Altered Gene Expression in Cerulein-Stimulated Pancreatic Acinar Cells: Pathologic Mechanism of Acute Pancreatitis

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Acute pancreatitis is a multifactorial disease associated with the premature activation of digestive enzymes. The genes expressed in pancreatic acinar cells determine the severity of the disease. The present study determined the differentially expressed genes in pancreatic acinar cells treated with cerulein as an in vitro model of acute pancreatitis. Pancreatic acinar cells were stimulated with 10^-8 M cerulein for 4 h, and genes with altered expression were identified using a cDNA microarray for 4,000 rat genes and validated by real-time PCR. These genes showed a 2.5-fold or higher increase with cerulein: lithostatin, guanylate cyclase, myosin light chain kinase 2, cathepsin C, progestin-induced protein, and pancreatic trypsin 2. Stathin 1 and ribosomal protein S13 showed a 2.5-fold or higher decreases in expression. Real-time PCR analysis showed time-dependent alterations of these genes. Using commercially available antibodies specific for guanylate cyclase, myosin light chain kinase 2, and cathepsin C, a time-dependent increase in these proteins was observed by Western blotting. Thus, disturbances in proliferation, differentiation, cytoskeleton arrangement, enzyme activity, and secretion may be underlying mechanisms of acute pancreatitis.

Key Words: Cerulein, Pancreatitis, Acinar cells, DNA microarray

INTRODUCTION

Acute pancreatitis is a multifactorial disease associated with the release of digestive enzymes to the pancreatic interstitial and the systemic circulation, as well as with increased cytokine production and release (Schoenberg et al., 1990). Cerulein pancreatitis is one of the best-characterized animal models of experimental pancreatitis and exhibits biochemical, morphological, and pathophysiological similarities to various aspects of human pancreatitis (Willemer et al., 1992). Doses of CCK or cerulein, a cholecystokinin (CCK) analog, beyond those that cause the maximum pancreatic secretion of amylase and lipase (Jensen et al., 1989; Sato et al., 1989) result in pancreatitis. The disease is characterized by dysregulation of the production and secretion of digestive enzymes, particularly the inhibition of pancreatic secretion and an elevation in serum levels, as well as cytoplasmic vacuolization, the death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas (Schoenberg et al., 1990; Lerc and Adler, 1995). The key events appear to be a premature, intra-pancreatic activation of digestive enzyme granules, but the earliest events that trigger acute pancreatitis are unclear.

Previously we showed that intravenous infusion of cerulein induces hyperamylasemia, inflammation, edema formation, and high production of lipid peroxide, an index of oxidative cell damage, in rat pancreas (Choi et al., 1985). Cytokine expression and secretory responses using CCK were determined in freshly isolated pancreatic acinar cells. Maximum stimulation of digestive enzymes and cytokines were achieved with 10^-8 M CCK (Kim et al., 1996) and 10^-9 M CCK (Yu et al., 2002; Yu et al., 2005; Ju et al., 2006; Yu et al., 2006), respectively. Stress or injury in acinar cells induces the activation of a signaling mechanisms and intracellular activation of digestive enzymes. These early events are translated into long-term responses by the expression of specific genes; these genes determine the ultimate severity of pancreatitis. We previously reported that NF-κB, AP-1, and mitogen-activated protein kinase are activated early and induce the expression of cytokines in cerulein-stimulated pancreatic acinar cells (Lee et al., 2003; Ju et al., 2006). We previously reported that cerulein (10^-8 M) induces the activation of Ras, NF-κB, AP-1, mitogen-activated protein kinase (p38, ERK, JNK), and JAK2/STAT3 to induce expression of cytokines (IL-6, IL-8, IL-1β, TGF-β) and vascular endothelial growth factor-D (VEGF-D) in pancreatic acinar AR42J cells (Yu et al., 2002; Lee et al., 2003; Yu et al., 2005; Ju et al., 2006; Yu et al., 2006; Lee et al., 2007; Yu et al., 2008). In addition, neutrophils activated pancreatic acinar cells to induce cytokine expression via NF-κB activation (Kim et al., 1999). Gene chip analysis using 8,000 genes for rat pancre-
atic acinar cells isolated from in vivo pancreatitis animal models using cerulein and taurocholate administration showed fifteen differentially expressed genes, including the pro-inflammatory mediators, MCP-1, IL-6, and gro-α as well as the transcription factor, EGR-1 (Ji et al., 2003). Cerulein (Grady et al., 1996) and taurocholate (Kim et al., 2002) activate stress kinases, including Jun kinase. Here we determined the gene expression changes after cerulein treatment of pancreatic acinar cells to understand of the pathophysiology of acute pancreatitis. Pancreatic acinar AR42J cells were stimulated with 10^{-8} M cerulein for 4 h. Alterations in gene expression were identified using a cDNA microarray for 4,000 rat genes and validated by real-time RT-PCR. Western blot analysis was performed to confirm changes in protein expression.

