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Molecular mechanisms of pancreatic injury

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Abstract

Purpose of review—The pathogenesis of acute pancreatitis (AP) is still not well understood. This articles reviews recent advances in our understanding of AP with emphasis on literature published during the last year.

Recent Findings—Zymogen activation was shown to be sufficient to induce AP. Another key early event, NFkB activation has previously been shown to induce AP. The relationship between these two key early steps is beginning to be clarified. The mechanisms responsible for zymogen activation- pathologic calcium signaling, pH changes, colocalization and autophagy; mechanisms of NFkB activation and potential therapeutic targets both upstream and downstream of these key events have been explored. Additional key findings have been elucidation of the dual role of oxidative stress in AP and role of bioenergetics in determining mode of cell death, recognition of endoplasmic reticulum stress as an early step and duct cells as important players in pancreatic injury.

Summary—Current findings have provided further insight into the roles and mechanisms of zymogen activation and inflammatory pathways in pancreatic injury. Future studies are being undertaken to establish the relative contributions of these pathways during acute pancreatitis which will be critical to identifying successful therapeutic targets.

Keywords

Acute pancreatitis; Trypsin; NFkB; ER stress

Introduction

The pathogenesis of Acute Pancreatitis (AP) remains to be elusive despite significant advances in the last 25 years. Beginning with proposition of Chiari (1) more than a century back that autodigestion by prematurely activated digestive enzymes is responsible for this debilitating disease, significant research has and continues to be focused on mechanisms responsible for this premature zymogen activation (2–5). Additionally, for over a decade now, NFkB activation has been observed early paralleling trypsinogen activation (6, 7). The relationship between these key events has been a matter of hot debate (7–10) and their relative contribution to pancreatic injury is currently not established. In this brief review, we first summarize current research on the role of zymogen activation and NFkB activation in pancreatic injury and then discuss recent literature exploring the mechanisms responsible for these key events and how these have guided our search for potential therapeutic targets.

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Zymogen Activation and NFkB activation- parallel players capable of pancreatic injury

Intracellular trypsinogen activation has consistently been observed early during the course of pancreatitis in experimental models (2, 3, 5, 11–16). The association of hereditary pancreatitis with mutations leading to high intracellular trypsin activity supports that trypsinogen activation is crucial in pancreatic injury (17, 18). *In-vitro* experiments using adenoviral gene transfer techniques to express active trypsin or mutated trypsinogens within pancreatic acini have provided further confirmation as well as mechanistic insights into acinar cell death induction by persistent intra-acinar trypsinogen activation (19–21).

In a landmark study published this year, Gaiser et al demonstrated that intra-acinar expression of active trypsin *in-vivo* was sufficient to induce cell death and inflammation resulting in AP (22). This study used a conditionally inducible pancreas acini-specific trypsinogen construct which was activated during post-translational modification. On the other hand, activation of NFkB, early event paralleling trypsinogen activation in time course, has also been shown to result in AP (23–25).

Both trypsinogen activation and NFkB activation are therefore sufficient to induce AP though their relationship has been debated for a long time (7–10). *In-vitro* expression of active trypsin failed to activate NF-kB (19) suggesting that these two events are independent which is further supported by our data (26). Thus it remains to be established whether these two independent and parallel players, each sufficient to result in pancreatic injury, are prerequisite for development of AP (figure 1). The elucidation of the relative contribution of these events is crucial in advancing our understanding of pancreatitis. Our group has recently developed novel knockout mice which lack trypsinogen7 gene, the mouse correlate of human cationic trypsinogen. These mice lack pathologic trypsinogen activation. Using these mice, studies are currently underway to study these crucial issues in the pathogenesis of AP.

Mechanisms of Zymogen activation

a) Pathologic calcium signaling

Cytosolic calcium influx [Ca^{2+}_i] is crucial in both physiological and pathological responses in the acinar cell, and understanding this differential response has been a great accomplishment in recent times. Unlike physiologic response which involves localized transient spikes in Ca^{2+}_i, pathologic response involves a sustained global rise in Ca^{2+}_i (27–29). This response is seen in experimental models of pancreatitis and its blockade leads to inhibition of trypsinogen activation (3, 11).

