Crambene induces pancreatic acinar cell apoptosis via the activation of mitochondrial pathway

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Submitted 7 November 2005; accepted in final form 16 March 2006

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Acute pancreatitis is a common disorder with potentially devastating consequences. The exact mechanisms by which diverse etiological factors induce the attack are still unclear; several recent studies, however, have shown that the severity of acute pancreatitis was inversely related to the extent of pancreatic acinar apoptosis (8, 13). Sparse information is known so far about the molecular mechanisms of pancreatic cell death, and only relatively few methods of inducing pancreatic acinar cell apoptosis have been identified (3, 6, 7, 27, 28). Crambene (1-cyano-2-hydroxy-3-butene), a plant nitrile, on pancreatic acinar cells. As evidenced by annexin V-FITC staining, crambene treatment for 3 h induced the apoptosis but not necrosis of pancreatic acini. Caspase-3, -8, and -9 activities in acini treated with crambene were significantly higher than in untreated acini. Treatment with caspase-3, -8, and -9 inhibitors inhibited annexin V staining, as well as caspase-3 activity, pointing to an important role of these caspases in crambene-induced acinar cell apoptosis. The mitochondrial membrane potential was collapsed, and cytochrome c was released from the mitochondria in crambene-treated acini. Neither TNF-α nor Fas ligand levels were changed in pancreatic acinar cells after crambene treatment. These results provide evidence for the induction of pancreatic acinar cell apoptosis in vitro by crambene and suggest the involvement of mitochondrial pathway in pancreatic acinar cell apoptosis.

caspases; cytochrome c; membrane potential

Apoptosis is a physiological or programmed form of cell death that has a characteristic and stereotypical morphology, including cell shrinkage, retention of organelles, and nuclear chromatin condensation (2, 15). The characteristic morphology and many biochemical features exhibited in apoptotic cells are largely mediated by a family of aspartate-specific cysteiny proteases (caspases), the central performers of the apoptotic pathway (9). Two separable pathways leading to caspase activation have been characterized, the extrinsic pathway and the intrinsic pathway (1, 2, 10). The former is initiated by ligation of transmembrane death receptors (CD95, TNF receptor, and TRAIL receptor) to activate “initiator” caspases (caspases-8 and -10), whereas the latter is characterized by the loss of mitochondrial membrane potential (ΔΨm), the release of mitochondrial cytochrome c, as well as the subsequent activation of initiator caspase (caspase-9). Both pathways converge to a common execution phase and result in activation of the “executioner” caspases (caspases-3, -6, and -7), which ultimately cause the demise of the cell.

Since the induction of apoptosis by crambene in pancreatic acinar cells has only been investigated in vivo, it is not clear whether their effects are direct effects of crambene or a secondary response; therefore, it is worth testing the effect of this nitrile in pancreatic acinar cells in vitro. However, no study has yet been reported on whether crambene could directly induce apoptosis on isolated pancreatic acinar cells, or, if so, what the mechanism of the induction of pancreatic acinar cell apoptosis by crambene is.

The current study aims to investigate the proapoptotic effect of crambene on pancreatic acinar cells in vitro and examine the possible apoptotic pathway activated by crambene in pancreatic acinar cells by evaluation with caspases activation and mitochondrial dysfunction.

Materials and Methods

Animals. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the animal ethics committee of National University of Singapore. Swiss mice (male, 25–30 g) were housed in a controlled environment with an ambient

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temperature of ~22–26°C and a 12:12-h light-dark cycle. The mice were fed a standard laboratory diet and given water ad libitum.

**Crambene.** Crambene was isolated from autolysed *Crambe abyssinica* meal using immiscible solvent extraction followed by high-performance liquid chromatography (27). Crambene purified by this method has been reported to be more than 99.5% pure.

**Preparation of pancreatic acini.** Pancreatic acini were obtained from mouse pancreas by collagenase treatment. Briefly, pancreata from mice were infused with buffer A (140 mM NaCl, 4.7 mM KCl, 1.13 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, and 10 mM HEPES; pH 7.2) containing 200 IU/ml collagenase and incubated in a shaking water bath for 10 min at 37°C. The digested tissue was passed through 50 mg/ml BSA and washed twice with buffer A for further experiments. Cell viability was determined by Trypan blue exclusion.

**Induction of pancreatic acinar cell apoptosis.** The prepared acini were distributed into microcentrifuge tubes containing buffer A. Crambene was added into these tubes with the working concentration of 2 mM. Acini were incubated with or without crambene at 37°C in a shaker water bath for 1, 2, 3, 4.5, and 6 h. In some experiments, caspase inhibitors were used together with crambene treatment.

