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Lipid Peroxidation, NF-κB Activation and Cytokine Production in Neutrophil-Stimulated Pancreatic Acinar Cells

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Reactive oxygen species (ROS), generated by infiltrating neutrophils, are considered as an important regulator in the pathogenesis and deveolpment of pancreatitis. The present study aims to investigate whether neutrophils primed by 4 β-phorbol 12 β-myristate 13 α-acetate (PMA) affect the productions H_2O_2 and lipid peroxide (LPO), NF-κB activation and cytokine production in pancreatic acinar cells, and whether these alterations were inhibited by an antioxidant, N-acetylcysteine (NAC) and superoxide dismutase (SOD). H_2O_2 (ferrithiocyanate method), LPO (as thiobarbiturate reactive substances), and cytokines (IL-1 β, IL-6, TNF-α; enzyme-linked immunosorbent assay) and NF-κB activation (electrophoretic mobility shift assay) were analyzed in acinar cells treated with or without PMA-primed neutrophils in the absence or presence of NAC (10 mM) or SOD (300 U/ml). As a result, the productions of H2O2, LPO and TNF-α were increased with the ratio of PMA-primed neutrophils to acinar cells while the productions of LPO, IL-1 β, IL-6 and TNF-α were increased with time. PMA-primed neutrophils resulted in the activation of NF-κB. Both NAC and SOD inhibited neutrophil-induced alterations in acinar cells. In conclusion, ROS, generated by neutrophils, activates NF-κB, resulting in upregulation of inflammatary cytokines in acinar cells. Antioxidants might be clinically useful antiinflammatory agents by inhibiting oxidant-mediated activation of NF-κB and decreasing cytokine production.

Key Words: Lipid peroxide, NF-κB, Cytokine, Pancreatic acinar cell, Neutrophil

INTRODUCTION

Infiltration of inflammatory cells, such as neutrophils, lymphocytes and monocytes, is quite common in damaged pancreatic glands of models of acute (Alder et al, 1979) and chronic (Okumura et al, 1982) pancreatitis. These phenomenon was also shown in those patients with acute pancreatitis (Aho et al, 1982) and chronic pancreatitis at early stage (Uys et al, 1973). Neutrophils are known to be the highest producer of reactive oxygen species (ROS) among those inflammatory cells. Actually ROS production of neutrophils obtained from the patients with acute pancreatitis was enhanced (Tsuji et al, 1994). Antioxidant status (plasma levels of glutathione and ascorbic acid) was decreased in the patients with chronic pancreatitis (Gut et al, 1994), which suggests the involvement of ROS in the pathogenesis of chronic pancreatitis as well as acute pancreatitis. ROS scavengers such as superoxide dismutase (SOD) and catalase diminished pancreatic injury in experimental pancreatitis (Sanfey et al, 1984). Even though ROS alone cannot initiate experimental pancreatitis (Fu et al, 1997), ROS are still considered as an important regulator in the pathogenesis and development of pancreatitis.

Clinical studies have shown the inflammatory cytokines such as IL-1 β, IL-6 and TNF-α in the serum of patients with acute pancretitis. The degree of cytokine elevation correlated with disease severity and overall morbidity (Gross et al, 1993; Heath et al, 1993). In experimental pancreatitis, blockade of the cytokine cascade using IL-1 receptor antagonist attenuated the rise in IL-6 and TNF-α expression (Fu et al, 1997). These studies suggest that inflammatory

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cytokines may play important roles in the process of pancreatitis. Although intrapancreatic cytokine levels during the early stages of acute pancreatitis have been examined recently (Norman et al, 1994), there have been no systematic studies comparing the activation of cytokine gene with that of transcription factors in the pancreas.

