I. Introduction: Focus of the Chapter

The major cell types defining exocrine functions of pancreas are pancreatic acinar and pancreatic ductal cells. Acinar cells secrete digestive enzymes, precursors of digestive enzymes (zymogens) and NaCl-rich fluid, whilst ductal cells secrete large volumes of bicarbonate-containing fluid and form a conduit for exocrine pancreatic secretion (reviewed in (35, 76, 128)). Both exocytotic secretion of proteins and fluid secretion are energy consuming processes. Furthermore, pancreatic acinar cells have high levels of protein synthesis (165)) supporting substantial protein export by secretion. The bioenergetics of this organ are designed to sustain these energy demands and adjust ATP generation when cells undergo shifts between resting and stimulated conditions. Disruption/insufficiency of ATP generation is an important feature of pancreatic damage in pathological conditions. In this chapter we will consider both physiological adaptation of bioenergetics in exocrine pancreas and its disruption in acute pancreatitis.

Pancreatic acinar cells constitute more than 80% of the volume of the exocrine pancreas (24) and are, thus, likely to make major contributions to bioenergetics parameters measured in experiments utilizing undissociated pancreas. Signaling mechanisms and bioenergetics of this cell type have been extensively investigated and a substantial part of this chapter therefore will be focused on the bioenergetics of pancreatic acinar cells. There has also been recently significant progress in characterizing bioenergetics changes in ductal cells, particularly in conjunction with the pathophysiology of acute pancreatitis; we will discuss this progress in section ‘Alteration of Bioenergetics in Pancreatic Pathophysiology’.

II. Mechanisms of ATP Generation

Information on the relative importance of different substrates (carbohydrates, amino acids and fatty acids) for the bioenergetics of exocrine pancreas is somewhat limited. Pancreatic acinar cells express a range of amino acid transporters (144), allowing accumulation of amino acids against a significant concentration gradient; this is beneficial for prominent protein synthesis in this cell type and could also support utilization of amino acids as substrates for ATP production. Notably, in in vivo and in vitro experimental models of acute pancreatitis expression of several amino acid transporters was significantly downregulated (144). A study by S. Araya and colleagues highlighted a high rate of glutamine transport in pancreatic acinar cells and compared CO₂ generation in response to addition of amino acids (glutamine and leucine), glucose and palmitoyl (3). The study concluded that glutamine is the preferred energy source for pancreatic acinar cells and that these cells operate an efficient conversion of glutamine to glutamate followed by glutamate utilization (via α-ketoglutarate) in the Krebs cycle (3). Information on the contribution of other amino acids and fatty acids, however, is sparse. Glucose can support
the bioenergetics of acinar cells and glucose-containing extracellular solution has been frequently utilized in studies of glycolysis and oxidative phosphorylation in this cell type. The relative contribution of oxidative metabolism and glycolysis to the net ATP generation in exocrine pancreas and pancreatic acinar cells is still a subject for debate. Mitochondria occupy a significant proportion of cell volume in pancreatic acinar cells (approximately 8% (24)). An early study by Bauduin and colleagues concluded that mitochondria are by far the major source of ATP production in acinar cells whilst glycolysis makes only minor contribution (14). This paper, however, reported that high concentration of Oligomycin (inhibitor of the mitochondrial F_1F_0-ATP synthase: abbreviated to ATP synthase) reduced nucleoside triphosphate (presumably mainly ATP) content to approximately 41% of the control value, suggesting a significant contribution of glycolysis to ATP generation. However, the same authors reported that antimycin (inhibitor of the mitochondrial electron transport chain) reduced nucleoside triphosphate content to 16% and anaerobiosis to 8% (14). H. Kosowski and colleagues from W. Halangk laboratory reported a decrease of ATP content to approximately 21% of the control value following 30 minuses of anoxia (98). Experiments with anoxia and antimycin could be interpreted as evidence for a rather modest contribution of glycolysis to ATP generation in the acinar cells. However, these treatments are expected to trigger the reverse mode of ATP synthase, which can convert this enzyme into a powerful ATP consumer (i.e. in this mode the enzyme functions as an ATPase consuming ATP and transporting protons into the matrix; reviewed in (32, 68, 101, 167)). The discrepancy between the ATP levels retained in the presence of oligomycin and after the inhibition of mitochondrial electron transport chain by anoxia or antimycin could be explained by a reverse mode of ATP synthase. ATP consumption by this reverse mode could be potentially prevented by the F_1F_0-ATP synthase inhibitory factor 1 (IF1) (32). However, it should be noted that normal pancreas contains relatively low levels of IF1 (e.g. in comparison with heart or pancreatic cancer tissue (158)) and therefore the effect of the reverse mode could be significant. Consequently, experiments with oligomycin or combination of oligomycin with an inhibitor of the electron transport chain should provide a better estimation of contribution made by glycolysis to ATP generation of the acinar cells. The early estimation of 41% (14) is consistent with more recent findings indicating a significant (at least 30-40%) contribution of glycolysis to ATP generation in acinar cells (158, 171). Furthermore, the balance of glycolytic and oxidative ATP production depends on external factors. In particular, secretagogues that produce moderate Ca^{2+} signals upregulate the rate of Krebs cycle and mitochondrial ATP generation ((39, 170, 171) and the next section), whilst application of insulin strongly potentiates glycolytic ATP production, which can sustain bioenergetics of acinar cells with damaged mitochondria (111, 145).