Recent research has focused on the sources of this pathologic calcium response. Our current understanding of the sources as well as elimination routes of Ca^{2+}_i and their relevance in pancreatic injury has been depicted in figure 2 (30–33). Endoplasmic reticulum (ER) membrane Ryanodine Receptors (RyR) (34) and plasma membrane store operated calcium channels (SOCs) (35, 36) have been implicated as important sources. In this context, pharmacologic antagonism of RyR using Dantrolene (37), and genetic and pharmacologic inhibition of TRPC3, a recently recognized SOC have been shown to reduce zymogen activation as well as pancreatic damage. Prolongation of the pathologic Ca^{2+}_i by inhibition of SERCA (38) (figure 2) or by ATP depletion (39) (figure 2 and 3) have been recognized as important mechanisms of pancreatic injury by bile acids and ethanol metabolites.

The downstream targets of Ca^{2+}_i are currently unknown though recently Calcineurin has been proposed as one such target (figure 3) (40). Calcineurin is a well recognized

downstream effector of Ca^{2+}_i in several inflammatory processes, especially in T-cell activation and its inhibitors have been clinically successful. Of note, Calcineurin inhibitor tacrolimus resulted in decreased zymogen activation and reduced parameters of pancreatic damage (41).

b) Colocalization of lysosomes and zymogens

We and others have shown that premature trypsinogen activation takes place in membrane-bound compartments of autophagic nature where zymogen and lysosomal contents colocalize (13, 42–44). Lysosomal cathepsin B is believed to activate trypsinogen in these colocalization vacuoles (figure 3) (13, 45). However, it appears that this process requires additional conditions, most likely low pH. Support for this hypothesis comes from the finding that missorting of cathepsin B into the secretory compartment alone failed to activate trypsinogen but only enhanced trypsin activity during AP (46). In fact, *in-vitro* experiments indicate that a low pH significantly enhances catalytic activity of cathepsin B to activate trypsinogen (43). The mechanism that could account for the lowering of pH in these vacuoles was recently identified as vacuolar ATPase (vATPase) which pumps protons into the vacuoles (figure 3) (4, 47).

c) Sensitizing effects of low extracellular pH (pHe)

Acidemia has been known to a precipitant of acute pancreatitis. But until now it was not clear how extracellular pH affects the acinar cell. RyR mediated increased the amplitude of pathologic Ca^{2+}_i (48), enhanced proton transfer into colocalization vacuoles by vATPase (49) and disruption of intercellular junctional coupling possibly facilitating spread of activated zymogens into intra-cellular spaces (50) are the so far described pathologic effects of low pHe (figure 4). Interestingly, Behrendorff et al recently showed that physiological secretion causes acidification of pancreatic lumen and the released protons appeared to function as a negative-feedback regulating acinar secretion (50). However, supramaximal stimulation with caerulein led to extreme prolonged acidification of lumen, thus causing low pHe (50). Further inhibited bicarbonate secretion by duct cells appears to be another mechanism leading to low pHe (described later).

d) Impaired autophagy, Zymophagy and lysosomal cathepsins

It is currently believed that the colocalization organelles are of autophagic nature as demonstrated by presence of LC3-II on the membrane of these organelles (51, 52). However the role of autophagy during pancreatitis has been controversial. Genetic inhibition of autophagy reduced trypsinogen activation and pancreatic damage indicating that autophagy is a harmful response (51). In contrast, Grasso et al described a selective autophagy which is a protective response to sequester and degrade potentially deleterious activated zymogens during early pancreatitis response (53). This protective autophagy was termed zymophagy. Inhibiting zymophagy reduced acinar cell survival while upregulating zymophagy led to reduced trypsinogen activation and improvement in all parameters of pancreatic injury (53). Another group suggested that during pancreatitis autophagy is retarded because of imbalance in cathepsin B (activates trypsinogen) and cathepsin L (degrades trypsin) due to misprocessing (52). Under physiological conditions, prematurely activated trypsinogen would undergo degradation by cathepsin L but during pancreatitis this autophagic degradation is retarded due to imbalance between cathepsins B and L (52).

Mechanisms of inflammatory pathways activation

NFkB activation has been observed in experimental models paralleling trypsinogen activation in time course (6–10, 54). Current data suggests that pathologic Ca^{2+}_i as well as activation of novel isoforms of protein kinase C are responsible for NFkB activation. The

