REVIEW

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Cerulein Pancreatitis: Oxidative Stress, Inflammation, and Apoptosis

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Cerulein pancreatitis is similar to human edematous pancreatitis, manifesting with dysregulation of digestive enzyme production and cytoplasmic vacuolization, the death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas. Reactive oxygen species are involved in nuclear factor-κB activation, cytokine expression, apoptosis and pathogenesis of pancreatitis. There is recent evidence that cerulein activates NADPH oxidase, which is a major source of reactive oxygen species during inflammation and apoptosis in pancreatic acinar cells. In addition, the Janus kinase/signal transducer and activator of transcription pathway has been suggested as being involved in inflammatory signaling in the pancreas. This review discusses the involvement of oxidative stress in inflammation and apoptosis in pancreatic acinar cells stimulated with cerulein as an *in vitro* model of pancreatitis. **(Gut and Liver 2008;2:74-80)**

Key Words: Cerulein; Pancreatitis; Inflammation; Apoptosis

INTRODUCTION

Acute pancreatitis is a multifactorial disease associated with the release of digestive enzymes to the pancreatic interstitium and to the systemic circulation with increased cytokine production and release, which can ultimately lead to deleterious local and systemic effects.[1-3] Evidence suggests that pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 act as mediators of the local and systemic manifestations of acute pancreatitis.[4-6] Activated pancreatic macrophages release IL-1β and TNF-α in response to local tissue damage. These cytokines act locally to aggravate acute pancreatitis and systemically with IL-6 to increase capillary permeability and promote leukocyte adherence and extravasation to cause multiple organ failure.[4-11]

Among several animal models of experimental pancreatitis that exhibit the biochemical, morphological, and pathophysiological similarities to various aspects of human pancreatitis, cerulein pancreatitis was shown to be one of the best-characterized and widely used experimental models.[12,13] Doses of a cholecystokinin (CCK) analog cerulein beyond those that cause the maximum pancreatic secretion of amylase and lipase[14,15] result in pancreatitis, which is characterized by a dysregulation of the production and secretion of digestive enzymes, particularly, the inhibition of pancreatic secretion and an elevation in their serum levels, cytoplasmic vacuolization and the death of acinar cells, edema formation, and an infiltration of inflammatory cells into the pancreas.[12,13,16]

Oxidative stress is regarded as a major pathogenic factor in acute pancreatitis.[17] 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.[18-20] Cerulein produces large amounts of reactive oxygen species (ROS) activates oxidant-sensitive nuclear transcription factor NF-κB and thus induces cytokine expression in freshly isolated pancreatic acinar cells without inflammatory cells *in vitro*.[21]

Apoptosis linked to oxidative stress has been shown in the pancreas of the patients with acute pancreatitis. Supraphysiologic concentrations of cerulein induce apoptosis in the rat pancreatic acinar AR42J cells.[22] High concentration of cerulein promoted the expression of pro-apoptotic gene bax and p53 and DNA fragmentation in AR42J cells, which was mediated by intracellular Ca^{++}.[23,24]

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Janus kinase/signal transducers and activators of transcription (Jak/Stat) signaling pathways have been reported to be involved not only in the immune response of numerous cytokines but also in the actions of primarily non-immune mediators such as growth factors and hormones. The Jak/Stat pathway is well known to be activated by the family of cytokine receptors and to mediate a wide variety of biological effects, such as immune response, differentiation, cell survival, proliferation, or oncogenesis.[25] Recently, it was reported that cerulein induced Jak2/Stat3 activation in pancreatic acinar cells.[26]

Therefore, the investigation of the relationship among oxidative stress, inflammation and apoptosis of pancreatic acinar cells stimulated with cerulein may provide clues for determining the pathophysiologic mechanism of pancreatitis.

