

# Tumor Necrosis Factor Alpha Down-regulated Human GSTA1 and GSTA4 Expression Through The NF- $\kappa$ B Signaling Pathway in Human Hepatoma HepG2 Cells\*

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**Abstract** Glutathione S-transferase Alpha1 and Alpha 4 (GSTA1 and GSTA4) are crucial for detoxifying a variety of endogenous and exogenous toxic compounds. However, GSTA1/4 expression is reduced in cholestatic patients. The molecular mechanism of GSTA1/4 down-regulation remains elusive. Here, we treated human hepatoma HepG2 cells with tumor necrosis factor alpha (TNF $\alpha$ ) and measured the expression of GSTA1/4, nuclear factor kappa B (NF- $\kappa$ B) and NF-E2 related factor 2 (Nrf2) by quantitative real-time quantitative polymerase chain reaction (qPCR) and Western blotting. We found that expression of GSTA1/4 was repressed by TNF $\alpha$  at both the mRNA and the protein level in a dose- and time-dependent manner. Furthermore, inhibiting the NF- $\kappa$ B signaling pathway could attenuate the TNF $\alpha$  induced reduction in GSTA1/4 expression in the HepG2 cells. Our findings indicate that down-regulation of GSTA1/4 expression in HepG2 cells is likely triggered by TNF $\alpha$  and mediated by activation of the NF- $\kappa$ B signaling pathway.

**Key words** GSTA1/GSTA4, TNF $\alpha$ , NF- $\kappa$ B, cholestasis

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Cholestasis is best defined as an impairment of bile flow from the liver into the intestine and results in the intracellular retention of toxic bile constituents, including bile salts<sup>[1-2]</sup>. The consequent accumulation of bile salts in the hepatocytes leads to oxidative stress, pro-inflammatory cytokine production, apoptosis and severe hepatocellular injury<sup>[3-6]</sup>. The hepatic clearance of bile acids can be divided into the following 4 phases: phase 0, hepatic uptake; phase I, metabolism (e.g., hydroxylation); phase II, detoxification (e.g., conjugation); and phase III, excretion<sup>[7]</sup>. However, some studies have confirmed that the activities of the detoxification enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, and glutathione S-transferase (GSTs), were decreased in the livers of bile duct-ligated (BDL) rats<sup>[8-10]</sup>.

It is well established that GSTs are crucial for the

detoxification of a variety of endogenous or exogenous toxic compounds by conjugating lipophilic electrophiles to glutathione (GSH)<sup>[11]</sup>. The GSTs can bind non-substrate ligands, including bile acids and bilirubin, which makes them less toxic and better substrates for alternative elimination pathways<sup>[12]</sup>. As phase II detoxifying enzymes, the GST functions include the repair of macromolecules that are oxidized *via* reactive oxygen species, anti-apoptosis, biosynthesis of physiologically important metabolites, and regeneration of S-thiolated proteins<sup>[13-15]</sup>. Among

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the GSTs, the alpha-class GSTs contribute the majority of phospholipid hydroperoxide activity and the ensuing toxicity<sup>[16-18]</sup>. Moreover, GSTA1 and GSTA4 play key roles in the oxidative stress protection mechanisms that catalyze the conjugation of glutathione with peroxide<sup>[19-20]</sup>. GSTA1 is crucial for the binding of hydrophobic ligands and steroid hormones<sup>[21]</sup>, and it plays an important protective role in JNK-associated apoptosis by suppressing JNK signaling activation<sup>[22]</sup>. Some recent studies confirmed that Gsta4-null mice have a reduced ability to conjugate 4-HNE, increased susceptibility to CCl<sub>4</sub>, mitochondrial dysfunction, increased protein carbonylation, reduced antioxidant capacity and increased apoptosis<sup>[23-25]</sup>. However, the activity and expression of GSTs were reportedly reduced under obstructive cholestasis conditions<sup>[8, 10, 26]</sup>. The molecular mechanism of this reduction remains unclear.