evidence for the role of pathologic Ca^{2+}_i comes from experiment using pharmacologic agents to block Ca^{2+}_i which led to inhibition of NFkB (7). Recent studies have established that activation of novel protein kinase C (PKC) isoforms ε and ζ are involved in NFkB activation using caerulein and ethanol based models of pancreatitis (55–59). These novel PKC isoforms also appear to be involved in zymogen activation (56, 60). It is currently unknown if the effect of pathologic Ca^{2+}_i and PKC in activation of these early pancreatitis responses are independent. The signal transduction steps leading to PKC activation and pathologic Ca^{2+}_i have been depicted in figure 3 based on the caerulein model of pancreatitis. The downstream targets of PKC as well as Ca^{2+}_i have so far been unknown. Protein kinase D1 has been proposed as the downstream target of PKC isoforms (61) recently, though PKD1 activation might also occur by other PKC independent mechanisms (62). Interestingly, pharmacologic inhibition of PKD1 resulted in reduced trypsinogen activation and NFkB activation but cell injury was not affected (63). Similarly, as described earlier, calcinerin has been proposed as downstream target of Ca^{2+}_i though its effects on NFkB activation during pancreatic is not known.

Mechanisms other than Calcium or PKC dependent pathways have also been put forth, notable among these being angiotensin 2 acting through AT2 receptors (64) and p38/MAPK pathways (65, 66). In a recent study, the role of acinar cell injury by itself to activate inflammatory cascades was explored. It was found that cell injury may lead to activation of damage-associated molecular pattern (DAMP) receptors (TLR-9 and P2X-7 recognized as important ones). Activation of DAMP receptors induces formation of a cytosolic complex termed the inflammasome that is implicated in initiating inflammatory cascade during pancreatic injury (67).

Bioenergetics, oxidative stress, determination of cell fate and its effects on pancreatic injury

Apoptosis is known to eliminate damaged cells without eliciting much inflammation while overwhelming cell injury shifts the cell death to necrosis which leads to widespread inflammation. However, apoptosis is an active process requiring energy. Therefore bioenergetics of the acinar cell is crucial in determining whether apoptosis or necrosis occurred during pancreatic injury (figure 4). Though it is misleading to say that apoptosis is a protective response, severity of pancreatitis is considerably less when apoptosis is the predominant mechanism compared to when necrosis is predominant (68). In an interesting study, Voronina et al found that physiological secretagouges (CCK, Acetyl choline, Bombesin) led to increase in ATP production while bile acid and non-oxidative metabolites of alcohol led to ATP depletion (figure 4) (39). This novel finding explains why bile acid and ethanol metabolites cause severe pancreatic injury (low ATP state leading to necrosis). In fact, intracellular administration of ATP abolished acinar injury in response to ethanol metabolites (69). On the other hand, positive bioenergetics resulting from physiological secretagouges would support normal physiological function of enzyme secretion (39). Thus bioenergetics alterations by toxic agents seem to be crucial in determining extent of pancreatic injury.

Oxidative stress is generally believed to be detrimental and going by this logic, several antioxidants have been tried in pancreatitis although with limited success (70, 71). A recent study investigating the role of Reactive Oxygen Species (ROS) in pancreatitis produced surprising findings (72). In this study, ROS induction in the acinar cells promoted apoptosis while inhibition of ROS generation led to increase in necrosis accompanied by reduced ATP (figure 4) (72). These findings suggest that ROS generation within acinar cells may be a protective response during pancreatitis. At the same time, oxidative stress in the neutrophils activated during inflammatory response to acinar injury may be responsible for further

propagation of local and systemic inflammation (figure 4) (72). Therefore oxidative stress appears to have a dual role in pancreatic injury.

Endoplasmic Reticulum (ER) Stress- an early event

ER stress is observed early paralleling trypsinogen activation in caerluein model as well as L-arginine model of AP (73, 74). It is currently unknown whether ER stress is dependent on trypsinogen activation or is an independent event. However ER stress is known to be able to induce cell death pathways as well as activate inflammatory pathways in other cell systems (75). Szmola and Sahin-Toth recently showed that expression of mutant chymotrypsin (CTRC) associated with hereditary pancreatitis in acinar cells led to accumulation of misfolded CTRC in the ER leading to ER stress activation and eventually cell death by apoptosis (76). This study proposes ER stress as a novel mechanism of pancreatic injury. Reducing ER stress using tauroursodeoxycholic acid, a chemical chaperone, or by using genetic manipulation of GRP78 showed protective effect in AP (77) (78).

The role of ER stress responses, also collectively also known as Unfolded Protein Response (UPR), has been explored in alcohol related pancreatitis in considerable detail (79). It appears that a functional UPR effectively clears ethanol induced oxidative ER stress and provides resistance to ethanol induced pancreatic damage (79). However, XBP1+/− mice fed with ethanol had significantly increased pancreatic damage (79). XBP1 is an essential component in the induction of UPR, and its partial deletion in XBP1+/− led to a defective UPR. Thus a defective UPR unmasked ethanol induced pancreatic damage (79). The role of the functional ER responses in other forms of pancreatic injury is currently unclear.