CERULEIN AND RECEPTOR

Cerulein, a CCK analogue (pGlu-Gln-[Met^3]-CCK8, sulphated), acts on two distinct CCK receptor subtypes, namely CCK_1 (previously named CCK_A) and CCK_2 (previously named CCK_B) receptors.[27] CCK receptors are G-protein-coupled receptors initiating transient Ca^{++} oscillations by activating phospholipase C and induction of inositol triphosphate (IP_3)-dependent Ca^{++} release from endoplasmic reticulum in pancreatic acinar cells.[28,29] While CCK_1 receptors are highly discriminating with respect to the presence of a sulphate moiety on the tyrosine residue in the sequence of CCK and peptide analogues including cerulein, CCK_2 receptors (CCK2R) bind sulphated and non-sulphated analogues with much less difference in affinity.[30,31] CCK1R mediates for example the secretion of pancreatic digestive enzymes and may also be involved in the regulation of satiety and feeding behavior, while CCK2R stimulate gastric acid production.[32] Increase in blood flow induced by sulphated CCK-8 analogue and nonsulphated gastrin-17 in the gastric mucosa of rats have been demonstrated to be due to the activation of CCK2R.[33] CCK1R has a role in the exocrine effects of cerulein such as amylase secretion. The CCK receptors (CCKR) are seven-helix membrane-spanning receptor principally coupled to G proteins.[34] Initially, this type of receptor was implicated in the secretory effects of digestive peptide hormones. However, CCK2R is now recognized to mediate the mitogenic and anti-apoptotic effects of gastrin on gastrointestinal and pancreatic cells. In a pancreatic tumor cell line expressing the endogenous CCK2R, the proliferative effects of the CCK2R have been shown to be induced by the activation of the Jak2/Stat3 pathway by this receptor.[35]

ROS, NF-κB, AND CYTOKINE

ROS are the important mediators in the initiation and development of pancreatitis.[36-38] Cerulein induced NF-κB activation and cytokine expression, which may be mediated by ROS that are produced by NADPH oxidase in pancreatic acinar cells. ROS generated from cerulein is the main contributor to cytokine production in acinar cells by directly activating the oxidant-sensitive transcription factor, NF-κB (Fig. 1). ROS production may disturb the integrity of cytoskeleton[39,40] and induce mitochondrial dysfunction of the cells.[41,42] This hypothesis was supported by our previous proteomic analysis of cerulein-stimulated pancreatic acinar cells.[43,44] The expression of cytoskeletal protein, tubulin beta chain and mitochondrial ATP synthase (beta chain precursor and subunit D) was induced by cerulein in pancreatic acinar cells. *In vivo* studies suggest that the intrapancreatic IL-1β, IL-6 and TNF-α are rapidly and coordinately increased during the early stages of acute pancreatitis.[45,46] In the experimental pancreatitis *in vivo*, the inflammatory cytokines are produced from the infiltrating inflammatory cells. However, our previous studies showed the production of inflammatory cytokines in pancreatic acinar cells by stimulation with secretagogue[21] and PMA-primed neutrophils.[47]

NF-κB belongs to the Rel family of transcription factors that regulate the activation of the cellular stress-related and early-response genes such as cytokines, growth factors, adhesion molecules, and acute-phase proteins.[48,49] The human Rel proteins include NF-κB subunit p50 (p50), p52, Rel (c-Rel), Rel A (p65) and Rel B. The classic form of the activated NF-κB is a heterodimer consisting one p50 and one p65 subunit. The induction of a nuclear translocation of a p50/p65 NF-κB heterodimer and a p50 NF-κB homodimer was previously identified in pancreatic acinar cells exposed to stimulated neutrophils.[47]

Rebamipide is an anti-ulcer agent that scavenges ROS[50] and inhibits the production of superoxide in the activated neutrophils.[51] Rebamipide treatment at a concentration that efficiently inhibited NF-κB activation, suppressed IL-6 expression induced by cerulein. These results suggest that antioxidant might inhibit the expressions of the inflammatory cytokines by suppressing NF-κB activation and/or the NF-κB interaction with the upstream regulatory binding site of the cytokines such as IL-6. ROS production is mediated by NADPH oxidase, which induces inflammation of pancreatic acinar cells during pancreatitis. Therefore, inhibition of NF-κB activation and cytokine expression by scavenging ROS and inhibi-

