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Suppressing progress of pancreatitis through selective inhibition of NF-κB activation by using NAC

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Abstract: Objective: To explore the characteristics of NF-κB activation in the progress of pancreatitis, the relationship with expression of TNF-*α* in the inflammatory reaction, and prevent the exacerbation of pancreatitis by using NAC. Method: Forty-eight rats were divided into three groups: therapy (group C), pancreatitis (group B) and control (group A). NAC served as the inhibitor of NF-κB activation. In the time intervals of 1.5, 3.0, 6.0, 12.0 hour, NF-κB activation was detected with flow cytometry (FCM) and the expression of TNF-*α* mRNA and protein with in situ hybridization (ISH) and enzyme-linked immuno-sorbent assay (ELISA) respectively. Meanwhile, the level of lipase and amylase in the serum was assayed and the pathological change was evaluated. Result: NF-κB activation in the pancreatitis group was higher than that in the control group ($P<0.01$), peaked at 3 hours, and was depressed by the inhibitor of NF-κB, NAC. The expression of TNF-*α* as well as the level of lipase and amylase in the serum also rose synchronously with activation of NF-κB. In contrast to group A, it was significantly different ($P<0.01$) in group B. After using NAC in group C, all of these values were decreased and the inflammatory reaction in the pancreas abated evidently. The pathology changes of the pancreas were shown to be alleviated in group C. Conclusion: First, NF-κB activity is intensively initiated in the course of pancreatitis and shown to have closely relationship with the release of cytokines. Second, use of NAC markedly depressed NF-κB activation. TNF-*α* expression is down regulated by cytokines. It is suggested that NAC probably acts as a useful agent for treatment of pancreatitis by indirectly inhibiting activation of NF-κB.

Key words: Pancreatitis, NF-κB, TNF-*α*, N-acetycysteine, Cytokine
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INTRODUCTION

Acute pancreatitis is believed to have intracellular activation of digestive enzymes and autodigestion of the pancreas as its central pathophysiologic cause. This noninfectious destruction of pancreatic parenchyma quickly induces an inflammatory reaction at the site of injury (Norman, 1998). Inflammatory media play a critical role in the pathogenesis of acute pancreatitis and is hot topic of research on the mechanisms of acute pancreatitis up to date (Lowry, 1993).

Nuclear factor-kappa B (NF-κB) serves as a transcriptor modulating large numbers of proteins. NF-κB molecule, activated by different initiators, causes a variety of cytokines to be released from inflammatory cells thus resulting in severe inflammation and immune reaction in the pancreatic tissue. In the current study, the function of activation of NF-κB on the progress of pancreatitis was

assessed by the flow cytometry (FCM). In situ hybridization (ISH) and enzyme-linked immunosorbent assay (ELISA) were used in this investigation of the level of TNF-*α* in gene transcription and protein expression showing that the levels of these cytokines were much higher than those of the control group. After the activation of NF-κB depressed by N-acetycysteine (NAC)-antioxidant, the expression of these cytokines was decreased remarkably and the pathological changes ameliorated simultaneously.

MATERIAL AND METHOD

Materials

Monoclone antibody for NF-κB p65 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IgG labeled FITC was purchased from Jackson company (USA). Sodium taurocholate (STC), propidium (PI), Triton X-100, DEPC was purchased from Sigma Corp. TNF-*α* mRNA hybridization kit in situ was purchased from Boster Corp (Wuhan, Hubei). TNF-*α* protein test kit was a product of Genzyme Corp in USA. Wistar rats, weighting 255±20 g were provided by animal center of Shanxi Medical University.

Experimental design

Forty-eight Wistar rats weighting 255±20 g were divided into 16-rats groups: Group 1 (controls), Groups 2 (AP) and group 3 (AP+NAC). The animal model of pancreatitis was prepared by the Aho *et al.*(1980) method. A tube was retro-inserted about 0.5 cm into the pancreatic duct and 5% STC (1 ml/kg) was injected into the tube at 0.1 ml/min. The control group received the same amount of saline solution instead of STC. Group 3 received injections of intraperitoneal NAC (500 mg/kg) simultaneously. Thereafter, NAC was given every 2 hours for 7 hours, while 8 animals were selected at random from each group and sacrificed at intervals of 1.5, 3.0, 6.0, 12.0 hour after blood was collected from them and stored at −20 ºC for serum amylase and lipase bioassay. The pancreas was harvested and dissected into three sections and stored at −80 ºC before use.

