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Effect of TLR4 on Apoptosis of HL-7702 Hepatocytes through PI3K/AKT /GSK3β Pathway

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ABSTRACT

Objective: To investigate whether toll-like receptor (TLR4) regulates apoptosis through PI3K/AKT/GSK3β signaling pathway in human HL-7702 hepatocytes and to investigate its regulatory mechanism.

Methods: The cultured human HL-7702 hepatocytes were divided into 5 groups, 3 of which were pretreated with TLR4 inhibitor (CLI-095), PI3K inhibitor (LY294002) and GSK3β inhibitor (LiCl) for 2h and then treated with LPS (10μg/mL) for 24h, respectively, to establish the hepatocyte apoptosis model, and one of the remaining two groups treated with LPS for 24h only was used as the model group, and the other group of untreated HL-7702 cells was used as the control group, with three replicates in each group. Cell viability was detected by CCK8, apoptosis rate by flow cytometry, expression of TLR4, AKT, p-AKT, GSK3β, p-GSK3β by Western-Blot, nuclear translocation of GSK3β by immunofluorescence, and expression of TNF-α and IL-6 mRNA by real-time fluorescence quantitative PCR.

Results: The CCK8 assay revealed that compared with control group, the cell viability was significantly lower after different times (1h, 3h, 6h, 12h and 24h) of LPS treatment of HL-7702 cells ($P< 0.05$); the inhibitors CLI-095, LY294002 and LiCl could significantly protect HL-7702 cell ($P < 0.01$). The cytometry showed that the apoptosis rate of LPS-treated HL-7702 cells was significantly higher than that of the control group ($P< 0.01$), and the inhibitors CLI-095, LY294002 and LiCl could significantly decrease the apoptosis rate of HL-7702 cells. Western-Blot assay revealed that the level of GSK3β phosphorylation was significantly increased in the model group; the inhibitors CLI-095, LY294002 and LiCl could all significantly lower GSK3β phosphorylation, while the level of AKT phosphorylation was increased in the LiCl group. The immunofluorescence detection results showed that GSK3β was mainly expressed in the cytoplasm of HL-7702 cell in the control group, and GSK3β aggregated toward the nucleus after 24 h of LPS treatment; the inhibitor CLI-095, LY294002, and LiCl could return GSK3β to the cytoplasm for aggregation. The expression level of TNF-α and IL-6 mRNA in the supernatant of HL-7702 cells in the model group was significantly increased compared with the control group ($P< 0.05$); the CLI-095, LY294002, and LiCl could significantly inhibited the expression of TNF-α and IL-6 mRNA in the supernatant of HL-7702 cells ($P< 0.05$).

Conclusions: TLR4 in human HL-7702 hepatocytes regulates apoptosis through phosphorylation of AKT, GSK3β in the PI3K/AKT/GSK3β signaling pathway.

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It was found that Toll-like 4 receptor (TLR4) expression on hepatocyte membranes was increased in acute liver failure (ALF) and inhibition of TLR4 signaling improved organ damage and systemic inflammatory responses in an animal model of ALF, suggesting that TLR4 plays an important role in mediating organ damage in ALF, but its mechanism of action has not been elucidated, Animal experiments have found that inhibition of the PI3K/AKT signaling pathway promotes hepatocyte proliferation and inhibits hepatocyte apoptosis, thus playing a protective role in ALF and inhibition of GSK3β-mediated hepatocyte apoptosis attenuates D-GalN/LPS-induced ALF, which all indicate that hepatocyte apoptosis plays an important role in the pathogenesis of ALF [1-6]. Earlier studies have demonstrated that TLR4 promotes angiogenesis in pancreatic cancer through PI3K/AKT signaling, suggesting that there is a signaling link between TLR4 and PI3K/AKT [7]. The PI3K/AKT/GSK30 signaling pathway is a pathway that transmits signals from the cell membrane to the nucleus, and it has a simple, linear, and unitary transduction characteristic, which plays a key role in the protection of the liver from injury [8]. So does TLR4 regulate apoptosis in ALF hepatocytes through the PI3K/AKT/GSK3p signalling pathway?