A key regulator of cytokine induction is the pleiotropic transcription factor, nuclear factor-κB (NF-κB). NF-κB represent a family of proteins sharing the Rel homology domain, which bind to DNA as homo- or hetero-dimers, and activates a multitude of cellular stress-related and early response genes such as the genes for cytokines, growth factors, adhesion molecules, and acute phase proteins (Wulczyn et al, 1996; Barnes & Karin, 1997). NF-κB is kept silent in the cytoplasm via interaction with inhibitory proteins of IκB family and activated by a variety of agents, including cytokines, mitogens and ROS such as hydrogen peroxide (Meyer et al, 1993; Wulczyn et al, 1996; Barnes & Karin, 1997). Several antioxidants such as N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate potentially inhibit NF-κB activation and/or NF-κB interaction with its upstream regulatory binding site thereby preventing NF-κB-mediated transcriptional activation (Schreck et al, 1991; Schenk et al, 1994; Blackwell et al, 1996). These studies suggest the hypothesis that antioxidants might inhibit cytokine production by inhibiting oxidant-mediated activation of transcription factors. Recently NF-κB activation was observed in cerulein-induced acute pancreatitis (Gukovsk et al, 1998; Steinle et al, 1999). We have shown that NF-κB regulates IL-8 production in *Helicobacter pylori*-stimulated gastric epithelial cells and hydroxyl radical scavengers (mannitol and dimetylthiourea) inhibit IL-8 production (Kim et al, 1999). Consequently, NF-κB represents a potential target for pharmacological therapy of inflammation. Treatment of antioxidants may play a role in dampening the inflammatory response by suppressing inflammatory NF-κB target genes.

The purpose of the present study on pancreatic acinar cells is to investigate whether neutrophils primed with 4β-phorbol 12β-myristate 13α-acetate (PMA) affect the productions H_2O_2 and lipid peroxide (LPO) as an indication of oxidative stress, NF-κB activation and cytokine production, and whether these alterations are inhibited by an antioxidant NAC and superoxide dismutase (SOD).

METHODS

Isolation of pancreatic acinar cells

Male Sprague-Dawley rats, inbred in the Yonsei University Medical Center animal unit, weighing 150~200 g were used. Acinar cells were isolated by enzymatic digestion of pancreas as described by Oliver et al (1987). Acinar cells were cultured in Ham's nutrient mixture F12 medium, with 15% fetal bovine serum, 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO, USA), and streptomycin at 0.1 mg/ml and penicillin at 100 U/ml. Cell viability was determined by the trypan blue dye exclusion test; more than 95% of the cells survived at 12 h-culture.

Preparation of neutrophils

Neutrophils were isolated from the peripheral blood of male Sprague-Dawley rats, weighing 150~200 g, with the use of discontinuous gradient separation on percoll. The procedure yields a neutrophil population that is 95% viable (trypan blue exclusion) and 98% pure (acetic acid crystal violet staining) (Szucs et al, 1994).

Experimental protocol

Acinar cells were plated at a density of 2×10^5/ml in a 12 well tissue culture plate (Falcon 3047, Becton Dickinson Labware, Lincoln Park, NJ, USA) and rat neutrophils were plated at a density of 2×10^6/ml with or without PMA (1 μg/ml) in a 0.45 μm culture plate insert (Millipore Products Division, Bedford, MA, USA) placed in each well. The inserts were used to prevent direct interaction between acinar cells and neutrophils. For time response of acinar cells to PMA-primed neutrophils, the cells were cultured for 2, 4 and 6 h. For concentration response of acinar cells to PMA-primed neutrophils, the cells were cultured for 2 h at the ratio of PMA-primed neutrophils : acinar cells, 1 : 1, 5 : 1, 10 : 1, 25 : 1, 50 : 1 and 75 : 1. To investigate the effect of NAC and SOD, acinar cells treated with PMA-primed neutrophils were cultured for 2 h in the absence or presence of NAC (10 mM) or SOD (300 U/ml). The levels of H_2O_2, LPO, IL-1β, IL-6 and TNF-α in the medium were determined. Prior to the experiment, the productions of H_2O_2, LPO and cytokines were compared

between acinar cells treated with neutrophils alone and those with PMA-primed neutrophis.

