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Review

Reactive oxygen species, Ca^{2+} stores and acute pancreatitis; a step closer to therapy?

David N. Criddle

Department of Cellular and Molecular Physiology, Institute of Translational Medicine, and NIHR Liverpool Pancreas Biomedical Research Unit, University of Liverpool, L69 3BX, UK

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ABSTRACT

Disruption of Ca^{2+} homeostasis can lead to severe damage of the pancreas, resulting in premature activation of digestive enzymes, vacuolisation and necrotic cell death, features typical of acute pancreatitis (AP). Therefore a fine balance between Ca^{2+} release from internal stores, Ca^{2+} entry and extrusion mechanisms is necessary to avoid injury. Precipitants of AP induce Ca^{2+} overload of the pancreatic acinar cell that causes mitochondrial dysfunction, *via* formation of the mitochondrial permeability transition pore (MPTP), loss of ATP production and consequent necrosis. Oxidative stress has been shown to occur in the development of AP and may modify Ca^{2+} signalling events in the acinar cell. However, the precise pathophysiological involvement is currently unclear and antioxidant therapy in the clinic has largely proved ineffective. Possible reasons for this are discussed, including evidence that ROS generation may determine cell death patterns. In contrast, recent evidence has indicated the potential for AP therapy *via* the prevention of Ca^{2+}-dependent mitochondrial damage. Multiple approaches are indicated from preclinical findings; 1) inhibition of Ca^{2+} release by IP_3R blockade, 2) inhibition of Ca^{2+} entry through Orai1 blockade and 3) prevention of MPTP formation. Clinical trials of drugs which prevent mitochondrial dysfunction induced by Ca^{2+} overload of pancreatic acinar cells are imminent and may provide patient benefit for a disease that currently lacks specific therapy.

Contents

1. Introduction: acute pancreatitis

The exocrine pancreas is a highly specialised secretory organ capable of synthesising, storing and releasing large quantities of digestive enzyme precursors into the small intestine, necessary for the breakdown of food. Homeostasis of the functional unit, the acinar cell, is therefore paramount for the smooth running of physiological processes; disruption can lead to severe damage of the pancreas, resulting in premature activation of zymogens, vacuolisation and necrotic cell death, features typical of acute pancreatitis (AP). This severe inflammatory disease, which currently affects approximately 50 per 100,000 individuals per year, is triggered predominantly by alcohol excess and gallstones,

E-mail address: criddle@liv.ac.uk

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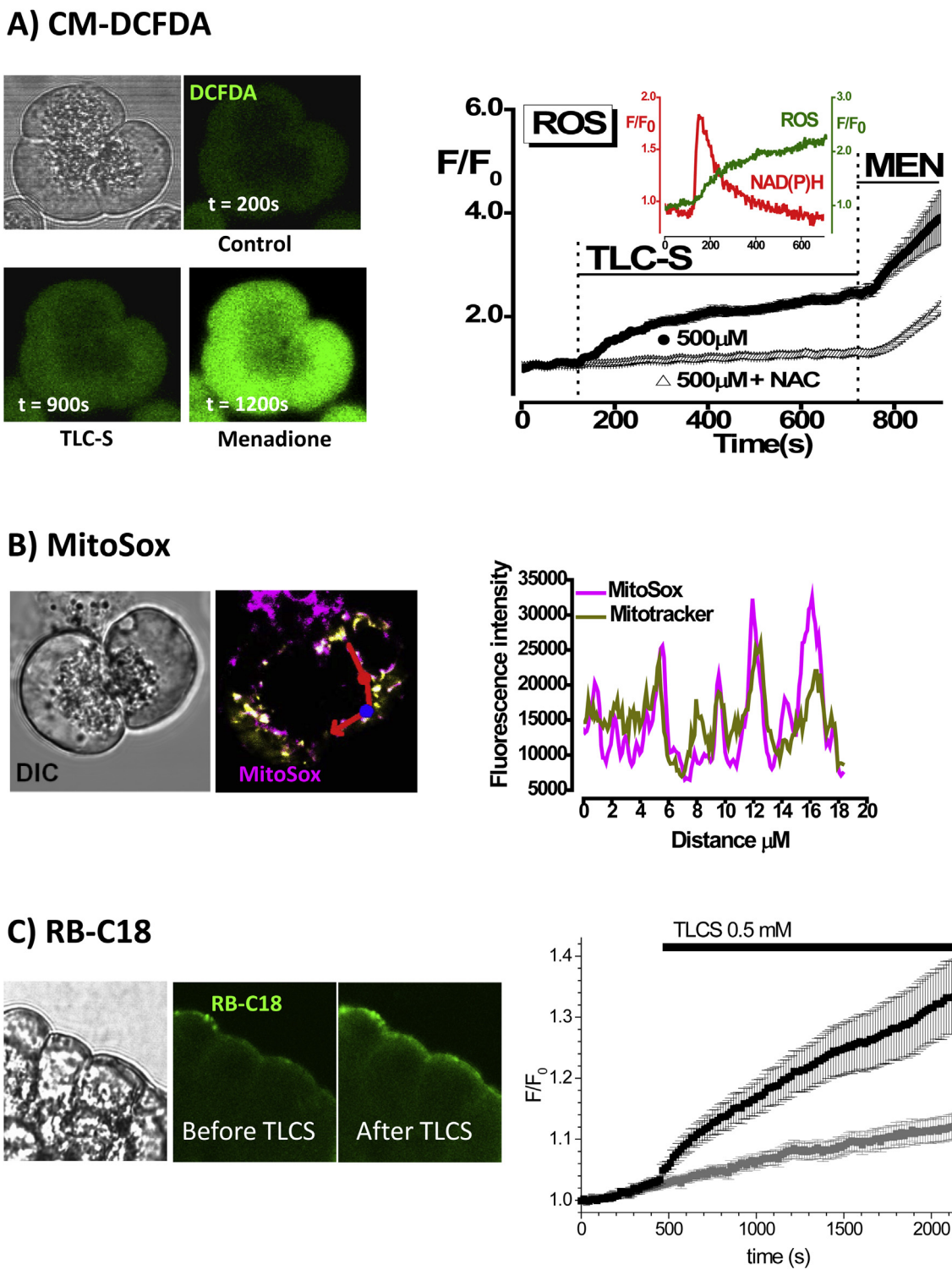

