NADPH Oxidase Mediates Cerulein-Induced Apoptosis in Pancreatic Acinar AR42J Cells

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The doctoral dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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December 2006

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December 2006

ACKNOWLEDGEMENTS

This thesis would not have been completed without the help and support from many people. First, I would like to thank my advisor Dr. Kyung Hwan Kim who is actively involved in the work of all his students, and always has their best interest in mind.

I also would like to express my appreciation to Dr. Hyeyoung Kim, for her advice during my doctoral research endeavor for the past seven years. I learned a lot from her enthusiasm, his inspiration.

I am always indebted to Dr. Joo Weon Lim for his encouragement and good teaching.

I thank my other committee members, Dr. Min Goo Lee, Jeon Han Park, Jong Eun Lee for their helpful suggestions and comments on my work.

I thank Dr. Young Soo Ahn, Dr. Dong Goo Kim, for the support, sensible suggestions.

I also would like to thank my lab mates and friends who were there with me through thick and thin. All your generous input, constructive and laughter are in here somewhere.

I wish to acknowledge the department of Pharmacology and Brain Korea 21 project for medical science for providing scholarships to pursue this study.

I thank my uncle, Jong Won Lim and aunt, grandmother, cousins for their continuous love, support.

Finally I would like to thank those who are very dear to my heart: my parents, Yoon Soo Yu, Young Ja Park, my sisiter, Hee Young Yu for their continuous love, support and prayers.

December, 2006

Ji Hoon Yu

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Abstract

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Apoptosis linked to oxidative stress has been known to be implicated in pancreatitis. NADPH oxidase has been considered as a major source of reactive oxygen species during apoptosis in pancreatic acinar cells. However, the source of reactive oxygen species in pancreatic acinar cells has not been clarified. Cerulein rapidly induces acute and edematous form of pancreatitis. It was investigated that NADPH oxidase mediates apoptosis in cerulein-stimulated pancreatic acinar AR42J cells. Treatment of cerulein resulted in the activation of NADPH oxidase, as determined by reactive oxygen species production, translocation of cytosolic subunits $p47^{phox}$ and $p67^{phox}$ to the membrane, and interaction between NADPH oxidase subunits. Cerulein induced Ca^{++} oscillation, the expression of apoptotic gene bax, p53, AIF and apoptotic indices (DNA fragmentation, TUNEL staining, caspase 3 activity, decrease in cell viability) in AR42J cells. Treatment with a NADPH oxidase inhibitor diphenyleneiodonium (DPI), Ca^{++} chelator BAPTA, or transfection with antisense oligonucleotides for NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ inhibited cerulein-induced reactive oxygen species production, translocation of NADPH oxidase cytosolic subunits $p47^{phox}$ and $p67^{phox}$ to the membrane, and the expression of apoptotic genes and apoptotic indices, as compared to the cells without treatment and those

transfected with the corresponding sense oligonucleotides. These results indicate that NADPH oxidase may mediate reactive oxygen species-induced apoptosis in pancreatic acinar cells.

Key words: NADPH oxidase, apoptosis, reactive oxygen species, pancreatic acinar cells

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Ⅰ. INTRODUCTION

Oxidative stress is regarded as a major pathogenic factor in acute pancreatitis[1]. Studies on experimental models of pancreatitis indicate that pancreatic oxidative stress occurs during an early stage of induction[2,3]. Scavenger therapy for reactive oxygen species has attained some success in experimental pancreatitis, including cerulein pancreatitis[4,5]. High dose of cerulein, a cholecystokinin (CCK) analogue[6], results in pancreatitis, which is characterized by a dysregulation of the production and secretion of digestive enzymes. In particular, inhibition of pancreatic secretions, an elevation in serum enzyme levels, cytoplasmic vacuolization, death of acinar cells, edema formation, and an infiltration of inflammatory cells into the pancreas are the result of such dysregulation[7]. In human acute pancreatitis, the increased levels of lipid peroxide in the bile or pancreatic tissue and subnormal levels of

antioxidant vitamins in the blood were reported[8]. Once produced, reactive oxygen species can act as a molecular trigger of pancreatitis. They can attack the biological membranes directly and trigger the accumulation of neutrophils and their adherence to the capillary wall[9,10]. Therefore, it is probable that reactive oxygen species play a central role in perpetuating pancreatic inflammation and in the development of extra pancreatic complications[11]. Inflammation and death of pancreatic acinar cells are the hallmarks of both human and experimental pancreatitis[12,13]. Death can occur by either necrosis or apoptosis. Some evidence indicated that mild acute pancreatitis was characterized by very little necrosis but a high degree of apoptosis, while severe acute pancreatitis was found to involve extensive acinar cell necrosis[14-17]. Sandoval et al.[18] indicated that cerulein stimulates pancreatic production of platelet-activating factor (PAF). PAF mediates both apoptosis and neutrophil chemotaxis in the pancreas. Neutrophils in turn may convert acinar cells undergoing apoptosis into necrotic cells.

There is an increasing evidence that a major source of reactive oxygen species during inflammation and apoptosis is NADPH oxidase, and that reactive oxygen species is produced in a Ca^{++}-dependent manner in leukemic and HL-60 cells[19,20]. In phagocytic cells, NADPH oxidase is composed of the membrane-bound subunits $gp91^{phox}$ and $p22^{phox}$, as well as the cytosolic subunits $p67^{phox}$ and $p47^{phox}$. Upon activation of the enzyme, the cytosolic subunits translocate to the membrane to form an NADPH oxidase complex which facilitates NADPH-dependent formation of superoxide (O_2^-), and in turn gives rise to the production of other secondary reactive oxygen species (H_2O_2). Several non-phagocytic cells, such as vascular endothelial cells and pulmonary and systemic smooth muscle cells, contain NADPH oxidase for the production of toxic reactive oxygen species[21,22].

It was hypothesized that pancreatic acinar cells may contain NADPH oxidase, which may be activated to produce reactive oxygen species in the course of pancreatitis. Recently it was suggested that the source of reactive oxygen species is NADPH oxidase in the stimulated neutrophils while pancreatic acinar cells do not produce reactive oxygen species in cerulein pancreatitis *in vivo*[23]. Previous study showed that cerulein produces large amounts of reactive oxygen species, activates oxidant-sensitive nuclear transcription factor NF-κB and thus induces cytokine expression in freshly isolated pancreatic acinar cells in the absence of inflammatory cells *in vitro*[24]. Recently it was detected that the presence of NADPH oxidase in pancreatic acinar AR42J cells[25]. Cerulein induced NF-κB activation and IL-6 expression were inhibited by treatment with the antioxidant rebamipide, diphenylene-iodonium (DPI) (nonspecific inhibitor for NADPH oxidase), or by transfection with antisense oligonucleotides (AS ODNs) for NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ in AR42J cells. Furthermore, it has been reported that exogenous hydrogen peroxide resulted in the nuclear loss of DNA repair proteins Ku 70 and Ku 80 and thus, induced apoptosis in pancreatic acinar AR42J cells[26].

In the present study, it used pancreatic acinar AR42J cells (pancreatoma cell line) to investigate the involvement of NADPH oxidase in apoptosis. The AR42J cell line is the only currently available cell line that maintains many characteristics of normal pancreatic acinar cells, such as the synthesis and secretion of digestive enzymes[27]. AR42J cells show receptor expression and signal transduction mechanisms parallel to those of normal pancreatic acinar cells[23]. Thus, this cell line has been widely used as an *in vitro* model to study cellular secretion, growth, proliferation, and apoptosis of the exocrine pancreas[27-31]. In addition, it was sought to transfect the cells with AS ODNs for NADPH oxidase subunits to directly inhibit NADPH oxidase activation.

