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Experimental acute pancreatitis: *In vitro* models

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1. Introduction

Acute pancreatitis is an extremely painful and life-threatening inflammatory disease of the exocrine pancreas (57, 100). A sobering point for both clinicians and researchers is that the treatment of acute pancreatitis remains largely supportive. Further, there is a lack of therapies that target primary mechanisms underlying the initiation or propagation of the disease. Thus reliable, relevant, and, importantly, convenient experimental animal models that resemble the human disease are crucial to developing an understanding of the pathobiology of pancreatitis (34, 35, 104, 105, 108, 110). In this chapter, we will review the current *in vitro* (i.e. *ex vivo*) models that serve as surrogates for experimental acute pancreatitis. We will specifically discuss: (1) the standard process of preparing pancreatic acinar cells or pancreatic tissue components; (2) assays for assessing *in vitro* injury and inflammatory precursors; and (3) the array of non-alcoholic and alcoholic *in vitro* models of pancreatitis.

The pancreatic acinar cell is the main parenchymal cell of the pancreas. It comprises roughly 90% of the pancreatic parenchyma, and functions to synthesize and secrete digestive enzymes in response to hormonal stimulation (24, 30, 36, 48).

The acinar cell is considered the initiating site of pancreatic injury, leading to pancreatitis, and thus *in vitro* preparations of acinar cells have been used for decades to define the molecular events that occur during the early stages of the disease (1, 49, 134). The advantage of these models are that they provide a high throughput (or at least rapid) system to examine whether cellular pathways or molecular targets modulate injurious *in vitro* corollaries to *in vivo* events during pancreatitis, including aberrant Ca^{2+} signaling, activation of digestive proteases, NF-kB activation, mitochondrial dysfunction, and cell death through apoptosis or necrosis. A disadvantage is that these systems lack the full inflammatory or systemic components and, therefore, subsequent *in vivo* validation of *in vitro* findings is crucial.

Recent use of adenovirus-mediated gene transfer has enabled researchers to manipulate acinar cell function in the presence of pathological agents (89, 146). Another powerful genetic approach for studying pancreatitis *in vitro* is to isolate acinar cells from the pancreas of gene-targeted knockout or transgenic mice (43, 51, 82, 144).

2. Acinar Cell Preparations

Single, Double, and Large Cluster Acinar Cell Preparations

Isolated pancreatic acini and acinar cells can be prepared from rat, mouse, and guinea pig pancreas using a collagenase digestion protocol (13, 93, 101, 116, 140). Depending on the stringency of the isolation protocol, single, double, and large cluster acinar cells are obtained **(Figure 1A-B)**. The greatest determinant in the stringency of acinar cell preparation is the concentration and duration of collagenase digestion. Nonetheless, there are several collagenases to choose from, including Sigma Type II (142), IV or V (80), and Worthington type IV (29). A newer collagenase P from Roche can be used to prepare smaller acini for electrophysiology (75, 147). Liberase (Roche) is another option for acinar cell isolation. Some authorities use collagenase NB1 (Serva) to perform human islet cell isolation, which also yields acinar cells (and duct cells) for experimental use (12, 63). Acinar size and integrity are highly dependent on the type of collagenase used and the application of shearing forces (99). After digestion of pancreatic tissue, acinar cells can be purified away from ducts, islets, and blood vessels by filtration and bovine serum albumin (BSA) density sedimentation. Following this method, acini can be maintained in culture for 24-48 hours but start to lose their polarity and secretory capability after several hours.

Lobules

To assess the direct as well as indirect effects of agonists on acinar cell secretion, *in vitro* preparations should ideally contain not only acinar cells but also nerves and islets.

