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Interleukin-6 upregulates paraoxonase 1 gene expression via an AKT/NF-κB-dependent pathway

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ABSTRACT

The aim of this study is to investigate the relationship between paraoxonase 1 (PON1) and atherosclerosis-related inflammation. In this study, human hepatoma HepG2 cell line was used as a hepatocyte model to examine the effects of the pro-inflammatory cytokines on PON1 expression. The results showed that IL-6, but not TNF-α and IL-1β, significantly increased both the function and protein level of PON1; data from real-time RT-PCR analysis revealed that the IL-6-induced PON1 expression occurred at the transcriptional level. Increase of IκB kinase activity and IκB phosphorylation, and reduction of IκB protein level were also observed in IL-6-treated HepG2 cells compared with untreated culture. This event was accompanied by increase of NF-κB-p50 and -p65 nuclear translocation. Moreover, treatment with IL-6 augmented the DNA binding activity of NF-κB. Furthermore, pharmacological inhibition of NF-κB activation by PDTC and BAY 11-7082, markedly suppressed the IL-6-mediated PON1 expression. In addition, IL-6 increased the levels of phosphorylated protein kinase B (PKB, AKT). An AKT inhibitor LY294002 effectively suppressed IKK/IκB/NF-κB signaling and PON1 gene expression induced by IL-6. Our findings demonstrate that IL-6 upregulates PON1 gene expression through an AKT/NF-κB signaling axis in human hepatocyte-derived HepG2 cell line.

1. Introduction

PON1 is an HDL-associated protein and is a major anti-atherosclerotic component of HDL [1]. PON1 is initially characterized as an organophosphorus hydrolase specific for hydrolyzing organophosphorus compounds, including insecticides and toxic nerve agents [2]. The PON1 protein is primarily synthesized in the liver and is secreted into the plasma [3], where associated with HDL. Recent studies demonstrated that PON1 is involved in protecting LDL and HDL from oxidative modifications [4]. The ability of HDL can inhibit the oxidation of LDL and promote macrophage cholesterol efflux through the action of its associated proteins include PON1 reduces the inflammation associated with atherosclerosis [5].

Atherosclerosis is considered to be an inflammatory disease with an epidemiological association between an increase in serum acute-phase reactants and the development of cardiovascular disease [6]. In the acute-phase reaction, inflammatory cells secrete many cytokines into the bloodstream. The most remarkable cytokines in the acute-phase reaction include IL-1, IL-6, and TNF-α. A previous study demonstrates that TNF-α and IL-1, which mediate the acute-phase response, also reduced the serum PON1 activity and the PON1 mRNA levels in Syrian hamster livers [7]. In addition, TNF-α and IL-1 treatment of HepG2 cells results in a decrease in PON1 mRNA levels, indicating that these cytokines are capable of directly affecting the regulation of PON1 expression in liver cells [8]. Besides, the oxidized phospholipids found in oxidized LDL alter the expression of PON1 through the inflammatory cytokine IL-6 [9]. Kumon et al. demonstrate that the difference in regulation of PON1 gene in HepG2 cells in response to various cytokines may contribute to the alteration of serum PON1 concentration according to the disease reflected by cytokine network [10]. However, there are still limited data to define the regulation of PON1 by

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certain inflammatory cytokines. In this study, the pro-inflammatory cytokine IL-6 could upregulate the function and gene expression of PON1 via an AKT–IKK–NF-κB axis in human hepatoma HepG2 cells.

2. Materials and methods

2.1. Materials

Pyrryolidine dithio-carbamate (PDTC) and anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Anti-PON1 antibody was purchased from Abcam (Cambridge, UK). Anti-phosphorylated-ERK, anti-phosphorylated-JNK, anti-phosphorylated-p38, anti-phosphorylated-AKT Ser473, anti-phosphorylated-AKT Thr308, anti-phosphorylated-IKKα/β antibodies, 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) (U0126) and LY294002 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IκBα, anti-IKKα/β, anti-NF-κB-p50, and anti-NF-κB-p65 antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). BAY 11-7082 was purchased from Calbiochem (CN Biosciences Notts, UK).

2.2. Cell culture

Human hepatoma HepG2 cells (obtained from American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate and antibiotics incubated at 37 °C in a humidified atmosphere of 5% CO_2 incubator. Cell growth curves were determined as described below. Cells were seeded at a density of 4×10^4 cells/well in a 12-well plate, incubating 24 h prior to drug treatment. IL-6 was added to the serum free medium with various concentrations for indicated time points.

