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Survivin signaling is regulated through nuclear factor-kappa B pathway during glycochenodeoxycholate-induced hepatocyte apoptosis

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ABSTRACT

Hepatocytes in primary culture undergo apoptosis upon exposure to glycochenodeoxycholate (GCDC). The signaling mechanisms of GCDC-induced apoptosis remain unclear. To investigate the role of antiapoptotic genes, we compared apoptotic response in primary hepatocytes following GCDC treatment. The hepatocytes from adult Sprague–Dawley rats were cultured in collagen-coated dishes and treated with GCDC in varying concentrations, or the same concentration at different time intervals. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, DNA fragmentation assay, and caspase assays. Expression of apoptosis-related genes and proteins was evaluated by RT-PCR, quantitative real-time PCR (qRT-PCR), and Western blotting, respectively. The DNA-binding property of a nuclear protein was assessed by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay. An interesting result was that GCDC caused hepatocyte apoptosis to display a biphasic phenomenon at a dosage of 50 μM, whereas it was not found at higher dosages such as 200 μM. GCDC stimulated the expression of antiapoptotic Survivin, which also presented a biphasic response. The activation of nuclear factor-kappaB (NF-κB) corresponded with the up-regulation of Survivin. The inhibitor of NF-κB, BAY 11-7082, suppressed the expression of Survivin and simultaneously eliminated the biphasic response. The expression of Survivin was transcriptionally mediated by the activation of NF-κB, as shown by EMSA and ChIP assay. Conclusions: These results demonstrated that a low dosage of GCDC induced the hepatocyte apoptosis to exhibit the biphasic response, which was regulated by the expression of Survivin through NF-κB signaling pathway.

1. Introduction

Glycochenodeoxycholate (GCDC) is synthesized in the liver through conjugating glycine with chenodeoxycholic acid. Intracellular retention of cytotoxic GCDC contributes to apoptosis in hepatocytes [1–3]. GCDC induces hepatocyte apoptosis through both death-receptor pathway and the mitochondrial pathway [4,5]. Some pathways such as Fas death receptor, TRAIL-Receptor 2/DR5, protein kinase C, activation of cathepsin B, have been investigated [6,7]. Fas receptor ligation activates procaspase-8, which either directly causes activation of caspase-3 to induce apoptotic cascade or in turn cleaves the *bcl-2* family protein, bid, to a truncated form that translocates to mitochondria and initiates mitochondrial pathways through activation of downstream effector caspases [8,9]. In addition, GCDC induces hepatocyte apoptosis through Fas-independent mechanisms which could be demonstrated by the increased expression of TRAIL-R2/DR5 in HuH-7 cells (Fas deficient) with GCDC treatment. Mitochondrial cytochrome *c* release and apoptosis are inhibited by transfection with dominant negative FADD constructs, or treatment with the selective caspase-8 inhibitor [10].

Bile acids induce apoptosis of hepatocytes through oxidative stress and mitochondrial dysfunction *in vitro* [8]. Generation of reactive oxygen species, induction of mitochondrial permeability transition, and release of cytochrome *c* are critical steps in the induction of apoptosis by bile acids. GCDC-induced apoptosis was associated with lipid peroxidation as demonstrated by an increase in 8-isoprostane release. Antioxidants inhibited both lipid peroxidation and apoptosis [1]. Antioxidants reduced bile acid-induced liver injury through preventing the generation of oxidant stress, subsequent stimulation of the mitochondrial permeability transition, and release of cytochrome *c* from mitochondria [8]. *In vivo* cytokines are key mediators within the complex interplay of intrahepatic immune cells and hepatocytes, as they can activate effector functions of immune cells as well as hepatocytic intracellular signaling pathways controlling cellular homeostasis [11]. Kupffer cells and liver-infiltrating monocyte-derived macrophages may

Abbreviations: GCDC, glycochenodeoxycholate; NF-κB, nuclear factor kappa B; cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; XIAP, X-linked Inhibitor of Apoptosis Protein

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be the primary sources of cytokines such as TNFα, IL6, IL-1, and prostaglandin E_2 [12]. TNFα can activate specific intracellular pathways in hepatocytes that influence cell fate in different manners, e.g., proapoptotic signals via the caspase cascade, survival signals through NF-κB pathway [13]. NF-κB is a key player in the inflammatory pathway, e.g., in cytokine-mediated hepatocyte apoptosis [14].

NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens [15]. NF-κB is a vital regulator for oxidative stresses, which is well known for its role in inflammation, immune response, control of cell division and apoptosis [16]. The function of NF-κB is primarily regulated by IκB family members, which ensure cytoplasmic localization of the transcription factor in the resting state. Upon stimulus-induced IκB degradation, the NF-κB complexes move to the nucleus and activate NF-κB-dependent transcription [17]. NF-κB is an oxidative stress responsive transcription factor and plays a significant role in the process of apoptosis [18]. The activation of NF-κB belongs to intracellular survival pathway in the context of death-receptor-induced apoptosis in the liver. At present we partially understand the correlation between NF-κB and anti–apoptotic genes, such as cIAP1, cIAP2, XIAP, Survivin, A1/Bfl-1, and iNOS [19]. NF-κB regulates important functions in cholestatic liver pathology [14]. During obstructive cholestasis, NF-κB is activated in hepatocytes and functions to reduce liver injury [20]. NF-κB is also required for hepatocyte proliferation in bile duct ligated mice [21]. Moreover, NF-κB mediates the upregulation of iNOS that is further augmented by oxidative stress [22].

In an effort to understand the mechanisms through which GCDC induces hepatocyte apoptosis, we performed a direct comparison of the signaling response of caspase, NF-κB, and antiapoptotic genes. Primary hepatocytes were utilized in this study to pursue the following objectives: (i) the apoptotic effect of time- and dose-dependent alterations following GCDC treatment; (ii) the interaction of caspase, NF-κB, and antiapoptotic genes during GCDC-induced apoptosis. A striking result was that GCDC not only induced hepatocyte apoptosis, but also caused a biphasic response under low dosage of 50 μM. Moreover the biphasic response was correlated to the alteration of caspase activity, NF-κB activation, and Survivin expression. These findings indicate that NF-κB and Survivin are important signal factors that regulate GCDC-induced apoptosis.

