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C/EBPα and C/EBPβ binding proteins modulate hepatocyte apoptosis through iNOS signaling pathway

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ABSTRACT

Inducible nitric oxide synthase (iNOS) and nitric oxide (NO) involve many pathophysiologic conditions. The expression of iNOS is regulated at multiple stages. Presently, the regulatory details of iNOS signaling are still unclear. This study aimed to investigate the regulatory role of C/EBPα and C/EBPβ in iNOS signaling pathway. By employing the techniques such as EMSA, ChIP assay, site-directed mutagenesis, and siRNA silencing, the relationship between iNOS and C/EBPα/C/EBPβ in rat hepatocytes was clarified. iNOS promoter was the direct transcriptional targets of the C/EBPα, C/EBPβ, and NF-κB binding proteins. There was the interactive influence between NF-κB and C/EBPα/C/EBPβ. The expression of iNOS was modulated by C/EBPα/C/EBPβ transcription factors. Moreover, the iNOS expression mediated glycochenodeoxycholate (GCDC)-induced apoptosis in hepatocytes. C/EBPα/C/EBPβ binding proteins could affect the GCDC-induced apoptosis through iNOS cascade. These findings indicate that C/EBPα and C/EBPβ regulate the iNOS expression, which may further modify cell responses such as apoptosis and cell survival.

1. Introduction

iNOS/NO plays an important role in the regulation of many physiological processes, such as in the immune system against pathogens and in the cardiovascular system to dilate blood vessels through the activation of guanylate cyclase [1,2]. In liver, the effect of iNOS/NO is hepatic-protective as demonstrated in sepsis and ischemia reperfusion [3]. The cytoprotective role of iNOS/NO can be best exemplified in a rodent model of endotoxemia. The addition of the iNOS inhibitors significantly increases hepatic damage [4]. The iNOS/NO is apoptosis-resistant. NO suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction [5]. NO protects hepatocytes from TNF-alpha-induced apoptosis and hepatotoxicity *in vivo* and *in vitro*. NO inhibits apoptosis through the increment in cyclic GMP formation and the suppression of caspase activity by the S-nitrosylation of the active site thiol [6]. The mitochondria-dependent apoptotic signaling pathway is also suppressed by the S-nitrosylation of caspase-8 and the inhibition of Bid cleavage by NO in hepatocytes [7]. Besides having anti-apoptotic function, NO is pro-apoptotic as well [8]. NO can increase TRAIL-induced cytotoxicity by facilitating the mitochondria-mediated caspase signal transduction pathway [9]. Obviously, iNOS/NO plays a considerable role under pathophysiologic conditions.

Although the expression of iNOS is involved in chronic liver disorders [10], regulation of iNOS expression in hepatocytes has not been addressed thoroughly. The regulation of iNOS expression occurs at multiple stages along the signaling pathway. Generally, the regulation of iNOS can be classified into upstream transcriptional factors and downstream posttranscriptional mechanisms. It is known that iNOS expression requires the transcription factor NF-κB [11]. The iNOS expression is down-regulated by steroids, TGF-β, the heat shock response, p53, and NO itself [4]. *In vivo* hepatic iNOS induction is differentially regulated by the typical acute-phase reactants [12]. The constitutively generated NO maintains the hepatic microcirculation and endothelial integrity. iNOS expression protects liver from apoptotic cell death in sepsis and hepatitis, whereas NO potentiates the hepatic oxidative injury in warm ischemia/reperfusion [13]. iNOS influences liver function in different degrees under a variety of pathophysiologic situation. iNOS/NO production can be either beneficial or detrimental. Accordingly, numerous mechanisms have evolved to regulate iNOS expression during hepatocellular injury. Study on iNOS/NO signaling may provide potential targets for the treatment of liver injury.

The iNOS/NO signaling regulates the GCDC-induced hepatocyte apoptosis, but some detailed links remain unclear. If iNOS is thought of as a middle point during GCDC-caused apoptotic process, what are the upstream transcriptional factors? What are the downstream targeting molecules? It is crucial to know how iNOS is regulated in hepatocytes. The current study emphasizes the relationship between iNOS expression and regulatory transcription factors. It has been known that NF-κB could bind the iNOS promoter to activate iNOS

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expression [11]. GCDC-induced hepatocyte injury could be modulated by other transcriptional nuclear factors such as AP-1, cJun, cFos, and so on [14]. However, we do not know whether there are other regulatory factors and how they function. Hepatocyte is a good cell type to study apoptosis, but still has some limitations, e.g. the primary culture of hepatocytes is hard to elucidate the interactions from *in vivo* hepatocytes, Kuffer cells, and endothelial cells. Additionally, the primary hepatocytes de-differentiate quickly and lose their ability to transport bile acids fast. Thus, the actual experiments that address GCDC-induced apoptosis in primary hepatocytes have to be performed within limited hours after cell isolation. This short time frame is not feasible for some experiments and increases difficulty in reproducibility.

To investigate the regulation of iNOS signaling, GCDC was utilized as an inducer to trigger apoptotic injury in rat hepatocytes. The iNOS expression was analyzed at different levels. Endogenous iNOS, transcriptionally regulated by C/EBPα and C/EBPβ, could modulate the GCDC-induced apoptosis in primary hepatocytes. C/EBPs — iNOS signaling pathway may be a future target for designing therapeutic intervention in liver disease.

2. Materials and methods

2.1. Hepatocyte isolation and culture

Hepatocytes were isolated from adult Sprague–Dawley rats along standard liver perfusion procedure and cultured as described previously [15,16]. Briefly, hepatocytes were isolated through collagenase perfusion, purified with repeated centrifugation, and followed by further purification over 30% Percoll. Highly purified hepatocytes (>98% viability) were suspended in Williams medium E containing 10% heat-inactivated calf serum. The cells were plated on collagen-coated dishes at a density of 5×10^5 and placed at 37 °C in 5% CO_2/95% air. The cell treatment included iNOS-specific inhibitor N-(3-(aminomethyl)benzyl)acetamidine (1400 W) (50 μM), NF-κb inhibitor BAY 11-7082 (30 μM), or GCDC (50 μM) as indicated.

