Influence of Nuclear Factor κB Activation on Inflammatory Mediators of Alveolar Macrophages in Rats With Acute Necrotizing Pancreatitis

Yalikun Sailai, MSc, Xiao Yu, MD,† Paerhati Baiheti, MSc,* Huayong Tang, MSc,† Yongguo Li, MD,† and Ming Xu, MD‡*

Aim: To investigate the potential influence of nuclear factor κB (NF-κB) activation on the inflammatory mediators secreted by alveolar macrophages (AMs) in rats with acute necrotizing pancreatitis (ANP) and to evaluate the effect of an inhibitor of NF-κB–*N*-acetylcysteine (NAC).

Methods: Ninety male Sprague-Dawley rats were randomly divided into 3 groups, 30 of each: control, ANP, and ANP plus NAC groups. The ANP rat models were established by a retrograde injection of 5% sodium taurocholate into the pancreatic duct. In addition to sodium taurocholate, the ANP plus NAC group received intravenous infusion of NAC (25 mg/100 g). At the sixth hour after modeling, the protein content of the bronchoalveolar lavage fluid, the myeloperoxidase in the lung tissue, and the transforming growth factor α and the nitric oxide (NO) secreted by AMs were determined. The histopathologic changes of the pancreas and the lung were observed under light microscope, and NF-κB activation of AMs was detected.

Results: The protein content of the bronchoalveolar lavage fluid and the myeloperoxidase level of the lung tissue showed a significant increase in the ANP group as compared with the NAC-administered group. The levels of transforming growth factor α and NO secreted by AMs in the ANP and the ANP plus NAC group rose significantly over that in the control group, and there was a significant difference between them. Although they were still higher than those in the control group, the pancreas destruction and the lung injury were slighter in the ANP plus NAC group and the activation of NF-κB was lower in the ANP plus NAC group as compared with that in the ANP group.

Conclusions: The correlation between the NF-κB activation, the upregulation of the inflammatory mediators secreted by AMs, and the tissue damage suggests a key influence of NF-κB in the pathogenesis of ANP. Inhibition of NF-κB activation may reverse the lung injury of ANP.

Key Words: pancreatitis, nuclear transcription factor κB, alveolar macrophages, inflammatory mediators

(*J Investig Med* 2010;58: 38–42)

Acute necrotizing pancreatitis (ANP) is a life-threatening necroinflammatory disease with high morbidity and mortality, and it is often complicated by systemic inflammatory response syndrome and multiple organ failure.[1,2] Within the first few days after the onset of ANP, acute lung injury (ALI) occurs as a frequent consequence of ANP.[3] Sepsis is a dominant cause for ALI and mortality in the later phase of the disease process.[4] However, the exact mechanisms of ALI are indefinite, but once the disease process is initiated, monocyte macrophages are stimulated excessively. Simultaneously, common inflammatory and repair pathways are invoked.[5] By far, in-depth understanding of the pathogenesis of ALI in ANP has not turned up trumps, and none of the clinical trials using novel therapeutic agents has indicated an improvement in patient outcome. Consequently, effective therapeutic interventions are thus called for.

Nuclear factor κB (NF-κB) is one of the most important transcription factors that control proinflammatory gene expression during ANP and known to be involved in inflammatory and immune responses.[6] It plays an important role in physiologic and pathologic conditions as an inducible NF.[7] In most cells, NF-κB is normally sequestered in the cytoplasm in an inactive form associated with a class of inhibitory proteins called IκBs. During ANP, NF-κB is rapidly activated and translocated to the nucleus, binds to specific κB sequences in the promoter regions, and transactivates the downstream genes, including interleukins, chemokines, adhesion molecules, receptors, and specific inducible isoform of nitric oxide synthase enzymes.[8] Recent experimental studies appeared to have shed some light on the intracellular signaling pathway in the inflammatory cascade in ANP.[9] The role of NF-κB in ANP has attracted more and more attention. For example, it has been shown to play a critical role in the pathogenesis of ANP by regulating the expressions of many proinflammatory genes in the pancreas.[10]

Hence, on the basis aforementioned, this study was conducted to evaluate the potential influence of NF-κB activation on the inflammatory mediators secreted by alveolar macrophages (AMs) in rats with ANP and to evaluate the effect of an inhibitor of NF-κB–*N*-acetylcysteine (NAC).

