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Original Article

TAB3 overexpression promotes NF-κB activation and inflammation in acute pancreatitis

Lili Gao[1], Hongmei Zhao[1], Hong Sun[1], Ying Huang[2]

[1]Department of Emergency, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University, Huai'an, Jiangsu Province, China; [2]Department of Intensive Care Unit, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University, Huai'an, Jiangsu Province, China

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Abstract: Acute pancreatitis (AP) is a clinically common inflammatory disease, NF-κB activation and the secretion of pro-inflammatory mediators have been considered as the main events of AP. According to reports, TAB3 is essential for NF-κB activation and participates in inflammatory responses. In this study, we used caerulein to establish an AP rat model and cell model. The expression of TAB3 was measured both in control group and AP group by Western blot and Immunohistochemistry. We firstly found the expression of TAB3 was significantly increased in caerulein-induced AP rat and cell model compared with control group, especially at 8 h. Furthermore, the increasing expression of TAB3 in AP group was also accompanied by increased levels of pro-inflammatory mediators (TNF-α, IL-6 and LDH). In addition, the decreasing expression of TAB3 in TAB3-siRNA transfected AP AR42J cells was accompanied by reduced levels of pro-inflammatory cytokines and inhibited the production of p-P65. These findings suggested that TAB3 may accelerate the inflammatory responses of AP through NF-κB activation.

Keywords: TAB3, NF-κB, AR42J cells, acute pancreatitis, inflammation, caerulein

Introduction

Acute pancreatitis is a common clinical condition with higher incidence and mortality [1-3]. The generally accepted pathogenesis is the activation of trypsinogen, which could provoke pancreatic auto-digestion and subsequently systemic inflammatory response [4]. Nevertheless, its precise pathogenesis has not yet been fully elucidated.

Several animal models have been used to study the pathogenesis of acute pancreatitis, one of the most common models is caerulein-induced AP model [5-8]. Caerulein is a gastric regulatory molecule similar to cholecystokinin, which could stimulate the secretion of pancreas. The mechanisms involved in are NF-κB activation and pro-inflammatory response.

According to many related literatures, TAB3 (transforming growth factor β-activated kinase binding protein 3) is a binding protein of TAK1 and can link TAK1 to TRAFs, subsequently activating NF-κB pathway. TAK1 first activates IKKs, subsequently IKKs phosphorylate IκBs and lead to the activation of NF-κB [9-14]. In this study, we found that TAB3 was overexpressed in AP. However, its molecular mechanism is not clear.

In this study, we established a caerulein-induced AP cell and animal model to analyze the expression level of TAB3 and to evaluate its potential effect on pro-inflammatory cytokines. Furthermore, we explored the role of TAB3 in NF-κB activation. Since NF-κB activation has a positive correlation with the inflammatory responses of AP, so we speculated that TAB3 may participate in AP through NF-κB pathway. To test our hypothesis, we transfected TAB3-siRNA into AP AR42J cells and found TAB3 siRNA could inhibit NF-κB activation in caerulein-induced AP, as well as reduce the levels of inflammatory mediators. Our study firstly demonstrated that the upregulation of TAB3 accelerated the AP progression via facilitating NF-κB activation. This study may provide a new clinical treatment direction for AP.

Materials and methods

Cell culture

Rat pancreatic AR42J acinar cells were purchased from ATCC and maintained at 37°C in humidified air with 5% CO_2 and 95% oxygen. The 100 nM caerulein (Sigma, #C9026) and PBS (phosphate buffered saline) was respectively added to the caerulein group and the control group at different time (0-24 h).

Western blot analysis

The protein samples were analyzed by PAGE and transferred to a PVDF membrane. The PVDF membrane was firstly blocked with 5% skim milk and then incubated with antibody against TAB3 (1:500, Santa Cruz Biotechnology, USA), IKKβ (1:500, Cell Signaling), P65 (1:500, Santa Cruz), p-P65 (1:500, Santa Cruz), β-actin (1:1000, Santa Cruz). After washing with TBST, the transfer membrane was placed into the second antibody and incubated for 2 h in the dark. Subsequently, ECL system (enhanced chemiluminescence system, Thermo Scientific Pierce, Rockford, USA) was used to detect the immunocomplexes, and the gray value of bands was measured by Bio-Rad image processing system. Finally, the density of bands was compared by ImageJ (NIH).

siRNA and transfection

The TAB3 siRNA target sequence was as follows: 5'-CTGAGGAAATGACAAGATT-3' (siRNA#1), 5'-GGTTGAAGTCTGAAGTTAA-3' (siRNA#2), 5'-TCCTTCATACATGCACATA-3' (siRNA#3). AR42J cells were planted in dishes and replaced with fresh culture for transfection siRNA. The 100 nM caerulein was added to AR42J cells after transfection for 40 h and were collected to carry on Western blot after transfection for 48 h.