Commonly used DPI is a nonspecific inhibitor for NADPH oxidase and also inhibits other flavoprotein-dependent oxidases[32]. Pancreatic acinar AR42J cells were more efficiently transfected with AS ODNs than freshly isolated pancreatic acinar cells in preliminary experiments.

This study investigates whether cerulein induces the activation of NADPH oxidase, the expression of apoptotic genes bax, p53, AIF and apoptotic indices in pancreatic acinar AR42J cells.

Ⅱ. Materials and Methods

1. Cell lines and culture condition

Rat pancreatic acinar AR42J cells (pancreatoma, ATCC CRL 1492) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

2. Experimental protocol

Reactive oxygen species production, the expression and interaction of NADPH oxidase subunits (Nox1, $p27^{phox}$, $p47^{phox}$ and $p67^{phox}$), translocation of cytosolic subunits $p47^{phox}$ and $p67^{phox}$ to the membrane, Ca^{++} oscillation, the expression of apoptotic genes bax, p53, AIF and apoptotic indices (DNA fragmentation, TUNEL staining, caspase 3 activity, and a decrease in cell viability) were determined in the cells cultured in the presence or absence of cerulein (10^{-8} M). The cells were either treated with or without 10 μM DPI and BAPTA or transfected with AS ODNs or S ODNs. The cells were treated with DPI and BAPTA for 30 min before the stimulation with cerulein. The cells were stimulated with cerulein for 15 min (ROS generation, translocation of NADPH oxidase subunits, or interaction between NADPH oxidase subunits), 8 h (mRNA expression of apoptotic genes) and 24 h (the levels of apoptotic proteins and apoptotic indices).

3. Measurement of reactive oxygen species generation

Reactive oxygen species production by suspensions of permeabilized cells (5 x 10^5/ml) was detected by lucigenin chemiluminescence using a Berthold Micro

Lumat LB 96V luminometer (Nashua, NH, USA). Lucigenin chemiluminescence was recorded continuously over 30 to 45 min from a test tube of the cells with 10 μM lucigenin in HEPES-buffered saline (HBS), before the addition of cerulein. Chemiluminescence counts were integrated over 15 min from the time of the addition of 10^{-8} M cerulein to plateau. The signal was recorded as relative light units per second (RLU/s). For measurement of intracellular reactive oxygen species by another useful assay for reactive oxygen species generation, the cells (5 x 10^5/ml) in a chamber slide (Nalge Nunc, Naperville, IL, USA) were cultured in the absence or presence of cerulein (10^{-8} M) for 15 min, washed with HBS and then loaded with 5 μM of 2', 7'-dichloro-fluorescencin diacetate (DCF-DA; Molecular Probes, Eugene, OR, USA) or 5',6'-carboxy 2',7'-dichlorofluorescencin diacetate (carboxy DCF-DA; Molecular Probes, Eugene, OR, USA) as a redox insensitive control for 5 min. The fluorescent dicholorofluorescenin was detected using a laser scanning confocal microscope (Leica TCS-NT) with excitation and emission wavelengths of 488 nm and 520 nm, respectively.

4. Preparation of whole cell extract, cytosolic fraction and membrane fraction

The cells were trypsinized and washed with PBS buffer and then centrifuged at 500 rpm for 5 min. The cells were resuspended with lysis buffer containing 10 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 50 μM leupeptin, 25 μM pepstatin, 25 μM aprotin, and 1 μM PMSF, lysed by drawing the cells through a 1 ml syringe with several rapid strokes, and centrifuged at 2,000 x g for 10 min. The supernatant was collected and used as a whole cell extract. To prepare the cytosolic and membrane fractions, the supernatant was separated further by centrifugation at 100,000 x g for 1 h. The membrane fraction was obtained by resuspending the pellet with lysis buffer.

The supernatant was used as the cytosolic fraction. The protein concentration was determined by the Bradford method[33].

5. Reverse transcription-polymerase chain reaction

The cells were treated with or without DPI and BAPTA or transfected with AS ODNs or S ODNs for NADPH oxidase subunits $p22^{phox}$, $p47^{phox}$, and cultured in the absence or presence of cerulein for 8 h or the indicated time periods. To determine the constitutive NADPH oxidase, the cells were cultured in the presence of cerulein for 1 h. Gene expressions of specific mRNAs were assessed using Reverse transcription -polymerase chain reaction standardized by coamplifying with GAPDH, which served as an internal control. Total RNAs isolated from the cells were reverse transcribed into cDNAs and used for PCR with rat specific primers for bax, p53, AIF and GAPDH. The sequences of primers used in the PCR were as follows: bax forward GATCCTGATTCCTGT-GTGTCGAA, reverse GCTGCATACATCACTGTCACGTTT; p53 forward GAT-CCTGATTCCTGTGTGTCGAA, reverse GCTGCATACATCACTGTCACGTTT; AIF forward ACTCCAAGAAGTCTGTCTGCTATCGA, reverse CTGACTCCAA-CTGATTGTACAATTGC; GAPDH forward ACCACAGTCCATGCCATCAC, reverse TCCACCACCCTGTTGCTGTA. The predicted sizes of the PCR products were 306 bp for bax, 306 bp for p53, 338 bp for AIF and 460 bp for GAPDH. After coamplification with 32 to 35 cycles, the PCR products were separated on 1.5% agarose gels and visualized by UV transillumination.

6. Western blotting

The cells were treated with or without DPI and BAPTA or either transfected with AS ODNs or S ODNs for NADPH oxidase subunits $p22^{phox}$, $p47^{phox}$ and cultured in the absence or presence of cerulein for 15 min, 24 h or the indicated time periods. Western blot analysis was performed with a

previously described method[26]. In brief, the whole cell extract, cytosolic fraction, and membrane fraction were loaded, separated by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred onto nitrocellulose membranes. After blocking of nonspecific binding with 5% nonfat dry milk, the membranes were incubated with polyclonal antibodies for Nox1, $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, bax, and p53 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at $4^{o}C$ overnight. The immunoreactive proteins were visualized using anti-goat secondary antibodies conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Actin was used as a loading control.

7. Co-immunoprecipitation

The cells (5×10^5/ml) were cultured in the presence of cerulein (10^{-8} M) for 15 min. Membrane fractions prepared from the cells were incubated with a specific polyclonal antibody or goat IgG as a negative control and protein G immobilized on agarose overnight at $4^{o}C$ in RIPA buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.25% NP-40 and 0.5% sodium deoxycholate. The protein G-antibody-antigen complex was collected by washing three times with ice-cold RIPA buffer. The final pellet was resuspended with 50 μl SDS-sample buffer and boiled for 5 min. This preparation was subjected to western blot analysis with the specific antibody.

8. Measurement of intracellular Ca^{++}

The free cytosolic concentration of Ca^{++} was measured using Fura-2 as described previously[24]. Briefly, Fura-2 loaded cells were plated on coverslips that formed the bottom of a perfusion chamber. After 2 to 3 min of incubation, the cells were superfused with standard perfusate and then perfused

again with standard perfusate containing 10^{-8} M cerulein alone or cerulein in the presence of 10 μM BAPTA and DPI. Fura-2 fluorescences excited at 350 nm and 380 nm were measured using a photon counting system (PTI Delta Ram, Monmouth Junction, New Jergy, USA).

9. Transfection with antisense or sense oligonucleotides

Phosphothioate-modified oligonucleotides (ODNs) were produced commer -cially (GIBCO-BRL, Grand Isand, NY). The sequences of $p22^{phox}$ antisense (AS) and sense (S) ODNs were GATCTGCCCCATGGTGAGGACC and GGT-CCTCACCATGGGGCAGATC. The sequences of the $p47^{phox}$ AS and S ODN were CTGTTGAAGTACTCGGTGAG and CTCACCGAGTACTTCAACAG. The cells were treated with ODNs using transfection reagent DOTAP (Boehringer Mannheim, Mannheim, Germany). When DOTAP was employed, the appropriate amounts of ODNs were incubated with DOTAP (15 g/ml) to achieve the final concentration of ODNs at 0.5 μM for 15 min. Then the mixture was added directly to the cells. The cells were incubated for 24 h and then used.

