Research Article

Interleukin-6 Induces the Down Regulation of Human Peroxisome Proliferator Activated Receptor Alpha via the MAPK-induced STAT Pathway in Human Hepatocytes

Guat-Siew Chew[a,b,*], **Stephen Myers**[a], **Kheng Leong Ooi**[b], **Alexander Chong Shu-Chien**[b], **Tengku Sifzizul Tengku Muhammad**[c,*]

Abstract: IL-6 plays a crucial role in the development of acute phase response. One of the important regulators 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 the gene expression of acute phase proteins. However, studies into the molecular mechanisms and signaling pathways in mediating the effects of IL-6 on the expression of PPARα are extremely limited. In this study, we determined that MAPK pathways, p38 and ERK but not JNK were involved in the down regulation of PPARα by IL-6 via the activation of SHP2. ERK may exert its effect by activating the downstream transcription factor, C/EBPα and -β. By contrast, ATF2 was not activated by p38 suggesting that p38 may produce its effect via other transcription factors. In this study, we also identified that, p38 and ERK pathways were involved in the DNA binding of STAT1 and -3, and were responsible for mediating the inhibitory action of STAT1 and -3 on PPARα promoter activity. Accordingly, this study has unravelled novel pathways by which IL-6 inhibits PPARα gene transcription, involving the modulation of p38 and ERK-C/EBP by activating the binding of STAT1 and -3 to STAT binding site on PPARα promoter. These findings represent a new model of IL-6-induced suppression of PPARα expression by the induction of STAT1 and STAT3 phosphorylation and the subsequent down-regulation of PPARα mRNA expression.

1. Introduction

The acute phase response (APR) is the immediate inflammatory reactions mediated by cytokines in response tissue injury, infection and trauma [1], including remarkable changes in the concentrations of many plasma proteins, known as the acute phase proteins (APPs) [2]. One of the most important cytokines responsible in the activation of APR is IL-6. This pro-inflammatory cytokine regulates hepatocyte APPs genes via the activation of certain signal transduction pathways, which in turn, stimulate transcription factors, such as STATs, NFκB and C/EBP [3]-[8].

Recently, there is accumulating evidence that a member of ligand-activated nuclear hormone receptors, PPARα may play an important role in the development of APR. The expression of PPARα was found to be decreased in liver treated with cytokines [9]-[11]. Indeed, we recently, demonstrated that cytokines produced an inhibitory effect on PPARα gene expression [12]. Interestingly, Gervois [4] showed the suppression of APPs gene expression was mediated by PPARα following the IL-6-induced APR in mice.

In light of the recent evidence that PPARα gene expression is regulated during IL-6-induced APR, which in turn regulates the expression of several key APPs, it is crucial that a detailed understanding of the molecular mechanisms of the signaling pathways underlying the response is studied. Recently, we reported that IL-6 decreased the PPARα gene expression at the transcriptional level via the action of C/EBP members [12]. In this study, we further explore the mechanisms involved in the

[a]School of Biomedical Science, University of Ballarat, Victoria, Australia
[b]School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
[c] Faculty of Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia, University of Louisville School of Medicine, HSC A-1115, 500
*Corresponding authors: E-mail: h.chew@ballarat.edu.au (GSC); sifzizul@umt.edu.my (TSTM)

modulation of PPARα gene expression and binding activity by IL-6-triggered signal transduction pathways using human hepatocarcinoma HepG2 cell line as a model system.

2. Materials and methods

2.1. Materials

The recombinant human IL-6 was obtained from National Institute for Biological Standards and Control, UK and the human HepG2 cell line was purchased from American Type Culture Collection (ATCC). All the cell culture reagents were purchased from Gibco/BRL. Rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Pharmacological inhibitors, NSC87877 was purchased from Tocris Bioscience (St. Louis, MO, USA), SB203580, U0126 and SP600125 were obtained from Calbiochem (Pacific Center Court San Diego, CA, USA).

2.2. 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. Cells were maintained at 37˚C in a humidified atmosphere with 5% (v/v) CO_2. Prior to treatment with 1000 U/ml of IL-6 or various inhibitors, the cells were pre-incubated in reduced concentration of HI-FBS (0.5%) for 4 hr [13].

2.3. 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 instruction. Real-time RT-PCR was performed using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) [12]. The quantity of PPARα mRNA was normalized against the housekeeping gene, β-actin, which acted as the internal control to determine the relative PPARα mRNA expression.

