Nelfinavir/ritonavir reduces acinar injury but not inflammation during mouse caerulein pancreatitis

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The mechanisms leading to cell death in acute pancreatitis are controversial (15, 17), and theories include abnormal vesicular trafficking leading to activation of zymogens (39), toxic effects of intracellular calcium (17, 27), and fatty acid ethyl esters (14), as well as activation of transcription factors (4), inflammatory mediators (6, 36, 50), cytoskeletal rearrangement (45), production of reactive oxygen species (5, 42), and mitochondrial damage (9, 11, 12). During experimental mouse pancreatitis, both necrosis and apoptosis occur (3, 10, 12, 13), although their roles during pancreatitis are controversial. Some studies suggest that apoptosis may be protective during pancreatitis (3); agents such as 1-cyano-2-hydroxy-3-butene (CHB), which induce apoptosis, also cause pancreatitis (47), suggesting a more causal role for apoptosis during pancreatitis.

A common organelle involved in both necrosis and apoptosis is the mitochondrion (7, 37, 43). During apoptosis, mito-

chondria lose transmembrane potential (9), resulting in cytochrome c release into the cytoplasm and formation of the apoptosome (12, 18, 26). The mitochondrial permeability transition pore complex (PTPC) controls transmembrane potential during apoptosis (13). PTPC opening is also implicated in necrotic cell death induced by ischemia reperfusion injury (1, 28), reactive oxygen species, and calcium (1, 28). Since the human immunodeficiency virus (HIV) protease inhibitors (PI) nelfinavir (NFV) and retonavir (RTV) prevent loss of mitochondrial transmembrane potential (29) by binding to and preventing opening of the mitochondrial PTPC (49), they may also prevent cell death. Indeed, NFV plus RTV (NFV/RTV) treatments reduce cell death and improve functional outcomes during mouse models of Fas-induced hepatitis, cerebral ischemia (49), sepsis (cecal ligation and perforation) (48), and retinal degeneration following retinal detachment (22).

Additional clinical clues that PI might benefit pancreatitis come from observations that the incidence of drug-induced pancreatitis decreased coincident with introduction of PI therapy, including NFV (8, 32). For these reasons we opted to assess whether PI might alter the outcome of experimental mouse pancreatitis.

MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee at Mayo Clinic, Rochester, MN. Male C57Bl/6 mice (Harlan Laboratories, Indianapolis, IN), 18–20 g, were housed and fed under standard conditions. The CCK analog caerulein and Boc-Glu-Ala-Arg-methyl-coumaryl-7-amide were purchased from Research Plus (Bayonne, NJ). All other reagents were purchased from Sigma (St. Louis, MO). Terminal deoxynucleotide dUTP transferase nick-end labeling (TUNEL) and active caspase 3 staining were performed at Molecular Histology (Little Rock, AR).

PI treatment and Z-VAD-fmk treatment. NFV has a short half-life in mice. RTV, another PI and CYP 3A inhibitor, increases plasma levels of NFV in mice with its own levels being undetectable; therefore we used NFV/RTV (48, 49). At 17 h before the first caerulein injection, animals were given 125 mg/kg of pediatric NFV suspension from Agouron Pharmaceuticals (La Jolla, CA) and 13 mg/kg of liquid RTV from Abbott Pharmaceuticals (Abbott Park, IL). NFV/RTV in distilled water or vehicle control (water) was given every 8 h by oral gavage, as previously described (48). Water given by gavage was used as the vehicle. Z-VAD-fmk was dissolved in DMSO at 10 mg/ml and administered intraperitoneally 30 min at 5 mg/kg (0.1 ml) as previously published (24).

Blood and tissue preparation. Animals were given hourly intraperitoneal injections of saline (control) or caerulein (50 μg/kg) in saline for 12 h, as previously described (41). They were euthanized.

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1 h after the last injection, and blood and tissue samples were harvested and frozen or fixed in formalin. For lung histology, lungs were inflated with neutral buffered formalin via tracheal puncture at 20 cm water and clamped for 10 min in situ before being harvested.

**Morphological examination.** The 5-μm sections of paraffin-embedded pancreas or lung tissue were stained with hematoxylin and eosin (H&E), active caspase 3, or TUNEL and examined by an experienced morphologist blinded to the sample identity. Acinar cell injury and/or necrosis and TUNEL positivity were quantitated by morphometry as described (3). Briefly, these and active caspase-3 were measured by imaging the whole section sequentially with a ×10 lens and storing JPEG images by a person blinded to its identity and the morphologist examining these. Nonacinar areas were excluded, and extent of injured acinar area was expressed as the percent of the total acinar tissue. Injury criteria, as previously described (2, 16), were the presence of acinar-cell ghosts with loss of cellular outline, spilage of intracellular contents, appearance of a diffuse pinkish appearance, and/or the destruction of the histology of whole or parts of the acini.