MATERIALS AND METHODS

Animal Model and Groups

Ninety adult male Sprague-Dawley (SD) rats weighing between 280 and 300 g were provided by the Experimental Animal Center, Xiangya Medical School, Changsha, China, and were housed under standardized conditions with free access to food and water. The animals were randomly divided into a control, an ANP, and an ANP plus NAC group. The rat ANP model was established by the retrograde injection of 5% sodium taurocholate (Sigma, St. Louis, MO) at 0.1 mg/100 g into the pancreatic duct as described previously.[11] In the control group, rats were given a saline injection. In addition to sodium taurocholate, rats in the ANP plus NAC group received intravenous infusion of NAC (Sigma) at 25 mg/100 g, whereas the control and the

From the *Department of Emergent Surgery, First Teaching Hospital of Xinjiang Medical University, Xinjiang Province; and †Department of General Surgery, Third Xiangya Hospital, and ‡Laboratory of Molecular Pharmacology, Xiangya College of Medicine, Central South University, Hunan Province, China.

Received April 21, 2009, and in revised form June 2, 2009.
Accepted for publication June 20, 2009.

Reprints: Xiao Yu, MD, Department of General Surgery, Third Xiangya Hospital, Central South University, Changsha 410013, Hunan Province, China. E-mail: yuxiaoyx4@163.com.

Supported by the Hunan Provincial Natural Science Foundation of China (No. 02jjk3026).

ISSN: 1081-5589
DOI: 10.231/JIM.0b013e3181b91bd6

ANP groups received the same amount of saline instead. All studies were performed with the approval of the experimental animal committee at our university.

Histopathologic Examination

Six hours after the ANP modeling, rats were killed by dislocation of the cervical vertebrae. The pancreas and the lung tissues were sectioned and then fixed in 10% formalin and embedded in paraffin, followed by hematoxylin-eosin staining. Pulmonary lesion was scored after the criteria of Lei et al.[11]

Protein Content of the Bronchoalveolar Lavage Fluid

After killing the rats, a thoracotomy was performed. Then, the whole lungs together with the trachea were taken out, the left bronchus was ligated, and the left lung was harvested for the measurements of the activity of myeloperoxidase (MPO); the right lung together with the trachea was used for lavage purpose. The surface blood was washed away with isotonic sodium chloride. A total of 5 mL of isotonic sodium chloride was repeatedly lavaged 5 times. The lavage fluid was collected and centrifuged, and the supernatant was obtained for protein content measurement.

Myeloperoxidase Activity in the Lung Tissues

Myeloperoxidase activity in the lung tissues of the 3 groups was assessed by the modification of the method described previously.[12] Frozen tissues were thawed and washed twice with 50-mmol/L potassium-phosphate buffer (pH 6.0), then homogenized in the hexadecyltrimethyl ammonium bromide suspension buffer (50-mmol/L potassium phosphate and 0.5% hexadecyltrimethyl ammonium bromide at pH 6.0; 50 mg of tissue/1 mL of buffer). It was homogenized at 13,500 rpm on ice for 1 minute. The homogenate was frozen and thawed on 2 consecutive occasions before a final 30-second homogenization and then centrifuged at 20,000*g* at 4°C for 15 minutes. The upper layer was incubated at 60°C for 2 hours and centrifuged at 2000*g* at 4°C for 15 minutes. The clear supernatants were collected for the assay of MPO. To measure MPO activity, 100 μL of supernatants was incubated at 37°C with 100 μL of 0.1% tetradimethylbenzidine dissolved in dimetylsulfoxide and 750 μL of 0.5-mmol/L hydrogen peroxide dissolved in 50-mmol/L potassium phosphate buffer (pH = 5.4). The enzyme activity was assessed photometrically at 655 nm by using an MPO calibration curve (Cat No. 475911; Calbiochem, La Jolla, CA). The within-run coefficient of variations was 5.8 for the lung tissues. The protein concentrations of the supernatants were measured by the Lowry method. The MPO activity was expressed as units per gram of protein.