Fig. 1. Detection of ROS in primary human and mouse pancreatic acinar cells using confocal microscopy. A) Typical images and graphs showing Ca^{2+}-dependent rises of ROS induced by the bile acid taurolithocholic acid sulphate (TLCS), measured with CM-DCFDA in human and murine acinar cells, that were inhibited by the antioxidant N-acetylcysteine (NAC) [7], B) Co-localisation studies using MitoSox and Mitotracker indicated that bile acid- induced ROS generation occurred in the mitochondria [7], C) TLCS-induced ROS increases measured with a novel lipophilic probe, RB-C18, recently developed for detecting near-membrane responses [16].

although diverse precipitants are recognised potentially suggesting a common mechanism [1]. Severe cases of AP involve a systemic inflammatory response syndrome (SIRS) that may result in multiple organ damage and death of the patient. A prominent feature of the development of AP is a disruption of calcium signalling within the acinar cell [2–4]; overload of cytosolic calcium ($[Ca^{2+}]_c$) leads to significant damage of the mitochondria, critically affecting their ability to produce ATP, thereby promoting cell death [5]. Importantly, the extent of pancreatic necrosis, which may develop in patients within days of symptom onset, is a major determinant of disease progression; the presence of necrosis dramatically raises the mortality rate in AP [1,6]. Sustained rises of $[Ca^{2+}]_c$ have been

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shown to cause maintained elevations of reactive oxygen species (ROS) within the acinar cell that promote cell death [7]. However, the precise involvement of oxidative stress in the development of AP is still unclear [8], mirrored by a disappointing translation of antioxidant treatment to the clinic to-date [9]. This review will focus on current evidence relating to the actions of ROS and Ca^{2+} in determining cell death in the exocrine pancreas, with particular relevance to the development of AP and promising new therapeutic approaches.

2. ROS generation in pancreatic acinar cells

Multiple sources and sites of action of ROS are likely to be relevant to AP pathogenesis. For example, ROS are generated locally within the acinar cell, where they can affect cell death patterns [7]. However, they are also produced and released by circulating neutrophils during in the inflammatory response, thereby influencing the development of pancreatic injury and systemic complications of AP [8]. In cells ROS are generated during normal respiration, a large proportion derived from complexes I and III of the mitochondrial electron transport chain [10]. Increasing evidence indicates that ROS may exert signalling roles in diverse physiological processes [11], rather than simply constituting an unwanted by-product. However, excessive generation of ROS has been shown to promote damage in a variety of cell types *via* disruption of lipid membranes, proteins and DNA [12]. Therefore a fine balance exists between ROS generation and endogenous scavenging of free radicals; the outcome of oxidative stress is likely to reflect the extent and severity of the insult that is applied to the pancreas.

Our group has demonstrated that the bile acid taurolithocholic acid sulphate (TLCS) induces ROS generation in human and murine pancreatic acinar cells, measured with confocal microscopy using the fluorescent probe CM-DCFDA (Fig. 1 a) [7]. This elevation of ROS was inhibited by the antioxidant N-acetylcysteine and use of co-localisation analysis with MitoSOX and Mitotracker indicated that generation occurred within the mitochondria (Fig. 1b). Bile acids have been shown to cause sustained elevations of $[Ca^{2+}]_c$ in pancreatic acinar cells [13,14] and the ROS response to TLCS was Ca^{2+}-dependent, consistent with a rise of mitochondrial Ca^{2+} detected using Rhod-2 [7]. Previously the importance of ROS generation in determining cell fate had been demonstrated in pancreatic acinar cells; the oxidant menadione induced a rise of ROS that promoted apoptotic cell death [15]. Pharmacological manipulation of cellular ROS levels, by inhibiting menadione-induced rises with the general antioxidant N-acetylcysteine (NAC) or potentiating the effect by blocking a detoxifying enzyme NAD(P)H:Quinone oxidoreductase 1 (NQO1), inhibited or potentiated the apoptotic cell death response, accordingly. Furthermore, in the same study similar manipulations of ROS levels induced by application of the bile acid TLCS modified acinar cell death accordingly by either promoting or inhibiting apoptosis. These observations suggested the potential importance of ROS to influence local pancreatic injury during AP. More recently we have developed a novel ROS probe that is localised in cellular membranes, thereby increasing sensitivity in the detection of free radicals by potentially avoiding quenching effects of endogenous antioxidants; diverse AP precipitants, including TLCS, produced detectable elevations of ROS in murine acinar cells [16] (Fig. 1c).

