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ARTICLE

Remifentanil upregulates hepatic IL-18 binding protein (IL-18BP) expression through transcriptional control

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Abstract

Interleukin (IL)-18 plays an important role in liver ischemia/reperfusion (I/R) injury. We have previously demonstrated that remifentanil protects against liver I/R injury by upregulating the hepatic expression of IL-18-binding protein (IL-18BP), a natural IL-18 inhibitor. The current study was performed to further clarify the effects of remifentanil on IL-18BP expression in the liver as well as investigate the underlying mechanisms. In Sprague-Dawley (SD) rats, we demonstrated that remifentanil significantly increased the expression of IL-18BP in normal rat liver tissue over a 24-h time period with maximal expression at 24 h after treatment. The upregulation of remifentanil on IL-18BP expression displayed similar trends in in vitro cellular studies, including mouse primary hepatocytes, normal human hepatocyte LO2, and mouse hepatoma cells Hep1-6. In LO2 cells, preexposure of the cells to remifentanil significantly inhibited IL-18-activated p65 NF-κB phosphorylation, and the inhibition was absent when the cells were transfected with IL-18BP siRNA, indicating the functional effects of IL-18BP induced by remifentanil. Pretreatment with actinomycin D abolished remifentanil-induced upregulation of IL-18BP mRNA, suggesting that the induction occurred at the transcriptional level. This was further supported by the luciferase reporter assay, which demonstrated that remifentanil treatment significantly increased transcription of the IL-18BP promoter. Both western blot analysis and ChIP assays showed that STAT1 and C/EBP β were activated by remifentanil. Furthermore, remifentanil failed to upregulate IL-18BP expression after silencing STAT1 or C/EBP β gene expression. These findings demonstrate that remifentanil could upregulate hepatic IL-18BP expression through transcriptional activation of the IL-18BP promoter, and STAT1 and C/EBP β are two key transcriptional factors involved in this process.

Introduction

Interleukin (IL)-18, a proinflammatory cytokine belonging to IL-1 family, acts as an important regulator of innate and acquired immune responses by its potency to immediately initiate a cascade of reactions, resulting in the production of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-1β [1, 2]. In accordance with its proinflammatory nature, elevated IL-18 levels have been detected in immune-related diseases and sterile inflammation, such as lupus nephritis and septicopyemia [3], and IL-18 levels are linked to the disease severity [4]. Moreover, IL-18 has been reported in multiple studies to play a prominent role in the pathophysiology of liver ischemia–reperfusion (I/R) injury [5, 6]. Depending on the potential pathogenicity related to constitutive expression in liver Kupffer cells (KC) and activated macrophages, regulation of IL-18 bioactivity is a crucial target of liver I/R injury modulation [7].

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The activity of IL-18 is mainly regulated by its natural inhibitor, IL-18-binding protein (IL-18BP), which binds IL-18 with high affinity and effectively blocks the interaction between IL-18 and its receptor, inhibiting the IL-18-induced IFN-γ from Th1 cells and decreasing the IL-18-stimulated nuclear factor-κB (NF-κB) activity [8, 9]. Both inducing the expression of endogenous IL-18BP and exogenously injecting recombinant-IL-18BP protein can effectively inhibit IL-18 activity and provide tissue protection [10–12]. Notably, IL-18BP is upregulated in inflammatory liver disease by immunoactivation in both experimental models [13] and clinical patients [4, 14]. A recent study shows that binding of IL-18 with recombinant-IL-18BP protects the murine liver from I/R injury by intervening in critical inflammation-associated pathways and KC apoptosis [6]. Therefore, IL-18BP may provide a potential target for inhibiting IL-18 activity. Like many other proteins, IL-18BP expression is mainly controlled at the transcription level [15, 16], and signal transducer and activator of transcription 1 (STAT1) and CCAAT/enhancer binding protein β (C/EBP β) have been reported to be critical transcription factors in regulating IL-18BP gene promoter activity [15, 17].

We found in a previous study that remifentanil, a μ opioid receptor (OR) agonist used widely in clinical patients to provide analgesia and sedation, can upregulate the expression of hepatic IL-18BP, and modulate the IL-18/IL-18BP balance in animal models of liver I/R injury [5]. Furthermore, compared with saline, remifentanil significantly inhibited IL-18 signaling in I/R livers and attenuated I/R-induced liver injury, suggesting the contribution of remifentanil-induced IL-18BP bioactivity in this injury. To further clarify the effects of remifentanil on IL-18BP expression in livers as well as the underlying mechanisms, the current study was performed in both normal rats and a human hepatic cell line, LO2. We report here that remifentanil upregulates IL-18BP expression in normal hepatocytes by regulating IL-18BP gene transcription. Our results show that this progress involves the activation of transcription factors STAT1 and C/EBP β. These findings provide mechanistic insight into the protective effects of remifentanil in liver I/R injury [18, 19].

Methods

Animals

The animal experiments were approved by the Shanghai Jiaotong University School of Medicine Animal Care and Use Committee. All of the procedures were performed in accordance with the guidelines of the National Institutes of Health (NIH) for animal care. Efforts were undertaken to minimize suffering and the number of animals used. Male Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co. Ltd, Shanghai, China) weighing 250 ± 20 g were used in the experiments. Animals were fed rat chow with free access to water and housed in a temperature and humidity-controlled room with a 12-h light/dark cycle.

Experimental protocol

Rats were anesthetized with pentobarbital sodium (50 mg kg^{-1}, intraperitoneally). After tracheal intubation, mechanical ventilation was provided with a rodent ventilator (Kent Scientific Corporation, Torrington, CT) with 50% O_2 and 50% room air at 60–70 breaths min^{-1}. The left femoral vein was cannulated to infuse remifentanil or 0.9% saline. Rats received 10 μg kg^{-1} min^{-1} of remifentanil for 30 min and were then sacrificed at 1, 6, and 24 h after finishing remifentanil infusion, respectively. Rats in the control group received same volume of 0.9% saline infusion as those in the remifentanil-treated groups and were sacrificed at 24 h. Euthanasia was carried out with CO_2 asphyxiation at indicated time points and the liver lobes were collected for future analysis. Throughout the whole process, the rectal temperature was controlled at 35–36 °C with a heating pad.