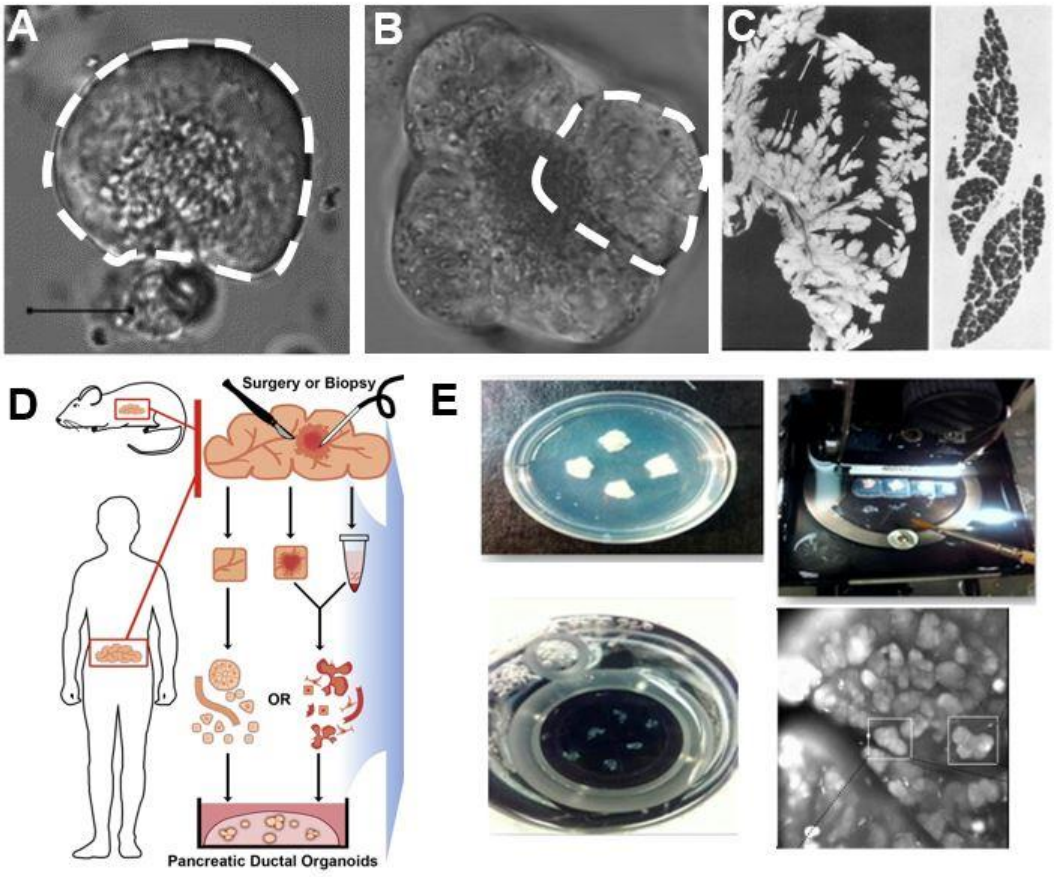

Figure 1. ***In vitro preparations of the pancreas*** **include (A) single acinar cell preparations, (B) acini, (C) pancreatic lobules, (D) pancreatic organoids, or (E) pancreatic slices. Adapted from (99), (93), (113), (10), (45), respectively.** Republished with permission.

For this reason pancreatic lobules are useful **(Figure 1C)**. In the original description by Scheele and colleagues, pancreatic lobules were spread apart by injecting Krebs-Ringer bicarbonate (KRB) buffer into the loose connective tissue of the pancreas and then individually excised by micro-dissection under a stereomicroscope (27, 113). This procedure minimizes damage to acinar cells since most of the surgical trauma is limited to ducts and vessels. The excised lobules preserve the overall acinar architecture of the tissue and their small size and allows for easy penetration of oxygen and solutes from the incubation medium. Following this method, lobules can be maintained for several hours in culture (6, 7, 66, 114).

