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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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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 (PPAR α). Currently, there is a growing interest in determining the role of PPAR α in regulating APR; however, studies on the molecular mechanisms and signaling pathways implicated in mediating the effects of IL-6 on the expression of PPAR α are limited. We previously revealed that IL-6 inhibits PPAR α 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 PPAR α . Treatment of cells with pharmacological inhibitors of JAK2, PI3K, AKT, and mTOR attenuated the inhibitory effect of IL-6 on PPAR α protein in a dose-dependent manner. These inhibitors also decreased the IL-6-induced repression of PPAR α mRNA expression and promoter activity. Overexpression of STAT1 and STAT3 in HepG2 cells cotransfected with a reporter vector containing this PPAR α promoter region revealed that both the expression

plasmids inhibited the IL-6-induced repression of PPAR α 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 PPAR α protein and mRNA was also inhibited. This study has unraveled novel pathways by which IL-6 inhibits PPAR α 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 PPAR α promoter. Together, these findings represent a new model of IL-6-induced suppression of PPAR α expression by inducing STAT1 and STAT3 phosphorylation and subsequent down-regulation of PPAR α mRNA expression.

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

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

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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, α_1 -antichymotrypsin, ceruloplasmin, haptoglobin, α_1 -acid glycoprotein (gp), and α_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 (PPAR α). PPAR α 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 PPAR α agonist, fenofibrate, the actions of IL-6 on modulation of gene expression on APPs are suppressed. Furthermore, this proved that the effect of PPAR α prevents the IL-6-induced expression of the APR genes via PPAR α -dependent mechanisms, directly at the transcriptional level [26]. Studies also demonstrated that regulation of PPAR α 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 PPAR α during APR is poorly understood. Although the expression of PPAR α 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 PPAR α gene expression in liver cells are limited. Recently, we reported that IL-6 decreased PPAR α 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 PPAR α 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 PPAR α expression. Furthermore, our results also suggest that IL-6-induced STAT1 and STAT3 proteins were essential for mediating the IL-6-inhibitory action on PPAR α 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), PPAR α , β -actin; and the phosphorylated forms Tyr⁷⁰¹ STAT1, Tyr⁷⁰⁵ STAT3, Ser⁷²⁷ STAT1, Ser⁷²⁷ STAT3, Tyr^{458/199} PI3K, Tyr³⁰ AKT, and Ser²⁴⁴⁸ 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 μ g/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5 % (v/v) CO₂. 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 μ l of 2 $\times$ SYBR Green RT-PCR Reaction Mix, 1 μ l of each forward and reverse primers (10 μ l), and 1 μ l of iScript Reverse Transcriptase in a final volume of 30 μ l. 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 PPAR α 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 μ g of proteins were used in SDS-PAGE to determine the levels of protein content for phosphorylated Tyr⁷⁰¹ STAT1, Tyr⁷⁰⁵ STAT3, Ser⁷²⁷ STAT1, Ser⁷²⁷ STAT3, Tyr^{458/199} PI3K, Tyr³⁰ AKT, and Ser²⁴⁴⁸ mTOR; and for PPAR α and β -actin, respectively. Subsequently, proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with blocking solution [1 $\times$ 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 $\times$ PBS and 0.1 % (v/v) Tween-20] and incubated with primary antibodies (rabbit anti-human Tyr⁷⁰¹ STAT1, Tyr⁷⁰⁵ STAT3, Ser⁷²⁷ STAT1, Ser⁷²⁷ STAT3, Tyr^{458/199} PI3K, Tyr³⁰ AKT, Ser²⁴⁴⁸ mTOR, PPAR α , and β -actin), which was diluted 1/1,000 in 1 $\times$ 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 $\times$ 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., Songpaju, 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'-TAACTCTTGAGGAAGAATTCT-3'. The oligonucleotides were labeled using biotin 3'-End DNA Labeling kit (Pierce) according to manufacturer's instructions. For binding reactions, 20 μ g 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 μ l 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.

