Menadione-induced Reactive Oxygen Species Generation via Redox Cycling Promotes Apoptosis of Murine Pancreatic Acinar Cells*

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Oxidative stress may be an important determinant of the severity of acute pancreatitis. One-electron reduction of oxidants generates reactive oxygen species (ROS) via redox cycling, whereas two-electron detoxification, e.g. by NAD(P)H:quinone oxidoreductase, does not. The actions of menadione on ROS production and cell fate were compared with those of a non-cycling analogue (2,4-dimethoxy-2-methylnaphthalene (DMN)) using real-time confocal microscopy of isolated perfused murine pancreatic acinar cells. Menadione generated ROS with a concomitant decrease of NAD(P)H, consistent with redox cycling. The elevation of ROS was prevented by the antioxidant N-acetyl-l-cysteine but not by the NADPH oxidase inhibitor diphenyliodonium. DMN produced no change in reactive oxygen species per se but significantly potentiated menadione-induced effects, probably via enhancement of one-electron reduction, since DMN was found to inhibit NAD(P)H:quinone oxidoreductase detoxification. Menadione caused apoptosis of pancreatic acinar cells that was significantly potentiated by DMN, whereas DMN alone had no effect. Furthermore, bile acid (tauro lithocholic acid 3-sulfate)-induced caspase activation was not affected by menadione. These results suggest that acute generation of ROS by menadione occurs via redox cycling, the net effect of which is induction of apoptotic pancreatic acinar cell death. Two-electron detoxifying enzymes such as NAD(P)H:quinone oxidoreductase, which are elevated in pancreatitis, may provide protection against excessive ROS and exert an important role in determining acinar cell fate.

Acute pancreatitis is a severe and debilitating inflammatory disease with a steadily increasing incidence and significant mortality (1, 2). Oxidative stress has been implicated in the development of acute pancreatitis in diverse animal experimental models, including fatty acid infusion, ischemia, pancreatic duct obstruction, gallstone pancreatitis, and alcohol ingestion (3–6), and measured in patients with mild and severe acute pancreatitis (7). While evidence suggests that oxidative stress may be an important determinant of disease severity (7, 8), the precise underlying mechanisms are still poorly understood and the extent of involvement unclear (9).

We have previously shown that the oxidant menadione (2-methyl-1,4-naphthoquinone) induces Ca2+-dependent apoptosis in pancreatic acinar cells via opening of the mitochondrial permeability transition pore (10). A variety of reductive enzymes, including microsomal NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase (complex I), are known to metabolize quinones by one-electron reduction reactions (11). The resultant unstable semiquinones can readily enter into a redox cycle when molecular oxygen is present, causing a reformation of the quinone, with the concomitant generation of reactive oxygen species (ROS). Since these enzymes use NAD(P)H as an electron donor, mitochondrial levels of these co-substrates should decrease as ROS are generated by metabolism of oxidants. Other reductive enzymes, such as NAD(P)H:quinone oxidoreductase 1 (EC 1.6.99.2, NQO1; DT-diaphorase), metabolize quinones via a two-electron reduction in which a stable hydroquinone is formed without ROS generation. NQO1 is thought to constitute an endogenous cellular detoxifying mechanism (12, 13) and is increased in both acute pancreatitis and pancreatic adenocarcinoma (14, 15).

The role of apoptosis in pancreatitis remains controversial. It has been suggested that while acinar cell necrosis is a key contributor to disease severity, apoptosis protects against the disease (16). Thus, prior induction of pancreatic acinar cell apoptosis protected mice against cerulein-induced pancreatitis (17), while inhibition of caspase activity elicited necrotizing pancreatitis (18). An important potential link between ROS

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3 The abbreviations used are: ROS, reactive oxygen species; DMN, 2,4-dimethoxy-2-methylnaphthalene; CM-H2DCFDA, 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; FITC, fluorescein isothiocyanate; TLC-S, tauro lithocholic acid 3-sulfate; NAC, N-acetyl-l-cysteine; DPI, diphenyliodonium chloride; NQO1, NAD(P)H:quinone oxidoreductase 1.
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generation and apoptosis has been suggested from evidence in T-cells (19) and a leukemia cell line (20).