2.2. Rat iNOS promoter plasmid construct

The rat iNOS-Lucif construct was made as described (GenBank accession number AF042085) [17]. A 2.1-kb fragment was amplified from Sprague–Dawley liver genomic DNA by PCR using the published primers (forward, 5′-CAG CCA AGT ATT CCA AAG CAA-3′; reverse, 5′-AGT CCA GTC CCC TCA CCA A-3′) and its identity was confirmed by DNA sequence analysis. This 2.1-kb fragment was ligated into the pGL4 basic luciferase vector for transient transfection. Renilla luciferase was used as the internal control from Promega. The QuikChange site-directed mutagenesis kit (Stratagene) was utilized to delete the exact NF-κb, C/EBPα, or C/EBPβ binding sites as described previously [18].

2.3. Transient transfection and luciferase assay

Transient transfection was accomplished using a kit from Qiagen according to the manufacturer's protocol. Subconfluent cultures of HepG2 cells (2×10^5) were transfected with 2 μg of each reporter iNOS-Lucif vector and a plasmid expressing the enzyme Renilla luciferase as an internal control. After overnight recovery, the cells were lysed using a commercially available kit (Promega). Firefly and Renilla luciferase activities were assayed by the use of the Dual Luciferase Reporter System (Promega). The firefly luciferase activity was normalized by Renilla luciferase activity present in each sample and expressed as -fold induction relative to control.

2.4. siRNA and transfection

The double stranded siRNAs against C/EBPα (GenBank accession no. NM_012524) or C/EBPβ (GenBank accession no. NM_024125) were consisted of pools of three target-specific 19–25 nt siRNAs in accordance with the Ambion web-based criteria. The scrambled siRNA, 5′-AUUGUAUGCGAUCGCAGAC-3′, was applied for nonspecific silencing effects [19]. Hepatocytes were seeded in 6-well plate at 5×10^5 cells for 6 h prior to transfection. The cells were transfected with 200 nM double-stranded siRNA and lipophilic transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA). The Williams E medium was replaced 16 h post transfection. The cells were exposed to 50 μM of GCDC and harvested in 4 h.

2.5. Caspase assay

100 μg of cell lysate was utilized to assay the activities of caspase-3. Caspase assay kit was purchased from Calbiochem. The reaction system employed the colorimetric substrate IETD-pNA and the caspase-3 activity was calculated as pmol/min [15].

2.6. TUNEL assay

The TdT-FragEL™ DNA fragmentation detection kit was obtained from Calbiochem (#QIA33), following instruction to perform TUNEL assay. Briefly, slides were treated with proteinase K (20 μg/mL) in 10 mM Tris–HCl (pH 8.0). Endogenous peroxidase was inactivated through incubating the sections for 5 min in 3% H_2O_2. The slide sections were covered in a moisture chamber for 1 h at 37 °C with 3.0 μl terminal deoxynucleotidyl transferase (TdT). The biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase conjugate. Diaminobenzidine reacted with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. After the sections were counterstained with 0.3% methyl green, the slides were then used in the morphological evaluation and characterization of normal and apoptotic cells.

2.7. Western blotting assay

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. β-actin was used as equal loading control.

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from hepatocytes with ready-to-use TRIZOL Reagent (Gibco, Invitrogen). cDNA was synthesized from 1 μg of total RNA by use of the cDNA synthesis kit (Qiagen, Valencia, CA). For qRT-PCR, gene was amplified with appropriate primer set in triplicate. Level of gene expression was calculated by a mathematical delta–delta method developed by PE Applied Biosystems. The level of gene expression was normalized to the expression level of 18S rRNA.

2.9. Electrophoretic mobility shift assay (EMSA)

DIG Gel Shift Kit (Cat. No. 03 353 591 910, Roche) was used for detecting sequence-specific DNA-binding proteins. Nuclear extracts were prepared with the modified Dignam protocol [15]. 4 μg of nuclear proteins and 2 μg of the nonspecific competitor poly [d(I–C)] or poly [d(A–T)] were incubated with 20 fmol/μl DIG-labeled oligonucleotides in binding buffer [100 mM Hepes, pH 7.6, 5 mM

EDTA, 50 mM $(NH4)_2$ S04, 5 mM dithiothreitol, Tween 20, 1% (w/v), 150 mM KCl]. Binding reactions were achieved through incubating the samples for 15 min at 22 °C. Protein–DNA complexes were separated from the unbound DNA probe by electrophoresis through 6% native polyacrylamide gels containing 0.5 × Tris borate/EDTA.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out as described [20]. Cells were homogenized, fixed in 10 ml 1% formaldehyde. The cell pellet 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%. For the immunoprecipitation, 4 μg of rabbit antiserum was used. Crosslinks were reversed on all samples, including 20% input, by addition of 400 μl Elution Buffer (1% SDS, 0.1 M $NaHCO_3$). DNA is extracted with an equal volume of phenol/chloroform/isoamyl alcohol. 2 μl of a total 50 μl ChIP-DNA was amplified using a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad).

2.11. Statistical analysis

All results represent at least three experiments using cells or extracts from a minimum of three separate isolations. Data are expressed as means ± SD unless otherwise indicated. Comparisons were performed using Student's *t* test or one-way analysis of variance followed by Bonferroni correction where appropriate. Differences were considered significant at the 95% confidence interval ($p<0.05$).

