Apoptotic Mechanisms of Peroxisome Proliferator–Activated Receptor-γ Activation in Acinar Cells During Acute Pancreatitis

Ping Xu, MD, PhD,* Xiao-Li Lou, MD,† and Cheng Chen, MD*  

Objective: The objective of this study was to determine the mechanism by which activation of peroxisome proliferator–activated receptor-γ promotes apoptosis of acinar cells in pancreatitis.  

Methods: AR42J cells pretreated with the peroxisome proliferator–activated receptor-γ agonist pioglitazone were activated by cerulein as an in vitro model of acute pancreatitis. Inflammatory cytokines and amylase were detected by enzyme-linked immunosorbent assay. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell apoptosis was measured by flow cytometry and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining. Activity of caspases was determined. Bax and Bcl-2 levels were assayed by Western blot.  

Results: Cytokines, amylase, and cellular proliferation decreased in pioglitazone-pretreated cells. Pioglitazone increased the activity of caspases 3, 8, and 9 in cerulin-activated AR42J cells as well as in the pancreas of rats 3 hours after induction of severe acute pancreatitis. Acinar cell apoptosis was induced by reducing the mitochondrial membrane potential in the pioglitazone group. Pioglitazone increased expression of proapoptotic Bax proteins and decreased antiapoptotic Bcl-2 in cerulin-induced AR42J cells and decreased Bcl-2 levels in pancreatic tissue of severe acute pancreatitis rats 1, 3, 6, 7, and 8 hours after induction.  

Conclusion: Pioglitazone may promote apoptosis of acinar cells through both intrinsic and extrinsic apoptotic pathways in acute pancreatitis.  

Key Words: PPARγ, acute pancreatitis, apoptosis, pioglitazone  

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cute pancreatitis (AP) is characterized not only by inflammation but also by parenchymal cell death.1,2 Apoptosis and necrosis are observed in clinical and experimental pancreatitis.3 Furthermore, apoptosis seems to be beneficial in controlling the severity of AP.4–5 However, the mechanisms regulating apoptosis in AP are not clear. It was recently reported that peroxisome proliferator–activated receptors (PPARs) play a modulatory role in the inflammatory responsiveness of different organs. We have found that pioglitazone, a PPARγ agonist, attenuates the severity of severe acute pancreatitis (SAP) and promotes apoptosis of pancreatic cells in SAP rats.6,7 However, the apoptosis signaling pathway in acinar cells stimulated by pioglitazone in AP is unknown.  

Classical apoptosis can be initiated via 2 major pathways: the intrinsic or mitochondria-mediated pathway and the extrinsic or death receptor–mediated pathway. Stimulation of either pathway results in the activation of caspases.8–10 In this study, we investigated the mechanisms regulating the apoptosis signaling pathway in AP using 2 different models: AR42J cells activated with cerulein as an in vitro model of AP and pancreatic cells taken from rats with SAP. We demonstrate how pioglutazone participates in the development of apoptosis by regulating mitochondria-mediated apoptotic pathways.  

MATERIALS AND METHODS  

Cell Culture and Treatments  

AR42J cells (ATCC, Rockville, MD) were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin in an atmosphere of 5% CO2 at 37°C and were routinely passaged by trypsinization with a change of medium twice weekly. Cells were plated at a density of 5 × 105 cells/mL in 6-well culture plates and allowed to attach for 12 hours. Cells were divided into 4 treatment groups: control, cerulin (cerulein alone), pioglitazone (cerulin + pioglitazone), and GW9662 (pioglitazone + cerulin + PPARγ antagonist GW9662). Drugs were diluted in dimethylsulfoxide (DMSO) and added at the following concentrations: pioglitazone, 40 μM; cerulin, 100 nM; GW9662, 10 μM. Control cells received 1 μL of DMSO only. Where cerulin was coincubated with other drugs, cerulin was added 30 minutes after the start of pioglitazone or pioglitazone + GW9662 incubations.  

Animal Model of SAP  

Adult male Sprague-Dawley rats (average weight, 200–250 g) were used in this study. All experiments were performed in accordance with the Shanghai Jiao Tong University Animal Care Guidelines. The experimental animals were housed at standard room temperature (22°C) in a 12-hour light/dark cycle and fed with standard pellet rat food (210 kcal/100 g per day) and tap water. The animals were fasted overnight before each experiment but allowed free access to water. Severe acute pancreatitis was induced as previously described.11 At the end of each experiment, pancreatic samples were collected and stored in liquid nitrogen for Western blot. Animal care and treatment were humane and in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Medical Scientific Research of the First People’s Hospital, Shanghai Jiao Tong University (Permit Number: 2012KY041). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.
Enzyme-Linked Immunosorbent Assay