2.4. SDS-PAGE and Western blot analysis

Total cellular protein was isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce) following manufacturer's instructions. The cytoplasmic extracts were prepared from the control and treated cells. Total cellular proteins were then size-fractionated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Milipore). The blotted membranes were incubated with blocking solution [1XPBS containing 5% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20] for 1 hr at room temperature with shaking. Blocking solution was removed by washing the membrane three times for 10 min each, in washing solution [1XPBS and 0.1% (v/v) Tween-20] and the membrane was incubated with individual primary antibodies against the component of signal transduction pathways which was diluted 1/1000 in 1XPBS containing 1% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20, for 1 hr at room temperature, with shaking. The membrane was then washed and immersed in secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) diluted 1/2000 in 1XPBS 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 described in the instructions supplied with the ECL kit (GE Healthcare) and Fuji Medical X-ray film.

2.5. Electrophoretic mobility shift assay (EMSA) and antibody supershift assay

Nuclear extracts used in EMSAs were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce). The sequences of the oligonucleotides containing STAT binding site (-612 to -604) [12] were as follows: 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 mixed together 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) non-denaturing polyacrylamide gels (29:1, acrylamide:bisacrylamide). DNA-protein complexes were transferred to nylon membrane (Hybond N+) and subsequently, detection of the biotin-labeled DNA on the membrane was carried out using Fuji Medical X-ray film. For antibody super-shift assays, 1 μg of STAT1 or STAT3 antibody (Cell Signaling Technology) was mixed with the binding reactions and the mixture was incubated on ice for 20 min followed by room temperature for another 20 min before the labeled oligonucleotides were added.

2.6. Transient transfection assay

The construction of the human PPARα promoter -765/+34 in pGL3-Basic Vector was described previously [12]. The HepG2 cells were transfected with the plasmids using Lipofectin transfection reagent (Invitrogen® Life Technologies) according to manufacturer's instruction with slight modification as previously described [12], [14]. Cells were re-plated 12 hr before transfection at a density of $9x10^5$ cells in 2 ml of fresh culture medium in a 6-well plastic dish. The cells were transfected with 2 μg of wild type PPARα promoter luciferase construct or the STATmut PPARα promoter luciferase construct and 0.5 mg tymidine kinase/Renilla (pRL-TK) vector as the control for transfection efficiency. Luciferase activity was measured by using the Dual-Glo luciferase assay system (Promega).

2.7. 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. Statistical significance was considered significant (p<0.05), very significant (p<0.01) or highly significant (p<0.001).

3. Results

3.1. IL-6-inhibitory effects on PPARα mRNA expression are mediated by AMPK pathways

Our previous study showed that IL-6 down-regulated the expression of PPARα at the levels of mRNA, protein content and binding activity [12]. In order to determine the role of MAPK pathways in mediating the inhibitory action of IL-6 on PPARα mRNA expression, various inhibitors were used. The cells were pre-treated individually either with NSC87877, an inhibitor against tyrosine phosphatase 2 (SHP2), an upstream component of MAPK pathway that is linked to the IL-6 signaling via gp130 [15], [16] as well as SB203580, U0126 or SP600125, the specific inhibitors of p38, MEK, and MKK4/7-JNK, respectively. As shown in Fig. 1D, NSC87877 attenuated the inhibitory effect of IL-6 on PPARα mRNA

expression in a dose-dependent manner with maximal increase of 20% as compared to IL-6 treatment alone achieved at 40 µM. Figure 1, panel A-C, showed that SB203580 and U0126 inhibited the IL-6-induced suppressive effect on PPARα mRNA levels with the highest inhibitory effect at 20 µM and 40 µM, respectively. By contrast, no significant attenuation effect was observed when SP600125 was used. These results suggested that IL-6 exerted its inhibitory effect on PPARα mRNA expression in HepG2 cells through the activation p38 and ERK, but, not JNK pathways.