Cell culture

Human normal hepatocytes LO2 cells and mouse hepatoma cells Hep1-6 (kindly provided by the Department of Liver Surgery, Ren Ji Hospital, Shanghai, China) were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin and incubated at 37 °C in 5% CO_2 in a humidified incubator. Primary mouse hepatocytes were isolated using the collagenase perfusion method as described previously [20]. Briefly, under pentobarbital anesthesia (50 mg kg^{-1} intraperitoneally), liver was perfused with 50 ml of calcium- and magnesium-free Hanks' balanced salt solution (Sigma Chemical, St. Louis, MO) supplemented with 0.5 mM EGTA, 5.5 mM glucose, and penicillin–streptomycin (Sigma), followed by 40 ml of Hanks' solution supplemented with 1.5 mM calcium chloride, 5.5 mM glucose, penicillin–streptomycin, and 0.02 g of Type IV collagenase (Sigma). After that liver was removed, and the digested product was centrifuged at 50 × *g* for 2 min. Then washed three times with Williams' medium E (Invitrogen, Carlsbad, CA) and cultured in Williams' medium E containing 10% FBS and penicillin–streptomycin. After 3 h of attachment, the unattached cells were removed and fresh medium was added. Cells were cultured for around 16 h before experiments.

The cells were co-cultured with 100 ng ml^{-1} of remifentanil or equal amount of phosphate-buffered saline (PBS) for the indicated time after 12 h of starvation in serum-free medium (SFM), or pre-treated with naloxone at 1000 ng ml^{-1} for 2 h, followed by incubation with remifentanil at 100 ng ml^{-1} for another 24 h. For ActD treatment, LO2 cells were pre-treated with 2 ng ml^{-1} ActD for 1 h, followed by another 24 h treatment with 100 ng ml^{-1} of remifentanil.

To transiently knockdown STAT1 or C/EBP β, LO2 cells were transfected with siSTAT1, siC/EBP β, or a negative control siRNA (siNC) using Lipofectamine 2000 (Invitrogen) in SFM according to the manufacturer's instructions. Forty-eight hours after transfection, cells were further treated with 100 ng ml^{-1} of remifentanil for 24 h. Pools of four siRNAs targeting STAT1 and two siRNAs targeting C/EBP β (GenePharma, Shanghai, China) were transfected, respectively. The sequences used were as follows:

5′-GCGUAAUCUUCAGGAUAAUTT-3′ (sense),
5′-AUUAUCCUGAAGAUUACGCTT-3′ (antisense) (siSTAT1 -human-647);
5′-GCUGGAUGAUCAAUAUAGUTT-3′ (sense),
5′-ACUAUAUUGAUCAUCCAGCTT-3' (antisense) (siSTAT1 -human-575);
5′-GCACCUGCAAUUGAAAGAATT-3′ (sense),
5′-UUCUUUCAAUUGCAGGUGCTT-3′ (antisense) (siSTAT1 -human-1601);
5′-GCAUCCUAGAACUCAUUAATT-3′ (sense),
5′-UUAAUGAGUUCUAGGAUGCTT-3′ (antisense) (siSTAT1 -human-2065);
5′-GCCCUGAGUAAUCGCUUAATT-3′ (sense),
5′-UUAAGCGAUUACUCAGGGCTT-3′ (antisense) (si C/EBP β -human-1888);
5′-GUUGAUGCAAUCGGUUUAATT-3′ (sense),
5′-UUAAACCGAUUGCAUCAACTT-3′ (antisense) (si C/EBP β -human-1810); and
5′-UUCUCCGAACGUGUCACGUdTdT-3′ (sense),
5′-ACGUGACACGUUCGGAGAAdTdT-3′ (antisense) (si NC).

To investigate the bioactivity of remifentanil-induced IL-18BP, LO2 cells were transfected with IL-18BP siRNA [5′-CUGGGUGAGUCCAUAUUCU-3′ (sense) and 5′-AGAAUAUGGACUCACCCAG-3′ (antisense)] or si NC (Genomeditech, Shanghai, China) using Lipofectamine™ RNAiMAX and transfection medium (Opti-MEM I Medium; Gibco) according to the manufacturer's instructions. Six hours later, the medium was replaced with RPMI 1640 containing 10% FBS. Twenty-four hours after transfection, the cells were treated with 100 ng ml^{-1} of remifentanil or equal amounts of PBS for 24 h, followed by incubation with 10 ng ml^{-1} recombinant human IL-18 protein (R&D, Minneapolis, MN) for another 24 h. The cells were then harvested to detect phosphorylation of p65 NF-κB.

Western blot

Liver tissue samples were homogenized with RIPA lysis buffer (Beyotime, Jiangsu, China). Cell samples were prepared with RIPA lysis buffer for protein extraction. Protein concentrations were quantified by BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg) were separated by 8–12% bis-tris polyacrylamide gel electrophoresis according to the molecular weight of the protein. After transferred onto polyvinylidene difluoride membranes, the blots were blocked with 5% dry milk or bovine serum albumin and incubated at 4 °C overnight with primary polyclonal antibodies: anti-IL-18BP (1:200, sc-134364, Santa Cruz), anti-p65 (1:1000, 4764; Cell Signaling Technology, Danvers, MA), anti-pp65 (Ser536, 1:1000, 3033; Cell Signaling Technology), anti-STAT1 (1:1000, 9172; Cell Signaling Technology), anti-pSTAT1 (Tyr701, 1:1000, 9171; Cell Signaling Technology), anti-C/EBP β (1:200, sc-150; Santa Cruz), anti-pC/EBP β (Thr 235, 1:1000, 3084; Cell Signaling Technology), anti-GAPDH (1:5000; Cell Signaling Technology), and anti-β tubulin (1:5000, AB0039; Abways Technology Inc., Shanghai, China). Then the stripes were washed three times with Tris-buffer saline containing 0.1% Tween-20 (TBST) and incubated with secondary antibodies: anti-rabbit (1:4000, 7074; Cell Signaling Technology), anti-mouse (1:5000, 7076; Cell Signaling Technology) or anti-goat (1:4000, sc-2768; Santa Cruz). After three times washing by TBST, the bands were scanned with Bio-RAD ChemiDoc™ XRS+ machine using chemiluminescence (Pierce), and quantified by densitometry using Image J software. Results were described as the ratio between phosphorylated and total proteins, or between target and housekeeping proteins expression.

Immunohistochemistry

To assess the expression of IL-18BP, paraffin-embedded liver sections were deparaffinized, blocked in 3% albumin from bovine serum in PBS for 30 min, and incubated with an antibody against IL-18BP (1:50, sc-9464; Santa Cruz) at 4 °C overnight followed by horseradish peroxidase-linked anti-goat IgG (Santa Cruz). Color was developed with 3,3′-diaminobenzidine (Dako).