2.3. Secreted protein preparation

The cultured medium was collected by centrifugation at 12,000 rpm, 4 °C for 15 min. The medium was further concentrated (50-fold) using an Amicon Ultra-4 centrifugal filter device (Millipore, Billerica, MA, USA) according to the manufacturer's protocol.

2.4. Measurement of PON1 enzymatic activity

The PON1 arylesterase activity was measured using phenylacetate as substrate. PON1 activity was measured at 37 °C for 40 min. Blanks were obtained with serum free medium. The activity was measured using spectrophotometer under OD 270 nm. The data were normalized to the protein content of the cells and expressed as arbitrary unit compared to untreated control.

2.5. Western blot analysis

Total cell lysate was isolated and protein concentration was determined using the Bradford method. Equal amounts of sample lysates were separated by SDS–PAGE, and transferred onto PVDF membrane, and then blocked with 5% nonfat milk, and incubated overnight at 4 °C with specific primary antibodies. Subsequently, the membrane was washed and incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG). Determination was performed using enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

2.6. Real-time PCR

Total RNA was extracted using RNA-Bee™ RNA isolation kit (TEL-TEST, Friendswood, TX, USA). The RNA was reverse-transcribed using oligo (dT) primer and SuperScriptIII (Invitrogen). For real time-PCR analysis, the total volume of the reaction was 15 μl containing 1 μl of cDNA, 0.3 μl of each primer with concentration 10 μM and 7.5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reaction was run on the ABI StepOnePlus™ instrument (Applied Biosystems, Foster City, CA, USA). Cycling conditions were 95 °C for 10 min., 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Triplicates were performed for each sample. The C_T was defined automatically by the instrument using automate of C_T determination. Primer sequences were as follows: PON1 forward primer 5′-TATTGTTGCTGTGGGACCTGA-3′; reverse primer 5′-CACGCTAAACCCAAATACATC-3′. β-Actin forward primer 5′-GCAAATGCTTCTAGGCGGACTAT-3′; reverse primer 5′-TGCGCAAGTTAGGTTTTGTCA-3′.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from HepG2 cells after treatment without or with 10 ng/ml of IL-6 for the indicated time periods. For the electrophoretic mobility shift assay, oligonucleotides containing NF-κB binding sites in the PON1 promoter were used (5′-biotin-AGCAGAGGGTATTCCTTATCCC-3′ and 5′-biotin-GGGATAAGGAATACCCTCTGCT-3′). The single-stranded sense and antisense strands were boiled and annealed to generate a double-stranded oligonucleotide. The protein-DNA binding reaction was performed at 30 °C for 20 min. in a final volume of 20 μl. The binding reaction contained 5 μg of nuclear extract, 5 pmol biotin labeled NF-κB specific oligonucleotide with the relevant PON1 gene sequence or a common NF-κB binding oligonucleotide, 20 μg poly(dI/dC), 2 μl buffer A and 4 μl buffer B. The DNA–protein complexes were separated on a 6% non-denaturing polyacrylamide gel, and then transferred onto nitrocellulose membrane followed by UV light cross-linking. The complexes were detected using horseradish peroxidase-conjugated streptavidin and SuperSignal Chemiluminescent substrate kit (Pierce, Rockford, USA), and the membranes were exposed to X-ray film.

2.8. Immunostaining of cellular NF-κB

Cells seeded in a 12-well plate were treated with IL-6 10 ng/ml for 2 h. After treatment, the cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocking with 1% BSA and treated with RNase. The fixed cells were then incubated with anti-human NF-κB-p50 and -p65 antibodies and exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG), followed by DNA staining with PI. The processed plates were analyzed using the ImageXpress Micro Widefield High Content Screening System (Molecular Devices Inc., USA). This System involves an automated inverted fluorescence-imaging microscope that identifies stained cells and reports the intensity and distribution of the fluorescence in individual cells. Images were acquired to identify cells according to their nuclear PI staining, fluorescence intensity and localization based on the staining from the FITC-conjugated goat anti-mouse IgG at a 1:200 dilution. Suitable filters and a 20× objective were used. A total of 800 cells were analyzed in each well. The morphology of the nucleus/cytoplasm was characterized by the intensity of the immunofluorescence staining with the anti-NF-κB-p50 and anti-NF-κB-p65 antibodies. Within each well, multiple cellular images were acquired by moving the position of the plate by the width of one image field (350 mm) in a square pattern of locations centered on the center of the well.

Images were acquired in each well until a preselected number of cells had been imaged and analyzed with MetaXpress System.

2.9. Statistical analysis

All of the data were presented as the means ± S.D. The statistical analysis used Student's *t* test for pairs, with the following significance levels: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All of the figures were represented data from at least three repeated experiments with similar patterns.