**Annexin V-FITC/propidium iodide staining detection.** Extent of apoptosis and necrosis was determined by annexin V-FITC/propidium iodide (PI) staining using a BD ApoAlert annexin V-FITC apoptosis kit. After treatment with 2 mM crambene, the acini were incubated with 5 μl annexin V (20 μg/ml in Tris-NaCl) and 5 μl PI (50 μg/ml in 1 x binding buffer) for 15 min in the dark at room temperature. Cells were observed by fluorescence microscopy (Carl Zeiss) using a "dual-band pass" filter designed to simultaneously detect fluorescein (excitation, 490 nm; emission, 520 nm) and rhodamine (excitation, 540 nm; emission, 570 nm). The cells showing visible annexin V staining (with no PI staining) were categorized as apoptotic cells. Samples were also quantified by a Gemini EM microplate spectrophotometer measuring red fluorescence (excitation, 535 nm; emission, 617 nm) and green fluorescence (excitation, 488 nm; emission, 530 nm). In some experiments, caspase inhibitors were used together with crambene treatment.

**Caspase assay.** Caspase-3, -8, and -9 enzyme activities were quantified using a fluorometric assay by measuring the extent of cleavage of enzyme-specific fluorometric peptide as previously described. After treatment, cells were incubated with a fluorometric substrate for either caspase-3 (Ac-DEVD-AFC, BD Pharmingen), caspase-8 (Ac-IETD-AFC, BD Pharmingen), or caspase-9 (Ac-LEHD-AFC, Calbiochem). The acini were centrifuged, and 100 μl of lysis buffer was added. Freeze and thaw steps were carried out three times by transferring from liquid nitrogen to a 37°C water bath. The lysed cells were centrifuged at 4°C for 30 min at 13,000 rpm. The supernatant was used for the assay. Fluorescence was measured at respective wavelengths (caspase-3, excitation, 394 nm and emission, 535 nm; caspases-8 and -9, excitation, 400 nm and emission, 505 nm) using a fluorescence plate reader (Tecan) every 5-min interval for 75 min. Caspase activity was expressed as relative fluorescent unit per hour per microgram DNA per microliter as calculated using the linear range of the assay. The caspase activity in untreated cells was considered to be 100%. For caspase inhibitor assays, caspase-3-
specific inhibitor Z-DEVD-FMK, caspase-8-specific inhibitor Z-IETD-FMK, and caspase-9-specific inhibitor Z-LEHD-FMK (R&D Systems) were used at the working concentration of 100 nM according to the manufacturer's instructions.

Mitochondrial membrane potential detection. After treatment with 2 mM crambene for 3 h, acinar cells were washed in fresh isolation buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 250 mM sucrose; pH 7.5) and homogenized in a 7-ml glass tissue Dounce homogenizer using up and down strokes of pestle. The homogenate was centrifuged at 800 g for 10 min at 4°C, and then the supernatant was retained and centrifuged at 10,000 g for 10 min at 4°C. Pellet was washed twice and resuspended in wash buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, and 100 mM sucrose; pH 7.5), and mitochondria were isolated by sequential centrifugation at 800 g for 10 min at 4°C and 10,000 g for 10 min at 4°C.

JC-1 (relative ratio of red to green fluorescence). The level of JC-1 retained by untreated cells at 3 h was considered to be 100%. In some experiments, caspase inhibitors were used together with crambene treatment.

Measurement of cytochrome c release from mitochondria. After treatment with 2 mM crambene for 3 h, acinar cells were incubated with or without crambene for different times. The homogenate was centrifuged at 10,000 g for 10 min at 4°C, and then the supernatant was retained and centrifuged at 100,000 g for 10 min at 4°C. Pellet was washed twice and resuspended in wash buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, and 100 mM sucrose; pH 7.5), and mitochondria were isolated by sequential centrifugation at 800 g for 10 min at 4°C and 10,000 g for 10 min at 4°C.

Fig. 2. Detection of caspase-3 activity in pancreatic acinar cells. Isolated pancreatic acini were incubated with or without 2 mM crambene (CHB) in a 37°C water bath for 1, 2, 3, 4.5, and 6 h. A: time-course assay on caspase-3 activity induced by crambene. B: effect of 3 h crambene with or without Z-DEVD-FMK on caspase-3 activation. C: effect of 3 h crambene with or without Z-DEVD-FMK on annexin V-FITC binding. The caspase-3 activity in corresponding incubation control was considered to be 100% (n = 6, †P < 0.002 compared with 3-h untreated samples; *P < 0.001 compared with 3-h crambene-treated cells).