3. Results

3.1. iNOS gene promoter contains potential binding sites for C/EBPα, C/EBPβ, and NF-κB

iNOS gene promoter includes putative nuclear protein binding sites. Some binding sites such as NF-κB and stat-1α had been proved [21]. They can act on the iNOS promoter to regulate iNOS expression. Other potential binding sites and their function still need to be demonstrated. The iNOS promoter region embodies conservative DNA-binding domains. A similarity can be observed in the rat, mouse, and human genomes (Table 1). Within −2.1 kb of the rat iNOS promoter upstream of the transcription start site, there are 11 C/EBPα, 4 C/EBPβ, 1 C/EBPδ, 2 C/EBP-like (do not belong to any C/EBP subgroup), and 7 NF-κB potential binding sites, respectively. In this study we focused on the binding sites of C/EBPα and C/EBPβ, with the known NF-κb as a positive reference. Two rat NF-κB potential binding sites, with the DNA sequences being gggactctcc (−104/−95) and ggggactctc (−105/−96), are overlapped in only one nucleotide difference. Another two NF-κB potential binding sites, with sequences gattttccct (−960/−951) and gggattttcc (−962/−953), have two nucleotide difference. All of them are conserved in the mouse. The similar overlap zone occurs in rat C/EBPα potential binding sites with sequences tgacgtaata (−169/−160) and gtgacgtaat (−170/−161), but sequences tgtcaatatt (−921/−912) and actttcataa (−909/−900) are connected in series two nucleotides apart. The C/EBPα potential binding sequence tgtcaatatt is completely conservative within rat, mouse, and human genomes. iNOS promoter has potential binding sites for C/EBPα, C/EBPβ, and NF-κB, which are evolutionarily conserved. Some binding motifs may be regulators of the iNOS expression.

3.2. C/EBPα, C/EBPβ, and NF-κB can bind iNOS promoter as demonstrated by EMSA

The multiple binding sites for C/EBPα, C/EBPβ, and NF-κB were found in rat iNOS promoter region. We only chose the potential binding domains with high conservation and near to the transcription start site for the following investigation. EMSA was run to examine whether these nuclear proteins could directly bind iNOS promoter regions, based on the differential mobility of free DNA and DNA-protein complexes in non-denaturing polyacrylamide gel. Oligonucleotide probes contained the identical sequences for C/EBPα, C/EBPβ, and NF-κb binding sites were designed and optimized (Table 2). The probe sequences are 5′-CCTACTGGGGACTCTCCCTTTG-3′ (NF-κb, -111/−90), 5′-CTGTCAA-TATTTCACTTTCATAAT-3′ (C/EBPα, −922/−899), and 5′-CAGTGACTT-TATGCAAAACAGCTC-3′ (C/EBPβ, −78/−69), in which the iNOS binding consensus was underlined. Results showed all of these *in vitro*-synthesized probes could bind nuclear proteins (Fig. 1). When consistent antibody was added into probe/nuclear protein complex, the binding signal was further inspected with gel super shift assay. The gel super shift revealed similar results to EMSA (data not shown). Through EMSA and gel super shift assay, we demonstrated there existed specific DNA sequences in iNOS promoter to bind C/EBPα, C/EBPβ, or NF-κB proteins, which suggested that the expression of iNOS might be mediated by C/EBPα, C/EBPβ, or NF-κB nuclear proteins.

Table 1
The potential nuclear binding sites may be regulators of iNOS expression. DNA-binding domains are identified in the rat, mouse, and human iNOS promoter regions.

Name	Total	Sequence[a]	Position in rat[b]	In mouse[c]	In human[d]
C/EBPα	11				
		tcttttccaa	−1782/−1773		
		gatgatgagt	−995/−986		
		tgtcaatatt	−921/−912	−963/−954	−440/−431
		actttcataa	−909/−900	−951/−942	
		aggaaattat	−867/−858		
		ttgtttgttt	−844/−835	−884/−875	
		ctttttttcc	−441/−432		−1155/−1146
		ctgcaaatga	−379/−370		
		gtgacgtaat	−170/−161		
		tgacgtaata	−169/−160		
		ttgcacacc	−119/−110	−133/−125	
C/EBPβ	4				
		aagcaaagta	−2082/−2073		
		actatctcac	−1236/−1227		
		tgtgccacag	−542/−533		
		tttatgcaaa	−78/−69		−68/−59
C/EBPδ	1				
		aaatgagggt	−576/−567		
C/EBP-like	2				
		tttggaagca	−1972/−1963		
		atttggaacc	−455/−446		
NF-κB	7				
		gtggctttcc	−1746/−1737		
		gggattttcc	−962/−953	−1004/−995	
		gattttccct	−960/−951	−1002/−993	
		tggaaaatcc	−899/−890		
		ggaaaatccc	−898/−889		
		ggggactctc	−105/−96	−119/−108	
		gggactctcc	−104/−95	−118/−109	

[a] Lower case letters represent the putative DNA sequence of iNOS promoter that is potentially bound by nuclear factors.
[b] Rat iNOS accession number NM_012611.2.
[c] Mouse iNOS accession number NM_010927.2.
[d] Human iNOS accession number NM_000625.4.

3.3. iNOS promoter is direct transcriptional targets of C/EBPα, C/EBPβ, and NF-κB as determined by ChIP assay

In order to find more evidence about the relationship between iNOS and nuclear proteins, ChIP assay was carried out to determine

Table 2
Oligonucleotide probes for electrophoretic mobility shift assay.

C/EBP α	CTGTCAATATTTCACTTTCATAAT
C/EBP β	CAGTGACTTTATGCAAAACAGCTC
NF-κb	CCTACTGGGGACTCTCCCTTTG

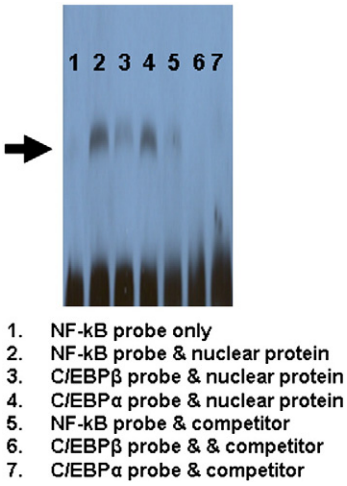

Fig. 1. Through the electrophoretic mobility shift assay, the three *in vitro*-synthesized oligonucleotide probes could bind identical nuclear proteins.

nuclear protein/DNA binding ability in their native chromatin context. Hepatocytes were homogenized in sterile cold PBS and fixed in formaldehyde to form protein:DNA complexes. The chromatin was then mechanically sheared by sonication to generate small protein: DNA fragments. These fragments were immunoprecipitated with antibodies specific for the nuclear protein or rabbit IgG as negative control. Immunoprecipitated protein was subjected to Western blot analysis to test whether the protein was antibody-specific (Fig. 2A). After reversing the cross-links and removing proteins from the immunoprecipitated protein:DNA complexes, the DNA samples were isolated. The purified DNA was analyzed by primers to cover potential regulatory regions (Table 3). The relative ratios from specific antibody/IgG could reflect the results of ChIP assay for NF-κb, C/EBPα, and C/EBPβ, respectively (Fig. 2B, C and D). The expression of iNOS could be modulated with C/EBPα, C/EBPβ, and NF-κB binding proteins.