We, and others, have found that the levels of tumor necrosis factor alpha (TNF $\alpha$ ) are significantly increased in both cholestatic patients and BDL rats<sup>[10, 27]</sup>. TNF $\alpha$  exerts a considerable positive effect on the hepatic inflammatory response and causes severe hepatic tissue damage<sup>[28]</sup>. A recent study indicated that TNF $\alpha$  decreases GSTA4 expression in 3T3-L1 adipocytes<sup>[23]</sup>. In addition, curcumin, the main polyphenolic active compound, was found to significantly decrease hepatic TNF $\alpha$  levels and increase GST enzyme activities<sup>[10]</sup>.

Therefore, we hypothesized that the down-regulation of hepatic GSTA1 and GSTA4 expression in cholestatic patients may be mediated by TNF $\alpha$ . Here, we investigate the molecular mechanism of GSTA1/GSTA4 reduction in human hepatoma HepG2 cells.

## 1 Materials and methods

### 1.1 Animal studies

Forty-two healthy male Sprague-Dawley (SD) rats, aged seven to eight weeks and weighing between 200 and 250 g, were obtained from the Center of Laboratory Animals of the Third Military Medical University, Chongqing, China. The rats were individually acclimated in plastic cages in temperature controlled (20°C ~23°C) rooms with humidity ranging between 40% and 60%, light cycles of 12-hour light and 12-hour dark, and fed a standard diet of rodent pellets and purified water. Before starting the experiments, the animals were allowed 1 week to

adapt to the new environment. The experimental protocols were reviewed and approved by the Ethics Committee of the Third Military Medical University, Chongqing, China. The 42 rats were randomly divided into two groups: the BDL group and the sham operation group. The animals were anesthetized using 50 mg/kg intraperitoneally injected sodium pentobarbital (21 rats per group). After the midline abdominal incision of the BDL group, the common bile duct (CBD) was isolated and doubly ligated with 4-0 silk sutures. In the sham operation group, rats received a sham-operation without ligation of the CBD. Seven rats in each group were sacrificed, according to standard protocol, after 3, 7 and 14 days post-surgery. The rat livers were immediately cut into small pieces and then kept in liquid nitrogen until use.

### 1.2 HepG2 cells culture and treatment

Human hepatoma HepG2 cells were purchased from the American Type Culture Collection USA (Catalogue No. ATCC HB-8065) and cultured as previously described<sup>[29]</sup>. The cells were starved with MEM containing 2% charcoal-stripped FBS (Biological Industries, Kibbutz Beit Haemek, Israel) for at least 12 h, and then treated with the indicated dose of chemicals for the indicated times. For the NF- $\kappa$ B signaling inhibition experiments, HepG2 cells were pre-treated with BAY 11-7082((E)-3-[4-methylphenylsulfonyl]-2-propenenitrile), obtained from the Beyotime Institute of Biotechnology, China, for 1 h before the addition of TNF $\alpha$ , obtained from the PeproTech Inc, USA(Catalogue No. 300-01A).

### 1.3 Cell viability analysis

The effect of different TNF $\alpha$  concentrations on cell viability was measured using the MTT assay, according to standard protocols. Briefly,  $5 \times 10^4$  cells were seeded per well in a 96-well plate. After 12 h of pre-incubation in complete medium, TNF $\alpha$  was added to the culture medium at various concentrations and the cells were incubated for 24 h. At 20 h post-treatment, 0.5 g/L of MTT was added to each well for the final 4 h. The absorbance was read at 570 nm. Five replicate wells were used for each concentration in three separate experiments.

### 1.4 RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA from rat livers and cultured HepG2 cells was extracted with Trizol reagent (Invitrogen, San Diego, CA). Total RNA was reverse transcribed into complementary DNA (cDNA) using the

PrimeScript RT reagent Kit with gDNA Eraser (Takara Biotechnology, Tokyo, Japan), and then real-time quantitative polymerase chain reaction (qPCR), using the SYBR premix Ex Taq II kit (Takara Biotechnology, Tokyo, Japan), was performed using a Bio-Rad CFX96 real-time system machine (Bio-Rad, Hercules, CA) to determine the mRNA levels of specific genes<sup>[27, 30]</sup>. The primers used in this study were used as previously described<sup>[26, 31]</sup> and are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization.