3. Results

3.1. IL-6 upregulates the function and gene expression of PON1

HepG2 cells were incubated in serum-free medium with various concentrations of TNF-α, IL-1β, and IL-6 for 48 h. After treatment, cell viability was measured by trypan blue dye exclusion method. The results revealed TNF-α, IL-1β, and IL-6 did not affect the proliferation and cell viability of HepG2 cells (data not shown). Next, we examined the effect of TNF-α, IL-1β, and IL-6 on the function and expression of PON1, HepG2 cells were treated with these cytokines for 48 h, the levels of secreted PON1 and its arylesterase activity in the culture medium were evaluated. As shown in Fig. 1A and B, exposure to IL-6 increased the arylesterase activity and expressed level of PON1 compared to untreated culture. In contrast, TNF-α and IL-1β could not modulate the function and protein level of PON1 in HepG2 cells. To further examine the regulation of PON1 gene expression by three tested cytokines, real-time PCR analysis was carried out. As illustrated in Fig. 1C, treatment with IL-6, but not TNF-α or IL-1β, significantly enhanced the PON1 mRNA expression in HepG2 cells. These results revealed that IL-6 induced the expression of PON1 at the transcriptional level. Since the expression levels of PON1 protein and mRNA had not significant difference ranged from 10 to 100 ng/ml of IL-6. In the following experiments, 10 ng/ml of IL-6 was used to investigate the molecular action of IL-6 on PON1 gene regulation.

3.2. IL-6 induces NF-κB activation

IL-6 is one of the most highly expressed mediators of inflammation and considered as a regulator of acute phase inflammatory responses. Several transcriptional factors, including signal transducer and activator of transcription and NF-κB involve in IL6-mediated signaling pathway. To gain insights into the transcriptional factors involved in IL-6-mediated PON1-upregulation, we applied pharmacological inhibitors of different signaling pathways to examine their effect on PON1 expression. The results demonstrated that the selective NF-κB inhibitors PDTC and BAY-11-7082 markedly reduced IL6-induced PON1 expression (Fig. 2A).

NF-κB is a transcription factor expressed in numerous cell types and plays a key role in the expression of many pro-inflammatory genes. It is also activated in response to pro-inflammatory cytokines [11]. Notably, there is a putative NF-κB binding element in the upstream region of the PON1 promoter, located at nucleotides −810 and −819. To analyze the effect of IL-6 on the NF-κB-activating signal cascade, the levels of IκBα, IKKα/β, and phosphorylated IKKα/β were examined by immunoblotting analysis. Fig. 2B shows that the amount of phosphorylated IKKα/β increased whereas the level of IκBα decreased in HepG2 cells in response to IL-6 treatment. Activated IKK can release NF-κB from IκB and allow the p50 and p65 subunits translocation from cytosol to nucleus [12]. To examine the subcellular distribution of NF-κB-p50 and -p65 subunits during IL-6 treatment, immunofluorescence staining was performed. As indicated in Fig. 2C and D, the nuclear NF-κB-p50 and -p65 increased upon IL-6 treatment compared to control.