2. Experimental procedures

2.1. Cell culture

Hepatocytes were isolated from adult Sprague–Dawley rats along the standard liver perfusion procedure [23] and cultured as previously described [24]. Dead cells were removed by Percoll (Sigma) gradient centrifugation. Following the last wash, hepatocytes were resuspended in cold William's E medium, diluted to a density of 5.5×10^5, and plated into collagen-coated dishes (Falcon). The cells were incubated at 37 °C for 6 h before GCDC treatment.

2.2. Caspase assay

Caspase activity was determined from the GCDC-treated hepatocytes. 100 μg of cell lysate was utilized to assay the activities of caspase-3 or caspase-8. Caspase assay kit was purchased from Calbiochem. The reaction system employed the colorimetric substrate IETD-*p*NA and calculated the activity as pmol/min.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was isolated with ready-to-use TRIZOL Reagent (Gibco, Invitrogen). RT-PCR kit was obtained from Qiagen. The cDNA product was amplified with Taq DNA polymerase using standard protocols. The 5′ forward and 3′ reverse complement PCR primers for Survivin were CTGATTTGGCCCAGTGTTTT and TCATCTGACGTCCAGTTTCG, respectively. For cIAP2, PCR primers were ACATTTCCCCAGC TGCCCATTC and CTCCTGCTCCGTCTGCTCCTCT. For cIAP1, PCR primers were CCAGCCTGCCCTCAAACCCTCT and GGGTCATCTCCGGGTTCCCAAC. For XIAP, PCR primers were CGCGAGCGGGGTTTCTCTACAC and ACCAGGCACGGTCACAGGGTTC. For A1/Bfl-1, PCR primers were ATCCACTC CCTGGCTGAGAACT and ACATCCAGGCCAATCTGCTCTT. For iNOS, PCR primers were CGAGGAGGCTGCCTGCAGACTGG and CTGGGAGGAGCTGATGGAGTAGTA. All PCR primers were synthesized from Integrated DNA Technology. The relative mRNA levels of Survivin were confirmed by real-time PCR. The data were normalized to the expression level of 18S rRNA.

2.4. TUNEL assay

TdT-FragEL™ DNA fragmentation detection kit was obtained from Calbiochem (#QIA33) and tissue slides were prepared according to instructions. Briefly, slides were treated with proteinase K (20 μg/mL) in 10 mM Tris–HCl (pH 8.0). Endogenous peroxidase was inactivated by incubating the sections for 5 min in 3% H_2O_2 at room temperature. Cells were incubated in a moisture chamber for 1 h at 37 °C with 3.0 μl TdT enzyme. The reaction was stopped by 0.5 M EDTA, detected by DAB, and counterstained by 0.3% mythyl green.

2.5. Immunoblot analysis

Protein samples were made from cold RIPA buffer, boiled for 10 min in Laemli sample buffer, resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies at dilution of 1:500–1000. Peroxidase-conjugated secondary antibodies were incubated at a dilution of 1:2000–3000. Bound antibody was visualized using chemiluminescent substrate (ECL; Amersham Biosciences) and exposed to Kodak X-Omat film. At least three independent experiments were performed.

2.6. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extracts were prepared as described by Dignam et al. with modification [25]. 4 μg of nuclear protein and 2 μg of the nonspecific competitor poly(dI·dC) were incubated in binding buffer (100 mM Hepes, pH 7.6, 5 mM EDTA, 50 mM (NH4)2 S04, 5 mM DTT, Tween 20, 1% (w/v), 150 mM KCl) with 20 fmol/μl of double-stranded DNA oligonucleotide containing an NF-κB consensus binding sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) that was labeled with digoxin using terminal transferase. For super gel shift, NF-κB p50 antibody (Santa Cruz sc-7178) was added into an oligonucleotide-protein complex.

2.7. Chromatin Immunoprecipitation (ChIP) Assays

The liver tissue was homogenized and then fixed at 1% Formaldehyde. The fixed liver tissue was resuspended in 2 ml Lysis Buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40, Protease Inhibitor Cocktail, pH 8.0). The crude nuclear extract was re-suspended in Lysis Buffer High Salt (1 × PBS, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS and Protease Inhibitor Cocktail), and subsequently sonicated at a power setting of 30%. NF-κB p50 and p65 antibodies (Santa Cruz) were applied for immunoprecipitation. Crosslinks were reversed by addition of 400 μl Elution Buffer (1% SDS, 0.1 M $NaHCO_3$) and 0.2 M NaCl overnight. 2 μl of ChIP DNA was amplified using a MyiQ Single Color Real-Time PCR Detection System (Biorad, Carlsbad, CA). The 5′ forward and 3′ reverse PCR primers were 5′-CGCAGAGCACGTGGGACCTG-3′ and 5′-GGCGACGACTGTAGCGCCTC-3′.

2.8. Statistical Analysis

All data represent at least three experiments using cells, or extracts from a minimum of three separate isolations and are expressed as means ± SD unless otherwise indicated. Statistical significance of differences is determined using either repeated measures for the ANOVA followed by Bonferroni correction or Student's *t*-test for independent samples. *P* values < 0·05 were considered significant.