**Table 1 Primers used for Real-time qPCR (SYBR Green)**

Gene	Species <sup>1)</sup>	Sense primer (5'→3')	Antisense primer (5'→3')
GSTA1	H	aatggttgagattgatgggatg	gggctctctcttattgtctttc
Gsta2	R	gcaggagtggagtttgatgag	tttggtggcagatgtagtga
Gsta3	R	ggctaaggaaatgatggagtt	tggtggcaatgtagttgagaa
GSTA4	H	gagtcctgagatgggttttag	ttggaacagcaggtggttac
	R	gctggagtggagttgaaagaa	gatggctctggtctgtgca
Gstm1	R	cccatctctcaacctcac	gggcagacctcaaatcacag
Gstm2	R	tgctcccactatgacagaa	cgtccacacgaatcctctc
Gstm4	R	tcacactggctctgctctct	ccctgcctatccaactgaat
GAPDH	H	accacagtcacatgccatcac	tccaccacctgttctgtga
	R	acagcaacacgggtggtggac	tttgagggtgcagcgaactt

<sup>1)</sup>H: human; R: rat

### 1.5 Protein extraction and Western blotting analysis

The total and nuclear protein from HepG2 cells was extracted using a Total Protein Extraction Kit and Nuclear Protein Extraction Kit (Keygen Biotech, Nanjing, China). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA). The dilutions of primary antibodies were as follows: GSTA1 (1 : 1 000) (Santa Cruz Biotechnology, Santa Cruz, CA), GSTA4 (1 : 1 000) (Proteintech Group, Chicago, IL, USA), Nrf2 (1 : 10 000) (Abcam, Cambridge, MA, USA), NF- $\kappa$ B p65 subunit (1 : 1 000) (Santa Cruz Biotechnology, Santa Cruz, USA) and phosphorylated NF- $\kappa$ B subunit p65 (pS536; 1 : 10 000; Epitomics). SH-PTP1 (1 : 1 000) (Santa Cruz Biotechnology, Santa Cruz, USA) and GAPDH (1 : 1 000; Santa Cruz Biotechnology, Santa Cruz, USA) were used as the loading reference for the data analyses.

### 1.6 Statistical analysis

Statistical analyses were conducted with the SPSS 19.0 software package (SPSS, Chicago, USA). The data were expressed as the mean  $\pm$  SD from at least three experiments and were analyzed by the one way variance analysis (ANOVA) test, where  $P < 0.05$  was considered statistically significant.

## 2 Results

### 2.1 Reduction of Gst levels in the liver of BDL rats

To test the expression of Gsta/Gstm, we collected liver samples from the sham-operated and the BDL rats, after 3, 7 and 14 days post-surgery, and then we measured the expression of Gsta2, Gsta3, Gsta4, Gstm1, Gstm2 and Gstm4 by qPCR. Our results demonstrated that Gsta2, Gsta3, and Gsta4 mRNA levels in the liver samples of the BDL rats were significantly decreased starting 3 days post-surgery compared with the sham group (Figure 1a). The mRNA levels of Gstm1, Gstm2 and Gstm4 were significantly decreased starting 7 days post-surgery compared with the sham group (Figure 1b).

