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Inhibition of GSK-3β Decreases NF-κB-Dependent Gene Expression and Impairs the Rat Liver Regeneration

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Abstract Serine-threonine protein kinase glycogen synthase kinase (GSK)-3 is involved in regulation of many cell functions, but its role in regulating liver regeneration is unknown. Here we investigated the effects of GSK-3β inhibition on liver regeneration after partial hepatectomy in the rat. The potent and selective GSK-3β inhibitor SB216763 (0.6 mg/kg intravenously) or vehicle (10% dimethyl sulfoxide) was administered 30 min before 70% partial hepatectomy. Liver regeneration was estimated by the cell proliferation, apoptosis, and the related cell signaling and cycling proteins. In 30 min after hepatectomy in the rat, GSK-3β was found to be translocated to the nucleus, but GSK-3β inhibitor SB216763 that could phosphorylate residue Ser9 on GSK-3β did not attenuated the accumulation. Consequently, the inhibition of GSK-3β decreased the nuclear factor-κB activity, the NF-κB-dependent gene expression, and COX2 expression, but enhanced $p21^{WAF1/Cip1}$ transcription. Moreover, the injection of SB216763 impaired the proliferation cell nuclear antigen (PCNA) index and increased the apoptosis of liver compared to the vehicle. GSK-3β plays an important role in rat liver regeneration. We conclude it may partially result from the inhibition of the NF-κB pathway and enhancement of $p21^{WAF1/Cip1}$ expression. J. Cell. Biochem. 102: 1281–1289, 2007.

Key words: GSK-3β; liver regeneration; NF-κB; cell proliferation; apoptosis

INTRODUCTION

GSK-3 was discovered over 20 years ago as one of several protein kinases that phosphorylated and inactivated glycogen synthase and, hence, regulate glucose metabolism [Embi et al., 1980; Frame and Cohen, 2001]. However, increasing knowledge has changed the image of GSK-3 to that of a broadly influential enzyme that is crucial regulator of many cellular functions [Jope and Johnson, 2004]. The cellular processes, that GSK-3 participates, range from cell membrane-to-nucleus signaling, gene transcription to cell cycle progression, and survival [Cohen and Abraham, 1999; Woodgett, 2001; Kuma et al., 2004; Dugo et al., 2005]. There are two known isoforms, GSK-3α and GSK-3β [Gross et al., 2004].

GSK-3β is considered a constitutively active enzyme. The regulatory mechanisms consist of its activity and subcellular distribution, which can be subjected to cell signaling cascades. By site-specific phoshorylation, the residue of Tyr216 increases its activity while Ser9 inhibits it [Bijur and Jope, 2001; Gross et al., 2004]. The nuclear level of GSK-3β is also dynamically changed in response to intracellular cues [Bijur and Jope, 2001; Jope and Johnson, 2004]. GSK-3β has many substrates, such as β-catenin, Axin, Tau, cyclin D1, AP-1, C/EBP, p53 and NF-κB [Frame and Cohen, 2001; Jope and Johnson, 2004]. By activating or inactivating these proteins, GSK-3β exerts broad functions on cell growth [Alt et al., 2000; Shao et al., 2000],

Abbreviations used: NF-κB, nuclear factor-κB; PCNA, proliferating cell nuclear antigen; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; IL-6, interlukin-6.

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mobility [Fagotto et al., 1996; Orsulic and Peifer, 1996], and dual function on apoptosis [Watcharasit et al., 2002; Rao et al., 2004]. Recently, a most landmark study showed that GSK3β-knockout mice die during development due to massive hepatocyte apoptosis, because of a defect in NF-κB activation [Hoeflich et al., 2000]. At the same time, GSK-3 protects primary hepatocytes from TNF-α-induced apoptosis through upregulation of NF-κB activation [Schwabe and Brenner, 2002].