**METHODS**

**Cell culture**

Rat pancreatic acinar AR42J cells (pancreatoma, ATCC CRL 1492) were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum (GIBCO-BRL, Grand Island, New York, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) under 44 mM sodium bicarbonate and 10% CO2 environment as recommended (Freshney et al., 1994).

**Experimental protocol**

Acinar cells were plated at 2×10^6/ml in a 100-mm culture plate (Falcon 3,047, Becton Dickinson Labware, Lincoln Park, New Jersey, USA) and allowed to attach for 12 h. The cells were treated with cerulein (10^{-8} M) and cultured for 4 h. The dose and duration of cerulein treatment induced activation of NF-κB and Janus kinase (Jak)/signal transducer and activator of transcription (Stat), inflammatory cytokine expression, and hypersecretion (Kim et al., 1996; Yu et al., 2002; Yu et al., 2005; Ju et al., 2006; Yu et al., 2006).

**Probe preparation and cDNA microarray**

Total RNA was prepared from cells stimulated with or without cerulein for 4 h by guanidine thiocyanate extraction method (Chomczynski and Sacchi, 1987). Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK) was incorporated when 50 μg total RNA was reverse transcribed into cDNA and primed with oligo (dT) primer. A cDNA probe from cells cultured without cerulein was incorporated with Cy3 while that from the cells with cerulein was incorporated with Cy5. Cy3- or Cy5-labeled cDNA probe was purified with Chroma-spin 100 columns (Clontech Laboratories, Inc., Palo Alto, California, USA) following the manufacturer’s instructions. A rat gene chip (4,000 genes and 2 housekeeping genes; Geno Check, Ansan, Kyung-ki-do, Korea. http://www.genochek.com) cDNA microarray was prehybridized at room temperature for 2 h in prehybridization buffer (6× SSC, 0.2% SDS, 5× Denhardt’s solution and 1 mg/ml salmon sperm DNA). Different fluorescent-labeled cDNA probes were mixed and applied on the microarray following incubation at 62°C for 16 h under humidified conditions. The fluorescent images of the hybridized microarray were scanned with a fluorescent laser confocal slide scanner (GMS 418 array, Wallac Laboratories, Atlanta, Georgia, USA). Images and quantitative gene expression levels were analyzed by ImaGene® II (BioDiscovery, Inc., Marina de Rey, California, USA).

**Real-time PCR analysis**

Real-time PCR analysis was performed with a SYBR® Green Realtime PCR master mix kit (Toyobo, Osaka, Japan).

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene Description</th>
<th>Primer sequences</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Regeneration protein, lithostatin (Pancreatic stone protein)</td>
<td>(F) ACACCTTGTATCTGTGCTCAATGTAG</td>
<td>Cy5/Cy3</td>
</tr>
<tr>
<td>2</td>
<td>Guanylate cyclase 2C</td>
<td>(F) GTGACATTGTGGCGTTTTCCAG</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Myosin light chain kinase 2</td>
<td>(F) CTCAGCAAGCAGGACATCAG</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Cathepsin C</td>
<td>(F) TCAAGCCCCAATCCTGAGTC</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Progestin-induced protein</td>
<td>(F) CTTGACAAAAACACAGAAGCA</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Pancreatic trypsin 2</td>
<td>(F) AGCATCGGCATCTGAACACTT</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Stathin 1</td>
<td>(F) FGGAGGATACACCTGCAAAGA</td>
<td>Cy3/Cy5</td>
</tr>
<tr>
<td>2</td>
<td>Ribosomal protein 513</td>
<td>(F) AAGGAGCCCTTCCCGGGAGAGGA</td>
<td></td>
</tr>
</tbody>
</table>