Activation of NF-κB in pancreatic tissue assay

Single cell suspension of pancreatic acinar was prepared as described in Oliver *et al.* (1987) and the cells were adjusted to a concentration of 2×10^6 cells/ml. The above solution 50 μl was supplemented with Rnase 1 μl and then incubated at 37 ºC for 10 mins following chilling in ice-water. After the solution was washed with PBA and NF-κB p65 McAb 40 μl was added into it, it was incubated 20 mins at room temperature. After 1 μl labeled FITC IgG was added into the solution, it was incubated 20 mins at room temperature. After 20 μl PI solution was added to the incubated solution, it was kept in a darkroom for 30 mins, and then washed with PBA solution and the activation of NF-κB was assayed with the flow cytometry (Elite-ESP, Coulter. Co., USA). The labeled FITC IgG served as negative control.

TNF-*α* mRNA in pancreatic tissue assay

In situ hybridization was conducted according to the manufacture's protocol. Sequential sections (6 μm thick) were cut from formalin fixed paraffin blocks and deparaffinized by hydration. The sections were incubated in 3% H_2O_2 for 10 mins at room-temperature to inhibit endogenous peroxidase activity and the TNF-*α* mRNA fragment was exposed by gastrin digestion at 37 ºC for 30 min. Hybridization was implemented at 41 ºC overnight. Diaminobenzidine (DAB) was used for color development and hematoxylin was used for counterstaining. Brown-yellow particles observed in the cytoplast served as positive. According to the ratio of positive cell numbers in the ten high visual fields, TNF-*α* mRNA expression was graded as follows: <10%, 10%–25%, 26%–50% and >50%, which, represented negative, weakly positive, medium positive and strongly positive respectively.

Evaluation of TNF-*α* protein in pancreatic tissue

The separated pancreatic tissue was homogenated at −20 ºC and centrifugated, the supernatant was collected for the test. TNF-*α* protein in the supernatant was measured by the enzyme-

linked immuno-sorbent assay. The coating antibody (anti-TNF-*α* McAb) was put into a 96-well plate and allowed to incubate overnight at 4 ºC temperature; after which, the plate was blocked with assay buffer for 30 mins at 37 ºC. The supernatant and standards were placed in the wells in duplicate with the horse-radish-peroxidase labeled antibody and allowed to incubate for 1 hour at 37 ºC. Following this step, 5 ml substrate containing 15 μl 3% H_2O_2 was added to the wells at 100 μl/well for 20 minutes, and the reaction was stopped with 2 mol/L H_2SO_4. Absorbency of the plate was read at 410 nm. Levels, compared with the standard curve, are reported as picograms per milliliter±standard error.

Biochemical assays

After the serum was diluted 10 fold with saline solution, amylase and lipase were determined using SYNCHRON LX^{TM} 20 biochemical analyzer (Beckman Coulter Co., USA).

Histological studies

Pancreas in each group were harvested at 3 hours and 6 hours respectively and placed in 10% buffered formalin. The tissues were then embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin for histological evaluation. For histological examination of the pancreatic tissue, the entire pancreas (10 random fields on each slide) of eight rats from each group was examined and scored for necrosis, vacuolization, inflammation, and edema by an experienced pathologist who was blind as to the treatment according to Schmidt's standard (1992) method.

Statistical analysis

The expression TNF-*α* mRNA in the pancreas tissue of the rat model was expressed reported as the mean±standard deviation (SD). Analysis of variance (ANOVA) was performed on all parametric data and significance was set at $P\leq 0.05$ to determine the significance of data within each group; Student's *t* test was performed to determine significance between different groups.

RESULT

Activation of NF-κB in the rat pancreatic tissue

To determine the expression of activation of NF-κB in pancreas, we performed flow cytometry (FCM) to determine changes in NF-κB activity after acute pancreatitis in different time course. It was found that an increased amount of activated NF-κB was detectable at 1.5 h and that the highest levels of activated NF-κB were observed in the rat pancreatitis model at 3 h after which the level of NF-κB activity decreased (Fig.1). Compared with the control group (group A), the value of activation of NF-κB was higher significantly ($P<0.01$) in group B at each interval. After administrating NAC, the activation of NF-κB was suppressed markedly (group C) ($P<0.01$ vs group B).