Quantitative real-time RT-PCR analyses

Cell samples were washed twice with PBS, and then harvested with TRIzol (Invitrogen, Carlsbad, CA). Total RNA was extracted by the TRIzol method, quantified, and reverse-transcribed to cDNA with PrimeScriptTM RT reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. SYBR green-based QPCR was

carried out with the following primers: Human IL-18BP, 5′-CAAACTCCATTCCCACCTACC-3′ (sense) and 5′-GCA AGGCTAAGGCATCAACA-3′ (antisense); Human β-actin, 5′-TTGCCGACAGGATGCAGAA-3′ (sense) and 5′-GCC GATCCACACGGAGTACT-3′ (antisense). Rat IL-18BP, 5′-GCTCTGGGATGGATTGAAGAC-3′ (sense) and 5′-G GTCAAGGTCATGCTGTGGT-3′ (antisense); Rat β-actin, 5′- TGTCACCAACTGGGACGATA-3′ (sense) and 5′-GG GGTGTTGAAGGTCTCAAA-3′ (antisense). Mouse IL-18BP, 5′-ACAACTGCCACTGTCTTAACTGGA-3′ (sense) and 5′-AGGTGCTCAATGAAGGAACCAT-3′ (antisense); Mouse β-actin, 5′-GTGACGTTGACATCCGTAAAGA-3′ (sense) and 5′-GTAACAGTCCGCCTAGAAGCAC-3′ (antisense). The target gene expression of each sample was normalized to its corresponding β-actin gene value. Final results were expressed as *n*-fold change in gene expression relative to the control group, calculated with the ΔΔCt method as described previously [21].

Luciferase assays

A 667 basepairs fragment (−657 ~ +10) containing the promoter region as well as the upstream of the transcriptional start site of the human IL-18BPa gene was amplified using primers

-F(Kpn I) 5′-CGG GGTACC AATCTGGTTTTTCTA CAAGAAGTTTGAG-3′

-R(Bgl II) 5′-GA AGATCT CCCCTCTGGCCA GAGCCA-3′ as described previously by Hurgin and Bachmann [15, 17]. After sequencing the PCR product was cloned into the luciferase expression vector pGM-Lu to construct luciferase reporter plasmid IL18BP-Luc. LO2 cells were cotransfected with 1.6 μg of the IL18BP-Luc or control empty vector pGM-Lu reporter plasmid and 0.4 μg of the internal control plasmid pRL-TK (Rellina, Renilla reniformis luciferase plasmid) using Lipofectamine™ 2000. Twenty-four hours later, cells were treated with 100 ng ml^{-1} remifentanil for another 24 h and harvested. The activation of remifentanil on the promoter region was assayed with Dual Luciferase reporter assays (Promega, E1960) according to the manufacturer's instruction. Firefly luciferase intensity was normalized by the corresponding Renilla luciferase intensity.

ChIP and ChIP-QPCR assays

Chromatin immunoprecipitation (ChIP) was performed with a Pierce™ Agarose ChIP Kit (Thermo Scientific). LO2 cells treated with or without remifentanil for 24 h were fixed in 1% formaldehyde at room temperature for 10 min. Cells were collected, lysed, sonicated, and split into two portions; one was used for control input and the other was incubated with antibody. Anti-STAT1(1:50, 9172; Cell Signaling Technology) or anti-C/EBP β (1:100, ab15050; Abcam) antibodies were used for immunoprecipitation (IP), and a nonspecific IgG antibody (Abcam) was used as a negative control. DNA from input or immunoprecipitated samples was assayed by PCR after reversing cross-linking and DNA purification. The primers used were as follows: IL-18BP promoter primer sequence (STAT1), forward primer 5′-TGCTGTTTATCAGGCTGTATCC-3′ and reverse primer 5′-ATGCAAATTAACCATGTACTCTTCA-3′; IL-18BP promoter primer sequence (C/EBP β), forward primer 5′-TGAGCATGGAGTGGGGTGTG-3′ and reverse primer 5′-ACGAGGCAGCTTGCTATGGA-3′. Identical analyses on the human GAPDH promoters were included as a control: forward primer 5′-TACTAGCGGTTTTACGGGCG-3′ and reverse primer 5′-TCGAACAGGAGGAGCAGAGAGC GA-3′. DNA recovered by ChIP was also quantitated by real-time quantitative PCR (QPCR) with the LightCycler 480 PCR System (Roche, Basel, Switzerland) for 40 cycles, and normalized to values obtained after amplification of unprecipitated (input) DNA. Relative enrichment was calculated with respect to the control levels. Primer sequences were as follows: IL-18BP promoter primer sequence (STAT1), forward primer 5′-AGGCTTCGTGGAAGC-TATCG-3′ and reverse primer 5′-GCCTCTGTTCA-CACTGCTCT-3′; IL-18BP promoter primer sequence (C/EBP β), forward primer 5′-ACCAGGGAAAGGA-CATGAGC-3′ and reverse primer 5′-CACGTCCCAATC-TAAGCCCA-3′.

Statistical analysis

All data were presented as mean ± SD. Data analysis was performed with statistical software SPSS 19.0 (IBM, Armonk, NY). Comparison between two groups was analyzed with two-tailed Student's *t*-test, and comparison among multiple groups was analyzed with one-way ANOVA, followed by Student–Newman–Keuls test. Statistical differences were considered significant when *P*-value was less than 0.05.

Results

Remifentanil increased IL-18BP expression level in normal rat livers

We demonstrated in our previous study that various doses of remifentanil can upregulate the IL-18BP levels in I/R livers [5]. To test whether remifentanil affected IL-18BP expression in normal livers, male SD rats were treated with 10 μg kg^{-1} min^{-1} of remifentanil for 30 min and sacrificed 1, 6, and 24 h later. As shown in Fig. 1a, remifentanil significantly increased the IL-18BP protein expression in livers