**Assays.** Serum amylase activity was measured colorimetrically using the Phadebas assay (Pharmacia Diagnostics, Portage, MI). Neutrophil sequestration in pancreas and lung was quantitated by measuring tissue MPO activity as described previously (20). Trypsin activity was measured fluorometrically, as described previously (23). Cytokines were measured by the Cytokine Core Laboratory (University of Maryland). For cytochrome c, pancreatic subcellular fractions were prepared, as previously described (26), and analyzed by Western blot using cytochrome c antibody from Oncogene (Cambridge, MA). Heat shock protein (HSP)70 was used as a mitochondrial marker, and PCNA as a cytoplasmic marker, both from Oncogene (Cambridge, MA) as previously described (29, 30).

**Preparation of acini and measurement of DiOC6 retention.** Acini were harvested in 20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, 10 mM sodium pyruvate, 0.1% bovine serum albumin, and 0.01% soybean trypsin inhibitor, as previously described (19), and filtered through a 70-μm mesh, then a 40-μm mesh, to get clusters 40–70 μm in size. Viability prior to use was >98% by Trypan blue exclusion. NFV or DMSO was added 30 min before 10 nM caerulein or saline (control) and incubated for 4 h. Retention of 3,3′-dihexyloxacarbocyanine (DiOC6) from Molecular Probes (Carlsbad, CA) was used for measuring mitochondrial membrane potential as previously described (12), despite theoretical concerns that it might not be as sensitive as other assays of mitochondrial potential.

**Measurement of TNF-α and KC mRNA.** RNA was extracted from mouse acini by use of the ToTally RNA kit from Ambion (Austin, TX, Texas). Semiquantitative PCR was done on cDNA extracted from 5 μg of RNA by use of the Platinum PCR supermix purchased from Invitrogen (Carlsbad, CA). Universal 18S internal standards (yielding a 315-bp band, used in case of keratinocyte chemoattractant (KC)), and Classic 18S internal standards (yielding a 489-bp band, used in case of TNF-α) obtained from Ambion (Austin, TX) were used at a 1:7 ratio along with KC and TNF-α primers. The KC and TNF-α primers (see sequences below) were synthesized at Sigma-Genosys (St. Louis, MO). The primers used for KC were forward 5′-TCCCCCTTCATCGTCTTATG GCCC and 5′-AACCTGGGAGTAGACAAGGTACAC, respectively. PCR was done in a 24-well thermocycler purchased from Perkin Elmer (Waltham, MA), as described previously (38), using 28 cycles (based on optimization protocol recommended in the 18S RNA Kit) with an annealing temperature of 60°C. Cyber Gold purchased from BioWhittaker (Walkersville, MD) was used to stain the bands and the intensity was measured by using The Gel Doc system from Bio-Rad (Hercules, CA).

**Analysis of data.** The results reported represent means ± SE of values obtained from three or more in vitro experiments and compared by Student’s t-test when the data consisted of only two groups or ANOVA when comparing three or more groups. Results from in vivo experiments were generated by use of six mice per group. If ANOVA indicated a significant difference, the data were analyzed by using Tukey’s method as a post hoc test for the difference between groups. A P value of 0.05 was considered significant.

**RESULTS**

NFV prevents caerulein-induced loss of mitochondrial membrane potential in mice acinar cells. We treated isolated acini with NFV alone or vehicle, followed by caerulein (10 nM), and measured mitochondrial transmembrane potential using DiOC6, as previously described (34). Although NFV did not alter mitochondrial transmembrane potential in resting acini (92.2 ± 8.0% of controls, P = 0.48), it prevented caerulein-induced (60 ± 2.6% control) loss of mitochondrial transmembrane potential (Fig. 1, P < 0.0001).

NFV prevents cytochrome c leakage and protects from caerulein injury. We next assessed whether NFV/RTV treatment of mice altered the postmitochondrial signaling events of caerulein-induced pancreatitis in vivo. Six mice per group were analyzed.

First, we measured active caspase 3 generation 12 h following caerulein since at this time point caspase 3 activation has been previously shown to be increased (10). Under control conditions there was negligible active caspase 3 staining (Fig. 2A), which increased to 13.5 ± 1.8% cells in caerulein treated mice (Fig. 2B) vs. 0.1 ± 0.02% in controls (P < 0.0001). Vehicle did not significantly affect this increase (14.4 ± 1.2%, P = 0.88, Fig. 2C); however, both Z-VAD-fmk (Fig. 2D) and NFV (Fig. 2E) reduced this significantly to 5.4 ± 1.9% (P < 0.02) and 7.6 ± 1.9% (P < 0.005), respectively (Fig. 2F).