Organoids

The most recent advance in studying pancreatic physiology *in vitro* involves the generation of pancreatic organoids (10, 46) **(Figure 1D)**. By definition, organoids are three-dimensional organ buds which arise from stem cells. With the use of growth factors, stem cell populations used to develop organoids can be coaxed into forming balls of terminally differentiated cells that self-organize into distinctive layers. As described by Boj and colleagues, pancreatic organoids can be rapidly generated from resected pancreatic tumors and biopsies following manual digestion with collagenase II and seeded in growth factor-reduced Matrigel (10). Conditioning the medium with the growth factor R-spondin promotes a predominantly duct cell population. These pancreatic organoids survive cryopreservation and exhibit ductal- and disease stage-specific characteristics. Further, pancreatic organoids from wild-type mice accurately recapitulated physiologically relevant aspects of disease progression *in vitro*. Following orthotopic transplantation, pancreatic organoids were capable of regenerating normal ductal architectures. This technique is particularly useful for studying duct cell phenotypes (10).

Pancreas Slice

To preserve the integrity of the pancreatic milieu for at least two days in culture, the novel method of culturing pancreas slices is useful (44, 45) **(Figure 1E)**. This technique allows for both *in situ* imaging of cellular events relevant to pancreatitis and genetic manipulation. To obtain a pancreas slice, Gaisano and colleagues gently infused a low melting agarose gel into the pancreatic duct of an anesthetized mouse via a transduodenal puncture and cannulation of the common bile duct (44, 45). The pancreas was then excised and trimmed. The agarose renders the pancreas firm enough to then slice, using a vibratome, at a thickness of 80-140 µm. Moreover, agarose is porous and thus provides free exchange of tissue with buffer, ensuring optimal health in culture for up to two days. Further, this technique permits transfection of cells as well as real time imaging.

Acinar cell lines

The most commonly used cell line to study the exocrine pancreas is the rat pancreatic acinar cell line AR42J **(Figure 2)**. These cells were derived from a transplantable tumor for the rat exocrine pancreas. The AR42J cells differ from primary pancreatic acinar cells in at least two ways: (1) they proliferate rapidly; and (2) although they synthesize, store, and secrete digestive enzymes, they express atypical receptors and conduct atypical inositol phosphate metabolism and cytoskeleton rearrangement (33). Dexamethasone favors their differentiation toward the acinar phenotype, including agonist-stimulated Ca^{2+} signaling (5, 15, 67, 124). The cell line is incubated for 48-72 hours in culture medium supplemented with 100 nM dexamethasone prior to experimental treatment or induction. AR42J cells are easily cultured in a RPMI 1640 medium supplemented with glutamine, FBS, and antibiotics at 37^0C under a humidified condition of 95% air and 5% CO_2. AR42J cells can be routinely plated at a density of 10^5 cells/ml in 75 cm^2 flasks and cultured for 7-10 days.

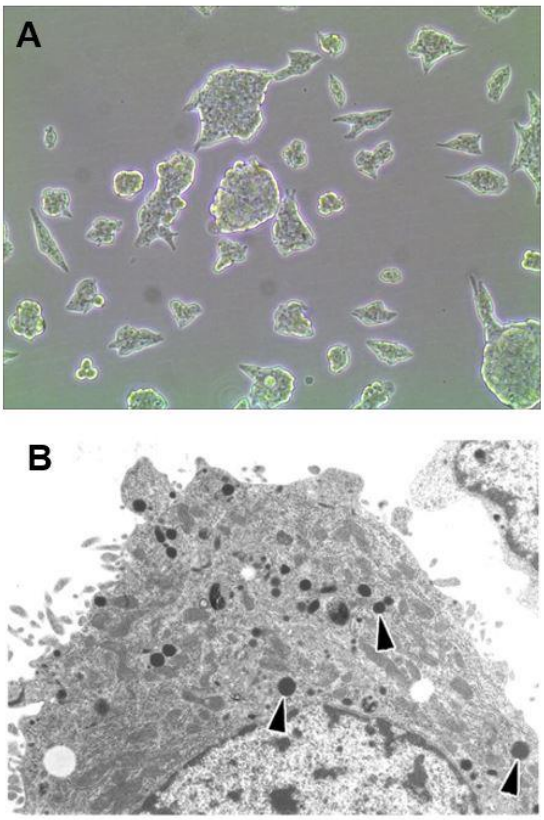

Figure 2. Morphological characteristics of the AR42J acinar cell line. AR42J cells primed with dexamethasone (100 nM) and visualized by (A) light microscopy using a 20X objective or (B) electron microscopy (arrow heads point to zymogen granules). Adapted from (33) and (20), respectively. Republished with permission.