To investigate the “redox” effects of menadione, a potentially non-cycling analogue of menadione was designed in which the 1,4-dione groups of menadione were protected to give the corresponding 2,4-dimethoxy-2-methylnapthalene (DMN; Fig. 1A). DMN was modeled into the three-dimensional structure of NQO1 to ensure that it could counteract the reductive potential of menadione at the active site (Fig. 1B). In doing so, it was envisaged that menadione would then be commandeered by one-electron reductive pathways in live pancreatic acinar cells rather than 2-electron (detoxification) pathways, thereby causing greater ROS production (Fig. 1C). Using an experimental approach, which allowed simultaneous measurement of ROS generation and mitochondrial NAD(P)H levels, we show that acute generation of ROS, due to metabolism of menadione via a redox cycle, promotes apoptosis in pancreatic acinar cells and that inhibition of NQO1 enhances this process.

EXPERIMENTAL PROCEDURES

Cell Preparation—Freshly isolated pancreatic acinar cells and acinar clusters of two or three cells were prepared from the pancreases of adult CD1 mice using collagenase (Worthington Biochemical Corp., Lakewood, NJ) as in our previous work (21). All experiments were performed at room temperature (23–25 °C), and cells were used within 4 h of isolation. The extracellular solution contained (mm): 140 NaCl, 4.7 KCl, 1.13 MgCl₂, 1 CaCl₂, 10 d-glucose, 10 HEPES (adjusted to pH 7.35 using NaOH) (all chemicals highest grade available from Sigma, Gillingham, UK).

Measurements of ROS, Cytosolic Calcium ([Ca²⁺]c), and Mitochondrial NADH—Confocal imaging of cells loaded with fluorescent dyes was performed using a Zeiss LSM510 system (Carl Zeiss Jena GmbH). Freshly dispersed cells were loaded with either 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA; 2.5 μM) for 10 min at 37 °C, or Fluo 4-AM (3 μM) for 30 min at room temperature, to measure reactive oxygen species and [Ca²⁺]c, respectively. The fluorescence of CM-H₂DCFDA or Fluo 4 was excited by an argon 488 nm laser line and emitted light collected from 505–550 nm. The images obtained were composed of 256 × 256 pixels and the optical section selected to be 5–6 μm. A C-Apochromat × 63 objective with a numerical aperture of 1.2 was used in all experiments and image analysis carried out using Zeiss confocal LSM510 image software. In [Ca²⁺]c measurement experiments 50 μM acetylcholine was applied as a positive control to ensure that the cells were capable of generating a Ca²⁺ response.

Mitochondrial metabolism was assessed contemporaneously by monitoring changes of NADH autofluorescence in the perigranular region of the acinar cells using the fast switching multichannel mode of the Zeiss LSM510 confocal microscope system (excitation 363 nm, emission 390–450 nm) as reported previously (22). All fluorescence measurements are expressed as changes from basal fluorescence (F/F₀ ratio), where F₀ represents the initial fluorescence recorded at the start of the experiment and F the fluorescence recorded at specific time points (“n” represents the number of cells studied for each experimental protocol).

Detection of Apoptosis—Detection of apoptosis was performed using the annexin V FITC apoptosis kit (CloneTech, Palo Alto, CA) and propidium iodide (10 μM) exclusion employed to eliminate necrotic cells; early apoptotic and non-apoptotic cells do not show staining with propidium iodide. Isolated cells were divided into a number of samples: 1) control cells, 2) cells incubated with 30 μM menadione for 30 min, 3) cells incubated with 30 μM DMN analogue for 30 min, 4) cells incubated with both 30 μM menadione and 30 μM DMN analogue for 30 min, and all incubation procedures were carried out in the dark at room temperature.

Cells from each sample were washed using standard buffer and centrifuged at 1000 × g for 1 min. The pellet was then re-suspended in 150 μl of binding buffer, and incubated with 1.25 μl of annexin V FITC conjugate for 15 min, in the dark, at room temperature. The cells then washed with binding buffer and used in analysis. Fluorescence measurements were performed using a Leica TCS SP2 confocal microscope with a 63× water immersion objective, aperture set at 1.2. Annexin was measured at 488 nm excitation and 504–553 nm emission. Propidium iodide was measured at 488 nm excitation and 630–693 nm emission. Levels of apoptosis were expressed as annexin FI intensity (arbitrary units).

