Reversibility of caspase activation and its role during glycochenodeoxycholate-induced hepatocyte apoptosis

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Abstract

The accumulation of glycochenodeoxycholate (GCDC) induced hepatocyte apoptosis in cholestasis. However, many hepatocytes still survived GCDC-induced apoptosis. The molecular mechanism for the survival of hepatocytes remains unclear. In present study, isolated rat hepatocytes were cultured in William'E medium and treated with 50µM of GCDC. DNA, RNA, cell lysate and nuclear proteins were collected in different intervals for DNA fragmentation assay, RT-PCR, Western blotting and gel mobility shift assay respectively. GCDC-induced active caspases were detected as early as at 2 h by Western blotting and kinetic caspase assay; whereas hepatocyte apoptosis was found at 4 h by DNA fragmentation and TUNEL assay. When GCDC was removed, the increased caspases as well as NF-κB could be restored to control level. A1/Bfl-1 and iNOS were up-regulated in 2 h GCDC-stimulation. After GCDC was removed, hepatocytes decreased expression of A1/Bfl-1 to the control level, but not iNOS. NF-κB activation coincided with the change of A1/Bfl-1. Survivin, cIAP1, cIAP2, XIAP, A1/Bfl-1 except iNOS were down-regulated by pan-caspase inhibitor Z-VAD-FMK. In addition, Z-VAD-FMK inhibited release of cytochrome c, and suppressed NF-κB activation. Our data suggested that caspase pathway is an important regulatory factor during hepatocyte apoptosis. GCDC-induced caspase response is reversible, which may activate anti-apoptotic genes to protect hepatocytes from apoptosis.

Key words: apoptosis, hepatocyte, caspase, bile acid
Introduction

Bile acids are accumulated in cholestasis (1). The major bile acids include glycocholate and taurocholate, which are crucial for digestion and absorption of fat (2). Pathophysiologically, ursodeoxycholate and its taurine-conjugated form are protective to hepatocytes by directly suppressing disruption of mitochondrial membrane structure (3); whereas GCDC is cytotoxic and contributes to hepatocellular injury (4). GCDC may cause hepatocyte damage by both acute necrosis and chronic apoptosis (5, 6). GCDC-induced hepatocyte apoptosis includes death-receptor and the mitochondrial pathways (7, 8). Moreover two apoptotic pathways are mediated by caspase cascade activation (9). Caspases, comprised a unique family of cysteine proteases, involve in cytokine activation and in the execution of apoptosis (10).

Apoptosis, a cellular suicide program, is essential for correct development and homeostasis of multicellular organism (11). Caspases are central initiators and executioners of apoptosis (10). Once activated, the caspases are responsible for cleavage of selective protein substrates, which have been widely implicated in many models of apoptosis (11, 12). In particular, caspase 8 has been found to be a prominent signaling caspase involved in initiation of apoptosis by the Fas, tumor necrosis factor (TNF) type I, and DR3 receptors (13, 14). Caspase 8 is abundant in the liver, allowing caspase to function as an initiator protease upstream of cathepsin B in bile salt–mediated hepatocyte apoptosis (15). Apoptosis is generally considered a fast process, since activation of caspases rapidly is followed by cell fragmentation and phagocytosis. However, different cells have a distinct variance for caspase activation. Hepatocytes are sensitive to the
activation of caspase to cause apoptosis, but neurons show a relative resistance toward caspase activity that may allow a low level of caspase activity (16, 17). There are many anti-apoptotic genes such as c-IAP 1 and 2, TRAFs 1 and 2, IEX-1L, and A1/Bfl-1 (18-20). Some genes play the antiapoptotic role by direct activation, not by caspase pathway (21). The relationship between anti-apoptotic genes and caspase activation is not yet clearly understood.

