Wedelolactone exhibits anti-fibrotic effects on human hepatic stellate cell line LX-2

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**Abstract**

Wedelolactone is a major coumarin of *Eclipta prostrata*, which is used for preventing liver damage. However, the effects of wedelolactone on hepatic fibrosis remained unexplored. The purpose of this study was to demonstrate the anti-fibrotic effects of wedelolactone on activated human hepatic stellate cell (HSC) line LX-2 and the possible underlying mechanisms by means of MTT assay, Hoechst staining, as well as real-time quantitative PCR and western blot. The results showed that wedelolactone reduced the cellular viability of LX-2 in a time and dose-dependent manner. After treatment of wedelolactone, the expressions of collagen I and α-smooth muscle actin, two biomarkers of LX-2 activation, were remarkably decreased. The apoptosis of LX-2 cells was induced by wedelolactone accompanied with the decreasing expression of anti-apoptotic Bcl-2 and increasing expression of pro-apoptotic Bax. In addition, phosphorylated status of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) and p38 was up-regulated, but not in p38. Moreover, wedelolactone significantly repressed the level of phosphorylated inhibitor of nuclear factor-κB (IκB) and p65 in nucleus in spite of tumor necrosis factor-α stimulation. In conclusion, wedelolactone could significantly inhibit the activation of LX-2 cells, the underlying mechanisms of which included inducing Bcl-2 family involved apoptosis, up-regulating phosphorylated status of ERK and JNK expressions, and inhibiting nuclear factor-κB (NF-κB) mediated activity. Wedelolactone might present as a useful tool for the prevention and treatment of hepatic fibrosis.

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**1. Introduction**

Hepatic fibrosis is the essential pathophysiological consequence of liver injury, and represents the common underlying mechanism of liver diseases (Moreira, 2007). As hepatic fibrosis progresses, excessive extracellular matrix (ECM) such as collagen I is produced and accumulated, leading to liver dysfunction and irreversible cirrhosis. Hepatic stellate cells (HSC) have been identified as the principle cellular source of the ECM (Yang et al., 2012). Normally, HSC which is in its quiescent state serve as the main storage site for vitamin A is essential in the regulation of retinoic acid homeostasis (Wynn, 2008). However, when liver injury occurs, HSC undergoes a remarkable transformation to a myofibroblast like phenotype. The biological change of HSC is closely associated with hepatic fibrosis, producing a variety of growth factors and cytokines and remodeling of ECM as well (Parsons et al., 2007). Previous studies indicated that apoptosis of activated HSC could effectively control fibrosis in the liver (Kisseleva and Brenner, 2011). Anti-fibrotic agents have been shown to induce apoptosis through diverse regulatory pathways, involving the mitochondrial-induced apoptosis members of the bcl-2 and bax families, and phosphorylation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) and p38 (Pan et al., 2012). Moreover, the activation of nuclear factor-κB (NF-κB) plays a critical role in HSC activation. Recent research demonstrated that increased hepatic NF-κB activity contributed to the inflammation and fibrosis in HSC (Saugspier et al., 2012).

Wedelolactone (7-methoxy-5,11,12-trihydroxy-coumarstan, Fig. 1) is a major coumarin isolated from *Eclipta prostrata*, which is used for preventing liver damage due to alcohol overdose, jaundice and inflammation (Deng and Fang, 2012; Singh et al., 2001). As a folk medicine, *E. prostrata* is also used for the treatment of infective hepatitis in India (Patel et al., 2008) and septic shock in China (Kobori et al., 2004). Wedelolactone has been reported to exhibit diverse biological effects, such as anti-hepatotoxicity and liver protection (Jayathirtha and Mishra, 2003; Singh et al., 2001; Wong et al., 1988). However, the effects of wedelolactone on hepatic fibrosis remain unexplored.

