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FORUM REVIEW ARTICLE

Calcium and Reactive Oxygen Species in Acute Pancreatitis: Friend or Foe?

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Abstract

Significance: Acute pancreatitis (AP) is a debilitating and, at times, lethal inflammatory disease, the causes and progression of which are incompletely understood. Disruption of Ca^{2+} homeostasis in response to precipitants of AP leads to loss of mitochondrial integrity and cellular necrosis. ***Recent Advances:*** While oxidative stress has been implicated as a major player in the pathogenesis of this disease, its precise roles remain to be defined. Recent developments are challenging the perception of reactive oxygen species (ROS) as nonspecific cytotoxic agents, suggesting that ROS promote apoptosis that may play a vital protective role in cellular stress since necrosis is avoided. ***Critical Issues:*** Fresh clinical findings have indicated that antioxidant treatment does not ameliorate AP and may actually worsen the outcome. This review explores the complex links between cellular Ca^{2+} signaling and the intracellular redox environment, with particular relevance to AP. ***Future Directions:*** Recent publications have underlined the importance of both Ca^{2+} and ROS within the pathogenesis of AP, particularly in the determination of cell fate. Future research should elucidate the subtle interplay between Ca^{2+} and redox mechanisms that operate to modulate mitochondrial function, with a view to devising strategies for the preservation of organellar function. *Antioxid. Redox Signal*. 15, 2683–2698.

Pancreatitis: Incidence and Problem

Acute pancreatitis (AP) is a devastating and debilitating disease, the pathophysiology of which still remains unclear. It currently affects 50–100 per 100,000 people annually, and is predominantly caused by gallstones or alcohol excess. Importantly, the incidence of AP is rapidly increasing, a phenomenon that is likely to be at least partly due to increased alcohol consumption. Approximately 20% of patients with AP develop a severe form of the disease, characterized by pancreatic necrosis and multiple organ failure, which carries substantial morbidity and mortality. Although overall pancreatitis has a mortality rate of ~5% (5), the presence of necrosis raises this to 17% (118). Complications such as infected necrosis may further raise the risk of mortality. Despite the considerable social and economic burden associated with the disease, there remains no specific therapy for AP, highlighting the fundamental need for detailed research into the pathogenesis of pancreatitis to identify potential drug targets for translational approaches.

The exocrine pancreas is composed primarily of pancreatic acinar cells, which comprise over 90% of the gland and are considered the primary site of local injury in AP. Over a decade ago, we proposed that disruption of acinar cell Ca^{2+} homeostasis underlies the development of pancreatic injury (167). Subsequent studies have confirmed the central role of Ca^{2+} in the pathogenesis of AP as induced by various precipitants, including caerulein hyperstimulation (82, 133), bile salts (78, 164), nonoxidative alcohol metabolites (fatty acid ethyl esters [FAEEs]), and fatty acids (FAs) (27, 28). Sustained, global elevations of Ca^{2+} induced by these diverse agents led to premature Ca^{2+}-dependent activation of zymogen granules (ZG), vacuole formation, and cell death (82, 133).

Under conditions in which tissue antioxidant levels are low, or the production of reactive oxygen species (ROS) exceeds the normal range of antioxidant capacity, oxidative stress develops (59). Several decades ago a role for ROS was proposed in AP, based on results derived from the application of various precipitants, including infusion of oleic acid and ductal obstruction, in a canine model (139). Subsequent studies have demonstrated increased oxidative stress linked to AP, including that induced by ductal obstruction (160) and taurocholate (134), the latter study concluding that ROS were involved in mediating tissue damage but were not a trigger for development of AP *per se*. Further, scavenging of ROS by antioxidants did not reduce acinar cell damage in two models of AP, with only a reduction of oedema observed (150). Thus, the precise involvement of ROS in the pathogenesis of AP has remained unclear.

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Interestingly, extensive evidence suggests that Ca^{2+} signaling and ROS are intimately linked (16), with many facets of Ca^{2+} homeostasis sensitive to the cellular redox status (Fig. 1). In view of the fundamental role that Ca^{2+} overload plays in the development of AP, this review will discuss the roles of Ca^{2+} and ROS in the pathophysiology of the exocrine pancreas, their interaction to modulate cell death pathways, and potential for therapeutic manipulation.

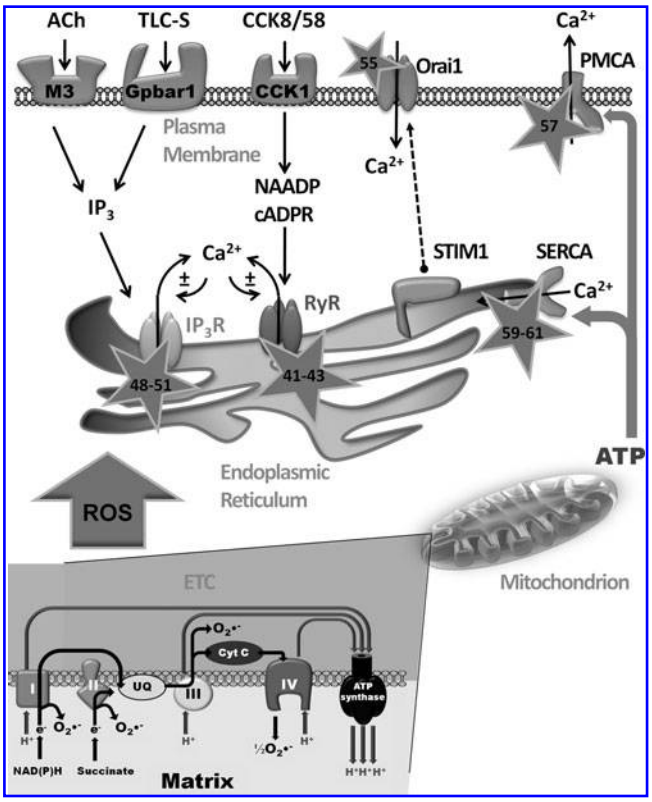

FIG. 1. Interplay between reactive oxygen species (ROS) and Ca^{2+} signaling. Physiological stimulation of pancreatic acinar cells by acetylcholine (ACh) and cholecystokinin (CCK), acting at muscarinic receptors (M_3) and cholecystokinin (CCK_1) receptors, respectively, raises intracellular Ca^{2+} levels. Bile acids, such as taurolithocholic acid 3-sulfate (TLC-S), act at Gpbar1 receptors. In response to receptor stimulation, the second messengers inositol 1, 4, 5 trisphosphate (IP_3), nicotinic acid adenine dinucleotide phosphate (NAADP), and cyclic ADP ribose (cADPR) are generated releasing Ca^{2+} from intracellular stores *via* the IP_3 receptor (IP_3R) and the ryanodine receptor (RyR); Ca^{2+} release may further precipitate Ca^{2+}-induced Ca^{2+}-release (CICR) contributing to depletion of the endoplasmic reticulum (ER) store. The fall in ER Ca^{2+} is sensed by stromal interaction molecule 1 (STIM1) protein that translocates to the plasma membrane and couples with Orai units, allowing store-operated Ca^{2+} entry to ensue and refilling of internal stores. Sustained rises of cytosolic Ca^{2+} are normally limited by clearance of Ca^{2+} to the cell exterior *via* the plasma membrane Ca^{2+}-ATPase (PMCA) or taken up into the ER store by the sarcoendoplasmic Ca^{2+}-ATPase (SERCA). ROS produced by the mitochondrial electron transport chain (ETC) may interact with targets involved in cellular Ca^{2+} homeostasis, thereby modifying their activity (stars: numbers represent reference material; see text for details).