Gene sequences used as forward (F) and reverse (R) primers for real-time PCR; fold is the ratio of Cy5/Cy3 for up-regulated genes and Cy3/Cy5 for down-regulated genes.
using a Roche Light cycler (Roche Molecular Biochemicals, Mannheim, Germany). Two micrograms of total RNA were reverse transcribed using the M-MLV reverse transcription system (Promega, Madison, Wisconsin, USA) in 20 \( \mu l \) in a thermocycler (Applied Biosystems GeneAmp PCR System 9700, Foster City, USA). Then 1/10 volume of each RT reaction was amplified with SYBR Green master mix (Toyobo, Osaka, Japan) containing 10 \( \mu M \) of customized primers and GAPDH (Table 1); the reactions were measured in a Light Cycler real-time PCR detection system (Roche Molecular Biochemicals). PCR was conducted using the following cycling conditions: pre-incubation and denaturation at 95°C for 10 min, followed by amplification with 40 cycles of: denaturation at 95°C for 30 s with a thermal ramp rate of 20°C/s; annealing at 60°C for 5 s with a thermal ramp rate of 20°C/s; amplification at 72°C for 30 s with a thermal ramp rate of 20°C/s. The mRNA levels of target genes were normalized to GAPDH. The primers used in real-time PCR were listed in Table 1. The primers for GAPDH were forward, ACCACAGTCCATGGCATCAC and reverse, TCCACACCCGTGTTGCTGTA, giving a 460 bp PCR product.

**Western blot analysis for guanylate cyclase, myosin light chain kinase 2, and cathepsin C**

Cells were treated with cerulein (10 \( -8 \) M) and cultured for 6 h. The cells were harvested and lysed in Tris-HCl buffer (pH 7.4) containing 0.5% Triton X-100 and a protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, Indiana, USA) for the determinations of guanylate cyclase, myosin light chain kinase 2, and cathepsin C. The protein concentration of each sample was determined by Bradford assay (Bio-Rad laboratories, Hercules, CA, USA). Protein (50 \( \mu g \)) was separated on 8∼10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, IL) by electroblotting. The transfer of protein and equality of loading in all lanes was verified using reversible staining with Ponceau S. The membranes were blocked using 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.15% Tween 20) for 3 h at room temperature. The proteins were detected with antibodies for guanylate cyclase (1 : 1,000; sc-34428), myosin light chain kinase 2, and cathepsin C (1 : 1,000; sc-12450), cathepsin C (1 : 1,000; sc-74590) and actin (1 : 1,000; sc-1615) (all from Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS-T containing 5% dry milk, and incubated at 4°C overnight. After washing in TBS-T, the immunoreactive proteins were visualized using goat anti-rabbit and donkey anti-mouse secondary antibodies conjugated to horse radish peroxidase, followed by enhanced chemiluminescence (Amersham). Actin was used as a loading control.

**RESULTS**

**cDNA microarray**

To characterize changes in mRNA expression induced by cerulein, rat pancreatic acinar AR42J cells were stimulated with or without cerulein for 4 h, and then total RNA was extracted. cDNA prepared from total RNA were labeled with Cy5 fluorochrome (with cerulein, red) and Cy3 (without cerulein, green) (Fig. 1A) to indicate relative expression levels. A Cy5/Cy3 ratio of 1 indicates identical expression.

**Up- and down-regulated genes**

Most genes showed only small differences after cerulein stimulation, indicated by Cy5/Cy3 ratios between 2 and 0.5. We extracted genes with expression levels more than 2.5 fold higher or lower after cerulein (Table 1). Two house-keeping genes, GAPDH and tubulin, were used as internal controls to correct for mRNA abundance. These genes showed similar intensities of signals in hybridized microarray, and the mean of those control genes were used to normalize the target genes. Cerulein elevated the expression of lithostatin, guanylate cyclase, myosin light chain kinase 2, cathepsin C, progestin-induced protein, and pancreatic trypsin 2. Cerulein down-regulated stathin 1 and ribosomal protein S13. These genes have a variety of functions, including cell proliferation and differentiation (lithostatin, progestin-induced protein, stathin 1, guanylate cyclase 2, trypsin 2), carcinogenesis (lithostatin, progestin-
induced protein, ribosomal protein S13, trypsin 2), enzyme activity and secretion (myosin light chain kinase 2, cathepsin, trypsin 2, guanylate cyclase 2), and cytoskeleton arrangement (myosin light chain kinase 2, stathin 1) (Fig. 1B).