Immunohistochemistry

Firstly, the rat pancreas specimens were respectively fixed and embedded by 10% formalin and paraffin wax, then all sections were successively dewaxed, rehydrated, and processed in citrate buffer for antigen retrieval. Subsequently, the slices were successively treated with 3% H_2O_2 and antibody against TAB3. After cleaning with PBS, the slices were incubated with DAB. Finally, the sections were respectively dehydrated, cleared, covered and examined under a Leicalight microscope.

Animal model

SD male rats (weighing from 180 g to 200 g) were purchased. 50 μg/kg caerulein was injected into the abdominal cavity of SD rats every four hours to induce AP model. Meanwhile the control groups were treated with PBS. Finally, all treated rats were killed from 4 h to 24 h.

Enzyme linked immunosorbent assay

After treatment with caerulein, the supernatant fluid was collected. We use ELISA kits to measure the level of pro-inflammatory mediators in the supernatant fluid.

Statistical analysis

The data were expressed as mean plus standard deviation. The statistical differences between groups were analyzed by One-way ANOVA with SPSS 15.0 software. $P<0.05$ is considered statistically significant. Each test was duplicated more than three times.

Results

Elevated TAB3 expression in caerulein-induced acute pancreatitis rat model

To study the level of TAB3, an AP rat model was established [15]. We performed immunohistochemistry analysis to study the expression of TAB3 in normal tissues and caerulein-induced pancreatitis tissues. As **Figure 1A** demonstrated, the TAB3 expression level was obviously increased in AP group compared with the control. Next, Western blot analyses was carried out to explore the expression of TAB3 in control and caerulein-induced pancreatitis tissues. As expected, the expression of TAB3 was significantly upregulated in AP group compared with the control, especially at 8 h (**Figure 1B**, **1C**). Thus, further experiment was required to clarify the possible function of TAB3 in the occurrence and development of AP.

Caerulein stimulated TAB3 expression and pro-inflammatory cytokine production in AR42J cells

In order to further study the role of TAB3 in AP, an *in vitro* AP cell model was established by treating AR42J cells with caerulein. As **Figure**

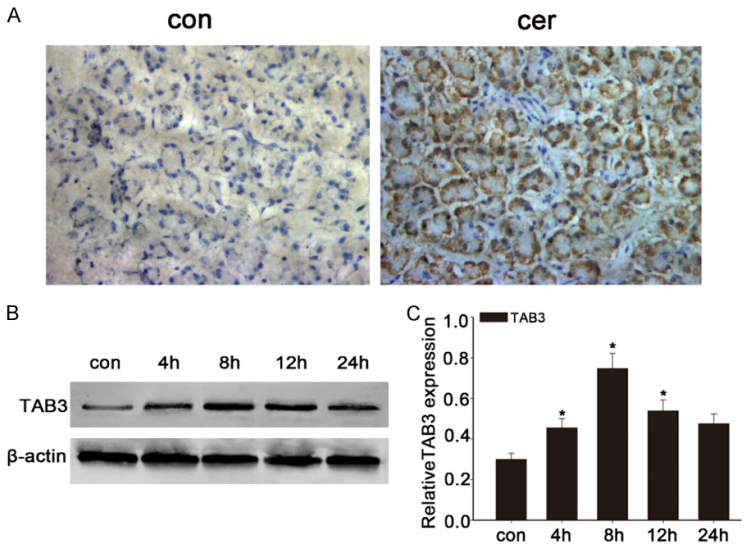

Figure 1. Elevated TAB3 expression in caerulein-Induced AP rat model. A. Representative images of rat pancreatic tissue from the control and AP group (n = 7 per group), immunostained for TAB3 (×200 magnification). B. The protein levels of TAB3 in the control and AP tissue was determined by Western blot analysis. C. The relative expression of TAB3 was quantified by referring to the amount of β-actin (*P<0.05).