10. Cell counting

The cells (4×10^4 /well) were cultured with various concentrations of cerulein for 24 h. Cell viability was directly determined by counting with a hemocytometer, using a trypan blue exclusion test.

11. DNA fragmentation assay

The cells (1×10^5 /well) were cultured in the presence or absence of cerulein for 24 h (Figs. 4B and 4C). DNA fragmentation was determined by the amount of oligonucleosome-bound DNA in the cell lysates. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. The nucleosomes were quantified using a

sandwich ELISA (Cell Death Detection ELISA ^{plus} kit; Boehringer Mannheim GmbH, Germany).

12. TUNEL assay

DNA fragmentation associated with cell death was detected by using an in situ terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) apoptosis detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). For the TUNEL analysis, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4°C and subjected to permeabilization for 20 min at room temperature with 0.1% sodium citrate containing 0.1% Triton X-100. The fixed and permeabilized AR42J cells were labeled with the TUNEL reaction mixture for 60 min at 37°C. The nuclei of these AR42J cells were also stained with 4', 6'-diamino-2-phenylindole (DAPI). Fluorescein-labeled DNA, indicating DNA fragmentation, was analyzed by using a laser- scanning confocal microscope (Leica TCS-NT). The percentage of TUNEL labeling was expressed as the number of TUNEL-positive nuclei divided by the total number of nuclei stained with DAPI.

13. Measurement of Caspase 3 activity

Caspase 3 activity was determined by measuring cleavage of the specific caspase 3 substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVDAFC) using a commercial kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). The immunosorbent fluorescence enzyme assay was performed, according to the manufacturer's protocol.

14. Statistical analysis

The statistical differences were determined using one-way ANOVA and Newman-Keul's test. All values were expressed as a mean ± S.E. of four separate experiments.

Ⅲ. RESULTS

1. Reactive oxygen species production and NADPH oxidase activation in AR42J cells cultured in the presence of cerulein

The effect of cerulein on reactive oxygen species production was investigated using lucigenin-enhanced chemiluminescence, which reflects mainly intracellular O_2^- production in AR42J cells[34]. A very low level of reactive oxygen species, such as O_2^-, was detected in the cells cultured in the absence of cerulein. Treatment with 10^{-8} M cerulein for 15 min resulted in substantial production of reactive oxygen species in the cells (None, Fig. 1A). Confocal microscopic monitoring of reactive oxygen species production demonstrated that the oxidized DCF fluorescence was increased inside the cells cultured in the presence of cerulein (Fig. 1B).

The necessary step for the activation of NADPH oxidase is the translocation of cytosolic subunits, including $p47^{phox}$ and $p67^{phox}$, to the membrane[19,20]. The $p47^{phox}$ and $p67^{phox}$ in membrane fractions were markedly increased 15 min after stimulation with cerulein, whereas those in cytosolic fractions were decreased (Fig. 1C). It is well documented that cytosolic subunits of NADPH oxidase, including $p47^{phox}$ and $p67^{phox}$, are translocated to the membrane upon stimulation, where they form a complex with the transmembrane Nox1 and $p22^{phox}$ to assemble the activated NADPH oxidase. After 30 min of stimulation with cerulein, the translocation of cytosolic subunits $p47^{phox}$ and $p67^{phox}$ to the membrane was reduced, and the subunits showed a tendency to reside in the cytosol. $p47^{phox}$ transferred more rapidly back to the cytosol, as compared to $p67^{phox}$. It was performed co-immunoprecipitation to examine whether membrane -associated $p67^{phox}$ and $p47^{phox}$ could form a complex with Nox1 or $p22^{phox}$ upon stimulation with cerulein. As shown in Fig. 1D, $p67^{phox}$ or Nox1 was detected in anti-Nox-1 or anti-$p67^{phox}$ immunoprecipitation derived from

membrane fraction of the cells cultured in the presence of cerulein. $p47^{phox}$ or $p22^{phox}$ was detected in immune-complexes derived from the membrane fraction of the cells cultured in the presence of cerulein, using a specific antibody for $p22^{phox}$ or $p47^{phox}$. $p47^{phox}$ associated with $p22^{phox}$ was not detected, and a small amount of Nox1 complexed with $p67^{phox}$ in the membrane fraction of the cells cultured in the absence of cerulein. The results demonstrate the interactions between cytosolic subunits and membrane subunits of NADPH oxidase by the stimulation of the cells with cerulein. Non-immune IgG was not associated with either Nox1 or $p22^{phox}$ in the cells cultured in the presence of cerulein (Fig. 1D, lower panel).