Determinations of cytokines, LPO and H_2O_2

Cytokine levels (IL-1 β, IL-6, TNF-α) were determined by enzyme-linked immunosorbent assay kits (R&D System, Minneapolis, MN, USA). LPO was measured as thiobabiturate reactive substance with a spectrofluorometer (SPF-500C, SLM Instruments, Urbana, IL, USA) at 515 nm excitation and 553 nm emission (Yagi, 1976). H_2O_2 was assayed by the modification of ferrithiocyanate method (Thurman et al, 1972).

NF-κB activation and electrophoretic mobility shift assay

Acinar cells were seeded at 5 x 10^6 cells onto 10-cm petri dish and treated with neutrophils (neutrophils : acinar cells, 10 : 1) primed with PMA (1 μg/ml) for 1, 2, 3 and 4 h. Two hour-stimulation was used to evaluate the effects of NAC (10 mM) and SOD (300 U/ml) on NF-κB activation. For electrophoretic mobility shift assay, nuclear proteins were extracted from acinar cells treated with PMA-primed neutrophis in the absence or presence of NAC or SOD and performed electrophoretic mobility shift assay by the method of Dignam et al (1983). Briefly, nuclear proteins were incubated with ^{32}P-labeled DNA corresponding to the NF-κB binding site of the *igk* gene (GATCGAGGGGGACTTTCCC TAGC; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and poly (dI-dC) (Boehringer Mannheim, Indianapolis, IN, USA). The mixtures were loaded onto a nondenaturing polyacrylamide gel and exposed to the radiography film for 6~18 h at −70°C. Quantitation of NF-κB-DNA binding activity was estimated by densitometry for the radioactivity of ^{32}P in NF-κB band. Control value was set to 100%. In competition experiment, nuclear proteins from acinar cells treated with PMA-primed neutrophils were treated with 100-fold excess of unlabelled probe (wild type NF-κB oligonucleotide), mutant type NF-κB oligonucleotide or unrelated oligonucleotide (Oct-1 or AP-1; Santa Cruz Biotechnology) at the start of 30 min-incubation. Supershift assay was performed using antibodies against two Rel proteins (p50, p65) to determine the Rel protein composition of the activated NF-κB dimers. Each anti-p50 or anti-p65 antibody was added for 30 min before the addition of ^{32}P-labeled oligonucleotide probe.

Statistical analysis

The statistical differences were determined one way analysis of variance (ANOVA) and Newman-Keul's test (Zar, 1984). All values are expressed as mean± SE, and statistical significance was set at $p<0.05$.

RESULTS

Oxidative stress indices and cytokine production in acinar cells

Neutrophils alone did not increase the productions of H_2O_2, LPO and cytokines in acinar cells (Table 1). However, PMA-primed neutrophils highly increased the productions of these oxidative stress indices and cytokines in the cells. For concentration response of acinar cells to PMA-primed neutrophils, the productions of H_2O_2, LPO and TNF-α were increased with

Table 1. Oxidative stress indices and cytokine production in acinar cells treated with or without neutrophils at 2 h-culture

	None	Neutrophils	PMA-primed neutrophils
H_2O_2 (nmol/ml)	1.5±0.2	1.8±0.1	12.7±1.1*
LPO (nmol/ml)	0.5±0.1	0.7±0.1	12.9±0.4*
IL-1 β (pg/ml)	2.6±0.1	3.5±0.3	22.4±2.0*
IL-6 (pg/ml)	3.9±0.3	4.3±0.3	52.0±4.0*
TNF-α (pg/ml)	3.1±1.6	4.0±1.8	45.2±5.1*

Values are mean±SE of triplicate samples from six different experiments.
* $p<0.05$ vs none.
PMA, 4 β-phorbol 12 β-myristate 13 α-acetate