3. ROS interactions with Ca^{2+}-Signalling in exocrine pancreas

A central role for Ca^{2+} in the pathophysiology of AP is well documented, with disruption of homeostasis in pancreatic acinar cell considered key to development [4,17–19]. Many studies have indicated the importance of inositol trisphosphate (IP_3) [13,14,20] and ryanodine [21,22] stimulated Ca^{2+} release channels on internal stores to pancreatic injury induced by AP precipitants. Sustained elevations of $[Ca^{2+}]_c$ in response to cholecystokinin hyperstimulation [4] bile salts [13,14], alcohol and its non-oxidative metabolites (fatty acid ethyl esters, FAEEs and fatty acids) [25–27] induce features of AP damage in acinar cells, including premature Ca^{2+}-dependent activation of zymogen granules, vacuole formation and cell death [3,4] . Emerging evidence also points to the pathophysiological importance of Ca^{2+} signalling in pancreatic ductal epithelial cells in AP, which has recently been reviewed [26].

3.1. Ca^{2+} release channels

Control of cytosolic Ca^{2+} levels within discrete microdomains is crucial to physiological function, requiring a fine coordination of Ca^{2+} release, entry and exit mechanisms [27]. The intracellular redox status may affect many fundamental aspects of Ca^{2+} signalling in the acinar cell. For example, a sensitisation of Ca^{2+}-release channels by exogenously applied oxidants can induce cytosolic oscillations in the absence of physiological stimulation [28,29]; such an action may assist maintenance of IP_3R-dependent Ca^{2+} oscillations that promote exocrine secretion [30]. IP_3Rs and RyRs, intracellular calcium release channels present on the endoplasmic reticulum (ER) and acidic stores, possess ROS-sensitive reactive cysteine residues [31–33], however, their modulation by oxidative stress is complex. For example, ROS have been reported to sensitize IP_3-mediated activation of the IP_3R but also to inhibit channel function [34,35]. ROS-induced oxidation of thiol groups, present on IP_3Rs and RyRs, is considered excitatory since it inhibits calmodulin binding to the ion channel thereby relieving an inhibitory mechanism [36–38]. Recently, cell-permeable calmodulin activators have been shown to stimulate a protective effect of calmodulin by inhibiting excessive Ca^{2+}release from the internal stores through IP_3Rs and RyRs [39]. Thus increased excitation of IP_3Rs and RyRs by localised oxidative stress could deregulate Ca^{2+}release; such modulation may have important consequences for development of AP, since both channels have been implicated in mediating pathological changes in experimental AP models [21,40]. The importance of IP_3R subtypes to acinar cell physiology was demonstrated using a knockout mouse model; whereas individual subtype 2 and 3 IP_3R knockout minimally affected Ca^{2+} signals and amylase secretion induced by ACh and CCK, dual knockout abolished such responses [41]. The IP_3R was implicated in mediating Ca^{2+} signals induced by toxic non-oxidative alcohol metabolites [24] and subsequent work using knockout mice showed that subtypes 2 and 3 were necessary for Ca^{2+}release, trypsinogen activation and acinar cell death [18]. Recently, the potential for AP therapy by inhibition of IP_3R-mediated Ca^{2+}release was demonstrated [40]. Caffeine and its dimethylxanthine metabolites inhibited IP_3R-mediated, sustained cytosolic Ca^{2+} elevations, loss of mitochondrial membrane potential and necrotic cell death pathway activation in pancreatic acinar cells (Fig. 2a). Importantly, caffeine and its dimethylxanthine metabolites also ameliorated pancreatic injury in multiple experimental AP models through IP_3R-mediated signalling inhibition. Interestingly, coffee drinking was previously found to be associated with a reduced risk of alcoholic AP [42]. Current evidence supports inhibition of Ca^{2+} overload and its consequences as a potential approach for AP therapy, and methylxanthine-based structures would appear suitable starting points for drug discovery and development.

3.2. Ca^{2+} entry mechanisms

Of particular recent interest in the field has been an improved understanding of important Ca^{2+} entry mechanisms present in

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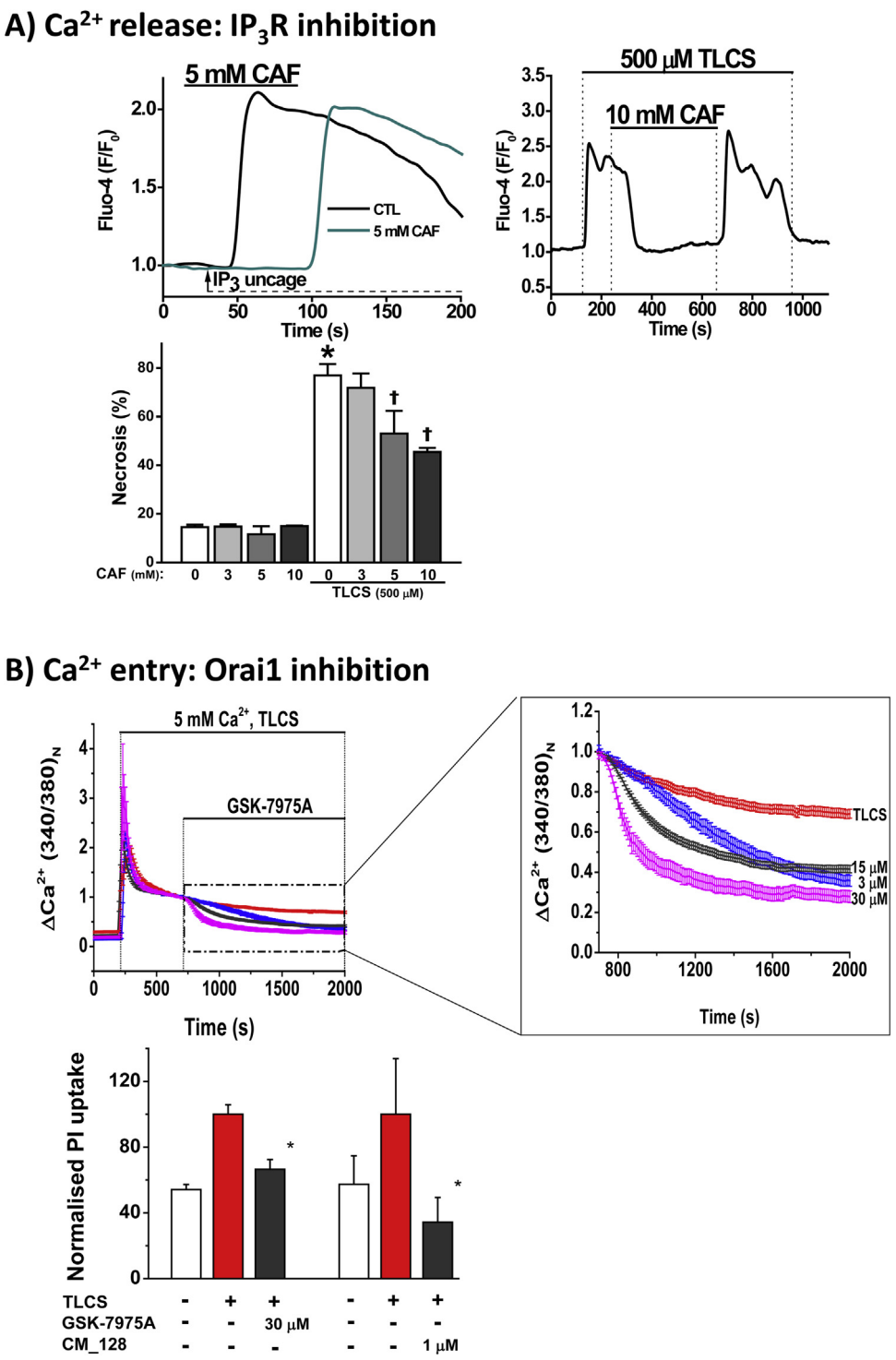