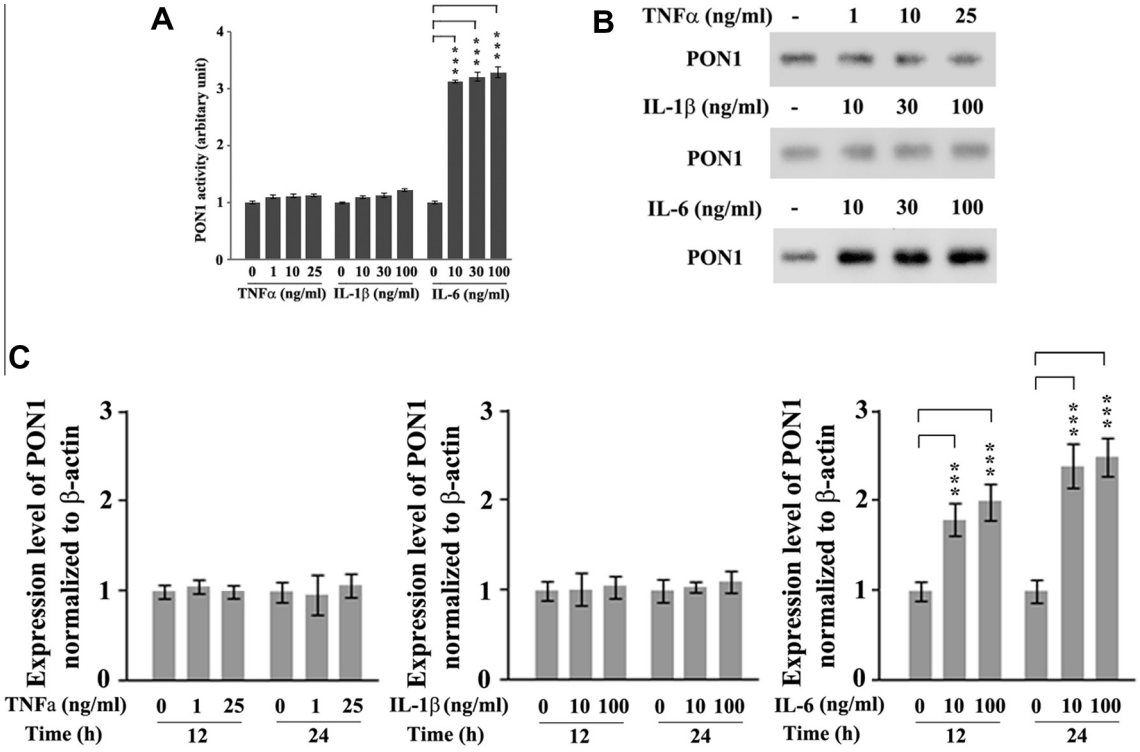

Fig. 1. The effects of TNF-α, IL-1β and IL-6 on the enzyme activity, protein and mRNA level of PON1. HepG2 cells were stimulated without or with various concentrations of TNF-α (1, 10 or 25 ng/ml) IL-1β (10, 30, or 100 ng/ml) and IL-6 (10, 30, or 100 ng/ml) for 48 h, (A) the arylesterase activity of PON1 was determined as described in Section 2; (B) the level of secreted PON1 was detected by Western blot. (C) IL-6 upregulates PON1 mRNA expression. HepG2 cells were treated with TNF-α (1 and 25 ng/ml) IL-β (10 and 100 ng/ml) and IL-6 (10 and 100 ng/ml) for 12 and 24 h after treatment the levels of mRNA was measured by real time RT-PCR.