Changes in interleukin (IL-1β), IL-6, tumor necrosis factor α (TNF-α), and amylose levels in cell culture supernatants were determined with an enzyme-linked immunosorbent assay (ELISA) kit (R&D). The supernatants were collected at the time point of 6 hours. Briefly, ELISA was performed in flat-bottom 96-well plates, which permit high-throughput results. The supernatants were incubated in wells for 2 hours at room temperature and then removed. The wells were washed with a series of buffer rinses. Biotin-labeled antibody was added and incubated for 1 hour at room temperature. The plate was washed 3 times and avidin-peroxidase was added for 30 minutes. The final step was the addition of the enzyme substrate and the production of colored product in the wells. The secretion rate of amylose is the ratio of amylose in supernatant to cell amylose.

3-[(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide Assay

Cells were seeded in 96-well plates (1 × 10^4 cells/well) and treated as described above for 3, 6, 12, and 24 hours, respectively. Cell growth was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Thiazolyl blue tetrazolium bromide (Sigma) was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/mL and filtered as a stock solution. Ten microliters of stock solution was added to 100 μL of medium in each well and incubated for 4 hours at 37°C. After removal of the supernatant, 200 μL of DMSO was added to each well to solubilize the formazan product, and the plates were read at an optical density of 470 nm using a microplate reader (Sigma). Triplicate experiments were performed in parallel at each time point and the mean (SD) values were calculated.

Annexin V-Fluorescein Isothiocyanate/Propidium Iodide Double Labeling for Flow Cytometry–Assessed Apoptosis

Apoptosis was determined by detection of phosphatidylserine exposure on cell plasma membranes using the fluorescent dye Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ), according to the manufacturer's protocol. Cells were seeded in 6-well plates (5 × 10^4 cells/well) and were treated with the previously described drug protocol for 3, 6, 12, and 24 hours, respectively. Cells were harvested by trypsinization and washed twice in ice-cold PBS, resuspended in 300 μL of binding buffer, and incubated with 5 μL of Annexin V-FITC solution for 30 minutes at 4°C under dark conditions. This was followed by a further incubation with 5 μL of propidium iodide (PI) for 5 minutes and then immediately analyzed by bivariate flow cytometry using FACScan (BD Biosciences) and Cell Quest software (BD Biosciences). Cells (1 × 10^5) from each drug exposure and time point were analyzed in triplicate.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed on AR42j cells cultured in chamber slides and pancreatic tissue slides using a Dead End Colorimetric TUNEL System (BD) in accordance with the manufacturer's instructions. Cells were treated as previously described. Cells were fixed for 15 minutes in 4% paraformaldehyde, and the TUNEL reaction was performed according to the manufacturer's instructions. The average number of apoptotic cells was determined by counting the number of TUNEL-positive cells in 10 randomly selected fields per slide (×400) using an optical microscope (Olympus, Japan), and the percentage of apoptotic cells was designated as the apoptotic index. At least 3 slides were counted for each group.

Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential (MMP/ΔΨm) assay was performed using the JC-1 Mitochondrial Membrane Potential Assay Kit (KeyGEN, Nanjing, China). JC-1 fluorochrome is a lipophilic cationic mitochondrial vital dye that becomes concentrated in the mitochondrion in response to ΔΨm. The dye exists as a monomer at low concentrations with an emission at 530 nm (green fluorescence, FL1-H), but at higher concentrations, it forms J-aggregates after accumulation in the mitochondrion, with an emission at 590 nm (red fluorescence, FL2-H). To evaluate the ΔΨm for each experimental condition, AR42j cells were seeded in 6-well plates, treated as described above, and washed twice with PBS after experimental treatment; then, 1 mL of staining dye/well (culture medium: JC-1 working dye = 1:1) was added and cells were incubated at 37°C for 20 minutes. Cells were washed twice with cold JC-1 staining buffer and harvested by trypsinization and then examined by flow cytometry.

Caspase Activity

The activity of caspases 3, 6, 8, and 9 was measured in AR42j cells. Cells were treated as described above, washed twice with ice-cold PBS, and then ruptured with RIPA lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. The supernatant was collected and assayed for caspase activity after centrifugation at 15,000g for 30 minutes. Equal amounts of protein were assayed in duplicate for each sample. The protein in supernatant samples was quantified by the method of Bradford assay and determined by use of a spectrophotometer at an optical density of 450 nm, and expression of cytokines was normalized by total protein. The assays were performed according to the manufacturer's instructions (KeyGEN).