Levels of Transforming Growth Factor α and NO Secreted by AMs

The whole bronchoalveolar lavage fluid (BALF) was centrifuged at 280 rpm for 10 minutes at 4°C. Cell pellets were resuspended (1×10^5 cells/mL) in the Royal Park Memorial Institute 1640 medium (Nikken, Osaka, Japan). Cell suspension was then placed in plastic Petri dishes (Nunc, Roskilde, Denmark) and incubated at 37°C for 1 hour in a CO_2 incubator (50-mL/L CO_2 + 95% air). The levels of transforming growth factor α (TNF-α) and NO secreted by AMs were measured by the enzyme-linked immunosorbent assay (ELISA) method according to the instruction of the kits (TPI Ltd, St. Louis, MO). Nonadherent cells were removed from adherent macrophages by washing with the Royal Park Memorial Institute 1640 medium. Purified AMs were recovered by gently rubbing the dishes with a rubber policeman. Nuclear protein was

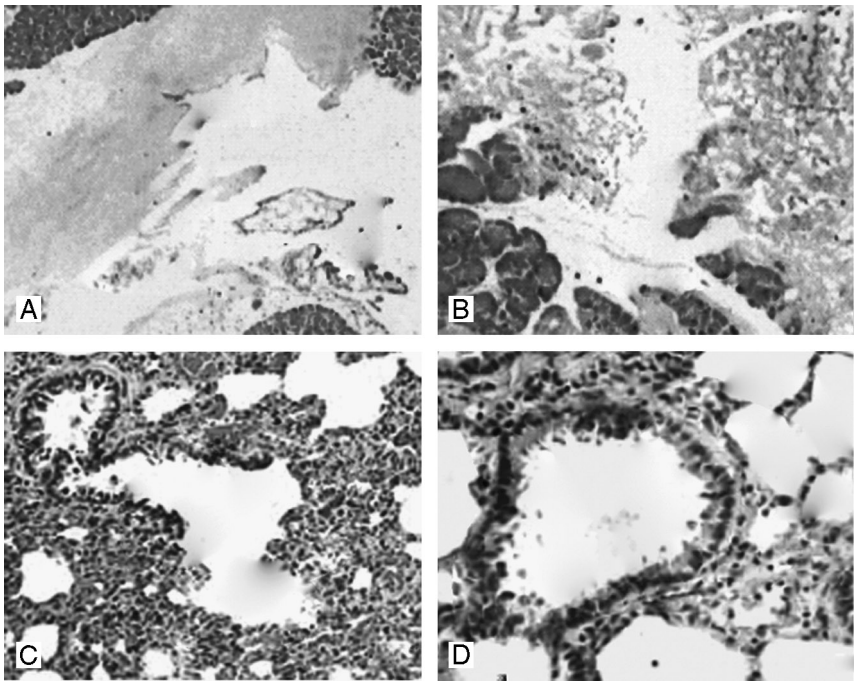

FIGURE 1. Light microscopic photograph of the histopathologic changes in the different groups (original magnification ×100). A, Extensive necrosis, intense edema, and inflammatory infiltrate in the ANP group. B, Extensive necrosis and intense edema in the ANP plus NAC group. C, Alveolar septal thickening, interstitial edema, diffuse alveolar blood stasis, infiltration of inflammatory cells, mostly neutrophils, and destruction of the alveolar wall in the ANP group. D, Mild edema of the alveolar walls and mild alveolar blood stasis with slight infiltration of neutrophils in the ANP plus NAC group.