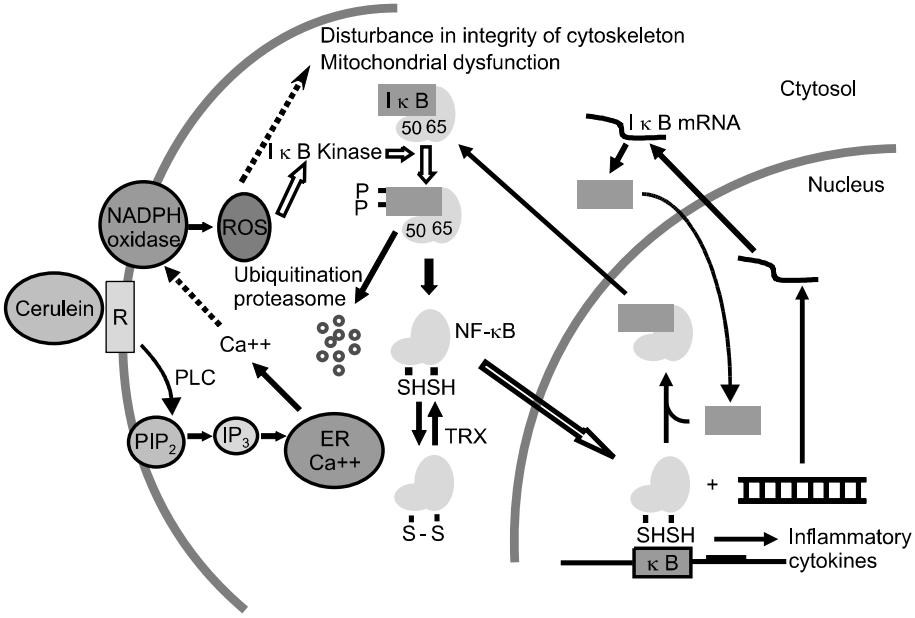

Fig. 1. Proposed mechanism of how cerulein induces nuclear factor (NF)-κB activation and cytokine expression in pancreatic acinar cells. Cerulein binds the CCK receptor (R), which is a G-protein-coupled receptor. Ligand-receptor binding initiates transient Ca^{++} oscillations by activating phospholipase C (PLC) and the induction of inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{++} release from the endoplasmic reticulum (ER) in pancreatic acinar cells. Ca^{++} can activate NADPH oxidase, which produces reactive oxygen species (ROS) that induce the activation of IκB kinase, which in turn phosphorylates IκB in the cytosol. IκB is an inhibitory subunit bound to NF-κB, a p65/p50 heterodimer in the cytosol. Phosphorylated IκB is ubiquitinated and degraded in a proteasome-dependent manner. NF-κB translocates to the nucleus and regulates cytokine expression. NF-κB is recycled after binding to IκB in the nucleus and transported to the cytosol. Therefore, ROS generated from cerulein appears to be the main contributor to cytokine production in acinar cells by directly activating the oxidant-sensitive transcription factor, NF-κB. ROS can disturb the integrity of the cytoskeleton and induce mitochondrial dysfunction in the cells. In the cytosol, the disulfide form of NF-κB is reduced to NF-κB by theoredoxin (TRX). PIP2, phosphatidylinositol 4, 5-bisphosphate; NF-κB subunit p50; 65, NF-κB subunit p65.

tion of NADPH oxidase might alleviate the inflammatory response in pancreatic acinar cells.