PPAR α promoter–reporter plasmids

The construction of human PPAR α promoter –765/+34 in pGL3-Basic Vector was described previously [20]. The PPAR α 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 $\times$ reaction buffer, 50 ng double-stranded DNA template, and 125 ng of complementary oligonucleotides [5'-CAAGAATTCTTGCCACAAGAGTTACAG-GTCAGT-3' and 5'-ACTGACCTGTAACCTTGTGGcAAGAATTCTTG-3'] of the human PPAR α promoter. The mutated construct was designated as STATmut PPAR α 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 9×10^5 cells in 2 ml of fresh culture medium in a six-well plastic dish. The cells were transfected with 2 μ g of wild-type (wt) PPAR α promoter luciferase construct or the STATmut PPAR α promoter luciferase construct and 0.5 μ g thymidine kinase/Renilla (pRL-TK) vector as a control for transfection efficiency. For the over expression studies, HepG2 cells were cotransfected with 2 μ g of PPAR α promoter luciferase construct, 0.5 μ g pRL-TK plasmid, and either 2 μ g of STAT1 or STAT3 expression plasmids or 2 μ g 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 $\pm$ 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 μ l of passive lysis buffer per well. Luciferase activity was determined as instructed by the manufacturer (Promega). In brief, 20 μ l of cell extracts were mixed at room temperature with 100 μ l 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 Tyr⁷⁰¹ and STAT3 Tyr⁷⁰⁵ within 15–30 min but not the total STAT1 and STAT3 (Fig. 1a). The levels of phosphorylated STAT1 Ser⁷²⁷ and STAT3 Ser⁷²⁷ 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 Tyr³⁰, and mTOR Ser²⁴⁴⁸ (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 (Tyr⁷⁰⁵) and STAT3 (Tyr⁷⁰⁵) 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 μ M 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 PPAR α mRNA expression are mediated by JAK–STAT and PI3K signal transduction pathways

Previously, we showed that IL-6 down-regulated the expression of PPAR α 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 PPAR α gene expression. We examined the effects of JAK–STAT and PI3K inhibitors on IL-6-inhibitory action on PPAR α protein content. The inhibitors dose dependently inhibited the IL-6-suppressed PPAR α protein expression (Fig. 2a). AG490 produced a statistically significant inhibition on the action of IL-6 with the highest level at 500 μ M, 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 PPAR α 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. 2 IL-6-inhibitory effects on PPAR α 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 PPAR α protein. **b** The effects of AG490, wortmannin, AKTIV, and rapamycin on IL-6-mediated suppression of PPAR α mRNA. Total cellular RNA was isolated and subjected to real-time PCR, assigning the signals for PPAR α / β -actin ratio in unstimulated cells as 100 %, the expression of PPAR α for each dose response treatment was relative to this control value. Effect of inhibitors on the IL-6-treated PPAR α 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 PPAR α 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 PPAR α 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 PPAR α suppression in HepG2 cells.

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

Previously, we demonstrated that the human PPAR α 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 PPAR α mRNA expression at the levels of transcription [20]. Since IL-6 decreased PPAR α mRNA and protein via JAK2-STAT1/3 and PI3K/AKT/mTOR pathways, we investigated the role of similar pathways in modulating the PPAR α 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 PPAR α transcriptional activity, HepG2 cells were preincubated with 500 μ M AG490 for 2 h followed immediately by IL-6 treatment for 24 h on PPAR α promoter transiently transfected cells. In agreement with our previous study [20], the activity from wt PPAR α promoter was reduced by 50 %

when the cells were treated with IL-6. Interestingly, pretreatment with AG490 at 500 μ M almost abolished the suppression caused by IL-6 by increasing the transcriptional activity of the PPAR α promoter by 25 %, suggesting the involvement of the JAK2–STAT1/3 pathway in mediating the suppression of IL-6-mediated PPAR α gene transcription (Fig. 3). The inhibitors used to elucidate the PI3K/AKT/mTOR pathway increased the PPAR α 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 PPAR α 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 PPAR α promoter activity. This pattern of attenuation also indicates that the wortmannin inhibitor at the upstream cascade blocked the IL-6 suppression on the PPAR α promoter activity more effectively than the inhibitor used at the downstream cascade of PI3K pathway.

The STAT-binding site in PPAR α promoter (from –612 to –604) is responsible for IL-6 suppression of PPAR α activity

The role of JAK2–STAT1/3 pathway may indicate a pivotal role for STAT in modulating the activity of PPAR α promoter by IL-6. In addition, scanning the PPAR α 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

Fig. 3 Effects of AG490, wortmannin, AKTIV, and rapamycin on IL-6-mediated suppression of PPAR α promoter activity. HepG2 cells were transfected with luciferase reporter construct containing the promoter of PPAR α 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 %

determine the importance of the STAT site, we transiently transfected HepG2 cells with wt PPAR α promoter (–765/+34) or with STATmut PPAR α 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 (mut) of STAT-binding site almost abolished the suppression induced by IL-6 on the PPAR α promoter activity (Fig. 4b). In addition, cotransfection with STAT1 and STAT3 expression plasmids in untreated HepG2 cells significantly reduced the transcriptional activities of the PPAR α 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 PPAR α promoter in mediating the IL-6 inhibition of PPAR α gene expression.