Real-time PCR analysis

To confirm these changes in gene expression, cells were stimulated with cerulein for up to 4 h. Real-time PCR analysis showed a time-dependent increase in 6 genes (lithostatin, guanylate cyclase, myosin light chain kinase 2, cathepsin C, progestin-induced protein, and pancreatic trypsin 2) and a time-dependent decrease in 2 genes (stathin 1 and ribosomal protein S13) (Fig. 2). At 4 h, cerulein increased mRNA levels of lithostatin, guanylate cyclase, and myosin light chain kinase 2 almost 10-fold, higher than in microarray analysis. Cerulein increased cathepsin C, progestin-induced protein, and pancreatic trypsin 2 about 2.5-fold. Cerulein decreased stathin 1 and ribosomal protein S13 levels about 2.5 fold, similar to changes in the microarray.

Western blot analysis of guanylate cyclase, myosin light chain kinase 2, and cathepsin C

To confirm changes in protein expression, Western blot analysis was performed using commercially available antibodies (Fig. 3). Cells were cultured in the presence of cerulein for 6 h, harvested, and lysed. Cerulein increased lev-
a trypsin-sensitive cleavage site that is conserved in several reg protein, expressed in the endocrine compartment of the species. Tryptic cleavage produces the amino-terminal de-

Lithostatin is a 144-residue protein that is identical to the Lithostatin is the only protein secreted from the pancreas and lipase, and hormones, including insulin and glucagon. The pancreas secretes primarily two types of metabol-

in cerulein-induced pancreatitis. 

volved in the pathogenesis of edema and vacuole formation intracellular calcium and calpain activation may be in-

Adler, 1995). Cerulein-induced acute pancreatitis shows pro-

cathepsin C and trypsin 2 (Willemer et al., 1992; Lerch and 

of inflammatory cells into the pancreas (Willemer et al., 

of pancreatic acinar cells, indicating a potential connection between pancreatitis and the development of pancreatic cancer. 

Guanylate cyclase (GC) has two forms, soluble and partic-

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characteristic events of pancreatitis include the dysregulation of digestive enzyme production, cytoplasmic vacuolization, the death of acinar cells, edema formation, and an infiltration of inflammatory cells into the pancreas (Willemer et al., 1992; Lerch and Adler, 1995). The premature activation of digestive enzymes is indicated here as the up-regulation of cathepsin C and trypsin 2 (Willemer et al., 1992; Lerch and Adler, 1995). Cerulein-induced acute pancreatitis shows pro-