We took advantage of the fact that caspases were activated by GCDC to examine the apoptotic mechanism of caspase cascade activation (7). The present study was designed to investigate the role of caspases in GCDC-induced hepatocyte apoptosis. Pan-caspase inhibitor, Z-VAD-FMK, was utilized to further elucidate the direct relationship between antiapoptotic genes and GCDC-induced caspase response. Our data suggest GCDC-induced hepatocyte apoptosis is modulated by caspase cascade activation. GCDC-induced caspase response is reversible, which may activate anti-apoptotic genes to protect hepatocytes from apoptosis.

**Experimental Procedures**

**Materials.** William'E medium and fetal calf serum were purchased from GIBCO. Goat polyclonal antibody against Survivin, donkey anti-goat horseradish peroxidase and mouse anti-β-actin were obtained from Santa Cruz Biotechnology. Rabbit anti-mouse horseradish peroxidase was acquired from Cell Signaling Technology. All other chemicals were purchased from Sigma except indicated.
**Cell culture.** Primary hepatocytes were isolated from adult Sprague-Dawley rats by standard liver perfusion procedure (22), removed dead cells by Percoll (Sigma), and then cultured in collagen-coated dishes (Falcon). Cells at density of $5.5 \times 10^5$ were incubated in William'E medium containing 10% fetal bovine serum for varying intervals.

**Caspase assay** Cells were grown in 35-mm dishes and harvested with cell lysis buffer. Protein concentration of cell lysate was determined with the bicinchoninic acid assay (BCA) method (Pierce). 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-pNA, and calculated the activity as pmol/min.

**Reverse transcriptase – polymerase chain reaction (RT-PCR) and real-time quantitative PCR** The RT-PCR kit was gotten from Qiagen. After reverse transcription, the cDNA product was amplified by PCR with Taq DNA polymerase using standard protocols. The amplified products (10 µl) were separated on 2% agarose gels, stained with ethidium bromide, and photographed using ultraviolet illumination. The 5’ forward and 3’ reverse-complement PCR primers for amplification of Survivin were CTGATTTCGCCAGTGGTTT and TCATCTGACGTCAGGTTTCG respectively. For cIAP2, PCR primers were ACATTCCCAGCTGCCATTC and CTCCTGCTCCGTCTGCTCTCT. For cIAP1, PCR primers were CCAGCCTGCCCTCAACCCTCT and GGGTCATCTCCGGGTTCCCAAC. For XIAP, PCR primers were CGCGAGCGGGTTTCTCTACAC and
ACCAGGCACGGTCACAGGGTTC. For A1/Bfl-1, PCR primers were ATCCACTCCCTGGCTGAGAACT and ACATCCAGGCCAATCTGCTCTT. For iNOS, PCR primers were CGAGGAGGCTGCCTGCAGACTGG and CTGGGAGGAGCTGATGGAGTAGTA. For GAPDH, PCR primers were CCATCACCATCTTTCCAGGAG and CCTGCTCACCACCTTCTTG. All PCR primers were synthesized from Integreted DNA Technology. The relative mRNA levels of Survivin were confirmed by real-time PCR. The data were normalized to the expression level of 18 S rRNA.

**TUNEL assay** TdT-FragEL™ DNA fragmentation detection kit was obtained from Oncogene Research Products, following instruction to prepare tissue slide. In this assay, terminal deoxynucleotidyl transferase (TdT) bound to exposed 3’-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacted with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. The counterstaining with methyl green aided in the morphological evaluation and characterization of normal and apoptotic cells.

**Western blotting** Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane (Millipore), and blotted with appropriate primary antibodies at dilution of 1:1,000. Peroxidase-conjugated secondary antibodies were incubated at a dilution of 1:3,000. Bound antibody was visualized using
chemiluminescent substrate (ECL; Amersham Pharmacia Biotech) and exposed to Kodak X-Omat film. At least three independent experiments were performed.

*Gel Mobility Shift Assay* Nuclear extract was prepared with the modified Dignam protocol. The concentration of nuclear protein was measured with Bio-Rad Protein Assay. 4µg of nuclear proteins 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 SO4, 5 mM DTT, Tween 20, 1 % (w/v), 150 mM KCl) with 20 fmol/µl of double-stranded DNA oligonucleotide. NF-κB consensus binding sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was labeled with digoxin. Binding reactions were performed by 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. The gel was transferred to Nytran membrane and exposed to Kodak films. At least three independent experiments were performed.