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

Fig. 4 STAT-binding site in PPAR α promoter played a significant role in IL-6 suppression of PPAR α activity. **a** Identification of the *cis*-acting element responsible for IL-6 response on PPAR α gene expression. The predicted STAT-binding site on PPAR α 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 PPAR α promoter activities. **c** The influence of STAT1 and STAT3 expression plasmids on PPAR α promoter. HepG2 cells were cotransfected with the (B2) PPAR α promoter luciferase constructs (–765/+34), along with 2 μ g 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

Fig. 5 Analysis of the DNA-binding proteins to the STAT-binding site of PPAR α promoter. **a** Time course of DNA-binding proteins to the STAT-binding site of PPAR α 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 μ g 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 μ g) 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)

(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 PPAR α promoter transcriptional activity. Interestingly, these results may also suggest that STAT1 and STAT3 proteins may form homo- and heterodimer between themselves.

STAT1 and STAT3 are responsible in mediating the IL-6-induced suppression of PPAR α 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 μ M 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 PPAR α expression levels. These results suggest that the binding of STAT1 and STAT3 is essential for mediating the IL-6-induced suppression effect on PPAR α 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 PPAR α activity. **a** IL-6-induced direct STAT1 and STAT3 DNA-binding protein of PPAR α promoter to regulate the IL-6 suppression of PPAR α gene expression. EMSA SS analysis was used to determine the presence of STAT1 and STAT3 proteins' complexes. IL-6-treated nuclear extracts (20 μ g) 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, α_1 -acid gp, α_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 PPAR α 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 PPAR α are limited.

The present study demonstrated that JAK-STAT and PI3K pathways are involved in the STAT-mediated IL-6-inhibition of PPAR α 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 PPAR α by IL-6. Second, this study proposed that the PI3K-AKT-mTOR pathway mediated the IL-6-inhibition of PPAR α 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 PPAR α promoter activity in transient transfection assay. Finally, IL-6-inhibition of PPAR α expression at the transcriptional level was regulated via the STAT-binding site of PPAR α 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 PPAR α 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 PPAR α mRNA, protein, and promoter activity, it is likely that the IL-6-induced PPAR α suppression was mainly activated by Tyr⁷⁰¹ STAT1 and Tyr⁷⁰⁵ 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 PPAR α mRNA, PPAR α protein, and PPAR α 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 PPAR α 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 PPAR α 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 PPAR α 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 PPAR α 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 PPAR α gene expression. Furthermore, cotransfection analysis of human PPAR α promoter with STAT1 and STAT3 expression plasmids considerably reduced the constitutive expression of PPAR α , further suggesting that both STAT1 and STAT3 homo- or heterodimers may serve as repressors in PPAR α 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 PPAR α 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 PPAR γ 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 PPAR α 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 α_2 -macroglobulin promoter was demonstrated in rat ovary [80]. The induction pattern of STAT-binding activity also closely mirrored the induction of phosphorylation of Tyr⁷⁰¹ STAT1 and Tyr⁷⁰⁵ 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 PPAR α 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 PPAR α gene transcription, involving the modulation of JAK2–STAT1/3 and PI3K–AKT–mTOR pathways, as well as a possible cross-talk 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 PPAR α mRNA expression. Altogether, these findings represent a new model of IL-6-induced suppression of PPAR α 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 PPAR α in a liver cell culture model; determining which regulatory pathways are activated by PPAR α may have therapeutic implications and may help clarify whether targeting these pathways is an effective approach in the management of inoperable or recurrent APR.