In separate experiments, cells were loaded with R110-aspartic acid amide (20 μM), a fluorescent indicator-linked general caspase substrate, at room temperature for 30 min. After loading, cells were washed and re-suspended in calcium-free buffer solution. A portion of cells were then treated with 300 μM taurilolcholic acid 3-sulfate (TLCS-S) alone, 300 μM TLC-S + 30 μM DMN, or 30 μM DMN alone, for 30 min at room temperature before fluorescence was recorded (excitation 488 nm, emission >505 nm) with a Leica SP2 confocal microscope. Changes in fluorescence have been normalized from basal R110-aspartic acid amide intensity values (F/F₀).

NQO1 Inhibition Assay—The ability of DMN to inhibit NQO1 was determined by measuring the reduction of cytochrome c at 550 nm on a Beckman DU650 spectrophotometer using a modification of a method described previously (23). Briefly, each assay contained cytochrome c (70 μmol) and recombinant human NQO1 (1 μg) in Tris-HCl buffer (50 mM, pH 7.5) containing NADH (100 μM), with varying concentrations of DMN and menadione at a final volume of 1 ml. The reactions were performed at room temperature over a period of 30 s (rates of reduction were calculated from the initial linear part of the reaction curve). All reactions were carried out in triplicate. The IC₅₀ value of DMN was obtained from the enzyme inhibition curve.

Computational Chemistry—The three-dimensional structures of the ligands (menadione and DMN) were constructed using ChemSketch freeware (Advanced Chemistry Development version 8.0) and energy-minimized. The x-ray crystallographic crystal structure of human NQO1 (Protein Data Bank file 1H69) was downloaded from the RCSB Protein Data Base and edited accordingly to provide a monomer of the protein which included the bound FADH₂ as cofactor. Both ligands (menadione and DMN) were flexibly docked using the MolDock algorithm (24). The docking procedure was randomized with a minimum of
When molecular oxygen is present. Menadione-induced Reactive Oxygen Species

**RESULTS**

Since three-dimensional molecular modeling studies suggested that DMN would inhibit menadione metabolism by the NQO1 enzyme (Fig. 1B), the effects of DMN (0.5–250 μM) as an inhibitor of purified recombinant human NQO1 were evaluated *in vitro* using menadione as the substrate, as reported previously (23). It was found that DMN inhibited NQO1 in a concentration-dependent manner over the micromolar range, with an IC₅₀ value of 46.7 ± 9.1 μM (Fig. 2). The actions of menadione and DMN were then investigated on generation of ROS, mitochondrial metabolism, and cell fate.

Stable baseline fluorescence was observed in cells loaded with CM-H₂DCFDA, indicating no auto-oxidation of the dye. Application of menadione (30 μM) induced a clear and significant (p < 0.05) rise of fluorescence that was generalized throughout the cell, indicative of an elevation of ROS (Fig. 3A; 8 of 8 cells). This increase in ROS started immediately after exposure to menadione and continued until a maximum level was attained at ~5 min after application.

Mitochondrial NAD(P)H levels may be measured concomitantly in pancreatic acinar cells as autofluorescence in the perigranular region of acinar cells, using the fast switching mode of the Zeiss LSM510 confocal system (22). Menadione caused a decrease of mitochondrial NAD(P)H autofluorescence (Fig. 3C) that was associated with the rise of ROS, consistent with substrate usage by reductive enzymes during redox cycling. When menadione was washed out the NADH levels began to recover, indicating the reversible nature of this effect (Fig. 3C).

The antioxidant N-acetyl-L-cysteine (NAC; 10 mM), applied 3 min before and during menadione application, induced a slight decline of basal H₂DCFDA fluorescence *per se* which was more evident in the basolateral regions of the cells, indicative of a scavenging action on basal ROS production (Fig. 4A). The menadione-induced elevation of ROS was completely prevented by the presence of NAC (Fig. 4, A and B; 16 of 16 cells, p < 0.05). After withdrawal of both NAC and menadione from the perfusion medium, a “rebound” increase in production of ROS was observed, with greater intensity in the perigranular mitochondrial region of the cells (Fig. 4, A and B). In contrast, the NAD(P)H oxidase inhibitor diphenyliodonium chloride (DPI; 10 μM), induced no change of basal H₂DCFDA fluorescence.
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FIGURE 5. Effects of DMN on ROS generation in pancreatic acinar cells. A, representative traces showing the lack of increase in CM-H$_2$DCFDA fluorescence expressed as changes from basal fluorescence ($F/F_0$) in an acinar doublet exposed to 30 μM DMN alone. In the presence of DMN, however, 30 μM menadione-induced ROS generation was potentiated. B, mean data from eight cells indicating a significant increase in CM-H$_2$DCFDA fluorescence with DMN-menadione combination compared with menadione alone (* indicates $p < 0.05$; data are expressed as mean ± S.E.). C, typical traces showing the increase of NAD(P)H autofluorescence in an acinar doublet induced by 30 μM DMN. In the presence of DMN, 30 μM menadione sharply decreased NAD(P)H autofluorescence, the rate of which was significantly greater than that obtained by menadione alone. Mean data from 8 cells are shown in D (* indicates $p < 0.05$).