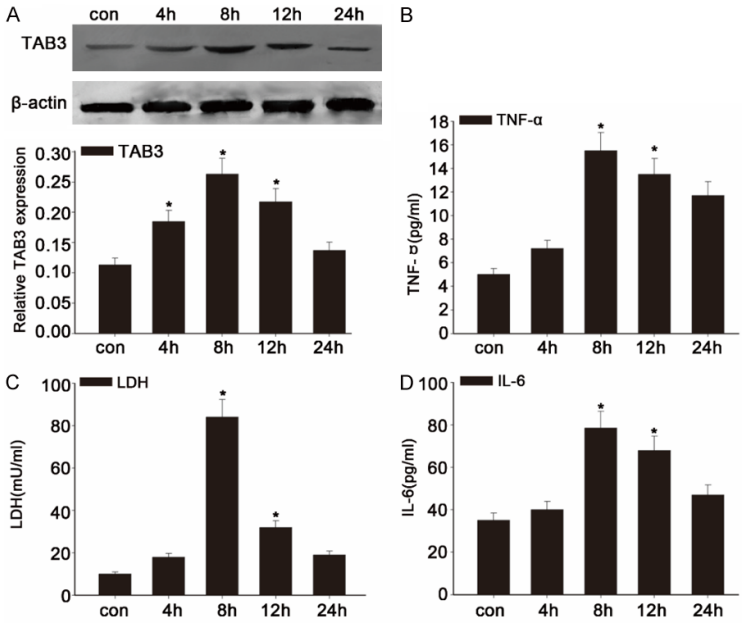

Figure 2. Caerulein stimulated TAB3 expression and pro-inflammatory cytokine production in AR42J cells. The AR42J cells were treated with 100 nM caerulein for different time (from 4 h to 24 h). The protein levels of TAB3 was determine by Western blot analysis and the relative expression of TAB3 was quantified by the intensity of staining of TAB3 to β-actin (A). The levels of TNF-α, IL-6 and LDH (B-D) in treated AR42J cells were measured by ELISA (*P<0.05).

2A demonstrated, the TAB3 level was lowered in the control group, while the TAB3 level was gradually increased in AP group, especially at 8 h, and subsequently slowly declined. Meanwhile, ELISA assay was carried out to detect the levels of pro-inflammatory cytokines in the supernatant, including TNF-α, IL-6 and LDH (**Figure 2B-D**). The levels of TNF-α, IL-6 and LDH were lowered in the control group, slightly elevated in AP group, especially at 8 h, and then slowly declined. Since the expression level of TAB3 in parallel with the production of inflammatorymediators after caerulein stimulation, we infered that TAB3 might play a positive role in the inflammatory process of AP.

Inhibition of TAB3 attenuated the caerulein-Induced pro-inflammatory mediator production in AR42J cells

The up-regulation of TAB3 in AP tissues indicates that the inhibition of TAB3 could mitigate caerulein-induced acinar cell injury. Firstly, we observed the inhibiting effect of TAB3 in AR42J cells *in vitro by* small interfering technology. As shown in **Figure 3A**, compared with control siRNA, siRNA#1, siRNA#2 and siRNA#3 reduced the level of TAB3, and the siRNA#2 had the best interference effect. Thus, we used TAB3 siRNA#2 to observe the endogenous levels of TAB3 in regulating caerulein-induced inflammatory response. To value the potential impacts of TAB3 inhibition on inflammatory response in AP, the levels of TNF-α and IL-6 were measured. Compared with the normal group, TAB3 inhibition apparently weakened the levels of TNF-α and IL-6 in caerulein-induced AR42J cells (**Figure 3B**, **3C**). Then, we further measured the levels of LDH. As depicted in **Figure 3D**, the elevated level of LDH in non-transfected AR42J cells was sharply declined in the TAB3 siRNA transfected AR42J cells. These results indicated that inhibition of TAB3 can