Duct cells – not silent bystanders

The elucidation of pathophysiology of ductal cells and its relevance during pancreatitis has been a major advance in recent years (80). Current research suggests that injurious stimulus alter normal physiological functions of duct cells as much as it does for the acinar cells. For example, bile acids at lower doses leads to enhanced ductal secretion while at higher doses ductal secretion is inhibited (81). Similar response occurs in response to ethanol related models (82). Based on these findings, Hegyi et al have put forth an interesting hypothesis that enhanced ductal fluid secretion during early stages of pancreatitis may defend against damage by washing out toxic agents and digestive enzymes while overwhelming of this ductal defense mechanism leads to pancreatic injury (80). Inhibited bicarbonate secretion may also lead to reduction in luminal pH which has recently been shown to contribute to intra-acinar zymogen activation, as described in figure 4 (48, 50, 80). In addition to inhibited bicarbonate secretion, it was recently shown that non-conjugated chenodeoxycholate at high doses led to mitochondrial injury and ductal ATP depletion (83). While membrane GPBAR1 receptors may mediate noxious effects of bile acids on acinar cells (38), duct cells lack these membrane receptors for bile acids (84). Instead duct cells have strong expression of large-conductance Ca^{2+} activated potassium channels (BK channels) which regulate bicarbonate secretion and mediate the bile acid induced responses in duct cells (84).

The search for therapy

Inflammation has been a major target of developing therapy for pancreatitis. Numerous anti-inflammatory agents – thalidomide (believed to target TNFα) (85, 86), panhaematin (which decreases leukocyte infiltration and chemokine release) (87), leukotriene receptor 1 antagonist montelukast (88), leukocyte function antigen (which was shown to inhibit neutrophil chemotaxis) (89), MCP1 inhibitors (90), COX-2 inhibiotor flavocoxid (91), calcineurin inhibitor tacrolimus (41, 92), calcium channel RyR antagonist Dantrolene (37), Rho-kinase inhibitor (93), activator of stress kinases and apoptotis Betacellulin (94), vitamin

K3 (shown to inhibit autophagy) (95) and broad anti-inflammatory agents like quercetin (96), resveratrol (97), pentoxyphylline (98), circumin (99, 100), and even human bone marrow derived clonal mesenchymal stem cells (101, 102) have all been tried within the last two years in experimental pancreatitis, and remarkably each one of them has shown beneficial response.

The biggest challenge in the field of pancreatitis research lies in finding clinical utility of these potential targets. A recent trial of Activated protein C (103, 104) failed to show any benefit reminding of the failure of the much-hyped Lexipafant in clinical trial more than a decade ago (105). The failure of valdecoxib in combination with glyceryl trinitrate in preventing post ERCP pancreatitis is another such example (106). These are constant reminders that there is a lot still to be learnt about the pathogenesis of pancreatic injury.

Conclusion

The understanding of mechanisms of pancreatic injury has advanced considerably though there is a lot more that remains to be explored. Lack of any therapy for this debilitating disease so far has been a serious setback in the field. Targeting multiple targets may be more effective than the current approach of single target directed approach. Further insight into the mechanisms and contributions of the key events - activation of zymogen and inflammatory pathways activation will be crucial in our understanding of pancreatitis.