Ca^{2+} Signaling in Health and Disease

Physiological Ca^{2+} signaling

The pancreatic acinar cell is a polarized, nonexcitable secretory unit that displays perfectly organized morphology so that spatiotemporally defined Ca^{2+} signals are generated in response to appropriate physiological neuro-hormonal stimulation (Fig. 1) (121, 122). Thus, cholecystokinin (CCK) and acetylcholine (ACh) stimulate their respective membrane receptors on human (104) and rodent acinar cells (24) to cause discrete oscillatory elevations of Ca^{2+} that are restricted to microdomains (123), which lead to fusion of ZG membranes with the apical membrane, (41) resulting in the exocytosis of inactive digestive enzyme precursors into the pancreatic duct. Recent evidence, however, suggests that transient global Ca^{2+} signals may be required for physiological enzyme secretion, whereas local signals generate primarily fluid secretion (89). A combination of type 2 and 3 inositol 1, 4, 5 trisphosphate (IP_3) receptors (IP_3R) may be essential for Ca^{2+} signals linked to exocrine secretion in pancreatic acinar cells, since responses to CCK and ACh were absent in double knockout mice, but not in single knockouts for each individual IP_3R subtype (44).

Physiological Ca^{2+} signals are generally confined to the apical pole by a buffer barrier of perigranular mitochondria. These organelles temporarily uptake Ca^{2+} released from the endoplasmic reticulum (ER), upregulating ATP production, and inhibiting global waves from permeating to the basolateral area (157, 165). When stimulation reaches sufficient intensity, or when mitochondria are inhibited, this barrier is overwhelmed and local repetitive Ca^{2+} responses in the granular area are transformed into global Ca^{2+} waves, underlining the complex role of mitochondria in physiological acinar cell function.

It is paramount that homeostatic mechanisms are maintained to prevent sustained global Ca^{2+} elevations and preserve mitochondrial function. Therefore, Ca^{2+} must be cleared continuously from the cytosol to restore resting state. Thus, Ca^{2+} is returned to the ER stores by the sarcoendoplasmic Ca^{2+}-ATPase (SERCA) or extruded by the plasma membrane Ca^{2+}-ATPase (PMCA), processes that consume ATP and are dependent upon fully functioning mitochondria (Fig. 1). Mitochondrial ATP production and bioenergetic regulation of the cell are influenced by Ca^{2+} continually leaked from the IP_3R into the mitochondria (19) and the resting Ca^{2+} is dictated by a balance between leak, extrusion/reuptake mechanisms, and Ca^{2+} entry. Ca^{2+} must periodically be allowed into the cell to refill intracellular stores *via* store-operated Ca^{2+} entry (SOCE), which has recently been shown to occur by the interaction of the ER sensor protein stromal interaction molecule 1 (STIM1) and the plasma membrane channel Orai1, which form a complex at ribosome-free ER plasma membrane junctions in acinar cells (90) (Fig. 1).

Abnormal Ca^{2+} signaling

Disruption of normal Ca^{2+} signaling was postulated as a trigger for pancreatitis over a decade ago (167). Hyperstimulation of acinar cells with CCK-8 caused large sustained cytosolic Ca^{2+} elevations, premature intracellular digestive enzyme activation, and necrosis (133). Subsequently, it was shown that major precipitants of AP, such as bile salts (Fig. 1) (78, 164) and nonoxidative metabolites of ethanol (27, 28), also generated toxic elevations of Ca^{2+} that resulted in cellular necrosis.

Ca^{2+} release from internal stores in response to these precipitants was dependent on IP_3R opening (27, 46, 164). However, sustained Ca^{2+} entry triggered by Ca^{2+} store depletion was required to raise cytosolic Ca^{2+} to detrimental levels; abrogation of the cytosolic Ca^{2+} rise with intracellular Ca^{2+} chelators prevented deleterious changes such as trypsinogen activation, vacuolization, and necrosis (29, 78, 133). The mechanism of cellular necrosis was further defined with respect to nonoxidative ethanol metabolites, which produced a Ca^{2+}-dependant inhibition of mitochondrial function, resulting in loss of membrane potential ($\Delta\Psi_m$), NAD(P)H, and ATP production (29). Experiments using mitochondrially targeted luciferase in pancreatic acinar cells have demonstrated that bile acids and nonoxidative ethanol metabolites cause a profound decrease of mitochondrial ATP, in addition to cytosolic reductions (29, 166). Recently, we have demonstrated that the bile acid taurolithocholic acid 3-sulfate (TLC-S) induced sustained cytosolic Ca^{2+} increases (Fig. 2) accompanied by an initial rise in NAD(P)H autofluorescence as previously described (120). However, the stimulatory effect on mitochondrial function was quickly lost in the presence of sustained cytosolic Ca^{2+} elevation, with NAD(P)H autofluorescence rapidly decreased, an action accompanied by a large, sustained increase in mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) (14) (Fig. 2); such signals are detrimental to mitochondrial function, thereby inhibiting ATP production. A reduction in ATP output would modify IP_3R function (7) and inhibit ATP-driven Ca^{2+} pumps, thereby impeding clearance of elevated cytosolic Ca^{2+} from the cell (Fig. 1) (123). In accord with this hypothesis, supply of intracellular ATP *via* a patch pipette abrogated the development of sustained Ca^{2+} elevations and consequent necrosis in response to nonoxidative ethanol metabolites (27, 29) and TLC-S (14). Evidence derived from such experiments highlights the fundamental importance of normal mitochondrial function for Ca^{2+} homeostasis and cell integrity under conditions of pancreatic insult (102).

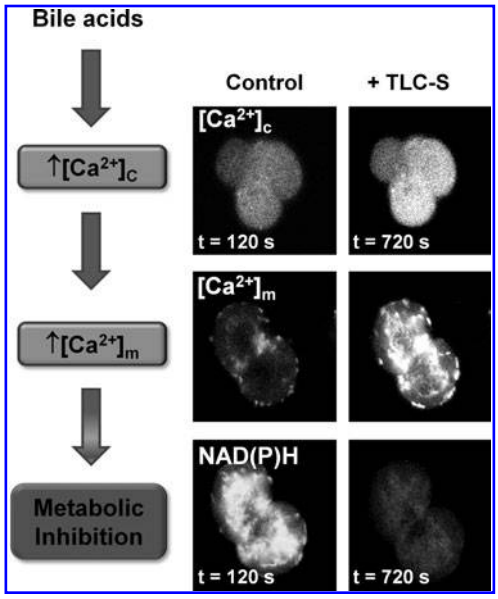

FIG. 2. Bile acid-induced mitochondrial metabolic inhibition: the importance of sustained Ca^{2+} elevations. Confocal images showing that the bile acid TLC-S induces sustained increases of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) in a triplet of pancreatic acinar cells loaded with the Ca^{2+} indicator Fluo4 (*upper panel*). The result of sustained $[Ca^{2+}]_c$ increases is uptake of Ca^{2+} by mitochondria. Simultaneous measurements of NAD(P)H autofluorescence (*lower panel*) and mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) using the mitochondrial Ca^{2+} probe Rhod2 (*center panel*) show that application of TLC-S (500 μM) to a doublet of pancreatic acinar cells induces a large sustained rise of $[Ca^{2+}]_m$ and marked loss of NAD(P)H autofluorescence intensity as mitochondrial function is inhibited. Note the predominantly perigranular distribution of both NAD(P)H and Rhod2 localized to mitochondria [adapted from Ref. (14)].

Influence of ROS on Ca^{2+} signaling

Given the importance of restriction of cytosolic Ca^{2+} levels within physiological limits and discrete microdomains, the cell must finely coordinate Ca^{2+} entry, release, and exit mechanisms to ensure that mitochondrial dysfunction does not ensue. Studies in other cell types suggest that many of these Ca^{2+} homeostatic processes are sensitive to the intracellular redox environment. For example, both IP_3R and ryanodine receptor (RyR) contain multiple cysteine residues that are sensitive to ROS, implying a modulatory role for free radicals on Ca^{2+} release channels (Fig. 1) (40, 96, 152), potentially influencing events such as deleterious intracellular activation of trypsinogen (68). Further, oxidation of thiols prevents negative regulation of the IP_3R by calmodulin by inhibiting its binding to the receptor (42, 60, 175). The general effect of RyR oxidation is to increase channel activity, a process mediated by enhanced subunit assembly and inhibition of calmodulin binding, a negative regulator of the receptor (60). The sensitivity of the IP_3R appears more complex, since ROS may sensitize the IP_3R to activation by IP_3 but inhibit channel function *per se* (73, 74). It has been shown that thiol oxidizing agents sensitize Ca^{2+}-release channels in a manner sufficient to induce cytosolic oscillations in the absence of physiological stimulation (15, 100) and thus function to preserve IP_3R-linked Ca^{2+} oscillations necessary for exocrine secretion (18). However, the situation is likely to be complex. A study in vascular smooth muscle cells has demonstrated that while exogenously applied ROS enhanced the release of IP_3-mediated Ca^{2+}, this did not occur when nonhydrolysable analogs of IP_3 were used instead of IP_3, potentially suggesting an additional role for ROS in the breakdown of IP_3 (155).