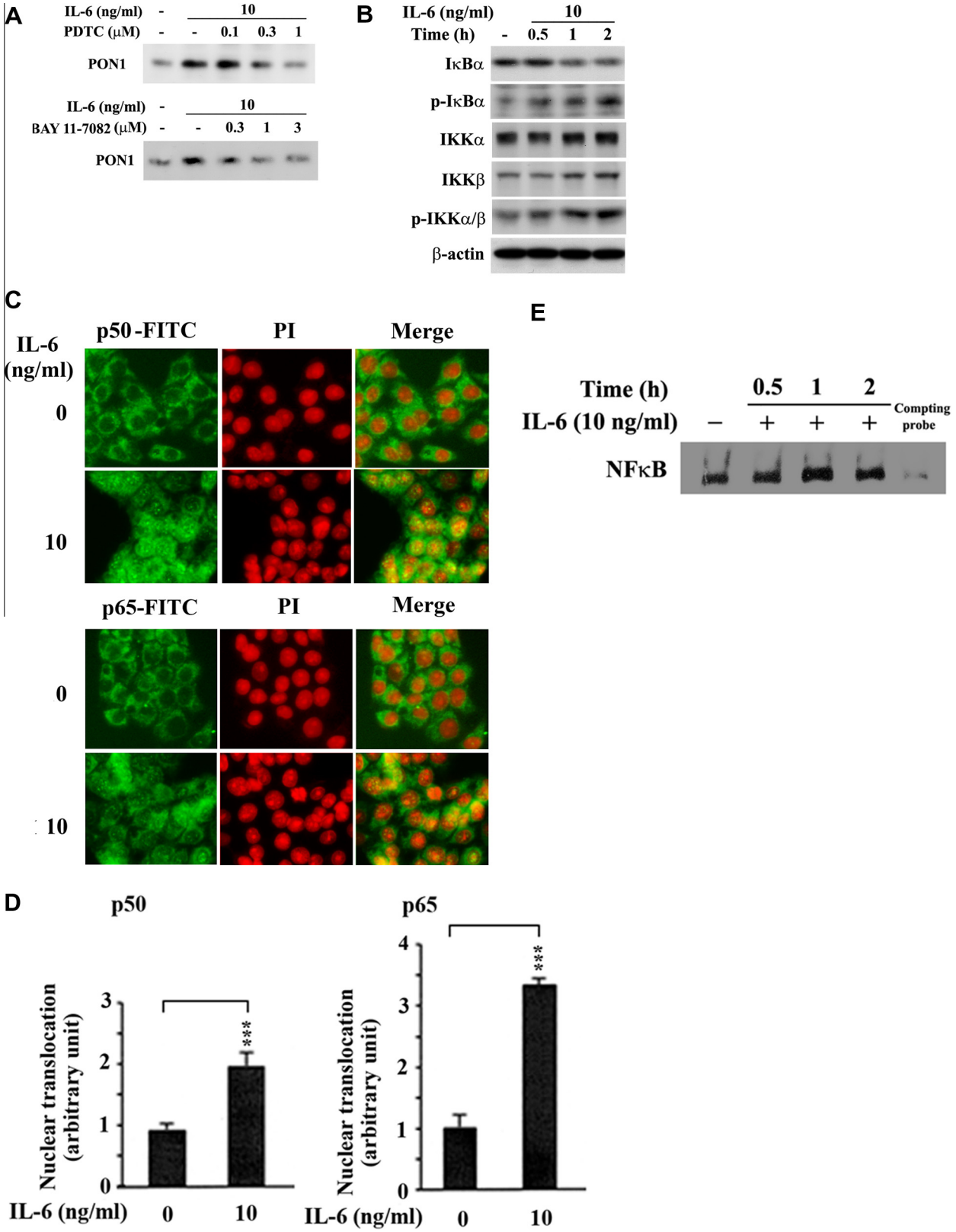

Fig. 2. NF-κB activation is required for IL-6-induced PON1 expression. (A) HepG2 cells were treated with 10 ng/ml of IL-6 in the presence or absence of PDTC or BAY 11-7082. After 48 h incubation, the levels of secreted PON1 were determined by Western blot. (B) IκBα and IKKα/β were activated in IL-6-treated HepG2 cells. Following treatment of cells with 10 ng/ml of IL-6 for 0.5, 1 and 2 h, the protein levels of IκBα, IKKα and IKKβ and the phosphorylation levels of IκBα and IKKα/β in the cell extracts were examined by Western blot analysis. (C) HepG2 cells were incubated with 10 ng/ml of IL-6 for 2 h. The cellular distribution of NF-κB was investigated by immunofluorescence assay; the images were acquired using the ImageXpress Micro Widefield High Content Screening System. (D) The images were calculated using the MetaXpress Software System. (E) IL-6 promotes DNA binding activity of NF-κB. HepG2 cells were treated without or with 10 ng/ml of IL-6 for indicated time points. After incubation, nuclear extracts were prepared, and the DNA binding activity of NF-κB was analyzed by EMSA.

Next, gel mobility shift assay was performed to determine whether IL-6 modulated the DNA-binding activity of NF-κB. The results revealed that IL-6 markedly increased the binding of NF-κB to the putative binding sequences located at upstream of the PON1 promoter (Fig. 2E). These results suggest that NF-κB transactivation was needed for IL-6-induced PON1 gene expression.

3.3. Involvement of AKT in IL-6-induced upregulation of the PON1 gene

It is well documented that NF-κB is one of the nuclear targets of several kinases, including MAPKs and AKT. To characterize the activated status of MAPKs and AKT upon IL-6 treatment, immunoblot analysis was carried out. As indicated in Fig. 3A, the levels of phosphorylated ERK and AKT, but not JNK and p38, were increased by IL-6 treatment in HepG2 cells. To investigate the role of ERK and AKT in IL-6-induced PON1 upregulation, the ERK inhibitor U0126 and AKT inhibitor LY294002 were added prior to IL-6 stimulation. Fig. 3B shows that the AKT inhibitor LY294002, but not ERK inhibitor U0126, dose-dependently decreased IL-6-induced PON1 expression. Moreover, the increased PON1 mRNA upon IL-6 treatment was significantly decreased by treated LY294002 (Fig. 3C). These results indicate that IL-6-elicited AKT activation plays an important role in IL-6-induced PON1 gene expression.

3.4. AKT activation acts as an upstream signal of NF-κB

Previous report demonstrated that IKK is one of the targets of AKT [13], to address whether the activation of IKK was regulated by AKT, HepG2 cells were incubated with IL-6 in the presence or absence of PI-3K/AKT inhibitor LY294002, the levels of IκBα and phosphorylated IKKα/β and AKT were determined by Western blot analysis. Fig. 4A shows, LY294002 effectively inhibited the phosphorylation of AKT in IL-6-treated HepG2 cells; as well as LY294002 significantly reduced IL-6-induced IKKα/β phosphorylation and IκBα degradation. To examine the effect of LY294002 on subcellular distribution of NF-κB-p50 and -p65 subunits during IL-6 treatment, immunofluorescence staining was performed. As indicated in Fig. 4B and C, the nuclear NF-κB-p50 and -p65 increased upon IL-6 treatment while LY294002 significantly decreased IL-6-induced NF-κB-p50 and -p65 nuclear localization. These results suggest that AKT might act as an upstream regulator of IKK/NF-κB signaling pathway to regulate the expression of PON1 gene expression in IL-6-treated HepG2 cells.