Fig. 2. Protective effects of Ca^{2+} release and Ca^{2+} entry pathway inhibition in human and murine pancreatic acinar cells. A) Effects of the IP_3R blocker caffeine to inhibit Ca^{2+} elevations induced by (i) IP_3 uncaging, (ii) TLCS application, and (iii) resultant necrosis of mouse pancreatic acinar cells [40]. B) Effects of new Orai1 blockers, GSK-7975A (GlaxoSmithKline) and CM_128 (CalciMedica), on sustained $[Ca^{2+}]_c$ elevations induced by TLCS and necrosis of isolated human pancreatic acinar cells [19].

pancreatic acinar cells, that mediate sustained pathophysiological elevations of $[Ca^{2+}]_c$ causing mitochondrial injury and cell death. Several ion channels have been implicated in pancreatic acinar cell pathophysiology. For example, TRPC3 channels have previously been shown to partially mediate sustained $[Ca^{2+}]_c$ rises triggered by bile acids and FAEEs in pancreatic acinar cells and to ameliorate experimental AP [43]. Recent evidence has demonstrated a role for the STIM1-Orai complex as the principal Ca^{2+} entry channel in this cell type [19,44,45]. The STIM1 protein is sensitive to the Ca^{2+} content of the ER store; when stores are depleted this protein clusters at the ER-plasma membrane junction where it interacts with Orai1 subunits to form a functional complex that allows entry of Ca^{2+} into the cell [46–48]. Pharmacological inhibition of Orai1 channels reduced pathological Ca^{2+} entry into murine pancreatic acinar cells, necrotic cell death and trypsin activation induced by alcohol non-oxidative metabolites, suggesting the ther-

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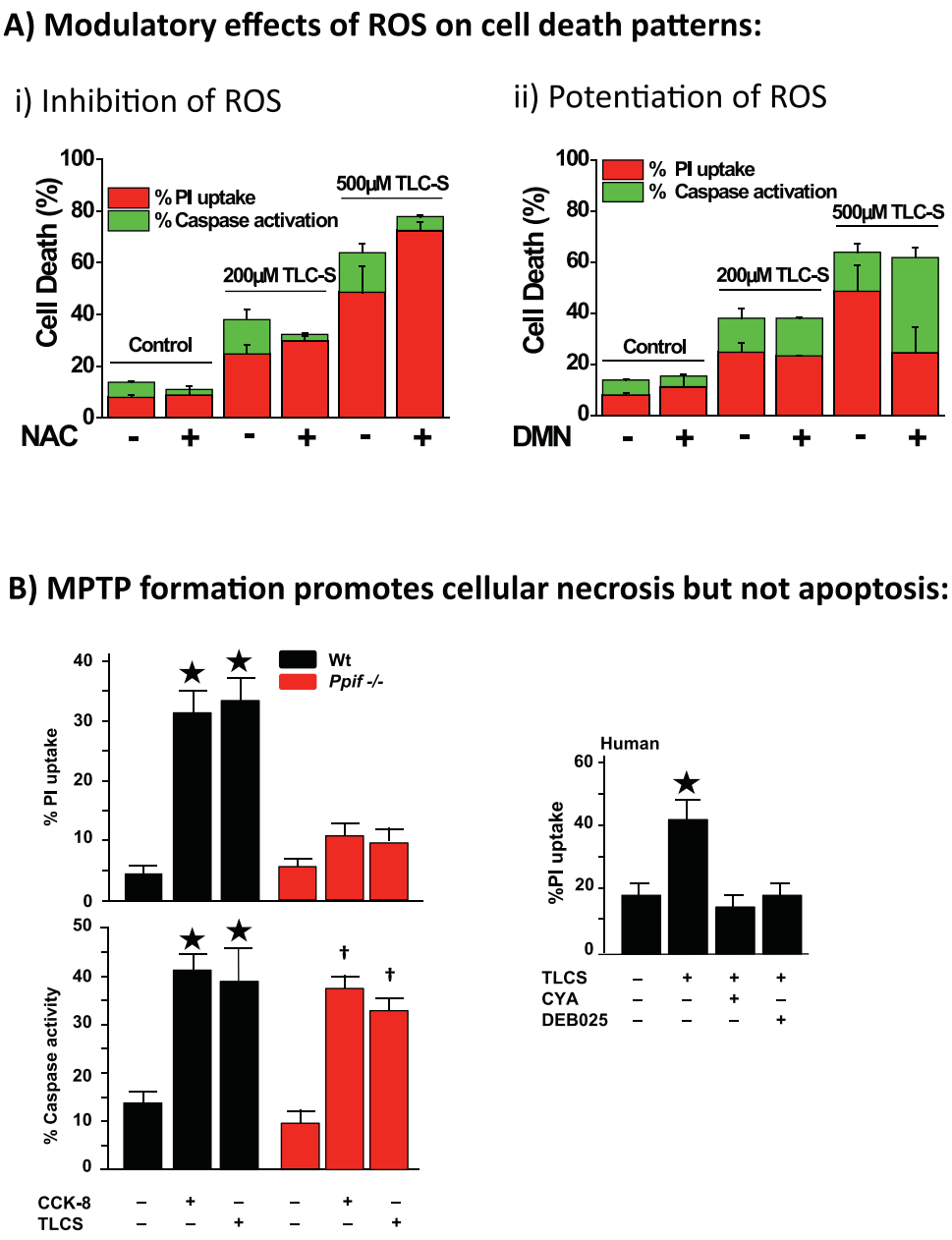