3.4. Site-directed mutagenesis of NF-κB, C/EBPα, and C/EBPβ binding sites in the iNOS promoter

To confirm the differential effect of every potential binding site on the iNOS promoter, a specific site was deleted and then the activity of the mutated iNOS promoter was measured with luciferase reporter assay. Site-directed mutagenesis at NF-κb, C/EBPα, or C/EBPβ binding sites were performed in the iNOS promoter and designated as mt-NF-κb, mt-C/EBPα-A, mt-C/EBPα-B, mt-C/EBPα-AB, and mt-C/EBPβ, respectively. The deleted DNA fragments in the iNOS promoter region were exactly identical to the sequence of C/EBPα, C/EBPβ, or NF-κB binding sites (Table 4). For the luciferase assay, HepG2 cells were transfected with 2 μg of each luciferase vector for 36 h. The deletions of NF-κb, C/EBPα, or C/EBPβ binding sites significantly altered iNOS promoter activity in different degrees (Fig. 3). According to data from EMSA, super gel shift, ChIP assay, and site-directed mutagenesis, it is concluded that iNOS is regulated by transcription factor C/EBPα, C/EBPβ, and NF-κB.

3.5. Effect of NF-κB inhibition

Because the deletion of NF-κb binding site significantly decreased iNOS promoter activity (Fig. 3), we therefore treated the primary hepatocytes with NF-κb inhibitor BAY 11-7082 to investigate the role of NF-κb following the utilization of the NF-κb inhibitor. The BAY 11-7082 decreased NF-κb-DNA binding activity as demonstrated through EMSA with oct1 as control (Fig. 4A). Also, the activation of NF-κb could be indirectly represented by IκB degradation as shown by Western blotting of IκBα protein (Fig. 4B). When NF-κb was brought down, the expression of iNOS was down-regulated as proved through qRT-PCR and Western blotting (Fig. 4C and D). These results clearly showed that NF-κB was a mediator for the expression of the iNOS in rat hepatocytes. Moreover, NF-κB inhibitor BAY 11-7082 not only reduced iNOS, but also altered C/EBPα- and C/EBPβ-DNA binding abilities (Fig. 4E). Levels of C/EBPβ and C/EBPα were measured with real-time PCR (Fig. 4F and G). The BAY 11-7082 could up-regulate C/EBPα, but down-regulate C/EBPβ expression through an unknown

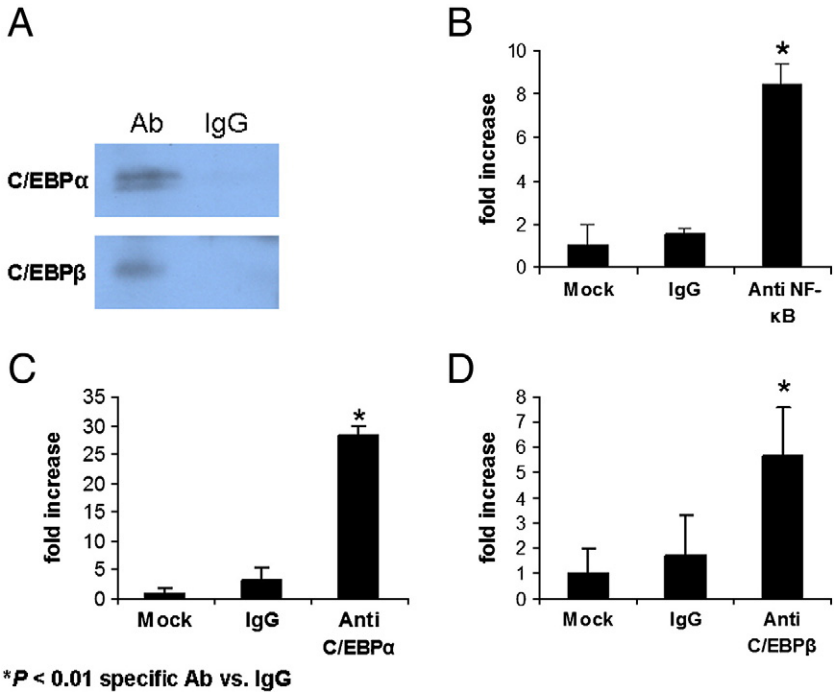

Fig. 2. A. Immunoprecipitated protein was subjected to Western blot analysis. B, C, D. The relative ratios from specific antibody/IgG could reflect the results of ChIP assay for NF-κb, C/EBPα, and C/EBPβ, respectively. *P<0.01 specific antibody vs. IgG antibody.

Table 3
Primers for ChIP assay.

NF-kB	Forward	TGGGGACCAGGAAGAGGTGGC
	Reverse	GCTGTGGCCCTGACAGTAGCC
C/EBP α	Forward	GTGGACCCTGGCGGGATATGC
	Reverse	ACAGATCCCTGAGTTTGGGCCAGT
C/EBP β	Forward	GAGGCCACTCGCTGCCAAGG
	Reverse	GTCCCGTGGGGCCAGAGTCT

Table 4
The deleted codes for site-directed mutagenesis in rat iNOS promoter.

NF-κb	GGGGACTCTC
C/EBPβ	TTTATGCAAA
C/EBPα-A	TGTCCATATT
C/EBPα-B	TTGCACACC
C/EBPα-AB	TGTCAATATT and TTGCACACC

mechanism. It appeared that there were interactions between NF-κb and C/EBPα or C/EBPβ.