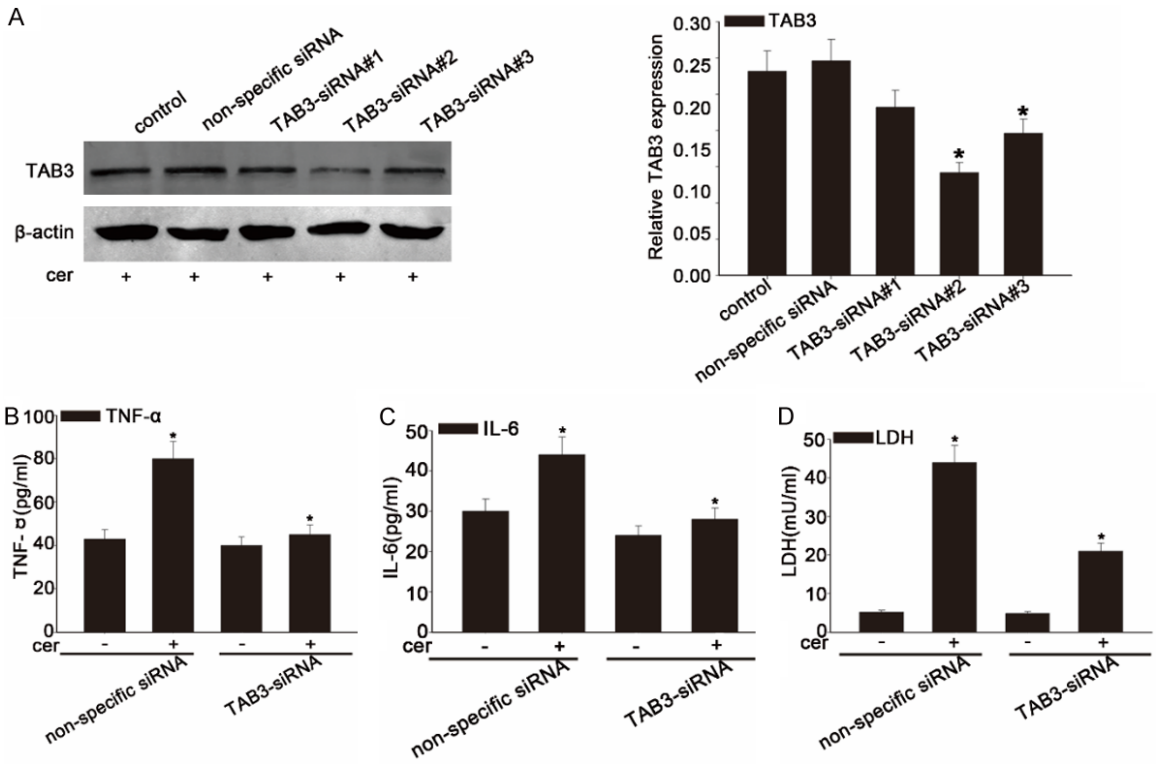

Figure 3. Effects of TAB3 inhibited by siRNA on caerulein-induced pro-inflammatory mediator production. A. The protein levels of TAB3 in caerulein-induced AR42J cells transfected with non-specific siRNA or TAB3-siRNA and quantification of TAB3 levels. B-D. The levels of TNF-α, IL-6 and LDH in treated AR42J cells were measured by ELISA (*P<0.05).

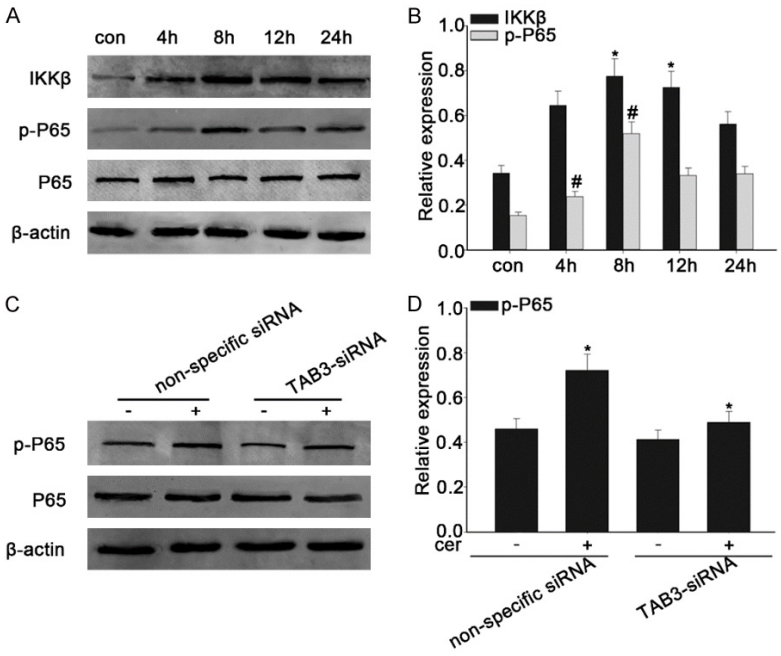

Figure 4. TAB3-siRNA cells alleviated the caerulein-triggered inflammatory response through NF-κB pathway. Non-transfected and transfected AR42J cells were respectively treated with 100 nM caerulein for different time points, including 0 h, 4 h, 8 h, 12 h, 24 h. A. The expression of IKKβ and phosphorylated P65 in non-transfected AR42J cells after caerulein treatment was measured by Western blot analysis. B. Relative quantitation analysis of IKKβ and phosphorylated P65 protein expression, β-actin was used as a loading control. C, D. The protein levels of activating phosphorylation of P65 in TAB3-siRNA transfected cells were measured by Western blot analysis and quantitative analysis of the intensity of protein expression relative to β-actin in the indicated groups (*, #P<0.05).