Recently, we verified the mechanism of TLR4 regulation of apoptosis in ALF hepatocytes by in vitro cellular experiments, which provides experimental basis for TLR4 as a potential therapeutic target. The report is as follows.

Materials and Methods

Experimental Material

Experimental Cells

Normal human-derived hepatocytes HL-7702 hepatocytes (SNL-141) were purchased from Wuhan Shangen Biotechnology Co., Ltd.

Main Reagents

LPS (for ALF model establishment) was purchased from Sigma Aldrich, USA. CLI-095 (HY-11109) was purchased from MCE(Shanghai). LiCl(S24113) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. Annexin V-FITC/PI Apoptosis Kit (70-AP10) was purchased from Hangzhou United Technologies Co., Ltd.Trizon and RNA extraction kit were purchased from Jiangsu Cowin Biotech Co., Ltd. GSK3β antibody was purchased from Proteintech Group (Wuhan), Inc. Cy3 Anti- Rabbit IgG was purchased from ABclonal Biotech (Wuhan) Co., Ltd. DAPI was purchased from KeyGen Biotech. Co. Ltd. HiScript IIQRT Super Mix for qPCR/+gDNA wiper and ChamQ Universal SYBR qPCR Master Mix were purchased from Vazyme Biotech (Dalian) Co., Ltd.

Main Instruments

Fluorescence quantitative PCR and ultra-high sensitivity chemiluminescence imaging system were purchased from Bio-Rad Laboratories (Shanghai) Co., Ltd. NovoCyte™ flow cytometry was purchased from Acea Bio (Hangzhou) Co., Ltd. PCR amplifier was purchased from Hangzhou BORI Co., Ltd. Enzyme labelling instrument was purchased from SpectraMax Medici Co., Ltd. Fluorescence microscope was purchased from OLYMPUS (Japan) Co., Ltd.

Experimental Methods

Experimental Grouping

- **Control Group:** HL-7702 was not treated in any way
- **Model Group:** HL-7702 cells were treated with LPS (10 ug/mL) for 24h
- **Three Pre-Treatment Groups:** HL-7702 cells were treated with CLI-095 (10 ug/mL), LY294002 (50 ug/mL) and LiCl (10 ug/mL) were treated for 2h, and then LPS (10 ug/mL) was added for 24h.

Cell Pretreatment and Modelling

HL-7702 cells were routinely cultured, passaged and diluted,96-well plates were laid with 3000 cells per well, and the culture was continued until the cells were completely attached to the wall, and then inhibitors (CLI-095, LY294002, LiCl) were added to treat the cells for 2h, and then LPS was added to treat them for 24h, to replicate the model of apoptosis, and the cells were collected for freezing and storage for examination.

CCK8 Assay

The modelled cells were digested,resuspended,counted and spread in plates with a cell density of 5x103 cells/well, and continued to be cultured for 24h before being changed to scrum-free medium, and LPS stimulation (10ug/mL) was added,and the cells in 96-well plates to be tested were changed to the same medium at different times after stimulation (1, 3,6, 12, 24h), with 100ul in each well; 10ul CCK reagent was added to each well; the absorbance value of each well was detected by an enzyme marker at 450nm and the survival rate was calculated. 10ug/ml CCK8 reagent to each well, and incubate in the incubator for 2h; the absorbance value of each well was detected at 450nm by an enzyme marker and the survival rate was calculated.

Flow Assay for Apoptosis

Collect 1×10^6~3×10^6 cells, add 1ml PBS, centrifuge at 1500 rpm for 3 min, and wash twice; take 300ul pre-cooled 1xBinding Buffer to resuspend the cells; add 5ul Annexin V-FITC and 10uL propidium iodide to each tube; mix slightly, and then incubate for 10 min at room temperature away from light; Add 200ul pre-cooled 1xBinding Buffer to each tube; mix well and then assay on a flow meter.