3. Results

3.1. GCDC induced apoptosis in rat hepatocytes with biphasic phenomenon

Rat hepatocytes were cultured in William's E medium containing 10% FBS and treated with GCDC in different intervals. GCDC could induce apoptosis with biphasic phenomenon as shown by TUNEL assay (Fig. 1A, B). Apoptosis began at 2 h after incubating the cells with 50 μM GCDC. More cells died of apoptosis at 4 h and 14 h. Apoptotic rates were 21% at 4 h and 17% at 14 h, respectively. In addition, GCDC-induced apoptosis could be reflected by caspase activation. Caspases are divided into two groups, based on their assumed role in the apoptotic cascade. Long-prodomain caspase-8 and short-prodomain caspase-3 were chosen to estimate their involvement during GCDC-induced hepatocyte apoptosis [3]. Activities of caspase-3 and caspase-8 were significantly elevated (Fig. 1C, D). The time-course of caspase response showed the kinetic process of apoptosis in rat hepatocytes. Activities of caspase-3 and caspase-8 reached the highest levels at 2 h, then reduced gradually, and increased again at 14 h. A biphasic response was observed in the 2 h–14 h duration at the concentration of 50 μM GCDC. Western blotting further confirmed the protein expression of caspase-8 and caspase-3 (Fig. 1E, F). Quantitation of Western blots by using β-actin as the loading control was consistent with that of caspase enzyme kinetics (Fig. 1G, H). GCDC could induce hepatocyte apoptosis, which showed the biphasic response from 2 h to 14 h at the concentration of 50 μM GCDC.

3.2. The biphasic phenomenon was GCDC dosage-dependent

The biphasic response was found at the concentration of 50 μM GCDC, but we did not know whether the biphasic response would change as GCDC dosage was adjusted. Next, the experiments were designed to measure differences in apoptosis following the varying concentrations of GCDC. TUNEL assay, histological staining, DNA fragmentation assay, and caspase assay were utilized to evaluate apoptosis in rat hepatocytes. In fact, 200 μM of GCDC caused acute necrosis of primary hepatocytes. Necrotic cells broke into debris or underwent cell lysis (Fig. 2A, B). Microscopic viewing showed a large

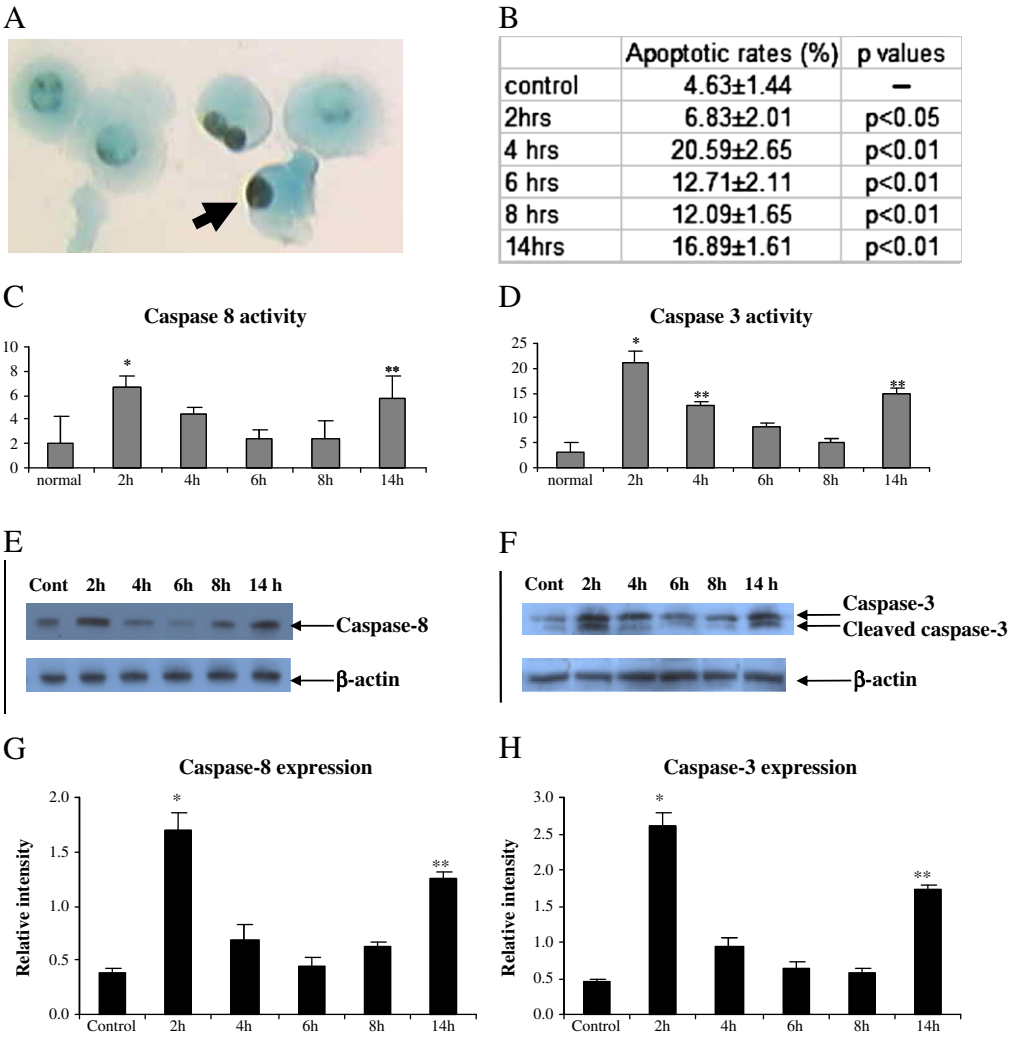

Fig. 1. Hepatocytes were exposed to 50 μM of GCDC. (A) TUNEL staining-GCDC treatment for 6 h. Arrow stands for nuclear condensation, a type of apoptotic cells. (B) Apoptotic rate (%). (C) Caspase-8 activity. 100 μg of cell lysates was utilized to measure enzyme activity (pmol/min), based on the concentration of *p*-nitroaniline in the calibration standard (50 μM). The extinction coefficient for *p*-nitroaniline is 10,500 M^{-1} cm^{-1}. Results are showed as mean ± SD for five independent experiments; $^{*}p<0.01$ and $^{**}p<0.05$ compared with control. (D) Caspase-3 activity. (E) Activated Casepase-8 protein by immunoblotting. (F) Activated Casepase-3 protein by immunoblotting. (G) Quantitation of Caspase-8 western blot signals over beta-actin expression. (H) Quantitation of Caspase-3 western blot signals over beta-actin expression.