LX-2 cell line is a stable and unlimited source of human HSC, which has been extensively characterized as a valuable cell-based model for studies of human hepatic fibrosis (Xu et al., 2005). LX-2 retains the key features of activated HSC, such as cytokine
signaling, neuronal gene expression, retinoid metabolism, and fibrogenesis. Therefore, in the current study, we employed the LX-2 cell line to investigate the anti-fibrotic effects of wedelolactone and the possible underlying mechanisms.

2. Materials and methods

2.1. Materials

Human HSC LX-2 cell line was purchased from BioHermes Inc. (Jiangsu province, China). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), 0.25% Trypsin and penicillin-streptomycin were purchased from Gibco (USA). Recombinant tumor necrosis factor-α (TNF-α) was purchased from R&D systems (USA). Wedelolactone was purchased from National Institutes for Food and Drug Control (NIFDC, China). α-smooth muscle actin (α-SMA) antibody, dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-aldrich (USA). The antibodies against collagen I, phospho-ERK, phospho-JNK, phospho-p38, ERK, JNK, p38 and NF-κB/p65 were purchased from Abcam Inc. (USA). The antibodies against phospho-inhibitor of NF-κB (IκBα), IκBα, Bcl-2, Bax, GAPDH, PCNA, anti-mouse and anti-rabbit IgG antibody conjugated with horseradish peroxidase were obtained from Cell signaling technology Inc. (USA). Western blot detection reagents were obtained from Beyotime institute of biotechnology (China). Dulbecco Modified Eagle’s medium (DMEM) was purchased from Gibco (USA). Recombinant tumor necrosis factor-α was purchased from R&D systems (USA). Hoechst staining kit, Enhanced chemiluminescence (ECL) and PVDF membrane were purchased from Millipore (USA). Hoechst staining kit, Enhanced BCA protein assay kit, RIPA lysis buffer and Nuclear and cytoplasmic protein extraction kit were obtained from Beyotime institute of biotechnology (China). Trizol reagent was purchased from Invitrogen (USA). Prime script RT reagent kit and SYBR premix taq were purchased from Takara (Japan).

2.2. Cell culture

LX-2 cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and the medium was changed every other day.

2.3. Measurement of cell viability

Cell viability was determined by MTT. A total of 1 × 10⁴ cells were seeded in 96 well plates for 24 h and made quiescent by incubating in FBS free DMEM overnight. Wedelolactone was dissolved in DMSO and reached the final culture concentration of 0.1% in DMEM. After treating with different concentrations (0, 1, 5, 10, 20, 40, 80, 120 and 160 μmol/l) of wedelolactone for 24 or 48 h, 5 mg/ml MTT was added to the wells and incubated for additional 4 h at 37 °C. The optical density of the dissolved material was measured at 490 nm. The assays were performed in three independent experiments with n = 3.

2.4. Apoptosis analysis

Apoptosis was determined by Hoechst staining. Cells were seeded on sterile cover glasses. After treated with vehicle or 10, 20 and 40 μmol/l wedelolactone in FBS free DMEM for 48 h, cells were washed twice with PBS and stained with Hoechst 33258 staining solution according to the manufacturer’s instructions. Stained nuclei were observed under a fluorescence microscope. Apoptotic cells were defined on the basis of nuclear morphology changes, such as chromatin condensation and fragmentation.

2.5. Real-time quantitative PCR

Total RNA was extracted from the cells using Trizol reagent, reverse-transcribed to complementary DNA (cDNA) using Prime script RT reagent kit and amplified with SYBR premix taq reagent using SYBR Greener qPCR Universal System (Applied Biosystems 7500). The fold induction was calculated as previously (Xu et al., 2009). The sequences (5’ to 3’) for the primers were shown in Table 1.