3.6. iNOS expression following the transfection of C/EBPα and C/EBPβ siRNAs

As C/EBPα or C/EBPβ was silenced by individual siRNA (Fig. 5A), iNOS was up-regulated by C/EBPα siRNA and down-regulated by C/EBPβ siRNA (Fig. 5B and C). We do not know what causes these opposite effects subsequent to the transfection of C/EBPα and C/EBPβ siRNAs at present. A further investigation is under way, in order to clarify how C/EBPα/C/EBPβ to regulate the iNOS expression.

3.7. C/EBPα and C/EBPβ can regulate GCDC-induced apoptosis

GCDC induced apoptosis in hepatocytes. The iNOS level modified the profile of GCDC-induced hepatocyte apoptosis as demonstrated by the iNOS-specific inhibitor 1400 W (Fig. 6A and B). Because the expression of iNOS was stimulated by the transfection of C/EBPα siRNA and reduced by C/EBPβ siRNA (Fig. 5B and C), we thus supposed that C/EBPα/C/EBPβ might affect the apoptotic process of GCDC-treated hepatocytes through the iNOS pathway. To demonstrate this hypothesis, we treated hepatocytes with iNOS inhibitor 1400 W (50 μM) or transfection of C/EBPα or C/EBPβ siRNA for 16 h. Thereafter hepatocytes were challenged with GCDC for another 4 h. The hepatocyte apoptosis was reflected with caspase-3 activity and TUNEL assay (Fig. 6C, D, E and F). As expected, the transfection of C/EBPα or C/EBPβ siRNA could alter the GCDC-induced apoptosis. The effects of

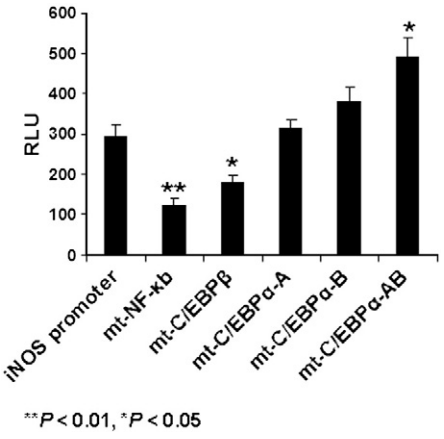

Fig. 3. The HepG2 cells were transfected with various iNOS promoter constructs. Intracellular luciferase activities were determined and normalized to the activity of control vector. Data represent mean ± SD of four different experiments. *P<0.05; **P<0.01 deletion vs. non-deletion.

C/EBPα or C/EBPβ siRNAs were eliminated by iNOS inhibitor 1400 W. Through the iNOS signaling pathway, C/EBPα/C/EBPβ played a regulatory role during the GCDC-induced apoptosis in hepatocytes.

4. Discussion

Our data demonstrate the endogenous iNOS is regulated by *cis*-acting elements such as C/EBPα, C/EBPβ, and NF-κB binding sites. C/EBPα down-regulates the iNOS expression, whereas C/EBPβ up-regulates the iNOS expression. The molecular basis for this observation remains unknown. The inhibition of NF-κB mediates the expression of C/EBPα and C/EBPβ. There is a certain relationship between NF-κB and C/EBPα/C/EBPβ transcription factors, but the mechanism needs to be scrutinized. Both C/EBPα and C/EBPβ binding proteins modulate the GCDC-induced apoptosis in hepatocytes through the iNOS signaling pathway. We do not know if C/EBPs — iNOS is the only pathway to regulate the GCDC-induced hepatocyte apoptosis. Considering the complicated regulation and various effects of C/EBPs [22], no simple rule governs the mechanism of transcriptional regulation in iNOS expression. The pathway of C/EBPs — iNOS may be one connection of multiple regulatory networks. Furthermore, the anti-apoptotic ability of the endogenous iNOS is limited. The high concentration of GCDC or low concentration of GCDC in long time exposure can completely neutralize the resistance of the endogenous iNOS. A continued advance will yield new insights on iNOS role during hepatocyte apoptosis.

The iNOS expression is controlled by a variety of mechanisms that can be classified into one of these four categories: transcriptional, posttranscriptional, translational, and posttranslational [23]. Nuclear transcription factors such as C/EBPs bind to specific base sequences within regions of the promoter and the enhancer to mediate gene transcription. The coordinated expression of different C/EBPs constitutes a critical component of the tissue-specific and cell stage-specific functions [24]. Modular protein C/EBPs were consisted of an activation domain, DNA binding domain, and leucine zipper dimerization region. C/EBPα and C/EBPβ are made of multiple polypeptides [25]. All C/EBP proteins contain a highly conserved C-terminal basic region-leucine zipper (bZIP) DNA binding domain, which can bind as homo- or hetero-dimers to certain promoters and enhancers. The bZIP domain via the adjacent basic regions also mediates a large number of the protein–protein interactions [26]. The N-terminal domains of C/EBPs are rather diverse, which are involved in contact with the basal transcriptional apparatus (TBP/TFIIB), transcriptional coactivators (CBP/p300), and sumoylation [27–29]. C/EBPα and C/EBPβ have multiple functions. C/EBPα binds to the promoter to facilitate the expression of the genes such as leptin, a protein that plays an important role in body weight homeostasis [30]. Also, the C/EBPα can interact with CDK2 and CDK4, thereby inhibiting these kinases and causing growth arrest [31]. The co-crystal structure of the C/EBPα bZIP domain is a continuous α-helix with residues 286–300 entering the major groove to make direct contact with the base pairs and the phosphate backbone [32]. Phosphorylation of C/EBPα serine 21 by extracellular signal-regulated kinase suppresses its activity [33]. Constitutive expression of C/EBPβ is highest in the liver and lung and is also detectable in other tissues such as kidney, heart, and spleen. Stimulation with LPS, IL-6, or IL-1 strongly induces C/EBP expression in cultured cells, suggesting a potential C/EBP role in the mediation of the inflammatory response [34]. The transcription factors search along the DNA strand for promoter regions and the enhancer region, which bind and create a special cubic structure. Thereafter, this molecular conformation is recognized by RNA polymerase [35]. The transcription factors are imperative for RNA polymerase to attach, which is an important mechanism to regulate the gene expression. A lot of transcription factors have been discovered; each recognizes and binds with a specific nucleotide sequence in the DNA [36]. A specific combination of transcription