amount of dead cells floating in the medium, which was similar to the previous observation [8,26]. Lactate dehydrogenase (LDH), the most widely used indicator of cellular necrosis, was significantly released in the treatment of 200 μM GCDC (Fig. 2C). At a concentration of 200 μM GCDC, DNA fragmentation assay demonstrated that hepatocyte apoptosis began at 2 h (Fig. 2D). Thereafter, hepatocyte apoptosis decreased from 4 h to 24 h. Activities of both caspase-3 and caspase-8 were also increased to the highest level at 2 h and reduced thenceforth with only one single peak individually (Fig. 2E, F). No biphasic response was found at the concentration of 200 μM GCDC. Clearly, the apoptotic pattern in hepatocyte was affected by different concentrations of GCDC. GCDC induced more necrosis at higher concentrations (200 μM) of GCDC than at lower concentrations (25 μM) of GCDC that was associated with the main apoptosis (data not shown). These data confirmed that the high concentration of GCDC caused more necrosis than apoptosis in the primary culture of rat hepatocytes. Thus the biphasic response of GCDC-induced hepatocyte apoptosis was dose (or concentration)-dependent.

3.3. Expression of antiapoptotic genes following GCDC treatment

After the stimulation by the 50 μM of GCDC, some hepatocytes survived apoptosis. It could be hypothesized that the up-regulated antiapoptotic genes accounted for the cell survival. To prove this hypothesis, total RNA was isolated from cells and RT-PCR or real-time PCR was performed to determine the levels of antiapoptotic genes. Since antiapoptotic genes are a big family, we only selected cIAP1, cIAP2, XIAP, Survivin, A1/Bfl-1, and iNOS for the current study. Through RT-PCR we distinguished no differences among cIAP1, cIAP2, XIAP, A1/Bfl-1 (Fig. 3A), but differences were found in iNOS and Survivin from 2 h to 14 h. iNOS gene had a high expression at 2 h and then decreased gradually to the lowest level at 14 h through an unknown mechanism. Survivin was increased at 4 h, decreased at 6 h, enhanced at 8 h, and maintained at the same level up to 14 h. The expression of Survivin showed biphasic response. Furthermore, the gene expression of Survivin was confirmed by real-time PCR that showed a similar pattern to RT-PCR (Fig. 3B). By Western blotting, protein expression of Survivin (Fig. 3C) displayed the same biphasic pattern as gene expression. The aforementioned data were gathered from the combination of both cells floating in medium and cells attached to the dish. To clarify expression of Survivin from dish-attached cells, we removed the cells floating in the medium, isolated RNA from dish-attached cells, and then re-ran RT-PCR or real-time PCR (data not shown). The results were exactly consistent, which means the expression of Survivin came mainly from dish-attached cells (Fig. 3D). GCDC could up-regulate intracellular, antiapoptotic Survivin that might modulate hepatocyte apoptosis.

3.4. GCDC stimulated the activation of NF-κB

GCDC induced the activation of NF-κB in the current model (Fig. 4A). Moreover, the activation of NF-κB generated a biphasic response in the time-course of 2 h to 14 h, which coincided with the pattern of