### 2.2 The bile acid content did not correlate with GSTA1 and GSTA4 expression in HepG2 cells

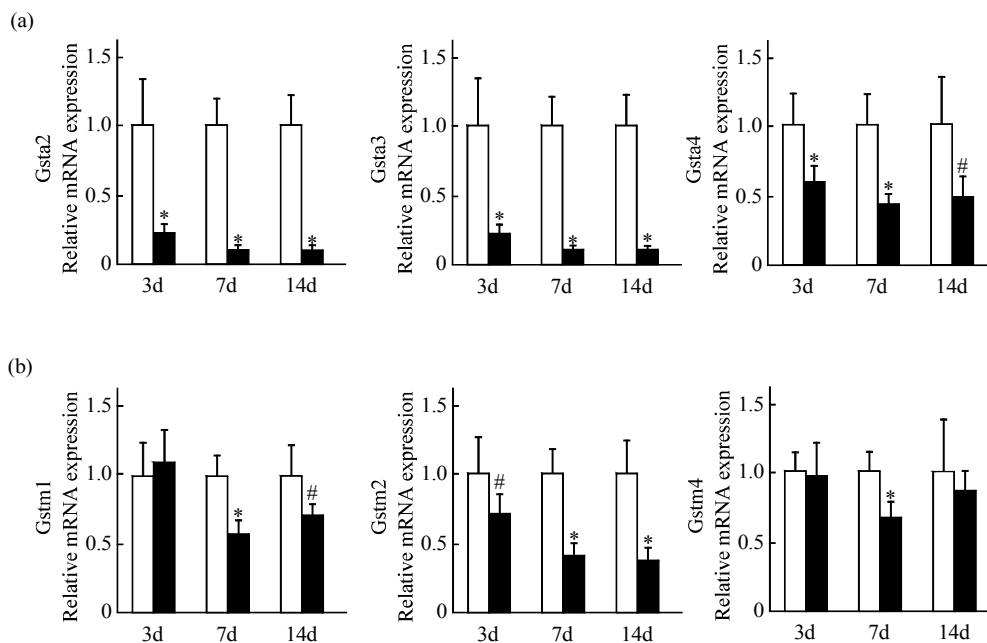
Considering that the accumulation of bile acid plays a major role in the pathogenesis of cholestasis, we tested whether bile acid levels regulate the GSTA1 and GSTA4 expression. We treated cultured HepG2 cells with cholic acid (CA) and chenodeoxycholic acids (CDCA), and Na<sup>+</sup>/taurocholate cotransporter (NTCP)-transfected HepG2 cells, allowing NTCP over-expression along with varying doses of taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDC) and glycochenodeoxycholic acid (GCDCA). Western blotting analysis revealed that GSTA1 and GSTA4 protein levels were not significantly changed by any of these treatments when compared with control.

### 2.3 GSTA1 and GSTA4 expression in HepG2 cells was repressed by TNF $\alpha$

We, and others, have confirmed that the TNF $\alpha$  levels are significantly increased in both cholestatic patients and BDL rats<sup>[10, 27]</sup>. To investigate the connection between TNF $\alpha$  levels and GSTA1/GSTA4 expression, we treated HepG2 cells with TNF $\alpha$  at varying doses. We quantified the GSTA1/GSTA4 mRNA and protein expression by qPCR and Western blotting respectively. Doses of TNF $\alpha$  ranging from

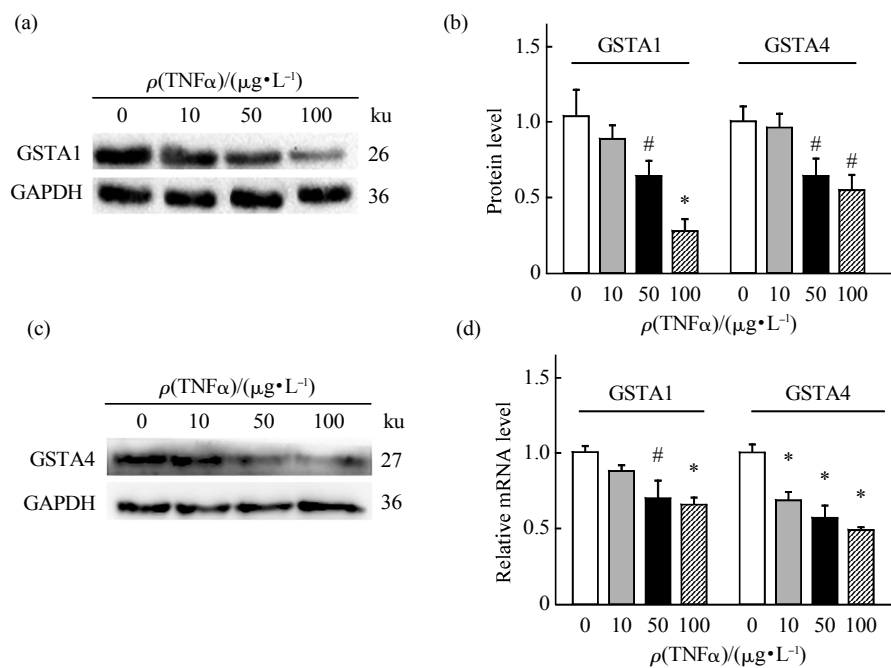
10~100  $\mu\text{g/L}$  were used. A dose-dependent inhibition in both mRNA (Figure 2d) and protein levels was

observed (Figure 2a, b, c). Furthermore, we investigated the time-dependent effect of  $\text{TNF}\alpha$  on