*Statistical analysis.* All data represent at least three experiments using cells, or extract from a minimum of three separate isolations and are expressed as means ± SD unless otherwise indicated. Differences between groups were compared using an ANOVA for repeated measures. All statistical analyses were performed with the statistical software SPSS.
Results

**GCDC-induced hepatocyte apoptosis** GCDC-induced hepatocytes apoptosis could be reflected by DNA fragmentation assay, caspase assay and western blotting (23-25). In present study, rat hepatocytes were isolated and cultured in collagen-coated dishes with William'E medium containing 10% FBS overnight. After stimulated with a concentration of 50 µM of GCDC in the different intervals, hepatocytes were harvested. DNA, RNA, and cell lysate protein were separately collected for further analysis. DNA fragmentation demonstrated hepatocyte apoptosis after treatment by 50 µM of GCDC for 16 h (Fig.1A). Caspases are divided into long-prodomain caspases (caspase-2, -8, -9, and -10) and short-prodomain caspases (caspase-3, -6, and -7) (26). In our study, caspase-8 and caspase-3 were selected as indicators to examine their involvement during GCDC-induced apoptosis. After 16 h, activities of both caspase-3 and caspase-8 were increased. Moreover extent of activated caspase-3 was higher than that of activated caspase-8 (Fig.1B). When the stimulating duration was decreased to 4 h in the presence of 50 µM of GCDC, hepatocyte apoptosis was still found by DNA fragmentation assay (Fig. 2A). At 4 h, anti-apoptotic gene Survivin was up-regulated (Fig. 2B). By Western blotting with crude cell lysate, the expression of caspase-3 and caspase-8 protein was initially increased at 2 h (Fig. 2C). Caspase activation was earlier than DNA fragmentation. The relative mRNA levels of Survivin were further confirmed by real-time PCR (Fig. 2D), which was identical to the level of Survivin protein by Western blotting (data not shown).
Reversibility of GCDC-induced caspase activation With 50 µM of GCDC caspase was activated at 2 h, but DNA fragmentation was enhanced at 4 h. These results suggested GCDC-induced hepatocyte apoptosis was duration-dependent. To address this issue, the cultured primary hepatocytes were stimulated with 50 µM of GCDC for 2 h, followed by removing medium containing GCDC, and re-cultured with fresh medium (without GCDC) for another 4 h. Thereafter, hepatocytes were re-stimulated with the 50 µM of GCDC for an additional 2 h. Caspase assays indicated that activities of caspase-3 and caspase-8 were increased in 2 h incubation with the 50 µM of GCDC, restored to control levels after removing GCDC, and elevated again for additional 2 h re-stimulation with 50 µM of GCDC (Fig.3A, B). No DNA fragmentation was found by unknown mechanism (Fig.3C). Apoptotic rate showed no difference among four groups by TUNEL assay (Fig.3D). However, apoptotic rate was increased significantly when GCDC was not removed (Fig.4). NF-κB was activated after 2 h GCDC stimulation, restored to control level after washing GCDC away, and reactivated by additional 2 h re-stimulation with 50 µM of GCDC (Fig.3E). Survivin expression was unchanged (Fig.3F). The data suggested that caspase response and NF-κB activation were reversible in a short treatment (2 h) by 50µM of GCDC. Under low concentration GCDC (50µM), the duration of GCDC plays a crucial role in hepatocyte apoptosis.