Important recent progress has been made with respect to elucidation of the vital Ca^{2+} entry mechanism in pancreatic acinar cells, which involves formation of an STIM1-Orai complex (90). Under conditions of store depletion, STIM1 senses Ca^{2+} changes within the ER and translocates to the plasma membrane forming a functional complex with Orai1 (98). Interestingly, the Ca^{2+} release (IP_3R) and Ca^{2+} entry mechanisms appear spatially distinct in the acinar cell, with ribosome-free portions of ER forming close associations with the plasma membrane (90). Recently, redox regulation of Orai1 has been reported in T-cells that may function to tune cellular Ca^{2+} signaling (12). This study showed that Orai1, which possesses an external cysteine residue, was sensitive to H_2O_2, whereas Orai3, in which this residue is absent, was insensitive. T-cells became progressively resistant to ROS after differentiation into effector cells, which correlated with an increased Orai3 expression. This might enable cells to proliferate, differentiate, and secrete cytokines when present in

oxidizing environments such as the inflamed pancreas. Whether ROS sensitivity plays a role in Orai-mediated Ca^{2+}-entry in cells of the pancreas has yet to be determined.

Despite the lack of voltage-sensitive Ca^{2+} channels in the pancreatic acinar cell, there is evidence to suggest that Ca^{2+} channel blockers may decrease the severity of experimental pancreatitis (65, 149) although postendoscopic retrograde cholangiopancreatography pancreatitis was unaffected by nifedipine treatment (125). The development of selective inhibitors of SOCE in pancreatic acinar cells, however, may provide an attractive proposition for amelioration of AP, and progress in this field is eagerly awaited.

Ca^{2+}-ATPase pumping mechanisms that lower the concentration of cytosolic Ca^{2+} play a critical role in homeostasis of the pancreatic acinar cell. Their importance is underscored by the fact that Na^+/Ca^{2+} exchangers, which have been shown to exert a homeostatic influence in other cell types, appear to be nonfunctional or absent. These energy-requiring pumps are also sensitive to the redox environment (Fig. 1). For example, H_2O_2 applied to pancreatic acinar cells profoundly altered the normal pattern of CCK or ACh evoked Ca^{2+} signals, increasing the proportion of global, sustained responses as the concentration of H_2O_2 increased (17), potentially *via* inhibition of the PMCA, thereby decreasing Ca^{2+} clearance from the cytosol through the plasma membrane. This effect appears linked to mitochondrial function, since mitochondrial depolarization and consequent ATP depletion correlated with a fall in PMCA activity (4). The SERCA pumps are subject to oxidative modification, possessing as many as six redox-sensitive cysteine residues (145). Current evidence suggests that mild oxidative conditions lead to oxidation of Cys674, upregulating pump activity (1), whereas prolonged exposure can cause suphonylation of the same residue and oxidation of other residues, eliciting irreversible SERCA inhibition and exacerbating Ca^{2+}-overload (1, 49, 154). Such divergent effects highlight the enormous complexity of the yet partially understood relationships between Ca^{2+} signaling and ROS; fine-tuning of the levels of localized, subcellular ROS may affect Ca^{2+} homeostasis positively or negatively.

Control of Redox Status

ROS generation

Before discussion of the involvement of ROS in cellular toxicity, it is pertinent to briefly highlight where and how ROS are produced, and what endogenous protective mechanisms are in place to deal with their excessive formation. In this respect, it should be emphasized that ROS are both generated under physiological conditions as an integral part of the mitochondrial ATP production and exert functions as signaling molecules in their own right, rather than simply being the widely reported mediators of undesirable toxicity. Growing evidence suggests that the balance between ROS-producing and ROS-scavenging systems underpins the proper functioning of the cell.

Mitochondria account for 90% of the total cellular oxygen consumption and are the principal source of physiological ROS (64). An estimated 1%–2% of all O_2 consumed is linked to production of radicals. To generate ATP, mitochondria oxidize NADH and $FADH_2$ produced by the tricarboxylic acid (TCA) cycle or *via* β-oxidation of FAs, in a series of reactions catalyzed by enzyme complexes I–IV located on the inner mitochondrial membrane (IMM), which constitute the electron transport chain (ETC). It is thought that 10 or more components of mitochondria produce ROS (2), but the main sources of superoxide ($O_2\bullet^-$) are complexes I and III of the ETC (Fig. 1). Nonmitochondrial sources of ROS are perhaps

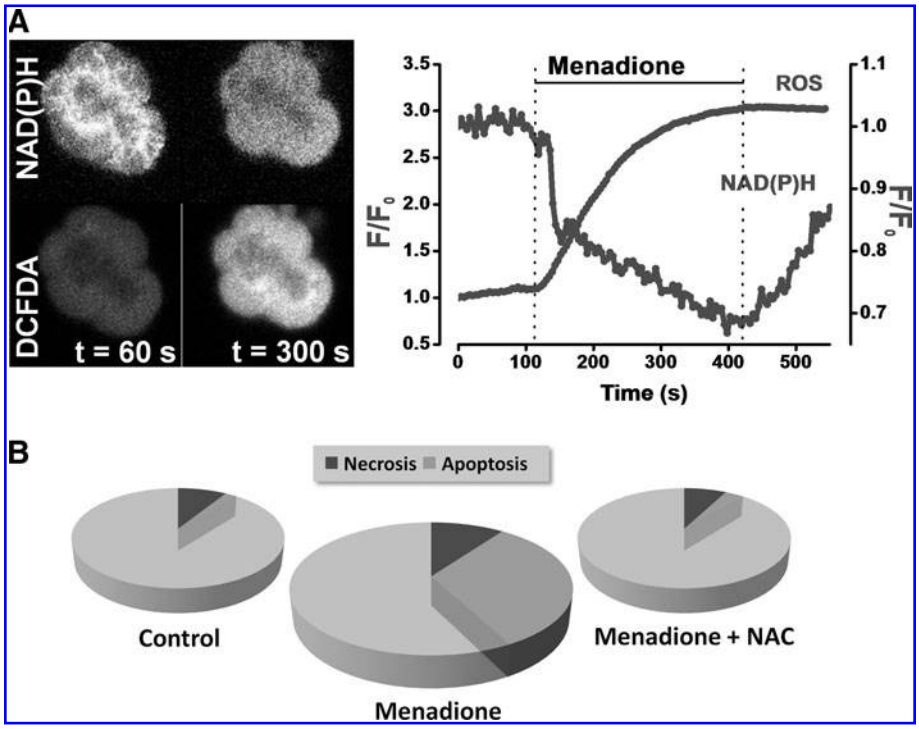

FIG. 3. ROS induce pancreatic acinar cell apoptosis. (A) Confocal images of a cluster of pancreatic acinar cells (*left*) showing intracellular ROS detected by 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-DCFDA; *lower panel*) and NAD(P)H autofluorescence (*upper panel*), and normalized fluorescence measurements (*right*) demonstrating that application of menadione (30 μM) markedly increased intracellular ROS, whereas NAD(P)H was concomitantly reduced as redox cycling of the quinone occurred. **(B)** Menadione induced apoptosis (*mid gray*; measured with rhodamine 110-aspartic acid amide, a general caspase substrate) but had no effect on cellular necrosis (*dark gray*; measured with propidium iodide uptake). Clearance of menadione-induced ROS with the antioxidant *N*-acetyl-*L*-cysteine abolished the increase in apoptosis demonstrating the specific involvement of ROS in pancreatic acinar apoptosis. Necrosis and apoptosis shown as a proportion of total cells counted [adapted from Refs. (25, 26)].

less well characterized; however, they may be prodigious in their ROS production. Specific ROS generation by NADPH oxidase and myeloperoxidase are critical to the immune response and are certainly involved in the development of the systemic complications of AP. We have previously measured substantial Ca^{2+}-independent ROS generation in freshly isolated murine pancreatic acinar cells in response to menadione (vitamin K3) (Fig. 3A) (26). Quinones such as menadione enter rapid redox cycles within the cell, consuming NAD(P)H and producing superoxide (Fig. 3A). Importantly, our findings indicated that the consequence of this acute ROS generation was promotion of acinar cell apoptosis, rather than necrosis; scavenging of radicals with *N*-acetyl-*L*-cysteine (NAC) prevented apoptosis (Fig. 3B).