3.2. IL-6 activates the components of p38-ATF2 and ERK-C/EBP signal transduction pathways in HepG2 cells

The activation of the key components of MAPK pathways by IL-6 in HepG2 cells was investigated using Western blot analysis. Fig. 2A showed that treatment of HepG2 cells over a period of 120 min produced a marked increase in the levels of phosphorylation of Tyr$^{180/182}$ p38 and Tyr^{71} ATF2 within 30-45 min but not total p38 and ATF2. A time-dependent increase in the levels of phosphorylated ERK p44/p42 and an increase in Ser^{21} C/EBPα, Thr$^{222/226}$ C/EBPα and Thr^{235} C/EBPβ (Figure 2B) were also observed. In all cases, the levels of
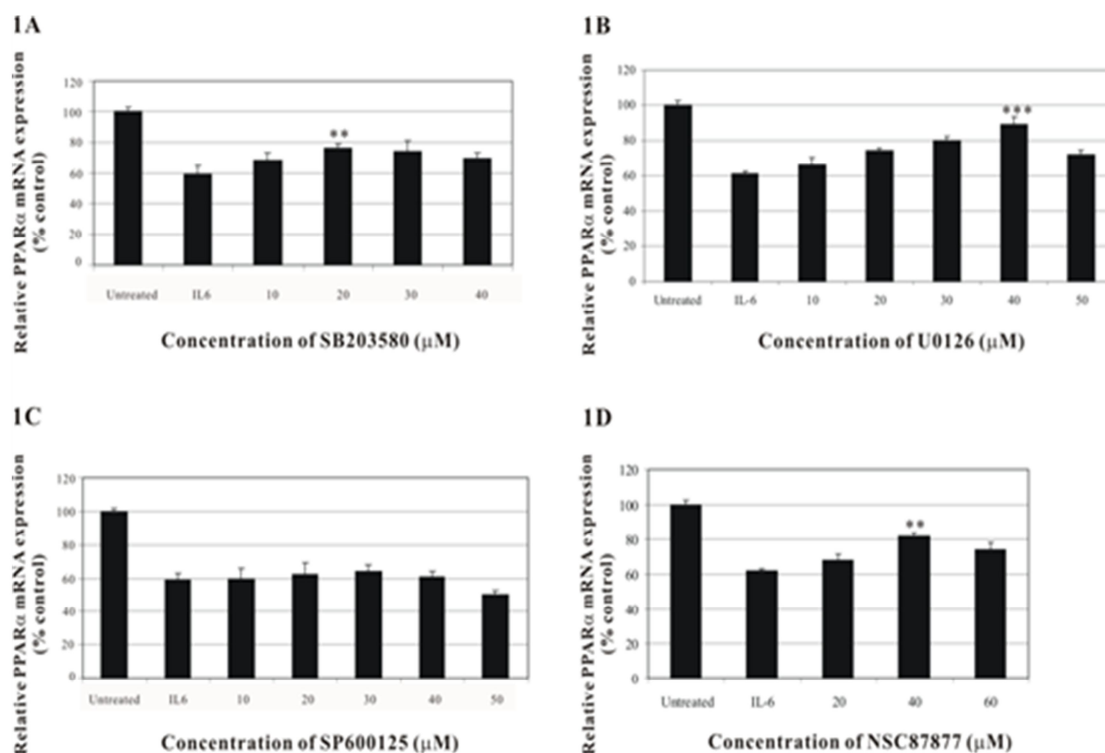