Fig. 3. Cell death patterns in human and murine pancreatic acinar cells. A) (i) Inhibition of bile acid TLCS-induced ROS generation by N-acetylcysteine (NAC) reduced apoptotic cell death and potentiated necrosis, whilst (ii) potentiation of ROS by inhibiting a detoxifying enzyme NQO1 with DMN produced the converse effect [7]. B) Genetic deletion of cyclophilin D ($PPIF^{-/-}$) or pharmacological inhibition of MPTP formation (with cyclosporin A (CYA) and Debio-025) reduced pancreatic acinar cell necrosis, whereas apoptosis was unaffected [73].

apeutic potential of Ca^{2+} release-activated Ca^{2+} channel blockade to ameliorate AP [45]. Subsequently, it was shown that inhibitors of Orai1 prevented Ca^{2+}-dependent injury of human pancreatic acinar cells (Fig. 2b) and experimental AP in three mouse models [19]. Importantly, the Orai1 blockers, GSK-7975A and CM_128, were applied as treatments i.e. post-induction of AP, emphasizing the translational potential for this novel therapeutic approach; CM_128 will shortly enter into Phase One clinical trials for AP. The Orai1 protein possesses an externally located cysteine residue which renders it sensitive to modulation by ROS. Sensitization of the Ca^{2+} entry process by ROS may therefore have implications for pancreatic acinar cell damage, however, this has not been evaluated so far. Differences between Orai channel subtypes have been reported; in contrast to Orai1, Orai3 is insensitive to oxidative stress since it lacks the external cysteine residue [49]. This may be of importance to the immune response in AP since T-lymphocytes become increasingly resistant to ROS after differentiation into effector cells, a feature linked to increased Orai3 expression. Under oxidizing conditions of the inflamed pancreas such a modification might facilitate proliferation, differentiation and cytokine production. Furthermore, a Ca^{2+}-redox feedback loop has recently been shown to control human monocyte immune responses [50]. Exposure to bacterial peptides or infection with pathogens promoted Orai/STIM mediated Ca^{2+} entry, the magnitude of which determined the rate of ROS production *via* NADPH oxidase 2 (NOX2). This study indicated that monocytes can optimize their ROS response by altering the Orai1/Orai3 ratio, thereby fine-tuning negative feedback from NOX2 to the Orai/STIM channel.

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3.3. Ca^{2+} extrusion/uptake mechanisms

The pancreatic acinar cell is critically dependent on the regular production and supply of intracellular ATP for $[Ca^{2+}]_c$ homeostasis. This situation contrasts with excitable cell types, such as those of cardiac and neuronal tissues, which possess a Na^+/Ca^{2+} exchanger that can decrease $[Ca^{2+}]_c$ elevations; this appears to be absent or non-functional in pancreatic acinar cells. As such, the integrity of mitochondria in pancreatic acinar cells is paramount to normal functioning of the exocrine unit; disruption of this organelle *via* formation of the mitochondrial permeability transition pore (MPTP) has strongly been implicated in the development of AP [51,73]. The acinar cell has two ATP-dependent $[Ca^{2+}]_c$ clearance mechanisms, the Sarco-Endoplasmic reticulum (SERCA) and Plasmalemmal (PMCA) Ca^{2+}-ATPases. Both pumps are modulated by oxidative stress, since they contain ROS-sensitive cysteine residues [52]. In pancreatic acinar cells oscillatory $[Ca^{2+}]_c$ responses to CCK or ACh were altered in a concentration-dependent manner by H_2O_2; sustained, global, $[Ca^{2+}]_c$ responses were promoted by this radical [53]. Since, mitochondrial impairment and consequent ATP depletion in response to H_2O_2 was correlated with a fall in PMCA activity [54], such transformation of $[Ca^{2+}]_c$ patterns might, at least in part, be mediated *via* decreased PMCA-mediated $[Ca^{2+}]_c$ clearance from the cytosol. However, the effects of ROS are likely to be highly concentration-dependent. Mild oxidative conditions can positively influence SERCA pump activity *via* oxidation of a specific cysteine residue (Cys674) [55], whereas prolonged exposure to oxidative stress may cause irreversible SERCA inhibition and overload of $[Ca^{2+}]_c$ *via* sulphonylation of this amino-acid together with oxidation of other residues [55–57]. Thus the extent and duration of oxidative stress is likely to determine outcome. Recently an important role of Bcl-2 in modulating PMCA activity and influencing cell death was demonstrated [58]. Ca^{2+} extrusion from AR42J cells was diminished by the overexpression of Bcl-2 and augmented by Bcl-2 silencing. Interestingly, a loss of Bcl-2 enhanced apoptosis induced by oxidative stress and dramatically decreased necrosis.