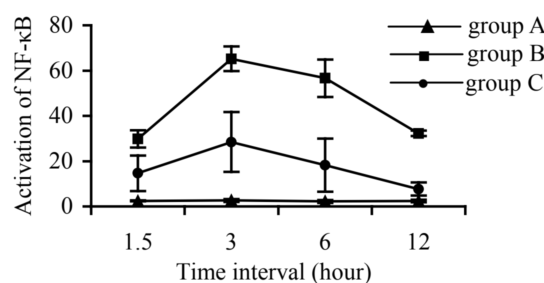


Fig.1 Activation of NF-κB in the rat pancreatitis model
Using flow cytometry, activation of NF-κB was assessed in the various time courses. Compared to group A, the activation of NF-κB was up-regulated significantly ($P<0.01$). This activity can be inhibited by NAC (see group C $P<0.01$ vs group B)

TNF-*α* level assay in the rat pancreatic tissue

TNF-*α* was a critical cytokine in the initiation of pancreatitis. The level in tissues was more significant than that in serum. So we detected TNF-*α* protein expression with enzyme-linked immunosorbent assay (ELISA) and the transcription of TNF-*α* mRNA with in situ hybridization (ISH) at the 6 hour point after establishing the animal model. The results showed that the level of protein and the mRNA transcription of TNF-*α* were up regulated distinctly ($P<0.01$) in the pancreatitis group and that these outcomes were inhibited to a certain extent by using NAC in group C (Table 1).

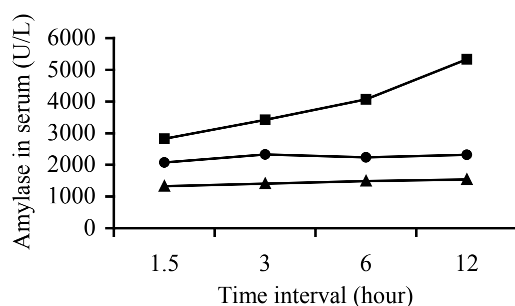
Table 1 At interval 6 hour, TNF-α expression and the pathological change score in pancreas in animal model

Groups	TNF-α		Pathological score			
	mRNA	Protein(ng/L)	Edema	Inflammation	Hemorrhage	Acinar necrosis
Group A	78.39	50.68±12.25	0.75±0.65	0.00±0.00	0.00±0.00	0.00±0.00
Group B	234.07▲	341.63±99.46▲	3.88±0.25▲	2.75±0.65▲	3.75±0.29▲	2.63±0.48▲
Group C	150.73▲■	133.60±19.18▲■	2.25±0.65▲■	2.18±0.48▲□	2.63±0.48▲■	1.23±0.48▲■

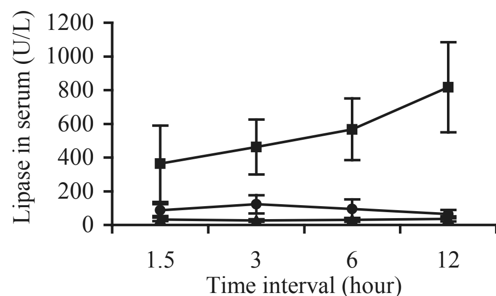
Note: ▲P<0.01 Vs Group A; □P<0.05, ■P<0.01 vs Group B

The interaction within the activation of NF-κB and pancreatic pathology

Compared with the control group, the serumal level of lipase and amylase in group B in serum rose continuously during the whole experimental course (P<0.01) (Fig.2). Serious pathological changes of inflammatory cell infiltration, edema and necrosis were observed in the pancreas at the 6 hour point and assigned to the third grade (severe). When the activation of NF-κB was inhibited by NAC, the level of lipase and amylase in serum fell significantly (P<0.05) and the inflammation in pancreatic tissue was attenuated (Table 1).



(a)



(b)

Fig. 2 The changes of lipase and amylase in serum with the course of rat pancreatitis model
From the sketch above, we see that the level of both of these digestive enzyme were higher in group B than that of in group A and it was inhibited by the NAC evidently.
(a) the changes of amylase; (b) the changes of lipase
—▲— group A —■— group B —●— group C

DISCUSSION

Acute pancreatitis (AP), which is associated with high mortality, is a serious inflammation of pancreas. The pathophysiology of acute pancreatitis is not well understood, and the clinical outcomes can be unpredictable. Various cytokines, such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), had been implicated in the local inflammatory changes of acute pancreatitis (Rongione *et al.*, 1997). In theory, inhibition of these cytokines production may result in decreased severity of acute pancreatitis. But the cellular mechanisms underlying cytokine production and acute inflammation in acute pancreatitis are unknown entirely.