We have recently shown that application of a high concentration of the bile salt TLC-S caused a significant increase in ROS generation in human and murine pancreatic acinar cells (Fig. 4) (14). Mitochondria were the site of TLC-S-induced ROS production; thin confocal sections showed a mitochondrial distribution of 2,7-dichlorodihydrofluorescein diacetate acetyl ester (DCFDA) fluorescence (Fig. 4B), whereas distribution of ROS-sensitive indicators colocalized with mitochondrial markers, and the inhibition of ETC with antimycin A and rotenone abolished all detectible ROS (Fig. 5) (14). The generation of ROS elicited by the bile acid was Ca^{2+}-dependent since pretreatment of cells with 1, 2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) abolished this effect, and thus likely to result from the overload of mitochondria due to sustained cytosolic Ca^{2+} elevations. The membrane-bound enzyme NADPH oxidase has been implicated in the generation of ROS by bile acids in AR42J cells, derived from a rat acinar cell tumor (172). In this study, caerulein stimulated the activation of NADPH oxidase, expression of pro-apoptotic factors, and subsequent apoptosis, effects that were Ca^{2+}-dependent and sensitive to diphenyliodonium (DPI) implicating NADPH oxidase in the production of ROS within AR42J cells (171). In freshly dispersed murine pancreatic acinar cells, however, we found that NADPH oxidase was not the source of bile acid- (14) or menadione-induced (26) ROS production since these effects were insensitive to DPI. Further, while AR42J cells express functional NADPH oxidase, the presence of this enzyme could not be confirmed by immunohistochemistry in primary pancreatic acinar cells and may even be absent (53).

During the course of AP there is significant recruitment of immune cells to the organ. Importantly, ROS induce both activation and proliferation of immune cells (70, 151, 153). Moreover, the recruited cells, particularly neutrophills, monocytes, and macrophages, generate copious ROS *via* the

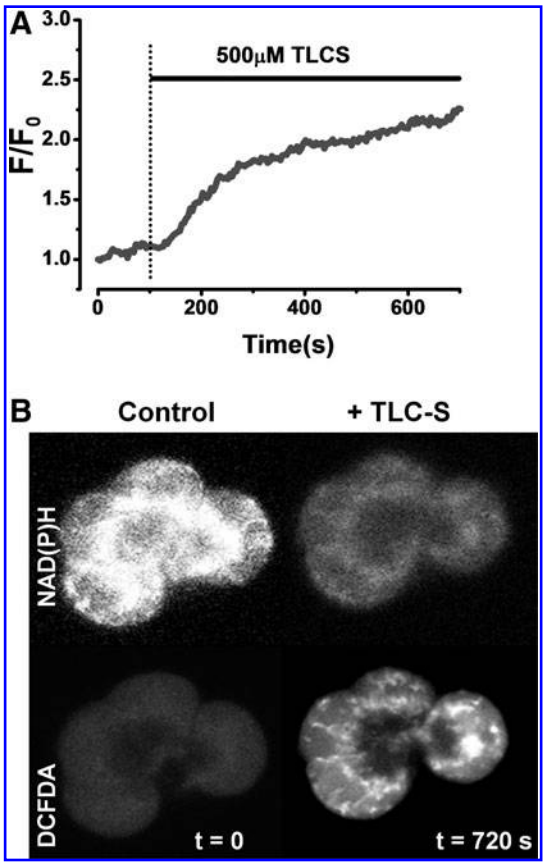

FIG. 4. Bile acid-induced mitochondrial ROS generation in human and murine pancreatic acinar cells. (A) Intracellular ROS generation by 500 μM TLC-S measured with 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-DCFDA) in a human pancreatic acinar cell. **(B)** Thin confocal sections (<2 μm) of a small cluster of murine pancreatic acinar cells reveal a typical mitochondrial distribution NAD(P)H autofluorescence (*upper panel*) at the beginning of the experiment. Application of TLC-S caused an increase of intracellular ROS, detected with DCFDA (*lower panel*), in the mitochondrial region, whereas NAD(P)H was concomitantly decreased [adapted from Ref. (14)].

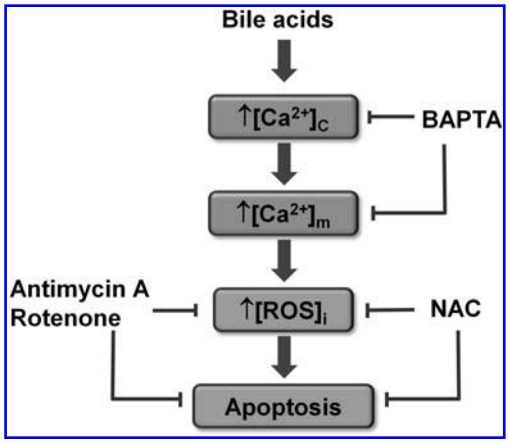

FIG. 5. Schematic representation of the proposed mechanism of bile-induced apoptosis within the pancreatic acinar cell. Bile acids stimulate the bile receptor Gpbar1, which elicits concentration-dependent increases in cytosolic Ca^{2+} ($[Ca^{2+}]_c$). Free $[Ca^{2+}]_c$ enters the mitochondrial matrix increasing the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$), resulting in mitochondrial ROS production and subsequent caspase activation. Clearance of ROS with the antioxidant *N*-acetyl-*L*-cysteine (NAC) or inhibition of mitochondrial ROS production with antimycin-A and rotenone, agents that block the ETC, abolishes apoptosis. Preincubation with the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), loaded in membrane-permeable-AM form, significantly dampens $[Ca^{2+}]_c$ elevations, abolishing both ROS production and apoptosis [adapted from Ref. (14)].

NOX family of NAD(P)H oxidases (6), which consist of flavoprotein, cytochrome b, and regulatory subunits (83). Upon activation, NAD(P)H oxidase assembles in the plasma membrane and reduces molecular oxygen to superoxide. Phagocytes employ NAD(P)H oxidase 2 to destroy pathogens within the phagosomal space (107). Within neutrophils, H_2O_2, formed from superoxide, is further converted to the extremely reactive hypochlorous acid by myeloperoxidase (169), a common marker of pancreatitis severity (10). While the activity of NAD(P)H oxidase and myeloperoxidase in the production of powerful oxidants is vital in the role of immune cells as host defense, under conditions in which these oxidants are directed against host tissue, worsening of inflammatory diseases such as pancreatitis results (126). ROS production is enhanced in neutrophils obtained from patients with AP (159) and ROS derived from neutrophil NADPH oxidase has been shown to implement damage in experimental AP (53).

ROS defense mechanisms

To regulate the concentration and localization of ROS, numerous antioxidant strategies have been developed by the cell. The proximal ROS generated within mitochondria is the superoxide anion (Fig. 1), which at neutral pH is a moderately stable radical generally confined to the mitochondrial interior provided organellar membrane integrity is uncompromised. Toxicity is reliant upon onward generation of further reactive species that may react directly with biomolecules such as proteins, lipids, and DNA (75, 86, 117). The deleterious effects of mitochondrial ROS are prevented by various antioxidant systems. For example, $O_2\bullet^-$ is enzymatically converted to H_2O_2 by a family of metaloenzymes, the superoxide dismutases (SOD) (43). While $O_2\bullet^-$ may dismutate to H_2O_2 spontaneously, the rate is slow and $O_2\bullet^-$ may either reduce transition metals (particularly Fe^{2+}), which in turn react with H_2O_2 to produce fiercely reactive NO•, or spontaneously react with HO• to produce peroxynitrite. Therefore, it is important that the cell maintains $O_2\bullet^-$ at the lowest concentration possible and it has been reported that SOD levels are decreased in cerulein-induced pancreatitis (30, 39, 110), suggesting compromise in the disease state. The role of SOD enzymes is to restrict dismutation to a rate limited only by the diffusion of the $O_2\bullet^-$ radical. The majority of $O_2\bullet^-$ is produced and released into the matrix that contains a specific form of SOD with manganese at its active site (MnSOD), which eliminates $O_2\bullet^-$ formed by the components of the ETC located on the IMM leaflet or within the matrix space itself (43). Expression of MnSOD can be upregulated by oxidative activation of pro-survival nuclear factor kappa B (NF-κB), thus limiting further ROS production.