Here we characterized how GSK-3β signaling affected cell function in many processes. The role of GSK-3β during liver regeneration has not yet been elucidated. During the priming phase of liver regeneration, many important immediate early genes are activated, including NF-κB, AP-1, C/EBP [Fitzgerald et al., 1995; Soriano et al., 1995; Iimuro et al., 1998; Mangnall et al., 2003; Schwabe et al., 2003], followed by their downstream gene expression and cell signaling. The induction of NF-κB, which results in IL-6 and iNOS induction and STAT-3 activation, can protect hepatocytes from TNF-α induced apoptosis [Diaz-Guerra et al., 1997; Iimuro et al., 1998]. Likewise, C/EBP correlates with COX2 [Casado et al., 2001]. Later on, these downstream proteins together with cyclins and kinases, contribute to the DNA synthesis, G1/S transition, and cell cycle progression.

Taking into account the preceding data, we investigated the localization and potential role of GSK-3β during liver regeneration by blocking GSK-3β with its selective inhibitor SB16763 (SB). In 30 min after PH we observed the translocation of GSK-3β to the nucleus. And inhibition of GSK-3β resulted in a diminished proliferation response and an increased apoptosis index, which may result from the decreased activity of NF-κB1/p105, the NF-κB-dependent gene expression, and COX2 induction.

MATERIAL AND METHODS

Chemicals

GSK-3β specific inhibitor SB was obtained from Sigma (St. Louis, MO) and was dissolved in DMSO. Both SB and vehicle were administered intravenously via the sublingual vein.

Animals

For PH, male Sprague-Dawley rats (180–210g) were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and injected with SB (0.6 mg/kg) or DMSO (10%) 30 min before PH. For sham operation (SH), rats undergoes abdominal surgery without liver resection. Then animals were subjected to 70% liver resection. Animals were cared for according to the Institutional Animal Care Instructions.

Proliferating Cell Nuclear Antigen (PCNA) Staining

Tissue was collected from DM and SB pretreated hepatectomized rats ($n = 3–5$) at indicated times. The livers were removed, cut into small blocks, and fixed in 10% buffered paraformaldehyde and embedded in paraffin. The blocks were stained with hematoxylin-eosin, according to standard protocols. The hepatocyte regeneration was analyzed by the proliferating cell nuclear antigen (PCNA). Immunohistochemistry for PCNA was performed using the labeled strept avidin biotin (LSAB) method. Monoclonal antibody for PCNA (sigma) was used at a 1:200 dilution as the first antibody. To calculate the PCNA index, the stained slides were examined under × 250 magnification, with eight randomly chosen fields of each slide. PCNA index = number of positive stained cells/total number of cells counted in each section. Four sections were evaluated at each time point for each experimental group.

TUNEL Assay

The TUNEL Test (TdT-mediated dUTP nick end labeling) was performed using the commercial kit from Boehringer-Mannheim (GmbH, Ingleheim, Germany) following the instructions of the manufacturer. Sections were analyzed with a fluorescence microscope (Olympus, Hamburg, Germany).

Preparation of Whole-Cell and Nuclear Extracts

Tissue samples were homogenized in cell lysis buffer (20 mmol/L Tris pH 7.4, 137 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, and 1% Triton X-100, 1 × protease inhibitor cocktail). The homogenates were centrifuged at 10,000*g* for 15 min at 4°C. The supernatants were obtained as whole-cell extract. Nuclear extracts were prepared according to the instruction of nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford). Protein concentration of the extracts was determined by the Bradford method and aliquots of the proteins were stored at −70°C.

Western Blot Analysis

The levels of phospho-(Ser9)-GSK-3β, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interlukin-6 (IL-6), and β-actin were determined in whole-cell extracts. The amount of $p21^{WAF1/Cip1}$, sp1, and GSK-3β were determined in nuclear extracts. Protein extracts were separated on a SDS–polyacrylamide gel, blotted onto a nitrocellulose membrane (Millipore Corp., Bedford), and incubated with anti-GSK-3β, anti-phospho-(Ser9)-GSK-3β, anti-β-actin (Cell Signaling Technology, Beverly), anti-sp1, anti-$p21^{WAF1/Cip1}$ (Santa Cruz Biotechnology, Santa Cruz), anti-COX2 (BioVision, Inc., Mountain View), anti-iNOS (BD Transduction Laboratories), or anti-IL-6 (Abcam Inc., Cambridge). Immunoblots were developed using horseradish peroxidase-conjugated goat anti-mouse or goat antirabbit IgG, followed by detection with enhanced chemiluminescence. Western blot analysis was performed for each protein of interest derived least at three times from different animals.