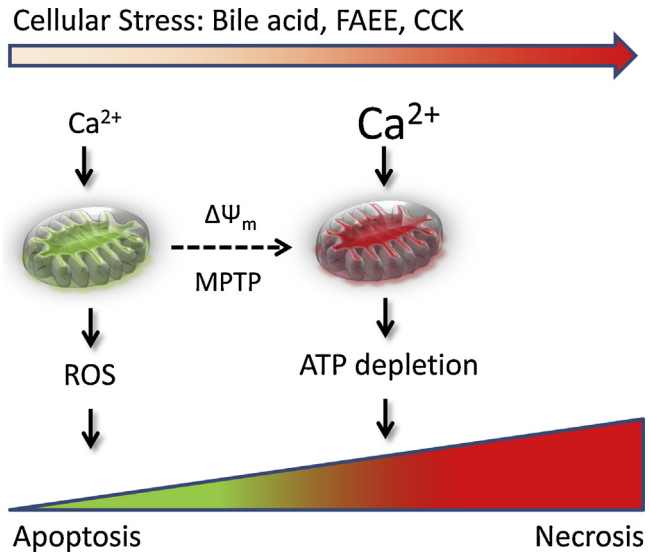

Fig. 4. A hypothetical model illustrating the outcome of increasing stress on pancreatic acinar cell fate. Low levels of stress induced by precipitants of acute pancreatitis (AP), including bile acids, non-oxidative ethanol metabolites (fatty acid ethyl esters: FAEEs) and cholecystokinin (CCK) hyperstimulation, induce sustained $[Ca^{2+}]_c$ elevations that raise mitochondrial 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. Formation of the MPTP causes mitochondrial depolarisation and a fall of ATP with resultant necrotic cell death. Strategies that reduce $[Ca^{2+}]_c$ overload or protect mitochondria by preventing MPTP formation are likely to be promising therapeutic avenues for the treatment of AP.

4. Involvement of ROS in AP: experimental models

Oxidative stress occurs in the pathogenesis of many diseases, including AP [59,60]; elevated levels of free radicals and/or their by-products, together with a reduction of antioxidant defences, have been reported in both animal experimental AP models and patient studies (recently reviewed; [9]). Several early clinical investigations suggested a prominent role for oxidative stress in AP development [61–64]. For example, oxidative stress in patients was detected early in the course of AP and persisted longer than clinical manifestations of the disease [61]. Such changes were linked to disease severity; significantly elevated ROS markers, together with reduced antioxidant levels, were detected in AP patients that were different between mild and severe patient groups.

Preclinical studies have also demonstrated that oxidative stress is present in experimental AP. For example, the extent of pancreatic injury in experimental caerulein-induced AP (CER-AP) correlated with reduced glutathione (GSH) depletion [65]. Subsequently it was shown that probiotics, which stimulated the biosynthesis of GSH, reduced oxidative stress in this model [66]. However, such a manipulation did not correlate with a positive outcome in the clinic; administration of probiotics to severe AP patients early after disease onset more than doubled the relative risk of mortality [67], although the study design has been questioned [68]. In the rat CER-AP model administration of SOD and catalase post-insult ameliorated ultrastructural and biochemical damage, however, pre-treatment was unprotective [69]. Furthermore, a comparison of agents applied to reduce oxidative stress in CER-AP, and in a more severe choline-deficient ethionine-supplemented diet AP model, only detected a decrease in pancreatic oedema; the study concluded that ROS might therefore play a role in development of pancreatic oedema but appeared unlikely to be instrumental in the development of acinar cell damage [70].

Although CER-AP is a convenient and reliable model of AP [71], other approaches may have more direct clinical relevance. For example, the ductal infusion of bile acids, such as taurolithocholic acid sulphate (TLCS), caused a more severe form of experimental AP [72,73]. In rats, bile acid-induced AP oxidative stress has been shown, including elevated MDA levels in pancreas and reduction of SOD activity [74]. In accord, local and systemic oxidative stress was also recently demonstrated in taurocholate-induced AP in obese rats [75]. Interestingly, although a significant protection of acinar cell damage and inflammation was observed by the scavenging of ROS in this model, such an intervention did not alter GSH, malondialdehyde (MDA), amylase or lipase levels or affect pancreatic oedema [76]. Furthermore, and perhaps of fundamental importance to the question of the role of ROS in AP, generation of ROS alone did not induce biochemical or morphological changes typical of AP, strongly implying that factors other than ROS are involved in triggering AP damage *in vivo*.