4. Discussion

IL-6 is a pleiotropic cytokine involved in inflammation with both pro- and anti-inflammatory effects [14]. Under pathological states, IL-6 and its regulating pathway may play a critical role in the pathogenesis of many diseases. A previous report shows that a substantial higher amount of IL-6 in both sera and tissues of patients with active atherosclerosis; its levels have been shown to correlate with the severity of disease [15]. However, the role of IL-6 in the development of atherosclerosis remains largely unclear. In this study, we found that IL-6 might upregulate the expression of an anti-atherosclerosis molecule PON1 gene at a transcriptional level through AKT/IKK/NF-κB activation pathway in human hepatocyte-derived HepG2 cells.

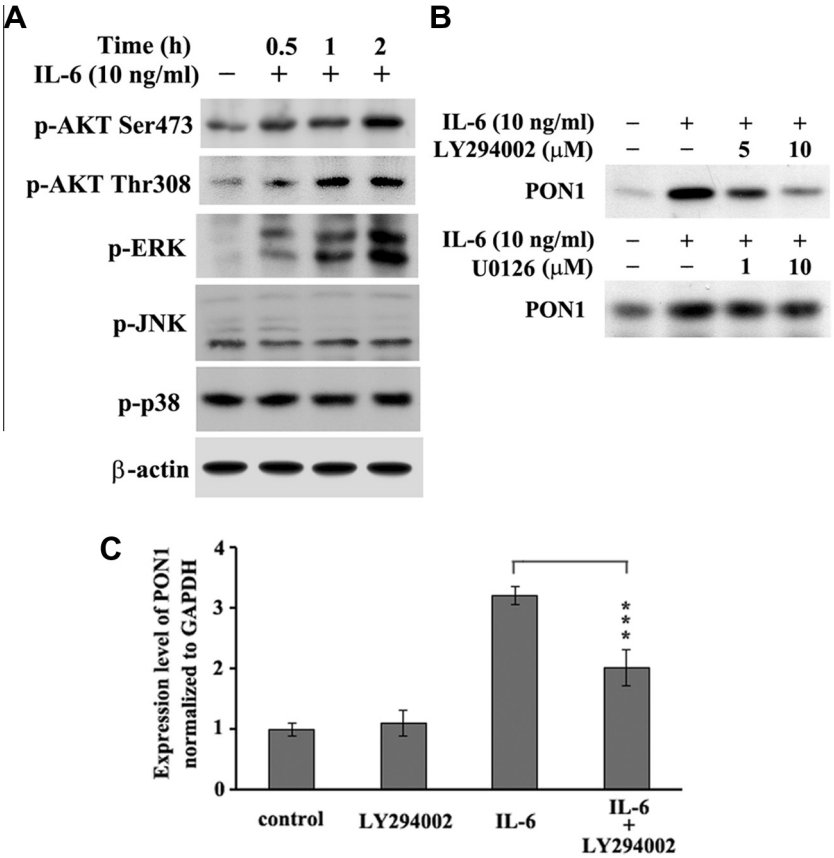

Fig. 3. AKT activation acts as an upstream regulator in IL-6-induced PON1 gene expression. (A) ERK and AKT are activated in IL-6-treated HepG2 cells. HepG2 cells were treated with 10 ng/ml of IL-6 for the indicated time points, the levels of phorsphorylated AKT, ERK, JNK and p38 in cell extract was detected by Western blot analysis. (B) Inhibition of AKT decreases IL-6-induced PON1 expression. HepG2 cells were pre-incubated with the AKT inhibitor (LY294002) and ERK inhibitor (U0126) for 1 h, and then treated with 10 ng/ml of IL-6 for another 48 h. After treatment, the expression of secreted PON1 was determined by Western blot. (C) Detection of mRNA. HepG2 cells were pre-incubated with the AKT inhibitor (10 μM LY294002) for 1 h, and then treated with 10 ng/ml of IL-6 for another 24 h the level of mRNA was measured by real time RT-PCR.