III. Stimulus-Metabolism Coupling

In the late 1970s, an important and surprising observation was made in experiments on isolated pancreatic acini (97, 177); upon stimulation with secretagogues (caerulein, cholecystokinin or carbachol) cellular ATP levels were stable or even slightly increased (97, 177). It would be expected that the increased energy demands imposed by the stimulation of secretion should reduce ATP content. The findings were therefore paradoxical and suggested existence of an effective mechanism(s), capable of compensating and even overcompensating for the increased ATP usage in the stimulated cells i.e. indicative of an efficient stimulus-metabolism coupling in this cell type. Early evidence for such stimulus-dependent upregulation of bioenergetics came from the study by M. Korc and colleagues demonstrating strong and rapid upregulation of glucose uptake in isolated pancreatic mouse acini stimulated with secretagogues ((97); see Figure 1A). Caerulein, CCK and Carbachol were tested and all produced clear and substantial increases in glucose uptake (2.5-3 times in comparison with control) (97). Crucially, the increase in the glucose uptake could be mimicked by the Ca^{2+} ionophore A23187. Furthermore, incubation of cells in the solution containing the Ca^{2+} chelator
EGTA suppressed the agonist-induced glucose uptake (97). The authors therefore concluded that intracellular Ca\(^{2+}\) may “…mediate hormone-stimulated glucose transport…” (97). The authors’ suggested role of cellular Ca\(^{2+}\) in the regulation of bioenergetics of the acinar cells was confirmed by the following 40 years of research. Important findings that characterized the role played by Ca\(^{2+}\) signaling in regulating bioenergetics were made at the end of the previous century in Denton’s laboratory. These experiments revealed the ability of Ca\(^{2+}\) to upregulate the rate of Krebs cycle; the effect of Ca\(^{2+}\) was mediated by rate-limiting dehydrogenases of the Krebs cycle (reviewed in (52, 119)). At the single cell level, the relationships between cytosolic Ca\(^{2+}\), mitochondrial Ca\(^{2+}\) and NAD(P)H (an important reducing equivalent produced in Krebs cycle that supplies electrons for the respiratory chain) were first demonstrated in hepatocytes (69). This study utilized cellular autofluorescence to monitor the NAD(P)H responses and revealed fast upregulation of the NAD(P)H production by Ca\(^{2+}\) transients (69). In isolated pancreatic acinar cells relationships between Ca\(^{2+}\) signalling (in cytosol and mitochondria) and NAD(P)H was first characterized by Voronina and colleagues (170). This study revealed fast and robust NAD(P)H transients associated with Ca\(^{2+}\) responses (Figure 1B and C), consistent with NAD(P)H responses recorded from perfused pancreas (87).

Subsequently, rises of NAD(P)H in response to CCK-induced Ca\(^{2+}\) transients were shown to occur in isolated human pancreatic acinar cells, confirming a similar stimulus-metabolism coupling to that previously elucidated in murine cells (125). Physiological concentrations of important secretagogues (e.g. CCK and ACh) induce oscillatory Ca\(^{2+}\) responses in acinar cells (reviewed in (139, 176, 179)). NAD(P)H responses were sufficiently fast to forms oscillations (reflecting oscillations in the rate of the Krebs cycle) closely associated with the oscillations of cytosolic Ca\(^{2+}\) (170)). Upon stimulation with Ca\(^{2+}\) releasing secretagogues pancreatic acinar cells generate local and global Ca\(^{2+}\) transients (e.g. (90, 160) reviewed in (137, 179)). All global Ca\(^{2+}\) transients were able to trigger NAD(P)H transients (i.e. increased rate of the Krebs cycle). The majority of local Ca\(^{2+}\) responses were also able to trigger resolvable NAD(P)H responses except for the very short local Ca\(^{2+}\) transients (less than 2.9 seconds) which are probably quenched by cytosolic calcium buffers before reaching the mitochondria (170). To upregulate activity of dehydrogenases of the Krebs cycle and accelerate its rate, Ca\(^{2+}\) must enter mitochondrial matrix. The phenomenon and properties of mitochondrial Ca\(^{2+}\) entry were extensively characterized in the second part of the previous century; however, the molecular mechanism was only discovered in 2011.

![Figure 1](image_url)

**Figure 1.** Stimulus-metabolism coupling in pancreatic acinar cells. (A) Changes in glucose uptake by pancreatic acini upon application of caerulein. (Adapted from Korc et al (97)). (B) NADH
oscillations in pancreatic acinar cells. Left panels show transmitted image of two pancreatic acinar cells and regions of interest for recording of fluorescence (corresponding to traces on the right panel). Lower left panel shows the distribution of NADH fluorescence. (Adapted from Voronina et al (170)). (C) Correlation between cytosolic Ca\(^{2+}\) responses (revealed by negative deflections on the trace of Ca\(^{2+}\)-dependent Cl\(^{-}\) current) and changes of NADH fluorescence. (Adapted from Voronina et al (170)). (D) Effect of mitochondrial Ca\(^{2+}\) uniporter (MCU) knockout on the NADH and FAD responses to CCK stimulation in pancreatic acinar cells. (Adapted from Chvanov et al (39)).