Western Blot Assay

Discard the cell culture medium in the petri dish, add 100ul cell lysate to each well, put it on ice for 20 min, then pipette it into the labelled EP tube, centrifuge it at 12,000 r/min for 10min, take the supernatant to a new EP tube (BCA assay), and put it at -20°C for storage. Determine the protein concentration according to the BCA kit, protein denaturation, the sample was subjected to SDS-PAGE electrophoresis for 1.5h, and then the membrane was flowed at a constant flow rate of 300mA for 1.5h. The primary antibody was incubated with a PVDF membrane overnight at 4°C, and the secondary antibody was incubated with the PVDF membrane at room temperature for 2h on the next day, the membrane was washed, and the PVDF membrane was wetted with luminescent liquid, and then placed in the sample placing area of the Ultra-high-sensitivity Chemiluminescent Imaging System and the procedure of developing the image was run. Imaging.

Immunofluorescence Detection of GSK3β

The operation was carried out according to the instructions of Immunofluorescence Detection of GSK3β Kit, which mainly included: HL-7702 cell fixation, punching, cell labelling (1:200 primary antibody GSK3β, 1:200 secondary antibody Cy3), re-staining of nuclei and sealing observation.

Real-Time Fluorescence Quantitative PCR (qPCR)

RNA was extracted according to the instructions of the kit, and the concentration and purity of RNA were determined by UV spectrophotometer (OD260/OD280 ratio in the range of 1.8-2.0). RNA was synthesized into micDNA by RNA reverse transcription kit, and fluorescence quantitative PCR was carried out. reaction system was as follows: 2xSYBR Green PCR Master Mix 10ul, cDNA 1ul upstream primer 0.4ul, downstream primer 0.4ul, and RNase Free ddH2O 8.2ul. the reaction steps were as follows: pre-denaturation at 95°C for 10 min; denaturation at 95°C for 10min; reaction step was as follows: pre-denaturation at 95°C for 10 min; denaturation at 95°C for 10min. and 10min; denaturation 95°C, 10 s; annealing 58°C,30 s; extension 72°C, 30 s; 40 cycles. The sequences are listed below. P-actin was used as an internal reference, and the relative expression of TNF-a and IL-6 was calculated according to the 2-AACt method.

Table 1: Primer Sequences and Annealing Temperature in qPCR

	Primer Sequences (5'-3')	Product Length (bp)	Annealing Temperature(°C)
Bax F	GACAGGGGCCTTTTTGCTACA	187	60.85
Bax R	CACGTCAGCAATCATCCTCTGC		
Bcl-2 F	AGGATTGTGGCCTTCTTTGA	161	57.03
Bcl-2 R	ACAAAGGCATCCCAGCC		
β-actin F	AGGGAAATCGTGCGTGAC	192	57.3
β-actin R	CATACCCAAGAAGGAAGGCT		

Statistical Methods

SPSS 20.0 software was applied for statistical analysis. All experiments were repeated three times, and quantitative results were expressed as mean ± standard deviation. Comparison of quantitative values between multiple groups was performed using one-way ANOVA, and two-by-two comparisons were performed using the LSD method. The test level a=0.05.

Results

Culture and Passage of HL-7702 Cell Line

Linder the light microscope, HL-7702 cells were observed to grow well attached to the wall, most of the cells were polygonal, tightly arranged, with intact cell membranes and clear intercellular boundaries; the nuclei were large, round and centered, with mononuclear predominating and cytoplasmic plasma abundance, as shown in Figure 1.