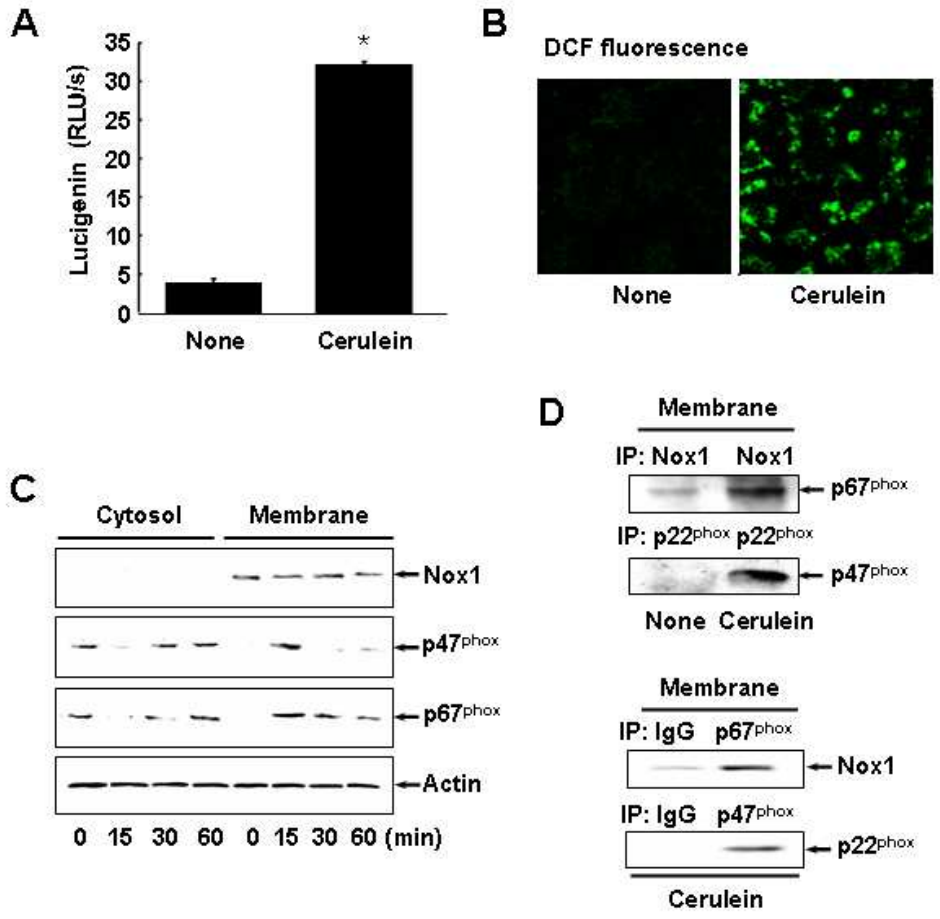


Figure 1. Reactive oxygen species production and the activation of NADPH oxidase in AR42J cells cultured in the presence of cerulein. (A) Reactive oxygen species production detected with lucigenin. The cells (5 x 10^5/ml) were treated with 10 μM lucigenin. Chemiluminescence was measured after the addition of 10^{-8} M cerulein for 15 min. Each vertical bar represents the S. E. of mean from four separate experiments. *p < 0.05 corresponding to the cells cultured in the absence of cerulein (None). (B) Reactive oxygen species production detected with DCF fluorescence in the cells. The cells were cultured in the absence or presence of cerulein (10^{-8} M) for 15 min and loaded with 5 μM DCF-DA for 5 min. DCF fluorescence was detected using a lasers canning confocal microscope. (C) Effect of cerulein on the localization of NADPH oxidase subunits. Cytosolic and membrane fractions were prepared from the cells stimulated with 10^{-8} M cerulein for the indicated time periods. Protein levels of Nox1, $p47^{phox}$ and $p67^{phox}$ were determined by western blotting. Actin was used a loading control. (D) Effect of cerulein on the interactions between NADPH oxidase subunits. The membrane fraction was prepared from the cells cultured in the absence or presence of 10^{-8} M cerulein for 15 min and immunoprecipitated with the antibodies for NADPH oxidase subunits (Nox1, $p22^{phox}$, $p67^{phox}$, $p47^{phox}$) or IgG as a negative control. The immunoprecipitates were analyzed with the indicated antibodies. None: the cells cultured in the absence of cerulein, Cerulein: the cells cultured in the presence of cerulein.

2. Intracellular Ca^{++}, reactive oxygen species production, and NADPH oxidase activation of AR42J cells treated with DPI and BAPTA and cultured in the presence of cerulein

Cerulein-evoked Ca^{++} oscillation was determined by the ratio of fluorescence excited at 350 nm and 380 nm. A relatively high cerulein concentration (10^{-8} M) evoked an initial peak in the Ca^{++} signal and induced the second Ca^{++}

signal by another addition of cerulein to the AR42J cells (Fig. 2C). A cerulein-induced increase in Ca^{++} signal was inhibited in the cells treated with Ca^{++} chelator BAPTA. By using BAPTA, it was investigated whether Ca^{++} oscillation contributes to reactive oxygen species production and the activation of NADPH oxidase of the cells cultured in the presence of cerulein (Figs. 2A and 2B). The activation of NADPH oxidase was determined by the translocation of cytosolic subunits to the membrane. As shown in Fig. 1A and 1C, cerulein markedly increased the lucigenin-enhanced chemiluminescence, an index of O_2^- production, and the translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane, which were inhibited in the cells treated with DPI and BAPTA (Figs. 2A and 2B).

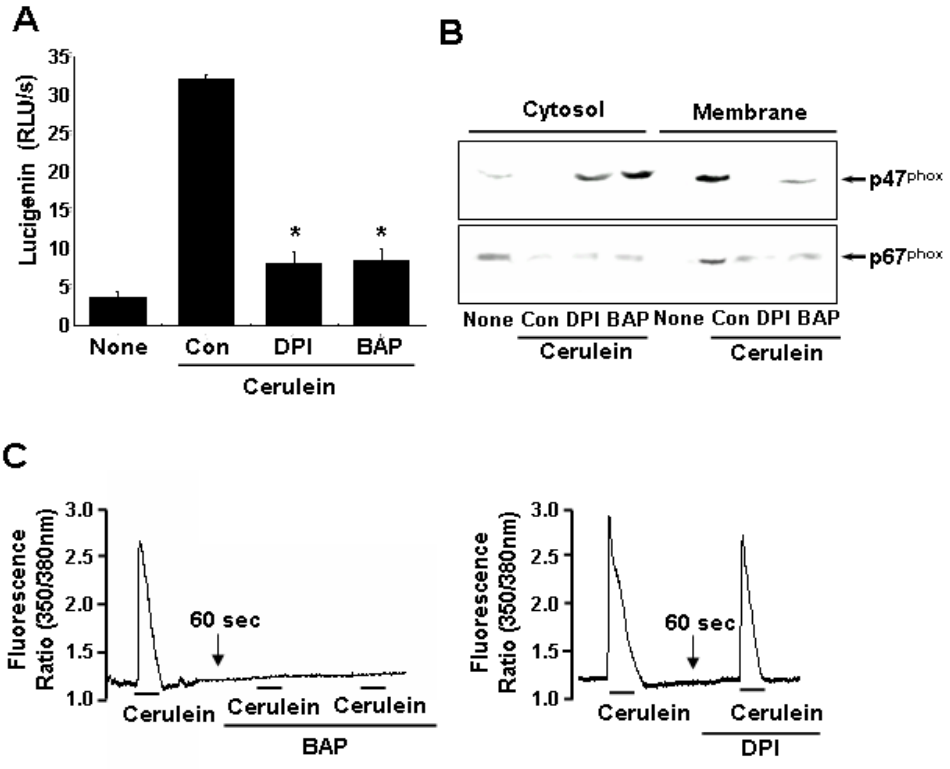


Figure 2. Effects of DPI and BAPTA on the cerulein-induced reactive oxygen species production, translocation of NADPH oxidase subunits and increased intracellular Ca^{++} in AR42J cells. DPI and BAP, the cells cultured in the presence of cerulein and treated with DPI and BAPTA. (A) Reactive oxygen species production detected with lucigenin in the cells. The cells (5 x 10^5/ml) were treated with 10 μM BAPTA for 30 min. Lucigenin (10 μM) was added, and chemiluminescence measured for 15 min after the addition of 10^{-8} M cerulein. Each bar represents the mean S. E. of four separate experiments. *p <0.05 compared to the cells cultured in the presence of cerulein (Con). None, the cells cultured in the absence of cerulein; (B) Membrane translocation of cytosolic NADPH oxidase subunits. The cells were treated with 10 μM BAPTA for 30 min and cultured in the presence of 10^{-8} M cerulein for 15 min. Protein levels of $p47^{phox}$ and $p67^{phox}$ in the cytosolic fraction or membrane fraction were determined by western blotting. Actin was used as a loading control. (C) Intracellular Ca^{++} levels. Fura-2-loaded cells were perfused with standard perfusate containing 10^{-8} M cerulein alone or cerulein with 10 μM BAPTA. The free cytosolic concentration of Ca^{++} was determined by Fura-2 fluorescence and expressed as the fluorescence ratio of 350 nm: 380 nm.

3. Reactive oxygen species production of AR42J cells transfected with AS ODNs for NADPH oxidase subunits and cultured in the presence of cerulein

To investigate the direct effect of NADPH oxidase on reactive oxygen species production, AS ODNs for $p22^{phox}$ and $p47^{phox}$ were transfected into the cells and reactive oxygen species levels were determined. RT-PCR analysis demonstrated that mRNA expression of $p22^{phox}$ and $p47^{phox}$ was substantially reduced in the cells transfected with AS ODNs for $p22^{phox}$ and $p47^{phox}$ (Fig. 3A). The result confirms the efficient transfection of AS ODNs into the cells. Cerulein-induced reactive oxygen species production was inhibited in the cells

transfected with AS ODNs, as compared to the corresponding S ODNs, determined by the lucigenin-enhanced chemiluminescence (Fig. 3B) and dicholorofluorescenin fluorescence (Fig. 3C). The results clearly demonstrate that cerulein-induced reactive oxygen species production is mediated by NADPH oxidase in AR42J cells