TABLE 1. Histopathologic Scores of Lung Injury ($\bar{X} \pm s$, n = 30)

Group	Pathologic Scores, Mean (SD)
Control	0.18 (0.03)
ANP	2.65 (0.67)*
ANP plus NAC	1.96 (0.34)†‡

*$P < 0.01$, the ANP versus the control group.
†$P < 0.05$, the ANP plus NAC versus the ANP group.
‡$P < 0.05$, the ANP plus NAC group versus the control group.

extracted from purified AMs as previously described.[13] Protein was stored at −70°C for subsequent examination of NF-κB activity.

Nuclear Factor κB Activation of AMs

Nuclear factor κB activation of AMs was determined using TransAM NF-κB p65 Chemi ELISA kit (Active Motif, Carlsbad, CA) by Central LB 960 microplate luminometer (Berthold, Bad Wildbad, Germany) and expressed as relative light units.

Statistics

The statistical package SPSS 10.0 (SPSS Inc, Chicago, IL) was used for all analyses. All values were expressed as mean (SD). Statistical differences among different groups were compared using analysis of variance with the Bonferroni/Dunn post hoc tests. The correlation of the NF-κB activation of AMs with the histopathologic score of the lung injury, the protein content of BALF, the MPO activity in the lung tissues, and the levels of TNF-α and NO secreted by AMs was analyzed using the Spearman rank correlation test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Histopathologic Analysis

The histopathologic study of the pancreas revealed an extensive necrosis of the pancreatic tissue, an intense edema, and an inflammatory infiltrate (Fig. 1A). The necrosis of the pancreatic tissue and the edema in the ANP plus NAC group were similar to those in the ANP group (Fig. 1B). The control group was normal. In addition, the representative light microscopic view of the lung injury, including the alveolar septal thickening, the interstitial edema, the diffuse alveolar blood stasis, the infiltration of the inflammatory cells, mostly neutrophils, and the destruction of the alveolar wall in the ANP group, was shown in Figure 1C. The lung histopathologic scores of the ANP group were significantly higher than those in the control group ($P < 0.01$, Table 1).

TABLE 2. Protein Content in BALF ($\bar{X} \pm s$, n = 30)

Group	Protein Content, Mean (SD), μg/mL
Control	276.33 (10.02)
ANP	1769.65 (220.16)*
ANP plus NAC	987.96 (70.53)†‡

*$P < 0.01$, the ANP versus the control group.
†$P < 0.05$, the ANP plus NAC versus the ANP group.
‡$P < 0.05$, the ANP plus NAC group versus the control group.

TABLE 3. Myeloperoxidase Activity in the Lung Tissues ($\bar{X} \pm s$, n = 30)

Group	MPO, Mean (SD), U/g
Control	1.07 (0.02)
ANP	8.86 (0.43)*
ANP plus NAC	3.29 (0.21)†‡

*$P < 0.01$, the ANP versus the control group.
†$P < 0.05$, the ANP plus NAC versus the ANP group.
‡$P < 0.05$, the ANP plus NAC group versus the control group.

In the ANP plus NAC group, the major histopathologic findings were mild edema of the alveolar walls and mild alveolar blood stasis with slight infiltration of neutrophils (Fig. 1D). The lung histopathologic scores of the ANP plus NAC group decreased significantly compared with those of the ANP group ($P < 0.05$).

Protein Content of BALF

After modeling, the protein content in BALF of the ANP group rose rapidly. Although it was significantly higher than that in the control group ($P < 0.05$), the protein content of the ANP plus NAC group was still significantly lower than that in the ANP group ($P < 0.05$; Table 2).

Myeloperoxidase Activity in Lung Tissues

Table 3 shows the MPO activity of the lung tissues in the 3 groups. The MPO levels in the ANP group were significantly higher than those in the control group ($P < 0.01$). In the ANP plus NAC group, the lung MPO levels were significantly decreased ($P < 0.01$).

Levels of TNF-α and NO Secreted by AMs

Levels of TNF-α and NO secreted by AMs in both the ANP (both $P < 0.01$) and the ANP plus NAC (both $P < 0.05$) group rose significantly over those in the control group, whereas the former was also significantly higher than the latter ($P < 0.01$; Table 4).