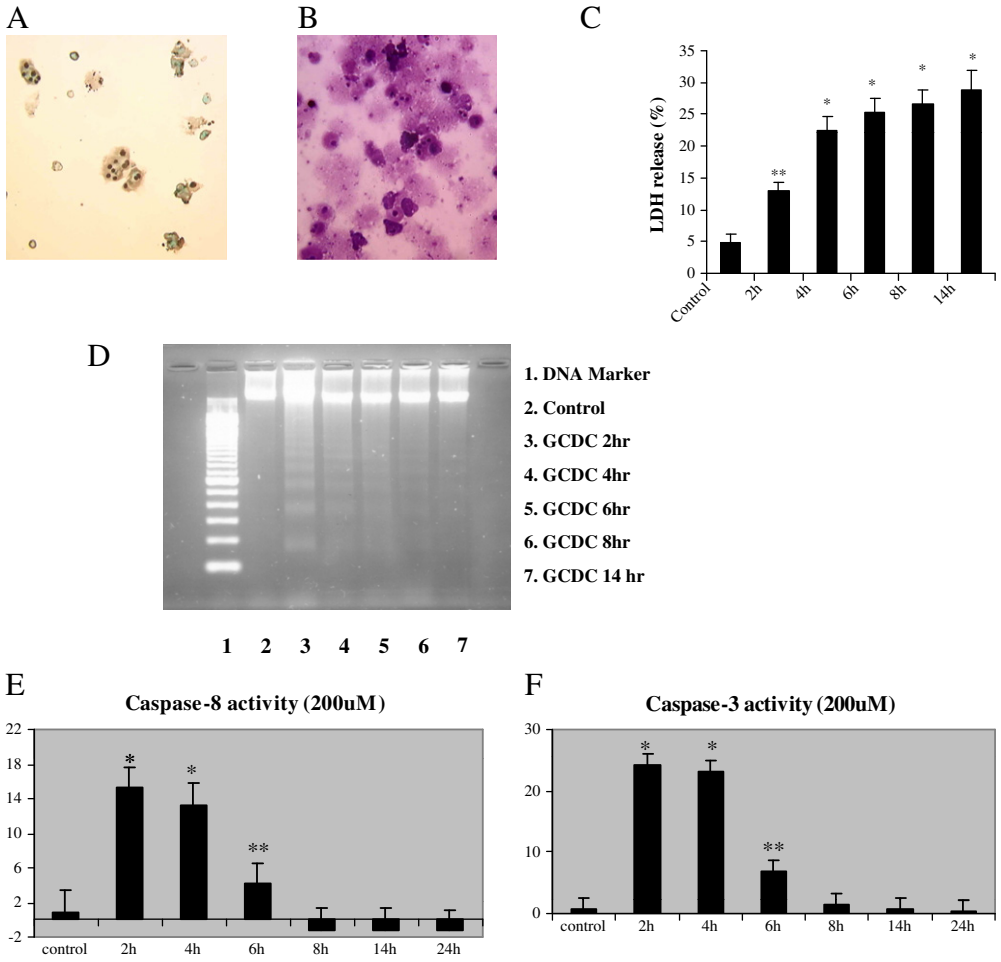

Fig. 2. Hepatocytes were treated with 200 μM of GCDC. (A) After GCDC treatment for 6 h, apoptotic and necrotic hepatocytes floating in medium by TUNEL assay. (B) Dead cells from 4 h medium were centrifugated and detected by H&E staining. (C) Effect of GCDC on LDH release. During the incubation of hepatocytes with or without GCDC, the release of LDH activity from cells was measured and expressed as the percentage of LDH released into buffer of the total activity present in hepatocytes. (D) DNA fragmentation assay. 10 μg of genomic DNA was run on 2% agarose gel. (E) Time-course of Caspase-8 activity. (F) Time-course of Caspase-3 activity. *$p<0.01$ and **$p<0.05$.

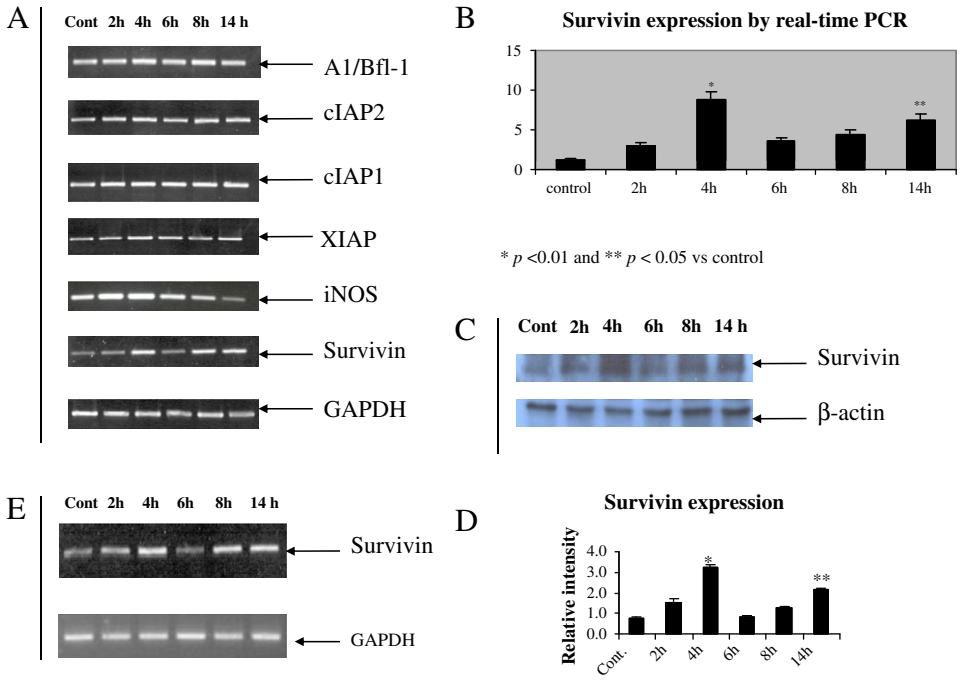

Fig. 3. (A) After the stimulation of 50 μM GCDC, the expression of cIAP1, cIAP2, XIAP, Survivin, A1/Bfl-1, iNOS, and GAPDH was determined by RT-PCR. (B) Survivin showed biphasic response as demonstrated by quantitative real-time PCR as well as RT-PCR. (C) By Western blotting, protein level of Survivin displayed the same biphasic pattern as that of gene expression. (D) The expression of Survivin mainly came from dish-attached cells.

hepatocyte apoptosis at the concentration of 50 μM GCDC. When a NF-κB inhibitor (30 μM of BAY 11-7082) was incubated with the primary hepatocytes, the biphasic activation of NF-κB was eliminated (Fig. 4B). Considering the expression of biphasic Survivin, it was speculated that GCDC-induced hepatocyte apoptosis was associated with the activation of NF-κB. The activated NF-κB further up-regulated the expression of Survivin. Perhaps because of the up-regulation of intracellular antiapoptotic Survivin in the G2-M phase hepatocytes, these cells could survive the GCDC-induced apoptosis. The surviving cells made a second apoptotic peak at 14 h under continuous treatment of 50 μM GCDC. NF-κB may be an important regulator that plays a pivotal role during GCDC-induced hepatocyte apoptosis.

3.5. Expression of Survivin mediated by NF-κB

The biphasic response was associated with NF-κB activation as well as Survivin expression. However, it was still unclear what the levels of antiapoptotic genes were when NF-κB was inactivated. By having employed 30 μM of BAY 11-7082 in culture medium for 60 min, hepatocytes were treated with 50 μM of GCDC in different intervals and then DNA, RNA, cell lysate, and nuclear proteins were collected. The biphasic activation of NF-κB was not found by gel mobility shift assay (Fig. 4B). Expression of antiapoptotic genes such as Survivin, XIAP, A1/Bfl-1, and iNOS was down-regulated with the lapse of incubation time, except iNOS and XIAP were up-regulated at 8 h with unknown significance (Fig. 5A). Western blotting for Survivin protein presented a similar pattern to its gene expression through RT-PCR (Fig. 5B). NF-κB inhibitor BAY 11-7082 could eliminate the biphasic phenomenon, as proven by DNA fragmentation assay (Fig. 5C). When total RNA was extracted from dish-attached cells, RT-PCR demonstrated that the antiapoptotic expression of Survivin was identical to that of the combination of both cells floating in medium and cells attached to dish (Fig. 5D, A). Morphological study by H&E staining and TUNEL assay showed that more apoptosis was