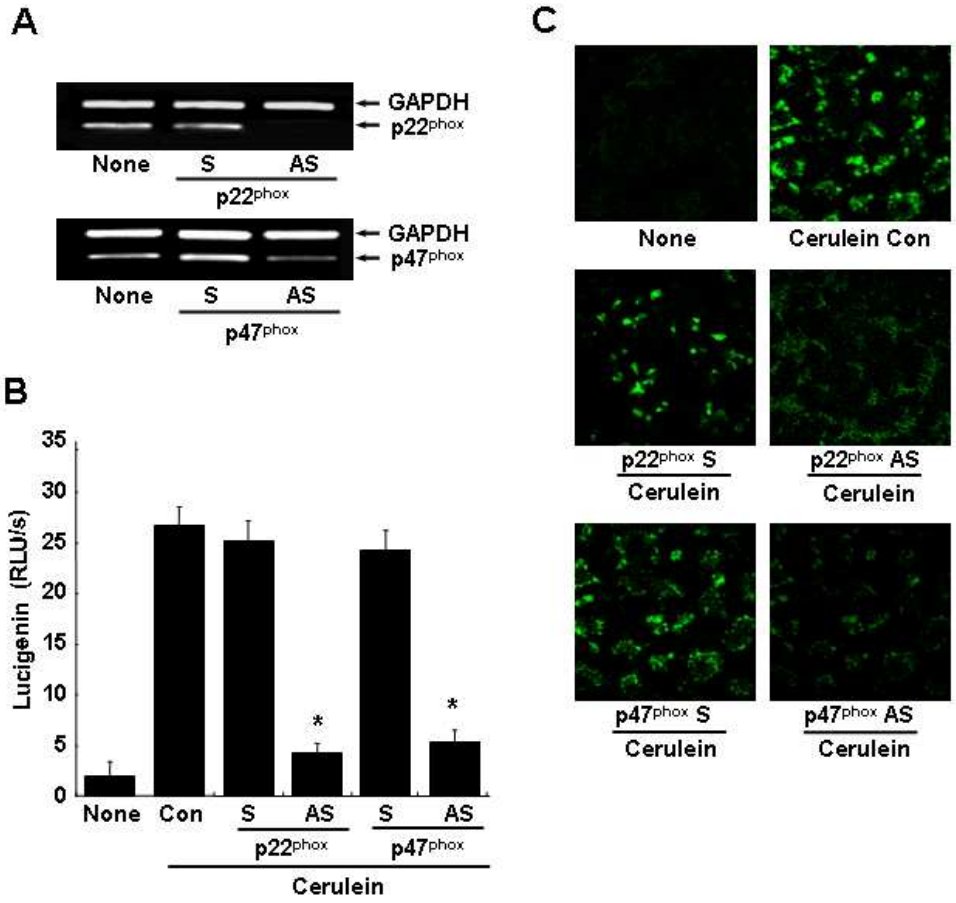


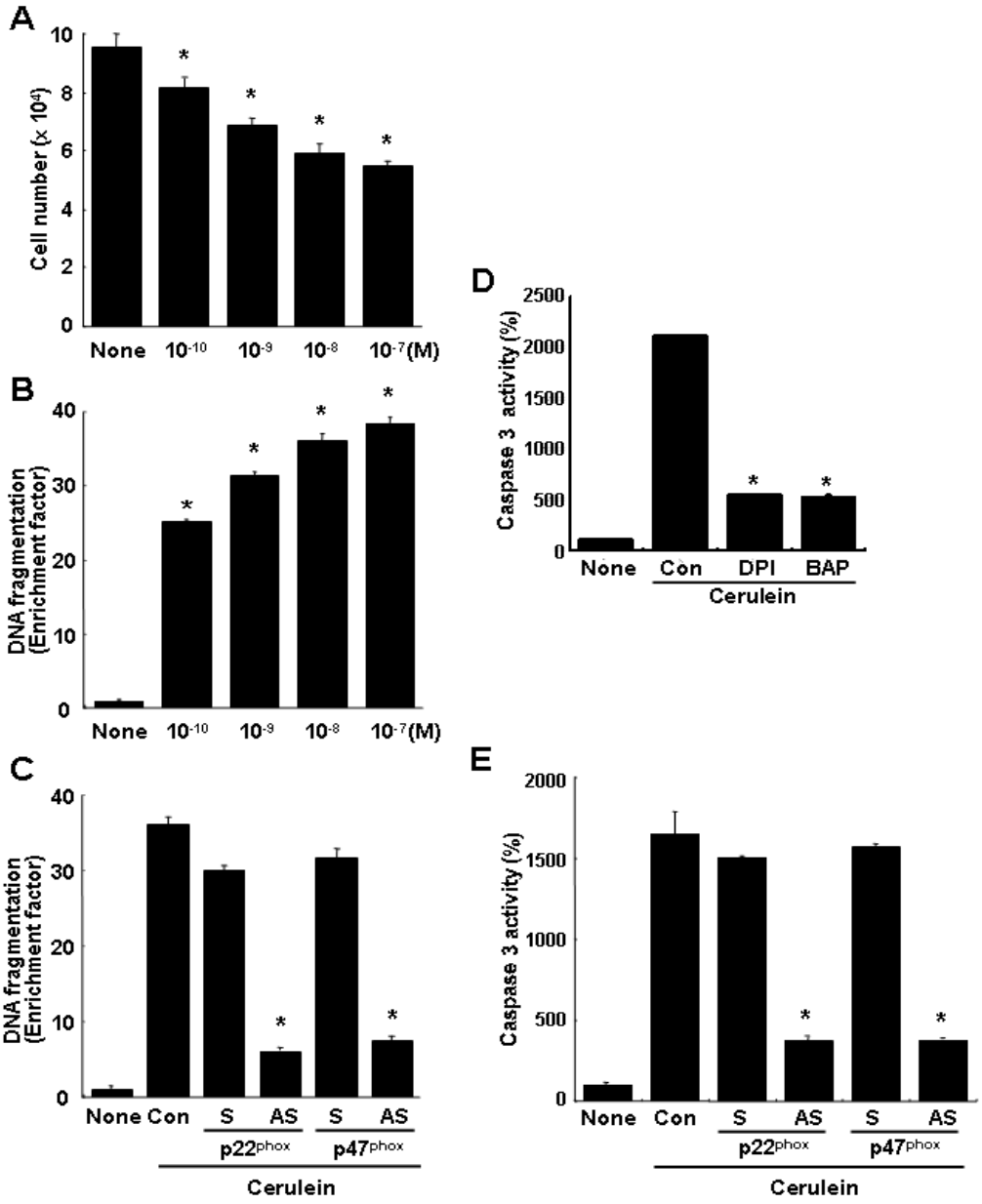
Figure 3. Cerulein-induced reactive oxygen species production in AR42J cells transfected with AS ODNs for $p22^{phox}$ and $p47^{phox}$. (A) mRNA expression of $p22^{phox}$ and $p47^{phox}$. The cells were transfected with 0.5 mM S or AS ODNs for $p22^{phox}$ or $p47^{phox}$ for 24 h using a DOTAP reagent and cultured in the

absence or presence of 10^{-8} M cerulein for 1 h. mRNA expression of $p22^{phox}$ and $p47^{phox}$ were determined by RT-PCR. The internal control (GAPDH) was coamplified with $p22^{phox}$ or $p47^{phox}$. (B) Reactive oxygen species production detected with lucigenin in the cells. Lucigenin (10 μM) was added to the transfected cells (5 x 10^5/ml) and chemiluminescence was measured for 15 min after addition of 10^{-8} M cerulein. Each bar represents the mean ± S. E. of four separate experiments. *p < 0.05 compared to the corresponding S ODN. (C) DCF fluorescence in the cells. The transfected cells were cultured in the presence of 10^{-8} M cerulein for 15 min and incubated with 5 μg/ml DCF-DA for 5 min. The DCF fluorescence was detected on a laserscanning confocal microscope. None: the cells cultured in the absence of cerulein, Con: the cells cultured in the presence of cerulein, S: the cells transfected with S ODN, AS: the cells transfected with AS ODN.