ROS, Ca^{++} AND NADPH OXIDASE

Once produced, ROS can act as a molecular trigger of pancreatitis. They can attack the biological membranes directly and trigger the accumulation of neutrophil and their adherence to the capillary wall.[52,53] Therefore, it is likely that ROS play a central role in perpetuating the pancreatic inflammation and the development of extrapancreatic complication.[54] Previously we demonstrated that oxidative stress induced upregulation of inflammatory cytokines in pancreatic acinar cells.[21]

There are increasing evidences that a major source of ROS during inflammation is NADPH oxidase in leukemic cells and HL-60 cells.[55-57] In phagocytic cells, the NADPH oxidase is composed of the membrane-bound subunits $gp91^{phox}$ and $p22^{phox}$ and the cytosolic subunits $p67^{phox}$ and $p47^{phox}$. Upon activation of the enzyme, a complex of the cytosolic subunits translocates to the membrane and facilitates NADPH-dependent formation of superoxide (O_2^-), which in turn gives rise to the production of other secondary ROS (H_2O_2). Recently, it was suggested that the source of ROS is the NADPH oxidase in the stimulated neutrophils in cerulein pancreatitis *in vivo*.[58] Our previous study showed that cerulein produces large amounts of ROS, activates oxidant-sensitive nuclear transcription factor NF-κB and thus induces cytokine expression in freshly isolated pancreatic acinar cells without inflammatory cells *in vitro*.[21] Since ROS are mainly produced in the activated inflammatory cells during inflammation *in vivo*, relatively small amounts of ROS which may be produced in pancreatic acinar cells could be considered as negligible production. However, since ROS can act as chemoattractants for inflammatory cells,[52,53] inhibition of ROS production in pancreatic acinar cells might be the first step to prevent the inflammatory process by inflammatory cells by inhibiting the infiltration of inflammatory cells into pancreas.

NADPH oxidase subunits Nox1, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ are expressed in pancreatic acinar AR42J cells. Cerulein

stimulated NADPH oxidase activity, and induced the translocation of cytosolic subunits $p47^{phox}$ and $p67^{phox}$ to the membrane in AR42J cells.[24] Ca^{++} may be involved in the translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane since a Ca^{++} chelator BAPTA-AM, completely blocked the translocation of cytosolic subunits of NADPH oxidase to the membrane.[24] Since cerulein-evoked Ca^{++} oscillation was not affected by an inhibitor of NADPH oxidase diphenyleneiodonium (DPI), DPI may directly inhibit the translocation of cytosolic subunits of NADPH oxidase to the membrane without disturbing Ca^{++} influx caused by cerulein in pancreatic acinar cells. DPI changes the redox state of cells not only by modulation of the level of ROS production, but also by induction of the efflux of glutathione to extracellular medium via specific transport channel in T24 cells.[59] Further study should be performed on the inhibitory mechanism of DPI on the activation of NADPH oxidase in pancreatic acinar cells.

CYTOKINE AND JAK/STAT PATHWAY

Although Stat proteins were discovered during analysis of interferon signaling, recent studies have revealed that Stat signaling can account for various cellular responses to several cytokines, growth factors, and hormones.[60,61] The Jak/Stat pathway is well known to be activated by the family of cytokine receptors and to mediate a wide variety of biological effects, such as immune response, differentiation, cell survival, proliferation, or oncogenesis.[25] The mechanism of Jak activation is well known for the cytokine receptors. Ligand binding induces oligomerization of the receptors' subunits, constitutively associated with Jaks, and a transphosphorylation of the tyrosine kinases. Activated Jaks in turn phosphorylate the receptor that recruits the Stat protein.[25]

Cerulein mediates the secretory and inflammatory events through CCK receptors. Therefore, cerulein-induced Jak2/Stat3 activation might be mediated by CCK receptor(s) even though we could not conclude which type of receptor is involved in this signaling. AG 490 has been known to inhibit the activation of Jak2[62-67] and Stat3[68-72] in various cells and tissues In cerulein pancreatitis model, AG 490 effectively inhibited Jak2 phosphorylation. Likewise, Stat3 phosphorylation was blocked by AG 490. Presumably it may be caused by inactivation of Jak2, which would be required to catalyze the event of Stat phosphorylation. These data provide a strong evidence to indicate that cerulein activates Stat3 through the activation of Jak2. Interestingly, AG490 inhibited phosphorylation of Jak2 and Stat3 and rapidly reduced the expression of IL-1β, regulators of immune responses for acute pancreatitis. AG490 may negatively regulate inflammation in the episodes of acute pancreatitis.