Mitochondrial Ca\(^{2+}\) entry is primarily mediated by the mitochondrial Ca\(^{2+}\) uniporter complex ((15, 51) reviewed in (110)). In mammals this complex is composed of the mitochondrial Ca\(^{2+}\) uniporter (MCU, pore forming protein localized in the inner mitochondrial membrane), its Ca\(^{2+}\) dependent regulators MICU1/MICU2 (47, 86, 132, 135), and other associated and regulatory proteins (reviewed in (110)). An important function of MICU1/MICU2 is to provide a threshold Ca\(^{2+}\) concentration that limits Ca\(^{2+}\) entry into mitochondria at low cytosolic Ca\(^{2+}\) concentration. The voltage difference between mitochondria and cytosol is substantial (approximately 140 mV); it is sufficient to drive Ca\(^{2+}\) entry into mitochondria even at low resting Ca\(^{2+}\) levels. This would form a futile Ca\(^{2+}\) cycle necessitating continuous energy dependent Ca\(^{2+}\) extrusion from mitochondria (20, 21, 47, 86, 109, 132). At higher Ca\(^{2+}\) concentrations MICU1 and MICU2 bind Ca\(^{2+}\) and undergo conformational change allowing Ca\(^{2+}\) entry through the MCU channel (47, 86, 109, 132). Threshold Ca\(^{2+}\) concentrations necessary for such ‘opening’ of the MCU complex are usually significantly higher than the resting cytosolic Ca\(^{2+}\) and usually also higher than Ca\(^{2+}\) concentrations in the areas distant from Ca\(^{2+}\) releasing channels in the cellular organelles or Ca\(^{2+}\) influx channels in the plasma membrane. Therefore, Ca\(^{2+}\) entry into mitochondrial matrix in many systems depends on the mitochondrial localization and specifically on the proximity of the organelles and their MCU complex to the Ca\(^{2+}\) releasing channels (reviewed in (159)). In pancreatic acinar cells mitochondria are found in three major groups - subplasmalemmal, perigranular and perinuclear (54, 83, 130, 161). These mitochondria are conveniently localized to respond to Ca\(^{2+}\) release from IP\(_3\) receptors (IP\(_3\)Rs) (161), Ryanodine Receptors (RyR) (158) and store operated Ca\(^{2+}\) (SOC) influx channels (103, 130). The mitochondrial Ca\(^{2+}\) uniporter is responsible for the bulk of Ca\(^{2+}\) entry into the mitochondrial matrix, however, other mitochondrial Ca\(^{2+}\) influx mechanisms have been reported (e.g. (25, 72, 147)). A recent study, utilizing MCU knockout (MCU KO) mice, demonstrated that the MCU complex is responsible for the major component of the mitochondrial Ca\(^{2+}\) entry in CCK-stimulated pancreatic acinar cells, however, very small mitochondrial Ca\(^{2+}\) signals were observed in CCK-stimulated acinar cells from MCU KO mice suggesting an alternative (and currently unknown) Ca\(^{2+}\) entry mechanism in pancreatic mitochondria (39). These observations were consistent with the results of measurements of mitochondrial NAD(P)H and FAD responses. During the Krebs cycle FAD is converted to FADH\(_2\); FAD is fluorescent and the decrease of its content/fluorescence in mitochondria suggests its reduction and could be interpreted (with caution) as an acceleration of Krebs cycle (57). NAD(P)H and FAD responses associated with Ca\(^{2+}\) signals have been measured in a number of cell types (e.g. (56, 69)); in these studies, transient increases in NAD(P)H fluorescence were usually accompanied by decreases in FAD fluorescence. The amplitudes of both NAD(P)H and FAD responses to CCK stimulation drastically decreased in the acinar cells from MCU KO animals in comparison with wild type (WT) mice ((39) and Figure 1D) This confirms the major role of Ca\(^{2+}\) signals and MCU-mediated mitochondrial Ca\(^{2+}\) responses in stimulus-metabolism coupling in pancreatic acinar cells. Notably small NAD(P)H and FAD responses were retained in the cells from MCU KO mice; this is consistent with the retention of small mitochondrial Ca\(^{2+}\) signals in cells of these animals (39). Another important parameter reflecting cellular bioenergetics is oxygen consumption. In line with the observed upregulation of glucose uptake (97) and NAD(P)H increase (39, 170), oxygen consumption significantly increases in perfused pancreas (87) and isolated acinar cells stimulated with Ca\(^{2+}\) releasing secretagogues
(39, 87, 112, 121). Importantly, this effect was diminished in acinar cells from MCU KO mice (39), again highlighting the role of mitochondrial $\text{Ca}^{2+}$ in the stimulus-metabolism coupling of this cell type. The notion of the efficient stimulus-metabolism coupling in pancreatic acinar cells is consistent with the outcome of experiments involving ATP and creatine phosphate measurements in perfused rat pancreas, which indicated that “ATP and PCr remained largely unchanged” in spite of maximal stimulation of the organ with 100 pM CCK producing robust protein and fluid secretion (117). In this study a higher concentration of CCK (1nM) produced a small decrease in ATP levels, which may be associated with tissue-level effects of supramaximal doses of this agonist and will be discussed in the role of bioenergetics in pancreatitis (see below). Whilst studies of the relationships between $\text{Ca}^{2+}$-releasing secretagogues and bioenergetics, conducted on isolated acinar cells and perfused pancreata, reported stable or minimally changed ATP levels, despite upregulation of energy consuming processes (97, 171, 177); results from experimental animal models, involving low “physiological” as well as supramaximal doses of secretagogues, are somewhat contradictory. In the study by R. Luthen and colleagues, all tested concentrations of caerulein (7 hourly intraperitoneal injections of caerulein each corresponding to 0.1, 1, 5 and 50 µg/kg body weight) produced significant and similar decreases in ATP concentration in mouse pancreata (by approximately 33% one hour after the first injection and 45% one hour after the last injection) (104). It is conceivable that in intact tissue the rate of ATP production is limited by tissue oxygen levels and nutrient supply (e.g. due to changes of pancreatic microcirculation, reviewed in (48)), which are usually not restricted in single cell preparations. It should be also noted that other studies involving animal models and stimulation with secretagogues reported stable pancreatic ATP levels (117, 127). This apparent contradiction emphasizes the importance of continuing investigation of stimulus-metabolism coupling at cellular and tissue levels as well as in animal models. The pathophysiology of acute pancreatitis is frequently associated with disruption of bioenergetics and loss of ATP in pancreatic tissue. In experimental models of acute pancreatitis this is particularly clear for bile-induced and fatty acid-induced acute pancreatitis; the relevant studies will be discussed below.