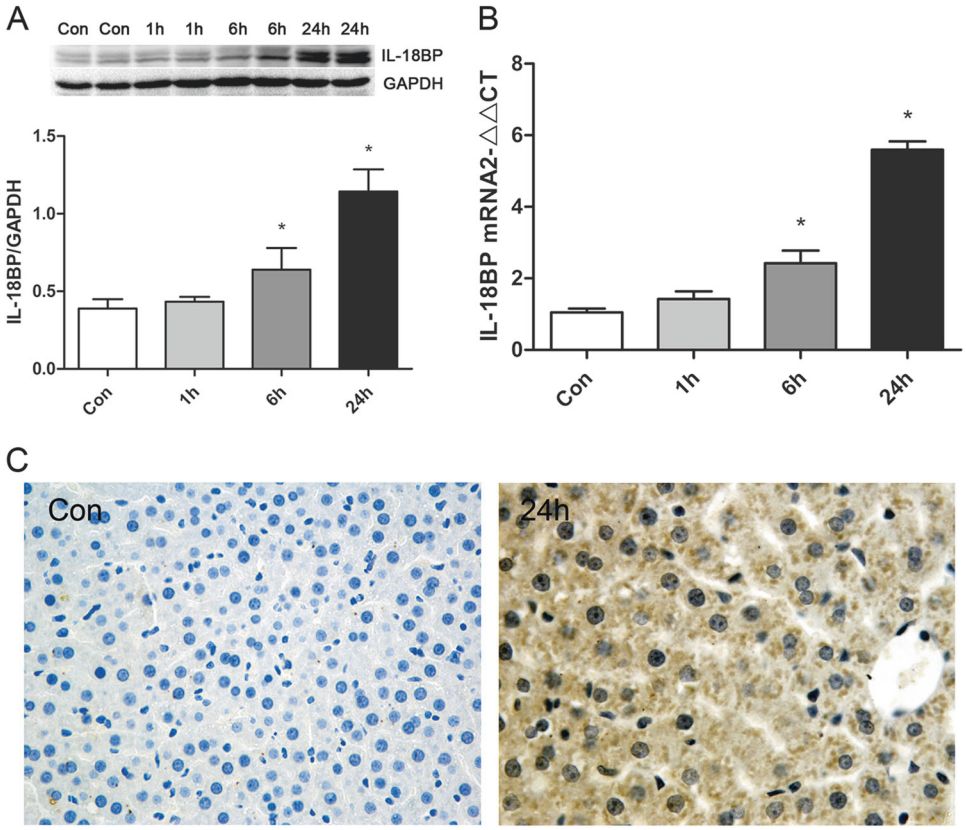

Fig. 1 Remifentanil increased the IL-18BP expression level in normal rat livers. Western blot (**a**) and QPCR (**b**) analyses showed that intravenous remifentanil treatment significantly increased the hepatic IL-18BP levels at 6 h and further induced a three-fold induction at 24 h in normal rats; $n = 5$ in each group, with a representative western blot shown. The data are expressed as the means ± SD. $^*P < 0.05$, at the indicated times, vs. the Control group (Con). **c** Immunohistochemical staining of liver samples from the control and 24-h treatment groups showed remifentanil-induced IL-18BP was mainly expressed by hepatocytes. Original magnification in C, ×40

by 6 h and further induced a three-fold induction by 24 h. The effects of remifentanil on hepatic IL-18BP mRNA levels displayed a similar trend (Fig. 1b). Immunohistochemistry analysis of liver samples from the 24-h treatment group showed that remifentanil-induced IL-18BP was mainly expressed by hepatocytes (Fig. 1c). These results indicate that remifentanil increases IL-18BP expression in normal liver tissues at both the message and protein levels and suggest that the upregulation mainly occurs in hepatocytes.

Remifentanil upregulated IL-18BP mRNA expression in hepatocytes

Mouse primary hepatocytes and two cell lines, normal human hepatocyte LO2 and mouse hepatoma cells Hep1-6, were then utilized to investigate the effects of remifentanil on IL-18BP expression in vitro. A modest but significant induction of IL-18BP mRNA was detected at 6 h after 100 ng ml^{-1} of remifentanil treatment (the selected dose was based on a previous report by Djafarzadeh et al. [22]), reaching a peak at 24 h and decreasing again by 36 h in all three kinds of cells (Fig. 2a–c). When LO2 cells were treated with an OR inhibitor naloxone before remifentanil, the induction of IL-18BP was not affected (Fig. 2d), suggesting this progress was OR-independent.

To investigate the functional effects of the upregulation of IL-18BP by remifentanil in hepatocytes, LO2 cells were exposed to recombinant human IL-18 after remifentanil treatment and the activation of p65 NF-κB was evaluated as a downstream target of IL-18. As shown in Fig. 2e, a 24 h exposure to IL-18 resulted in a significant increase in the level of phosphorylated p65 (pp65) in LO2 cells, whereas in cells pre-treated with remifentanil, this increase was absent. In addition, silencing the IL-18BP genes in LO2 cells significantly abolished the effects of remifentanil, indicating that the inhibition of IL-18-induced p65 phosphorylation by remifentanil was mediated by IL-18BP.

Remifentanil transcriptionally regulated the IL-18BP gene in hepatocytes by enhancing IL-18BP promoter activity

To elucidate the mechanism of remifentanil regulation of IL-18BP expression, LO2 cells were pre-treated with a transcription inhibitor actinomycin D (ActD) before exposure to remifentanil. ActD alone caused only a slight decrease in the IL-18BP mRNA level, but it significantly decreased remifentanil-induced upregulation of IL-18BP, indicating that the induction was at the transcriptional level (Fig. 3a). To determine whether remifentanil directly