EMSA

To determine NF-κB activation, NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGG) with biotin 3′ end labeling was carried out by electrophoretic mobility shift assay (EMSA) following the instruction of LightShift chemiluminescent EMSA kit (Pierce). The nuclear proteins (10 μg) were incubated for 20 min at RT with biotin-labeled DNA probes in the 20 μl of reaction mixture comprising 1 mM EDTA, 5 mM KCl, 10% glycerol, 1 μg of poly (dI · dC), 5 mM dithiothreitol, and 20 mM Tris-HCl, Ph 8.0. The following steps were prepared according to the instruction of the kit.

RNA Extraction and Reverse Transcription

Frozen tissue was grinded to a fine powder in a mortar and pestle cooled with liquid nitrogen. Total RNA was isolated from prepared liver samples by Trizol (Invitrogen, Carlsbad) reagent. And cDNA was synthesized from 4 μg of total RNA with random primers following the manufacturer's protocol (MBI Fermantas, Vilnius, Lithuania).

Quantitative Real-Time RT-PCR

The single-stranded cDNA was then used in a quantitative real-time PCR to evaluate the relative expression levels of iNOS (5′- GTCACCTATCGCACCCGAGAT and 5′-GCCACTGACACTCCGCACAAA), IL-6 (5′-CCCAACTTCCAATGCTCTCCTAAT and 5′-GCACACTAGGTTTGCCGAGTAGAC), $p21^{WAF1/Cip1}$ (5′-CAAAGTATGCCGTCGTCTGTTC and 5′-CATGAGCGCATCGCAATC) and COX2 (5′-AGAGAAAGAAATGGCTGCAGAGTT and 5′-AGCAGGGCGGGATACAGTT), compared to β-actin (5′-ATGGTGGGTATGGGTCAGAAG and 5′-TGGCTGGGGTGTTGAAGGTC). Six sets of primers were used for PCR. Real-time PCR was performed and primers were designed according to the ABI manufacturer's protocols (Applied Biosystems, Perkin-Elmer). All samples were examined in duplicate. PCR-specific amplification was performed in the Applied Biosystems (ABI7300) real-time PCR machine. The reactions were run for 40 cycles (96°C 15′, 60°C 60s). Fluorescence was analyzed by using the Light Cycler Software version 3.5 (Roche Diagnostics).

Statistical Analysis

The data shown are the means ± SE of three experiments. Statistical significance was estimated with Student's *t*-test for unpaired observations. A *P*-value of less than 0.05 was considered significant.

RESULT

Inhibition of GSK-3β by SB Reduces Liver Proliferation and Induces Apoptosis

SB is a potent and selective inhibitor of GSK-3β. It has been demonstrated that SB can phosphorylate residue Ser9 on GSK-3β and, hence, are likely to inhibit the activity of the kinase [Smith et al., 2001; Woodgett, 2001; Dugo et al., 2005]. To prove the inhibitory effect of SB on GSK-3β activity in regenerating liver, we analyzed the level of phosphorylation of Ser9 of GSK-3β. 30 min of SB treatment significantly enhanced phospho-Ser^9 GSK-3β, demonstrating an effective inhibition of GSK-3β by SB in regenerating liver models (Fig. 1A). To determine the possible role of GSK-3β on liver regeneration, we measured PCNA, caspase 3–like activity, and the TUNEL assay at 0, 16 and 24 h (Fig. 1B–E). Pretreatment of SB before PH increased caspase 3–like activity at 16 and 24 h after PH (Fig. 1C) and had an increased apoptotic index, as determined by TUNEL assay on 24 h, compared to the control (Fig. 1D).