2.6. Western blot analysis

Whole cell proteins were lysed on ice by RIPA lysis buffer with 1% phenylmethylsulfonyl fluoride (PMSF), centrifuged at 15,000 × g for 15 min and the supernatant was collected. Nuclear and cytoplasmic proteins of LX-2 were obtained using nuclear and cytoplasmic protein extraction kit according to the manufacturer’s instructions. Protein concentration was measured using enhanced BCA protein assay kit. Equal amounts of the protein (50 μg/lane) were separated by SDS-PAGE and transferred to PVDF membrane that were probed with anti-α-SMA, anti-collagen I, anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, anti-phospho-IκBα, anti-ERK, anti-JNK, anti-p38, anti-IκBα, anti-NF-κB/p65, anti-Bcl-2, anti-Bax, anti-GAPDH and anti-PCNA antibodies, respectively. Immunodetection was visualized using the ECL detection system according to the manufacturer’s instructions. Quantitative densitometric analyses of immunoblotting images were performed using ImageJ 1.44p software.

2.7. Statistical analysis

Data were presented as mean ± standard deviation (S.D.) of three independent experiments. The results were analyzed by one-way analysis of variance (ANOVA) using SPSS 17.0 software. Differences were considered as being significant at P < 0.05.

3. Results

3.1. Inhibitory effect of wedelolactone on cellular viability of LX-2

To explore the anti-fibrotic effects of the wedelolactone on HSC, the viability of LX-2 cells following wedelolactone treatment was
first determined by MTT assay. LX-2 cells were exposed to different concentrations of wedelolactone (0, 1, 5, 10, 20, 40, 80, 120 and 160 μmol/l) for 24 or 48 h, respectively. The cellular viability of LX-2 was decreased in a time-dependent manner. The concentrations of wedelolactone at 80 μmol/l exhibited a significant reduction in the cell number of LX-2 at each time point, the viability rate at the concentration of which for 48 h was (76.30 ± 4.62)%). The concentrations above 80 μmol/l showed stronger inhibition on cellular viability. Therefore, we chose concentrations of 10, 20 and 40 μmol/l at 48 h for the following analysis to avoid the cytotoxicity to the cells. The results suggested that wedelolactone could effectively inhibit cellular viability and exhibit cytotoxicity to LX-2 cells (Fig. 2).

3.2. Wedelolactone inhibited the activation of LX-2 in vitro

The activation of HSC is one of the central pathophysiological mechanisms of hepatic fibrogenesis, in which over-expressions of α-SMA and collagen I are considered as the biomarkers of HSC activation (Parsons et al., 2007). We next investigate the protective effect of wedelolactone against hepatic fibrosis in vitro. LX-2 cells were exposed to wedelolactone at 10, 20 and 40 μmol/l for 48 h, and total RNA and protein were isolated from LX-2 for subsequent analysis of gene expression and protein levels, respectively. We first analyzed α-SMA and collagen I gene expression levels using real-time quantitative PCR. Treatment with wedelolactone significantly reduced the gene expression levels of α-SMA and collagen I in a dose-dependent manner (Fig. 3A). We next evaluated the relative expression levels of α-SMA and collagen I protein in LX-2 cells. Total proteins were isolated and resolved on an SDS-PAGE gel for western blot analysis. Fig. 3B showed that the repression in the expression of both α-SMA and collagen I proteins by wedelolactone treatment were dose-dependent. Quantitative analyses of these data indicate that both α-SMA and collagen I protein levels were significantly reduced in LX-2 cells in a dose-dependent manner.

3.3. Wedelolactone induced LX-2 apoptosis through the family of Bcl-2 proteins

We next examined the effects of wedelolactone on LX-2 apoptosis using Hoechst 33258 staining. LX-2 exhibited significant morphology alteration following treatment of wedelolactone at 10, 20 and 40 μmol/l. Specifically, apoptotic bodies were formed in 20 and 40 μmol/l wedelolactone groups, whereas cell shrinkage was detected with wedelolactone treatment at 10 and 20 μmol/l (Fig. 4A). In order to elucidate the mechanism of wedelolactone induced apoptosis of LX-2 cells, the mRNA and protein expression of Bcl-2 and Bax, two mitochondrion-dependent apoptotic family members were determined by real-time quantitative PCR. (B) Collagen I and α-SMA protein expressions were determined by western blot. Data were presented as mean ± S.D. of three independent experiments with n = 3. *P < 0.05, **P < 0.01 compared with control.
3.4. Role of MAPKs in wedelolactone induced LX-2 apoptosis

Previous study indicated that HSC apoptosis was associated with the activation of MAPK signaling (Pan et al., 2012). Thus we examined the effect of wedelolactone on the phosphorylation status of MAPK family proteins ERK, JNK and p38 by western blot. As shown in Fig. 5, wedelolactone treatment significantly increased the phosphorylated levels of ERK and JNK, but not in p38, suggesting the important role of wedelolactone in activation of ERK and JNK signaling in wedelolactone induced apoptosis in LX-2 cells.