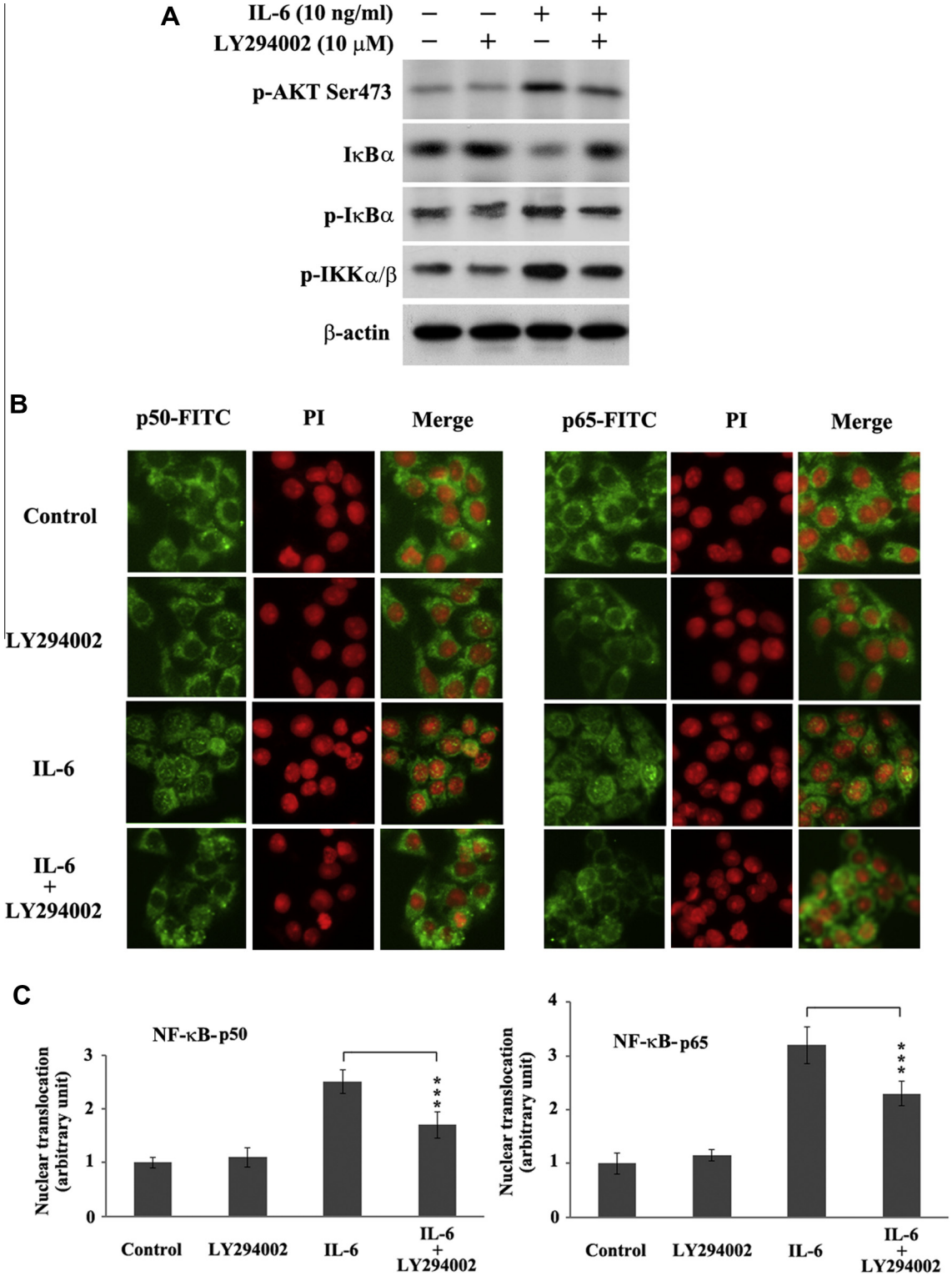

Fig. 4. AKT activation acts as an upstream regulator of NF-κB signaling. (A) Inhibition of AKT blocked IL-6-induced IKKα/β activation and IκBα degradation. HepG2 cells were pre-incubated with the AKT inhibitor (LY294002) for 1 h, and then treated with 10 ng/ml of IL-6 for another 2 h. After treatment, the levels of phorsphorylated AKT and IKKα/β as well as total IκBα in cell extract were measured by Western blot analysis. (B) The cellular distribution of NF-κB was investigated by immunofluorescence analysis; the images were acquired using the ImageXpress Micro Widefield High Content Screening System. (C) The images were calculated using the MetaXpress Software System.