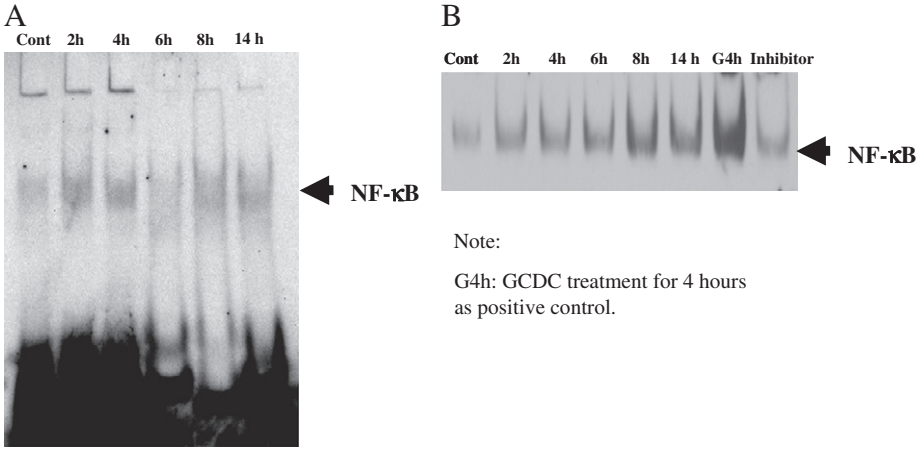

Fig. 4. GCDC stimulated the activation of NF-κB. (A) NF-κB activated by 50 μM of GCDC with a biphasic response. (B) The biphasic activation of NF-κB could be inhibited by 30 μM of BAY 11-7082.

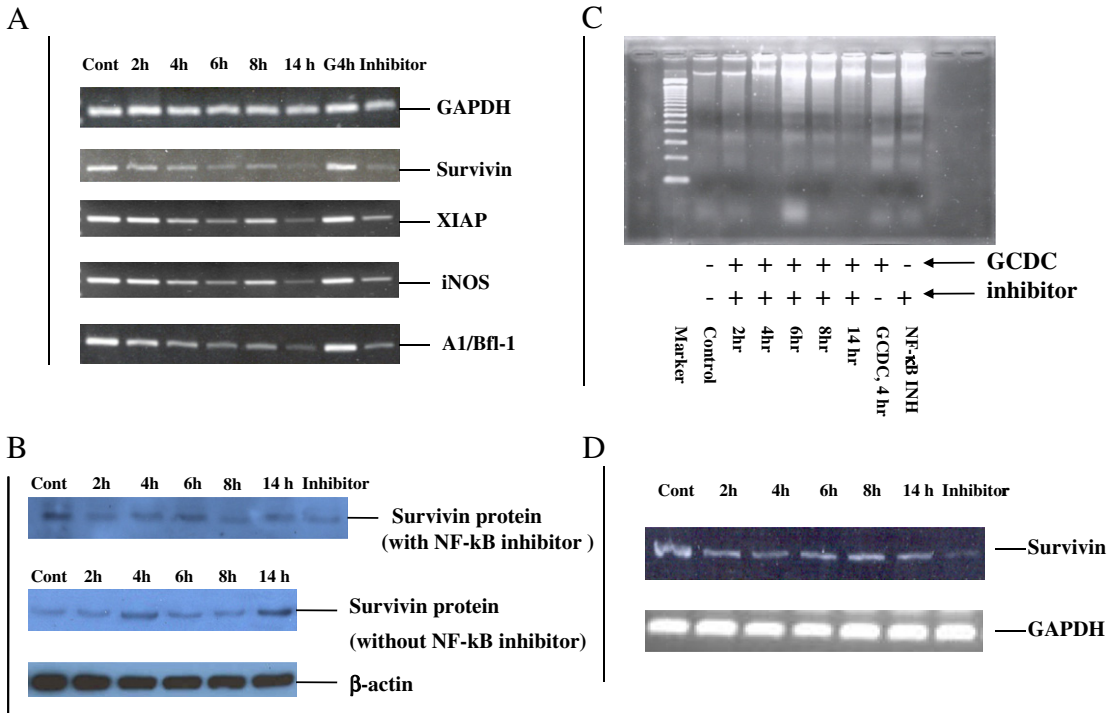

Fig. 5. NF-κB inhibitor eliminated the biphasic response. (A) By having employed 30 μM of BAY 11-7082 in the culture medium for 60 min, hepatocytes were then treated with 50 μM of GCDC in different intervals. Expression of GAPDH, Survivin, XIAP, iNOS, and A1/Bfl-1 was determined through RT-PCR amplification. G4h means GCDC treatment for 4 h as positive control. (B) Survivin protein was detected by Western blotting. (C) DNA fragmentation assay. 10 μg of DNA were run on 2% agarose gel. (D)Level of Survivin mRNA isolated from dish-attached cells was analyzed by RT-PCR.

found with NF-κB inhibitor BAY 11-7082 than without the NF-κB inhibitor (data not shown). NF-κB inhibitor BAY 11-7082 down-regulated the expression of antiapoptotic Survivin and eliminated the biphasic response of hepatocyte apoptosis at the concentration of 50 μM GCDC. NF-κB may have protected hepatocytes from apoptosis through the up-regulation of Survivin.