Like the cytosol, mitochondria possess small molecule antioxidants such as ascorbate, glutathione (GSH), and vitamin E (α-tocopherol), which are concentrated at levels above those of the cytosol (77, 162). Both ascorbate and GSH are actively transported into mitochondria, ascorbate in its oxidized form *via* Glut1 where it is recycled by the ETC and prevents oxidative damage to the mitochondrial DNA (77). A number of lipophillic antioxidants, such as α-tocopherol (Vitamin E), polyphenols, and carotenoids, are present within cellular membranes. Under physiological conditions polyphenols and carotinoids may perhaps be discounted as major sinks of radical species, since their concentration in plasma is rarely above 1% of that of α-tocopherol (45). The hydrophobic α-tocopherol radical is scavenged by ascorbate (84).

The oxidation of GSH to glutathione disulphide (GSSG) is an important step in the channeling of potentially harmful radicals (168). GSH is synthesized from the precursor NAC and consists of three amino acid residues, glutamate, cysteine, and glycine, in which the central thiol group accounts for the reducing activity of the molecule. The pancreas contains high levels of GSH, $\sim 2\,\mu$mol/g tissue, which represents the fourth highest among the visceral organs (61, 108). Further, the rate of metabolic GSH turnover is high in the pancreas, with only the liver and kidneys possessing higher activities (61). The pancreas therefore appears well adapted to deal with oxidative stress. The actions of GSH are dependent upon turnover between reduced (GSH) and oxidized (GSSG) forms; GSSG is created and can be shuttled back to GSH *via* GSH reductase. Crucially, this requires the donation of an electron from NAD(P)H primarily generated within the TCA cycle. However, although GSH is present in millimolar concentrations the rate constant for reaction with H_2O_2 is negligible, and therefore intracellular diffusion of H_2O_2 *per se* would not be markedly affected by GSH (22). It is the presence of the selenium-containing enzyme GSH peroxidase (GPx), which catalyzes the reduction of peroxides to water (or related alcohols) and the concomitant oxidation of GSH, that makes this a favorable reaction.

In addition to GPx, there are complementary enzyme systems that perform important antioxidant defense roles. Of particular relevance is NAD(P)H quinone oxidoreductase 1 (NQO1) an FAD-containing (flavoprotein) obligate two-electron reductase that functions as a cellular detoxifying mechanism (34, 136). It is ubiquitously expressed in all tissues and its expression is upregulated in both AP and pancreatic adenocarcinoma (61, 88, 91), suggesting a function to limit oxidative stress in disease states. NQO1 uses NAD(P)H derived from the TCA cycle as a reducing cofactor in multiple reactions such as the production of relatively stable hydroquinones from endogenously generated quinone species, for example, tocopherol and coenzyme Q_{10} (8, 146). NQO1 performs many protective functions, including direct scavenging of superoxide (147), maintenance of the reduced, antioxidant form of coenzyme Q_{10} (8), and stabilization of p53 (3), which controls the transcription of multiple genes involved in oxidative stress and cancer. We have recently highlighted the important influence of NQO1 in the pancreatic acinar cell to modulate the internal redox environment. Inhibition of this enzyme with the inhibitor 2,4-dimethoxy-2-methylnaphthalene (DMN) dramatically increased the generation of menadione-induced ROS (26), since one-electron reduction directly to the relatively stable hydroquinone was prevented, thereby shunting the quinone into two-electron reductive pathways that generate unstable semiquinone intermediates and superoxide within redox cycles. Further, DMN also potentiated bile acid-induced ROS production (14), an action that promoted apoptosis (Fig. 6) and consistent with previous observations underlining the importance of ROS in this modality of cell death.

Ca^{2+}, ROS, and Cell Death

Cell death mechanisms in the exocrine pancreas

The severity and/or duration of stress applied to pancreatic acinar cells is a major influence on cell death modality (25). Apoptosis and necrosis represent distinct ends of the cell death spectrum, believed to be composed of up to 11 types

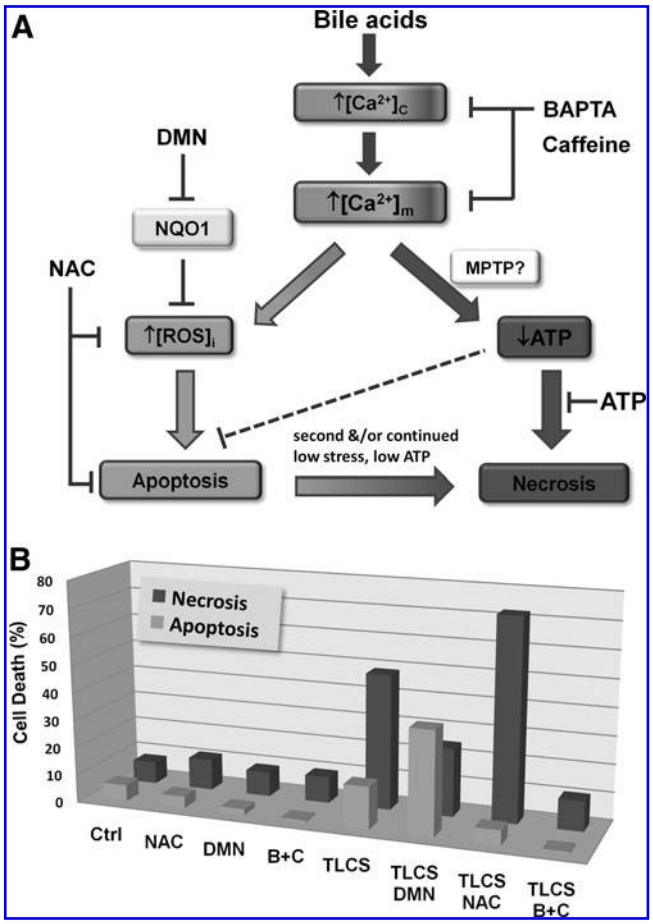

FIG. 6. Effects of intracellular ROS and cytosolic Ca^{2+} ($[Ca^{2+}]_c$) on cell death induced by bile acids. A schematic representation shows how manipulation of ROS and Ca^{2+} may influence cell fate. When mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) is elevated following bile acid-induced $[Ca^{2+}]_c$ rises, mitochondrial ROS generation is triggered, which promotes apoptosis. As $[Ca^{2+}]_m$ becomes further increased/sustained mitochondrial function is compromised, probably *via* opening of the mitochondrial permeability transition pore (MPTP), and ATP production falls leading to necrosis. The bar chart below shows the results of an *in vitro* cell death assay evaluating the effects of agents that manipulate either $[Ca^{2+}]_c$ or ROS on TLC-S induced toxicity. The antioxidant NAC markedly reduced TLC-S-induced ROS and apoptosis, whereas the NADPH quinone oxidoreductase 1 (NQO1) inhibitor 2,4-dimethoxy-2-methylnaphthalene (DMN) potentiated both ROS and apoptosis. Conversely, NAC augmented necrosis, whereas DMN reduced this form of cell death. A combination (B+C) of the Ca^{2+} chelator BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid and caffeine, an IP_3R blocker, completely prevented cell death caused by the bile acid, returning both apoptosis and necrosis to control levels. Patch pipette administration of intracellular ATP was able to prevent TLC-S-induced necrosis (27) [adapted from Ref. (14)].