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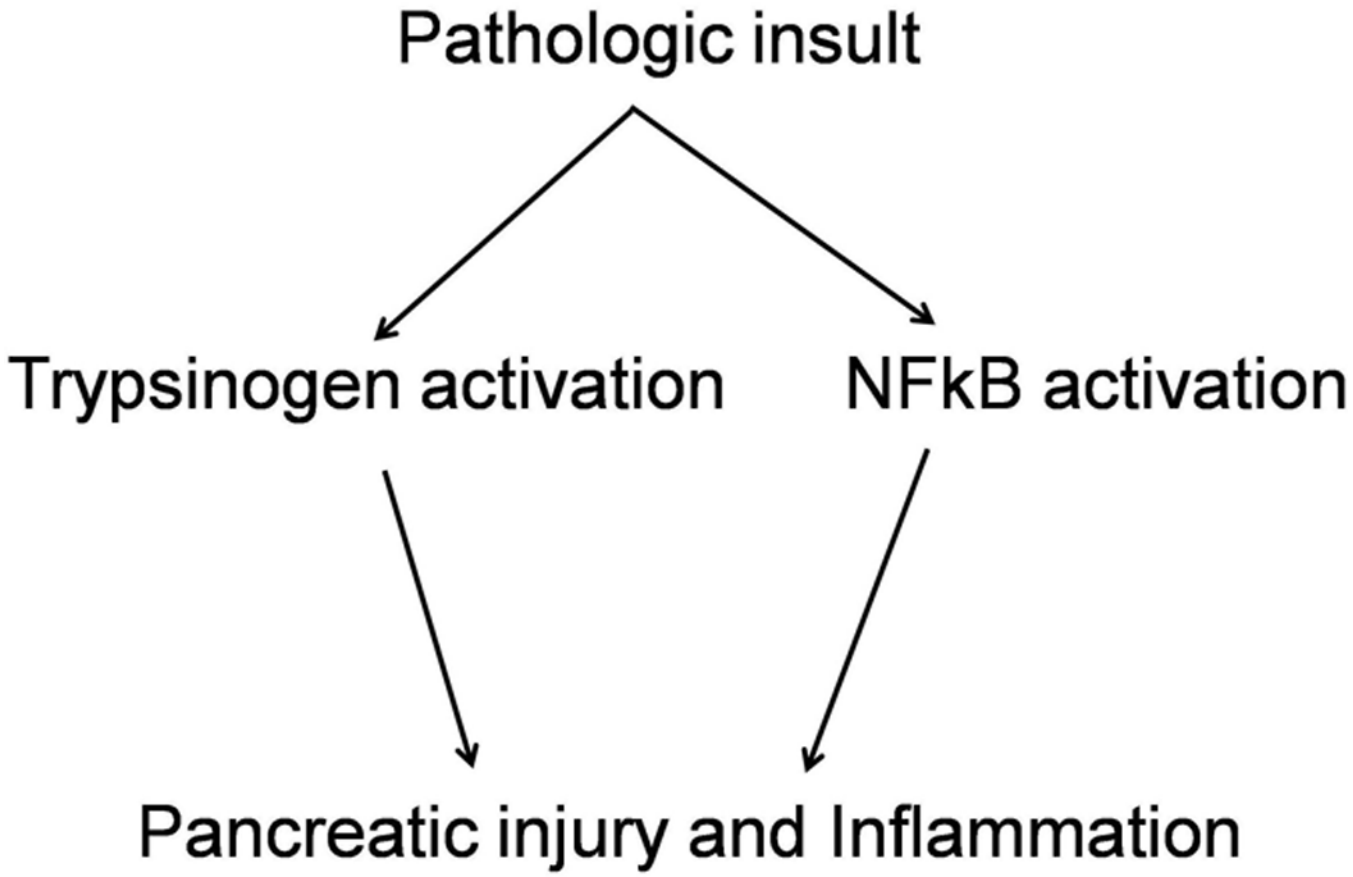

Figure 1. Two key parallel and independent events occurring early during pancreatitis

Both these events are capable of causing pancreatic damage leading to acute pancreatitis. The relative contribution of these events in acute pancreatitis is one of the central questions in the pathogenesis of pancreatic injury at present.