Figure 1. The effects of various inhibitors on IL-6-mediated suppression of PPARα mRNA. HepG2 cells were cultured in 0.5% (v/v) HI-FCS for 4 hr and then pre-treated with (A) SB203580 (B) U0126 (C) SP600125 and (D) NSC878777 for 2 hr prior to treatment with 1000 U/ml of IL-6 for 24 h. Total cellular RNA was then isolated and subjected to Quantitative Real-Time PCR. Assigning the signals for PPAR α/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.
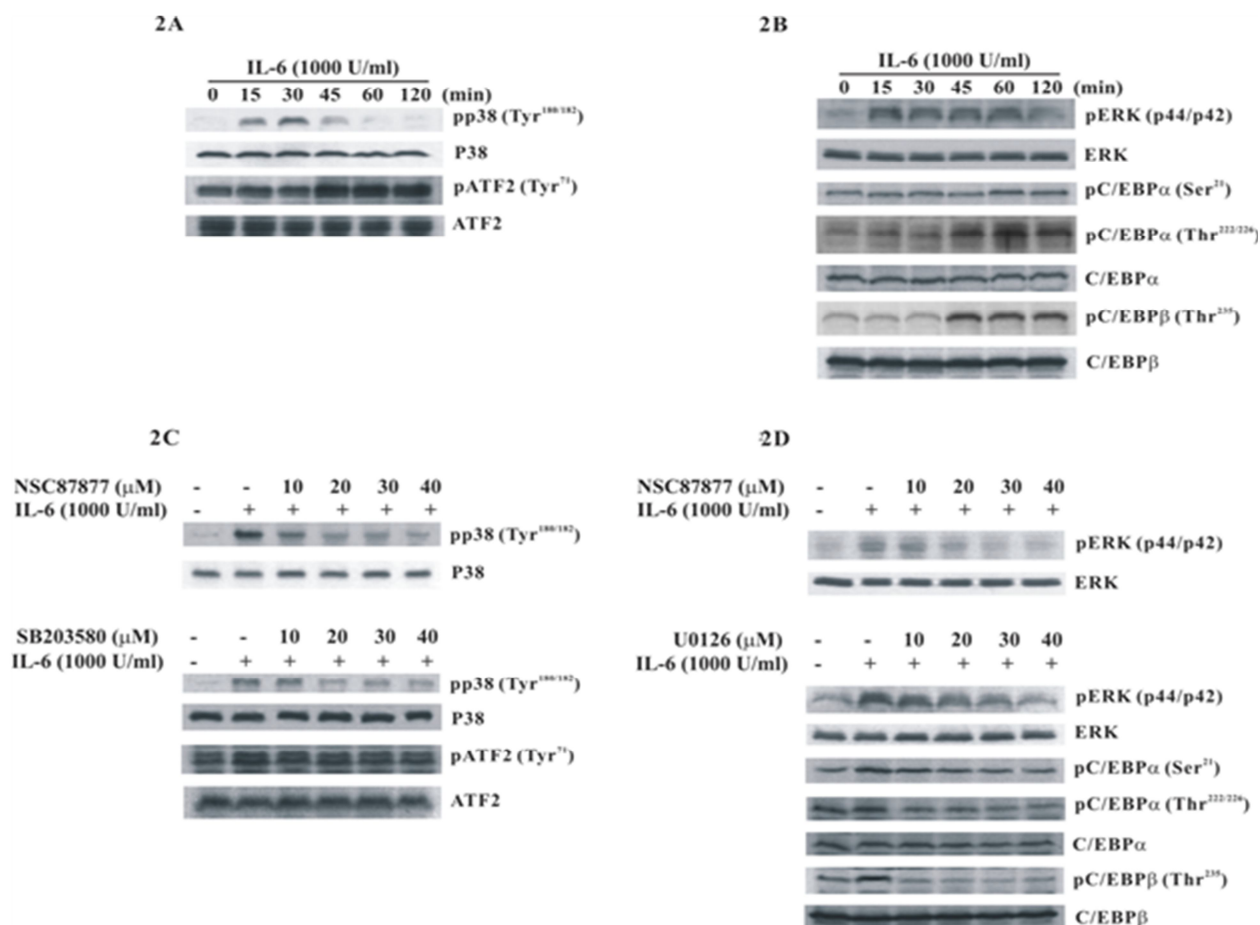
Figure 2. Time-dependent effect of IL-6 on the components of MAPK activity. Confluent HepG2 cells were treated with 1000 U/ml IL-6. The cytoplasmic extract were prepared and subjected to Western blotting using specific antibodies for phosphorylated form of (A) p38 and ATF2 (B) ERK and C/EBP. After stripping these membranes, each membrane was individually re-blotted with specific antibodies for p38, ATF2, ERK and C/EBP. Fig. 2C-D Cells were pre-treated with various concentrations of inhibitors for 2 h, followed by stimulation with 1000 U/ml of IL-6 for the prerequisite time. Cytoplasmic extracts were prepared and subjected to Western blotting using various antibodies as described.

phosphorylated proteins were increased after 15 min for ERK followed by C/EBPα and C/EBPβ after 45 min.

In order to corroborate that p38-ATF2 and ERK-C/EBP pathways were indeed activated in response to IL-6 treatment, the cells were pre-incubated with pharmacological inhibitors for 2 hr followed by IL-6 treatment for a requisite time. As shown in Figure 2C, the induction of phosphorylated p38 by IL-6 was inhibited by NSC87877. Similar attenuation affect was also produced by SB203580 on the phosphorylation level of p38 with the highest level produced at 20 µM. By contrast, similar concentration of SB203580 did not produce a significant inhibition on the phosphorylation of ATF2 (Figure 2C). In delineating the ERK-C/EBP pathway, it was demonstrated that NSC87877 abolished the phosphorylation level of p44/42 of ERK at 40 µM, corresponding to the attenuation effect of IL-6 suppression of PPARα mRNA expression. U0126 at concentration of 40 µM markedly inhibited the phosphorylation of C/EBPα and C/EBPβ (Figure

2D). Thus, these results clearly suggest that two components of MAPK pathway, p38, and, ERK via the activation of C/EBPα and C/EBPβ, may play an important role in mediating the inhibitory effect of IL-6 on PPARα gene expression.