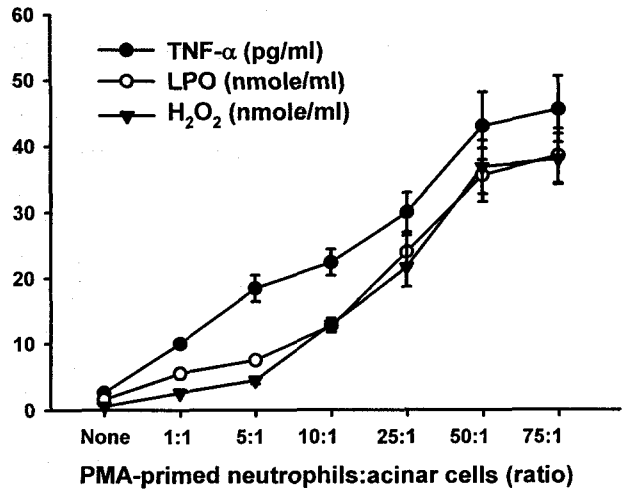

Fig. 1. Concentration response of acinar cells to PMA-primed neutrophils for the productions of LPO, H_2O_2 and TNF-α at 2 h-culture. Each point represents mean±SE of triplicate samples from six different experiments. PMA, 4β-phorbol 12β-myristate 13α-acetate.

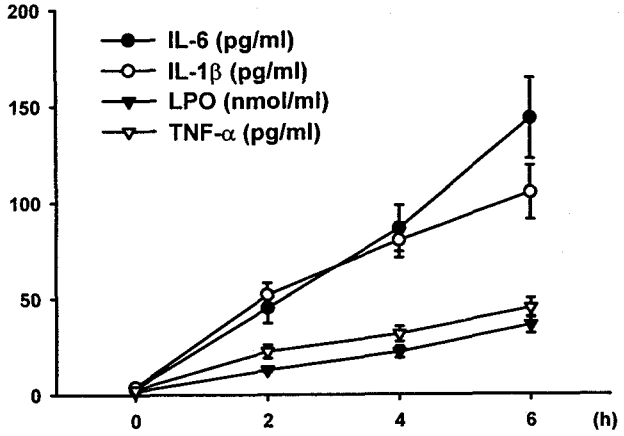

Fig. 2. Time response of acinar cells to PMA-primed neutrophils for the productions of LPO, IL-1β, IL-6 and TNF-α at the ratio of neutrophils : acinar cells, 10 : 1. Each point represents mean±SE of triplicate samples from six different experiments. PMA, 4β-phorbol 12β-myristate 13α-acetate.

the ratio of PMA-primed neutrophils to acinar cells up to the ratio of 50 : 1 and became plateau until that of 75 : 1 at 2 h-culture (Fig. 1). H_2O_2 levels in the medium (nmol/ml) released from acinar cells alone (none), treated with PMA-primed neutrophils at the ratio of neutrophils : acinar cells, 1 : 1, 5 : 1, 10 : 1, 25 : 1, 50 : 1 and 75 : 1 were 0.55±0.08, 2.6±0.1, 4.5±0.5, 12.9±1.0, 21.7±3.0, 36.8±4.0 and 38.5±4.1, respectively. LPO content in the medium (nmol/ml) released from acinar cells alone was 1.57±0.5, which increased to 5.5±0.7, 7.5±0.7, 12.7±1.0, 24.0±2.5, 35.6±4.0 and 38.5±4.1 at the ratio of neutrophils : acinar cells, 1 : 1, 5 : 1, 10 : 1, 25 : 1, 50 : 1 and 75 : 1. TNF-α (pg/ml) released from acinar cells alone (none), treated with PMA-primed neutrophils at the ratio of neutrophils : acinar cells, 1 : 1, 5 : 1, 10 : 1, 25 : 1, 50 : 1 and 75 : 1 were 2.6±0.1, 10.0±0.3, 18.5±2.0, 22.4±2.0, 30.0±3.0, 43.0±5.1, and 45.5±5.0, respectively. For the time response of acinar cells to PMA-primed neutrophils (at the ratio of neutrophils : acinar cell, 10 : 1), the productions of LPO, IL-1β, IL-6 and TNF-α were increased with time (Fig. 2). At the start of incubation, 2, 4, and 6 h-culture, LPO contents (nmol/ml) were 1.5±0.5, 12.7±1.0, 22.0±3.0 and 36.0±4.4 while TNF-α levels (pg/ml) were 2.6±1.0, 22.4±3.5, 31.4±4.0 and 45.0±5.1, respectively. IL-6 levels (pg/ml) were 3.1±1.6, 45.2±8.0, 86.6±12.0 and 143.6±21.0 and IL-1β levels (pg/ml) were 3.9±0.3, 52.0±6.3, 80.4±9.0 and 105.1±14.0 at 0, 2, 4 and 6 h-culture. Further studies on NAC and SOD for the productions of LPO and cytokines, 2 h-culture time and the ratio of PMA-primed neutrophils : acinar cells, 10 : 1 were used.