A less common derived acinar cell line is the 266-6. This cell line is derived from young adult mouse tumors induced with elastase I/SV-40 T-antigen fusion gene. Robert Hammer first described the line in 1985 (97). 266-6 cells retain a partially differentiated phenotype and express several digestive enzymes. They respond to carbachol and cholecystokinin (CCK) but do not respond to substance P, secretin, or vasoactive intestinal peptide (VIP). The culture method is the same as that described for AR42J cells, except that there is no dexamethasone priming.

3. Assays for *In Vitro* Surrogates of Pancreatitis

Ca^{2+} Signaling

Pancreatic acinar cells have served as an epithelial cell model for examining Ca^{2+} signaling for decades **(Figure 3)**. Consistent with the polarized nature of acinar cells, Ca^{2+} signals in these cells exhibit highly organized spatial characteristics (103). Most agonist-stimulated Ca^{2+} signals in acinar cells initiate in the apical region and propagate to the basolateral region (31, 48, 52). Single cell imaging of Ca^{2+} signals involves the use of fluorescent Ca^{2+} dyes and confocal microscopy. A number of Ca^{2+} sensing dyes are available, depending on the needs of the researcher (37, 77, 94, 127). The simplest dyes exhibit signature fluorescent properties upon binding Ca^{2+}; they are excited by a certain wavelength of light and emit photons at a certain emission wavelength (i.e. Fluo-3AM, Fluo-4AM). Ratiometric dyes (i.e. Fura-2), on the other hand, exhibit distinct spectral shifts upon Ca^{2+} binding, such that the Ca^{2+}-free form is excited maximally at 380 nm while the Ca^{2+} bound form is excited maximally at 340 nm. Both states emit peak fluorescence at 510 nm.