Nuclear Factor κB Activation of AMs

Figure 2 shows that the NF-κB activation of AMs in the ANP group was significantly higher than that in the control group ($P < 0.01$) and was significantly decreased in the ANP plus NAC group ($P < 0.01$).

Correlation Analysis

The NF-κB activation of AMs was positively correlated with the histopathologic score of the lung injury ($r = 0.66$ and 0.69, respectively; both $P < 0.01$), the protein content of BALF ($r = 0.72$ and 0.75, respectively; both $P < 0.01$), the MPO activity in the lung tissues ($r = 0.62$ and 0.65, respectively; both

TABLE 4. Levels of TNF-α and NO Secreted by AMs ($\bar{X} \pm s$, n = 30)

Group	TNF-α, pg/mL	NO, Mean (SD), μmol/L
Control	158.95 (26.10)	21.03 (1.52)
ANP	1662.71 (138.45)*	89.15 (7.03)*
ANP plus NAC	779.01 (56.27)†‡	42.23 (3.29)†‡

*$P < 0.01$, the ANP versus the control group.
†$P < 0.05$, the ANP plus NAC versus the ANP group.
‡$P < 0.05$, the ANP plus NAC group versus the control group.

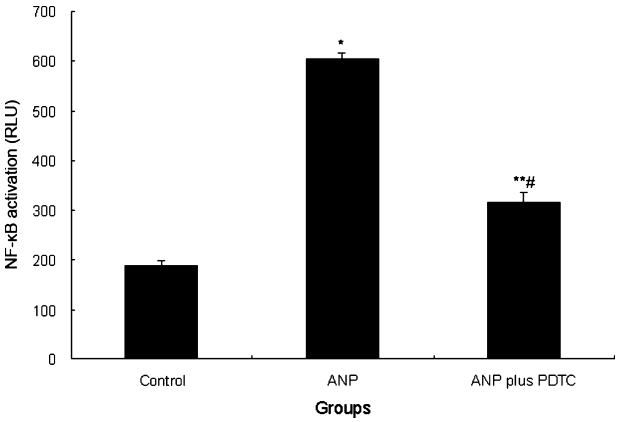

FIGURE 2. Nuclear factor κB activation of AMs in BALF in the different groups. *$P < 0.01$, the ANP versus the control group. †$P < 0.01$, the ANP plus NAC versus the ANP group. ‡$P < 0.05$, the ANP plus NAC versus the control group.

$P < 0.01$), and the levels of TNF-α and NO secreted by AMs ($r = 0.83$ and 0.80 and $r = 0.85$ and 0.83, respectively; both $P < 0.01$).

DISCUSSION

Acute necrotizing pancreatitis as an inflammatory disorder may cause various systemic complications. Among them, ALI is the most dreadful and impending catastrophe that leads to a mortality rate of 25% and is difficult to deal with clinically.[14] It has been indicated that oxygen-free radicals, platelet-activating factor, cyclooxygenase-2, cytokines, and arachidonic acid metabolites play an important role in the progression of ALI in ANP.[15] In addition, pancreatic proteolytic enzymes or activated phospholipase A2 released into the circulatory system determines the development of lung injury.[16] Furthermore, other mediators in the lung tissue such as the platelet-activating factor and the arachidonic acid metabolites can stimulate inflammatory cell activation. In addition, the interaction of polymorphonuclear granulocytes, endothelium, and endothelium-derived mediators seems to be important to amplify lung damage.[17] However, the mechanisms of the development of this disease and the effective therapies for preventing or reversing it remain obscure. Randomized studies of ANP in the clinical setting do have limitations. In this regard, reliable ANP animal models are of paramount importance. Therefore, this study was conducted to investigate a mechanism of the progression of ALI—the potential influence of NF-κB activation on the inflammatory mediators secreted by AMs and the histopathologic changes in rat models with ANP—which has not been reported previously.