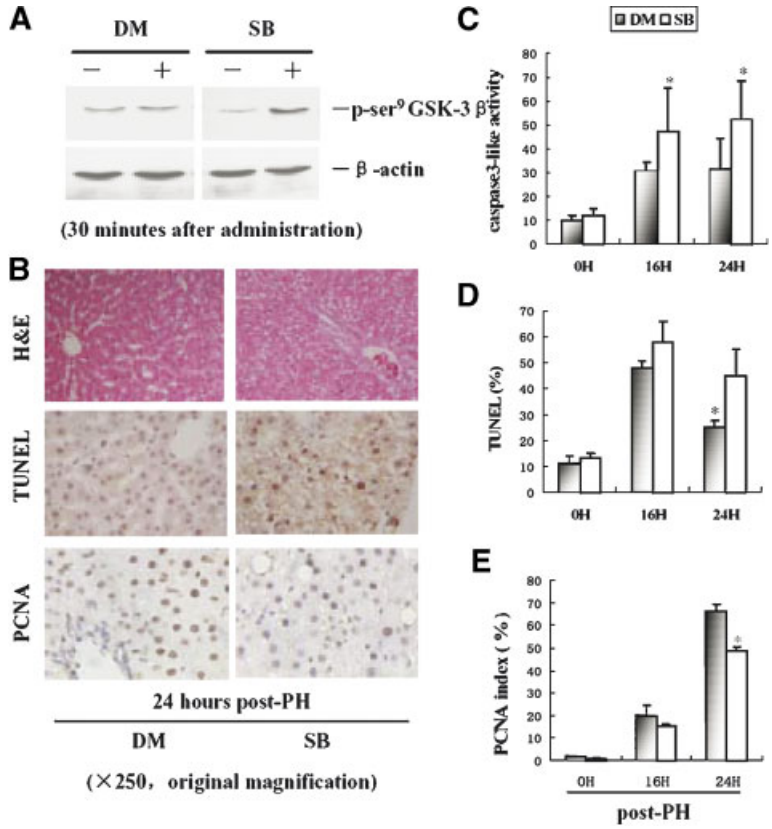

Fig. 1. SB pretreatment caused GSK-3β phosphorylation and regulated hepatocyte proliferation and apoptosis in 16h and 24h after PH in rat. **A**: SB phosphorylated GSK-3β on Ser9 30 min after injection (whole-cell extract). **B**: Liver sections were stained with H and E, TUNEL or immunohistochemically with PCNA (×250 magnification) on 24h after PH. **C**: Caspase 3-like activity was determined by 7-amino-4-trifluoromethyl coumarin (AFC) release assay 16h and 24h after PH and is shown as AFC release (nmol) per μg protein to evaluate apoptosis. **D**: Percentages of apoptotic cells among total hepatocytes were determined by counting apoptotic cells by TUNEL staining as described in Material and Methods. **E**: Mitotic and PCNA-positive hepatocytes following PH were counted in triplicate using different sections. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Moreover, the inhibition of GSK-3β caused a lower rate of proliferation, as assessed by PCNA (Fig. 1E).

Partial Hepatectomy Induces Nuclear Accumulation of GSK-3β

To test whether the subcellular distribution of GSK-3β verifies along with pharmaceutic treatment or partial hepatectomy, immunoblots of nuclear fractions prepared from liver tissue were testified for GSK-3β and sp1. As it shows in Figure 2, we detected the samples at different indicated times after PH or sham operation, which has been pretreated with SB or control. In the DM treatment group, PH caused the nuclear accumulation of GSK-3β at 7 min after PH which lasted at least 30 min, while the samples of sham operation group did not alter in nuclear nor cytosolic level. And the SB group showed the same dynamic movement of GSK-3β in contrast to the DM (Fig. 2A,B). These results revealed that the nuclear level of GSK-3β is dynamic and is correlated with signal of liver resection, but independent of GSK-3β activity.