Interestingly, IL-6 and PON1 levels have been associated with liver protective effect in CCl_4-induced liver injury. A previous study demonstrates that IL-6 stimulates liver regeneration and promotes hepatoprotection after CCl_4 treatment in IL-6+/+ mice [16]. IL-6 expression after CCl_4 administration is suggested to play a hepatoprotective role in IL-6-deficient mice with liver injury [17]. Intriguingly, treatment of mice with CCl_4 to induce liver damage, high levels of PON1 expression could be observed in the hepatocytes surrounding the fibrous septa and inflammatory areas and the CCl_4-treated rats exhibit an increased hepatic PON1 concentration. These results imply that PON1 might protect against liver inflammation [18], and suggested that PON1 is not simply

upregulated by IL-6 once injury is established but rather may alleviate injury and inflammation, perhaps leading to hepatic protection. It is therefore hypothesized that physiologic IL-6 may therefore play an important role in limiting injury and possibly eventual chronic disorders via its upregulated effects on PON1 gene expression. In this study, we found that IL-6, but not TNF-α or IL-1β, could induce PON1 gene expression in HepG2 cells. Our findings reinforce a previously published data in which PON1 gene expression is upregulated by IL-6 in HepG2 cells [10]. Further study is needed to elucidate the potential role of IL-6-mediated PON1 expression in liver injury and atherosclerosis in vivo.

To characterize the precise mechanism by which IL-6 regulates PON1 gene expression, the observed effect on mRNA suggests that IL-6 regulates PON1 to some degree at the transcriptional level. Numerous studies demonstrate that IL-6 induces the anti-inflammatory response through JAK/STAT3 signaling pathway. Nevertheless, Wang and colleagues demonstrate that IL-6 induces NF-κB activation in the intestinal epithelia. In inflammation states, IL-6 could induce the expression of ICAM via the NF-κB transcriptional pathway [14]. These results reveal that IL-6 regulates downstream target gene expression may via different transcription factors, including STAT3 and NF-κB. NF-κB is a nuclear transcription factor and is considered to be a central mediator of immune and inflammatory responses, more than 100 genes are regulated by this transcription factor [19]. From the TFSEARCH web site (http://www.cbrc.jp/research/db/TFSEARCH.html), a putative NF-κB binding site located at nt −810 to −819 upstream of the PON1 promoter was identified, which is required for PON1 expression [20]. In this study, we showed that IL-6-induced PON1 gene expression was accompanied by an increase in IKKα/β phosphorylation and a decrease in IκBα protein level. In addition, IL-6 could induce translocation of both p50 and p65 subunits of NF-κB from the cytosol to the nucleus and increased the binding of NF-κB to PON1-specific NF-κB-response sequence. Moreover, treatment with NF-κB inhibitors, PDTC and BAY 11-7082, effectively abolished the IL-6-induced PON1 gene expression, suggesting that NFκB activation contributed to IL-6-mediated PON1 upregulation in HepG2 cells.

Accumulating evidence shows that NF-κB signaling is one of targets of phosphatidylinositol 3-kinase (PI-3K)/AKT pathway. AKT is activated by a large spectrum of cytokines, hormones, and growth factors [21]. Activation of AKT signaling triggers the activation of NF-κB system [22]. AKT-induced potentiation of NF-κB-p65 subunit transactivation capacity requires the activation of IKK [23]. These evidences show that through the IKK, the PI-3K/AKT signaling pathway can activate the nuclear translocation of NF-κB complexes as well as potentiate their transactivation efficiency. Additionally, previous studies reveal that the relation with AKT and the PON family. Shiner et al. report that PON2 gene expression is upregulated in unesterified cholesterol-enriched macrophages through activation of the PI-3K/AKT signal pathway [24]. Our previous study shows that ethanol extract of *Graptopetalum paraguayense* upregulates PON1 gene expression via an AKT/NFκB pathway [20]. Here, we found that IL-6 induced AKT phosphorylation at Ser473 residue in HepG2 cells. The time course of AKT phosphorylation is compatible with that of NF-κB activation in IL-6-treated cells. Pretreatment of cells with a PI-3K specific inhibitor, LY294002, attenuated IL-6-induced phosphorylation of AKT and IKK, as well as NF-κB nuclear translocation, and thus reduced PON1 gene expression, suggesting that PI-3K/AKT/NF-κB axis is required for IL-6-mediated PON1 gene upregulation. Our observations demonstrate for the first time that IL-6 induces IKKα/β phosphorylation by activating AKT and then promoting IκB degradation, subsequently induces NF-κB release and nuclear translocation to bind with the NF-κB response element located at upstream of PON1 gene promoter, leads to PON1 gene expression. Based on these results, it will be interesting to study whether IL-6 indeed upregulates PON1 gene expression in vivo, and whether this event plays a protective role in both injured liver and atherosclerosis in vivo.

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