GCDC-induced gene expression After 50 µM of GCDC stimulation, some cells died of apoptosis and floated in supernatant (Fig. 4A), but the other attached to dish and were alive (Fig. 4B). It was unclear whether anti-apoptotic genes were up-regulated and accounted for cell survival. Therefore, RNA was isolated from cells and RT-PCR was
performed to detect gene transcription. In anti-apoptotic gene family we selected cIAP1, cIAP2, XIAP, A1/Bfl-1, iNOS, and Survivin. Results showed that there were no changes among cIAP1, cIAP2, XIAP, and Survivin (Fig. 5A), but had different for A1/Bfl-1 and iNOS. A1/Bfl-1 gene was increased at 2 h and then decreased to the baseline level in another 4 h (without GCDC) extending culture. iNOS showed higher amplification at 2 h, but maintained at the same level in another 4 h (without GCDC) culture. To demonstrate gene expression from bottom attached cells, we washed away top dead cells, isolated RNA from bottom cells, and then repeated RT-PCR. The results are completely identical (data not shown), which means expression of genes from dish-attached cells. NF-κB activation was also increased at 2 h, and then restored to the baseline level in another 4 h (without GCDC) culture (Fig. 5B). Survivin expression by Western blotting was unchanged (data not shown). The relative expression levels of Survivin were measured by real-time PCR (Fig. 5C).

**Gene expression affected by caspase inhibitor.** Caspases mediate the intracellular activation of other caspases and selectively cleave distinct intracellular substrates, leading to dismantling of a cell’s architecture, signaling apparatus, and repair mechanisms. Eventually, caspases induce the activation of endonucleases that complete cellular suicide by internucleosomal DNA fragmentation (27). Our study found that activities of caspase-3 and caspase-8 were increased at 2 h in the presence of 50 µM of GCDC. However when GCDC-stimulation was removed at 2 h, activities of caspases could be decreased and restored to control level by an unknown mechanism. GCDC-induced caspase activation is reversible. Next we investigated the correlation of caspase activation and
anti-apoptotic gene expression. Pancaspase inhibitor (Alexis, Z-VAD-FMK, 260 020 M001) was utilized to treat hepatocytes. Caspase-3 and caspase-8 were significantly inhibited (Fig. 6). When 50 µM of GCDC was added into medium to stimulate hepatocytes for 4 h, activity of caspase-3 was increased to 11.40 pmole/min at 4 h compared to 3.98 pmole/min of control (without GCDC); whereas activity of caspase-8 was elevated from baseline 3.27 pmole/min increasingly to 8.53 pmole/min at 4 h. 50µM of Z-VAD-FMK inhibited caspase-3 and caspase-8 in 60.26% and 65.88% respectively. Expression of Survivin, cIAP1, cIAP2, XIAP, A1/Bfl-1 except iNOS was down-regulated (Fig. 7A). The protein level of Survivin was consistent to Survivin mRNA (Fig.7B).

**Pathway for GCDC-induced hepatocyte apoptosis** GCDC induced hepatocyte apoptosis by death-receptor or the mitochondrial pathway (28). GCDC cause direct activation of Fas that stimulates FADD, caspase-8 activation, and subsequent apoptosis either through Bid cleavage and translocation to mitochondria or by direct activation of downstream effector caspases. In mitochondria there are actually two pathways. One is GCDC directly stimulates generation of reactive oxygen species from the respiratory chain, which induces the permeability transition and cytochrome c release into cytosol. The other is GCDC causes translocation of Bax to mitochondria, which functions as channel for cytochrome release into cytosol and activation of downstream effector caspases (8). After the stimulation with a concentration of 50 µM of GCDC, cytochrome c was increased even at 4 h (Fig. 8A). When 50 µM of Z-VAD-FMK was used, cytochrome c release was decreased. As hepatocytes were treated by 50 µM of GCDC, caspase 9 was increased at 4 h as well. However under co-treatment of GCDC plus Z-VAD-FMK, the activity of
caspase 9 was reduced (Fig. 8B). In addition, Z-VAD-FMK inhibited NF-κB activation induced by 50 µM of GCDC (8C). It is worth to do further investigation for the role of cytochrome c and caspase 9. GCDC-induced hepatocyte apoptosis was a useful model to study mechanism of apoptosis and pathogenesis of cholestatic liver injury. Although it has been incompletely understood the role of anti-apoptotic genes and NF-κB activation, our study provides a clue to their protective function against GCDC-induced hepatocyte apoptosis.