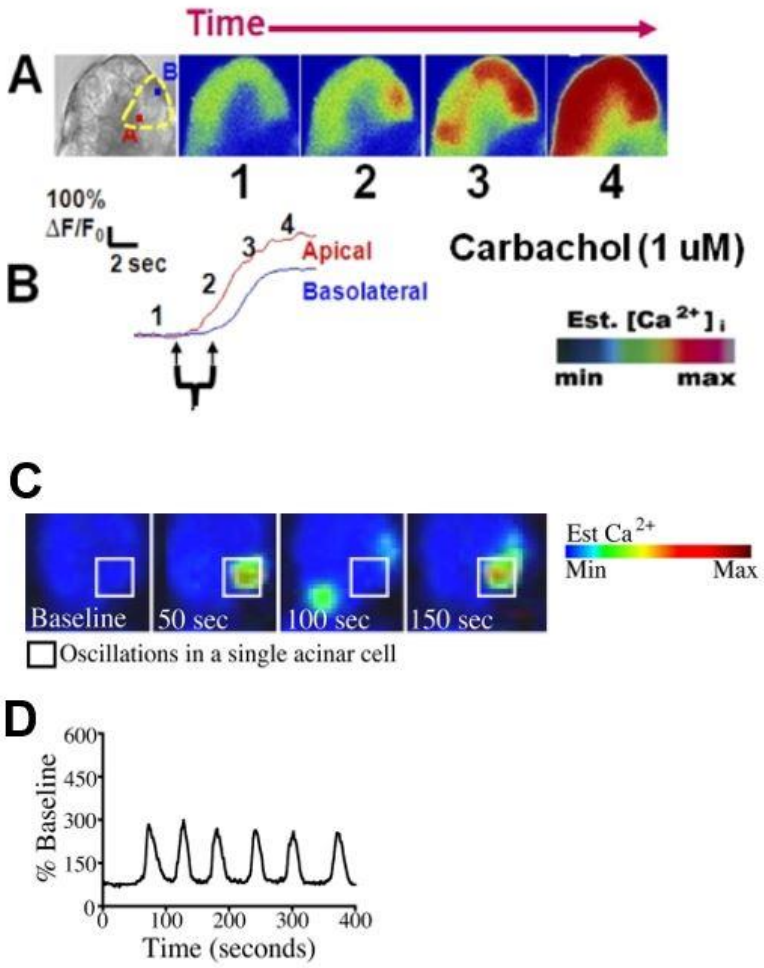

Figure 3. Typical Ca^{2+} transients upon stimulation with supraphysiologic concentrations of carbachol (1 µM) or physiologic concentrations of caerulein (10 pM). Changes in whole cell Ca^{2+} were measured by time-lapse confocal microscopy using the Ca^{2+} dye Fluo-4AM (5 µM). Images are represented in pseudocolor with a color scale. (A) From left to right; bright field view of an acinus labeled at the apical and basolateral regions of interest. Upon stimulation with physiologic carbachol (1 µM; Ach analog), subsequent images show the initiation of the Ca^{2+} signal in the apical region followed by propagation to the basal region. (B) Each paneled image (1-4), corresponds to a frame along a representative tracing of change in fluorescence over time for each region of interest. (C-D) Oscillating Ca^{2+} signals are observed in response to low-dose caerulein (10 pM; CCK analog). These figures were originally published in the J Biol Chem (96) and (106). Republished with permission.

Cells are loaded with the Ca^{2+} dye of choice, allowed to adhere to glass coverslips, and excited with the agonist of choice, while collecting real time images usually with a laser scanning confocal microscope (93).

Intra-Acinar Protease Activation

Premature intra-cellular activation of digestive proteases has long been thought to represent an early, initiating event in the pathogenesis of pancreatitis. The traditional method for examining intra-acinar protease activation involves probing pancreatic acinar cell lysates with a fluorogenic substrate for the protease of interest (58, 110, 118). The readout is obtained from a fluorimeter (e.g. a fluorescent plate reader or cuvette system, also termed a fluorimeter) in the form of a kinetic plot. These data can be normalized to total protein content or total DNA in order to control for cell loading. Since the initial description of these fluorogenic substrates in 1983 (64, 65), bisamide derivatives of rhodamine 110 have been used as

a sensitive and selective substrate for activated protease measurements. Proteolytic selectivity is achieved by using specific benzyloxycarbonyl-peptides. The tripeptide derivative bis-(CBZ-Ile-Pro-Arg)-R110 (BZiPAR) has been successfully used by some groups to measure trypsinogen activation by live microscopy (54, 55, 62, 105).

NF-κB Translocation

NF-κB activation is thought to be an early and critical component of the inflammatory response during acute pancreatitis (104). Traditional methods for examining NF-κB activity *in vitro* include protein determination of NF-κB pathway markers (i.e. phosphorylated IκB; p65 nuclear translocation; IKK upregulation), electromobility shift assay (EMSA), and immunohistochemistry for phosphorylated p65 (51, 125). Newer techniques include the transfection (or usually infection via viral vectors in pancreatic cells) of NF-κB-luciferase reporters **(Figure 4)**. With these techniques, binding of NF-κB subunits to a nuclear response element drives transcription of the luminescent protein luciferase. The commonly used luciferase reporters are firefly (21) and renilla (68) luciferases. The development of secreted luciferases such as gaussia (Gluc), secreted alkaline phosphatase (SEAP), and cypridina allows for serial determination from the media of NF-κB activity over time (3, 41, 87, 126).

Mitochondrial Damage

Mitochondrial dysfunction has been shown to play a critical role in the pathogenesis of pancreatic acinar cell injury, resulting in pancreatitis (73). Manifestations of mitochondrial dysfunction in pancreatitis include loss of mitochondrial inner membrane potential ($\Delta\Psi m$), generation of reactive oxygen species (ROS), release of the apoptosis, or programmed cell death mediator cytochrome c into the cytosol, and failure of ATP production; the events lead to varying degrees of acinar cell necrosis or apoptosis (92). Recent data show that preventing mitochondrial damage improves several aspects of pancreatitis and ameliorates disease severity (85, 119).