IV. Metabolism - Signaling Feedback

Several pathophysiologically-relevant substances (including fatty acids, reactive oxygen species, asparaginase, bile acids and non-oxidative alcohol metabolites) can deplete cellular ATP ((5, 26, 44, 111, 133, 134, 145, 171); and the next section). While $\text{Ca}^{2+}$ signaling can influence metabolism and mediate efficient stimulus-metabolism coupling in pancreatic acinar cells, there is also a powerful effect of metabolism on signaling. Interesting early manifestation of such feedback mechanism came from study by A. Hofer and colleagues which demonstrated inhibition of $\text{Ca}^{2+}$ leak from the ER lumen by ATP depletion (78). Further studies identified the prominent influence of ATP depletion on ACh-induced $\text{Ca}^{2+}$ oscillations in pancreatic acinar cells (12); a modest decrease in cytosolic ATP concentration (revealed by measuring luciferase bioluminescence in live cells) resulted in abrupt termination of $\text{Ca}^{2+}$ oscillations ((12) and Figure 2A). The molecular phenomenon underlying this cellular response was identified in studies from the Yule laboratory that characterized ATP sensitivity of $\text{IP}_3$ receptors responsible for the $\text{Ca}^{2+}$ oscillations in the acinar cells. All three types of $\text{IP}_3$Rs are expressed in pancreatic acinar cells (62), however, the expression of $\text{InsP}_3$R$_1$ is relatively low (62) and it is unlikely to play significant role in physiologically-relevant $\text{Ca}^{2+}$ responses. $\text{InsP}_3$R$_2$ and $\text{InsP}_3$R$_3$ are the major receptor subtypes in acinar cells (62). These are ATP sensitive and $\text{Ca}^{2+}$ fluxes via these channels are inhibited by ATP reduction in mM or sub-mM range (18, 129). Further study utilizing acinar cells from $\text{InsP}_3$R$_2$ knock-out mice revealed particular importance of this $\text{Ca}^{2+}$ releasing channel in determining ATP sensitivity of $\text{Ca}^{2+}$ responses ((129) and Figure 2B). Another vital component of calcium signaling, $\text{Ca}^{2+}$ influx via the plasma membrane, is also ATP-sensitive. In pancreatic acinar cells,
Ca\(^{2+}\) influx is primarily mediated by the store operated Ca\(^{2+}\) entry (SOCE) based on the interaction of the ER Ca\(^{2+}\) sensor STIM1 and the plasma membrane channel-forming protein Orai (1, 64, 103, 168, 172). A role for TRPC channels in SOCE has also been reported (92).

Stimulus-metabolism coupling and metabolism-signalling feedback are schematically illustrated in Figure 3. As expected, ATP depletion also suppressed Ca\(^{2+}\) uptake into the ER lumen via sarcoplasmic / endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) and Ca\(^{2+}\) extrusion via plasma membrane Ca\(^{2+}\) ATPase (PMCA) (12, 111, 145). In pancreatic acinar cells inhibition of SOCE by ATP depletion occurred in parallel with the inhibition of Ca\(^{2+}\) extrusion (12) suggesting an important and hitherto unidentified link between the two processes. The inhibition of Ca\(^{2+}\) influx by ATP depletion is, however, incomplete and prolonged depletion leads to Ca\(^{2+}\) overload and damage/death of pancreatic acinar cells (111, 145). Furthermore, some inducers of acute pancreatitis (e.g. bile acids and fatty acids) seem to be capable of generating an alternative Ca\(^{2+}\) influx pathway which is not inhibited by ATP depletion (12, 111, 145). A number of novel Ca\(^{2+}\) influx channels have been recently identified in pancreatic acinar cells (60, 143, 157) and the effects of mitochondrial inhibition and ATP depletion on these channels is currently unknown. Identifying the relationships between the recently identified Ca\(^{2+}\) entry pathways and cellular bioenergetics could be the subject of productive future investigation.

**Figure 2.** Metabolism-signaling feedback. (A) Inhibition of acetylcholine induced cytosolic Ca\(^{2+}\) oscillations in a pancreatic acinar cell by ATP depletion (induced by application of oligomycin and iodoacetate). (Adapted from Barrow et al (12). (B) ATP sensitivity of Ca\(^{2+}\) release from internal stores of InsP\(_3\) stimulated permeabilized pancreatic acinar cells. The figure compares ATP sensitivity of cells isolated from wild type mice (WT) and from mice with knocked out InsP\(_3\)R2. The figure was adapted with modifications from Park HS et al. The type 2 inositol (1,4,5)-trisphosphate (InsP\(_3\)) receptor determines the sensitivity of InsP\(_3\)-induced Ca\(^{2+}\) release to ATP in pancreatic acinar cells (129).

ATP depletion resulted in the efficient inhibition of the store operated Ca\(^{2+}\) entry (SOCE) in pancreatic acinar cells (12). Inhibition of mitochondria and/or ATP depletion have been shown to suppress SOCE entry in a number of other cell types (63, 66, 79, 114, 146) and could be therefore considered as a general phenomenon not limited to the pancreatic acinar cell. The specific molecular mechanism responsible for such inhibition is still debated.