**Fig. 1** Changes in the expression of Gsta2~4 and Gstm1, 2, 4 in the liver of BDL rats

(a) Real-time qPCR analysis of Gsta2, Gsta3 and Gsta4 mRNA levels in the liver of BDL rats after 3, 7 and 14 days post-surgery. (b) Real-time qPCR analysis of Gstm1, Gstm2 and Gstm4 mRNA levels in the liver of BDL rats after 3, 7 and 14 days post-surgery. ( $n = 7$  for each group).  $*P < 0.01$  and  $\#P < 0.05$  vs controls. □: Sham; ■: Bile duct ligation.



**Fig. 2**  $\text{TNF}\alpha$  repressed GSTA1/GSTA4 expression in a dose-dependent manner in HepG2 cells

It was detected by Western blotting analysis (a, b, c) and SYBR green qPCR (d) that different doses of  $\text{TNF}\alpha$  repressed GSTA1 and GSTA4 expression. Data is given as the mean  $\pm$  SD ( $n=3$ ).  $*P < 0.01$  and  $\#P < 0.05$  vs control.

mRNA and protein levels of GSTA1/GSTA4. TNF $\alpha$  (100  $\mu$ g/L) decreased the GSTA1/GSTA4 expression at both the mRNA (Figure 3d) and the protein level (Figure 3a, b, c).

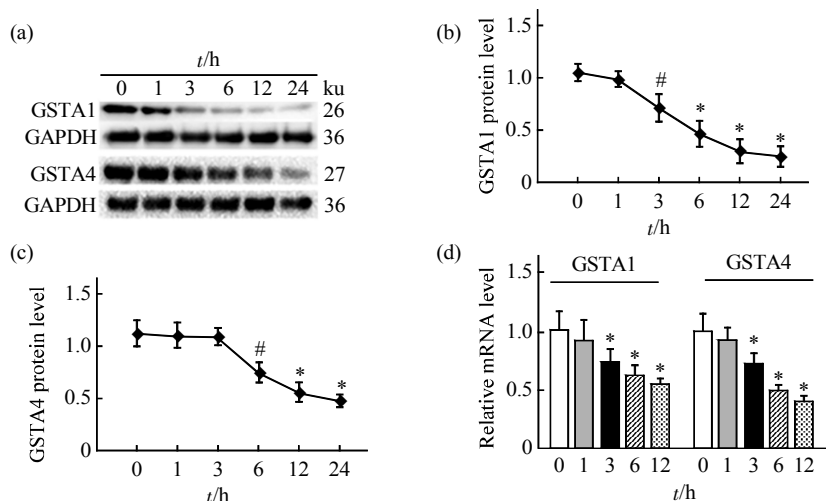
In addition, we tested whether the effect of TNF $\alpha$  on GSTA1 and GSTA4 was due to cytotoxicity of TNF $\alpha$  using MTT. The results demonstrated that the tested doses of TNF $\alpha$  have no influence on HepG2 cell viability. Taken together, these results confirmed a role for TNF $\alpha$  in repressing GSTA1/GSTA4 expression.

#### 2.4 TNF $\alpha$ activated NF- $\kappa$ B signaling and did not repress Nrf2 protein expression

Nrf2 is known to be essential for inducing

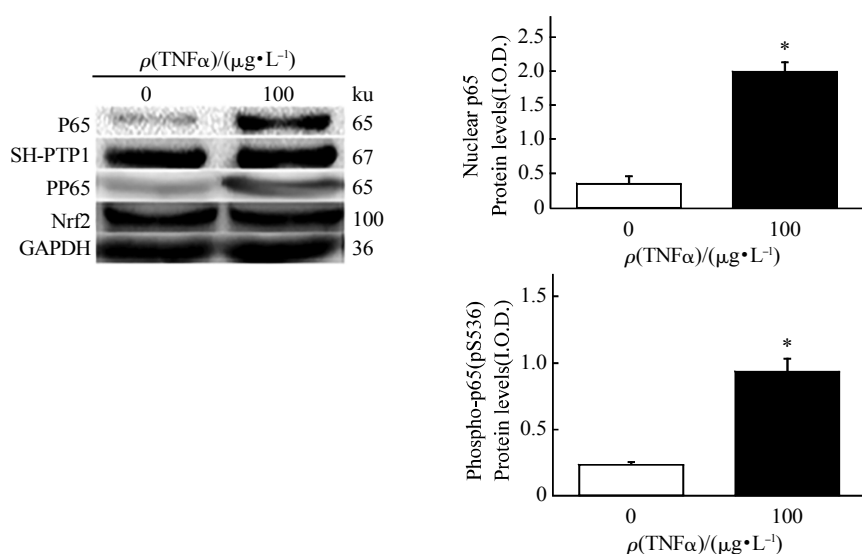
phase II detoxifying enzymes expression, including GSTA1 and GSTA4 [32-33]. Thus, we tested whether the GSTA1 and GSTA4 expression decrease was due to a change in Nrf2 expression. We found that there was no significant change in total Nrf2 protein levels (Figure 4).