The effect of pancreatitis on $\Delta\Psi m$ can be measured in isolated acinar cells using the $\Delta\Psi m$-sensitive fluorescence probe tetramethylrhodamine methyl ester (TMRM), which is a lipophilic cation dye whose accumulation in mitochondria is proportional to the amount of $\Delta\Psi m$. After preincubation with an agonist, cells are loaded with 1 μM TMRM for 10-20 min at 37^0C and transferred to a fluorimeter to measure fluorescence intensity at 543 nm/570 nm (90, 119). $\Delta\Psi m$ can also be detected using another $\Delta\Psi m$-sensitive fluorescence probe JC-1, which exists as a green monomer at low $\Delta\Psi m$. Because JC-1 forms red fluorescent J-aggregates at higher potentials, the ratio between red (550 nm/600 nm) and green (485 nm/535 nm) fluorescence is used to monitor changes in $\Delta\Psi m$. A loss of $\Delta\Psi m$ leads to depletion of intracellular ATP and subsequent necrosis. ATP levels in pancreatic acinar cells can be detected using a luciferin/luciferase luminescence-based assay that is normalized to protein content.

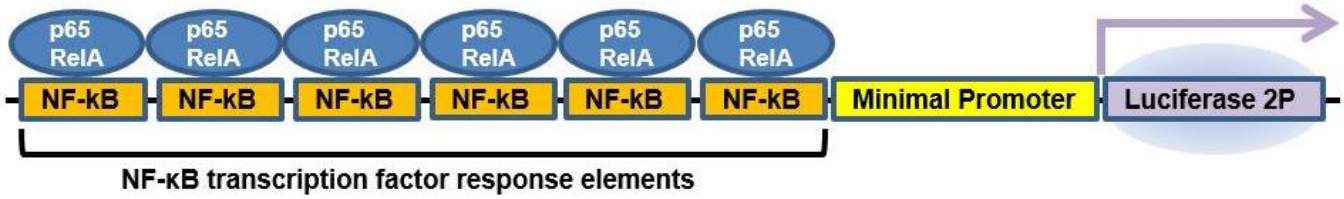

Figure 4. Schematic of the NF-κB-luciferase adenoviral construct. The NF-κB-luciferase adenoviral construct contains six tandem-repeat transcription factor response elements, a minimal promoter, and a luciferase coding region. Binding of NF-κB subunits to a nuclear response element drives transcription of the luminescent protein luciferase. Originally published in the J Biol Chem (95). Republished with permission.

Permeabilization of the mitochondrial outer membrane occurs through opening of the mitochondrial permeability transition pore (MPTP), and the event is integral to apoptosis in pancreatitis. MPTP opening and subsequent mitochondrial outer membrane permeabilization result in the release of the mitochondrial resident protein cytochrome c into the cytosol. The technique to detect cytochrome c release within acinar cells relies on examining immunoblots against cytochrome c from cellular fractions of mitochondria-enriched membrane versus cytosolic fractions (74, 91).

The mitochondria within acinar cells are highly susceptible to oxidative damage from ROS, and they in turn also serve as primary generators of ROS when the electronic transport chain within the inner mitochondrial membrane is perturbed (usually with loss of $\Delta\Psi m$). ROS can act as a molecular trigger of cytochrome c release and death responses in pancreatic acinar cells, thus also demonstrating the cross-talk in the mitochondria between necrosis and apoptosis triggers (85). Intracellular ROS levels (both mitochondrial and non-mitochondrial) are detected using 2,7-dichlorofluorescein (DCF) (91). ROS that is selectively generated by the mitochondria can be monitored by labeling the cells with the mitochondrial ROS-sensitive rhodamine-based fluorescent dye DHR123. Mitochondrial localization of DHR123 can be confirmed by co-staining the cells with the mitochondrial specific marker MitoTracker Red (CMXRos). Proper analysis of ROS production in living cells requires the combined use of several fluorescent ROS probes in parallel experiments, assessment of non-ROS related parameters that can induce artifacts (e.g. $\Delta\Psi$, pH), and the inclusion of adequate control conditions. For example, a common positive control that is known to cause the generation of mitochondrial ROS is rotenone, which inhibits complex I of the electron transport chain. A negative control is the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), which blocks mitochondrial ROS production.