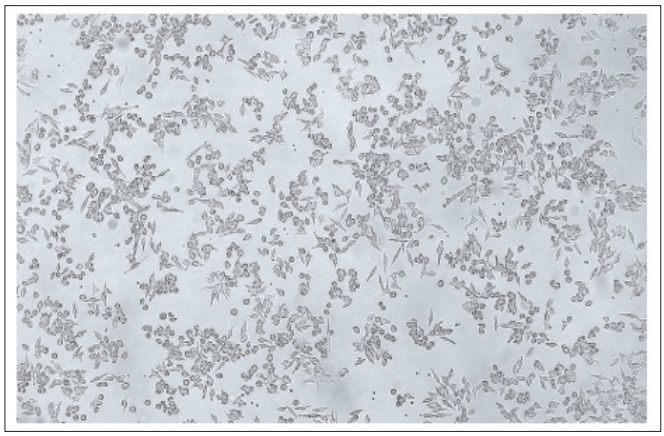

Figure 1: Normal HL-7702 Cell Culture

Detection of HL-7702 Cell Viability by CCK8 Method

CCK8 The results showed that the cell viability of HL-7702 cells decreased gradually (P< 0.05 or P< 0.001) after LPS was applied to HL-7702 cells for different times (1, 3, 6, 12, and 24h) compared with that of control, and in a certain time-dependent manner, and the viability of HL-7702 cells was 41% of that of the control after 24 h of LPS application (P < 0.001).The addition of LPS to HL-7702 cells pretreated with CLI-095, LY294002 and LiCl, respectively, significantly reduced the decrease in cell viability (P< 0.001), and the cell viability was still 79% of that of the control after 24h. The results are shown in Figure 2.

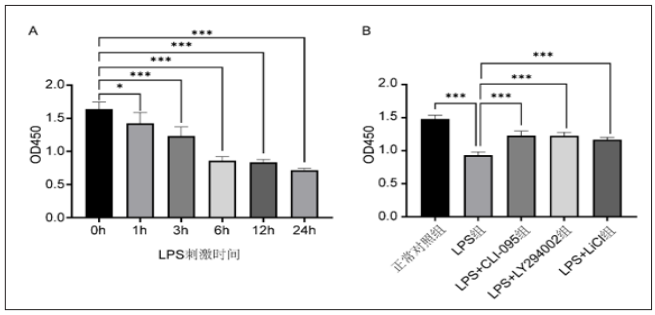

Figure 2: Viability of HL-7702 Cells Detected by CCK8 Assay

A. Changes in cell viability 1-24 h after LPS stimulation of HL-7702 cells

B. Improvement of cell viability of LPS-treated HL-7702 cells by pretreatment with different inhibitors

Effects of Different Inhibitors on Apoptosis of HL-7702 Cells

As shown in Figure 3, the apoptosis rate ofHL-7702 cells increased significantly after LPS treatment compared with the control group (P< 0.01). The apoptosis rate of HL-7702 cells pretreated with CLI-095, LY294002, and LiCl, respectively, was significantly decreased by the addition of LPS, and the decrease was more significant for CLI-095 and LY294002 (P< 0.01).

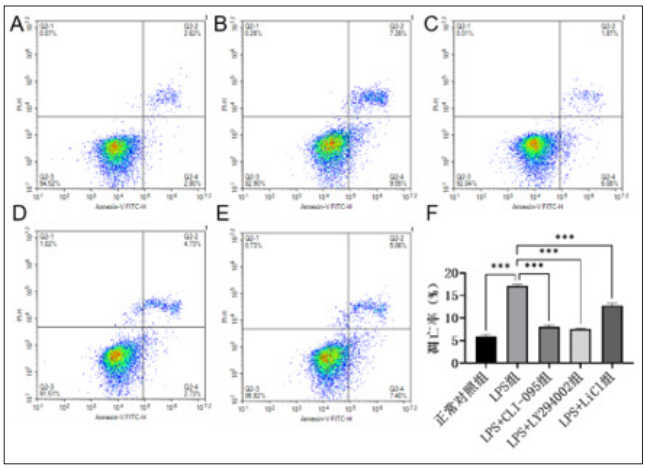

Figure 3: Apoptosis Rate of Cells in Each Group Detected by Flow Cytometry.
(A) (Normal Control Group (B) LPS Group (C) CLI-095 Treated Group (D) LY294002 Treated Group (E) LiCl Treated Group

Effects of Different Inhibitors on the Expression of TLR4, p-AKT/AKT, p-GSK3β/GSK3β in HL-7702 Cells

As shown in Figure 4, Wcstcrn-Blot assay revealed that the expression of TLR4 in HL-7702 cells of the model group gradually increased, and its expression was about 3.6 times higher than that of the control group after 24h (P< 0.01). GSK3β and AKT phosphorylation levels were significantly increased in the model group, with GSK3β being more significantly elevated; after LPS treatment of CLI-095, LY294002 and LiCl-pretreated HL-7702 cells, GSK3β phosphorylation was reduced in all of them compared with the model group (P< 0.01), whereas AKT phosphorylation levels were increased in the LiCl-pretreated group (P< 0.01).