Nuclear factor κB is a kind of pleiotropic regulative protein of transcription, which regulates the expressions of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. The activation of NF-κB is thought to be part of a stress response because it is activated by many divergent stimuli, including proinflammatory cytokines such as TNF-α, interleukin 1β, epidermal growth factor, T- and B-cell mitogens, bacteria and lipopolysaccharides, viruses, viral proteins, double-stranded RNA, and physical and chemical stresses. Its activation also takes part in the pathogenesis of ANP, and the inhibition of the action can ameliorate the rat with ANP.[18] Hypoxemia and ischemia can activate NF-κB and subsequently act on genes for proinflammatory cytokines, chemokines, immune receptors, and adhesion molecules. All the previously mentioned cytokines and inflammatory mediators can further aggravate the injury to multiple organs in severe acute pancreatitis, particularly the lung tissue, and thus accelerate acute respiratory distress syndrome or damage to other organs.[6,19] Moreover, in recent years, some researchers also reported that the activation of AMs may play an important role in lung injury associated with ANP and that TNF-α and NO secreted by AMs are increased significantly in rats with ANP.[20,21] Transforming growth factor α has been considered as a prognostic factor of ANP. Most authors believed that the increase in TNF-α activity was due to the excessive stimulation of the mononuclear macrophage by endotoxin.[22] Excessive production of NO causes vasodilatation and hypotension leading to organ hypoperfusion, edema, and organ dysfunction. The reaction of NO with superoxide causes the formation of peroxynitrite, which is a powerful oxidant and cytotoxic agent and may play an important role in the cellular damage associated with the overproduction of NO.[23,24]

Our study provides evidence that the injection of sodium taurocholate can cause ANP, with manifestation of the damage to the pancreas, and inflammatory infiltration. The rise of protein content in BALF, the increase of MPO activity, and the TNF-α and the NO levels secreted by AMs were also the indicators of lung damage caused by ANP. Accompanied with these, ELISA results also suggested that NF-κB was activated at the onset of ANP. The lower expression of the NF-κB p65 subunit in the ANP plus NAC group indicated that the treatment of NAC inhibited the NF-κB activation. Nuclear factor κB–*N*-acetylcysteine also led to a slighter histological damage to the pancreas and lung and a lower increase in the protein content in BALF, in MPO activity, and in TNF-α and NO levels secreted by AMs. All these results are consistent with previous studies, substantiating that NF-κB activation is involved in the pathogenesis of ANP and inhibition of the activation may reverse rat ANP.[25,26] Correlation between NF-κB activation, tissue damage, protein content in BALF, MPO activity, and TNF-α and NO levels secreted by AMs is a direct evidence that NF-κB activation plays a key role in the pathogenesis of lung injury in the ANP model.

In summary, we may draw a conclusion that the correlation between NF-κB activation, up-regulation of inflammatory mediators secreted by AMs, and tissue damage suggests a key influence of NF-κB in the pathogenesis of ANP. The use of NF-κB inhibitors can block the activation of the signal transduction pathway to block the synthesis of inflammatory cytokines, which may be the future direction of the treatment of lung injury occurring in ANP.

REFERENCES

1. Turkyilmaz S, Alhan E, Ercin C. Effects of caffeic acid phenethyl ester on pancreatitis in rats. *J Surg Res*. 2008;145:19–24.
2. Liu H-S, Pan C-E, Liu Q-G. Effect of NF-κB and p38 MAPK in activated monocytes/macrophages on pro-inflammatory cytokines of rats with acute pancreatitis. *World J Gastroenterol*. 2003;9:2513–2518.
3. Xia Q, Jiang JM, Gong X, et al. Experimental study of "Tong Xia" purgative method in ameliorating lung injury in acute necrotizing pancreatitis. *World J Gastroenterol*. 2000;6:115–118.
4. Makhija R, Kingsnorth AN. Cytokine storm in acute pancreatitis. *J Hepatobiliary Pancreat Surg*. 2002;9:401–410.
5. Pastor CM, Matthay MA, Frossard JL. Pancreatitis-associated acute lung injury: new insights. *Chest*. 2003;124:2341–2351.
6. Arnalich F, Garcia-Palomero E, Lopez J. Predictive value of nuclear factor–κB activity and plasma cytokine levels in patients with sepsis. *Infect Immun*. 2000;68:1942–1945.
7. Ethridge RT, Hashimoto K, Chung DH, et al. Selective inhibition of

NF-κB attenuates the severity of cerulein-induced acute pancreatitis. *J Am Coll Surg*. 2002;195:497–505.