**Figure 3.** Simplified diagram of stimulus-metabolism coupling and metabolism-signaling feedback in pancreatic acinar cells. The schematic illustrates the mechanisms of physiological adaptation of bioenergetics to modulate stimulation by secretagogues. In the diagram ΔΨ\(_m\) indicates mitochondrial membrane potential (major component of the proton-motive force), ETC – electron transport chain, AA - amino acids, FA - fatty acids. Dashed
green lines illustrate the stimulating effect of physiological Ca\textsuperscript{2+} signals (Ca\textsuperscript{2+} oscillations induced by ACh or CCK) on the Krebs cycle and of insulin on glycolysis. Dashed black lines highlight the role of ATP in the regulation of InsP\textsubscript{3} receptors (InsP\textsubscript{3}Rs) and store operated Ca\textsuperscript{2+} entry (SOCE). Both IP\textsubscript{3}Rs and the SOCE pathway are ATP-sensitive mechanisms. In resting unstimulated cells and those stimulated by physiological concentrations of secretagogues, ATP levels are sufficient to sustain both InsP3Rs -mediated Ca\textsuperscript{2+} release from the endoplasmic reticulum and SOCE via the STIM/Orai mechanism. However, ATP depletion (e.g. triggered by the inducers of acute pancreatitis) can suppress the InsP3R -mediated Ca\textsuperscript{2+} release and SOCE. This is also accompanied by the inhibition of Ca\textsuperscript{2+} leak from the ER, Ca\textsuperscript{2+} uptake into the ER by sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} pumps and Ca\textsuperscript{2+} extrusion by Ca\textsuperscript{2+} pumps of the plasma membrane (not shown). - For the sake of clarity/simplicity we have omitted some Ca\textsuperscript{2+}- mobilizing second messengers and corresponding intracellular Ca\textsuperscript{2+}-releasing channels. (For detailed reviews of Ca\textsuperscript{2+} signaling in this cell type, see Refs. (179) and (137)). We have also simplified the entry mechanisms of amino acid (AA) and fatty acid (FA) derivatives into the Krebs cycle. Feedback regulation of the rate of the Krebs cycle by ATP, ADP and NAD(P)H was also omitted on this diagram -.

The pronounced effect of cellular metabolism on Ca\textsuperscript{2+} signaling limits energy expenditure associated with actual signaling responses and downstream reactions of this critical for the acinar cells signaling cascade. It also offers some protection against Ca\textsuperscript{2+} overload and associated Ca\textsuperscript{2+} toxicity. This protection is however incomplete and can be overwhelmed by the inducers of acute pancreatitis.

V. Alteration of Bioenergetics in Pancreatic Pathophysiology

Ca\textsuperscript{2+}-dependent mitochondrial dysfunction is a core feature of acute pancreatitis. In contrast to the spatially restricted, oscillatory Ca\textsuperscript{2+} signaling events that occur in response to physiological levels of secretagogues in isolated pancreatic acinar cells, supramaximal stimulation causes damaging global, sustained calcium elevations (136, 141, 173). The ability to cause overload of cytosolic Ca\textsuperscript{2+} and thereby disrupt Ca\textsuperscript{2+} homeostasis in acinar cells is a principal pathological feature common to known precipitants of acute pancreatitis, including bile acids, non-oxidative ethanol metabolites and fatty acids (46, 92) (see Figure 4A), and is an important trigger for mitochondrial damage that compromises cellular ATP production (45). The importance of altered energy levels within the pancreas relevant to its pathology has been recognized for many years, although in vivo studies have produced somewhat varied results. In the early 1990s, substantial falls of cellular ATP in the pancreas were detected in experimental AP models (using non-invasive NMR spectrometry of \textsuperscript{31}P) (127). Interestingly, there was heterogeneity in responses dependent on the model employed. Thus, whereas significant falls of ATP were detected in AP induced by fatty acid (oleic acid) infusion or regional ischemia, levels were unchanged in caerulein hyperstimulation and partial ductal obstruction models. In contrast, a contemporaneous study in rat pancreas showed that ATP levels were more than halved from controls in the caerulein AP model (77). A subsequent investigation by W. Halangk and colleagues supported these findings, demonstrating a reduction of phosphorylating respiration in acinar cells and fall of ATP to 57% in caerulein AP after 24 hours (using a Clark-type electrode) (70). The authors concluded that caerulein-induced acute pancreatitis is accompanied by “…a drastic and long-lasting reduction of the capacity for mitochondrial ATP production…”.

It should be noted that this phenomenon developed relatively slowly i.e. at early time points (up to 5 hours) the cellular ATP content reported was not significantly diminished from controls and therefore slower than other manifestations of experimental acute pancreatitis like vacuolization (37) and intracellular trypsinogen activation (108). In contrast, a recent study found that high levels of L-lysine caused mitochondrial damage which occurred before activation of trypsinogen and NFkB, potentially via impairment of electron transport chain coupling or a disbalance between hydrolysis and synthesis of ATP (22). To-date, the balance of evidence from in vivo models has demonstrated loss of pancreatic ATP as acute pancreatitis develops, indicating that early mitochondrial damage is a critically important factor in disease progression; variability between studies may reflect methodological differences, etiology and/or severity of the experimental model and
further comparative studies are warranted. The in vivo findings are, therefore, consistent with ATP measurements on isolated acinar cells showing rapid declines of ATP concentration in cells treated with fatty acid (POA) or bile acid (TLC-S) (45, 171). In addition, the bile acid chenodeoxycholate has been shown to decrease intracellular ATP levels in pancreatic ductal cells, with inhibition of both oxidative and glycolytic metabolisms linked to impaired bicarbonate secretion (91, 107). With respect to in vivo models, the bioenergetics of acinar and ductal cells in pancreatic tissue will be influenced by changes in local blood flow. This is particularly relevant to the pathophysiology of acute pancreatitis, which is associated with impairment of microvascular perfusion (e.g. (120, 139, 148, 174) reviewed in (48)). Inducers of acute pancreatitis have been shown to increase the permeability of pancreatic capillaries (e.g. (120, 139, 148), reduce microvascular blood flow (162) and pancreatic oxygen tension (93, 162)). Notably, different models of experimental acute pancreatitis are associated with distinct changes in the microcirculation, ranging from minimal responses and even modest increases of capillary perfusion in mild acute pancreatitis (95) to drastic decreases of capillary perfusion associated with more severe models (95, 162). The intrinsic abilities of the exocrine cells to adjust metabolism and signaling in response to secretagogues and the inducers of acute pancreatitis (discussed in the previous section) will be therefore “superimposed” on changes in oxygen supply and nutrient availability determined by the microcirculation.