NF- $\kappa$ B plays an important and well-established role in many inflammation processes and is one of the main TNF $\alpha$  signaling pathways. NF- $\kappa$ B is normally sequestered in the cytoplasm by the inhibitor molecule I $\kappa$ B. Once activated, the I $\kappa$ B kinase (IKK) signalosome is activated and I $\kappa$ B is ubiquitinated and subsequently degraded, allowing the phosphorylation



**Fig. 3** TNF $\alpha$  repressed GSTA1/GSTA4 expression in a time-dependent manner in HepG2 cells

It was detected by Western blotting analysis (a, b, c) and by SYBR green qPCR (d) that TNF $\alpha$  (100  $\mu$ g/L) repressed GSTA1/GSTA4 expression in a time-dependent manner. Data is given as the mean  $\pm$  SD ( $n=3$ ). \* $P < 0.01$  and # $P < 0.05$  vs control.



**Fig. 4** The effect of TNF $\alpha$  (100  $\mu$ g/L) on inducing of NF- $\kappa$ B signaling and Nrf2 protein expression in HepG2 cells

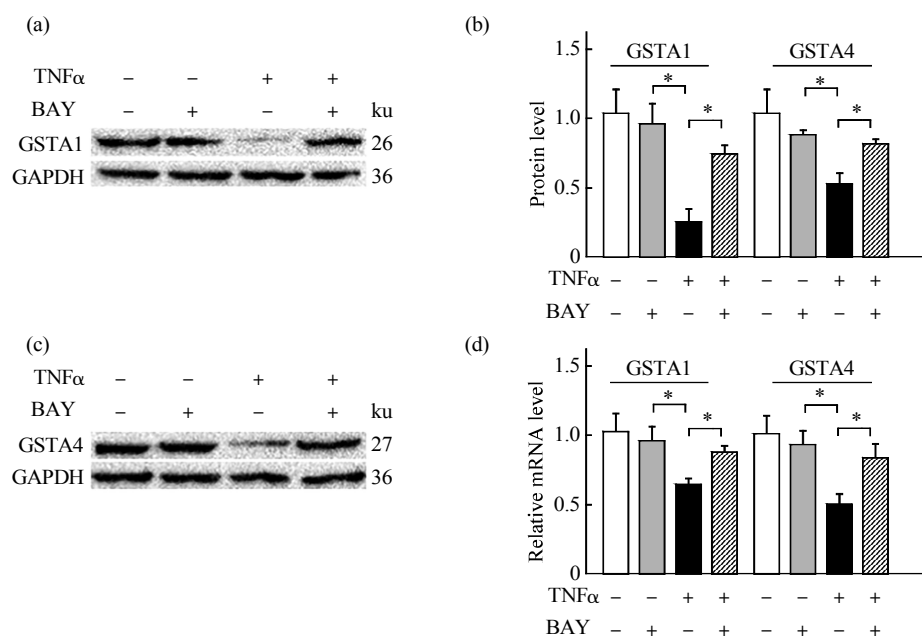
TNF $\alpha$  (100  $\mu$ g/L) increased levels of the p65 in the nuclear fraction and the phosphorylated p65 in total protein; whereas had no effect on Nrf2 protein expression. Data is given as the mean  $\pm$  SD ( $n=3$ ). \* $P < 0.01$ .

of the p65/50 hetero-dimer, which is the main form of NF- $\kappa$ B. The phosphorylated p65/p50 moves to nucleus and induces target gene expression. We examined whether NF- $\kappa$ B signaling was activated by TNF $\alpha$  in the HepG2 cells. We treated HepG2 cells with 100  $\mu$ g/L of TNF $\alpha$  for 12 h and then extracted both the total and the nuclear protein. The total p65 in the nuclear fraction and the phosphorylated p65 in total protein were significantly increased after TNF $\alpha$  treatment (Figure 4). Taken together, these data indicated that TNF $\alpha$  activated NF- $\kappa$ B signaling in the HepG2 cells but did not reduce Nrf2 protein expression.