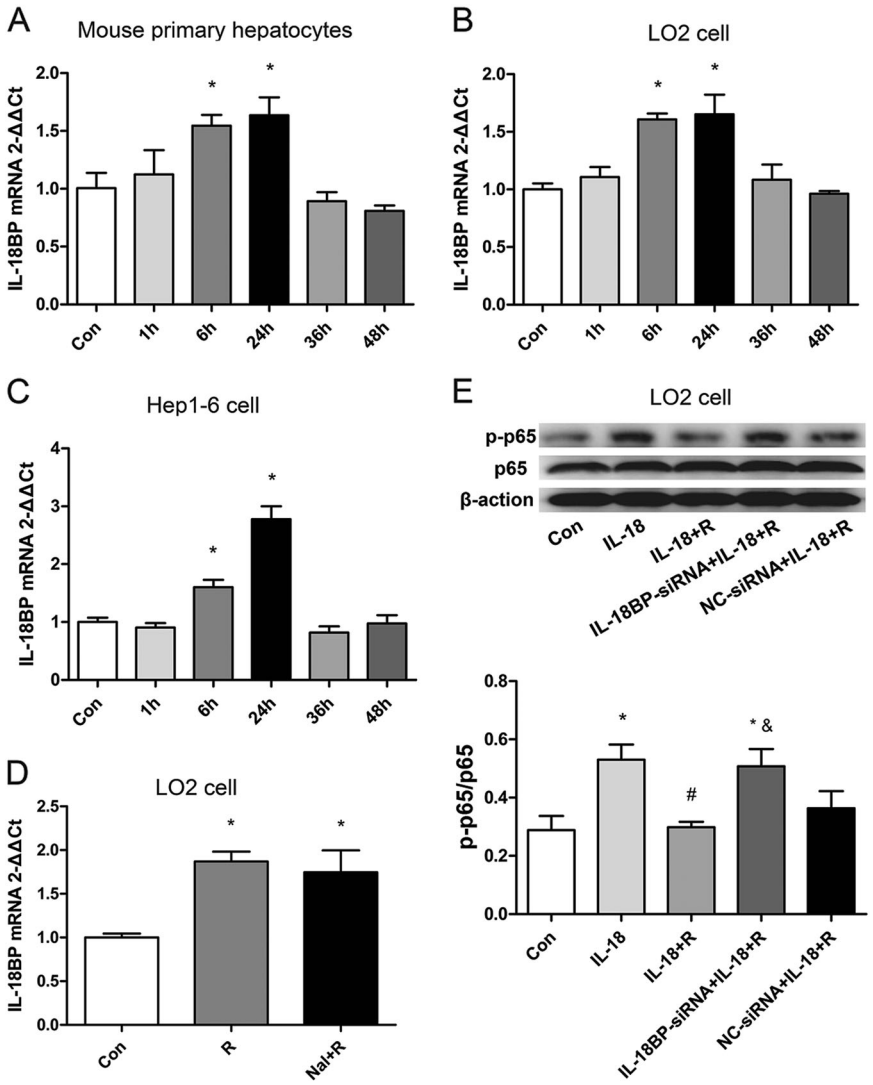

Fig. 2 Remifentanil upregulated IL-18BP mRNA expression in hepatocytes. QPCR analyses showed significantly increased expression of IL-18BP mRNA levels 6 and 24 h after treatment with 100 ng ml^{-1} of remifentanil (R) in **a** mouse primary hepatocytes, **b** LO2 cells, and **c** Hep1-6 cells. **d** Naloxone (Nal) did not affect the effects of remifentanil on IL-18BP mRNA expression in LO2 cells. **e** Western blot analyses showed that preexposure of LO2 cells to remifentanil for 24 h significantly inhibited IL-18-activated p65 phosphorylation. The inhibition was absent when the IL-18BP genes in LO2 cells were silenced with IL-18BP siRNA. $n = 3$ in each group in **a**, **c**, **d**, **e**. $n = 5$ in each group in **b**. The data are expressed as the means ± SD. $^{*}P < 0.05$ vs. the Control group (Con). $^{\#}P < 0.05$ vs. the IL-18-treated group. $^{\&}P < 0.05$ vs. the IL-18+R group

controlled the transcription of the IL-18BP gene promoter, a dual luciferase reporter assay was utilized. Since IL-18BPa is the most abundant isoform of human IL-18BP [8, 23], it was concentrated on and referred to as IL-18BP in the following study. The luciferase activity in LO2 cells transfected with a luciferase reporter construct containing the proximal promoter of the IL-18BP gene was approximately three-fold higher than that obtained with the empty pGM-Lu (Fig. 3b), indicating the presence of basal promoter activity. Treatment with 100 ng ml^{-1} remifentanil further increased the luciferase activity ($P < 0.05$ compared with IL18BP-Luc vector alone), although it did not induce luciferase expression with the empty pGM-Lu vector. These data suggest that remifentanil increases IL-18BP expression in hepatocytes by transcriptionally activating the proximal promoter of the IL-18BP gene.

Remifentanil induced activation of the STAT1 and C/EBP β transcription factors

It is reported that the IL-18BP promoter contains γ-activated sequence (GAS) and C/EBP β sites, both of which are essential for basal and IFN-γ-induced promoter activity [15, 17]. We next analyzed whether STAT1 and C/EBP β transcription factors played a role in the IL-18BP gene activation in response to remifentanil. As shown in Figs. 4a and 5a, STAT1 and C/EBP β expression levels remained consistent, whereas the activation of both transcription factors was significantly increased after remifentanil treatment. The increase peaked between 30 min and 1 h and decreased again by 2 h. To identify the interactions between these transcription factors and DNA in the IL-18BP promoter, Chromatin immunoprecipitation (ChIP) and ChIP QPCR assays were used. After amplification of anti-STAT1

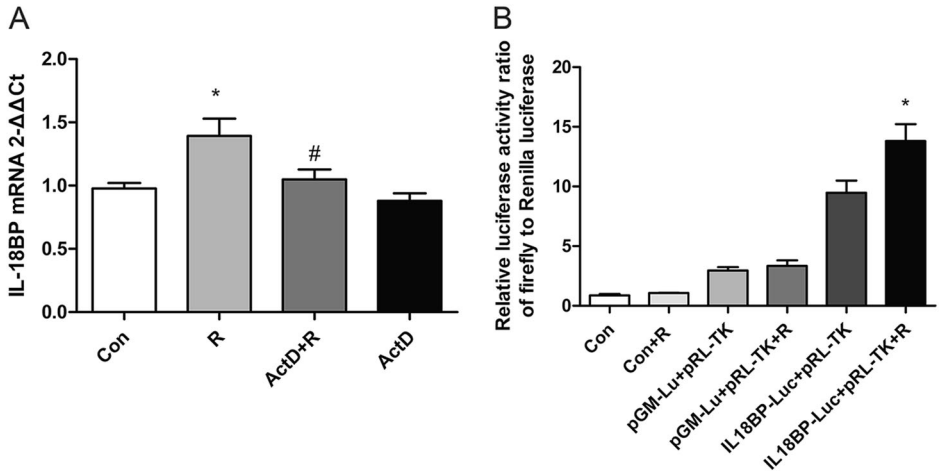

Fig. 3 Remifentanil transcriptionally regulated the IL-18BP gene. **a** Pretreatment with ActD (2 μg ml^{-1}) for 1 h before remifentanil (R) administration significantly abolished remifentanil-induced upregulation of IL-18BP in LO2 cells. $n = 5$ in each group. The data are expressed as the means ± SD. $^{*}P < 0.05$ vs. the Control group (Con). $^{\#}P < 0.05$ vs. the remifentanil-treated group. **b** LO2 cells were cotransfected with IL-18BP promoter-driven luciferase reporter plasmid IL18BP-Luc or control empty vector pGM-Lu and the internal control plasmid pRL-TK. Twenty-four hours after transfection, cells were treated with 100 ng ml^{-1} of remifentanil for another 24 h. Remifentanil treatment significantly increased the IL18BP-Luc luciferase activity. $n = 3$ in each group. The data are expressed as the means ± SD. $^{*}P < 0.05$ vs. the IL-18BP-Luc + pRL-TK group

or anti- C/EBP β antibody-immunoprecipitated DNA samples, both assays revealed that treatment with 100 ng ml^{-1} remifentanil for 30 min significantly stimulated binding of STAT1 and C/EBP β to the promoter area (Figs. 4b, c and 5b, c).

Suppression of STAT1 or C/EBP β inhibited remifentanil-induced IL-18BP mRNA expression

To further investigate the role of STAT1 or C/EBP β in the remifentanil-mediated induction of IL-18BP, we transfected LO2 cells with STAT1 or C/EBP β-targeting siRNAs before exposing the cells to remifentanil. Effective inhibition of both STAT1 and C/EBP β resulted in significant decreases of remifentanil-induced IL-18BP expression (Fig. 6). Notably, although both siRNAs downregulated the target protein expression by more than 80% (quantitative data not shown), the inhibition on IL-18BP expression was modest, indicating that additional mechanisms contribute to the effects of remifentanil. The data in Figs. 4–6 suggest that both STAT1 and C/EBP β are involved in remifentanil-induced transcription of the IL-18BP promoter.