In accord, we have recently evaluated the effects of a mitochondrial-targeted antioxidant Mitoquinone (MitoQ) in bile acid- and caerulein-induced AP in mice. MitoQ has been shown to be protective in diverse pathologies associated with oxidative stress, including diabetes [77], cardiac ischaemia-reperfusion injury [78] and sepsis [79]. In the severe TLCS-AP model, however, no beneficial effects of MitoQ treatment were detected, although MitoQ was effective in abolishing H_2O_2-induced increases of ROS in isolated pancreatic acinar cells [80]. Some limited protection was observed in the milder CER-AP model, however, such effects were shared by the non-antioxidant analogue control decylTPP, suggesting non-specific effects of MitoQ. Furthermore, certain parameters

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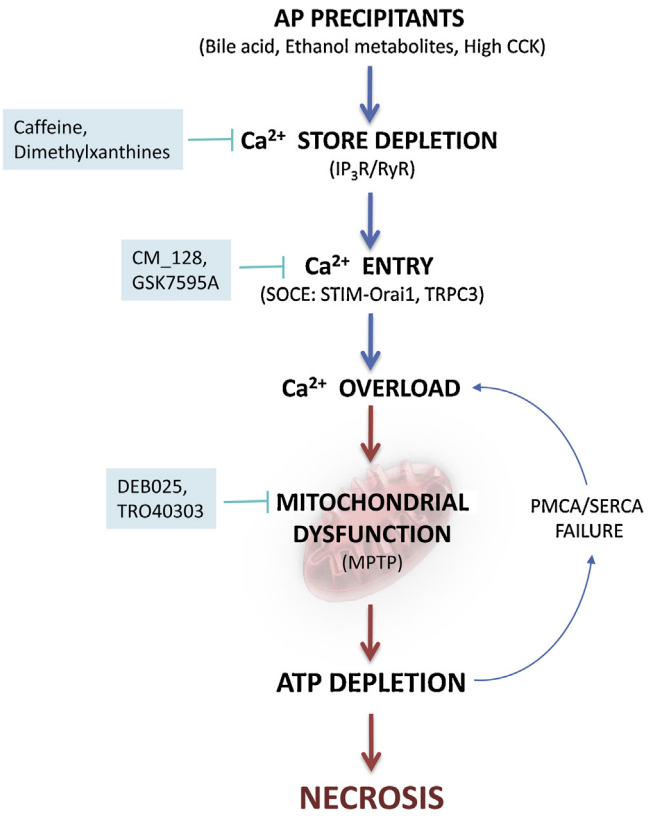

Fig. 5. Schematic illustrating translational approaches for treatment of acute pancreatitis (AP). Precipitants of AP deplete internal Ca^{2+} stores in pancreatic acinar cells which elicits store-operated Ca^{2+} entry (SOCE) and consequent cytosolic Ca^{2+} overload. Mitochondrial dysfunction ensues, *via* the formation of the mitochondrial permeability transition pore (MPTP), which depletes cellular ATP, the pivotal trigger for necrotic cell death. Furthermore, ATP rundown inhibits Ca^{2+} clearance from the cytosol *via* the plasmalemmal and sarco-endoplasmic Ca^{2+}-ATPase pumps (PMCA and SERCA) culminating in a vicious cycle that perpetuates Ca^{2+} overload and acinar cell death. Recent evidence has demonstrated that prevention of cytosolic Ca^{2+} overload or direct inhibition of MPTP formation using specific pharmacological agents protects against acinar cell necrosis and ameliorates experimental AP (see main text for further details).

i.e. interleukin-6 and lung MPO increases in CER-AP were exacerbated by MitoQ indicating complexity of antioxidant effects. Our recent results contrast with prior evidence suggesting a protective action of antioxidants in the bile-acid-induced AP model [81]. The general antioxidant N-acetylcysteine (NAC), which prevents oxidant-induced ROS increases in pancreatic acinar cells [7,15], ameliorated taurocholate-induced AP in rats. However, it should be noted that in this study NAC was administered prior to induction of AP, which does not accurately reflect the clinical situation in which disease has already undergone significant progression on patient admission. Consistent with this, a study in mice clearly showed that only prophylactic treatment with NAC i.e. before AP induction, was successful in limiting the severity of experimental AP, whereas antioxidant therapy was ineffective when given post-insult [82].

5. Involvement of ROS in AP: a translational gap

Whilst the importance of $[Ca^{2+}]_c$ overload to development of acinar cell damage has been firmly established [4,18,83], and potential treatment strategies for protection *via* inhibition of Ca^{2+} release [40] and entry [19,45] demonstrated in preclinical *in vivo* models, the translational value of ROS inhibition with antioxidants has appeared less convincing [9]. Clinical evaluations of antioxidant therapy in AP patients have produced variable outcomes, despite encouraging results from some early studies. For example, a combination of selenium, vitamins C and E, β-carotene and methionine improved AP in a small patient study [84], whilst selenium alone reduced AP mortality in a non-randomised trial [85,86]; no severe AP patients, however, were examined in these investigations. Additionally, treatment of patients with an intravenous high dose vitamin C, which decreased oxidative stress markers in blood, caused a faster recovery from AP symptoms compared to low dose vitamin C and healthy control patients, although the fate of the severe AP patients was incompletely documented [87]. Time-dependent increases in ROS have been demonstrated in a preclinical study in rats in response to ductal ligation [88] and some benefit of antioxidant therapy in post-ERCP AP was reported in a prospective, double-blinded, placebo-controlled trial [89]; high dose allopurinol, applied prior to the surgical procedure, reduced AP incidence. Such a protective effect is therefore consistent with the efficacy of antioxidants in preclinical experimental AP models when administered as pre-treatments.