### 2.5 Inhibition of NF- $\kappa$ B activation attenuated TNF $\alpha$ -mediated decrease in GSTA1 and GSTA4 expression

We next examined whether NF- $\kappa$ B signaling participated in the TNF $\alpha$ -induced GSTA1 and GSTA4 down-regulation. HepG2 cells were treated with 50  $\mu$ mol/L of BAY 11-7082 (NF- $\kappa$ B signaling inhibitor) for 1 h, and then 100  $\mu$ g/L of TNF $\alpha$  for another 24 h.

The results demonstrated that TNF $\alpha$  treatment alone significantly decreased the GSTA1 mRNA and protein levels in HepG2 cells to 64.3% (Figure 5d) and 25.4% (Figure 5b), respectively, when compared with the control cells. However, pre-treatment with BAY 11-7082 prior to TNF $\alpha$  were significantly increased the GSTA1 mRNA and protein expression by 23.2% (Figure 5d) and 49% (Figure 5b), respectively, when compared with TNF $\alpha$  treatment alone. Similarly, treatment with only TNF $\alpha$  significantly decreased the GSTA4 mRNA and protein levels to 50.5% (Figure 5d) and 52.6% (Figure 5b), respectively, when compared to the control cells. However, in the HepG2 cells stimulated with TNF $\alpha$  in the presence of BAY 11-7082 the GSTA4 mRNA and protein levels were significantly increased by 33.8% (Figure 5d) and 29.1% (Figure 5b), respectively, when compared with the TNF $\alpha$  treatment alone. Taken together, these results suggested that NF- $\kappa$ B is a crucial factor in the TNF $\alpha$ -induced down-regulation of GSTA1 and GSTA4 expression.



**Fig. 5 Inhibition of NF- $\kappa$ B activation suppresses TNF $\alpha$ -mediated decrease in GSTA1/GSTA4 expression in HepG2 cells**

After 1 h of treatment with 50  $\mu$ mol/L BAY 11-7082, HepG2 cells were cultured for 24 h with TNF $\alpha$  (100  $\mu$ g/L). GSTA1/GSTA4 expression was measured by Western blotting analysis (a, b, c) and by SYBR green qPCR (d). Data is given as the mean  $\pm$  SD ( $n=3$ ). \* $P < 0.01$  and  $^{\#}P < 0.05$  vs controls.

### 3 Discussion

The induction of detoxification enzymes is one of the essential adaptive protective responses in cholestatic hepatocytes [27, 30, 34-36]. However, GSTA1/

GSTA4 expression is reduced in cholestatic patients and rodents. The molecular mechanism of GSTA1/GSTA4 down-regulation remains elusive.

The accumulation of toxic bile acids in hepatocytes likely plays a role in triggering cholestatic

liver injury. However, the results obtained in this study indicated that bile acids were not involved in GSTA1/GSTA4 down-regulation. Obstructive cholestasis is always accompanied by inflammatory cytokines, such as TNF $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 [37]. Among these inflammatory cytokines, TNF $\alpha$  is involved in regulating some transporter expression [27, 38-39]. We, and others, have found that the levels of TNF $\alpha$  are significantly increased in both cholestatic patients and BDL rats [10, 27]. In the present investigation, we demonstrated that GSTA1/GSTA4 expression was repressed by TNF $\alpha$  in HepG2 cells, which was similar to the results found in 3T3-L1 adipocytes [23].