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References

1. Bode JG, Albrecht U, Häussinger D, Heinrich PC, Schaper F (2012) Hepatic acute phase proteins—regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF- κ B-dependent signaling. *Eur J Cell Biol* 91:496–505
2. Gruys E, Toussaint MJM, Niewold TA, Koopmans SJ (2005) Acute phase reaction and acute phase proteins. *J Zhejiang Univ Sci* 6B:1045–1056
3. Lowenstein C, Matsushita K (2004) The acute phase response and the atherosclerosis. *Drug Discov Today Dis Mech* 1:17–22
4. Gomez CR, Goral J, Ramirez L, Kopf M, Kovacs EJ (2006) Aberrant acute-phase response in aged interleukin-6 knockout mice. *Shock* 25:581–585
5. Febbraio MA, Rose-John S, Pedersen BK (2010) Is interleukin-6 receptor blockade the Holy Grail for inflammatory diseases? *Clin Pharmacol Ther* 87:396–398
6. Matthews VB, Allen TL, Risis S, Chan MHS, Henstridge DC, Watson N, Zaffino LA, Babb JR, Boon J, Meikle PJ, Jowett JB, Watt MJ, Jansson JO, Bruce CR, Febbraio MA (2010) Interleukin-6-deficient mice develop hepatic inflammation and systemic insulin resistance. *Diabetologia* 53:2431–2441
7. Gabay C, Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 340:448–454
8. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C (2004) Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *J Lipid Res* 44:1169–1196
9. Kishimoto T (2010) IL-6: from its discovery to clinical applications. *Int Immunol* 22:347–352
10. Dhainaut JF, Marin N, Mignon A, Vinsonneau C (2001) Hepatic response to sepsis: interaction between coagulation and inflammatory processes. *Crit Care Med* 29:S42–S47
11. Hoffmeister A, Rothenbacher D, Bazner U, Frohlich M, Brenner H, Hombach V, Koenig W (2001) Role of novel markers of inflammation in patients with stable coronary heart disease. *Am J Cardiol* 87:262–266
12. Neurath M, Finotto S (2011) IL-6 signalling in autoimmunity chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev* 22:83–89
13. Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V (2000) Inflammation, obesity, stress and coronary heart disease: is interleukin the link? *Atherosclerosis* 148:209–214
14. Hoang T, Haman A, Goncalves O, Wong GG, Clark SC (1988) Interleukin-6 enhances growth factor-dependent proliferation of the blast cells of acute myeloblastic leukemia. *Blood* 72:823–826
15. Wang FW, Hao HB, Zhao SD, Zhang YM, Liu Q, Liu HJ, Liu SM, Yuan QH, Bing LJ, Ling EA, Hao AJ (2011) Roles of activated astrocyte in neural stem cell proliferation and differentiation. *Stem Cell Res* 7:41–53
16. Ganapathi MK, Rzewnicki D, Samols D, Jiang SL, Kushner I (1991) Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells. *J Immunol* 147:1261–1265
17. Daveau M, Davrinche C, Julien H, Hiron M, Arnaud P, Lebreton JP (1988) The synthesis of human α -2-HS glycoprotein is down-regulated by cytokines in hepatoma HepG2 cells. *FEBS Lett* 241:191–194
18. Koj A, Gordon HH, Gauldie J (1988) An alternative regulatory pathway of the acute phase response: the role of fibroblast-derived interferon-beta 2. *Experientia* 44:9–10