Discussion

In the present study, we reported that intravenous remifentanil significantly increased IL-18BP expression both in normal rat livers and human hepatocytes. Remifentanil contributed to IL-18BP upregulation by directly activating the IL-18BP promoter and increasing transcription of the IL-18BP gene. Furthermore, transcription factors STAT1 and C/EBP β were activated by remifentanil. Effective knockdown of either transcription factor by siRNA significantly reduced remifentanil-induced IL-18BP mRNA expression, suggesting that both STAT1 and C/EBP β played critical roles in remifentanil-induced IL-18BP expression.

The potential tissue protective effects of remifentanil during the perioperative time have generated significant interest in recent years. While the protection was first discovered in myocardium and nerve cells [24, 25], increasing evidence indicates its ability to ameliorate hepatic injuries [5, 19, 26]. For example, remifentanil-treated hepatocytes have significantly improved cell viability compared with control cells after hypoxia/reoxygenation (HR) [19]. Moreover, remifentanil pretreatment in vivo significantly decreases the serum aminotransferase levels and profoundly attenuates the liver histologic damages in rats subjected to acute hepatic I/R injury [5, 18, 19]. However, the underlying mechanism remains elusive.

IL-18 is a proinflammatory cytokine that plays a prominent role in the pathophysiology of liver I/R injury. Increased IL-18 levels are found in both I/R livers from animal models and serum from patients who develop hepatic failure after hepatectomy [6, 27]. Neutralizing IL-18 with anti-IL-18 antibodies profoundly alleviate liver I/R injury [6]. We previously demonstrated that remifentanil is a potent inducer of IL-18BP, a natural inhibitor of IL-18, in rat I/R livers [5]. Here we show that remifentanil also induced IL-18BP expression in normal hepatocytes both in vivo and in vitro and explore a direct transcriptional

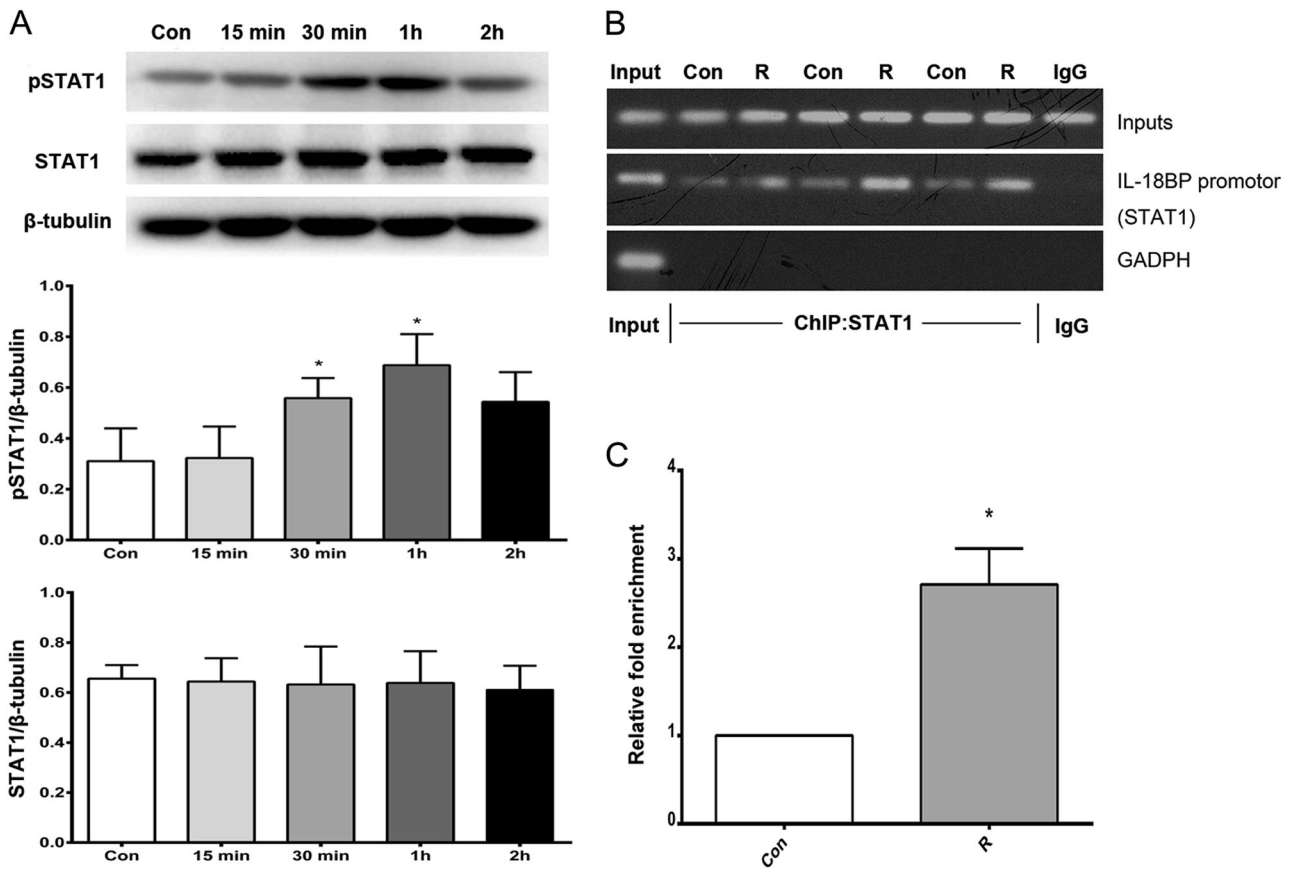

Fig. 4 Remifentanil induced activation of transcription factor STAT1. **a** LO2 cells were treated with 100 ng ml^{-1} of remifentanil for the indicated times. Immunoblot analysis showed that remifentanil significantly increased phospho-STAT1 (pSTAT1) levels by 30 min and 1 h, which were decreased again by 2 h. Representative western blots from three independent experiments are shown. **b**, **c** ChIP and ChIP-QPCR assays indicated enhanced STAT1 interaction with its response element in LO2 cells treated with 100 ng ml^{-1} remifentanil for 30 min. DNA isolated from cells before (input) and after immunoprecipitation with anti-STAT1 antibody was amplified with specific primer sets. An image of three independent samples from control (Con) and remifentanil (R)-treated groups after PCR amplification is shown in **b**. IgG was used as a negative control. $n = 3$ in each group in **a**–**c**. The data are expressed as the means ± SD. $^*P < 0.05$ vs. the Control group (Con)

mechanism for the upregulation. Our data suggest a link between an OR agonist, remifentanil, and IL-18 signaling inhibitor IL-18BP, providing a possible explanation for the protective effects of remifentanil in liver I/R injuries.