The expression of GSTA, as one of the phase II enzymes, is cooperatively controlled by the Nrf2 signaling pathway. To test whether the TNF $\alpha$ -induced reduction in GSTA1/GSTA4 was related to the decrease in Nrf2 expression, we analyzed total Nrf2 protein levels in the HepG2 cells after TNF $\alpha$  treatment. Interestingly, the data showed that the reduction in GSTA1/GSTA4 was not accompanied by a change in Nrf2 protein, which is consistent with our previously findings from clinical liver samples of obstructive cholestasis [26]. These results also suggest that the transcriptional activity of Nrf2 is impaired during obstructive cholestasis.

Because TNF $\alpha$  is well-known to induce NF- $\kappa$ B signaling, which is implicated in diverse pathological processes including infection, inflammation and cancer [40], we tested whether NF- $\kappa$ B signaling was activated by TNF $\alpha$  and involved in the regulation of GSTA1/GSTA4 in HepG2 cells. We observed that total p65 protein in the nuclear fraction and phosphorylated p65 in total protein were significantly increased after TNF $\alpha$  treatment alone (Figure 4). Then, we demonstrated that the NF- $\kappa$ B signaling pathway was involved in the TNF $\alpha$ -induced down-regulation of GSTA1/GSTA4 expression (Figure 5b and d). These findings indicate that NF- $\kappa$ B may decrease Nrf2 transcriptional activity under obstructive cholestasis conditions. As pathological stimuli, activate both the NF- $\kappa$ B signaling pathway and the Nrf2-ARE (the antioxidant-response element) pathway [41-43], there may be crosstalk between these two pathways in different pathological processes. For example, a recent study reported that the NF- $\kappa$ B subunits p50/p65 induce transcription of Nrf2 in AML cells, human monocytes and THP-1 cells at a specific promoter  $\kappa$ B-site [44]. Conversely, another study reported that NF- $\kappa$ B also

antagonizes Nrf2-ARE signaling [45], similar to our findings. Furthermore, some studies have confirmed that p65 can recruit co-activators in nucleus, such as CREB binding protein (CBP) [46]. Meanwhile, CBP and the small Maf proteins are well-established co-activators of Nrf2, and they are required for the transcriptional activity of Nrf2 [47-50]. Taken together, it is possible that the TNF $\alpha$ -induced reduction of GSTA1/GSTA4 in obstructive cholestasis is due to NF- $\kappa$ B inhibition of the Nrf2 interaction with its co-activators, resulting in impaired Nrf2-ARE binding. The specific molecular mechanism needs to be further studied.

In summary, we demonstrate that TNF $\alpha$  can transcriptionally repress the GSTA1/GSTA4 expression through the NF- $\kappa$ B signaling pathway and that inhibition of the NF- $\kappa$ B signaling pathway could reverse this repression. The NF- $\kappa$ B signaling pathway may be a possible therapeutic target to improve the liver's detoxification ability and to attenuate liver injury in human obstructive cholestasis.

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## HepG2 细胞中 TNF $\alpha$ 通过 NF- $\kappa$ B 信号通路 下调 GSTA1/GSTA4 表达 \*

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**摘要** 谷胱甘肽巯基转移酶  $\alpha 1/\alpha 4$ (GSTA1/A4)是体内重要的解毒酶, 可降低多种内、外源性毒性化合物的毒性。然而, 胆汁淤积病人肝细胞内 GSTA1/A4 的表达是下调的, 下调机制尚不清楚。本研究通过肿瘤坏死因子  $\alpha$ (TNF $\alpha$ )处理人肝癌细胞 HepG2 细胞, 利用实时荧光定量聚合酶链式反应(qPCR)和蛋白质印迹(Western blot)检测 GSTA1/A4、核因子  $\kappa$ B(NF- $\kappa$ B)和核因子 E2 相关因子 2(Nrf2)的表达。发现 TNF $\alpha$  在 mRNA 水平和蛋白质水平均抑制 GSTA1/A4 表达, 且呈剂量与时间依赖关系。干扰 NF- $\kappa$ B 信号通路, 可减弱 TNF $\alpha$  对 GSTA1/A4 表达的抑制作用。以上结果表明, 在 HepG2 细胞中, TNF $\alpha$  可通过激活 NF- $\kappa$ B 信号通路抑制 GSTA1/A4 表达。

**关键词** GSTA1/GSTA4, TNF $\alpha$ , NF- $\kappa$ B, 胆汁淤积

**学科分类号** Q2, Q7

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