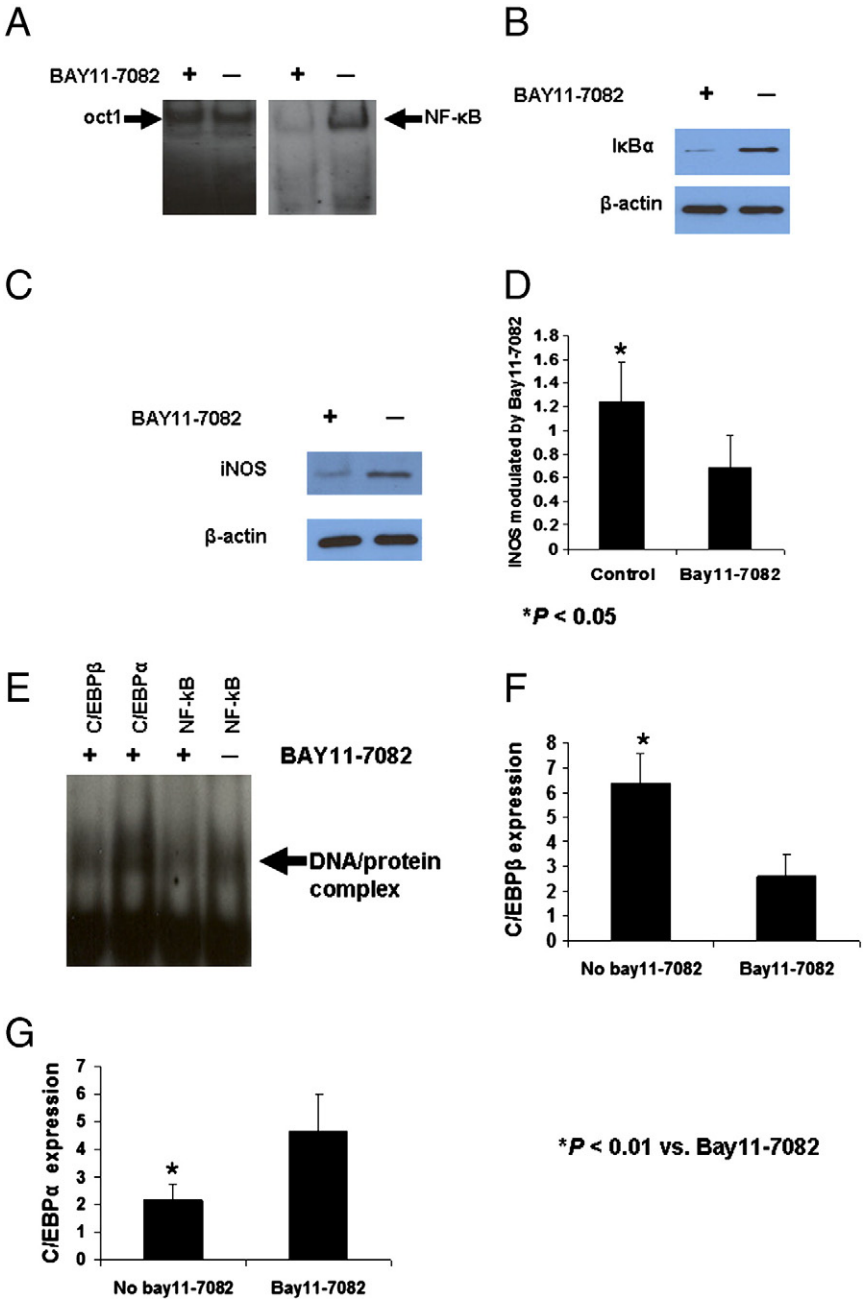

Fig. 4. A. The BAY 11-7082 decreased NF-κb-DNA binding activity as demonstrated through EMSA with oct1 as control. B. The activation of NF-κb could be indirectly represented by IκB degradation as shown by Western blotting of IκBα protein. C, D. After NF-κb was inhibited, the expression of iNOS was down-regulated as proved with Western blotting and qRT-PCR, *P<0.05. E. NF-κB inhibitor BAY 11-7082 reduced iNOS as well as altered C/EBPα- and C/EBPβ-DNA binding abilities. F, G. Expression of C/EBPβ and C/EBPα were measured with real-time PCR following BAY 11-7082 treatment. *P<0.01 no BAY 11-7082 vs. Bay11-7082.

factors is necessary to activate a gene. C/EBPs are classical transcription factors. Their function has been investigated in other cells, such as adipocytes, myeloid cells, lung epithelium, and so on [37,38]. This study uncovers C/EBPα has an opposite effect caused by C/EBPβ in the regulation of iNOS expression. Perhaps C/EBPα can work with the binding motifs of iNOS promoter to inhibit the attachment of RNA polymerase, whereas C/EBPβ stimulates the attachment of RNA polymerase and triggers an activation of transcription. Certainly, this hypothesis needs supports from the alternation of molecular conformation.

iNOS is included in complex immunomodulatory and inflammatory mechanisms [39]. iNOS expression can be induced in many cell types with suitable agents such as bacterial lipopolysaccharides, cytokines, and other compounds. iNOS can also be inhibited by a wide variety of immunomodulatory compounds [40]. iNOS is regulated very tightly. Modulation on both the transcriptional and post-transcriptional level is the major regulation mechanism for iNOS expression [41]. *Cis*-controlling elements and transcription factors are associated with iNOS expression. In the human iNOS promoter, mutation of a C/EBPβ-binding site within the region from −365 to the transcription initiation site markedly decreased the promoter activity [42]. C/EBPβ participated in DNA-protein complex formation and involved the activation of the human iNOS promoter by IL-1β [43]. Changes in the relative expression of different C/EBPs may affect global changes in hepatocyte gene expression, which can promote or inhibit hepatocyte survival. C/EBPβ is important in modulating the expression of various acute phase response genes such as iNOS. The activity of iNOS/NO is increased in hepatocytes during the mid-late