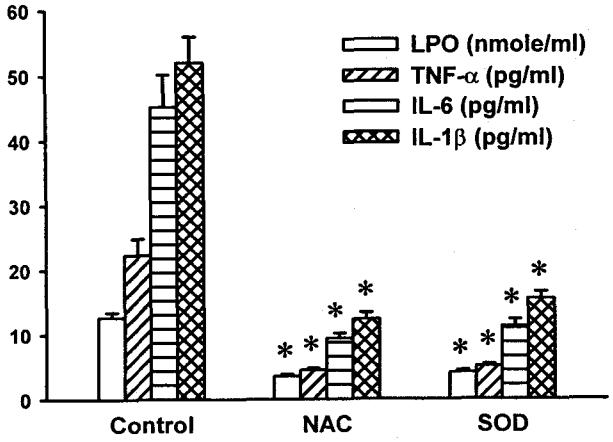

Fig. 3. Effects of NAC and SOD on the productions of LPO, TNF-α, IL-1β and IL-6 in acinar cells treated with PMA-primed neutrophils at 2 h-culture. Each point represents mean±SE of triplicate samples from six different experiments. * $p<0.05$ vs control. PMA, 4β-phorbol 12β-myristate 13α-acetate; NAC, N-acetylcysteine; SOD, superoxide dismutase.

Effects of NAC and SOD on the productions of LPO and cytokines in acinar cells

The levels of LPO (nmol/ml), TNF-α (pg/ml), IL-6 (pg/ml) and IL-1β (pg/ml) released from acinar cells treated with PMA-primed neutrophils were 12.7±0.8, 22.4±2.5, 45.2±5.0 and 52.0±4.0, respectively (Fig. 3). Treatments of NAC and SOD inhibited neutrophil-induced increases in LPO and cytokines. The levels of LPO, TNF-α, IL-6 and IL-1β released from acinar cells treated with PMA-primed neutrophils in the presence of NAC were 3.5±0.3, 4.4±0.3, 9.4±0.8 and 12.4±1.0 while those in the presence of SOD were 4.1±0.4, 5.1±0.3, 11.2±1.1 and 15.5±1.1, respectively.

NF-κB activation in acinar cells

Fig. 4 shows autoradiographs from the electrophoretic mobility gel shift assays for NF-κB in acinar cells. Two different NF-κB bands were shown in the cells treated with PMA-primed neutrophils, while the cells at the start of incubation contained a little activated NF-κB. An increased amount of activated NF-κB was detected at 1 h and even higher levels of activated NF-κB were observed at 2 h after treatment with PMA-primed neutrophils. Two different NF-κB bands reflects the presence of two species of activated NF-κB dimer. Supershift assay was performed using antibodies directed against two Rel proteins (p50, p65) to determine the Rel protein composition of NF-κB dimers of the cells. As shown in Fig. 5A, the upper activated NF-κB band undergoes a partial supershift with anti-p50 and a complete