(97). Apoptosis is genetically regulated (116), whereas necrosis is largely considered an uncontrolled default mechanism of cell death. More recently, autophagy has become of interest to the field of pancreatology. Classically viewed as a major intracellular degradation mechanism for both long-lived proteins and whole organelles, autophagy operates under stress conditions to promote survival under starvation conditions or cell death when apoptosis is inhibited (173). Degradation within autolysosomes is catalyzed by hydrolases, specifically cathepsins L and B (13), and functions to recycle vital nutrients such as amino acids (101). Although the role and significance of autophagy in disease states is incompletely understood, recent pioneering work has shown that mice deficient in Atg5, a protein central to autophagy, exhibited reduced trypsinogen activation and almost no AP with the exception of mild oedema (62). Subsequent work has reported impaired autophagic flux to mediate vacuolization and trypsinogen activation in rodent models of AP that may be due to an imbalance between cathepsins L & B (92). Future studies are required to elucidate the relative importance of autophagy in severe pancreatitis disease models that are characterized by significant necrosis.

The principal mechanism of pancreatic acinar cell death is necrosis (25, 80), the extent of which determines the severity of the disease. Whereas necrosis elicits inflammation, apoptosis involves a regulated cascade of signaling events that result in the clean removal of the dead cell from the tissue (97). These major forms of cell death coexist to differing extents in established models of AP induced by caerulein hyperstimulation, bile acids, choline-deficient ethionine-supplemented (CDE) diet, and pancreatic ductal obstruction (38, 76, 119). Induction of apoptosis reduces the severity of caerulein-induced pancreatitis (9), whereas inhibition of caspase activity (therefore, apoptosis) leads to severe necrotizing pancreatitis (93). In experimental AP at least, the severity correlates directly with the extent of measured necrosis and crucially bears an inverse correlation with apoptosis reviewed in (52). Thus clinically, the balance of cell death between apoptosis and necrosis may be critical for the outcome of the disease, a hypothesis that requires thorough testing.

Apoptosis: The role of ROS

The manner in which ROS mediate apoptosis is incompletely understood; however, there are direct interactions between radical species and apoptotic machinery. For example, H_2O_2 can mediate cytochrome c release, caspase-3 activation, and DNA fragmentation in a manner dependent upon translocation of Bax/Bak proteins, a crucial step in the initiation of apoptosis (23, 137, 141). Generation of ROS by the glucose oxidase system has also been demonstrated to induce apoptotic changes such as increasing the Bax/Bcl-2 ratio and subsequent DNA fragmentation.

In rat, the antioxidant melatonin prevented much of the apoptosis produced in an ischemia/reperfusion experimental pancreatitis model demonstrating the fundamental relationship between ROS and apoptosis (103). However, perhaps counter-intuitively, ROS and other oxidants may inactivate caspases (138) or activate pro-survival NF-κB, the latter shown in taurocholate (161), ethanol (51), and caerulein (54) models of pancreatitis. Thus, the participation of ROS in disease models appears multifactorial and complex. Importantly, when release of cytochrome c occurs, this interrupts the ETC, an action that causes further generation of ROS, damage to mitochondrial components, and augmentation of the oxidative burden (21, 174). Thus, once apoptosis is initiated, mitochondria become a persistent source of ROS but not

ATP. When excessively generated, the unstable nature of radicals means that cell injury by direct oxidative damage to subcellular components may occur. Lipid peroxidation is often used as a biomarker of oxidative stress and there are components of every cellular membrane that are extremely vulnerable. Polyunsaturated FAs are found abundantly throughout the ER, mitochondrial, and plasma membranes and are freely peroxidated by ROS, primarily hydroxyl free radicals. This may lead to disruption of membrane integrity, loss of secretory function, and necrosis (142).

The interplay between ROS and apoptotic signaling is intriguing. Pancreatic acinar cells generate significant levels of ROS *via* redox cycling when exposed to the oxidant menadione (Fig. 3A) (26). This ROS production induced apoptosis (measured by both caspase activation and phosphatidylserine translocation) in a manner completely abolished by the antioxidant NAC, intimately linking ROS and apoptosis within the pancreatic acinar cell (Fig. 3B). Recently, the role of ROS in modulating cytochrome C release and subsequent apoptosis has been elegantly demonstrated in isolated mitochondria and intact pancreatic acinar cells (114). The pathophysiological relevance of acute ROS production was further strengthened by recent findings that ROS were generated by the bile acid TLC-S (Fig. 4) and which also promoted apoptosis (14). Our findings suggest that $[Ca^{2+}]_c$ elevations induced by bile acids elicit Ca^{2+} uptake into the mitochondrial matrix increasing $[Ca^{2+}]_m$. This rise generates mitochondrial ROS with subsequent caspase activation and apoptosis; when mitochondrial ROS were decreased by NAC or by inhibition of the ETC with antimycin-A and rotenone, apoptosis was prevented. In support of the involvement of Ca^{2+} in the route to eventual apoptosis, preincubation with the Ca^{2+} chelator BAPTA-AM significantly dampened $[Ca^{2+}]_c$ elevations, abolishing both ROS production and apoptosis. The actions of bile acids were modulated by NQO1; NQO1 inhibition revealed ROS generation by TLC-S at concentrations that could not be detected under control conditions. Further, bile acid-induced apoptosis was potentiated when NQO1 was inhibited (Fig. 6) (26, 14), implicating NQO1 in the regulation of intracellular ROS and consequent apoptosis, a view consistent with current evidence in diverse cell types. For example, overexpression of NQO1 has been shown to decrease cellular ROS production (33), whereas the absence of NQO1 in KO mice has been shown to potentiate menadione-induced toxicity (132).

Importantly, when manipulation of bile acid-induced ROS was performed, the balance of cell death modality was affected. For example, potentiation of TLC-S-induced ROS by DMN augmented apoptosis while concomitantly reducing necrosis. Conversely, NAC treatment abolished apoptosis but potentiated necrosis (Fig. 6). Our current view is that ROS generation constitutes a crucial determinant of pancreatic acinar cell death, primarily in promotion of apoptosis, an action that may serve as a protective mechanism under conditions of acute cellular stress. Whether such an effect of ROS would be beneficial or detrimental in an *in vivo* setting, by shifting the apoptosis/necrosis balance in pancreatic acinar cells, has not yet been established in models of AP.

Necrosis: The importance of Ca^{2+}

Early studies have demonstrated the crucial part played by cytosolic Ca^{2+} in the induction of apoptosis (71, 170). Indeed, Ca^{2+} overload may be the common pathway that links all forms of cell death (135). However, it has been recognized that pathophysiological stimulation of pancreatic acinar cells with CCK, bile acids, or nonoxidative ethanol metabolites causes sustained elevations of cytosolic Ca^{2+} that primarily trigger necrosis (Fig. 6) (27, 78, 133). Coapplication of BAPTA-AM and caffeine, an IP_3R blocker, completely prevented the necrosis induced by the bile acid TLC-S in an *in vitro* toxicity model (Fig. 6) (14). A common feature of Ca^{2+} overload in pancreatic acinar cells is that mitochondrial function becomes severely inhibited, leading to loss of ATP production (27, 102, 166). The actions of AP precipitants at the cellular level are mirrored within *in vivo* models of pancreatitis, which have demonstrated reductions of cellular ATP (113), and ATP depletion has been proposed as a master switch for a transition from apoptosis to necrosis (85).

