongly suggested for mTOR effecting IL-6 signaling by regulating STAT3 phosphorylation and activation [62]. Hence, this study supports the potential presence of a novel crosstalk between the PI3K and STAT3 pathways (PI3K–AKT–mTOR–STAT) to down-regulate PPARα mRNA levels in response to IL-6. Concomitantly, studies have also shown that STAT3 is phosphorylated and activated in a PI3K-dependent manner in PI3K-transformed murine cells, thus providing a promising therapeutic target that could enhance the effectiveness of PI3K inhibitors [63].

STATs have been identified as a family of transcription factors that play an important role in gene regulation in response to cellular stimulation by growth factors (GFs) and cytokines [47]. In addition, STAT1 and STAT3 were also found to be activated by IL-6 in hepatocytes [64, 65]. Because of their size (90 kDa) that is far beyond the exclusion limit of nuclear pore, STAT needs to be phosphorylated and actively translocated into the nucleus to regulate the expression of certain genes involved in APR [66, 67]. As STAT is associated with other pathways and also plays a critical role in controlling and mediating IL-6-effects of APP genes, the STAT site in the human PPARα gene promoter may also play a crucial role in mediating the action of IL-6. Thus, the conventional mechanism of phosphorylated STAT dimers bound to STAT-binding site was proposed in this study. Transient transfection and EMSA analyses using wt and mutated STAT-binding sites on the PPARα promoter region (−765/+34) established that the STAT-binding site within the promoter was required for the interaction with STAT1 and STAT3 in mediating the IL-6 suppressive response on PPARα gene expression. Furthermore, cotransfection analysis of human PPARα promoter with STAT1 and STAT3 expression plasmids considerably reduced the constitutive expression of PPARα, further suggesting that both STAT1 and STAT3 homo- or heterodimers may serve as repressors in PPARα transcriptional activity. Although STAT was originally identified as an activator of transcription [68–70], STAT1 and STAT3 have been implicated as repressors to a vast number of genes [71–73]. For example, GF-activated transcription of STAT5 and STAT3 was found to negatively regulate the PPARα transcriptional activity in COS-1 cells [73]. In fact, other studies have shown that STATs activated by IL-6, IL-1, IFN-γ, and TNF-α have the ability to down-regulate PPARγ gene expressions [74–77]. Antibody SS assays further confirmed the interaction between STAT1 and STAT3 with the STAT-binding site. However, due to partial inhibition of complex C3 by antibodies, STAT1 and STAT3 may also form heterodimers with other STAT family members/transcription factors. For example, phosphorylation of STAT-induced SH2-mediated heterodimerization with STAT family members, followed by translocation of the STAT dimer for transcription activation and subsequent binding to specific consensus sequence of target-gene promoters in the nucleus [12, 47, 78]. Heterodimerization of STATs was achieved via reciprocal binding of this critical, phosphorylated tyrosine of one monomer, and SH2 domain of the partner dimer and were frequently observed in cytokine responses [44, 70]. Thus, our findings suggested that the effects of IL-6 suppression on PPARα promoter were dependent on tyrosine phosphorylation of STAT1 and STAT3 and subsequently the DNA-binding activity of STAT to its corresponding binding site via protein–protein interactions within the STAT family members or/and with other transcription factors [73, 79]. Activations and DNA-binding activities of STAT1 and STAT3 were increased reaching their peaks within 15–30 min after incubation with IL-6 (Fig. 5). Therefore, it can be hypothesized that STAT1 and STAT3 proteins were translocated to the nucleus within 15 min after the

treatment and were mostly deactivated after 2 h. These parameters were consistent with STAT activation by IL-6 in other cell types [80, 81]. For example, the transient binding of STAT protein to IL-RE of the $\alpha_2$-macroglobulin promoter was demonstrated in rat ovary [80]. The induction pattern of STAT-binding activity also closely mirrored the induction of phosphorylation of $Tyr^{701}$ STAT1 and $Tyr^{705}$ STAT3. Since the binding activity of STAT occurred at similar time points (15–30 min) as those of the phosphorylation, it is tempting to speculate that the binding activities of STAT1 and STAT3 to the PPARα promoter were transient and mainly regulated by phosphorylation. In agreement with this result, studies have shown that the prerequisite for STAT transcriptional activities depends on its phosphorylation resulting in dimerization which enables nuclear localization and DNA binding [82]. The duration of STAT phosphorylation, and therefore, the transcriptional activity, is regulated by the balance of receptor-driven JAK2 catalytic activity and constitutive nuclear dephosphorylation by a protein, tyrosine phosphatase [69, 83, 84]. Other studies demonstrated that the loss of phosphorylation of tyrosine residue upon DNA binding in the nucleus causes STAT protein to be transported back to the cytoplasm which may lead to the abolition of STAT activation and binding activity [70, 78].

In summary, this study has successfully unraveled pathways by which IL-6 inhibits PPARα gene transcription, involving the modulation of JAK2–STAT1/3 and PI3K–AKT–mTOR pathways, as well as a possible crosstalk between PI3K–AKT–mTOR and STAT pathways. The present study also underlines the significance of JAK–STAT as a dominant pathway due to crosstalks between JAK–STAT and PI3K via JAK activation and STAT transcription factors in down-regulating the PPARα mRNA expression. Altogether, these findings represent a new model of IL-6-induced suppression of PPARα expression.

Accordingly, given that the PPARs play a major role as lipid-sensing transcription factors that regulate cholesterol and fatty acid homeostasis [23–25] and the emerging evidence that this transcription factor family is also implicated in the development of APR in the liver and inflammation and atherosclerosis [25–29], future studies are essential to delineate the role of PPAR in the development of APR. Although our studies were focused on PPARα in a liver cell culture model; determining which regulatory pathways are activated by PPARα may have therapeutic implications and may help clarify whether targeting these pathways is an effective approach in the management of inoperable or recurrent APR.

**Acknowledgments** The authors are grateful to Dr. Chew Choy Hoong (UTAR) for providing the PPARα promoter plasmids. This project was supported by grants from the E-Science Fund, The Malaysian Ministry of Science, Technology and Innovation, and the Malaysian Toray Science Foundation (MTSF).

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