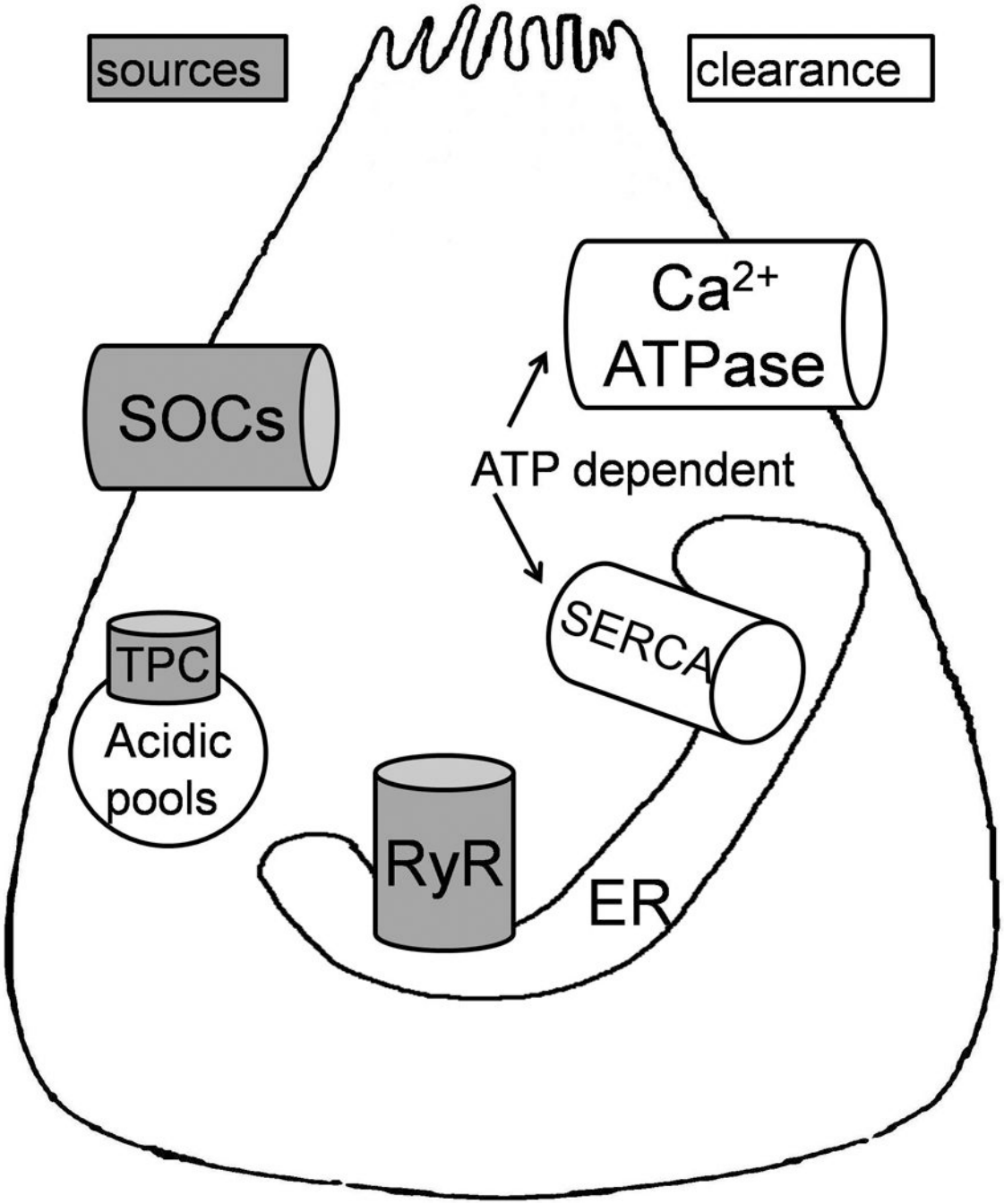

Figure 2. Sources and clearance routes of pathologic cytoplasmic calcium response [Ca^{2+}_i]

Ryanodine Receptors (RyR) (34) and store operated calcium channels (SOCs) (35, 36) are major sources of Ca^{2+}_i. RyRs are calcium sensitive channels, and open in response to mild rise in Ca^{2+}_i although cADPR and NADDP are also possible RyR ligands (27–29, 33). The identity of SOCs and the mechanism of their regulation by ER calcium signals has been a field of active research. Recently TRPC3 and ORAI channels have been identified as important SOCs (35, 36) and it is postulated that STIMs that sit on ER membrane sense calcium depletion within the ER (as would occur after opening of RyRs) and migrate to plasma membrane where they open the SOCs (35, 36). Acidic pools are thought to be important in alcohol induced injury (30) and include organelles with low pH such as

lysosomes, endososomes and zymogens. Recently recognized Two Pore Channels (TPCs) release calcium from acid pools (31–33). Mitochondria have been recognized as another source of calcium (not shown in the figure). Note that clearance of Ca^{2+}_i is an ATP requiring process, and ATP depletion or direct inhibition of SERCA prolongs Ca^{2+}_i, a mechanism thought to be important in pancreatic injury due to bile acids and ethanol metabolites.