3.6. NF-κB was the transcriptional factor regulating the expression of Survivin

During GCDC-induced apoptosis in primary hepatocytes, NF-κB is activated at the same time. The activation of NF-κB may be a protective response although the exact mechanism is still under investigation [24]. In rat there are two potential binding sites of NF-κB in the Survivin promoter region, which are at −197 (site 1: GGGACTTTCC) and −149 (site 2: TTTCCAGAGG) upstream of the ATG initiator codon respectively. Moreover the site 2 is highly conserved in mouse Survivin promoter at position −158 bp (Fig. 6A). EMSA was carried out to investigate whether NF-κB nuclear protein could directly bind to Survivin promoter region, based on the differential mobility of free DNA and DNA protein complexes in non-denaturing polyacrylamide gel. Oligo probes using the sequences 5′-CTCATGGGGCGGGACTTTCCCGGCTGACCT-3′ (Oligo 1) and 5′-AAGGCGACTTTTTCCAGAGGGCGTGGCCTG-3′ (Oligo 2) were designed, in which the NF-κB binding consensus was underlined. By EMSA, the two *in vitro*-synthesized oligo probes could bind nuclear protein (Fig. 6B). The only difference was the intensity at which oligo probes can bind the nuclear protein. It appeared that the probe at −197 bp distal site had a higher affinity for NF-κB nuclear protein than the one at −149 bp site. When the NF-κB p50 antibody (Santa Cruz sc-7178) was added into oligo1-protein complex to identify NF-κB specific protein through super gel shift, binding of the antibody resulted in further retardation of the band through the gel (Fig. 6C). Through EMSA and super gel shift, we demonstrated that there existed DNA sequences in the Survivin promoter region that can be bound by NF-κB nuclear protein. In order to find direct evidence to confirm the relationship between NF-κB and Survivin, ChIP assay was performed to study the protein:DNA interactions in their native chromatin context. Following immunoprecipitated protein:DNA complexes, reversed the cross-links, and removed proteins, DNA fragments were extracted. Then the isolated DNA was analyzed by using primers designed to amplify a potential regulatory region. The ratio of NF-κB antibody/IgG was 6.6-fold ($P=0.0136$). Through combining the data from the EMSA, super gel shift, and ChIP assay, we concluded that Survivin was regulated by the NF-κB nuclear protein (Fig. 6D, E).

4. Discussion

Hepatocyte apoptosis, a distinct process of cell death, can be observed in such conditions as viral infection, alcoholic hepatitis, cholestatic disorders and so on [27]. GCDC causes hepatocyte apoptosis that can be reflected by morphological markers (e.g. TUNEL assay) and functional indicators (e.g. caspase assay). TUNEL assay relies on the presence of nicks in the DNA that can be identified by terminal deoxynucleotidyl transferase, an enzyme catalyzes the addition of dUTPs that are secondarily labeled with a marker [28]. TUNEL assay is a classical method for detecting DNA fragmentation that results from apoptotic signaling cascades. During apoptosis, caspases execute the disassembly of cellular components by proteolytic cleavage of a variety of substrates. Some of the caspases identify and cleave a specific peptide substrate, whereas others recognize the same peptide substrate to activate caspase cascades [29]. The caspase substrate cleavage causes the characteristic 'DNA ladder' in apoptotic cells, a key feature of programmed cell death that can be measured by DNA fragmentation assay. However, it is more difficult to quantify DNA fragmentation. In the current study both TUNEL and caspase assays were utilized as hallmarks of apoptosis to estimate their involvement during GCDC-induced hepatocyte apoptosis.

Our data and previous studies have demonstrated that some hepatocytes were still alive after treatment with 50 μM GCDC, which means these cells have the intrinsic ability to resist apoptosis [1,30,31].

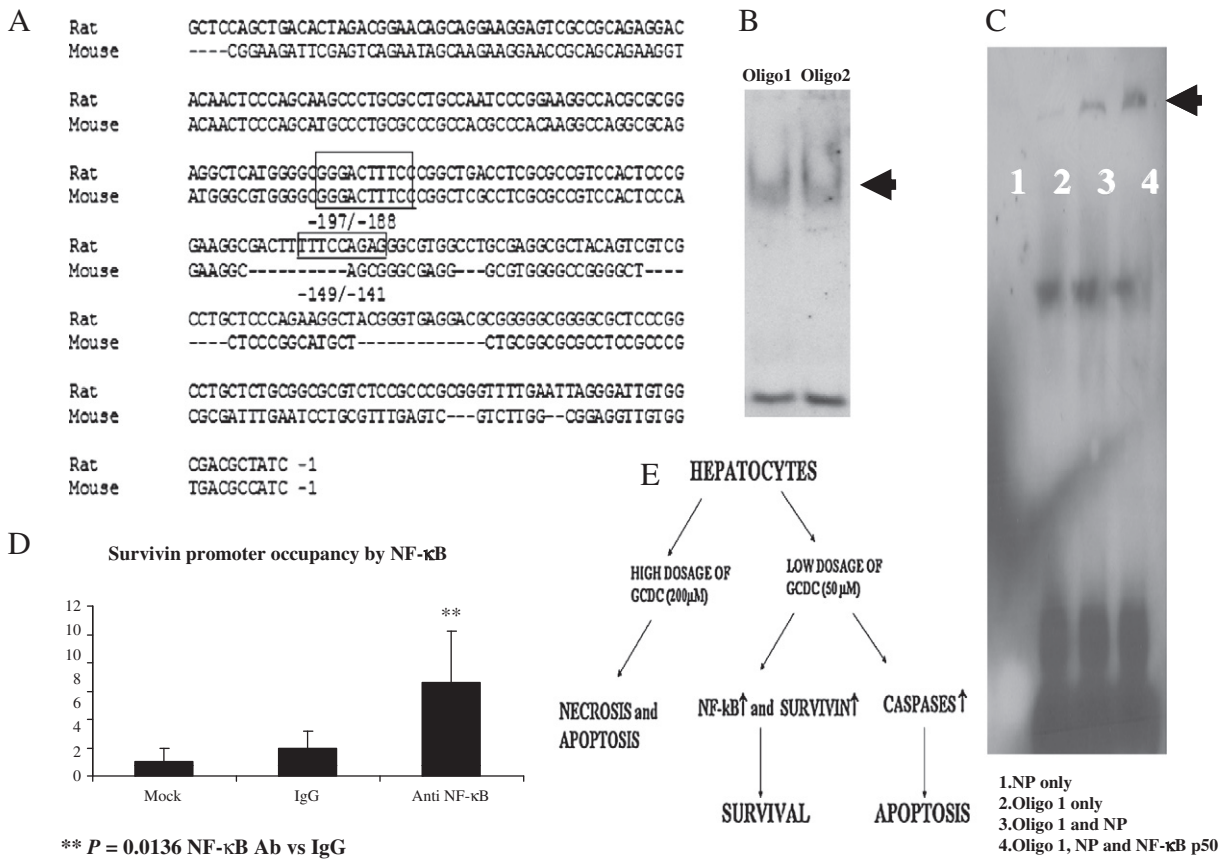

Fig. 6. Survivin is transcriptionally regulated by NF-κB. (A) Alignment of Survivin gene promoter between rat and mouse. (B) Two oligonucleotide sequences bearing NF-κB DNA-binding consensus in rat Survivin promoter. (C) DNA-binding property of NF-κB was assessed by EMSA and super gel shift. NP: Nuclear protein. (D) ChIP assay to access Survivin promoter occupancy by NF-κB. The ratio of NF-κB Ab/IgG was 6.6-fold ($P = 0.0136$). (E) Combined data from EMSA, super gel shift, and ChIP assay, it is concluded that Survivin is regulated by NF-κB nuclear protein.