GSK-3β Inhibitor Decreases NF-κB Activity but Exerts no Effect on p65 Translocation

The previous data suggests that GSK-3β has been linked to regulation of iNOS and IL-6 gene transcription. To test whether they were mediated by NF-κB pathway, we evaluated the effect of SB on NF-κB by electrophoretic mobility shift assays and Western blot. Nuclear extracts were obtained in both SB and DM treated rats in 30 min after PH. As shown in Figure 2D, liver resection stimulates NF-κB DNA binding activity, while GSK-3β inhibitor decreased it. On the other hand, translocation of p65 to nuclear immediately after PH was not affected by inhibition of GSK-3β (Fig. 2C).

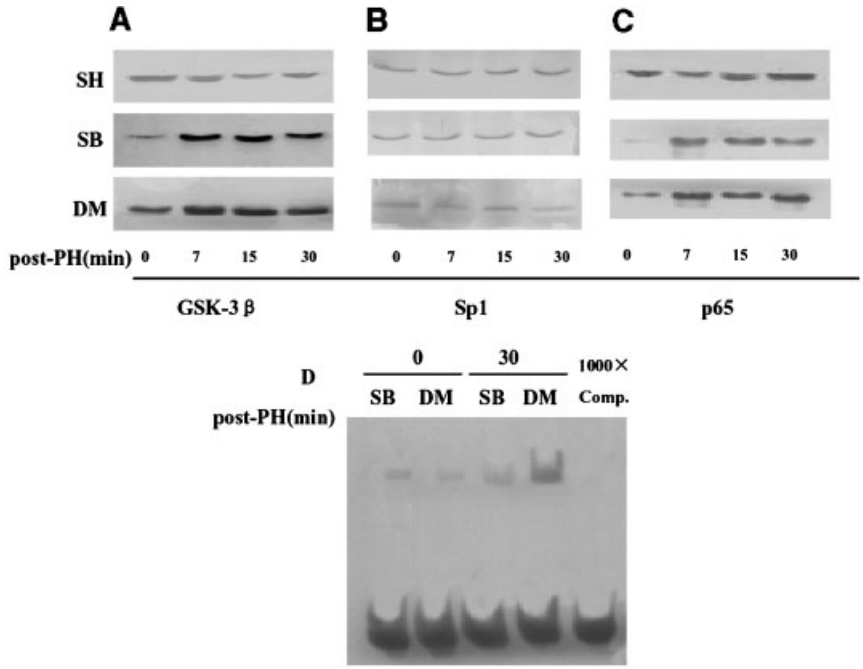

Fig. 2. PH induces nuclear accumulation of GSK-3β and inhibition of GSK-3β activity impairs NF-κB activity but exerts no effect on p65 translocation. (**A**–**C**): Partial hepatectomy with DM or SB 30 min pretreatment or sham operation (SH) causes different movement of GSK-3β. Nuclear extracts were immunoblotted for GSK-3β, sp1 and p65. **D**: NF-κB binding activity was analyzed by EMSA before or after 30 min of PH in DM or SB pretreated rats. Comp., competition.

Changes of the Expression of Important Genes Correlating to the Potential Substrates of GSK-3β in the Liver After PH

Previous observation showed that GSK-3β had various substrates including transcription factor NF-κB, AP-1, C/EBP [Fitzgerald et al., 1995; Soriano et al., 1995; Iimuro et al., 1998; Schwabe et al., 2003], which also played important roles during liver regeneration. To find the possible role GSK-3β may play in regulating these factors and liver regeneration, we detected their downstream gene expression and some important cell cycling genes. As shown in Figure 3, RNA expression of NF-κB targeted gene iNOS and IL-6 was significantly reduced by using inhibitor of GSK-3β in 6 and 12 h after PH (Fig. 3A, B), and accordingly the protein change of iNOS and IL-6 was confirmed by Western blots (Fig. 3C).