Fig. 3. Detection of caspase-8 and -9 activity in pancreatic acinar cells. Isolated pancreatic acini were incubated with or without 2 mM crambene in 37°C water bath for 1, 2, 3, 4.5, and 6 h. A: time-course assay on caspase-8 and -9 activity induced by crambene. B: effect of 3 h crambene with or without Z-IETD-FMK on caspase-8 activation. C: effect of 3 h crambene with or without Z-LEHD-FMK on caspase-9 activation. The caspase activity in untreated cells at 3 h was considered to be 100% (n = 6, †P < 0.002 compared with 3-h untreated samples; *P < 0.001 compared with 3 h crambene-treated cells).
5 mM HEPES; pH 7.4) to obtain the mitochondria suspension. Cytochrome c release from isolated mitochondria in acinar cells was then measured by the cytochrome c DuoSet IC ELISA kit (R&D Systems). Absorbance was measured at 450 nm with the reference wavelength at 540 nm (Tecan). Results were adjusted by total cytochrome c in mitochondria by addition of 0.1% Triton X-100. The level of cytochrome c determined in untreated cells at 3 h was considered to be 100%. Data were expressed as percentage increase of optical density.

**Statistical analysis.** All experiments were repeated at least three times. Values are means ± SE. Treatment effects were compared using Student’s t-test or one-way ANOVA followed by a post hoc analysis (Tukey test). A *P* value of <0.05 was regarded as a significant difference.

**RESULTS**

**Treatment of pancreatic acinar cells with crambeine induces apoptosis but not necrosis.** Treatment of pancreatic acinar cells with 2 mM crambeine for 3 h caused an increase of annexin V-FITC staining but not PI as evidence by fluorescence microscopy as well as spectrofluorometry. As shown by Fig. 1, annexin V-FITC binding was observed in crambeine-treated (3 h) pancreatic acinar cells but not in untreated samples (Fig. 1B). Fluorescence measurement using a plate reader showed that annexin V-FITC binding (Fig. 1A-a) was only statistically elevated in 3-h crambeine-treated cells compared with control. In terms of PI staining (Fig. 1A-b), during the early time points, no significant difference was found between crambeine-treated and untreated cells. However, in the 6-h treated group, crambeine was shown to increase PI staining significantly. These results indicate that treatment of pancreatic acinar cells with 2 mM crambeine for 3 h induced the early stages of apoptosis but not necrosis of pancreatic acinar cells.

The apoptosis induced by crambeine in pancreatic acinar cells was confirmed by determination of caspase-3 activity. As shown by Fig. 2A, after incubation of crambeine with a series of time points, caspase-3 activity in pancreatic acinar cells was significantly increased only at 3 h compared with the corresponding incubation control. When caspase-3 inhibitor (Z-DEVD-FMK) was added, the activation of caspase-3 by crambeine at 3 h was significantly reduced (Fig. 2B). Moreover, the increase in annexin V-FITC binding by crambeine was significantly attenuated in cells treated with Z-DEVD-FMK (Fig. 2C). These results suggest crambeine treatment induced an activation of caspase-3 in pancreatic acinar cells at 3 h.

**Activity of caspases 8 and 9 increases in crambeine induced pancreatic acinar cell apoptosis.** Crambeine treatment was also found to stimulate both activation of caspases-8 and -9 in pancreatic acinar cells after 3 h incubation (Fig. 3A). Moreover, such activation could be blocked respectively by corresponding inhibitors (Fig. 3, B and C). As shown by Fig. 3B, crambeine treatment significantly increased caspase-8 activity by 1.38-fold compared with untreated acini, and the activity was reduced by 28% when caspase-8-specific inhibitor Z-IETD-FMK was added. Caspase-9 activity was increased by 1.95-fold with crambeine treatment compared with untreated acini, and this activation was significantly decreased by 83% when caspase-9-specific inhibitor Z-LEHD-FMK was used (Fig. 3C).

Furthermore, we compared the blocking effect of both Z-LEHD-FMK and Z-IETD-FMK on activation of caspase-3. As shown by Fig. 4A, treatment of Z-LEHD-FMK blocked significantly more caspase-3 activity than that of Z-IETD-FMK. In terms of annexin V staining, both inhibitors significantly decreased annexin V-FITC binding by crambeine (Fig. 4B). Also, the reduction of annexin V by Z-LEHD-FMK was more pronounced than that of Z-IETD-FMK. These data suggest that both caspases-8 and -9 may be involved in pancreatic acinar cell apoptosis by crambeine.