19. Mackiewicz A, Kushner I (1989) Interferon β /B-cell stimulating factor 2/interleukin 6 affects glycosylation of acute phase proteins in human hepatoma cell lines. *Scand J Immunol* 29:265–271
20. Chew CH, Chew GS, Najimudin N, Tengku-Sifzizul TM (2007) Interleukin-6 inhibits human peroxisome proliferator activated receptor alpha gene expression via CCAAT/enhancer-binding protein in hepatocytes. *Int J Biochem Cell Biol* 39:1975–1986
21. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ (2001) Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 21:1393–1403
22. Wang Z, Burke PA (2007) Effects of hepatocyte nuclear factor-4 α on the regulation of the hepatic acute phase response. *J Mol Biol* 371:323–335
23. Lee CH, Olson P, Evans RM (2003) Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. *Endocrinology* 144:2201–2207
24. Myers SA, Wang S-CM, Muscat GEO (2006) The chicken ovalbumin upstream promoter-transcription factors modulate genes and pathways involved in skeletal muscle cell metabolism. *JBC* 281:24149–24160
25. Panzenboeck U, Kratzer I, Sovic A, Wintersperger A, Bernhart E, Hammer A, Malle E, Sattler W (2006) Regulatory effects of synthetic liver X receptor- and peroxisome-proliferator activated receptor agonists on sterol transport pathways in polarized cerebrovascular endothelial cells. *Int J Biochem Cell Biol* 38:1314–1329
26. Gervois P, Kleemann R, Pilon A, Percevault F, Koenig W, Staels B, Kooistra T (2004) Global suppression of IL-6-induced acute phase response gene expression after chronic in vivo treatment with the peroxisome proliferator-activated receptor- α activator fenofibrate. *J Biol Chem* 279:16154–16160
27. Duval C, Chinetti G, Trottein F, Fruchart JC, Staels B (2002) The role of PPARs in atherosclerosis. *Trends Mol Med* 8:422–430
28. van Raalte DH, Li M, Pritchard PH, Wasan KM (2005) Peroxisome proliferator-activated receptor alpha (PPAR α): a pharmacological target with a promising future. *Pharm Res* 21:1531–1538
29. Jonkers IJ, Mohrschlatt MF, Westendorp RG, Van der Laarse A, Smelt AH (2002) Severe hypertriglyceridemia with insulin resistance is associated with systemic inflammation: reversal with bezafibrate therapy in a randomized controlled trial. *Am J Med* 12:275–280
30. Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR (2000) The acute phase response is associated with retinoid X receptor repression in rodent liver. *J Biol Chem* 275:16390–16391
31. Fang C, Yoon S, Tindberg N, Jarvelainen HA, Lindros KO, Ingelman-Sundberg M (2004) Hepatic expression of multiple acute phase proteins and down regulation of nuclear receptors after acute endotoxin exposure. *Biochem Pharmacol* 67:1389–1397
32. Feingold K, Kim MS, Shigenaga J, Moser A, Grunfeld C (2004) Altered expression of nuclear hormone receptors and coactivators in mouse heart during the acute phase response. *Am J Physiol Endocrinol Metab* 286:E201–E207
33. Lee TI, Kao YH, Chen YC, Che YJ (2008) Proinflammatory cytokine and ligands modulate cardiac peroxisome proliferator-activated receptor. *Eur J Clin Invest* 39:23–30
34. Chew CH, Samian MR, Najimudin N, Tengku-Muhammad TS (2003) Molecular characterization of six alternatively spliced variants and novel promoter in human peroxisome proliferator activated receptor α . *Biochem Biophys Res Commun* 305:235–243
35. Elliot J (2008) Soc3 in liver regeneration and hepatocarcinoma. *Mol Interv* 8:19–21