Next, we measured cytochrome c release into the cytosol (Fig. 2, G and H) by Western blots of mitochondrial and plasma fractions of mice. The nuclear fraction was used as a control. Under control conditions there was negligible cytochrome c staining (Fig. 2G), which increased to 13.5 ± 1.8% cells in caerulein treated mice (Fig. 2H) vs. 0.1 ± 0.02% in controls (P < 0.0001). Vehicle did not significantly affect this increase (14.4 ± 1.2%, P = 0.88, Fig. 2I); however, both Z-VAD-fmk (Fig. 2J) and NFV (Fig. 2K) reduced this significantly to 5.4 ± 1.9% (P < 0.02) and 7.6 ± 1.9% (P < 0.005), respectively (Fig. 2L).

**Fig. 1.** Nelfinavir (NFV) stabilizes mitochondrial membrane potential in acinar cells. Acini were preincubated with saline (CON) or 30 micromolar NFV for 30 min (solid bars) followed by addition of 10 nM caerulein (Cer) for 3 h as indicated. 3, 3-Dihexyloxacarbocyanine (DiOC6) retention was measured fluorometrically, as described previously (23). Although NFV did not alter mitochondrial transmembrane potential in resting acini, it prevented caerulein-induced (60 ± 2.6% control) loss of mitochondrial transmembrane potential (Fig. 1, P < 0.0001).
cytoplasmic fractions of pancreatic tissues. PCNA and HSP70 were used to confirm the purity of the cytoplasmic and mitochondrial fractions. Cytochrome c was minimally present in the cytosolic fractions of control cells yet significantly increased in the tissues from mice with caerulein-induced pancreatitis. Prior administration of NFV/RTV reduced cytochrome c release to the level of untreated control cells (Fig. 2, G and H).

NFV/RTV also decreased the percentage of TUNEL-positive cells (2.78%) compared with the vehicle-treated animals (7.04%, \( P < 0.0005 \)) or caerulein-only groups (Fig. 3B) (6.71%, \( P < 0.04 \)). As a positive control for inhibiting apoptosis, ZVAD-fmk also reduced the number of TUNEL-positive cells to 0.5% (\( P < 0.02 \)) (Figs. 3, A–F).

In addition to reducing TUNEL positivity, acinar injury as determined by H & E staining was reduced (\( P < 0.03 \)) by NFV/RTV treatment (7.6%), compared with the caerulein (24.9%), or caerulein + vehicle (26.8%)-treated groups (Figs. 3, G–L).

NFV’s protection from acinar cell injury is independent of proinflammatory cascades and trypsin generation. The pathogenesis of pancreatitis is felt to involve local pancreatic injury followed by local and systemic inflammation. Having shown that NFV/RTV protect against acinar injury, we next studied their effects on systemic amylase levels and on inflammation. Reductions in cell death were accompanied by a reduction in serum amylase from 17.1 to 2.4-fold control in caerulein and 19.0 to 2.6-fold in caerulein+vehicle-treated animals to 12.4 to 1.5-fold in NFV-treated animals (\( P < 0.05 \)) (Fig. 4A).

NFV/RTV and Z-VAD-fmk did not alter the increase in pancreatic MPO levels (Fig. 4C) or pancreatic edema (Fig. 4B) during pancreatitis. In addition, since trypsin contributes to the pathogenesis of pancreatitis (35), we measured its activity in pancreatic homogenates and found that trypsin activity was unaffected (Fig. 4D) either by NFV/RTV or, predictably, by Z-VAD-fmk. In vitro, trypsin activity was not impacted by NFV (data not shown). Also, NFV/RTV did not affect the caerulein-induced degradation of IK-B\( \alpha \) (results not shown).
Systemic inflammation was measured by analyzing serum levels of IL-6, TNF-α, MCP-1, IL-10, IL-1β, or KC (data not shown). In all cases, serum levels of these cytokines were not altered by NFV pretreatment. Inflammation in a distal site was assessed in the lung by histology and MPO activation. Lung histologies were similar in caerulein-treated animals with or without Z-VAD or NFV/RTV (Fig. 5, A–E). Likewise, lung MPO activity remained similar in caerulein-treated animals irrespective of whether they received ZVAD or NFV/RTV (Fig. 5F).