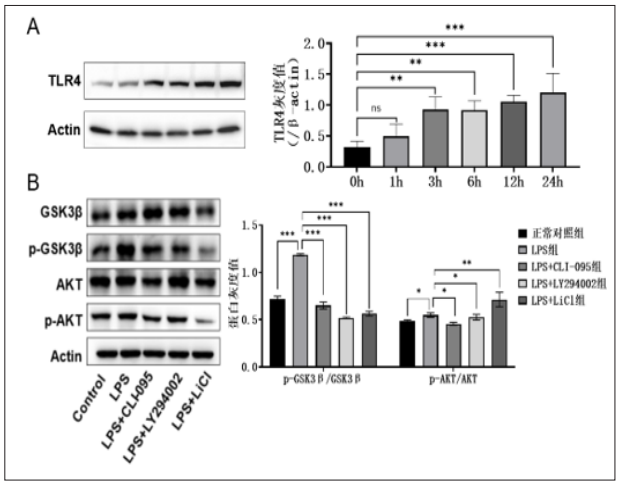

Figure 4: Western-Blot Detection ofTLR4/GSK3β/AKT Pathway Protein Expression Levels in Each Group of Cells

A. TLR4 Protein Expression Levels in Cells of Each Group

B. GSK3β,p-GSK3β, AKT, p-AKT Protein Expression Levels in Cells of Each Group

Effect of Different Inhibitors on GSK3β Nuclear Translocation in HL-7702 Cells

Immunofluorescence detection revealed that GSK3β was mainly expressed in the cytoplasm of HL-7702 cells in the control group, and only a very small amount of expression was found in the nucleus of the cells. After LPS treatment, GSK3β expression in HL-7702 cells increased significantly and tended to aggregate in the nucleus;in the CLI-095, LY294002 and LiCl pretreatment groups, the expression of GSK3β in HL-7702 cells decreased significantly (P<0,05), with CLI-095, LY294002 decreased more significantly (P< 0.01), and tended to aggregate in the cytoplasm, decreased (P< 0.05), with CLI-095 and LY294002 decreasing more significantly (P< 0.01) and tending to aggregate in the cytoplasm. See Figure 5.

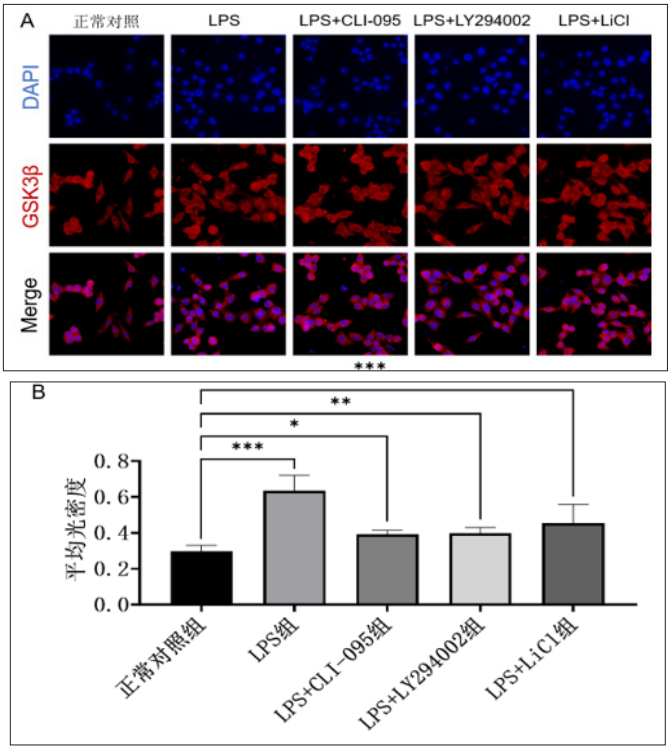

Figure 5: Immunofluorescence Detection of GSK3β Nuclear Ectasia

Effect of Different Inhibitors on mRNA Expression of Inflammatory Factors in HL-7702 Cells

As shown in Figure 6, the expression of inflammatory factors TNF-α and IL-6 mRNA in HL-7702 cells in the model group was significantly increased compared with that in the control group (P < 0.01). In the CLI-095, LY294002, and LiCl pretreatment groups, the expression of inflammatory factors TNF-α and IL-6 mRNA in HL-7702 cells was significantly decreased (P < 0.05).