Western Blotting Analysis

Changes in expression of Bcl-2 and Bax in AR42j cells and SAP pancreatic cells were detected by Western blotting. Briefly, cells were washed twice with ice-cold PBS and ruptured with lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. The supernatant was collected and assayed for caspase activity after centrifugation at 15,000g for 30 minutes. Equal amounts of protein were assayed in duplicate for each sample. The protein in supernatant samples was quantified by the method of Bradford assay and determined by use of a spectrophotometer at an optical density of 450 nm, and expression of cytokines was normalized by total protein. The assays were performed according to the manufacturer's instructions (KeyGEN).
buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. Cell extracts were centrifuged for 5 minutes at 12,000g and supernatants were collected and heated for 15 minutes at 100°C. Protein concentration was determined by Bradford assay using a commercial kit purchased from Bio-Rad Laboratories. Equal quantities of protein were separated electrophoretically on 10% sodium dodecyl sulfate/polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Roche). After blocking with 5% milk, the membrane was stained with the specific primary antibody followed by horseradish peroxidase-conjugated secondary antibody (Abcam). The specific bands were visualized by use of an ECL Plus Western blotting detection system (Bio-Rad). The gamma ratio value was calculated using Image Lab 6.0 software (Bio-Rad) from Chemi DocTM XRS+ system images (Bio-Rad). The optical density of imaged bands were normalized by using β-actin or GAPDH signal obtained on the same blot. Three independent experiments were performed to obtain the mean (SD).

RESULTS

Pioglitazone Reduces Production of IL-1β, IL-6, TNF-α, and Amylase

AR42j cells were activated by cerulein, and cytokine release into culture supernatants was measured by sandwich ELISA. Treatment of AR42j with cerulein alone resulted in an increase in production of IL-1β, IL-6, and TNF-α and secretion rate of amylase compared with the control group (P < 0.05, Figs. 1, 2B). Pioglitazone pretreatment significantly inhibited the cerulein-induced production of cytokines, compared with the cerulein group (P < 0.05, Figs. 1, 2B). However, cytokine secretion was restored when PPARγ signaling was inhibited by the antagonist GW9662. We detected that serum amylase of SAP rats in different groups, which showed that serum amylase was increased significantly in the SAP group compared with the control group but decreased in the pioglitazone-pretreated group (P < 0.05, Fig. 2A).

Pioglitazone Suppresses Proliferation of Cerulein-Activated AR42j Cells

The viability of AR42j cells treated with cerulein in the presence and absence of pioglitazone was assessed by MTT colorimetric assay at various time points after treatment (Fig. 3). Compared with cerulein group cells, pioglitazone significantly suppressed cellular proliferation at 6, 12, and 24 hours (P < 0.05). In contrast, cerulein alone did not affect proliferation at any time point. When
GW9662 was included in the incubations, the suppressive effect of pioglitazone on cellular proliferation was blocked.

**Detection of Apoptotic Cells**

Quantification of cell apoptosis and necrosis was performed using Annexin V-FITC binding analysis and PI staining. The number of apoptotic cells was significantly increased in cells treated with pioglitazone compared with the cerulein group cells at 6, 12, and 24 hours (Fig. 4). Cerulein alone had no effect on cell apoptosis. However, the presence of GW9662 reduced the number of Annexin V-FITC-positive cells to control levels.

**TUNEL-Based Detection of Programmed Cell Death in Pancreatitis Cells**

Apoptosis of AR42j cells was examined using TUNEL staining 6 hours after treatment with cerulein. In cerulein group cells, TUNEL-positive cells were infrequent (2.63% ± 0.51%). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling–labeled cells in the pioglitazone group were significantly increased to 8.30% ± 1.85% (P < 0.05, Fig. 5). However, the number of TUNEL-positive cells was markedly reduced in cells treated with GW9662 (3.87 ± 0.35%) compared with the pioglitazone group. Apoptosis was also examined in the pancreas of SAP rats, which showed that apoptotic cells in the pioglitazone-pretreated group were more than those in the SAP group (Figs. 6A–C). Hematoxylin-eosin (HE) staining revealed that necrosis was more serious in the SAP group than that in the pioglitazone group (Figs. 6D–F). Therefore, it tells us that pioglitazone can promote apoptosis but decrease necrosis in the pancreas of SAP rats.