GSK-3β Activity Influences the Pattern of Cell Cycle Molecules

To elucidate the underlying mechanism responsible for the ability of GSK-3β inhibition to suppress cell mitosis while augmenting apoptosis during liver regeneration, we assessed some cell cycling genes and COX2 expression, which has been defined to exert significant role in cell proliferation in liver regeneration [Casado et al., 2001] and GSK-3β-mediated apoptosis in renal cells [Rao et al., 2004]. $P21^{WAF1/Cip1}$, a cyclin inhibitor, was slightly upregulated at 12 h after PH and returned to normal at 48 h. And the accumulation of $p21^{WAF1/Cip1}$ was enhanced by administration of SB216763, which was testified by real-time PCR (Fig. 4B) and immunoblots (Fig. 4C upper panel). However an inhibition of COX2 RNA (Fig. 4A) and protein (Fig. 4C third panel) expression was observed because of SB216763 treatment at 12 h especially. In our study, expression of CDK2 and CDK4 was also investigated but both of them were not affected by SB216763 treatment (data not shown).

DISCUSSION

Liver regeneration after partial hepatectomy is considered to be divided into several stages, all of which are dependent on the presence or absence of a number of factors, acting in a cascade-like pattern [Court et al., 2002]. The regeneration response is orchestrated by

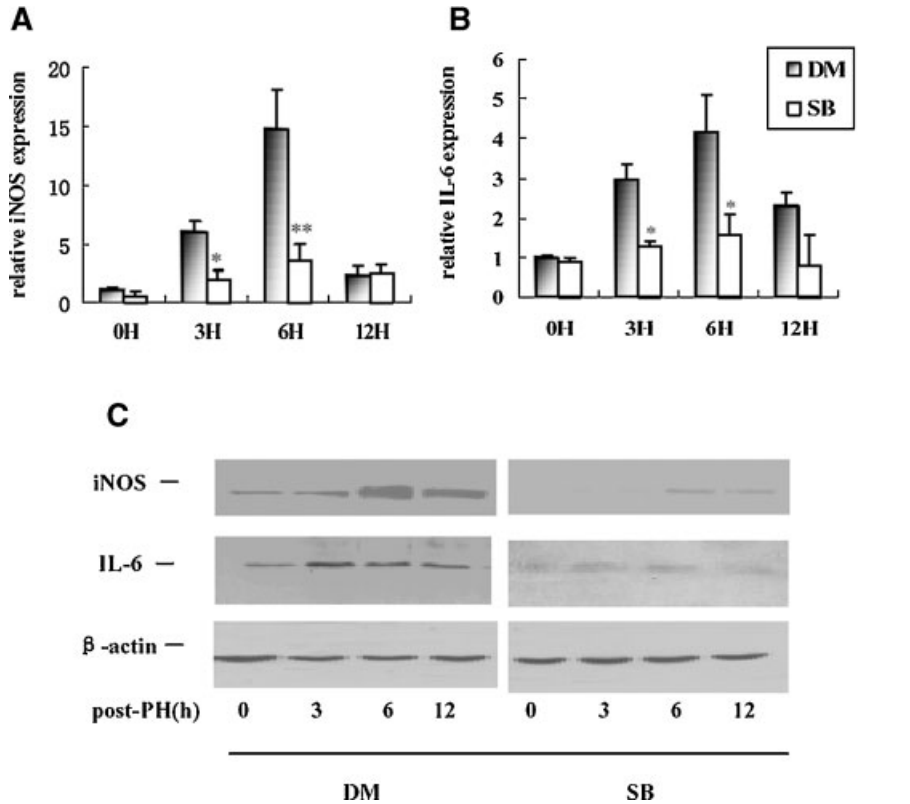

Fig. 3. NF-κB-dependent gene expression in DM or SB pretreated rats at indicated times after PH (**A**,**B**). To estimate mRNA expression of iNOS and IL-6, a real-time PCR method was employed, standardized by the amount of β-actin. (A) iNOS expression. (B) IL-6 expression. Values are expressed as means ± SE of 3 separate experiments. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$. **C**: To quantify the protein level, western blot were performed. The level of iNOS and IL-6 in SB pretreated rats showed obvious decrease compared to the control.

specific stimuli involving sequential changes in gene expression, growth factor production and, extracellular matrix remodeling [Michalopoulos and DeFrances, 2004]. Many growth factors cytokines (HGF, EGF, TGF-α, TNF-α, and IL-6) and transcription factors (c-Myc, c-Fos, c-Jun, p53, NF-κB) have been identified as important regulators of this process [Fausto et al., 1995; Casado et al., 2001].