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et al., 2006). This progestin effect is mediated by progeste-
one receptors in stromal cells (Kurita et al., 2000). In con-
trast, progestins control mammary gland tumorigenesis af-
ter binding to progesterone receptors (Carnevale et al., 2007).
The progesterone receptor functions either as a trans-
scription factor or as a signaling activator in a breast cancer cell 
line (Carnevale et al., 2007). Progestin initiates Wnt-beta-
catenin signaling for proliferation and differentiation in rat 
uterine stromal cells (Rider et al., 2006). A progesterone 
agonist prevented BRCAl-mediated mammary tumori-
genesis in mice, suggesting anti-progesterone treatment 
may be effect for breast cancer prevention in individuals 
with BRCAl mutation (Poole et al., 2006). Treatment of 
progesterone stimulates cell proliferation within the islets 
of Langerhans in rats (Nieuwenhuizen et al., 1999). There-
fore, cerulein-induced increases in progestin may increase 
cell proliferation and relate pancreatitis and pancreatic 
cancer. 
The pancreas is an important endocrine and exocrine se-
cretory organ in mammals. Many digestive enzymes are 
synthesized in pancreatic acinar cells (Gorelick and Otani, 
1999). Under normal conditions, these enzymes remain in-
active in isolated zymogen granules inside pancreatic aci-
ar cells (Kassell and Kay, 1973) and only become active 
after entering the small intestine. The activation of a key 
zymogen enzyme in zymogen granules, trypsin, requires proteolytic 
activation by cleavage of the propeptide, which can be com-
pleted in the duodenum through activation by the brush 
border endoprotease, enteropeptidase (Kassell and Kay, 
1973). This initial activation of trypsin can further activate 
trypsinogen into active trypsin and other zymogens, such as 
chymotrypsinogen, proteolastase, and prophospholipase 
to their active states (Gorelick and Otani, 1999). During 
acute pancreatitis, these digestive enzymes are pre-
mately activated before leaving the pancreas and start 
digesting the pancreas to lead to acute pancreatitis (Steer, 
1999). Lithostatin contains a trypsin-sensitive site, and up-regulated trypsin 2 may cleave lithostatin to tryptic 
cleavage products, including a carboxy-terminal peptide of 
133 amino acids. In addition, trypsin is activated in pancre-
atic cancer cells (Chen et al., 2009) to stimulate growth and 
adhesiveness in an autocrine manner (Giancotti and 
Mainiero, 1994). The stage and type of carcinoma is related 
to the level of trypsin associated with cell invasion and 
extracellular matrix degradation (Koivunen et al., 1991; Walz 
and Fenton, 1994). Therefore, up-regulation of trypsin 2 in 
pancreatic acinar cells may contribute to the development 
of pancreatic cancer. 
Stathmin, a major microtubule-destabilizing protein, is 
down-regulated by cerulein. In general, stathmin interacts 
directly with soluble tubulin to form a complex that seque-
ters free tubulin and impedes the polymerization of micro-
tubules (Belmont and Mitchison, 1996). The depolymerizing 
activity of stathmin is turned off upon its phosphorylation 
during the onset of mitosis, leading to formation of the mi-
otic spindle. Conversely, reactivation of stathmin by de-
phosphorylation is necessary before the cells exit mitosis 
and enter a new interphase (Rubin and Atweh, 2004). In 
addition to its role in mitosis and cell cycle progression, 
stathmin is also involved in diverse cell functions, such as 
cell proliferation and differentiation (Larsson et al., 1995). 
Stathmin is expressed in actively proliferating cells (Iancu 
et al., 2001), including liver regeneration after partial hepa-
tectomy (Koppel et al., 1993) and hepatic ischemia-re-
perfusion injury (Barone et al., 2005), whereas its expre-
sion is dramatically decreased upon the induction of differ-
etiation and cessation of proliferation of leukemia cells 
(Melhem et al., 1991), and in the later stages of mega-
karyocyte maturation (Rubin et al., 2003). Stathmin is 
abundantly expressed in fetal liver, but dramatically de-
creased in adult liver (Bièche et al., 2003). Cerulein may 
differentially cinditize and cessation of proliferation by de-
creasing stathmin expression, but cerulein also increased 
lithostatin and progestin, two genes that increase cell pro-
iferation, indicating an imbalance between cell proliferation 
and differentiation. 
Ribosomal protein S13 is found in the head region of the 
small subunit, where it interacts with the central protub-
erance of the large ribosomal subunit and with the P 
site-bound tRNA through its extended C terminus (Cukras 
and Green, 2005; Noller et al., 2005). The bridging inter-
actions between the large and small subunits are dynamic 
and are critical in the molecular motions of the translation 
cycle. S13 provides a direct link between the tRNA-binding 
site and the movements in the head of the small subunit 
seen during translocation, thereby providing signal trans-
duction (Cukras and Green, 2005). The expression level of 
ribosomal protein S13 was lower in NKT cell lymphoma 
than in normal lymphocytes, indicating that it plays a role in 
the development of the NKT cell lymphoma (Yang et al., 
2006). Cerulein decreases S13 expression, indicating 
disturbances in translation or signal transduction may be 
involved in the pathogenesis and/or development of 
pancreatitis. 
In our previous studies, cerulein induced the expression of 
cytokines (IL-6, IL-8, IL-1α, TGF-β) and VEGF-D by the 
activation of NF-κB, AP-1, Mitogen-activated protein kin-
ases, and Jak2/Stat3 in pancreatic acinar AR42J cells (Yu 
et al., 2002; Lee et al., 2003; Yu et al., 2005; Ju et al., 2006; 
Yu et al., 2006; Lee et al., 2007; Yu et al., 2008). Here, cer-
ulein up-regulated 6 genes (lithostatin, guanylate cyclase, 
myosin light chain kinase 2, cathepsin C, progestin-induced 
protein, pancreatic trypsin 2) and down-regulated 2 genes 
(stathmin 1, ribosomal protein S13) that are related to pro-
liferation, differentiation, carcinogenesis, cytoskeletal ar-
angement, enzyme activity, and secretion. These changes 
may accompany inflammatory events. Since lithostatin, 
progestin-induced protein, trypsin, and ribosomal protein 
S13 are involved in carcinogenesis, the relationship be-
tween pancreatitis and the development of pancreatic can-
cer requires further study. Additional in vivo studies should 
also be performed for comparison to human pathophysiology. 

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