**Changes in Caspase Protease Activity Induced by Pioglitazone in AR42j Cells**

To determine if apoptosis induced by pioglitazone in cerulein-activated AR42j cells is related to caspase protease activity, the enzymatic activity of caspase 3, caspase 6, caspase 8, and caspase 9 was measured using spectrophotometry in lysates of cerulein-activated AR42j cells treated with pioglitazone or pioglitazone + GW9552. After 6 hours of cerulein activation, activity of caspases 3, 8, and 9, but not caspase 6, was increased in cells after pioglitazone exposure, compared with cerulein group cells (P < 0.05, Fig. 7). The increase in caspase activity was prevented by the addition of GW9662 to the cell cultures. Although the increase in caspase 6 activity after pioglitazone exposure did not reach statistical significance, caspase 6 activity was nonetheless suppressed by GW9662 (P < 0.05, Fig. 7). These results implied that pioglitazone elevated the activity of caspase 3, caspase 8, and caspase 9 in cerulein-induced AR42j cells.

**Pioglitazone Increased Loss of ΔΨm in AR42j Cells**

To observe the effect of pioglitazone on MMP in cerulein-activated AR42j cells, we assayed for changes in ΔΨm by flow cytometry analysis using the potential-sensitive dye JC-1. Changes in ΔΨm in cells were determined using JC-1-base staining 6 hours after starting treatment with cerulein. As shown in Figure 8, the loss of ΔΨm (FL1-H%) in the control group and cerulein group was 11.27% ± 4.41% and 15.03% ± 3.67%, respectively. Loss of ΔΨm in the pioglitazone group was 28.20% ± 2.56%, significantly higher than that in the control group (P < 0.05). However, loss of ΔΨm was prevented when the cells were also treated with GW9662 (loss = 20.67% ± 2.20%, P < 0.05 vs. pioglitazone group). The results suggested that pioglitazone induced apoptosis
of AR42j cells by reducing the mitochondrial membrane potential (MMP or $\Delta \Psi_m$). A similar response may occur in acinar cells during the inflammatory process that occurs in AP.

### Changes in Caspase Activity Induced by Pioglitazone in the Pancreas of SAP Rats

We assessed the activity of caspase 3, caspase 6, caspase 8, and caspase 9 in pancreatic tissue of rats with SAP. The activity of each caspase was elevated in the pancreas of the SAP rats 3 hours after the SAP operation. Moreover, when the SAP rats were treated with pioglitazone, caspase activity significantly increased compared with nontreated SAP rats ($P < 0.05$, Fig. 9). The results suggested that pioglitazone also enhanced apoptosis in the pancreas of SAP rats.

### Effects of Pioglitazone on Changes in Bcl-2 and Bax Protein Expression in AR42j Cells and in the Pancreas of SAP Rats

Changes in Bcl-2 and Bax protein expression were evaluated by Western blot in cerulein-induced AR42j cells after pretreatment with 40 mM pioglitazone. Expression of antiapoptotic Bcl-2 protein was decreased 6 and 12 hours after the pioglitazone pretreatment, compared with cerulein group cells ($P < 0.05$, Fig. 10); in contrast, expression of proapoptotic protein Bax was increased by the same treatment ($P < 0.05$, Fig. 10). Analysis of $\beta$-actin expression confirmed that protein loading was similar across lanes on each gel. Expression of Bcl-2 and Bax returned to control levels when the PPAR$\gamma$ agonist GW9662 was added with pioglitazone (Fig. 10). Expression of Bcl-2 was significantly decreased at 1 and 3 hours after the SAP operation, compared with SAP group rats ($P < 0.05$, Fig. 11F). Bax expression increased at 1, 3, 6, and 12 hours (Fig. 11E). However, it has been reported that the ratio of Bax to Bcl-2, rather than Bcl-2 expression alone, is important for regulation of apoptosis.\(^{11}\)

### DISCUSSION

In our previous studies, we found that pioglitazone, an activator of PPAR$\gamma$, could induce apoptosis in pancreatic acinar cells in SAP rats.\(^6\) However, the mechanism by which pioglitazone...
induces apoptosis in acinar cells is still unknown. In this study, we determined how pioglitazone affects apoptosis in AR42j cells activated by cerulein and verified the effect of pioglitazone in pancreatic tissue of SAP rats.

The AR42j cell line maintains many characteristics of normal pancreatic acinar cells, including the synthesis and secretion of digestive enzymes. Receptor expression and signal transduction mechanisms in AR42j cells parallel those of pancreatic acinar cells. Thus, this cell line has been widely used as an in vitro model to study cellular secretion, proliferation, and apoptosis of the exocrine pancreas.

Peroxisome proliferator–activated receptor-γ, a subgroup of ligand-activated nuclear receptors, plays a critical role in cell cycle regulation, cell differentiation, cell invasion, and apoptosis. Pioglitazone, as a PPARγ agonist, has shown effectiveness in a series of independent studies. For example, in rheumatoid arthritis synovial fibroblasts, troglitazone, another synthetic PPARγ agonist, inhibited the production of TNF-α, IL-6, IL-8, and MMP-3 without causing apoptosis, whereas in several malignant cell lines, PPARγ activation was shown to induce apoptosis.