IL-18 is now known to be involved in the pathogenesis of a wide range of liver diseases in addition to liver I/R injury. IL-18 knockout mice have a significantly increased survival rate than wild-type mice after the administration of a lethal dose of acetaminophen (APAP) [28]. The serum IL-18 levels are elevated in chronic HBV and HCV patients and reflect the severity and activity of the viral infection [29]. Therefore, current data indicate that remifentanil might also have hepatoprotective effects in patients with these acute and chronic liver diseases because it effectively increases the IL-18BP expression in hepatocytes, providing a negative regulatory mechanism for attenuating IL-18 activity. Moreover, since previous studies show that IL-18 plays an important role in negative regulation of liver regeneration [30, 31], and that suppression of IL-18 with an antisense oligodeoxynucleotide significantly promotes proliferation of hepatocytes [20], our findings also indicate the possible protective effects of remifentanil on liver regeneration after hepatectomy, which merits further investigation.

Previous studies from our and other labs have shown that remifentanil may exert hepatic protective effects through inhibition of I/R-induced upregulation of oxidative stress [18, 19], elevation of inflammatory cytokines [18, 26], and infiltration of immune cells [5], etc. Actually, activation of IL-18 signaling has extensive crosstalk with the above-mentioned pathologic progresses. For example, oxidative stress has been shown as an important event in NLRP3 inflammasomes activation and IL-18 processing [21], whereas IL-18 at the same time could prime the oxidative burst of neutrophils [32]. IL-18 signaling also stimulates the production and expression of proinflammatory cytokines,

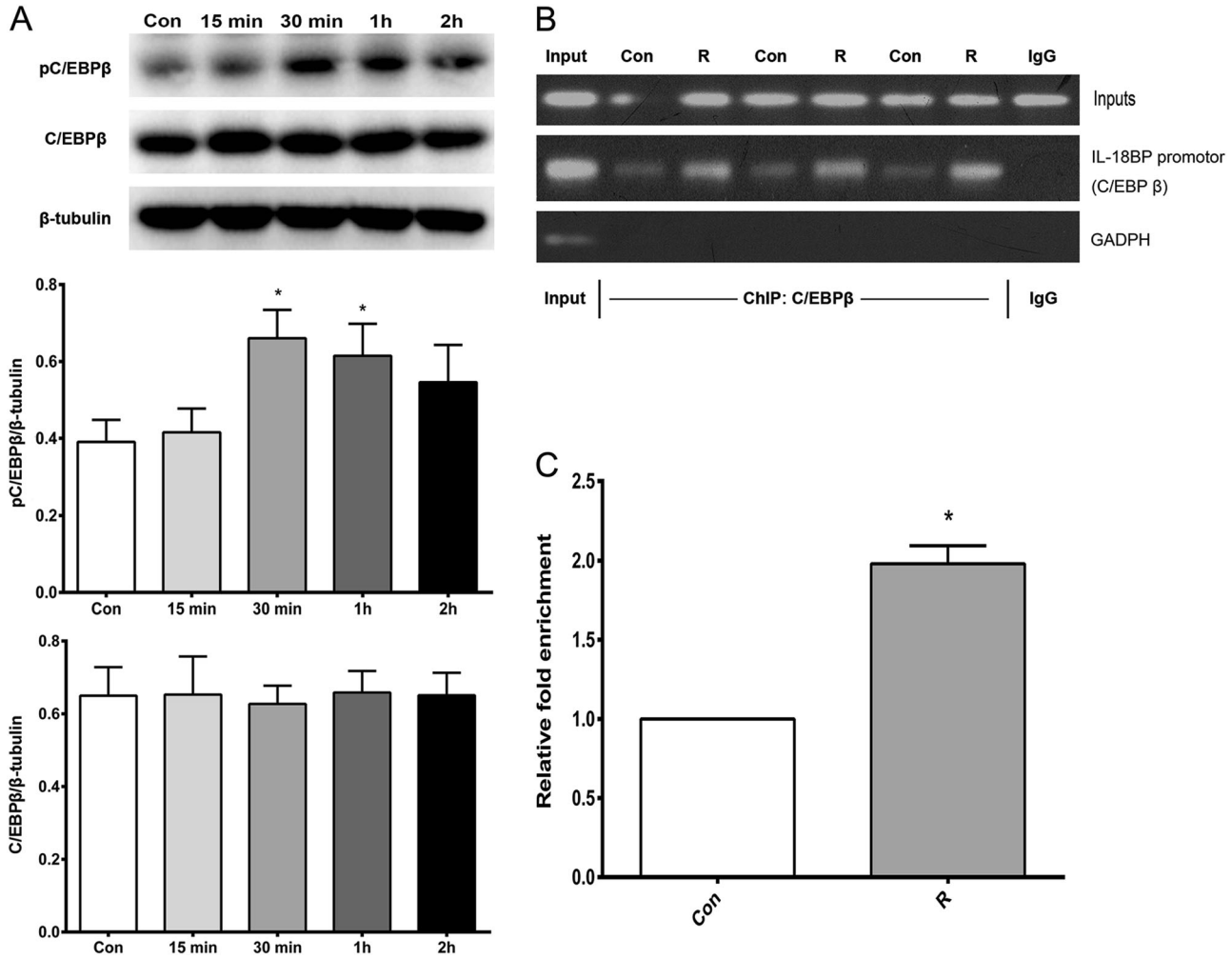

Fig. 5 Remifentanil induced activation of transcription factor C/EBP β. **a** LO2 cells were treated with 100 ng ml^{-1} of remifentanil for the indicated times. Immunoblot analysis showed that remifentanil significantly increased phospho-C/EBP β (pC/EBP β) levels by 30 min and 1 h. Representative western blots from three independent experiments are shown. **b**, **c** ChIP and ChIP-QPCR assays indicated enhanced C/EBP β interaction with its response element in LO2 cells treated with 100 ng ml^{-1} remifentanil for 30 min. DNA isolated from cells before (input) and after immunoprecipitation with anti-C/EBP β antibody was amplified with specific primer sets. An image of three independent samples from control (Con) and remifentanil (R)-treated groups after PCR amplification is shown in **b**. IgG was used as a negative control. $n = 3$ in each group in **a**–**c**. The data are expressed as the means ± SD. $^*P < 0.05$ vs. the Control group (Con)

such as TNF-α and IL-1β, and contributes to neutrophil recruitment in tissues [1]. Results from the current study indicate the possibility that upregulation of hepatic IL-18BP and inhibition of IL-18 signaling may contribute to remifentanil-mediated alleviation of I/R-induced complex pathologic responses. Future studies are needed to explore this issue.