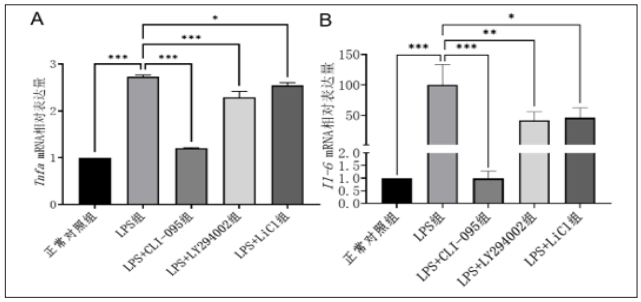

Figure 6: qPCR Detection of mRNA Expression of Inflammatory Factors in each Group of Cells

Discussion

ALF has a high morbidity and mortality rate and a very poor prognosis. Artificial liver and liver transplantation are the main therapeutic means, but they are difficult to be widely carried out due to the high cost, so it is of great practical significance to explore effective therapeutic means. Hepatocyte apoptosis has been confirmed to play an important role in the development of ALF, therefore, in-depth study of the mechanism of hepatocyte apoptosis has potential value for clinical treatment of ALF [9].

Replication of hepatocyte apoptosis using LPS has become a standard model for the study of hepatocyte apoptosis [10,11]. This model was used in this experiment, and the apoptosis rate of human HL-7702 cells was detected by flow cytometry at different times of LPS stimulation, and the results showed that the apoptosis rate gradually increased after different times of stimulation with the same concentration of LPS with a certain time-dependence, and the highest rate of apoptosis appeared in the 24h, which indicated that LPS had a direct toxicity effect on the in vitro-cultured HL-7702 cells, and could cause apoptosis of HL-7702 cells.

This experiment confirmed that the expression of TLR4 on the membrane of HL-7702 cells was significantly elevated and apoptosis was increased after LPS stimulation, suggesting that TLR4 was involved in HL-7702 cell apoptosis. We used flow cytometry to quantify the apoptosis of HL-7702 cells stimulated by LPS and found that the apoptosis rate was significantly higher; while the apoptosis rate of HL-7702 cells in the group pretreated with different inhibitors was significantly lower than that in the model group. These results indicated that TLR4 was able to regulate LPS-induced apoptosis in HL-7702 cells through the PI3K/AKT/GSK3β signaling channel.

We detected GSK3β nuclear translocation in each group of cells by immunofluorescence, and found that GSK3β was mainly expressed in the cytoplasm of HL-7702 cells, and after LPS treatment, GSK3β expression in HL-7702 cells increased significantly and tended to aggregate in the nucleus; however, after pretreatment with CLI-095, LY294002, and LiCl respectively, HL-7702 cell GSK3β expression decreased significantly, and CLI-095 and LY294002 decreased more significantly and tended to aggregate towards the cytoplasm, indicating that inhibition of upstream signaling molecules had a greater effect on the signaling pathway [12].

In this study, AKT, GSK3β and their phosphorylation were also detected, and the results showed that the phosphorylation levels of GSK3β and AKT in the model group were significantly elevated, with the elevated phosphorylation level of GSK3β being more pronounced; GSK3β phosphorylation was reduced in both CLI-095, LY294002, and LiCl-pretreated HL-7702 cells treated with LPS, whereas the LiCl AKT phosphorylation level was increased in the pretreatment group, indicating that not only upstream signals but also downstream signaling molecules of the TLR4/PI3K/AKT/GSK3β signaling pathway are involved in the regulation of LPS-induced apoptosis in HL-7702 cells. Finally, we detected the expression of effector molecules TNF-α and IL-6, and the results were completely consistent with the above results, suggesting that the TLR4/PI3K/AKT/ GSK3β signaling pathway was fully activated after LPS stimulation in HL-7702 cells [13].

In conclusion, this experiment confirmed that TLR4 in human HL-7702 hepatocytes regulates apoptosis through the phosphorylation of AKT and GSK3β in the PI3K/AKT/GSK3β signaling pathway.

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