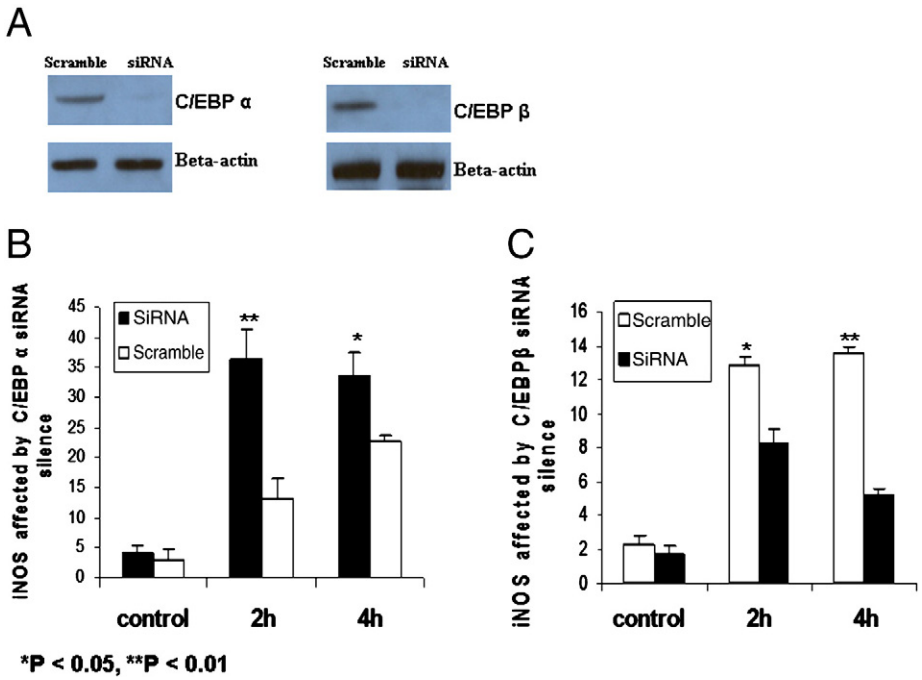

Fig. 5. A. Effective knockdown of C/EBPα or C/EBPβ was confirmed by Western blot analysis. B, C. The levels of iNOS were determined subsequent to C/EBPα or C/EBPβ siRNA treatment. *P<0.05; **P<0.01 scrambled RNA vs. siRNA.

pre-replicative period following partial hepatectomy [44]. NO has been shown to inhibit caspase-3 activation by TNFα [6]. Considering the pivotal role of caspase-3 in apoptosis, C/EBPβ-mediated NO production may be one of the mechanisms that prevent injury-related cytokines from causing hepatocyte apoptosis [7]. Our data about C/EBPs and iNOS were derived from rat primary hepatocytes, but these results were similar to those in other tissues or cells. Vascular endothelium participates in the control of vascular tone and function. iNOS expression, following induction by lipopolysaccharide or cytokines, results in endothelial cell activation and dysfunction. In rat pulmonary microvascular endothelial cells, C/EBP sites within the −205/+88 region are responsible for induction of the iNOS promoter.

There is an intricate interaction between C/EBPs and NF-κB. Mutation results show that mt- NF-κB and mt- C/EBPβ decreased the luciferase activities and mt- C/EBPα-AB significantly increased the promoter activity. However single mutation mt-C/EBPα-A or mt-C/EBPα-B did not alter the promoter activity. These data reveal the iNOS promoter has multiple *cis*-acting elements. Theses regulatory sites are interrelated and interact with each other to balance iNOS expression. Single C/EBPα mutation does not change promoter activity, suggesting either the presence of compensatory mechanisms masking the importance of C/EBPα loss or the likelihood that some C/EBPα sites are not essential to the promoter function. Two or more simultaneous mutations in C/EBPα sites can cause an alternation of the molecular conformation or cubic structure [38,43,45]. It is hardly surprising to get a substantial change in iNOS promoter function. In another study, mutations of the C/EBPβ- and NF-κB-binding site significantly decreased the iNOS promoter activity, but the iNOS promoter was activated subsequent to transfection of C/EBPβ liver-enriched activator protein and NF-κB. C/EBPβ stimulates iNOS gene expression synergistically with NF-κB in primary rat hepatocytes [42]. The human iNOS promoter is activated by IL-1β, which are regulated by C/EBP along with NF-κB binding sites in rat pulmonary microvascular endothelial cells [43]. Signaling pathways of iNOS expression vary in different cells or species, but activation of NF-κB and thereby activation of the iNOS promoter seems to be an essential step for the iNOS induction [21]. The interaction between C/EBPs and NF-κB is also seen in other tissues. C/EBPβ and NF-κB cooperatively bind and activate the IL-6, IL-8, G-CSF, serum amyloid, intercellular adhesion molecule 1, superoxide dismutase and Mediterranean fever promoters during the inflammatory response in myeloid and other cells [26]. C/EBPα or C/EBPβ is interacted with translated p50 or p65, mapping to the bZIP domain of C/EBP and to the Rel homology domain of p65 [46]. C/EBPα or C/EBPβ works with NF-κB p50 to induce bcl-2 transcription and inhibit apoptosis in hematopoietic cells [47]. A further study is still needed to elucidate the interaction between NF-κB and C/EBPs in hepatocytes.