**Treatment of crambeine collapses the mitochondrial membrane potential.** Following crambeine treatment, the mitochondrial integrity was assessed. As shown in Fig. 5A, a-f, control pancreatic acinar cells displayed the phenomenon of polarized mitochondria, whereas the crambeine-treated cells had depolarized mitochondria, which indicated that in crambeine-treated pancreatic acinar cells the ∆Ψm had collapsed.

We also quantified ∆Ψm by using a fluorescence plate reader (Fig. 5B). The relative ratio of red to green fluorescence was significantly decreased in crambeine-treated cells compared with untreated group. However, pretreatment with either Z-LEHD-FMK or Z-IETD-FMK in both crambeine-treated and untreated cells showed no alteration on the ∆Ψm.

**Crambeine treatment increases cytochrome c release from mitochondria.** We analyzed the effect of crambeine treatment on the release of cytochrome c from mitochondria in pancreatic acinar cells. As shown by Fig. 6, the release of mitochondrial cytochrome c in treated pancreatic acinar cells was significantly increased over 1.33-fold compared with the untreated
These results indicated that treatment of acini with 2 mM crambene for 3 h induces the release of cytochrome c by mitochondria in pancreatic acinar cells.

**DISCUSSION**

The extent of pancreatic acinar cell apoptosis has been shown to be inversely related to the severity of the acute pancreatitis, suggesting that apoptosis is a teleologically beneficial form of cell death in acute pancreatitis. Since prophylactic induction of apoptosis has been found to have a protective action against acute pancreatitis in animal models (3), it is reasonable to speculate that selective induction of pancreatic acinar cell apoptosis may be of value in clinical acute pancreatitis, especially in individuals with a high risk of pancreatitis (such as those undergoing endoscopic retrograde cholangiopancreatography). However, before one could take these studies into the clinic, more basic research is needed into the mechanisms of apoptosis of pancreatic acinar cells (2).

In the current study, we have investigated apoptosis of pancreatic acinar cells induced by crambene in vitro, through...
investigated the change of activating caspase-3. That both extrinsic and intrinsic pathways may be involved in caspase-8 and caspase-9 inhibitors as well. These data indicate moreover, caspase-3 activity was blocked significantly by treated pancreatic acinar cells, and these activated caspases were blocked when the corresponding inhibitor was used. All three caspases were significantly activated in crambene-quantifying caspase-3, -8, and -9 activities. Our data showed that (19, 21, 24).

Moreover, caspase-3 was measured, whereas the retained cytochrome c in the mitochondria was removed during washing. Our studies show that crambene induced a significant \( \Delta \Psi_m \) reduction and a significant release of cytochrome c by mitochondria as well. We did not observe any effect of caspase-9 inhibitor on mitochondrial membrane depolarization, which indicated caspase-9 activation occurs downstream of mitochondrial membrane depolarization. These results, together with the caspase-9 activation data, further supported that the intrinsic pathway is involved in crambene-induced apoptosis in pancreatic acinar cells.

To clarify the possible involvement of the extrinsic pathway, which is suggested by the activation of caspase-8, we analyzed the production of TNF-\( \alpha \) and Fas ligand levels in pancreatic acinar cells after treatment with crambene, but no increase in either of them was found (data not shown). We also evaluated the effect of caspase-8 inhibitor on \( \Delta \Psi_m \), but no blocking effect was observed. This data suggested that the extrinsic pathway does not play an important role in crambene-induced apoptosis. Even though we did not observe any effect of caspase-9 inhibitor on mitochondrial membrane depolarization either, this is not surprising, as caspase-9 activation occurs downstream of mitochondrial membrane depolarization. However, the current data do not exclude the possibility of the involvement of the extrinsic pathway. Recent studies have shown that the activation of caspase-8 is possibly independent of death receptors (20). Moreover, the activation of caspase-8 can be stimulated indirectly via the intrinsic pathway following activation of caspase-3 (29). Apart from caspase-8 activation, all our results point to the contribution of the mitochondrial pathway in crambene-induced apoptosis of pancreatic acinar cells.

Our studies demonstrate for the first time that crambene induces apoptosis in pancreatic acinar cells in vitro, as defined by the externalization of PS, increase of caspase activation, loss of \( \Delta \Psi_m \), and release of mitochondrial cytochrome c. In light of the data presented in this paper, it is reasonable to conclude that the intrinsic pathway of apoptosis mediated by loss of \( \Delta \Psi_m \), release of mitochondrial cytochrome c, as well as activation of caspase-9 is the primary pathway involved in crambene-induced pancreatic acinar cell apoptosis.

GRANTS

This work was supported by National Medical Research Council Grant R-184-000-078-213.
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