4. Cell viability, DNA fragmentation, and caspase 3 activation of AR42J cells treated with DPI and BAPTA or transfected with AS ODNs for NADPH oxidase subunits and cultured in the presence of cerulein

To determine the relationship between cerulein and apoptosis, the dose response of cerulein for cell viability, the nucleosome-bound DNA were determined in AR42J cells after 24 h treatment with cerulein (Figs. 4A and 4B). The number of viable cells, determined by a trypan blue exclusion test, was significantly reduced by treatment with cerulein in a dose-dependent manner. This paralleled the increases in nucleosome-bound DNA, an index of DNA fragmentation. Cerulein-induced DNA fragmentation was inhibited in the cells transfected with AS ODNs, as compared to S ODNs (Fig. 4C). Caspase 3 activity (Fig. 4D, 4E) was used as apoptotic indices, and treatment with DPI and BAPTA or transfection with AS ODNs significantly inhibited cerulein-induced increases in these apoptotic indices, as compared to the cells

without treatment or those transfected with the corresponding S ODNs. To evaluate the relation between intracellular Ca^{++}, activation of NADPH oxidase and apoptosis in the cells stimulated with cerulein, the cells were treated with DPI and BAPTA or transfected with AS ODNs for $p22^{phox}$ and $p47^{phox}$ and cultured in the presence of cerulein (10^{-8} M). Cerulein-induced DNA fragmentation was inhibited in the cells transfected with AS ODNs, as compared to S ODNs (Fig. 4C). TUNEL staining were used as apoptotic indices, and treatment with DPI and BAPTA or transfection with AS ODNs significantly inhibited cerulein-induced DNA fragmentation (Fig. 4F and 4I), as compared to the cells without treatment or those transfected with the corresponding S ODNs. Cerulein induced apoptosis in 86.5% ± 7.6% (Fig. 4G) and 87.6% ± 6.4% (Fig. 4I) respectively. Treatment with DPI and BAPTA resulted in 21.5% ± 5.4% and 21.9% ± 5.9% of apoptosis (Fig. 4G). Transfection with AS ODNs of p22phox and p47phox reduced apoptosis in 30.6% ± 3.6% and 32.6% ± 3.5% respectively (Fig. 4I).


A
Cell number (x 10⁴)
10
8
6
4
2
0
None
10-10
10-9
10-8
10-7(M)
B
DNA fragmentation (Enrichment factor)
40
30
20
10
0
None
10-10
10-9
10-8
10-7(M)
C
DNA fragmentation (Enrichment factor)
40
30
20
10
0
None
Con
S
AS
S
AS
p22phox
p47phox
Cerulein
D
Caspase 3 activity (%)
2500
2000
1500
1000
500
0
None
Con
DPI
BAP
Cerulein
E
Caspase 3 activity (%)
2000
1500
1000
500
0
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Con
S
AS
S
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p47phox
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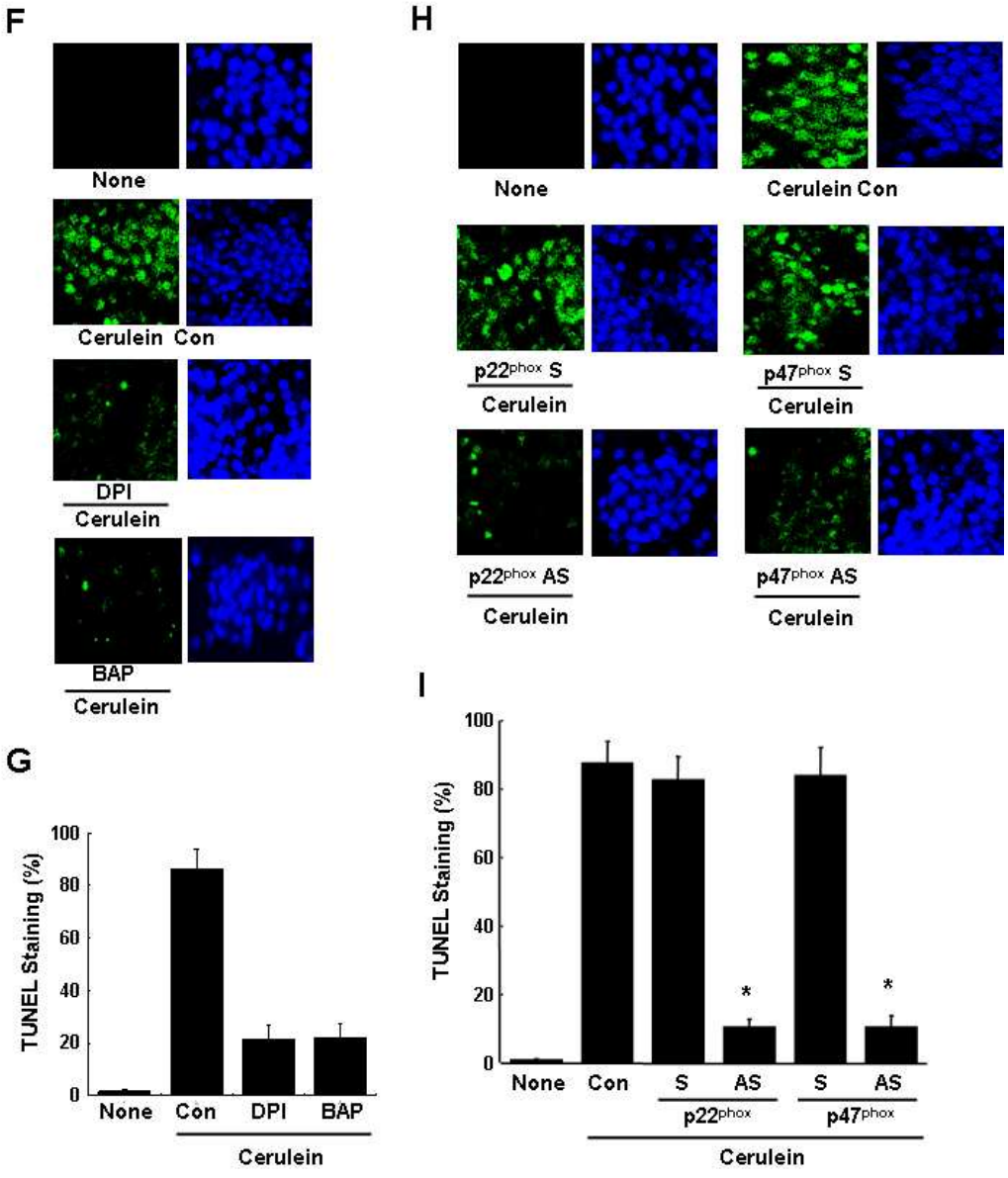

Figure 4. Effect of DPI and BAPTA and transfection with AS ODNs for $p22^{phox}$ and $p47^{phox}$ on cerulein-induced apoptosis and DNA fragmentation in AR42J cells. (A) Cerulein-induced cell death. The cells (4 x 10^4/well) were cultured with various concentrations of cerulein for 24 h. Viable cells were

identified using a trypan blue exclusion test. (B) Cerulein-induced DNA fragmentation. The cells (1 x 10^5/well) were cultured with various concentrations of cerulein for 24 h. DNA fragmentation was determined by the amount of oligonucleosome-bound DNA in the cell lysates. The relative increase of nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. (C) Effect of AS ODN on the cerulein-induced DNA fragmentation. The transfected cells (1 x 10^5 /well) were cultured in the presence of 10^{-8} M cerulein for 24 h. DNA fragmentation was determined by the amount of oligonucleosome-bound DNA in cell lysates. The cells treated with 10 μM BAPTA for 30 min (D) or either transfected with S or AS ODN (E) were cultured in the presence of 10^{-8} M cerulein for 24 h. Caspase 3 activity was determined using a commercial kit and expressed as the percentage of activity of the cells cultured in the absence of cerulein (none). The cells were treated with 10 μM DPI and BAPTA for 30 min and cultured in the presence of 10^{-8} M cerulein for 24 h (F, G). Prominent TUNEL staining in the cells cultured in the presence of cerulein, which was inhibited by treatment with DPI and BAPTA. The nuclei of the cells were also stained with DAPI (F, H). The percentage of TUNEL labeling was expressed as the number of TUNEL-positive nuclei divided by the total number of nuclei. Effect of transfection with AS ODNs for $p22^{phox}$ and $p47^{phox}$ on cerulein-induced apoptosis determined by TUNEL staining in AR42J cells. The cells were transfected with AS ODNs for $p22^{phox}$ and $p47^{phox}$ and cultured in the presence of 10^{-8} M cerulein for 24 h. (H, I) Prominent TUNEL staining in the cells cultured in the presence of cerulein, which was inhibited by transfection with AS ODNs for $p22^{phox}$ and $p47^{phox}$ as compared to the corresponding S ODNs. The nuclei of the cells were also stained with DAPI. (H, I) The percentage of TUNEL labeling was expressed as the number of TUNEL-positive nuclei divided by the total number of nuclei. Each bar represents the mean ± S. E.