NF-κB, which is closely related to the inflammatory response, is a family of proteins sharing the Rel homology domain that binds to DNA as homo- or hetero-dimers. It exists in an inactive form in the cytoplasm bound to an inhibitor I-κB. Phosphorylation of I-κB results in its dissociation from NF-κB and then translocation into the nucleus. The central role of NF-κB in the stress response and as a proximal cytokine gene regulator is well established (Pahl, 1999). This study's results suggested that the role of NF-κB in TNF gene expression in severe acute pancreatitis is a crucial step for initiating expression of the cytokines released from inflammatory cell and subsequently damaging other organs. Our study showed that activation of NF-κB rises significantly with time its peak at interval 3 hour compared with the control group. Consistent with the current result, Steinle *et al.*(1999) reported an early increase in NF-κB binding activity after hormone-induced pancreatitis in a rat model. Gukovsky *et al.*(1998) found that NF-κB was strongly activated in the rat pancreas within 30 minutes after an IV injection of cerulein. Collectively, those re-

sults identify that NF-κB activation would be an early event in the course of acute pancreatitis and act as transcriptive regulator of many cytokines. It is suggested that NF-κB may play an important role in the inflammatory changes and progression of this disease (Ethridge *et al*., 2002).

This study showed that severity of AP is closely associated with the level of TNF-α expression in the pancreatic tissue and suggested that TNF-α may be an important effective molecule in acute inflammation. Several studies also clarified the relationship between TNF-α and AP in humans (Kikucki *et al*., 1996; Norman, 1998). It is important to recognize that tissue levels of TNF-α, but not serum levels, are responsible for the vast majority of the biologic effects (Bhatnagar *et al*., 2001). Hence, there is need to assay TNF-α level at cellular level in AP because of its factual reflection of pancreatic pathological change. This study showed that expression of TNF-α at intracellular level of the pancreatitis model was much higher than that in the control group at 6 hours and coincided with the pathologic change. Since TNF-α is known to play a major role in the initiation of an immune response, it is tempting to speculate that the TNF-α producing cells contributed to releasing of pro-inflammatory cytokines during the early phase of the response and mediated many of the pathophysiological changes (Grewal *et al*., 1994). Up to now, all evidence suggested that this cytokine produced in the pancreas and lung propagate pancreatic necrosis and the development of the adult respiratory distress syndrome (ARDS), both of which are major causes of morbidity and mortality (Gross *et al*., 1993; Norman *et al*., 1997).

Reactive oxygen species (ROS), generated by infiltrating neutrophils, are considered important regulators in the pathogenesis and development of acute pancreatitis. A hallmark of the inflammatory response in acute pancreatitis is the induction of cytokine gene expression, which may be regulated by oxidant-sensitive transcription factor, nuclear factor-κB (NF-κB) (Kim *et al*., 2000). The translocation of activated NF-κB is believed to be responsible for the later cytokine activation cascade, which includes ROS activation. Among ROS, H_2O_2 is suggested to be the mediator of oxidant-mediated NF-κB activation and resulting in up-regulation of certain cytokines such as IL-1b, IL-6, and TNF-α which may mediate pancreatic inflammation. N-acetylcysteine (NAC), as an antioxidant, inhibits NF-κB activation by increasing the intracellular stores of glutathione in the cells and furthermore enhancing the endogenous antioxidative defense mechanism (Pinkus *et al*., 1996). NAC directly eliminated ROS (hydroxyl radicals, hypochlorous acid, etc.) produced by inflammatory cells. In this study, NAC evidently depressed the NF-κB activation (group 3) in pancreatic tissue, while the level of TNF-α, lipase and amylase was decreased simultaneously. Therefore, we believe that inhibition of a number of inflammatory molecules by targeting the NF-κB system represents an exciting and promising approach to the treatment of pancreatitis; antioxidants such as NAC might be useful anti-inflammatory agents in reducing oxidant-mediated NF-κB activation.

In summary, our study indicated that the activation of NF-κB plays a key role in the pathophysiology of pancreatitis and is known as an early event in the progress of pancreatitis. This study showed that NF-κB activation contributed obviously to the expression of numerous cytokines and modulates the interaction among these inflammatory mediators; the inflammatory reaction in pancreatitis could be attenuated through the inhibition of NF-κB activity by antioxidant-NAC; and that from which result, we can speculate that NAC might act anti-inflammatory agents for inhibiting activation of NF-κB and decreasing cytokine production in pancreatitis.

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