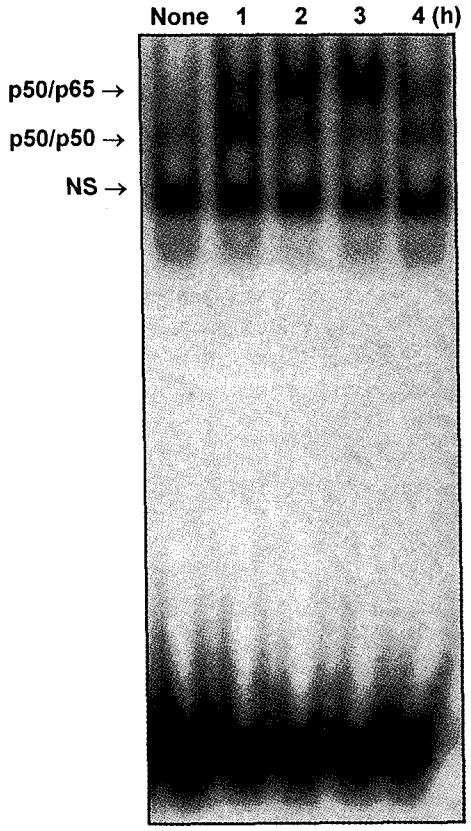

Fig. 4. A time course of NF-κB activation in acinar cells treated with PMA-primed neutrophils. Activated NF-κB bands (a p50/p65 heterodimer and a p50 homodimer) are indicated by arrows. PMA, 4β-phorbol 12β-myristate 13α-acetate; NS, nonspecific binding.

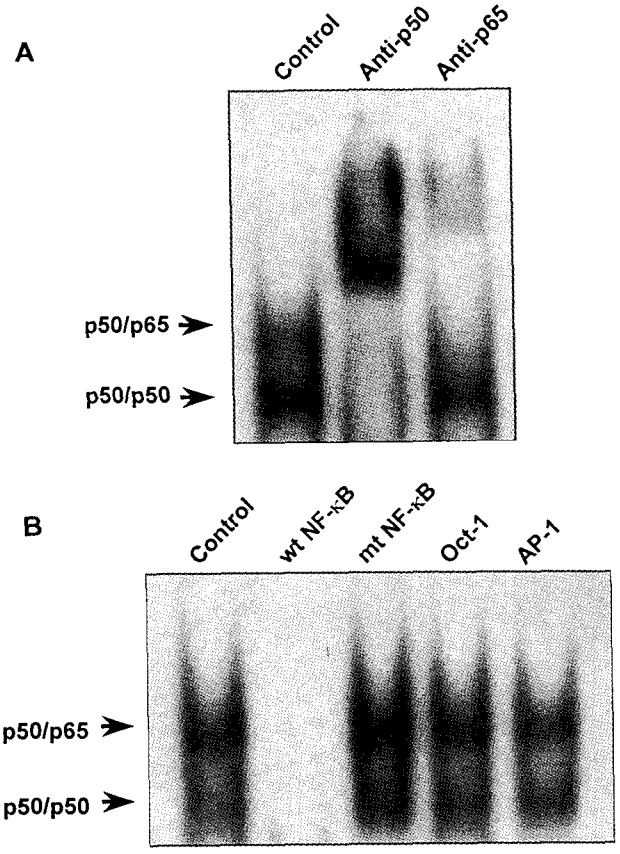

Fig. 5. Specific NF-κB complex formation in acinar cells treated with PMA-primed neutrophils at 2 h-culture. Binding reactions were performed using antibodies against two Rel proteins (p50, p65) (A) or an unlabeled wild type (wt), mutant type (mt) NF-κB probe, an unrelated oligonucleotide (Oct-1 or AP-1) or no reagent (control) (B). Activated NF-κB bands (a p50/p65 heterodimer and a p50 homodimer) are indicated by arrows. PMA, 4β-phorbol 12β-myristate 13α-acetate.

supershift with anti-p65. The lower activated NF-κB band undergoes a supershift only with anti-p50. Thus, treatment with PMA-primed neutrophils resulted in nuclear translocation of two activated NF-κB dimers in acinar cells; the classic p50/p65 NF-κB heterodimer (upper band) and a p50 homodimer (lower band). In competition assay using nuclear extracts prepared from acinar cells after 2 h-treatment of PMA-primed neutrophils, binding of ^{32}P-labeled oligonucleotide probe to NF-κB was blocked in the presence of 100-fold excess unlabed probe (wild type NF-κB oligonucleotide), but not by mutant type NF-κB oligonucleotide or unrelated oligonucleotide (Oct-1 or AP-1) (Fig. 5B).