Cell Injury

The three most common assays used to assess acinar cell injury include: (1) lactate dehydrogenase (LDH) release; (2) propidium iodide (PI) uptake; and (3) reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). LDH catalyzes the interconversion of pyruvate to lactate and NADH to NAD+ (72). Elevated levels of LDH are indicative of tissue injury and breakdown. LDH can be measured using colorimetric assays supplied by Promega (cat #G1780) (93). PI is a high affinity DNA-binding dye that is effectively excluded from live cells (59, 79, 123). Dead or dying cells have compromised plasma membranes and thereby allow the leakage of PI, which then enters the nucleus and binds to DNA. MTT reduction is a measure of mitochondrial function and cell viability (8, 16, 81). MTT is reduced to insoluble formazan by mitochondrial dehydrogenases. Water insoluble formazan can be solubilized using isopropanol or other solvents. The dissolved material is measured spectrophotometrically, yielding absorbance as a function of the concentration of the converted dye.

4. Non-Alcholic Models

Secretagogues

The peptide hormone CCK, or its analog caerulein, has been used in *in vitro* models to reproducibly induce acute pancreatitis-like responses in acinar cells (14, 58, 63, 111, 112, 134, 140). Pancreatic acinar cells express high and low affinity CCK receptors (CCKRs), which are activated by low and high concentrations of CCK, respectively (76, 139). Low concentrations in the picomolar range bind to high affinity CCK receptors and maximally stimulate physiological acinar cell enzyme secretion (138). High, or supra-physiological, concentrations in the nanomolar range bind to low affinity CCK receptors and result in a relative reduction in the

secretory response, a phenomenon that is thought to be pathological to the cell because it leads to the retention of the prematurely activated proteases and their missorting (62).

The activation of digestive proteases requires a rise in cytosolic Ca^{2+}, which occurs through release from intracellular Ca^{2+} pools (primarily the endoplasmic reticulum) that are gated by IP3 receptors and ryanodine receptors (48, 60, 105, 110). Another consequence of supraphysiological CCK, seen both *in vitro* and *in vivo*, is the emergence of large intra-acinar vacuoles (39, 105, 120).

There are other CCK analogues which do not lead to protease activation or pancreatitis, even at high concentrations because they elicit distinct phenotypic responses and distinct cell signals. They include the O-phenyl-methyl-ester analog of CCK (OPE) and JMV-180 (76, 139). These agonists can serve as physiological controls to differentiate between pathological signals. The agonist bombesin (also known as gastrin-related peptide) causes intra-acinar protease activation but no acinar cell injury because, unlike CCK, bombesin does not cause activated proteases to be retained in the acinar cell (34). Other secretagogues that stimulate acinar cell enzyme secretion include secretin, VIP, and pituitary adenylate cyclase-activating peptide (PACAP) (48, 115, 117).

Several investigations have questioned whether CCK hyperstimulation is relevant to human acinar cells (109, 112). Whereas CCK receptors are abundant on murine acinar cells, they have little to no expression in the human acinar cell (50, 133). Except for a notable recent report (86), CCK failed to elicit a Ca^{2+} signal or a secretory response in isolated human acini (50, 78, 122). By contrast, acetylcholine or its long-acting analog carbachol stimulates robust physiological and pathological (at high millimolar concentrations) responses in acinar cells from mouse, rat, and man (70, 98). Several clinical correlates of pancreatitis are associated with cholinergic overload, from exposure to scorpion toxin or organophosphates (which would prevent the degradation of acetylcholine by inhibiting endogenous acetylcholinesterases) (107, 121, 128, 132).