Further study should be performed either by using the cells transfected with dominant negative gene of Stat3 or by examining at the promoter level by using cytokine such as IL-1β promoter reporter construct to determine the transcriptional regulation of cytokines by Stat3. In addition, the possible involvement of Jak3 in signaling for cytokine expression in pancreatic acinar cells should be determined since recent studies showed that AG 490 achieved inhibition of Jak3 and its downstream substrates Stat5a/b *in vivo*.[73,74]

ROS AND APOPTOSIS

Apoptosis linked to oxidative stress has been shown in the pancreas of the patients with acute pancreatitis. Supraphysiologic concentrations of cerulein induce apoptosis in the rat pancreatic acinar AR42J cells.[22] High concentration of cerulein promoted the expression of pro-apoptotic gene bax and p53 and DNA fragmentation in AR42J cells, which was mediated by intracellular Ca^{++}.[23,24]

There is a possible relationship between cerulein-induced activation of NADPH oxidase and apoptosis because ROS produced during pancreatitis are important mediators of apoptosis.[75,76] In cerulein-induced pancreatitis, a high degree of ROS production and apoptosis were observed in pancreatic acinar cells.[77,78] Cerulein induced increases in apoptotic indices including the expression of apoptosis-inducing factor (AIF), DNA fragmentation, TUNEL staining, and caspase-3 activity in AR42J cells.[79] Apoptotic cell death is contributed by the activation of caspase-3 and the accumulation of p53, a known signaling molecule that acts upstream of caspase-3.[80] p53 is induced in cerulein-stimulated pancreatic acinar cells.[23] The signaling pathways leading to caspase activation during apoptosis involves the release of cytochrome c and other apoptogenic factors from injured mitochondria. AIF is conserved mitochondrial protein that is released into the cytoplasm and nucleus during apoptotic stimuli, inducing chromatin condensation and DNA fragmentation, in human and rodent cells. AIF induces apoptotic changes in purified nuclei, even in the presence of caspase inhibitors,[81,82] suggesting the mediator function of AIF for apoptosis in a caspase- independent fashion. Caspase-3 activation and AIF expression were induced by cerulein in pancreatic acinar AR42J cells, which suggests that cerulein affects mitochondria to cause the transcriptional activation and regulation of caspase-3 and thus induces the expression of apoptotic gene such as AIF.

Cerulein-induced up-regulation of AIF with an increase

in DNA fragmentation, TUNEL staining, and caspase-3 activity were inhibited in the cells treated with NADPH oxidase inhibitor DPI.[79] The study suggests that the activation of NADPH oxidase and an increase in ROS production mediate apoptotic cell death in caspase-dependent and caspase-independent manner in pancreatic acinar cells stimulated with cerulein.

CONCLUSION

ROS production in pancreatic acinar cells during pancreatitis may be mediated by NADPH oxidase, which induces cytokine expression and apoptosis of pancreatic acinar cells. ROS mediates NF-κB activation and Jak/Stat pathway, which regulates inflammatory gene and apoptotic gene expression in pancreatic acinar cells. Thus, the activation of NADPH oxidase, NF-κB and Jak2/Stat3 seems to be the molecular mechanisms underlying the pathogenesis of acute pancreatitis. Inhibition of NADPH oxidase, NF--κB and Jak/Stat may alleviate inflammation and apoptosis of pancreatic acinar cells during pancreatitis by suppressing the expression of inflammatory cytokines and apoptosis-associated gene and caspase-3 activity. Therefore, NADPH oxidase, NF-κB and Jak/Stat may serve as the potential therapeutic targets in the development of new drugs in the treatment of acute pancreatitis.

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