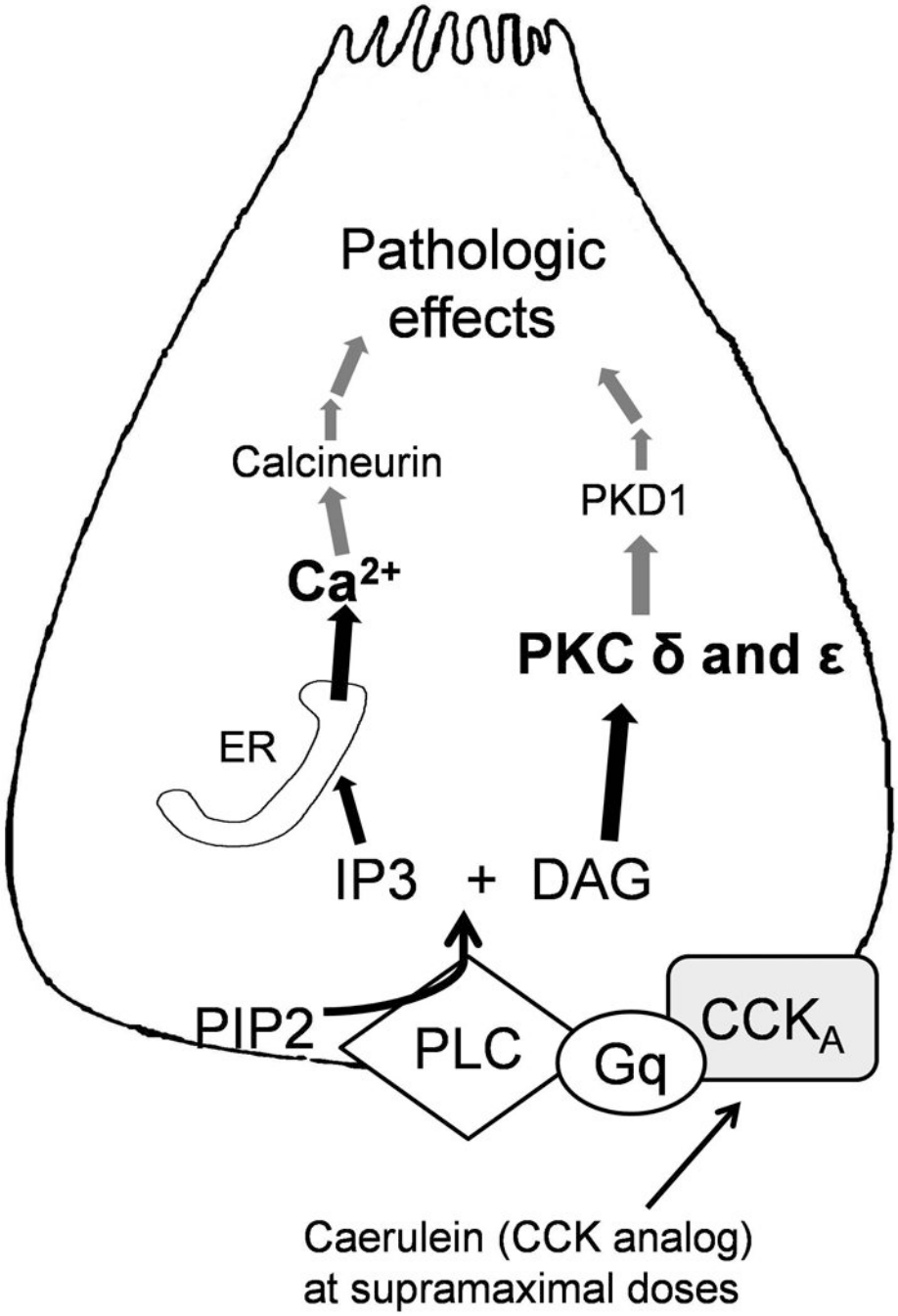

Figure 3. Signal transduction events resulting in pathologic trypsinogen activation and NFkB activation

Cholecystokinin analog Caerulein induced pancreatitis has been used as a model in this schematic. CCK_A: Cholecystokinin receptor subtype A, G_q: G-protein q subtype; PLC: phospholipase C, PIP2: Phosphoinositol 4-phosphate, IP3: Inositol-3 Phosphate, DAG: Diacylglycerol; PKC: protein kinase C, PKD: protein kinase D. Caerulein (CCK analog) binds to its receptor as shown and leads to generation of IP3 and DAG. IP3 opens ER membrane IP3 receptors which are implicated in physiologic calcium signaling. Calcium released through IP3R leads to opening of RyRs as described in figure 2. The grey lines in

the figure depict either unknown steps or proposed mechanisms awaiting verification in future studies.