From the anti-apoptotic family, we chose some members such as cIAP1, cIAP2, XIAP, Survivin, A1/Bfl-1, and iNOS to study their role during GCDC-induced apoptosis. There were no significant differences among cIAP1, cIAP2, XIAP, and A1/Bfl-1 except Survivin. The up-regulated Survivin protected the hepatocyte from GCDC-induced apoptosis at a dosage of 50 μM. If a high dosage (e.g. 200 μM) of GCDC was used, approximately 70% of the cells were dead within 4 h (Fig. 2). Yerushalmi et al. found the generation of reactive oxygen species played a significant role in high dosage necrosis [8]. The high dosage of GCDC probably caused a heavily acute necrosis that prevented the hepatocytes from amplification of anti-apoptotic genes such as Survivin. However in low dosages (e.g. 50 μM), cells had enough time to adjust anti-apoptotic levels to adapt to the environment. That might be the reason why the biphasic response of GCDC-induced apoptosis was only apparent at the concentration of 50 μM, not 200 μM. Therefore, the biphasic response was dose-dependent. Survivin might be responsible for the biphasic response during GCDC-induced hepatocyte apoptosis. This study will broaden our understanding of the mechanisms of apoptosis and its modulation, which are crucial factors for the liver injury/repair response toward the long term goal of designing future hepatoprotective strategies through potential use of the NF-κB/Survivin signaling pathway.

Survivin is the smallest member of the inhibitor of apoptosis protein family, which is expressed in several apoptosis-regulated fetal tissues, including lung, liver, heart, and gastrointestinal tract [32]. Survivin is a direct inhibitor of caspase-3 [33]. The activation of Survivin is critically required for suppression of GCDC induced-hepatocyte apoptosis [24,34]. Moreover Survivin is crucial for mitosis and cell cycle progression, which plays a significant role in the process of cell proliferation in human cancer [35,36]. Survivin is regulated by the cell cycle and its expression is found to be dominant only in the G2/M phase [36]. The biphasic response of hepatocyte apoptosis in our study may result from the surviving hepatocytes in the G2/M phase of the cell cycle. The additional evidence for Survivin regulating apoptosis can be found during angiogenesis [37]. Endothelial cells receive cues from growth factors to initiate mitosis, migration, and organization of endothelial cells into primitive angiotubes and patent vascular networks. Up-regulation of Survivin in endothelial cells protects endothelium from death-inducing stimuli. Furthermore, dominant negative Survivin negates the ability of Angiopoietin-1 to protect cells from undergoing apoptosis [38]. GCDC causes hepatocyte injury by oxidative stress, in which reactive oxygen species can act as signaling molecules to up-regulate the expression of Survivin. Up-regulation of Survivin can suppress apoptosis through two possible mechanisms. One is that it enables the hepatocytes to adapt to the GCDC-stressed microenvironment through amplification of Survivin-related genes [39]. This process of adaptation requires about 4 h or longer in our experimental system. The other is that the amplified Survivin directly inhibits activation of caspases since the hepatocytes in G2/M phase can express Survivin to survive the GCDC-induced apoptosis. Anyway, all of these speculations require further testing.

NF-κB is a critical regulator for gene expression induced by diverse stress signals that include the mutagenic and oxidative stresses [40]. The activation of NF-κB is likely to be involved in the induction of gene expression associated with hepatocyte adaptation [41]. This nuclear transcription factor appears to function both as a pro- and as an anti-apoptotic factor depending upon cell type and mode of stress [42]. Our study demonstrates the activation of Survivin plays an important role for hepatocyte to resist GCDC-induced apoptosis. The involvement of NF-κB is an essential component of the Survivin signaling pathway.

NF-κB could mediate hepatocyte apoptosis through up-regulation of Survivin. Our study also documented that NF-κB inhibitor (BAY 11-7082) could decrease Survivin expression and caused a significant increase in hepatocyte apoptosis. Schoemaker et al. had shown that bile acids did not activate NF-κB from 30 min to 1 h with 50 μM GCDC [43]. Rather the cytokines-activated NF-κB during cholestasis could protect hepatocytes against GCDC-induced apoptosis through the pathway of up-regulating A1/Bfl-1 and cIAP2 (4 days and 1, 2, 3 and 4 weeks). With differences, we harvested cells at 2 h, 4 h, 6 h, 8 h, and 14 h separately. The time frame was dissimilar from Schoemaker's study. Moreover, we did not find that the expression of both A1/Bfl-1 and cIAP2 was significantly up-regulated during 2 h–14 h treatment except Survivin (Fig. 3). More important, Schoemaker's results originated from in vivo study by employing Wistar rats subjected to bile duct ligation. Schoemaker's data had different conditions from our in vitro study. Thus, a further investigation needs to be performed to clarify the relationship between GCDC-induced hepatocyte apoptosis and NF-κB activation.

In summary, GCDC induced apoptosis in rat primary hepatocytes with a biphasic response. The biphasic response involved an interaction between the activation NF-κB and the expression of Survivin. The anti-apoptotic Survivin, regulated by NF-κB, might be a decisive factor of the biphasic response. NF-κB/Survivin is an essential signaling pathway that protects hepatocytes from GCDC-induced apoptosis.

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