Effects of NAC and SOD on NF-κB activation in acinar cells

Fig. 6 shows NF-κB complex of nuclear proteins extracted from the cells treated with PMA-primed neutrophils in the absence (control) or presence of NAC and SOD. Treatment of both antioxidants decreased NF-κB complex formation (Fig. 6A). The radioactivities of ^{32}P in each NF-κB bands of the cells treated with NAC and SOD were 45% and 40% of the control levels in p50/p65 heterodimers and 55% and 60% of the control levels in p50/p50 homodimers, respectively (Fig. 6B).

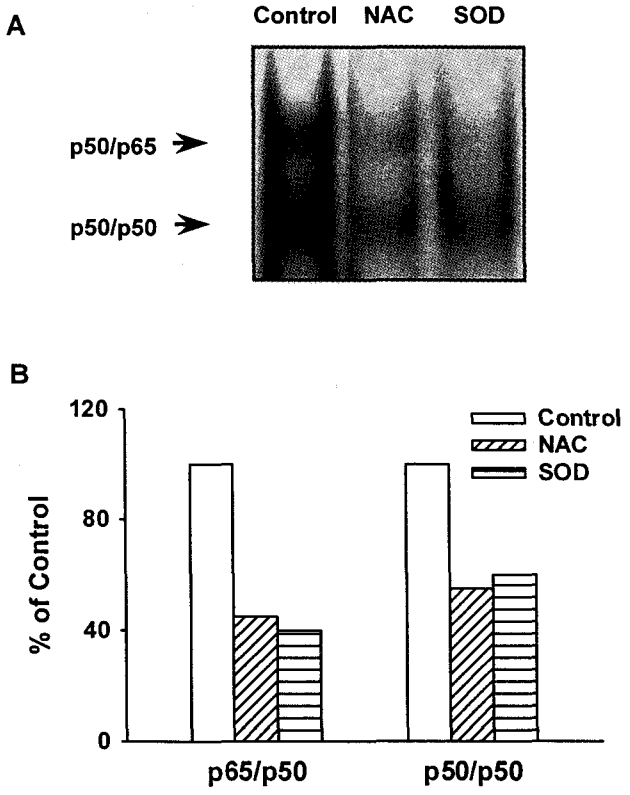

Fig. 6. Effects of NAC and SOD on specific NF-κB complex formation in acinar cells treated with PMA-primed neutrophils at 2 h-culture. Activated NF-κB bands (a p50/p65 heterodimer and a p50 homodimer) are indicated by arrows (A) and quantitated by densitometry as % of control (B).

DISCUSSION

The main finding of the present study is that (1) PMA-primed neutrophils stimulate the productions of H_2O_2 and LPO, NF-κB activation and the productions of inflammatory cytokines (IL-1β, IL-6, TNF-α) in pancreatic acinar cells and (2) an antioxidant NAC and SOD inhibit LPO production and oxidant-mediated activation of NF-κB and thereby decrease cytokine production by PMA-primed neutrophils.

Many previous reports have suggested that ROS may play an important role in the initiation and development of pancreatitis (Uys et al, 1973; Alder et al, 1979; Aho et al, 1982; Okumura et al, 1982). Actually the superoxide production by neutrophils treated with PMA in pancreatitis patients was significantly higher than in healthy controls (Tsuji et al, 1994). Among ROS, H_2O_2 is suggested to be the mediator of oxidant-mediated NF-κB activation. The essential cysteine residues of NF-κB are likely targets of modification and this modification together with phosphorylation events may be responsible for their complex signalling mechanism on immune and inflammatory processes (Schreck et al, 1991; Bauerle & Baltimore, 1996). Several unrelated stimuli like phorbol esters, IL-1β, IL-8 and ultraviolet light have been shown to activate NF-κB, which is in agreement with the pleitropic roles of NF-κB in many different cells (Neurath et al, 1998). In present study, ROS generation was detected in PMA-primed neutrophils, which may contribute to high levels of H_2O_2 and LPO in the medium from acinar cells treated with PMA-primed neutrophils. The present prompt activation of NF-κB is a combination of a direct effect of ROS, including H_2O_2, and an indirect effect mediated by cytokines stimulated by PMA-primed neutrophils in acinar cells since the productions of IL-1β, IL-6 and TNF-α increased in acinar cells treated

with PMA-primed neutrophils. However, neutrophils alone did induce neither oxidative damage, determined by the productions of H_2O_2 and LPO, nor cytokine production in acinar cells.