of four separate experiments. *p <0.05 compared to the cells cultured in the absence of cerulein (None) (A, B). *p <0.05 compared to the corresponding S ODN. None, the cells cultured in the absence of cerulein; Con, the cells cultured in the presence of cerulean S, the cells transfected with S ODN; AS, the cells transfected with AS ODN (C, E, I). *p <0.05 compared to the cells cultured in the presence of cerulein (Con). None, the cells cultured in the absence of cerulein; Con, the cells cultured in the presence of ceruelin; DPI and BAP, the cells cultured in the presence of cerulein and treated with DPI and BAPTA (D, G).

5. Apoptotic gene expression of AR42J cells cultured in the presence of cerulein

To determine the relationship between cerulein and apoptosis, the dose response of cerulein for apoptotic gene expression was determined in AR42J cells treated with cerulein (Fig. 5). RT-PCR for bax, p53 and AIF showed a significant induction of the genes in AR42J cells cultured in the presence of cerulein, both dose- and time- dependently (Fig. 5A, 5B and 5E). mRNA and protein expression of bax, p53 and AIF was reduced in the cells treated with DPI and BAPTA (Fig. 5C and 5F). mRNA and protein expression of bax, p53 and AIF was substantially reduced in the cells transfected with AS ODNs for $p22^{phox}$ and $p47^{phox}$ (Fig. 5D and 5G).

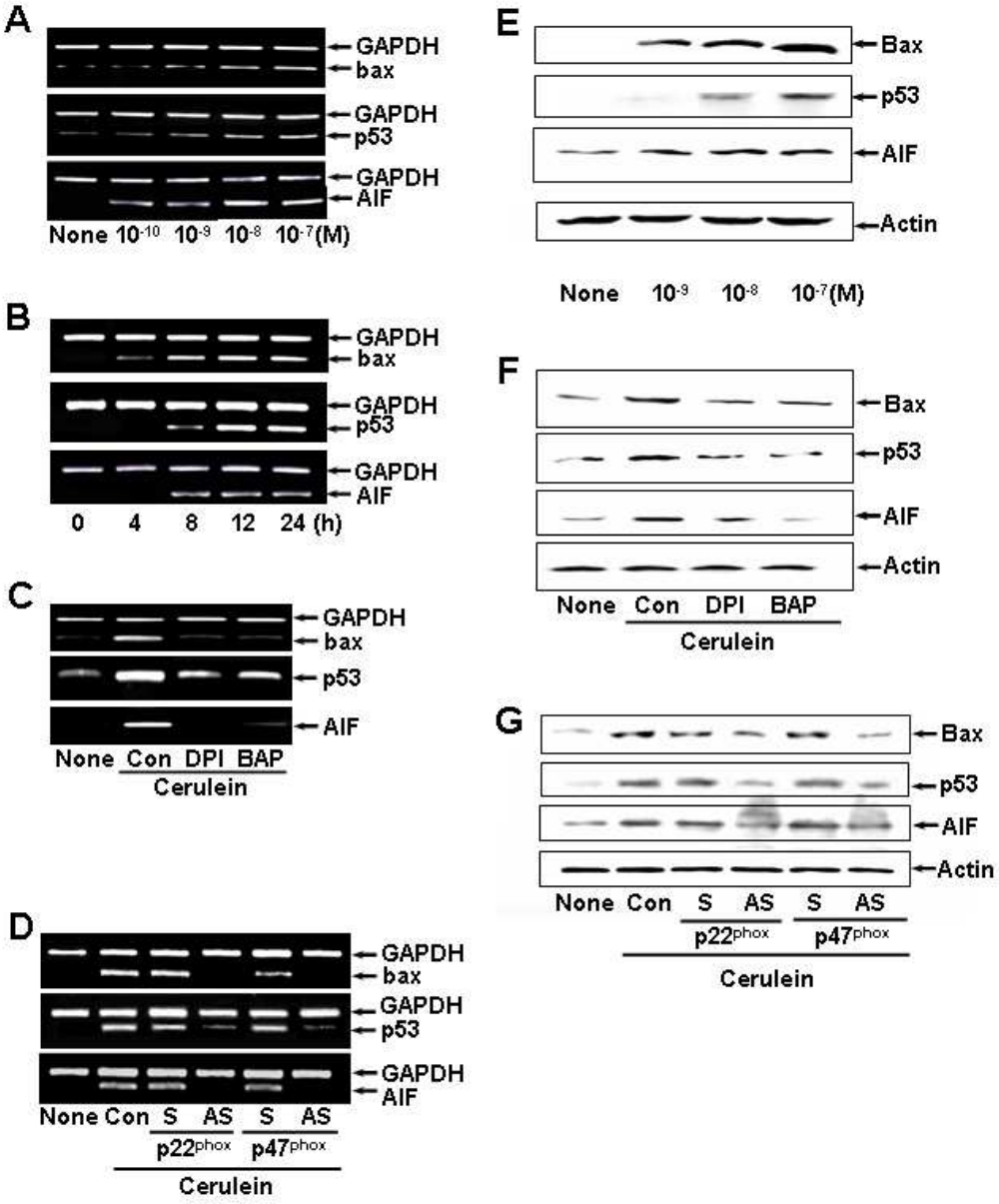

Figure 5. Effect of DPI and BAPTA and transfection with AS ODNs for $p22^{phox}$ and $p47^{phox}$ on cerulein-induced expressions of bax, p53 and AIF in AR42J cells. (A-G) The cells were cultured with various concentrations of

cerulein for 8 h (A) or with 10^{-8} M cerulein for various time periods (B). The cells treated with 10 μM DPI and BAPTA for 30 min (C) or transfected with S or AS ODNs (D) were cultured in the presence of 10^{-8} M cerulein for 8 h. mRNA expression of bax, p53 and AIF was determined by RT-PCR. The internal control (GAPDH) was co-amplified with bax, p53 and AIF. (A-G) The cells were cultured with various concentrations of cerulein for 24 h (E) or treated with 10 μM DPI and BAPTA for 30 min (F) or transfected with S or AS ODNs for $p22^{phox}$ or $p47^{phox}$ (G) and cultured in the presence of 10^{-8} M ceruelin for 24 h. Protein levels of bax, p53 and AIF in whole cell lysates were determined by western blotting. Actin was used a loading control. None, the cells cultured in the absence of cerulein; Con, the cellscultured in the presence of ceruelin; DPI and BAP, the cells cultured in the presence of cerulein and treated with DPI and BAPTA; S, the cells transfected with S ODN; AS, the cells transfected with AS ODN.