How might sustained elevations of cytosolic Ca^{2+} trigger eventual necrosis? It is known that as cytosolic Ca^{2+} rises, mitochondrial uptake of Ca^{2+} occurs *via* an electrochemical gradient driven by the ETC. To achieve this, movement across both mitochondrial membranes must occur. Originally, the outer mitochondrial membrane was thought freely permeable to small ions such as Ca^{2+}. However, this is likely mediated by the voltage-dependent anion channel (VDAC), a pore that regulates Ca^{2+} movement into the inter-membrane space (47). Transport across the IMM is primarily achieved *via* the mitochondrial uniporter, a channel currently poorly characterized (79, 127). Crucially, it is highly Ca^{2+}-selective and allows its entry into the matrix to stimulate ATP production. This is achieved by a number of synergistic mechanisms, including allosteric activation of three Ca^{2+}-dependent enzymes within the TCA cycle, α-ketoglutarate dehydrogenase, isocitrate dehydrogenase, and pyruvate dehydrogenase (94, 95), which elevate reductive intermediates such as NAD(P)H, feeding the ETC. The adenine nucleotide translocase (ANT), which transports ADP, and ATP synthase (Complex V) are both involved in ATP production and are regulated by Ca^{2+} (32, 99). Overall, the effect is to increase the supply of substrates to the ETC, allowing for a faster rate of oxidative phosphorylation and ATP production. This is highlighted by simultaneous measurements of cytosolic Ca^{2+} and mitochondrial NAD(P)H autofluorescence in human and rodent pancreatic acinar cells, in which physiological stimulation with CCK-8 and CCK-58 caused oscillatory Ca^{2+} rises that triggered associated oscillatory NAD(P)H elevations (24, 104, 165). Importantly, however, the movement of Ca^{2+} into the matrix is at the expense of $\Delta\Psi_m$ and large sustained Ca^{2+} elevations may completely collapse the proton motive force ceasing the production of ATP. Such transition from low permeability to high is likely to occur *via* the formation of a mega-channel, the mitochondrial permeability transition pore (MPTP).

Involvement of the MPTP in necrosis

Mitochondrial permeability transition was observed over 30 years ago as the formation of a nonspecific pore enabling the passage of low-molecular-weight solutes (63, 66, 67). This supramolecular complex, formed from pre-existing components assembled at the contact sites between the inner and outer membranes of the mitochondria, contributes to cell fate and may play a role in determining the severity of AP (102). The formation of the MPTP in response to sustained cytosolic

Ca^{2+} and ROS breaches the permeability barrier of the IMM, allowing free movement of all solutes < 1500 Da into and out of the mitochondrial matrix. This includes proton movement into the matrix and thus dissipates the proton gradient and prevents further oxidative phosphorylation and mitochondrial ATP generation. In addition, ROS generated by the ETC, usually confined within membrane structures, may exit. The exact regulation and composition of this pore are still under vigorous debate; however, current evidence suggests that the core machinery consists of the ANT and phosphate carrier, which together form a pore modulated by cyclophilin D (57). Short-term flickering of the MPTP may be a physiological phenomenon for the removal of Ca^{2+} from the matrix (69, 115) and function to match mitochondrial Ca^{2+} to specific substrates (37). However, sustained opening is triggered by high levels of mitochondrial Ca^{2+} taken up from the cytosol and other stimulants, principally ROS.

Interestingly, a recent study suggests that cyclophilin D may have a more wide-reaching role as a redox sensor within the mitochondria, with oxidation of the protein influencing its conformation and activity (87). Formation of the MPTP has been noted in a number of disease states, particularly in cardiac and neurological ischemia/reperfusion injury but awaits characterization in the pancreas.

Both Ca^{2+} and ROS appear central to the opening of the MPTP. Previous studies in hepatocytes have shown that MPTP opening occurs *via* menadione-induced oxidative stress (124), consistent with a model of ROS-sensitized Ca^{2+}-dependent MPTP induction (20, 156). Animals lacking the VDAC and ANT, apparent components of the MPTP, may still be able to form the MPTP (55, 58, 81), whereas those lacking cyclophilin D are markedly protected from necrosis but have normal apoptotic responses (106). Elevation of mitochondrial Ca^{2+} is a required step in the opening of the MPTP and subsequent loss of membrane potential and ATP synthesis (56). However, both Ca^{2+} and ROS exist in a positive feedback loop to uncouple $\Delta\Psi_m$. ROS were postulated to uncouple membrane potential over a decade ago (72) and subsequent evidence has demonstrated the involvement of specific mitochondrial uncoupling proteins in this process (36). This uncoupling is consistent with a model in which ROS is generated in an uncontrolled manner *via* a positive feed-forward loop (105).

Importantly, when considering the role of ROS in the pathophysiology of the pancreas, it should be appreciated that multiple targets of ROS exist. Interventions designed to inhibit radical generation or limit their effects by scavenging will necessarily affect diverse systems *in vivo*. In AP, localized inflammation can escalate to a full-blown systemic inflammatory response syndrome (SIRS) involving activation and infiltration of neutrophils, the latter ROS-producing cells exacerbate the progression of experimental pancreatitis (53). ROS also activates NF-κB, leading to an influx of neutrophils *via* chemokine and cytokine transcription; the neutrophils release further ROS and reactive nitrogen species during the respiratory burst (142). Thus, oxidative stress amplifies the initial tissue damage experienced in AP (Fig. 7).

Since a role for ROS was first suggested in AP (139, 140), studies have produced conflicting findings. Antioxidants were shown to be beneficial in ameliorating experimental AP induced by caerulein (50), pancreatic ductal obstruction (144), and taurocholate (48). However, reduction of ROS by scavengers did not decrease acinar cell damage in two models of AP (150). Further, Ebselen, a GPx analog, has shown beneficial effects in caerulein and CDE mouse models but was without effect in Na-taurocholate rat experimental pancreatitis (109). There may be a number of explanations for these disparate results and conflicting findings with other

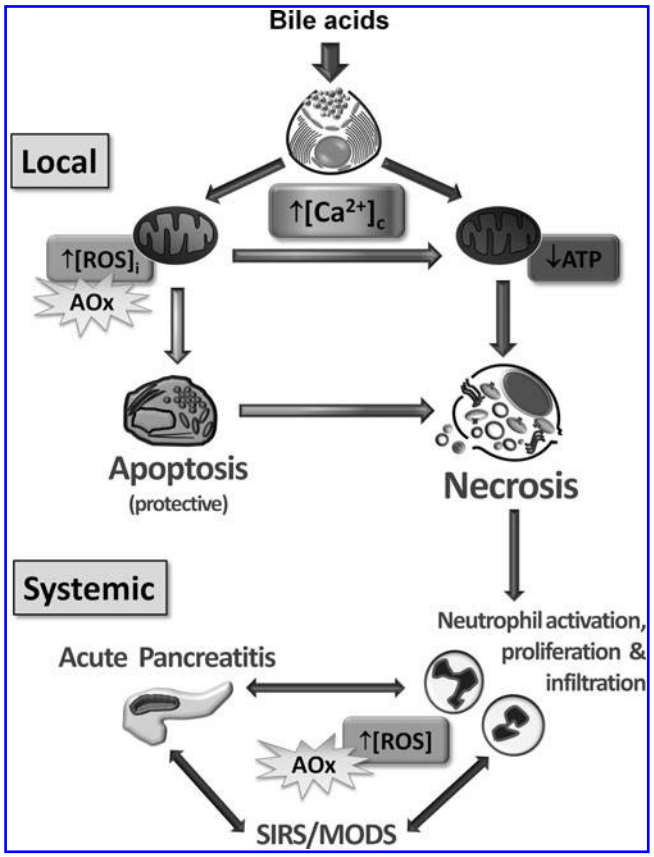

FIG. 7. Schematic model of the complex interplay between $[ROS]_I$, $[Ca^{2+}]_C$, and $[Ca^{2+}]_M$ in bile acid-induced pancreatitis. Sustained elevations of $[Ca^{2+}]_C$ in pancreatic acinar cells acutely affect mitochondrial function; the outcome would depend on the level and duration of these pathological signals. Mitochondria uptake Ca^{2+} from the cytosol raising $[Ca^{2+}]_m$ levels, an action that stimulates mitochondrial $[ROS]_I$ generation and promotion of apoptosis. This may constitute a protective mechanism since stressed cells may be successfully disposed of by macrophages, thereby removing the risk of releasing deleterious digestive enzymes. However, greater and/or more sustained stress by high $[Ca^{2+}]_C$ and $[Ca^{2+}]_m$ levels elicits depolarization of mitochondria with loss of ATP production; this fall in energy is the crucial trigger for necrosis. Leakage of intracellular contents, cytokine release, and inflammation may ensue that result in activation, proliferation, and infiltration of neutrophils, initiating a cycle that ultimately may lead to a systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS). Antioxidant (AOx) treatment may affect the development of acute pancreatitis at several places but which may have differing outcomes. First, inhibition of ROS released by neutrophils is likely to reduce the damage associated with the inflammatory response; however, a reduction of ROS at the local level might conversely prevent apoptosis of pancreatic acinar cells thereby increasing necrosis and organ damage [adapted from Refs. (14, 25)].

antioxidant treatments, including differences between combinations of agents, doses, species, and types of model. Importantly, antioxidant treatments have frequently been given simultaneously, or before, AP induction in animal models from which it would prove difficult to draw a human clinical parallel. The main focus of animal studies has tended toward proving the involvement of oxidative stress and not the standardized testing of the efficacy of specific antioxidant therapies. Thus, the present preclinical evidence evaluating a role for oxidative stress in pancreatitis models is complex and most likely subject to variation depending on the precise experimental conditions employed. The importance of ROS in the disease state should be placed firmly in the context of evidence obtained from human studies, specifically the assessment of antioxidant treatments for AP.