The mechanisms underlying ATP depletion induced by acute pancreatitis precipitants have been addressed in detail using isolated pancreatic acinar cells. Confocal microscopy investigations demonstrated that Ca\(^{2+}\) overload of acinar cells caused rapid depolarization of the mitochondrial membrane potential, accompanied by decreased NAD(P)H and increased FAD\(^{+}\) levels, with a resultant cellular ATP fall leading to necrosis. When applied at lower concentration (200 µM) the bile acid TLCS caused predominantly oscillatory calcium rises, accompanied by NADH elevations, in accord with stimulus-metabolism coupling (as described above). However, sustained Ca\(^{2+}\) elevations at higher concentration (500 µM) led to rises of mitochondrial Ca\(^{2+}\) with dramatic decrease of NAD(P)H (26, 169) (see Figure 4B). Similarly, the non-oxidative ethanol metabolite palmitoleic acid ethyl ester (POAEE) and POA, produced by hydrolysis of the fatty acid ethyl ester (53, 80), induced sustained elevations of cytosolic Ca\(^{2+}\) that resulted in mitochondrial dysfunction (45). Simultaneous measurements of cytosolic Ca\(^{2+}\) and Δψ\(_{m}\) revealed the temporal relationship of these events; as cytosolic Ca\(^{2+}\) progressively rose there was a mirrored mitochondrial depolarization and rapid decrease of NAD(P)H (42) (see Figure 4A). The consequence of mitochondrial impairment was a profound depletion of intracellular ATP (measured using with Magnesium Green); this effect was maximal and not further increased by the protonophore CCCP (45) (see Figure 4C). Interestingly, a Ca\(^{2+}\)-independent action of POA was also detected, revealed by pre-treatment with the Ca\(^{2+}\) chelator BAPTA which prevented the loss of Δψ\(_{m}\) although NAD(P)H decrease still persisted. The rundown of mitochondrial ATP production has dramatic effects on Ca\(^{2+}\) homeostasis in the pancreatic acinar cell, which appears particularly vulnerable to sustained cytosolic Ca\(^{2+}\) increases; unlike excitable cells such as cardiomyocytes, the contribution of the Na\(^+/Ca\(^{2+}\) exchanger to Ca\(^{2+}\) homeostasis in the pancreatic acinar cell appears weak or absent (123). The major routes for Ca\(^{2+}\) clearance, the SERCA and PMCA pumps, are fueled by cellular ATP and would be progressively impaired as energy levels fall during acute pancreatitis development (11, 29). This is likely to result in a vicious cycle in which Ca\(^{2+}\)-dependent mitochondrial dysfunction perpetuates sustained cytosolic Ca\(^{2+}\) elevation, resulting in augmented necrotic cell death (43). The importance of maintaining ATP levels for acinar cell function was demonstrated by the provision of intracellular ATP via a patch pipette (45); this prevented the damaging Ca\(^{2+}\) elevations induced by POA, confirming the crucial roles of the SERCA/PMCA for Ca\(^{2+}\) clearance in pancreatic acinar cells (45) (see Figure 4D). In accord, ATP provision prevented the sustained Ca\(^{2+}\) rises and consequent acinar
cell necrosis induced by bile acid (26), while supplemental ATP was able to mitigate the detrimental effects of ethanol and its non-oxidative metabolites on CFTR in ductal cells that promote acute pancreatitis (84, 105).

Figure 4. Pathological Ca²⁺-dependent mitochondrial bioenergetic dysfunction in pancreatic acinar cells. (A) Correlation between palmitoleic acid (POA)-induced sustained cytosolic Ca²⁺ response (Fluo4) and mitochondrial depolarization (TMRE). (Adapted from Criddle et al. (45)). (B) Correlation between bile acid (TLCs: tauroliothocholic acid sulphate)-induced sustained mitochondrial Ca²⁺ rises (Rhod2) and decrease of NAD(P)H (autofluorescence) in a doublet of pancreatic acinar cells (left panel) and corresponding traces (right panel). (Adapted from Booth et al. (26)). (C) Bioenergetics changes in response to POA in pancreatic acinar cells, shown as concurrent decrease of mitochondrial NADH and increase of FAD (autofluorescence: left panel) and depletion of ATP (Magnesium: Green). (D) Provision of ATP via a patch-pipette prevents toxic POA-induced sustained Ca²⁺ elevation in pancreatic acinar cells, highlighting the vital importance of ATP-dependent Ca²⁺ pumps to Ca²⁺ clearance and prevention of cell damage (C, D). (Adapted from Criddle et al. (45)).