Discussion

Accumulation of bile acids induces hepatocyte apoptosis and necrosis (29). Our study demonstrated that low dosage (50 µM) of GCDC caused hepatocyte apoptosis, not necrosis. The increase of caspases could be restored to control level after low dosage of GCDC was removed. The activities of caspases were reversible. GCDC also induced expression of anti-apoptotic genes, such as Survivin, A1/Bfl-1 and iNOS. Moreover activities of caspases were correlated to those genes, because pancaspase inhibitor (Z-VAD-FMK) may inhibit expression of Survivin, cIAP1, cIAP2, XIAP, A1/Bfl-1 except iNOS. At present we cannot completely understand the role of those genes during GCDC-induced hepatocyte apoptosis.

In inflammatory conditions hepatocyte survival is dependent on the protective function of anti-apoptotic genes (30). The expression of anti-apoptotic genes is mediated by caspase response which can be inhibited by Z-VAD-FMK (31). Many important anti-apoptotic genes such as IAP family, A1/Bfl-1, and iNOS have been identified, which have anti-
apoptotic role in mature hepatocytes by controlling the transcription of specific survival genes (32). Although our preliminary results did not indicate the altered levels of cIAP1, cIAP2, and XIAP in 2 h stimulation, the potential role for Survivin, A1/Bfl-1 and iNOS had been investigated. Survivin inhibits apoptosis via a pathway independent of bcl-2 (33). Survivin modulates caspase activation as well as Fas-mediated hepatic apoptosis, which is regulated via the mitochondrial pathway (34). Mitochondrial A1/Bfl-1, a member of the Bcl-2 family, is expressed in various tissues during development and adult life as well as cancer cell lines. A1/Bfl-1 was shown to protect cells from apoptosis (35). A1/Bfl-1 induced by cytokines suppresses the release of cytochrome c (36). Nitric oxide (NO) inhibits caspase-3 activity in hepatocytes (37). NO protects hepatocytes from TNF-alpha/ActD-induced apoptosis via the interruption of mitochondrial apoptotic signaling through S-nitrosylation of caspase-8 (38). NO production depends on the expression of iNOS in response to inflammatory cytokines (39, 40). It is known that iNOS gene transfer could suppress hepatocyte apoptosis (41). iNOS is heterogeneously distributed in liver (42, 43), which may represent a mechanism through which hepatocytes control the degree of apoptosis in the liver (44, 45).

NF-κB is the prototypic transcription factor in eukaryotic cells known to play a pivotal role in transactivation of promoters for genes involved in inflammation, immune responses, and anti-apoptotic mechanism (46). The anti-apoptotic molecules regulated by NF-κB include c-IAP 1 and 2, TRAFs 1 and 2, IEX-1L, and A20 (32, 33). IAP family has been suggested to act as direct inhibitors of caspases. NF-κB activation suppresses mitochondrial release of cytochrome c through the activation of a Bcl-2 homologue
The expression of iNOS can be regulated at the level of transcription and NF-κB activation is essential for its expression (48). NF-κB activates endogenous iNOS via IKKβ and provides protection from apoptosis (49).

In summary, GCDC-induced hepatocytes apoptosis is mediated by caspase activation, which is a highly regulated process. Caspase cascade may activate anti-apoptotic genes to prevent hepatocytes from apoptosis. GCDC-induced caspase response is reversible, which may be a useful approach to block GCDC-induced hepatocyte apoptosis.

Figure Legends

Fig. 1. Hepatocyte apoptosis induced by 50µM of GCDC for 16hr. A, genomic DNA fragmentation. 10µg of DNA were run on each lane on 2% agarose gel. Lane 1: DNA marker; Lane 2: control without GCDC treatment; Lane 3: treated cells with 50µM of GCDC for 16hr. B, Caspase activities in vitro. Protein concentration of cell lysate was determined with the bicinchoninic acid assay method (BCA, Pierce). 100µg of cell lysates was utilized to assay the activity of caspase-3 or caspase-8. The reaction system employed the colorimetric substrate IETD-pNA, and calculated the activity as pmol/min. Assay was set at at 37 °C for 60min. Cell lysate without GCDC treatment as control.