3.3. p38 and ERK activates STAT-1 and-3

In order to investigate p38 and ERK pathways were involved in the activation of STAT; inhibitors were used to block the STAT phosphorylation. It was found that, an increase in the concentration of SB203580 and U0126 partially inhibited the tyrosine phosphorylation residues of STAT1 and STAT3 (Figure 3). However, the level of attenuation of the serine phosphorylation by both inhibitors was not significant suggesting that p38 and ERK activated STAT1 and -3 via the phosphorylation of tyrosine residue.

3.4. p38 and ERK pathways are involved in IL-6-induced suppression on PPARα promoter activity

We previously demonstrated that the human PPARα promoter construct carrying the region from -765 to +34 displayed an approximately 50% decrease in luciferase activity in HepG2 cells-treated with IL-6 [12]. Because IL-6 decreased the PPARα mRNA levels via p38 and ERK pathways, we then investigated the role of similar pathways in modulating the PPARα promoter activity. As shown in Fig. 4A, NSC87877 produced the highest inhibition, followed by U0126 and SB203580. This pattern of attenuation activity also indicates that the inhibitors used at the upstream cascade blocked the IL-6 suppression on the PPARα promoter activity more

Figure 3. The effects of p38 and ERK inhibitors on the phosphorylation of STAT1 and -3. Pre-treatments of cells with SB203580 and U0126 were followed by incubation with IL-6 for requisite time to determine the levels of phosphorylated STAT1 and -3.

effectively than the inhibitor used at the downstream. As expected, the SP600125 (JNK inhibitor) did not show a significant inhibition of transcription of the promoter activity (Figure 4A).

3.5. STAT1 and -3 are responsible in mediating the IL-6-induced suppression of PPARα expression through p38 and ERK pathways

The scanning of the PPARα promoter using MatInspector [17] revealed the presence of the potential binding site for STAT transcription factor located between nucleotides -612 and -604. We, next, examined the interaction of nuclear extracts, prepared from HepG2 cells treated with IL-6 over the period of 120 min, with the STAT binding site. EMSA showed three major DNA-protein complexes designated as C1-C3 were present in untreated cells, with IL-6-treated nuclear extracts produced a significant induction in the DNA-protein complexes C2 and C3 at 15 min and 30 min (Figure 4B). The specificity of complexes C2 and C3 was confirmed when these complexes were significantly competed out using 400X of unlabeled STAT oligonucleotides (Figure 4C). Complex C1 may represent the non-specific interaction as the complex was not competed out by unlabeled oligonucleotides. Antibody supershift experiment showed that complexes C2 and C3 contained STAT1 and -3 (data not shown).