A. How does ATP depletion occur?

The precise mechanisms of ATP depletion are still unclear, although a crucial role of MPTP opening in mediating mitochondrial dysfunction has been identified in the pancreas (23, 124, 152), in common with other tissues such as heart, liver and brain (30, 89, 153). The formation of this mega-pore causes a profound permeabilization of the inner mitochondrial membrane that allows movement of solutes <1.5kD in and out of the organelle. Although the exact composition of the pore is contentious and currently unresolved (17, 31, 73, 74, 89), prevention of MPTP opening is achieved via inhibition of the modulatory protein cyclophilin D (CypD); this protein is therefore considered an important target for acute pancreatitis therapy (40, 82, 124, 150, 163). Our group has shown that knockout and pharmacological inhibition of CypD ameliorated damage in isolated murine and human acinar cells induced by acute pancreatitis precipitants and was protective in multiple in vivo experimental models reflecting the principal etiologies (including biliary: TLCs-AP (101) and alcoholic: FAEE-AP (81)) (124). Furthermore, CypD-sensitive MPTP formation mediated mitochondrial dysfunction in pancreatic ductal cells, indicating a common mechanism operating in multiple cell types implicated in the pathogenesis of acute pancreatitis (76, 163). The principal trigger for MPTP opening is the sustained elevation of mitochondrial Ca²⁺ (65), however, reactive oxygen species (ROS) have also been implicated in triggering MPTP channel formation (16, 59). In human and murine pancreatic acinar cells bile acid-induced effects are complex since sustained elevations of cytosolic and mitochondrial Ca²⁺ lead to sustained ROS increases; both Ca²⁺ and ROS formation are therefore likely to be important to the outcome of bioenergetics changes that underlie mitochondrial dysfunction in acute pancreatitis (26). Physiological and pathophysiological changes of acinar cell bioenergetics are schematically illustrated in Figure 5.
Figure 5. Diagram illustrating the effects of physiological and pathophysiological Ca\(^{2+}\) signals on mitochondrial bioenergetics that determine pancreatic acinar cell responses and fate. Physiological stimulation of pancreatic acinar cells with secretagogues cholecystokinin (CCK) and acetylcholine (ACh) elicits oscillatory Ca\(^{2+}\) signals that stimulate mitochondrial ATP production (stimulus-metabolism coupling) that fuels secretion. However, when subjected to stress, cellular bioenergetics are perturbed leading to instigation of cell death patterns. Precipitants of acute pancreatitis (AP), including bile acids, non-oxidative ethanol metabolites (fatty acid ethyl esters: FAEEs), fatty acids (FAs) and secretagogue hyperstimulation, cause sustained cytosolic Ca\(^{2+}\) elevations raise mitochondrial matrix Ca\(^{2+}\). Ca\(^{2+}\)-dependent ROS generation in the mitochondria preferentially promotes apoptotic cell death, which may constitute a local protective mechanism whereby necrosis is avoided. However, as the level and/or duration of stress increases, a shift from apoptotic to necrotic cell death ensues. Ca\(^{2+}\)-dependent formation of the mitochondrial permeability transition pore (MPTP) is a primary event that results in mitochondrial depolarization, altered NADH/FAD levels, ATP fall and necrotic cell death development. Elevated ROS levels also drive necrosis in a manner that is independent of the MPTP. Since clearance of cytosolic Ca\(^{2+}\) from the acinar cell is acutely dependent on ATP-requiring pumps (plasmalemmal Ca\(^{2+}\)-ATPase (PMCA) and sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)), the depletion of ATP evokes a vicious cycle which perpetuates Ca\(^{2+}\) overload and mitochondrial dysfunction. (Adapted from Criddle, DN (41)).

B. Role of ROS in bioenergetic alterations

Secretagogues and bile acids increase ROS generation in pancreatic acinar cells (26, 38); reviewed in (41)). In pancreatic acinar cells augmented ROS production promotes apoptosis (44), which may be an endogenous protective mechanism that enables the cell to deal with cellular stress by avoiding necrosis (19, 26, 44, 85). Necrosis is considered a largely uncontrolled form of cell death and in acute pancreatitis is associated with development of systemic inflammation (94); accordingly, inhibition of caspases caused a more severe necrotizing pancreatitis (19, 113). Our recent work has demonstrated that oxidative stress induced by exogenous \(\text{H}_2\text{O}_2\) or menadione, which generates intracellular ROS via redox cycling (44), caused bioenergetics changes that were independent of CypD-sensitive MPTP opening (6). Increasing levels of oxidants elicited an apoptosis to necrosis shift that was unaffected by knockout of CypD or pharmacological inhibition with cyclosporin A. This lack of sensitivity to CypD is consistent with evidence in primary hepatocytes showing that CypD deletion affected MPTP formation induced by Ca\(^{2+}\) but not by thiol oxidants (13) and a model in which there is a regulated (Ca\(^{2+}\) activated and cyclosporin-sensitive) and unregulated (cyclosporin-insensitive) MPTP (8, 75). Therefore, counteracting excessive ROS in the pancreas with antioxidants might be desirable. However, despite many clinical trials for acute pancreatitis, results have generally been disappointing or inconclusive (4). A more recent approach to
combat oxidative stress is the targeting of antioxidants to mitochondria in order to produce more localised, specific actions (126). For example, MitoQ, which accumulates in mitochondria due to its positively charged triphosphonium ion (TPP⁺) (10), prevented ROS-mediated damage in diverse cell types (33, 36, 49, 50, 102), although efficacy in clinical investigations so far has been disappointing (138, 154). In isolated acinar cells MitoQ reduced H₂O₂-mediated ROS elevations yet afforded no protection against loss of mitochondrial membrane potential induced by CCK or bile acid (81). Furthermore, MitoQ treatment produced mixed effects in in vivo acute pancreatitis models. When applied as a treatment after disease induction, it was unprotective in TLCS-AP but caused partial amelioration of histopathology scores in CER-AP, albeit without concomitant reduction of biochemical parameters (pancreatic trypsin and serum amylase). Interestingly, lung myeloperoxidase and interleukin-6 were concurrently increased by MitoQ in CER-AP, which may involve biphasic effects on ROS in isolated polymorphonuclear leukocytes, inhibiting acute increases but causing elevation at later time points. In pancreatic acinar cells, MitoQ exerted complex actions on mitochondrial bioenergetics that may in part mediated by the targeting TPP⁺ moiety (5). Thus, MitoQ and the general antioxidant n-acetyl cysteine caused sustained elevations of basal respiration and inhibition of spare respiratory capacity, an important indicator of the cell’s ability to respond to stress (149, 178), actions attributable to an antioxidant action. However, mitochondrial ATP turnover capacity and cellular ATP concentrations were also markedly reduced by both MitoQ and DecylTPP, a non-antioxidant control, suggesting non-specific actions of the positively-charged mitochondrial targeting moiety (67, 140, 142). Such events were associated with a compensatory elevation of glycolysis and induction of acinar cell death. The results of this study also indicated that ROS exert a significant negative feedback control of basal respiration, potentially by effects on uncoupling proteins thereby determining basal metabolic function (9, 27, 58). Increasing evidence points to important physiological signalling roles of ROS (55, 61, 71): therefore, an important consideration when addressing mitochondrial dysfunction by quenching free radicals is the potential consequences of disrupting normal physiological organellar function.