Fig. 2. Hepatocyte apoptosis induced by 50µM of GCDC. A, DNA fragmentation. 10µg of DNA were run on 2% agarose gel. B, Activated Survivin gene was examined with RT-PCR. C, Activated caspase-3 and 8 were examined by Western blotting. D, The relative
mRNA levels of Survivin were determined by real-time PCR. The data were normalized to the expression level of 18 S rRNA (*p<0.01, n = 3).

Fig. 3. Transient insults. The cultured primary hepatocytes were stimulated with 50 µM of GCDC for 2 hours, followed by removing medium containing GCDC, washed with PBS, and replaced with fresh medium (without GCDC) for another 4 hours culture. Thereafter hepatocytes were re-stimulated with the 50 µM of GCDC for an additional 2 hours. A, Caspase-3 activities. Values are mean ± SD (*p<0.01, n = 5). B, Caspase-8 activities (*p<0.01, ** p<0.05, n = 5). C, DNA fragmentation. 10µg of DNA were run on 2% agarose gel. D, TUNEL assay for apoptotic rate (%). E, Gel Mobility Shift Assay was performed with 4 µg of nuclear proteins. F, RT-PCR of Survivin gene.

Fig. 4. TUNEL assay was utilized to identify apoptotic hepatocytes treated by 50 µM of GCDC. A, Floating cells (including dead and apoptotic cells) at 4 h. B, Adherent cells at 4 h. C, TUNEL assay for apoptotic rate (*p<0.01, n = 4).

Fig. 5. Anti-apoptotic genes and hepatocyte apoptosis treated by 50 µM of GCDC. A, RT-PCR analysis of cIAP1, cIAP2, XIAP, A1/Bfl-1, iNOS, Survivin, and GAPDH. The amplified DNA was carried out on a 2% agarose gel, and was visualized by ethidium bromide staining. B, NF-κB activation by Gel Mobility Shift Assay. C, The relative expression levels of Survivin were determined by real-time PCR. The data were normalized to the expression level of 18 S rRNA (*p<0.01, n = 3).
Fig. 6. 50µM of pancaspase inhibitor (Alexis, Z-VAD-FMK, 260 020 M001) to treat hepatocytes. When 50 µM of GCDC was added into medium for 4 h, caspase-3 and caspase-8 were inhibited significantly. Values are mean ± SD (*p<0.01, n = 4).

Fig. 7 Pancaspase inhibitor and anti-apoptotic genes. A, 50µM of Z-VAD-FMK and expression of Survivin, XIAP, A1/Bfl-1, cIAP1, cIAP2 and iNOS. B, Z-VAD-FMK and Survivin expression by Western blotting.

Fig. 8. Pan-caspase inhibitor, Z-VAD-FMK (50µM), decreased activity of caspase-9, inhibited release of cytochrome c, and suppressed NF-κB activation during GCDC (50µM)-induced hepatocyte apoptosis. A, cytochrome c was detected by Western blotting. B, expression of caspase 9. C, NF-κB activation.

Reference


Fig. 3

A

Caspase 3 activity (50 μM GDC)

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B

Caspase 8 activity (50 μM GDC)

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C

D

Apoptosis Panel

E

F

Downloaded from http://www.jbc.org/ by guest on October 30, 2017
Fig. 4

A  Floating cells

B  Adherent cells

C

![Graph showing cell counts over time](image-url)
Fig. 5

A

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B

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Fig. 6
Fig. 7

A

1 2 3

- Survivin
- XIAP
- A1/Bfl-1
- cIAP2
- cIAP1
- iNOS
- GAPDH

B

1 2 3

- Survivin
- β-actin

1. Control
2. GCDC for 4 h
3. GCDC + pan-caspase inhibitor
Fig 8

A

1  2  3

Cytochrome c

β-actin

B

1  2  3

Caspase 9

β-actin

C

1  2  3

NF-κB

1. Control
2. GCDC for 4 h
3. GCDC+ pan-caspase inhibitor
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