Hepatocyte apoptosis is an essential change in hepatic pathology following various etiological factors such as viruses, alcohol, cholestasis, etc. [48]. For the treatment of hepatic injury, an effective method is to decrease or inhibit apoptosis in hepatocytes. C/EBP binding proteins could affect the hepatocyte apoptosis through the iNOS signaling pathway, which provided an important target to block apoptosis. However, this is a complicated process. The role of C/EBPs is required to be demonstrated *in vivo* study, because of complex regulation and the multiple effects of C/EBPs. Liver is rich with C/EBPs. Liver function is influenced by the levels of C/EBPs [49]. It is likely to improve the liver function through an intervention of C/EBPs — iNOS pathway. The present data show a possibility to link the basic research with the clinical relevance. GCDC-induced hepatocyte injury occurs in many hepatic diseases such as cholestatic disorder, hapertitis, primary sclerosing cholangitis, and so on [18], but the current treatments are less effective in clinical practice. The study on C/EBPs — iNOS signaling in hepatocytes aims at finding an effective approach for the treatment of toxic bile-induced liver injury. Several studies have provided useful information. Hypoxia enhanced rat iNOS promoter activity, but this hypoxia-induced promoter activity was abolished by the disruption of a C/EBP motif at −910 through substitution of GG for AA at −904 to −903 as well as deletion of −914 to −905 [50]. Human iNOS gene was transcriptionally regulated by IL-1β in rat pulmonary microvascular endothelial cells. C/EBP sites located downstream of −205 are involved in the activation of the human iNOS promoter by IL-1β [43]. The silencing of the C/EBPβ binding site may regulate inflammatory/immune response through the iNOS pathway.

The present data were obtained from rat primary hepatocytes. These results need to be further confirmed by means of *in vivo* animal

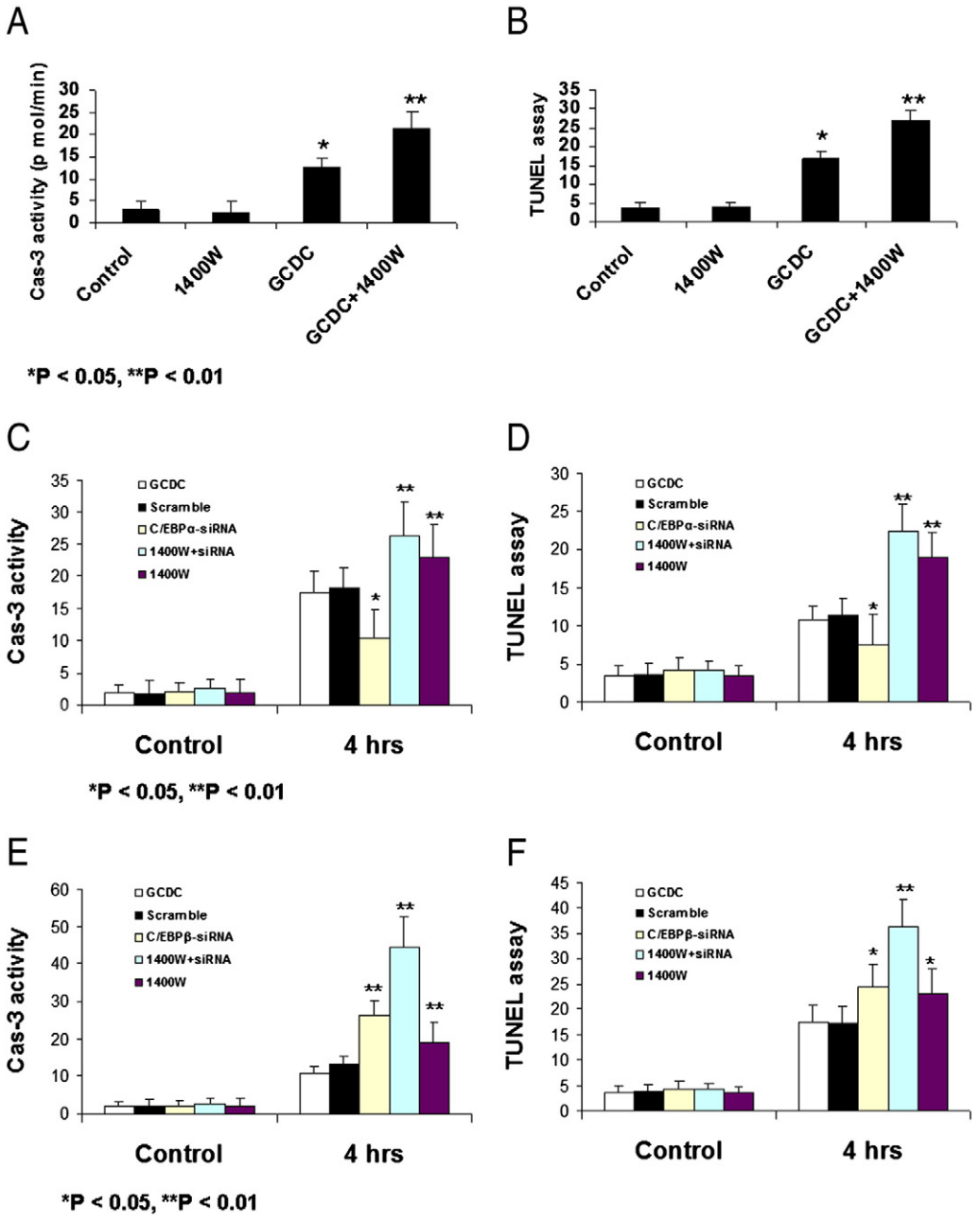

Fig. 6. A. 6B GCDC induced apoptosis in hepatocytes, but iNOS-specific inhibitor 1400 W (50 μM) altered the profile of GCDC-caused hepatocyte apoptosis as demonstrated with caspase-3 activity and TUNEL assay *P<0.05; **P<0.01. C, D, E, F. Hepatocytes were treated with iNOS inhibitor 1400 W or transfected with C/EBPα or C/EBPβ siRNAs for 16 h. Thereafter cells were challenged with GCDC for another 4 h. The hepatocyte apoptosis was reflected with caspase-3 activity and TUNEL assay. The transfection of C/EBPα or C/EBPβ siRNAs could affect the GCDC-induced apoptosis, but the effects of C/EBPα or C/EBPβ siRNAs were eliminated by iNOS inhibitor 1400 W.

studies such as bile duct ligation, hemihepatectomy, or other compound-induced liver injury models. The challenge lies in testing the feasibility, efficacy, and reliability in individual model. In summary, the C/EBPα and C/EBPβ binding proteins regulate the expression of iNOS. Through iNOS signaling pathway, the GCDC-induced apoptosis in hepatocytes is furthermore modulated. These data suggest that the C/EBPs/iNOS signaling pathways have a potential value in the treatment of hepatocyte apoptosis.

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