36. Qian Y, Deng J, Xie H, Geng L, Zhou L, Wang Y, Yin S, Feng X, Zheng S (2009) Regulation of TLR4-induced IL-6 response in bladder cancer cells by opposing actions of MAPK and PI3K signaling. *J Cancer Res Clin Oncol* 135:379–386
37. Kwak SY, Kim UK, Cho HJ, Lee HK, Kim HJ, Kim NK et al (2008) Methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) gene polymorphisms as risk factors for hepatocellular carcinoma in a Korean population. *Anticancer Res* 28:2807–2811
38. Chang KT, Tsai CM, Chiou YC, Chiu CH, Jeng KS, Huang CYF (2005) IL-6 induces neuroendocrine dedifferentiation and cell proliferation in non-small cell lung cancer cells. *Am J Physiol Lung Cell Mol Physiol* 289:446–453
39. Samardzija M, Wenzel A, Aufenberg S, Thiersch M, Remé C, Grimm C (2006) Differential role of JAK-STAT signaling in retinal degenerations. *FASEB J* 20:2411–2413
40. Tomita Y, Morooka T, Hoshida Y, Zhang B, Qiu Y, Hamada K et al (2006) Prognostic significance of activated AKT expression in soft-tissue sarcoma. *Clin Cancer Res* 12:3070–3077
41. Morgensztern D, McLeod HL (2005) PI3K/AKT/mTOR pathway as a target for cancer therapy. *Anticancer Drugs* 16:797–803
42. Yuan ZQ, Sun M, Feldman RI, Wang G, Ma X et al (2000) Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/AKT pathway in human ovarian cancer. *Oncogene* 19:2324–2330
43. Quandt K, Frech K, Karas H, Wingender E, Werner T (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 23:4878–4884
44. Yu H, Li X, Marchetto GS, Dy R, Hunter D, Calvo B et al (1996) Activation of a novel calcium-dependent protein-tyrosine kinase. Correlation with c-Jun N-terminal kinase but not mitogen-activated protein kinase activation. *J Biol Chem* 271:29993–29998
45. Bauzá G, Miller G, Kaseje N, Wigner NA, Wang Z, Gerstenfeld LC, Burke PA (2011) The effects of injury magnitude on the kinetics of the acute phase response. *J Trauma* 70:948–953
46. Zambon A, Gervois P, Pauletto P, Fruchart JC, Staels B (2006) Modulation of hepatic inflammatory risk markers of cardiovascular disease by PPAR α activators: clinical and experimental evidence. *Arterioscler Thromb Vasc Biol* 26:977–986
47. Schuringa JJ, Jonk LJ, Dokter WH, Vellenga E, Kruijer W (2000) Interleukin-6-induced STAT3 transactivation and Ser727 phosphorylation involves Vav, Rac-1 and the kinase SEK-1/MKK-4 as signal transduction components. *Biochem J* 347:89–96
48. Tron K, Samoylenko A, Musikowski G, Kobe F, Immenschuh S, Schaper F et al (2006) Regulation of rat heme oxygenase-1 expression by interleukin-6 via the JAK/STAT pathway in hepatocytes. *J Hepatol* 45:72–80
49. Yeoh CT, Ernst M, Rose-John S, Akhurst B, Payne C, Long S et al (2007) Opposing roles of gp130-mediated STAT-3 and ERK-1/2 signaling in liver progenitor cell migration and proliferation. *J Hepatol* 45:486–494
50. Brand S, Dambacher J, Beigel F, Zitzmann K, Heeg MHJ, Weiss TS et al (2007) IL-22-mediated liver cell regeneration is abrogated by SOCS-1/3 overexpression in vitro. *Am J Physiol Gastrointest Liver Physiol* 292:1019–1028
51. Markiewski M, DeAngelis R, Strey C, Foukas P, Gerard C, Gerard N, Wetse R, Lambris J (2009) The regulation of liver cell survival by complement. *J Immunol* 182:5412–5418
52. Darnell JE Jr, Kerr IM, Stark GR (1994) JAK/STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415–1421
53. Schaper F, Gendo C, Eck M, Schmitz J, Grimm C, Anhuof D et al (1998) Activation of the protein tyrosine phosphatase SHP2 via the interleukin-6 signal transducing receptor protein gp130 requires