GSK-3β phosphorylates and thereby regulates on many cell functions, including cell growth, mobility, and apoptosis. A recent study has demonstrated that GSK-3β −/− mice died of massive hepatocyte apoptosis during development [Heflich et al., 2000], which suggests GSK-3β has a profound influence on regulating liver development. In this study, we tried to demonstrate whether and how GSK-3β might affect liver regeneration, using a potent and selective GSK-3 inhibitor, SB. A lower rate of proliferation and an increase in apoptosis index was observed at 24h after liver resection in animals pretreated with SB. This may suggests that GSK-3β is involved in the priming and progression phase of liver regeneration. And the inhibition of GSK-3β activity may be harmful to the process.

To investigate the mechanism, we observed the subcellular distribution and gene expression of GSK-3β along with liver regeneration. Our results show that the signal of liver resection increases GSK-3β nuclear accumulation independent of its activity. However gene expression of GSK-3β showed no fluctuation (date not shown). Considering GSK-3β has a potent role in regulating many transcription factors such as NF-κB [Fitzgerald et al., 1995; Iimuro et al., 1998], which is a key regulator of liver regeneration, development and primary hepatocytes apoptosis [Iimuro et al., 1998; Hoeflich et al., 2000; Martin et al., 2005], we therefore detected translocation of p65 subunit of NF-κB and its DNA binding activity. We demonstrated that the inhibition of GSK-3β

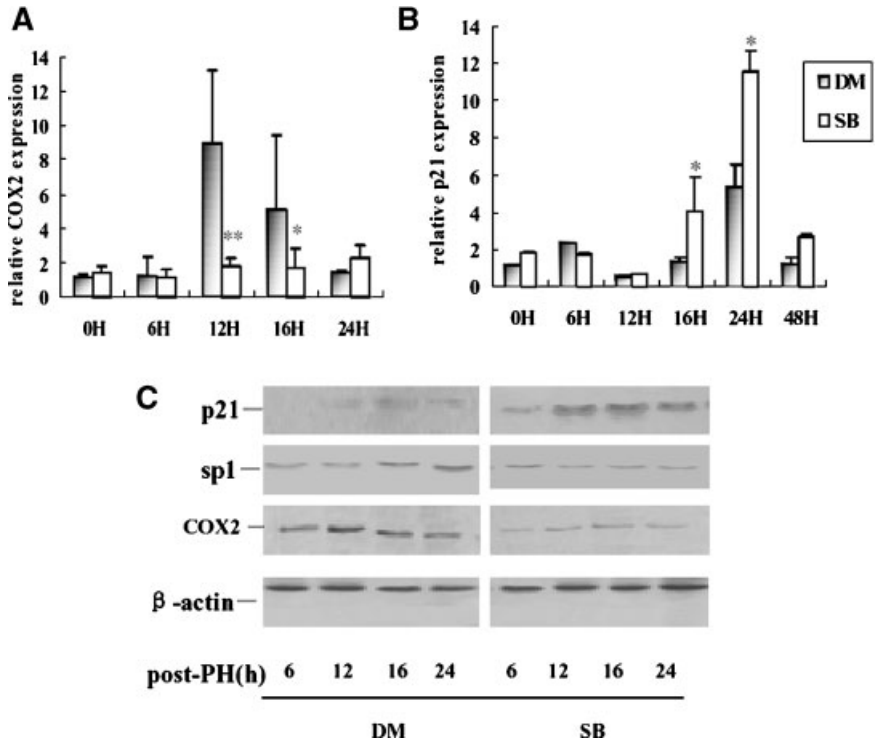

Fig. 4. COX2 and p21 expression in DM or SB pretreated rats at indicated times after PH. **A**,**B**: To estimate mRNA expression of COX2 and p21, a real-time PCR method was employed, standardized by the amount of β-actin. A: COX2 expression. B: p21 expression. Values are expressed as means ± SE of 3 separate experiments. **C**: To quantify the protein level, western blot were performed. The level of COX2 was diminished by GSK-3β inhibition, while the level of p21 was increased in compared to the control.