Ⅳ. DISCUSSION

Reactive oxygen species is regarded as an important factors in the pathogenesis of acute pancreatitis. An increase in intracellular Ca^{++} is involved in pancreatitis induced by CCK and its analog cerulein[35,36]. In this study, cerulein induced reactive oxygen species production and Ca^{++} oscillation in pancreatic acinar AR42J cells. Searching for the origin of reactive oxygen species production in pancreatic acinar cells, it was focused on a family of membrane-localized Nox1 homologous to the $gp91^{phox}$ component of the phagocytic NADPH oxidase. This study shows that NADPH oxidase subunits $gp91^{phox}$ homolog Nox1, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ are constitutively expressed in pancreatic acinar AR42J cells. Cerulein activated NADPH oxidase by inducing the translocation of cytosolic subunits $p47^{phox}$ and $p67^{phox}$ to the membrane in AR42J cells. It was determined here that cerulein-induced reactive oxygen species production could be derived from activated NADPH oxidase. This notion is further supported by the observation that cerulein-induced reactive oxygen species production was inhibited in the cells transfected with AS ODNs, but not with S ODNs for $p22^{phox}$ and $p47^{phox}$. NADPH oxidase inhibitor DPI, Ca^{++} chelator BAPTA inhibited cerulein-induced reactive oxygen species production and translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane. The results clearly demonstrate that the increase in intracellular Ca^{++} is involved in the activation of NADPH oxidase. Intracellular calcium activated NADPH oxidase in lymphocytes, endothelial cells and neutrophils[37-39]. NADPH oxidase-derived reactive oxygen species increased calcium signaling in endothelial cells and neutrophils[40,41]. The mechanism for calcium-dependent activation of NADPH oxidase is proposed as the conformational change of NADPH oxidase subunit Nox5 in lymphocytes[37]. Since it was demonstrated that the presence of NADPH oxidase in pancreatic acinar cells, further study should

be performed on the activation mechanism of NADPH oxidase by Ca^{++} oscillation in pancreatic acinar cells.

Reactive oxygen species produced during pancreatitis are mediators of apoptosis, which is involved in the pathogenesis and progression of acute pancreatitis[42,43]. In cerulein-induced pancreatitis, a high degree of reactive oxygen species production and apoptosis were observed in pancreatic acinar cells[44,45]. Therefore, it was focused on the possible relations among cerulein-induced activation of NADPH oxidase, Ca^{++} oscillation and apoptosis in the present study. Cerulein reduced cell viability and increased apoptotic indices, including DNA fragmentation, caspase 3 activity, and TUNEL staining in pancreatic acinar AR42J cells. Apoptotic cell death is mediated by stimulated caspase 3 activity, an inducer of apoptosis, and the accumulation of p53, a known signaling molecule that acts upstream of caspase 3[46]. The signaling pathways leading to caspase activation during apoptosis involve the release of cytochrome c and other apoptogenic factors from injured mitochondria. The release is mediated by the translocation of cytosolic bax, a pro-apoptotic member of the Bcl-2 family, to mitochondria in response to various apoptotic stimuli[47-49]. Several studies indicate that p53 regulates the function of bax and mitochondrial integrity[50,51]. p53 has a short half-life and the pro-apoptotic function of p53 is achieved by increased expression at the transcriptional level and by post-translational stabilization of the protein[52,53]. Genotoxic agents stimulate transcription of p53 via reactive oxygen species[54]. Oxidative stress induced up-regulation as well as mitochondrial translocation of bax during apoptosis in endothelial cells[55] and brain cortical cells[56]. Mitochondria play an important role in the regulation of apoptosis. When the mitochondrial pathway is triggered, pro-apoptotic protein, such as apoptosis-inducing factor (AIF), translocate from their mitochondrial locations into the cytosol[57]. In the

caspase-independent pathway, the cytosolic AIF translocates to the nucleus resulting in DNA fragmentation[58].

In the present study, cerulein induced the expressions of apoptotic genes such as bax, p53 and AIF in AR42J cells. Inhibition of NADPH oxidase-mediated reactive oxygen species production inhibited the expression of bax, p53 and AIF in AR42J cells. The results suggest that reactive oxygen species produced by activated NADPH oxidase up-regulate apototic genes and increase caspase 3 activity and thus induce apoptosis in cerulein-stimulated AR42J cells. Transfection of AS ODNs of NADPH oxidase suppressed cerulein-induced DNA fragmentation as compared to S ODNs for $p22^{phox}$ and $p47^{phox}$. Cerulein-induced up-regulation of bax, p53 and AIF, as well as an increase in caspase 3 activity and TUNEL staining, were inhibited in the cells treated with BAPTA or transfected with AS ODNs, but not with S ODNs for $p22^{phox}$ and $p47^{phox}$. These results indicate that an increase in intracellular Ca^{++} and activation of NADPH oxidase mediate apoptotic cell death mechanisms in cerulein-stimulated AR42J cells.

Ⅴ. CONCLUSIONS

The present study identified the presence and activation of NADPH oxidase, and characterized the role of NADPH oxidase in cerulein-induced apoptosis using molecular and biochemical approaches, the results are summarized as following

1. NADPH oxidase system is present in pancreatic acinar AR42J cells.
2. NADPH oxidase system generates reactive oxygen species in cerulein -treated AR42J cells
3. NADPH oxidase activation by cerulein is inhibited by DPI, BAPTA and AS ODNs of NADPH oxidase subunits
4. Reactive oxygen species generated by NADPH oxidase induces apoptosis by caspase 3 activation, DNA fragmentation.
5. Reactive oxygen species generated by NADPH oxidase upregulates bax, p53 and AIF.
6. DPI, BAPTA and AS ODNs of NADPH oxidase suppressed expression of bax, p53, AIF, caspase 3 activation and DNA fragmentation induced by cerulein.

In conclusion, reactive oxygen species production in pancreatic acinar cells during the course of cerulein-induced pancreatitis may be mediated by NADPH oxidase which may induce apoptosis of pancreatic acinar cells. These suggest that inhibition of NADPH oxidase as well as removing intracellular Ca^{++} might alleviate apoptosis in pancreatic acinar cells by inhibiting the expression of apoptosis-associated genes and caspase 3 activity.

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국문요약

췌장선세포에서 NADPH Oxidase에 유도된 Cerulein에 의한 세포고사

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세포고사는 췌장염에서 산화적 손상과 밀접한 관련이 있다. NADPH oxidase는 췌장 선세포에서 세포고사과정에서 활성산소의 중요한 원천으로서 고려되어지고 있다. 그러나, 췌장 선세포에서의 활성산소의 원천연구는 아직 정확히 알려진 바 없다. Cerulein은 급성, 부종형성을 보이는 췌장염을 빠르게 유발한다.

본 연구에서는 췌장 선세포에서 NADPH oxidase가 세포고사를 유도하는지를 보고자 하였다. Cerulein 자극에 의하여 NADPH oxidase의 세포질내 인자인 $p47^{phox}$와 $p67^{phox}$ 가 세포막으로 이동하여 결합함으로서, NADPH Oxidase가 활성화 되고, 이로 인해 활성산소가 생성되었다. Cerulein은 세포내 칼슘농도를 증가시고, 세포고사성 유전자인 bax, p53, AIF를 발현시키며, DNA 절단화, caspase 3 활성화, 생존 세포수를 감소시킨다. NADPH Oxidase 억제제인 DPI, 칼슘 킬레이터인 BAPTA, NADPH Oxidase의 $p47^{phox}$와 $p22^{phox}$ 인자의 AS ODN을 세포내 처치하여, cerulein에 의해 생성되는 활성산소, NADPH Oxidase의 활성화, 세포고사성 유전자 발현, DNA 절단화, caspase 3 활성화를 억제하였다. 이러한 결과로 NADPH Oxidase에 의해 생성되는 활성산소가 췌장 선세포에 세포고사를 유도함을 확인하였다.

핵심 되는 말 : NADPH Oxidase, 세포고사, 활성산소, 췌장 선세포