In our study, AR42j cells were activated by cerulein to produce an in vitro model of AP, as described in a previous report, which showed that cerulein induced secretion of amylase, TNF-α, IL-1β, and IL-6. Pioglitazone was shown in our previous studies to inhibit the inflammation of the pancreatic acinar cells. Here, we show by annexin V/PI flow cytometry and TUNEL immunohistochemistry that pioglitazone can promote apoptosis of pancreatic acinar cells activated by cerulein, which is the same in pancreatic tissue of SAP rats. A similar effect of pioglitazone on apoptosis has also been documented by Masamune et al. We also found that necrosis was decreased by pioglitazone by HE staining, which means that pioglitazone prevents necrosis by promoting pancreatic cell apoptosis.

We found in our experiments that caspase 3, caspase 6, caspase 8, and caspase 9 were elevated in cerulein-induced AR42j cells pretreated with pioglitazone. In addition, the activity of these caspases was also increased when SAP rats were treated by pioglitazone. The results suggest that caspase activity plays an important role in the facilitation of apoptosis in pancreatic acinar cells during AP. Our study also showed that the MMP or ΔΨm in pioglitazone-treated AR42j cells was decreased compared with control or cerulein-treated cells, suggesting that apoptosis induced by pioglitazone promoted pancreatic cell apoptosis.

![Graph showing OD (450nm) for caspase3, caspase6, caspase8, and caspase9](image)

**FIGURE 9.** Caspase 3, 6, 8, and 9 activity in the pancreas of SAP rats 3 hours after induction with and without pioglitazone treatment (*P < 0.05 vs control group, #P < 0.05 vs SAP group).
by pioglitazone was mediated in part by increased mitochondrial membrane permeability.

Based on the above findings, we analyzed the molecular mechanism mediating pioglitazone-induced AR42j cell apoptosis. Our data revealed regulation of proteins involved in intrinsic and extrinsic apoptotic signaling pathways including Bcl-2, Bax, caspase 3, caspase 6, caspase 8, and caspase 9, as well as changes in ΔΨm. It has been reported that reduction of MMP is among the very first intracellular events preceding the execution phase of apoptosis via the mitochondria-mediated death pathway.19,20 Our experimental results showed that pretreatment of cerulein-activated AR42j cells with pioglitazone caused a reduction of MMP (Fig. 6). The intrinsic, mitochondrial apoptotic pathway is regulated by the Bcl-2 family of proteins.21 Bcl-2 family proteins are classified as proapoptotic (Bax, Bak, Bad, Bid, Bik, and Bim) or antiapoptotic (Bcl-2, Bcl-XL, and Mcl-1).22 Proapoptotic proteins promote release of cytochrome c from the mitochondria, initiating the apoptotic cascade. Cytochrome c activates caspase 9, which cleaves and activates downstream effector proteases including caspase 3, leading to apoptosis.3,24 Our data showed increased expression of proapoptotic Bax proteins and decreased expression of Bcl2 in cerulein-induced AR42j cells pretreated with pioglitazone and decreased Bcl-2 expression in pancreatic tissue of SAP rats 1 and 3 hours after induction of AP. Unexpectedly, the expression of Bax was increased at 12 hours but was unchanged at 6 hours after induction in the pancreas of SAP rats. However, it has been reported that it is the ratio of Bax to Bcl-2, rather than Bcl-2 alone, which is important for the regulation of apoptosis.11

The extrinsic pathway is mediated by death receptors. The Fas ligand interacts with the Fas receptor, causing caspase 8 and caspase 10 activation.24-26 Activated caspase 8 and caspase 10 directly cleave and activate downstream effector proteases, including caspase 3, resulting in apoptosis.27 The present study showed that the expression of the caspase 8 was activated. Activated caspase 8 cleaves and activates downstream effector caspases, including caspase 9 and caspase 3, which were up-regulated in pioglitazone-treated AR42j cells and in pancreatic acinar cells from SAP rats. In addition, caspase 8 and caspase 10 have the ability to cleave the Bcl-2 family member Bid into truncated Bid, thereby resulting in disruption and release of cytochrome c.28,29

The most important enzymes involved in apoptosis are the caspases, which hydrolyze structural and functional proteins, ultimately leading to cell death.30-32 Our results suggest that pioglitazone promotes apoptosis of pancreatic acinar cells during AP via activation of caspases, which may involve both intrinsic and extrinsic apoptotic pathways.

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