Bile Acids

The most common cause of acute pancreatitis is impaction of gallstones or sludge in the distal common bile duct, a situation called biliary pancreatitis (4, 69, 71, 129). There are two hotly debated and non-mutually exclusive theories for biliary pancreatitis: (1) increased pressure in the pancreatic duct and (2) reflux of bile into the pancreatic duct (61). The latter can be recapitulated *in vitro* by exogenous administration of bile or its components. Bile is composed predominantly of the bile acids taurocholate (TC), taurochenodeoxycholate (TCDC), taurodeoxycholate (TDC), while taurolithocholic acid 3-sulfate (TLCS) comprises a small fraction of bile (26, 135). However, TLCS is most commonly used *in vitro* because it is the least hydrophilic and, therefore, most potent of the naturally occurring bile acids. It induces Ca^{2+} signals at low micromolar concentrations that are below the critical micellar concentration (42). Bile acids can be transported into pancreatic acinar cells through specific transporter, or they can bind to their cognate receptors, including the transmembrane G protein-coupled receptor TGR5 (also known as the G-protein coupled bile acid receptor 1, or GPBAR1) (53, 102). Bile acid administration triggers aberrant acinar cell Ca^{2+} signals leading to trypsinogen activation and cell death (47, 83, 84, 130). Rescuing ATP depletion by patching ATP into isolated acinar cells prevents necrotic cell death due to the bile acids (11, 130, 131).

Fatty Acids

Recent investigations into the role of obesity during acute pancreatitis have revealed that accumulation of intra-pancreatic fat is associated with greater tendency towards pancreatic necrosis during acute pancreatitis and that acute

pancreatitis is associated with multisystem organ failure in obese individuals (23, 88, 108). These findings provided the rationale to examine a direct role for fatty acids in acinar cell pathobiology *in vitro*. Unsaturated fatty acids, in particular, appear to exert a proinflammatory role; they trigger pathological intracellular Ca^{2+} signals, inhibit mitochondrial complexes I and V, and cause necrosis. Saturated fatty acids have no such effect.

5. Alcoholic Models

Alcohol is a major etiology of acute pancreatitis (28, 145). Chronic ethanol exposure appears to sensitize the pancreas to the pathologic effects of other concomitant stressors in the development of the disease (2, 57, 70, 96).

The mechanism of the sensitizing effect of alcohol is unclear. *In vitro* exposure to clinically relevant concentrations of ethanol (50-100 mM; for at least an hour of incubation, under sealed conditions) in combination with physiological concentrations of CCK or carbachol have been shown to trigger pathological responses of pancreatitis in acinar cells, including protease activation, intracellular activation of NF-kB, expression of pro-inflammatory cytokines, vacuolization, and necrosis (25, 32, 56, 96).

One mechanism of ethanol's toxic effects is through the actions of its metabolites, including the oxidative (acetaldehyde) and non-oxidative (fatty acid ethyl ester, FAEEs) metabolites (9, 17, 22, 136, 137, 143). Several studies have now demonstrated that both pathways in ethanol metabolism are evident in the pancreas and that exposure of pancreatic acinar cells to ethanol alone results in accumulation of both acetaldehyde and FAEEs (17, 18, 38). The non-oxidative metabolites FAEEs increase acinar cell lysosomal fragility and induce a rise in intracellular Ca^{2+} (19, 40, 141), along with premature intracellular digestive enzyme activation, acinar cell vacuolization, and loss of $\Delta\Psi m$, ATP depletion, and cell necrosis (17, 131).

6. Summary

In summary, we have described methods for the isolation of pancreatic acinar cells, lobules, organoids, and slices. In addition, we have provided a description of assays for critical surrogates of pancreatitis *in vitro*. Lastly, we have given an overview of the various types of secretagogues and naturally occurring agonists that can be used to stimulate pancreatic acinar cells *in vitro* for the purpose of studying pathologic surrogates of pancreatitis. The use of such tools is helping researchers, not only to elucidate the molecular mechanisms mediating acute pancreatitis, but also to test novel therapeutic agents on acinar cells, that could reduce cell damage caused by pancreatitis.

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