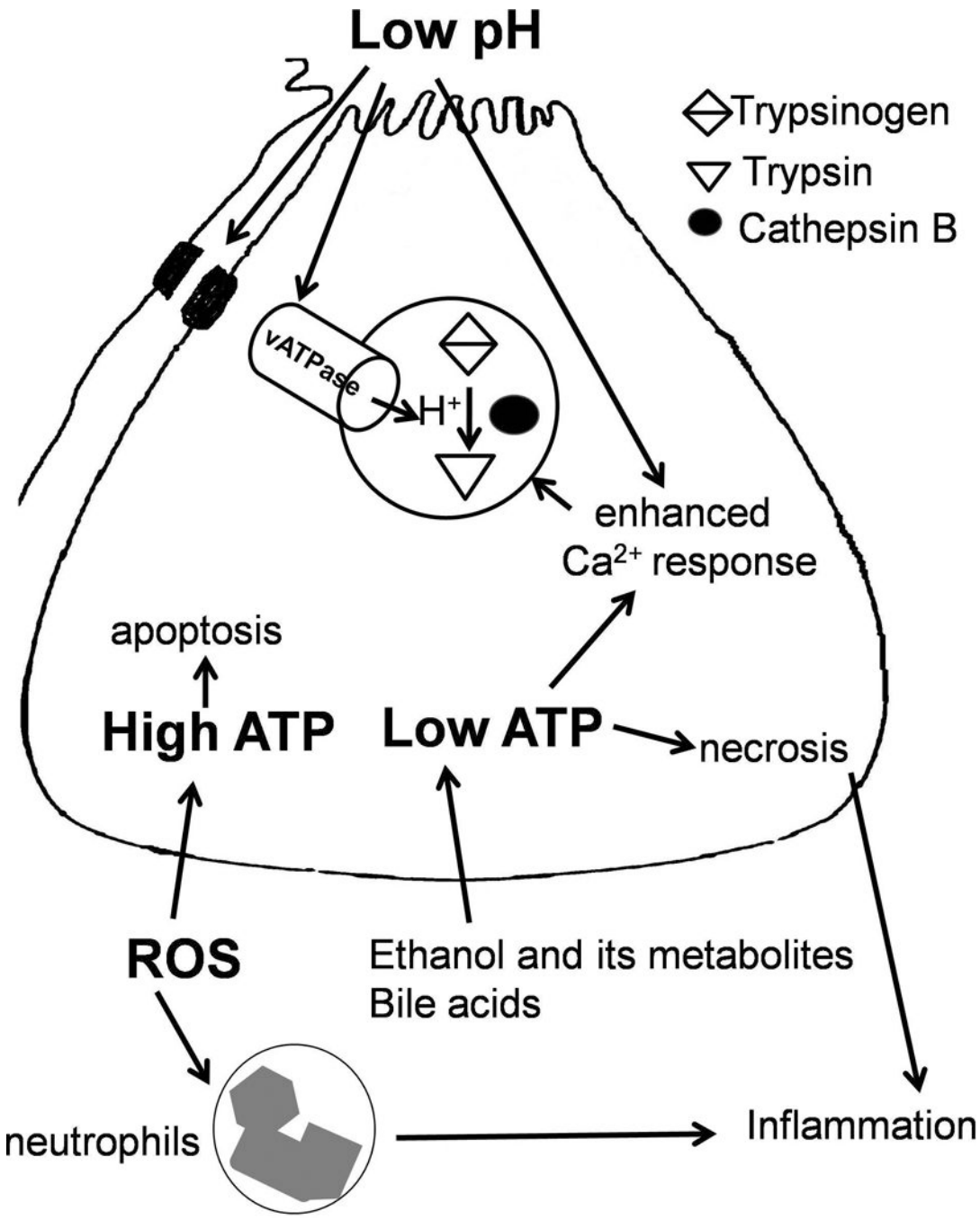

Figure 4. Effect of low extracellular pH, role of bioenergetics in determining cell fate and dual role of oxidative stress

Low pH in the lumen leads to enhanced activity of vATPase, enhanced pathologic calcium response and disruption of intercellular junctions leading to zymogen activation and spread of activated zymogens causing further damage (48–50). High ATP states favor apoptosis and this mode of death avoids inflammatory response leading to relatively less severe injury compared to necrosis which elicits intense inflammation (39). Necrosis is the mode of death during low ATP states and causes severe pancreatic injury. ROS depletion in the acinar cells leads to low ATP state and favors necrosis while ROS induction favors apoptosis avoiding severe pancreatic damage, and therefore seems to be protective to the acinar cell (72). At the

same time, ROS in the neutrophils leading to inflammation may contribute to pancreatic injury. Thus oxidative stress spears to have dual role in pancreatitis.