Recent studies (Heath et al, 1993; Norman et al, 1994) suggest that during acute pancreatitis activated pancreatic macrophages release inflammatory cytokines (IL-1, IL-6, TNF-α) in response to pancreatic damage. Intrapancreatic IL-1β, IL-6 and TNF-α are rapidly and coordinately increased during early stages of acute pancreatitis. In experimental pancreatitis, IL-1β and TNF-α were detected within the pancreas early in the course of pancreatitis, while IL-6 was produced in the pancreas after pancreatitis was more fully developed. Even though there is a time difference of cytokine production in experimental pancreatitis, similar timing of IL-1β, IL-6 and TNF-α production was shown in present *in vitro* study.

NF-κB belongs to a Rel family of transcription factors regulating the activation of a wide variety of genes that respond to immune or inflammatory signals (Wulczyn et al, 1996; Barnes & Karin, 1997). Human Rel proteins include p50, p52, Rel (c-Rel), Rel A (p65) and Rel B. The classic form of activated NF-κB is a heterodimer consisting one p50 and one p65 subunit. Present study demonstrates that PMA-primed neutrophils stimulate the transcription factor, NF-κB and induces nuclear translocation of a p50/p65 NF-κB heterodimer and a p50 NF-κB homodimer in pancreatic acinar cells. Although the mechanism of NF-κB activation is not fully understood, the key event appear to be degradation and dissociation of the inhibitory subunits to release NF-κB complexes (Wulczyn et al, 1996). Pancreas of control animal contains abundant amounts of inhibitory proteins, IκBα and IκBβ (Gukovsk et al, 1998; Steinle et al, 1999). Recent report shows that IκBα and not IκBβ is primarily responsible for controlling nuclear translocation of NF-κB in pancreas (Steine et al, 1999). Therefore, further study should be performed whether the onset and termination of NF-κB activation correlate with degradation and reappearance of IκBα in acinar cells treated with PMA-primed neutrophils or ROS directly.

To get the support for the hypothesis that ROS mediates NF-κB activation, which is responsible for the productions of inflammatory cytokines in acinar cells, we determined the effect of a known antioxidant, NAC and superoxide scavenging enzyme SOD on neutrophil-induced alterations. In present study, the inhibitory effect of NAC on cytokine production was in parallel with its suppression on LPO production in acinar cells treated with PMA-primed neutrophils. Since NF-κB binding sites were found in the promoters of IL-8 and IL-6, the expressions of these genes were inhibited by NAC (Ben-Baruch et al, 1995; Wulczyn et al, 1996). NAC could increase the intracellular stores of glutathione in the cells, thereby enhancing endogenous antioxidative defence mechanism (Morris & Bernard, 1994). In addition, NAC could directly scavenge ROS (hydroxyl radicals, hypochlorous acid) produced by inflamed cells (Gressier et al, 1994). More recently Villagrasa et al (1997) reported that NAC reduced superoxide generation in response to PMA and also replenishes cellular glutathione level in human neutrophils. Therefore, NAC might be clinically useful due to its ROS scavenging effect. Besides NAC, with treatment of SOD, we found a similar correlation between inhibition of pancreatic NF-κB activation and inhibition of cytokine production. These results confirm our hypothesis on relations among ROS, NF-κB activation and cytokine production in pancreatic acinar cells.

In conclusion, we can hypothesize that ROS, generated by infiltrating neutrophils into pancreas, activate NF-κB in acinar cells, resulting in upregulation of certain cytokines, like IL-1β, IL-6 and TNF-α, which may mediate pancreatic inflammation. We believe that inhibition of a number of inflammatory molecules by targetting NF-κB system represents an exciting and promising approach to the treatment of pancreatitis. By reducing oxidant-mediated NF-κB activation, antioxidants might be endowed with clinically useful antiinflammatory effect.

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