Pre-treatment of cells with SB203580 and U0126 markedly inhibited STAT binding activity, thereby decreasing the formation of complexes C2 and C3 (Figure 4C). In contrast, SP600125 showed no effect towards the formation of the complexes C2 and C3. Thus, it is conceivable that these observations indicate that the formation of complexes C2 and C3 were regulated via the activation of p38 and ERK.
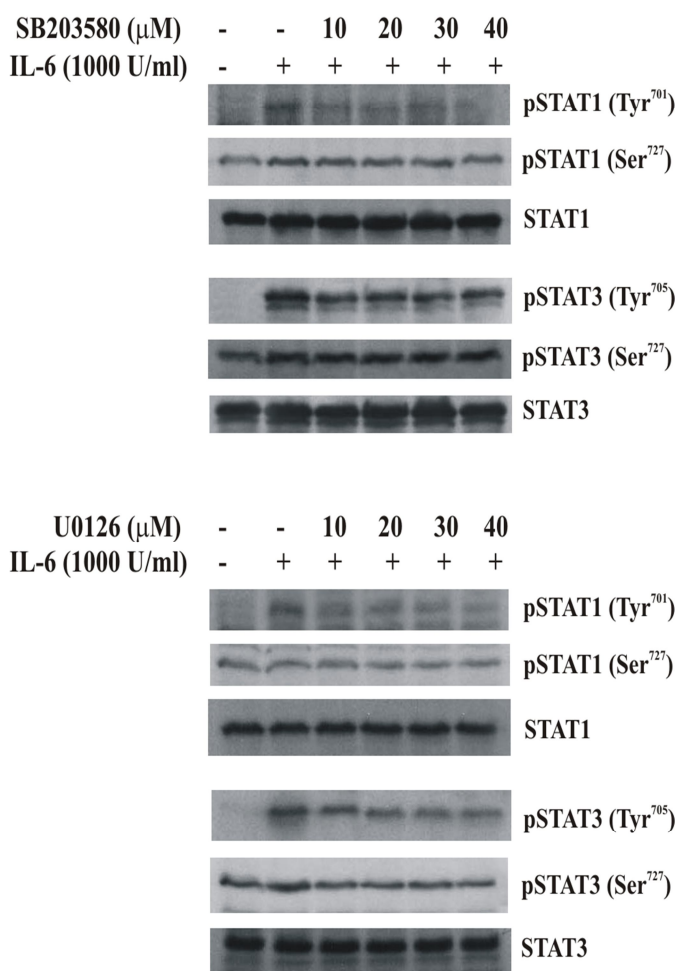
4. Discussion

PPARα is of crucial importance in many pathological conditions such as in the regulation of APPs [3, 4, 18-20]. Due to the critical role of PPARα in regulating APPs, the mechanisms involved in the modulation of PPARα suppression by IL-6 is clearly important. In this current study, we demonstrated for the first time a crucial role of SHP2 associated p38 and ERK but not JNK in mediating the inhibitory effect of IL-6 on PPARα expression and promoter activity in HepG2 cells.

An increasing body of evidence supports the role of SHP2 in inducing the phosphorylation of the components of MAPK [15]. It was demonstrated that phosphorylation of the different members of MAPK is regulated by SHP2-binding site Tyr[759] of gp130 at the membrane cell [21]. Interestingly, Olsnes [22] also found only two components of MAPK (ERK and p38) but not JNK are capable of producing the cellular effects by the mediator. For example, in sclerosant-treated monocytes, phosphorylated ERK and p38 alone are responsible in regulating the secretion of IL-6 and TNF-α.

Dong [23] elucidated that C/EBP signaling pathway was activated at the downstream level by IL-6 which contributed to increased expression of plasminogen activator inhibitor-1
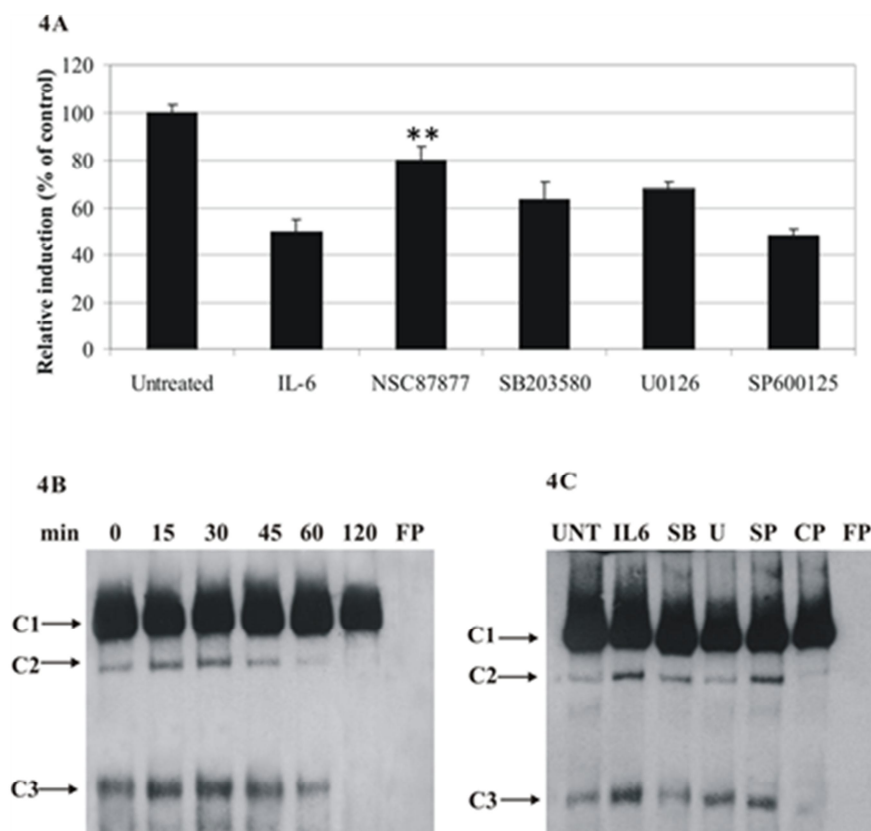
Figure 4. The effects of various inhibitors on the IL-6-mediated suppression of PPARα promoter activity in HepG2 cells. (A) HepG2 cells were transfected with luciferase-reporter construct containing the promoter of PPARα gene (-765/+34) in the absence and presence of IL-6. The transient transfected cells were then pre-treated with NSC87877, SB203580, U0126 and SP600125 for 2 hr prior to treatment with 1000 U/ml of IL-6 for 24 hr. Relative induction (%) of the promoter constructs activities in IL-6 treated cells inhibitors and without treatment of individual inhibitors, as compared to untreated cells (control) which has been assigned as 100%. (B) DNA-binding proteins of nuclear extracts to the STAT binding site of PPARα promoter in time-course upon IL-6 treatment. (C) HepG2 cells were either untreated or pre-treated with SB203580 (SB), U0126 (U) and SP600125 (SP), respectively for 2 hr before incubation in the absence or presence of 1000 U/ml of IL-6 (IL6) for 30min. Nuclear extracts were then isolated and 20 µg of nuclear extract were subjected to EMSA using biotin-labeled STAT (-612/-604) oligonucleotides. C represents patterns of DNA-protein complexes (C1, C2, and C3) detected, indicated by labeled arrows. UNT (untreated control) represents cells treated in the absence of IL-6, CP (cold probe) represents competition assay using 400-fold molar excess of unlabelled STAT oligonucleotide and FP (free probe) represents biotin-labelled STAT oligonucleotide alone.