Clinical experience with ROS

Antioxidants are grouped into agents that are free radical-scavenging enzymes (*e.g.,* SOD), enzyme cofactors (*e.g.,* selenium), enzyme substrates (*e.g.,* vitamins A, C, and E), or nonenzymatic antioxidants (*e.g.,* Ebselen and CV-3611). The rationale for the use of such therapy in the treatment of AP is essentially twofold. First, an increase in oxidative stress has been implicated in the pathogenesis of many systemic phenomena, including the SIRS and sequelae, such as acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS), all of which are major features of AP. Greater oxidative stress has been observed in patients with severe and mild AP compared to healthy volunteers (158). In remote organ injury, oxidative damage to plasma constituents such as proteins and lipids is a mortality predictor in patients with established ARDS (128–131). Second, a decrease in antioxidant levels has been demonstrated in a variety of animal AP models, including caerulein hyperstimulation, CDE diet, and Na-taurocholate (31, 111, 112, 143). Thus, a reduction in oxidative stress by antioxidant treatment should theoretically be beneficial in AP patients.

Unfortunately, the promise of antioxidant therapy has not been borne out by human clinical trials that have produced a range of conflicting findings. Most trials have concentrated on scavenging free radicals *via* the GSH pathway. Selenium, a cofactor required for efficient GPx function, and NAC have both featured in these studies. Other studies have used vitamins A, C, and E, to augment the antioxidant defense. Unlike animal studies, enzymes have not been directly administered and also such studies frequently used combinations of antioxidants, making it difficult to determine effects of individual compounds. A combination of antioxidants, including NAC and selenium, was assessed using an observational study on patients (163), showing that antioxidants were safe, with no reported side effects, and restored antioxidant levels toward normal, though only vitamin C and selenium were significantly improved. However, the authors failed to demonstrate a significant impact on mortality in severe AP. A further prospective study assessed efficacy of high dose vitamin C treatment in patients with AP (35). The high-dose group demonstrated greater levels of plasma antioxidants, and reduced levels of lipid peroxidation, and recovery from clinical symptoms was significantly quicker. This study demonstrated promising results; however, the fate of the patients with severe AP was not documented adequately and this must be a crucial endpoint for any potential treatment. A further major criticism of these trials and others is a lack of randomization and blinding, impeding objective, accurate conclusions regarding the potential benefits of antioxidant therapy.

Importantly, a recent single-center study in the United Kingdom has addressed the criticisms leveled at previous studies, including the lack of patient randomization. The first reported randomized, controlled trial of anti-oxidant therapy in AP (148) evaluated the effects of administration of a combination of NAC, selenium, and vitamin C to AP patients over 3 years. Relative serum levels of antioxidants rose, whereas markers of oxidative stress fell in the active treatment group during the course of the trial. However, at 7 days, there was no significant difference in the primary end point, organ dysfunction, between test and control groups or for any secondary end-point of organ dysfunction or patient outcome. Further, this study highlighted a trend toward a more deleterious outcome in patients given antioxidant therapy and the potential benefit to manipulate ROS in the treatment of AP remains far from clear. In accord, a recent meta-analysis of randomized clinical trials suggested that antioxidant supplements may increase mortality in several diseases (11). Our findings on the role of ROS in pancreatic acinar cells (14) are consistent with the lack of effect of antioxidant therapy in clinical AP and, furthermore, provide an explanation for the potential of antioxidant treatment to worsen AP since we found that ROS-scavenging shifted pancreatic acinar cell death from apoptosis to necrosis.

Conclusions

Tightly controlled, transient Ca^{2+} signals are integral to the transduction of neurohormonal stimulation that leads to exocrine secretion. Ca^{2+}-release channels, Ca^{2+}-entry mechanisms, and energy-dependent Ca^{2+}-pumps are sensitive to the cellular redox environment, and generation of ROS, principally by mitochondria, is likely to profoundly influence Ca^{2+} homeostasis. However, when sustained, global elevations of Ca^{2+} are generated by precipitants of AP, mitochondrial function is compromised. The acute Ca^{2+}-dependent generation of ROS in the pancreatic acinar cell promotes apoptosis, a process that allows for removal of dead cells without eliciting inflammation, and may constitute a protective mechanism; the level of stimulation is dependent on the balance between ROS-producing systems and the activity of multiple antioxidant defense mechanisms present in the cell.

A sustained mitochondrial Ca^{2+} increase, however, eventually causes rundown of ATP production since $\Delta\Psi_m$ is lost, probably *via* MPTP formation, with dramatic consequences for the cell. Inhibition of mitochondrial energy production leads to failure of homeostatic ATP-dependant Ca^{2+} pumps that normally return cytosolic Ca^{2+} to basal levels. Further, apoptosis is compromised since energy-dependant caspase activation is inhibited and cells are shunted into necrosis; abrogation of deleterious Ca^{2+} elevations or supply of intracellular ATP prevent necrosis induced by bile acids and FAEEs, and such approaches may prove promising for amelioration of AP. Necrosis triggers the SIRS that recruits neutrophils to the site of inflammation, causing further release of ROS and damage may escalate in a vicious cycle resulting in MODS (Fig. 7). Application of antioxidants may thus reduce systemic inflammation. However, concurrent inhibition of ROS-mediated apoptosis in the exocrine pancreas may also exacerbate organ injury by increasing necrotic cell death. In

accord, recent clinical findings have shown that not only is antioxidant therapy ineffective in the treatment of AP, but also it may actually worsen the outcome, a profile that may be common to other diseases. Thus, ROS cannot be simply viewed as villains of the piece, but are likely to exert far more subtle and complex actions in the body.

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Abbreviations Used

ACh = acetyl choline
ANT = adenine nucleotide translocase
AP = acute pancreatitis
ARDS = acute respiratory distress syndrome
BAPTA-AM = 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester)
cADPR = cyclic ADP ribose
CCK = cholecystokinin
CDE = choline-deficient ethionine-supplemented
CICR = calcium-induced calcium release
DCFDA = 2,7-dichlorodihydrofluorescein diacetate acetyl ester
DMN = 2,4-dimethoxy-2-methylnaphthalene
DPI = diphenyliodonium
ER = endoplasmic reticulum
ETC = electron transport chain
FA = fatty acid
FAEE = fatty acid ethyl ester
GPx = glutathione peroxidase
GSH = glutathione
GSSG = glutathione disulfide
IMM = inner mitochondrial membrane
IP_3 = inositol 1, 4, 5 trisphosphate
IP_3R = inositol 1, 4, 5 trisphosphate receptor
MODS = multiple organ dysfunction syndrome
MPTP = mitochondrial permeability transition pore
NAADP = nicotinic acid adenine dinucleotide phosphate
NAC = *N*-acetyl-*L*-cysteine
NF-κB = nuclear factor kappa B
NQO1 = NADPH quinone oxidoreductase 1
PMCA = plasma membrane Ca^{2+}-ATPase
ROS = reactive oxygen species
RyR = ryanodine receptor
SERCA = sarcoendoplasmic Ca^{2+}-ATPase
SIRS = systemic inflammatory response syndrome
SOCE = store-operated Ca^{2+} entry
SOD = superoxide dismutase
STIM1 = stromal interaction molecule 1
TCA = tricarboxylic acid
TLC-S = taurolithocholic acid 3-sulfate
VDAC = voltage-dependent anion channel
ZG = zymogen granule

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