3.5. Wedelolactone inhibited NF-κB activity in TNF-α induced LX-2 in vitro

The transcriptional factor NF-κB has been shown to be involved in the regulation of cytokine signaling and cellular apoptosis, which contributed to the activation of HSC. Activation of NF-κB is linked to phosphorylation and proteolytic degradation of IκB (Sun and Karin, 2008). TNF-α is known as one of the strongest stimuli of NF-κB signaling (Lee et al., 2011). We next examined the effects of wedelolactone on the expression of proteins involved in NF-κB activation in TNF-α treated LX-2 cells. As expected, the phosphorylated level of IκB was increased following TNF-α stimulation. However, treatment with wedelolactone significantly suppressed the phospho-IκB expression regardless of the TNF-α stimulation (Fig. 6A). The nuclear translocation is critical for NF-κB/p65 to exert its effects. We subsequently determined the effects of wedelolactone on NF-κB/p65 translocation from cytoplasm to nucleus following TNF-α stimulation. After wedelolactone treatment, the expression of p65 was significantly decreased in nucleus, but increased in cytoplasm with or without TNF-α stimulation (Fig. 6B). Taking together, these data indicated that the anti-fibrotic effects of wedelolactone in LX-2 cells might be mediated by NF-κB signaling.

4. Discussion

Hepatic fibrosis is a reversible wound-healing response against a variety of acute and chronic stimulation in the liver, including ethanol, viral infection, drugs and toxins, cholestasis, and metabolic disease, all of which would disturb the balance between repair and scar formation in the organ. HSC has been shown to play a pivotal role in hepatic fibrogenesis, which orchestrates the secretion, deposition and accumulation of ECM and eventually results in hepatic fibrosis (Mormone et al., 2011; Zhang and Yao, 2011). Therefore, inhibition of HSC activation and proliferation while inducing its apoptosis has evolved as the potential strategy for preventing or even reversing hepatic fibrosis (Kisseleva and
Brenner, 2011). Erzhi Pill, composed of Fructus ligustri lucidi and E. prostrare, is an herbal prescription (Registration number issued by State Food and Drug Administration of China: Z31020387) in the clinical practice of Traditional Chinese Medicine. It has been widely used for liver protection and therapy of hepatic diseases, especially in the treatment of jaundice and chronic hepatitis. Treatment with Erzhi Pill has also been shown to reduce serum alanine aminotransferase and intrahepatic triglyceride, promote the regeneration of hepatic cells, and prevent cirrhosis (He et al., 2012b) and vascular smooth muscle cells (Zhang et al., 2012). Moreover, different components of Eclipta prsstrate may influence activation and proliferation of HSC. Previous study reported that triterpenoids from Eclipta prostrata exhibited antiapoptotic activity on HSC (Lee et al., 2008). However, the effects of wedelolactone on hepatic fibrosis are largely unknown. In the current study, the inhibitory effects and underlying molecular mechanisms of wedelolactone on activated HSC were explored in vitro.

Wedelolactone at concentration of 80 μmol/l or higher was exhibited to reduce the proliferation of activated LX-2 cells significantly at each time point, suggesting that wedelolactone could inhibit HSC growth in a time and dose-dependent manner. After HSC being activated, they expressed biomarkers such as α-SMA, and transformed into hepatic myofibroblasts, which served as the principal collagen producing sites in the liver (Li et al., 2012). In the current study, the mRNA and protein expression levels of α-SMA and collagen I were reduced in a dose-dependent manner after the treatment of wedelolactone for 48 h, indicating that wedelolactone could dramatically inhibit the activation of HSC and further repress the development of hepatic fibrosis.