activity had no effect on p65 accumulation, but decreased its DNA binding activity, in agreement with previous study [Fitzgerald et al., 1995; Iimuro et al., 1998; Hoeflich et al., 2000; Martin et al., 2005]. It is very likely that phosphorylation of p65 by GSK-3β enhances its transactivation. Because multiple phosphorylation of p65 appears to be advantageous for its transactivation [Schwabe and Brenner, 2002], which may be irrelevant to p65 translocation.

We also studied some NF-κB dependent gene transcription, iNOS and IL-6, which are two critical genes during liver regeneration, regulated by NF-κB [Diaz-Guerra et al., 1997; Streetz et al., 2000; Zeini et al., 2004]. Our results suggested there was an obvious decrease in both gene expression levels. The induction of iNOS in liver in the course of several hepatic dysfunctions, such as hyperdynamic circulation and chemical aggression or during septic shock, appears to be a widely established response of damaged organ [Diaz-Guerra et al., 1997]. And IL-6 may attribute to induction of the acute phase response and triggering G0/G1 phase transition of hepatocytes after hepatectomy [Streetz et al., 2000; Zeini et al., 2004]. So the inhibition of GSK-3β may affect NO synthesis, acute phase response, cell cycling and consequently liver regeneration process through downregulating iNOS and IL-6 expression. However, another NF-κB downstream gene cyclin D1 showed no regulation after treated with GSK-3β inhibitor (data not shown), which may result from the compensation of other factors.

In order to find other important genes correlating to the potential substrates of GSK-3β after PH, we detected COX2 and some cell cycling genes. COX2, contributing to the synthesis of prostaglandins to liver regeneration [Casado et al., 2001; Zeini et al., 2004], has been demonstrated to be regulated by GSK-3β in medullary interstitial cells [Rao et al., 2004] and primary cortical astrocytes [Sanchez et al., 2003]. And in our experiment, we found the inhibition of GSK-3β diminished COX2 RNA and protein expression from 6 to 24 h. Casado et al. [2001] has reported that pretreated with NS398, a selective COX-2 inhibitor, the PCNA levels at 24 h was dramatically decreased.

However, our results showed that $p21^{WAF1/Cip1}$ was up-regulated at 24 h in animals pretreated with SB. p21 plays a role in controlling timing of G1-phase and p21 knockout animals show an earlier entry into S-phase than wt mice [Albrecht et al., 1998]. Some recent study discovered that GSK-3β can regulate p53-induced increase in the level of p21 [Watcharasis et al., 2003]. Whether GSK-3β exerts the same function on p21 during liver regeneration, remains unknown. Taking COX2 and p21 into account, after inhibiting GSK-3β, COX2 was decreased while p21 was increased. These results may partially explain the reason for the ability of GSK-3β inhibition to suppress cell mitosis while augmenting apoptosis during liver regeneration.

In conclusion, our study assesses the role GSK-3β may play in liver regeneration and how it affects some important target genes which were also key genes to liver regeneration. Observations in our laboratory show that the inhibition of GSK-3β decreased NF-κB DNA binding activity but not p65 subunit translocation, and consequently inhibited NF-κB dependent gene transcription, iNOS and IL-6. Moreover, the inhibiting group impaired COX2 expression and raised p21 level. We infer that these mechanisms are mostly likely to elucidate how GSK-3β contributes to liver proliferation and protecting hepatocytes from apoptosis.

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