C. Inflammatory response: bioenergetics changes in blood cells

While most work elucidating bioenergetics changes related to acute pancreatitis has been carried out in pancreatic cells and tissues, changes in blood cells linked to the generation and propagation of a systemic inflammatory response are likely to be of significance (118, 151). The bioenergetics changes in circulating leukocytes are only partially understood, not least because investigations encounter significant technical difficulties during isolation, including potential damage to cells that might induce artefacts. Previously, a modest (1.5-fold) increase in basal respiration was found in total peripheral blood mononuclear cells isolated from acute pancreatitis patients, although ATP production was unaltered (34). Our recent work has shown subset-specific bioenergetics alterations occur in leukocytes from acute pancreatitis patients that imply functional alterations linked to clinical disease progression (122). Separation of blood cell subtypes from acute pancreatitis patients was feasible for detailed bioenergetics evaluations using Seahorse XF flux analysis. The study found no difference in basal respiration in blood leukocyte subtypes between acute pancreatitis patients and healthy controls. However, when the mitochondria were challenged with a “stress test” (by manipulation of the respiratory chain with rotenone and antimycin (complexes I & III), oligomycin (ATP synthase) and FCCP (protonophore) (6, 7, 28)) it was revealed that patient lymphocytes possessed decreased maximal respiration and spare respiratory capacity, indicating functional impairment. Furthermore, a diminished oxidative burst was evident in neutrophils from acute pancreatitis patients, compared to healthy controls, that may indicate compromised activity in this cell type, whereas this was enhanced in both monocytes and lymphocytes; further work is necessary to
determine the bases for such altered bioenergetics in circulating blood cells and whether bioenergetics analysis might be predictive of acute pancreatitis severity and thereby assist prognosis.

D. Therapeutic aspects: maintenance of ATP provision

How may detrimental bioenergetics changes linked to development of acute pancreatitis be prevented? Recent attention has focused on inhibition of the Ca\(^{2+}\) overload machinery that causes mitochondrial dysfunction. For example, preclinical studies in murine pancreatic acinar cells have shown that inhibition of Orai1, expressed in pancreatic acinar cells (103), prevented sustained Ca\(^{2+}\) increases induced by fatty acid ethyl esters, coupled with maintenance of mitochondrial membrane potential and a reduction of necrosis (64). Prevention of the toxic effects of acute pancreatitis precipitants by Orai1 inhibitors GSK-7975A (GlaxoSmithKline) and CM4620 (CalciMedica) was further demonstrated in human pancreatic acinar cells, and the potential for acute pancreatitis treatment indicated from their efficacy in multiple in vivo experimental models (CER-AP, TLCS-AP and FAEE-AP) (175). More recently, important protective actions of the Orai1 inhibitor CM4620 on immune cells were shown that contribute to the beneficial outcomes in acute pancreatitis (172) and this agent is currently being evaluated in Phase II clinical trials (155). Interestingly, a recent study has also identified the involvement of TRPM2 channels in Ca\(^{2+}\) entry in pancreatic acinar cells and inhibition of these redox-sensitive channels may provide a novel approach to treat biliary acute pancreatitis (60). As mentioned above, prevention of the downstream effects of Ca\(^{2+}\) overload induced by acute pancreatitis precipitants on mitochondria is considered a promising therapeutic strategy, with new MPTP inhibitors in development and evaluation in preclinical models (2, 82, 124, 163, 164). Despite a lack of clarity about the composition of the MPTP, most effort has been directed towards inhibition of the modulatory protein CypD, a prime target in multiple pathologies (30). Genetic deletion of CypD or pharmacological inhibition with cyclosporin A or its analogue NIM811 treatment effectively reduced histological damage and biochemical alterations in multiple acute pancreatitis models (23, 124, 163). Furthermore, since ATP supplementation ameliorated Ca\(^{2+}\) clearance from the cytosol and prevented damage in acinar and ductal cells, there is potential to prevent necrotic damage in acute pancreatitis by boosting cellular energy provision (45, 84, 106). However, development of an effective means to achieve this in patients is challenging; ATP does not effectively cross cell membranes and is subject to degradation by enzymatic action. Nevertheless, studies using liposomes as a vehicle to deliver ATP have been shown to exert protective actions against damage in heart, liver and brain (96, 99, 166). Notably a recent study showed that enhancing cellular energy production with galactose ameliorated asparaginase- and FAEE- induced acute pancreatitis (134), indicating potential therapeutic value of manipulating energy levels in acute pancreatitis patients. Currently a multicentre randomised double-blind clinical (GOULASH) trial is being conducted to assess high versus low energy administration in the early phase of acute pancreatitis (116). Previously, enteral nutrition has been shown to significantly decrease mortality and frequency of multi-organ failure in severe acute pancreatitis (115, 131) and results of the GOULASH trial are awaited with interest.

VI. References


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