markedly alleviate the severity of inflammatory response in caerulein-induced AR42J cells.

TAB3-siRNA cells alleviated the caerulein-triggered inflammatory response through NF-κB pathway

NF-κB pathway is a classic signaling pathway in acute pancreatitis. In our experi-

ment, we verified that caerulein markedly increased the levels of IKKβ and phosphorylated P65 in AP cell model (**Figure 4A**, **4B**). Many literatures demonstrated that TAB3 is closely associated with the NF-κB pathway. Therefore, we guessed that NF-κB activity may be positively regulated by increasing TAB3 expression in caerulein-induced AR42J cells, and then regulate the inflammatory process. To test the above argument, the phosphorylation of P65 in siRNA transfected cells was determined by Western blot. Consistent with previous findings, caerulein remarkably augmented the production of p-P65 in non-specific siRNA cells. However, this augment was markedly decreased in the TAB3 siRNA transfected cells (**Figure 4C**, **4D**). By the above experiment results, we concluded that the high levels of TAB3 could accelerate the inflammatory responses of AP through NF-κB activation.

Discussion

AP is a common digestive condition and is generally related to excessive drinking and cholelithiasis gallstones in bile duct [16, 17]. All the time, the activation of digestive enzymes and the self-digestion of pancreatic tissue are regard as the important events of AP, which could cause systemic inflammatory response and organ dysfunction [18-22]. In recent years, more and more evidences have shown that NF-κB signal pathway is of great concern in AP [23-25]. NF-κB is a ubiquitous nuclear transcription factor and is composed of NF-κB1 (P50), NF-κB2 (P52), RelA (P65), RelB, c-Rel [26, 27]. Our study firstly confirmed that the up-regulated expression of TAB3 activated NF-κB pathway, and promoted the secretion of pro-inflammatory mediators in caerulein-induced AR42J cells, which may accelerate the occurrence of AP.

To verify this hypothesis, we established a caerulein-induced AP cell and animal model to investigate the role of TAB3 in AP. We confirmed that caerulein increased the levels of IKKβ and p-P65 in AP cell model. We found that the TAB3 expression level was obviously increased in AP group compared with the control by Western blot and Immunohistochemistry assays. Furthermore, we found that the elevated expression of TAB3 was related with an up-regulated levels of TNF-α, IL-6 and LDH. Subsequently, the inhibiting effect of TAB3 was evaluated by small interfering technology. TAB3 inhibition obviously reduced the levels of TNF-α, IL-6 and LDH in caerulein-induced AR42J cells. Moreover, the phosphorylation of P65 in siRNA transfected cells was determined by Western blot. We found that the level of p-P65 was remarkably decreased in the TAB3 siRNA transfected and caerulein-treated AR42J cells. Therefore, we firstly reported that caerulein induced TAB3 expression, and the up-regulation of TAB3 accelerated the AP progression via facilitating NF-κB activation.

Taken together, the results of the present study showed that the expression of TAB3 was significantly increased in AP and accompanied by increased levels of pro-inflammatory mediators. The TAB3 inhibition was accompanied by reduced levels of pro-inflammatory cytokines and p-P65. We concluded that TAB3 may contribute to the development of AP by activating NF-κB signaling pathway. However, its detailed molecular mechanisms still need further research.

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Disclosure of conflict of interest

None.

Address correspondence to: Hong Sun, Department of Emergency, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University, No. 1, The Yellow West Road, Huaiyin District, Huai'an 223300, Jiangsu Province, China. Tel: +86-13511-528858; E-mail: sh608858@126.com; Ying Huang, Department of Intensive Care Unit, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University, No. 1, The Yellow West Road, Huaiyin District, Huai'an 223300, Jiangsu Province, China. Tel: +86-15896182075; E-mail: huangying5249@163.com

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