(PAI-1) in HepG2 cells and primary mouse hepatocytes. Indeed, the finding that C/EBP proteins are located at the downstream of ERK upon stimulation of IL-6 was supported elsewhere. *U0126 was found to block the phosphorylation of C/EBP* by inhibiting the phosphorylation of ERK1/2. In addition, Ross [24] reported that the inhibition of granulopoiesis was due to the inhibition of C/EBP which was mediated by ERK1/2 pathway. In fact, we recently demonstrated the role of C/EBP in down-regulating the promoter and binding activity of PPARα in IL-6-treated HepG2 cell line [12].

Interestingly, although it has been reported that ATF2 was activated via p38 pathway upon stimulation with mediators such as insulin-like growth factor-1 [25], [26], this study did not support that finding. Therefore, it is tempting to speculate that phosphorylation of ATF2 by IL-6 in HepG2 cells may be mediated by other IL-6-activated pathways. In fact, ATF2 is known to be activated via pathways other than MAPK such as a novel Polo-like kinase 3 (Plk3) pathways in human corneal epithelial (HCE) cells [27].

A potential role of STAT as the downstream effector of p38 and ERK in binding and regulating the PPARα promoter activity in IL-6-treated liver cells was observed in this study. Previous studies also showed the role of IL-6-induced phosphorylation of STAT via the activation of p38 in modulating other promoter activity such as haptoglobin [28]. It was also reported that U0126 inhibited the phosphorylation of STAT3 in a series of end stage plasma B cell upon IL-6 treatment suggesting the role of ERK in activating STAT [29]. Therefore, STAT binding site may be predominant responsive element for IL-6 signaling and the binding of STAT protein to their DNA motif presence on the human PPAR□ promoter may have caused the suppression of PPARα upon IL-6 treatment.

From the previous study, the activation of STAT1 and -3 were discovered when HepG2 cells were treated with IL-6.

Accumulating evidence reported that an increase in activity and level of STAT was found to down-regulate the expression of PPARα during the acute phase response [30], [31] and instead of widely reported JAK pathway, other pathways may also played a critical role in mediating the cellular IL-6-effects via activating STAT. Indeed, EMSA demonstrated that the specific binding site of STAT (-612/-604) sequence in PPARα promoter was crucial for IL-6 suppression of PPARα synthesis and strongly suggested that MAPK pathways, p38 and ERK, were involved in the activation of STAT1 and -3.

Acknowledgments

Conflict of interest: None declared.

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