decided to investigate the effects of DMN on [Ca$^{2+}$]$_c$ for comparison. Acetylcholine, applied at a quasi-physiological concentration (50 nm) as a control, induced typical oscillatory elevations of [Ca$^{2+}$]$_c$ (Fig. 6A), which were associated with an increase in NADH autofluorescence (Fig. 6B), consistent with stimulus-metabolism coupling as reported previously (22, 26). In contrast, DMN elicited no change in [Ca$^{2+}$]$_c$ in the majority of cases (Fig. 6A; 20 of 31 cells), whereas the remainder exhibited only occasional, transient increases of [Ca$^{2+}$]$_c$ with no discernible pattern. In contrast, mitochondrial NADH levels were always increased in a sustained manner on application of DMN (31 of 31 cells; Figs. 6B and 5C), suggesting a Ca$^{2+}$-independent mechanism of action.

Menadione (30 μM) induced apoptosis in pancreatic acinar cells, as indicated by a significant increase of annexin staining compared with non-treated control cells that was predominantly in the cell membrane (Fig. 7, A and B, $n = 84$; $p < 0.01$), as described previously (10). In contrast, the non-redox cycling analogue DMN (30 μM) did not induce apoptosis, with annexin staining not significantly different from controls (Fig. 7B, $n = 81$; $p > 0.05$). However, in the presence of DMN, menadione-induced apoptosis was greatly potentiated (2-fold) compared with menadione alone (Fig. 7B, $n = 83$; $p < 0.01$).

To investigate whether inhibition of NQO1 enzyme would also modify the effects of bile acids, recognized precipitants of acute pancreatitis (27), on acinar cell fate, the activation of caspases by TLC-S was assessed. TLC-S (300 μM) induced caspase activation in pancreatic acinar cells, as indicated by a significant increase of R110-aspartic acid amide fluorescence compared with control levels (Fig. 8, A and B, $n = 57$). DMN (30 μM) alone produced no caspase activation; however, it greatly increased the response to TLC-S compared with TLC-S alone (Fig. 8, A and B, $n = 56$; $p < 0.01$).

DISCUSSION

Our present findings show a major link between the acute production of ROS induced by redox cycling of oxidants and apoptotic pancreatic acinar cell death. This generation of free radicals occurs within minutes of application of menadione and is fuelled by mitochondrial production of NAD(P)H co-substrates that serve as electron donors for reductive enzymes. Menadione-induced increases of ROS in the acinar cells were clearly abolished by the antioxidant N-acetylcysteine, a free radical scavenger and precursor of the ROS scavenger glutathione (28), which has been shown to block ROS production induced in rat pancreatic acinar cells by pancreatic duct obstruction (29) and in HepG2 cells by cytochrome P450 2E1 (30).

In contrast, such increases were unaffected by DPI, widely employed as a blocker of membrane NADPH oxidase (31, 32), although this compound does inhibit other flavoenzymes such as mitochondrial NADH dehydrogenase (32, 33). Recent studies have demonstrated NAD(P)H oxidase activity (34) and inhibition of superoxide production by DPI in a pancreatic cancer cell line (35). Current evidence suggests that ROS production is enhanced in neutrophils obtained from patients with acute pancreatitis (36), and ROS derived from neutrophil NADPH oxidase may play an important role in implementing damage in experimental acute pancreatitis (37). The lack of effect of DPI observed in the present study would strongly suggest that acinar cell NAD(P)H oxidase is not responsible for the generation of ROS by menadione.