8. Gukovsky I, Gukovskaya AS, Blinman TA, et al. Early NF kappa B activation is associated with hormone-induced pancreatitis. *Am J Physiol*. 1998;275:G1402–1414.
9. Kim H, Seo JY, Roh KH, et al. Suppression of NF-κB activation and cytokine production by *N*-acetylcysteine in pancreatic acinar cells. *Free Radic Biol Med*. 2000;29:674–683.
10. Algul H, Tando Y, Schneider G, et al. Acute experimental pancreatitis and NFkappaB/Rel activation. *Pancreatology.* 2002;2:503–509.
11. Lei WZ, Wei JJ, Shen WL, et al. The relationship between MODS of experimental necrotizing pancreatitis and endotoxinmia. *Zhongguo Shiyan Waike Zazhi*. 1995;12:131–133.
12. Luo WS, Guo ZG. Determine the neutrophil of the cardiac muscle using MPO method. *Zhongguo Yao Li Xue Tongbao*. 1990;6:264–266.
13. Schreiber E, Matthias P, Muller MM, et al. Rapid detection of octamer binding proteins with 'mini-extracts', pre-pared from a small number of cells. *Nucleic Acids Res*. 1989;17:6419.
14. Bhatia M, Moochhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol*. 2004;202:145–156.
15. Bhatia M, Wong FL, Cao Y, et al. Pathophysiology of acute pancreatitis. *Pancreatology.* 2005;5:132–144.
16. Hardman J, Shields C, Schofield D, et al. Intravenous antioxidant modulation of end-organ damage in L-arginine–induced experimental acute pancreatitis. *Pancreatology.* 2005;5:380–386.
17. Song AM, Bhagat L, Singh VP, et al. Inhibition of cylooxygenase-2 ameliorates the severity of pancreatitis and associated lung injury. *Am J Physiol Gastrointest Liver Physiol*. 2002;28:G1166–1174.
18. Sookhal S, Wang JJ, McCourt M. A novel therapeutic strategy for attenuating neutrophil-mediated lung injury in vivo. *Ann Surg*. 2002;235:283–291.
19. Satoh A, Masamune A, Kimura K. Nuclear factor–κB expression in peripheral blood mononuclear cells of patients with acute pancreatitis. *Pancreas*. 2003;26:350–356.
20. Cheng S, He SG, Zhang JL. The role of alveolar macrophage activation in rats with lung injury associated with acute necrotizing pancreatitis. *Zhonghua Waike Zazhi*. 2002;40:609–612.
21. Cheng S, Zhao J, He SG, et al. The role of nitric oxide in lung injury associated with acute necrotizing pancreatitis. *Zhonghua Waike Zazhi*. 2003;41:336–339.
22. Coelho AM, Machado MC, Cunha JE, et al. Influence of pancreatic enzyme content on experimental acute pancreatitis. *Pancreas*. 2003;26:230–234.
23. Long J, Song N, Liu X-P, et al. Nuclear factor-kappaB activation on the reactive oxygen species in acute necrotizing pancreatitic rats. *World J Gastroenterol*. 2005;11:4277–4280.
24. Jaworek J, Jachimczak B, Tomaszewska R, et al. Protective action of lipopolysaccharidesin rat caerulein-induced pancreatitis: role of nitric oxide. *Digestion*. 2000;62:1–13.
25. Yu X, Li YG, He XW, et al. Hyperbaric oxygen reduces inflammatory response in acute pancreatitis by inhibiting NF-κB activation. *Eur Surg Res*. 2009;42:130–135.
26. Liu H-B, Cui N-Q, Li D-H, et al. Role of Kupffer cells in acute hemorrhagic necrotizing pancreatitis–associated lung injury of rats. *World J Gastroenterol*. 2006;12:403–407.