Although IL-18BP seems to play a rather limited role as an inhibitor of IL-18, IL-18BP transcriptional regulation is complex and involves multiple regulatory sequences. We have focused on the upstream 657 bp region of the IL-18BP gene because previous studies demonstrated that this region is essential for both basal promoter activity and induction of the gene by other agents, such as IFN-γ and β-AR agonist isoproterenol (ISO) [12, 15, 17]. This proximal IL-18BP gene promoter area has several GAS sites and C/EBP β response elements (C/EBP E); therefore, the effects of remifentanil on transcription factors STAT1 and C/EBP β were investigated in the current study. Here we report that both STAT1 and C/EBP β were activated by remifentanil and had increased protein/DNA interactions with the GAS and C/EBP E sites, respectively, as detected by ChIP analysis. Interestingly, Hurgin et al. [15] previously demonstrated that IFN-γ-activated C/EBP β binds to both C/EBP E and GAS sites in Hep G2 cells, whereas STAT1 is not associated with the proximal GAS in their model. It is unknown whether remifentanil-activated C/EBP β competes with STAT1 for binding to the particular GAS element at the proximal IL-18BP promoter in the current model. However, with the use of siRNA technology, our data

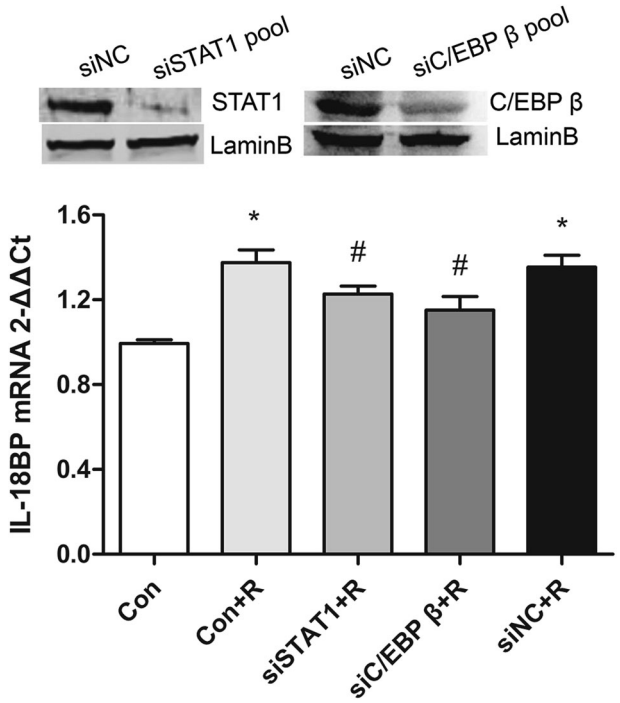

Fig. 6 Suppression of STAT1 or C/EBP β inhibited remifentanil-induced IL-18BP mRNA expression. LO2 cells were transfected with STAT1 or C/EBP β-targeting siRNAs before exposure to 100 ng ml^{-1} of remifentanil (R) for 24 h. Effective inhibition of either STAT1 or C/EBP β resulted in significant decreases of remifentanil-induced IL-18BP mRNA expression. si NC: negative control; si STAT1 pool: pools of four siRNAs targeting STAT1; and si C/EBP β pool: pools of two siRNAs targeting C/EBP β; $n = 4$ in each group. The data are expressed as the means ± SD. $^{*}P < 0.05$ vs. the Control group (Con). $^{\#}P < 0.05$ vs. the non-transfected remifentanil treated group (Con + R)

indicate that STAT1 activation plays a direct role on at least some of the GAS sites of the IL-18BP promoter and is essential to the biological activity of remifentanil.

In the current study, remifentanil increased the phosphorylation and DNA-binding activities of STAT1 and C/EBP β without affecting the expression levels in LO2 cells. The mechanisms through which remifentanil activates these two transcription factors remain unknown, but previous studies may provide a hint. Multiple members of the opioid family have been reported to be able to activate the extracellular signal-regulated kinase1/2 (ERK1/2) signaling pathway [33, 34], which is integral to the activation of transcription factors, such as STAT1 [35, 36] and C/EBP β [37, 38]. Since we revealed in current study that remifentanil induces IL-18BP expression via an OR-independent effect, it is possible that it activates receptors beyond the classical ORs, such as Toll-like receptors [39], to stimulate the ERK1/2 signaling and induce the activation of transcription factors. Those potential ERK1/2-related actions of remifentanil are currently under investigation in our lab.

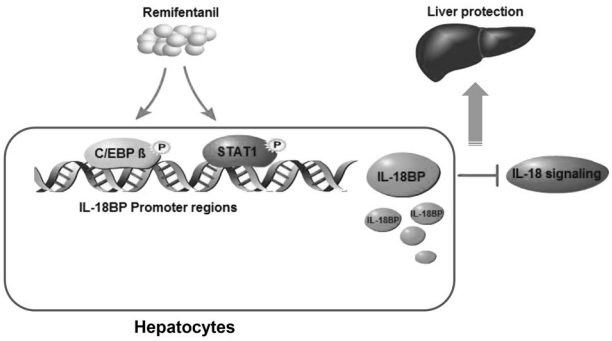

Fig. 7 Schematic diagram of the effects of remifentanil on hepatic IL-18BP expression and IL-18 dysfunction-mediated liver injury. Remifentanil significantly increases phosphorylation of transcription factors STAT1 and C/EBP β at the IL-18BP gene promoter region, therefore, transcriptionally activates the IL-18BP gene and upregulates the expression of IL-18BP protein in hepatocytes. Remifentanil-induced IL-18BP significantly inhibits IL-18 signaling in livers and alleviates IL-18 dysfunction-mediated liver injury

The present study has several limitations. Firstly, the molecular basis underlying the upregulation of IL-18BP by remifentanil was only studied in LO2 cell line. Whether the results can be applied to other hepatocyte cell lines is not known. Secondly, since current study is an experimental research with animals and cells, further clinical trials are warranted to confirm the beneficial effects of remifentanil in patients with hepatic IL-18 signaling activation.

Taken together, the data presented in the current study demonstrate that remifentanil upregulates IL-18BP expression in hepatocytes and identify a transcriptional mechanism of remifentanil that involves the activation of transcription factors STAT1 and C/EBP β (Fig. 7). These findings provide insight into the molecular mechanisms underlying the protective effects of remifentanil in liver I/R injuries that have been observed in experimental studies and clinical patients, expand our understanding of the pharmacological effects of remifentanil, and suggest its potential treatment benefits for other IL-18 dysfunction-mediated hepatic diseases, such as APAP overdose, HBV or HCV infection, and after hepatectomy when liver regeneration is required.

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Authors contributions X.L. and H.Y.: studies conduct, data analysis, and manuscript writing; Y.L.: studies conduct and data analysis; Y.J. and L.Y.: study design and data interpretation; X.W., W.Y., and D.S.: study design, data interpretation, and manuscript revision; J.T.: study design, data interpretation, manuscript revision, and financial support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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