Apoptosis, which is associated with fibrosis reversal, is considered as an effective process to eliminate activated HSC (Elshearkawy et al., 2005). It has been demonstrated that wedelolactone was capable of inducing apoptosis in prostate cancer cells (Sarveswara et al., 2012) and adipose tissue-derived mesenchymal stem cells (Lim et al., 2012a). Therefore the effect of wedelolactone on HSC apoptosis was evaluated. Hoechst 33258 staining analysis showed a small amount of apoptotic LX-2 cells without wedelolactone treatment, but wedelolactone significantly increased LX-2 apoptosis in a dose-dependent manner. Mitochondrial pathway involved Bcl-2 family plays a pivotal role in the cellular apoptotic process, in which Bcl-2 is an integral membrane protein with anti-apoptotic activity, whereas Bax is a pro-apoptotic member (Ding et al., 2011; Kim et al., 2011). Results from our study showed that the ratios of bcl-2/bax were decreased by wedelolactone treatment. Taking together, wedelolactone might exert protective role against hepatic fibrosis through bcl-2/bax involved apoptosis.

The MAPK signaling pathway has been shown to regulate diverse cellular processes such as proliferation, differentiation and apoptosis (Rubinfeld and Seger, 2005). Previous studies reported the regulation of MAPK induced apoptosis by several natural products (Gao et al., 2011; Liu et al., 2011). ERK-dependent activation of the mitochondrial pathway had a pro-apoptotic effect on HSC (Pan et al., 2012). Recently, wedelolactone had been shown to inhibit adipogenesis through the ERK pathway in human adipose tissue-derived mesenchymal stem cells (Lim et al., 2012a). JNK activation was involved in the apoptosis in prostate cancer cells by wedelolactone (Sarveswara et al., 2012). In the current study, treatment of wedelolactone significantly promoted the phosphorylation of ERK1/2 and JNK, but not in p38, implying that the wedelolactone induced apoptosis of LX-2 cells might be mediated through ERK and JNK signaling pathways.

NF-κB mediated transcriptional activation is known as a central pathophysiological mechanism in the process of HSC activation (Sun and Karin, 2008). The expression and secretion of various inflammatory cytokines and adhesion molecules are induced when NF-κB activity is up-regulated, which plays a major role in hepatic fibrosis (Elsharkawy and Mann, 2007; Jaruga et al., 2004). Upon stimulation by inflammatory cytokines such as TNF-α, lκB is phosphorylated by IKK and degraded. NF-κB is then released and translocates to the nucleus from cytoplasm, and activates the transcription of its target genes (Elsharkawy and Mann, 2007; Szuster-Ciesielska et al., 2011). Therefore, inhibition of NF-κB activity is considered as an underlying mechanism for anti-fibrosis (Luedde and Schwabe, 2011). Several studies reported that wedelolactone inhibited NF-κB mediated gene transcription by blocking the phosphorylation of lκB in hepatocarcinoma cells (Lim et al., 2012b) and vascular smooth muscle cells (Zhang et al., 2012). Our results showed that wedelolactone treatment significantly decreased the phosphorylated lκB expression and inhibited the translocation of NF-κB/p65 from cytoplasm to nucleus, suggesting that the anti-fibrotic effects of wedelolactone might be mediated by the inhibition of NF-κB activity.

5. Conclusions

In conclusion, the anti-fibrotic effects and the possible mechanisms of wedelolactone were demonstrated in human HSC LX-2 cell line. Wedelolactone dramatically inhibited the activation of HSC through inducing Bcl-2 family involved apoptosis, up-regulating phosphorylated status of ERK and JNK proteins, and inhibiting NF-κB mediated activity. The knowledge gained from the current study contributes to elucidating the mechanism of the anti-fibrotic effects of wedelolactone on LX-2 cell line, and is expected to
provide new opportunities for the development of novel therapeutic strategies against liver fibrosis.

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