Fig. 3. Terminal deoxynucleotide dUTP transferase nick-end labeling (TUNEL) staining and histology [hematoxylin and eosin (H&E)] showing pancreatic acinar apoptosis and injury are reduced by NFV pretreatment. Pancreas was harvested 1 h after the last of a series of 12 hourly intraperitoneal injections of saline (A), 50 μg/kg caerulein (B), animals pretreated with water before administration of caerulein (C), and animals pretreated with Z-VAD-fmk (D) or NFV/RTV (E) before administration of caerulein. These were fixed and stained for TUNEL as described in MATERIALS AND METHODS. F: quantitation of these apoptotic nuclei depicted as means ± SE. G–L: acinar injury as determined by H & E staining as indicated. *Significant (P < 0.05) reduction compared with untreated caerulein- or vehicle-treated groups.
Fig. 4. NFV/RTV reduce serum amylase without affecting the increase in trypsin, pancreatic water content, or neutrophil infiltration. Serum amylase (A), pancreatic water content (B), or pancreatic MPO levels (C) were measured in control animals or those which received 50 μg/kg caerulein intraperitoneal every hour for 12 h, caerulein after pretreatment with vehicle (Veh) before the induction of pancreatitis, or pretreatment with Z-VAD-fmk NFV/RTV. *P value <0.05 compared with vehicle-treated group. D: intrapancreatic trypsin activity measured in control mice or 30 min after receiving 50 μg/kg ip caerulein, or those pretreated with vehicle, Z-VAD-fmk, or NFV/RTV before the induction of pancreatitis. *P value <0.05 compared with vehicle-treated group.

Fig. 5. Lung inflammation as seen on histology and neutrophil infiltration as measured by MPO are unaffected by NFV/RTV. Representative ×250 images of lungs harvested, fixed, and stained with H&E 1 h after the last of a series of 12 hourly intraperitoneal injections of saline (A), 50 μg/kg caerulein (B), animals pretreated with vehicle before administration of caerulein (C), and animals pretreated with NFV/RTV (D) or Z-VAD-fmk (E) before administration of caerulein. F: bar graph of MPO activity per mg protein. The different groups are saline (CON), 50 μg/kg caerulein, animals pretreated with vehicle before administration of caerulein, and animals pretreated with Z-VAD-fmk or NFV/RTV before administration of caerulein.
Although it remains controversial what role apoptosis vs. necrosis play in the pathogenesis of pancreatitis, it is apparent that both processes do occur, although to different degrees in different experimental models. In this study we have used ZVAD-fmk treatments as a means of inhibiting the consequences of caspase activation that occur during apoptosis. Consistent with ZVAD-fmk selectively inhibiting caspases, mice receiving ZVAD-fmk in addition to caerulein had less caspase 3 activation, TUNEL positivity, and overall pancreatic injury compared with caerulein alone. These data suggest that, in this model, apoptosis contributes to pancreatic injury. Moreover, and consistent with our underlying hypothesis, treatment with PI of isolated acini in vitro, or mice in vivo, results in reduced acinar injury, caspase 3 activation, and TUNEL positivity. These observations are also consistent with observations in patients receiving PI: 1) The use of HIV PI such as NFV has reduced the incidence of pancreatitis in HIV-infected patients from as high as 14% in the pre-highly active antiretroviral therapy era to rates as low as 0.13, 0.35, and 0.85% since 1996 (8, 31, 33, 40). 2) Nucleoside reverse transcriptase inhibitors such as didanosine (ddI), stavudine (4dT), and agents such as azathioprine that are mitochondrialtoxic cause pancreatitis (25, 44). 3) NFV reduces incidence of pancreatitis caused by these agents (e.g., from 4.93% with ddI/d4T/efavirenz to 0% with NFV included, and from 6.25% in all regimens with ddI/d4T to 2.05% with NFV included in these) (32). In addition to our observations concerning the effect of NFV/RTV on acinar injury, we observed, somewhat unexpectedly, no effect on pancreatic inflammation, edema, serum cytokines, and pulmonary inflammation. This suggests that the processes that promote acinar cell injury and inflammation are separable, as has been previously speculated (46). This is further supported by observations that stimuli that cause inflammation do not necessarily cause pancreatitis (see Supplementary Fig. S1).1 It is therefore tempting to speculate that stimuli that induce pancreatic cell death might also independently induce inflammation in an unrelated way. This might be tested, for example, by determining whether caerulein causes systemic inflammation in mice who had previously received pancreatectomies.

In summary, NFV protects pancreatitis by preventing loss of mitochondrial membrane potential, thus reducing acinar cell injury, without interfering with caerulein-induced inflammatory pathways.

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1 Supplemental data for this article are available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website.

REFERENCES