- tyrosine kinase JAK1 and limits acute-phase protein expression. *Biochem J* 335:557–565
54. Bashkar K, Miller M, Chludzinski A, Herrup K, Zagorski M, Lamb BT (2009) The PI3K–AKT–mTOR pathway regulates A β oligomer induced neuronal cell cycle events. *Mol Neurodegener* 4:14–32
55. Cartot A, Armand JP, Soria JC (2006) PI3K–AKT–mTOR pathway inhibitors. *Bull Cancer* 93:19–26
56. Whittaker S, Marais R, Zhu AX (2010) The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* 26:1829–1839
57. Hong F, Nguyen VA, Shen X, Kunos G, Gao B (2000) Rapid activation of protein kinase B/AKT has a key role in antiapoptotic signaling during liver regeneration. *Biochem Biophys Res Commun* 279:974–979
58. Taub R (2004) Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 5:836–847
59. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2:489–501
60. Chiang PH, Wang L, Bonham CA, Liang X, Fung JJ, Lu L et al (2004) Mechanistic insights into impaired dendritic cell function by rapamycin: inhibition of JAK2/STAT4 signaling pathway. *J Immunol* 172:1355–1363
61. Nepomuceno RR, Balatoni CE, Natkunam Y, Snow AL, Krams SM, Martinez OM (2003) Rapamycin inhibits the interleukin 10 signal transduction pathways and the growth of Epstein Barr virus B-cell lymphomas. *Cancer Res* 63:4472–4480
62. Kim JH, Kim JE, Liu HY, Cao W, Chen J (2007) Regulation of interleukin-6-induced hepatic insulin resistance by mammalian target of rapamycin through the STAT3–SOCS3 pathway. *J Biol Chem* 283:708–715
63. Vogt PK, Hart JR (2011) PI3K and STAT3: a new alliance. *Cancer Discov* 1:481–486
64. Ernst M, Najdovska M, Grail D, Lundgren-May T, Buchert M, Tye H et al (2008) STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. *J Clin Invest* 118:1727–1738
65. Hong F, Kim WH, Tian Z, Jaruga B, Ishac E, Shen X, Gao B (2002) Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-x(L) proteins. *Oncogene* 21:32–43
66. de Wolf CJF, Cupers RMJ, Bertina RM, Vos HL (2006) Interleukin-6 induction of protein S is regulated through signal transducer and activator transcription 3. *Arterioscler Thromb Vasc Biol* 26:2168–2174
67. Ruminy P, Gangneux C, Claeysens S, Scotte M, Daveau M, Salier JP (2001) Gene transcription in hepatocytes during the acute phase of a systemic inflammation: from transcription factors to target genes. *Inflamm Res* 50:383–390
68. Heinrich PC, Behmann I, Hermanns HM, Muller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374:1–20
69. Levy DE, Darnell JE Jr (2002) STATs: transcriptional control and biological impact. *Mol Cell Biol* 3:651–662
70. Lim CP, Cao X (2006) Structure, function, and regulation of STAT proteins. *Mol Biosyst* 2:536–550
71. Kaelin CB, Gong L, Xu AW, Yao F, Hockman K, Morton GJ, Schwartz MW, Barsh G, MacKenzie RG (2006) Signal transducer and activator of transcription (STAT) binding sites but not STAT3 are required for fasting-induced transcription of agouti-related protein messenger ribonucleic acid. *Mol Endocrinol* 20:2591–2602
72. Roglans N, Vila L, Farre M, Alegret M, Sanchez RM, Vazquez-Carrera M, Laguna JC (2007) Impairment of hepatic STAT3 activation and reduction of PPAR α activity in fructose-fed rats. *Hepatology* 45:778–788
73. Zhou YC, Waxman DJ (1999) Cross-talk between Janus kinase-signal transducer and activator of transcription (JAK–STAT) and the peroxisome proliferator activated receptor α (PPAR α) signaling pathways. Growth hormone inhibition of PPAR α transcriptional activity mediated by STAT5b. *J Biol Chem* 274:2672–2681
74. Hogan JC, Stephens JM (2001) The identification and characterization of a STAT1 binding site in the PPAR γ 2 promoter. *Biochem Biophys Res Commun* 287:484–492
75. Mráček T, Cannon B, Houštěk J (2004) IL-1 and LPS but not IL-6 inhibit differentiation and downregulate PPAR gamma in brown adipocytes. *Cytokine* 26:9–15
76. Tanaka T, Itoh H, Doi K, Fukunaga Y, Hosoda K, Shintani M et al (1999) Down regulation of peroxisome proliferator-activated receptor γ expression by inflammatory cytokines and its reversal by thiazolidinediones. *Diabetologia* 42:702–710
77. Waite KJ, Floyd ZE, Arbour-Reily P, Stephens JM (2001) Interferon- γ -induced regulation of peroxisome proliferator-activated receptor gamma and STATs in adipocytes. *J Biol Chem* 276:7062–7068
78. Haspel RL, Darnell JE Jr (1999) A nuclear protein tyrosine phosphatase is required for the inactivation of STAT1. *Proc Natl Acad Sci USA* 96:10188–10193
79. Luo G, Yu-Lee LY (1997) Transcriptional inhibition by STAT5: differential activities at growth-related versus differentiation-specific promoters. *J Biol Chem* 272:26841–26846
80. Russel D, Norman R, Dajee M, Liu X, Henninghausen L, Richards JS (1996) Prolactin-induced activation and binding of STAT proteins to the IL-6RE of the α_2 -macroglobulin (2M) promoter: relation to the expression of α_2 M in the rat ovary. *Biol Reprod* 55:1029–1038
81. Zvonic S, Hogan JC, Arbour-Reily P, Mynatt RL, Stephens JM (2004) Effects of cardiotrophin on adipocytes. *J Biol Chem* 279:47572–47579
82. Krasilnikov M, Ivanov VN, Dong J, Ronai Z (2003) ERK and PI3K negatively regulate STAT transcriptional activities in human melanoma cells: implications towards sensitization to apoptosis. *Oncogene* 22:4092–4101
83. Lee HK, Seo IA, Shin YK, Park JW, Suh DJ, Park HT (1997) Capsaicin inhibits the IL-6/STAT3 pathway by depleting intracellular gp130 pools through endoplasmic reticulum stress. *Biochem Biophys Res Commun* 382:445–450
84. Decker T, Kovarik P (1999) Transcription factor activity of STAT proteins: structural requirements and regulation by phosphorylation and interacting proteins. *Cell Mol Life Sci* 55:1535–1546