Chemical modification of the menadione molecule to form DMN blocked the capacity of this oxidant to enter into a redox cycle and led to significant physiological consequences; ROS were no longer generated in the acinar cells nor was apoptosis induced. Interestingly, DMN elicited a reversible, Ca$^{2+}$-independent increase of NAD(P)H in the mitochondrial region of the cell per se, indicating that it might block an endogenous reductive process. Since DMN did not inhibit menadione-induced ROS production but rather potentiated it, it would appear that it does not compete for one electron reductive enzyme target(s) of menadione but instead inhibits a two-electron detoxifying enzyme such as NQO1, expressed in pancre-
acicinar cells (15) and which utilizes NADH and NADPH as co-factors (38). Analysis of human recombinant NQO1 in vitro confirmed an inhibitory action of DMN on this enzyme (Fig. 2).

NQO1 is protective against menadione-induced oxidative stress in P19 embryonal carcinoma cells (39) and may be an endogenous mechanism that protects cells against the toxicity of electrophiles and reactive forms of oxygen in general (12, 40). This enzyme is overexpressed in many tumors and constitutes a potential target for cancer chemotherapy (41). Interestingly, a recent study has demonstrated that this reductive enzyme is elevated in pancreatitis (14) and may thus play a significant role in pancreatic acinar cell protection. The affinity of menadione for hepatic NQO1 is thought to be more than ten times greater than its affinity for microsomal NADPH-cytochrome P450 reductase (40), and further investigation is clearly required to clarify the potential importance of such an endogenous detoxifying mechanism to counter oxidative stress in the pancreas.

Molecular modeling of potential interactions between DMN and NQO1 predicts that three important hydrogen-bonding sites exist that would stabilize the presence of DMN within the enzyme, inhibiting the entry and subsequent metabolism of menadione (Fig. 1, B and C). Inhibition of such a two-electron detoxifying enzyme in the present experiments would have two major predicted consequences, both of which would be expected to potentiate ROS production. First, NAD(P)H levels would be elevated since enzymatic usage of these electron donors by two-electron reductive enzymes would be diminished, thereby providing greater reductive potential to drive one-electron metabolism of menadione. Second, menadione would now be preferentially shunted through one-electron reductive system(s) releasing more ROS. Both a potentiation of ROS generation and accelerated usage of NAD(P)H were observed in the combined presence of menadione and DMN in isolated pancreatic acinar cells, consistent with a model in which two-electron metabolism of menadione is inhibited by DMN (Fig. 1C). In accordance, treatment of pancreatic cancer cells with the nonspecific NQO1 inhibitor dicoumarol induced both oxidative stress and apoptosis (42). Interestingly, the potentiation of apoptosis by inhibition of NQO1 in the present study was not confined to the oxidant menadione, since DMN also greatly augmented caspase activation induced by TLC-S. This bile acid has been shown to induce apoptosis of hepatocytes via activation of caspases 8, 9, and 3 (43), an effect associated with oxidative stress (44), although this action of bile has previously not been reported in pancreatic acinar cells. Our current data therefore underscore the potential relevance of the detoxifying enzyme NQO1 as an important determinant of pancreatic acinar cell fate in pathological situations, since bile acids are recognized precipitants of acute pancreatitis (27), and whose detrimental actions may involve oxidant stress in vivo (6).
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Previously we have demonstrated that Ca\(^{2+}\)-dependent opening of the permeability transition pore by menadione is linked to the development of apoptosis (10). In the present study, the pro-apoptotic action of menadione was greatly potentiated by the presence of DMN, in association with an enhanced ROS production. It is thought that opening of the permeability transition pore is favored by oxidative stress via the oxidation of intracellular glutathione and other sulfydryl groups (45, 46), and our present data are in accord with this notion. Furthermore, a recent study in HepG2 cells suggests that cadmium-induced ROS generation may trigger apoptosis via a caspase-dependent pathway (47). Whether the entry of cells into apoptosis is beneficial in the development of acute pancreatitis is currently unresolved, however, several lines of evidence suggest that this may inhibit the formation of cellular necrosis, thereby limiting organ damage and improving disease outcome (17, 18).

In conclusion, the present data suggest that acute endogenous generation of ROS in live pancreatic acinar cells may constitute a natural pathway by which acinar cells promote programmed cell death. The production of ROS by menadione occurs via redox cycling, carried out by one-electron reductive enzymes. Two-electron detoxifying reductive enzymes such as NQO1, which are elevated in pancreatitis (14), may provide protection against excessive ROS generation and therefore play an important role in determining acinar cell fate.

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