However, other clinical trial outcomes have been less positive. For example, a prospective randomised, controlled study failed to demonstrate a reduction of hospital stay or complication rates in AP patients, although antioxidant treatment was effective in decreasing oxidative stress [90]. Similarly, combined antioxidant therapy did not reduce in-hospital mortality in patients with predicted severe AP, despite a significant restoration of vitamin C and selenium levels towards normal following treatment [91]. More recently, a carefully controlled randomised, double-blind placebo trial of combined antioxidant therapy in severe AP patients detected no difference in organ dysfunction or patient outcome [92]. In this study, serum antioxidant levels were elevated whilst markers of oxidative stress fell during treatment. Importantly, this study revealed a trend towards more deleterious outcome in patients given antioxidant therapy. A subsequent study showed no significant difference in organ dysfunction and length of hospital stay associated with combined antioxidant treatment in severe AP patients, despite restoration of redox balance [93].

6. Current status?

How might the variable effects of antioxidants in the clinic be explained? It is important to consider that ROS are generated in multiple places during AP and a blanket inhibition with general antioxidants may influence manifold processes. In addition to local generation of ROS within the acinar cell, there is likely to be a significant involvement of ROS produced during the inflammatory response; both activation and proliferation of immune cells are promoted by ROS [94–96] and ROS production is enhanced in neutrophils obtained from AP patients [97]. Since recruitment of immune cells to the pancreas occurs during the course of AP [98], generation of ROS *via* neutrophil NADPH oxidase may exert a significant influence on AP damage; in accord, depletion of neutrophils was protective in experimental AP [99]. Furthermore, interactions between oxidative stress and pro-inflammatory cytokines *via* MAP kinases and protein phosphatases may potentiate the inflammatory response and worsen tissue injury in AP [100,101]. In this context application of antioxidants to specifically target the inflammatory response in AP may be beneficial. However, the antioxidant MitoQ exerted biphasic effects on ROS production in polymorphonuclear leukocytes, generated *via* activation of NADPH oxidase [80]. Thus, phorbol ester-induced acute ROS production was inhibited, whereas a later phase was potentiated, highlighting complexity of antioxidant action. Current evidence suggests that the contribution of ROS in AP *via* NADPH oxidase is likely to be restricted to production from inflammatory cells, since this enzyme was not detectable in primary pancreatic acinar cells [99], while bile acid- or

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menadione-induced ROS rises were not altered by pharmacological inhibition using diphenyliodonium [7,15].

Perhaps most importantly in relation to the observed negative outcomes of antioxidants in clinical trials is their action to inhibit ROS-induced apoptotic cell death [7,15]. Inhibition of bile acid-induced ROS generation shifted the balance of pancreatic acinar cell death from apoptosis toward necrosis (Fig. 3a) [7]. Activation of the apoptotic cell death pathway is considered less damaging than necrosis, since this allows cell debris to be effectively removed by macrophages [102]; necrosis can instigate an inflammatory response in tissues surrounding the pancreas [5,103]. Inhibitory actions of antioxidants on ROS may therefore prejudice an endogenous protective mechanism to cope with cells undergoing stress. In agreement, a reduction of apoptosis *via* inhibition of caspase activity has been shown to produce a more severe necrotizing pancreatitis [104–106]. In pancreatic acinar cells necrosis ensues when ATP production is compromised due to Ca^{2+}-dependent mitochondrial dysfunction, mediated by MPTP opening [51,73,107]. Supplementation of intracellular ATP to acinar cells stimulated by palmitoleic acid or bile acid was protective in acinar [7,24] and ductal [108] cells. It has been proposed that ATP depletion is the trigger for necrotic cell death [109] and a gradation of cell death from apoptosis to necrosis may exist that is dependent on the extent and severity of the insult (Fig. 4). In a recent study, we showed that pharmacological inhibition or genetic deletion of cyclophilin D ($PPIF^{-/-}$), which modulates the MPTP, reduced necrotic, but not apoptotic, cell death in response to AP precipitants (Fig. 3b). Furthermore, pharmacological inhibition of cyclophilin D ameliorated AP in a variety of *in vivo* experimental models, including a significant reduction of pancreatic necrosis, suggesting the potential for therapeutic intervention in the clinic [73]. These results are consistent with earlier work demonstrating that the absence of cyclophilin D markedly protected mice from necrosis, whereas normal apoptotic cell death responses were retained [114]. Recent evidence shows that oxidative stress can modulate cyclophilin D activity, consistent with a model of ROS-sensitized Ca^{2+}-dependent MPTP induction [111,112] and this protein may act as a general redox sensor within the mitochondria [113]. However, the actions of oxidative stress on MPTP formation in exocrine pancreas have yet to be elucidated. Interestingly, differences between the properties of mitochondria in different cell types have been reported [114]; modulation of MPTP formation may thus vary between tissues and further investigation is warranted.

7. Conclusions: therapeutic avenues?

Ca^{2+} homeostasis is paramount for normal physiological functioning of the exocrine pancreas; this is achieved by a fine balance between Ca^{2+} release from internal stores, Ca^{2+} entry and extrusion mechanisms. Disruption results in Ca^{2+} overload of the acinar cell that causes mitochondrial dysfunction, *via* formation of the MPTP, loss of ATP production and consequent necrosis. Oxidative stress, at low levels, preferentially promotes apoptosis which may be beneficial in AP by avoiding necrosis that can trigger severe inflammation; application of antioxidants may therefore prejudice an endogenous protective mechanism and this approach has proven unsuitable as AP therapy in the clinic. In contrast, recent evidence has indicated that prevention of Ca^{2+}-dependent mitochondrial damage appears a promising strategy for AP therapy [73,115]. Multiple approaches have been suggested from preclinical findings (Fig. 5); 1) inhibition of Ca^{2+} release by IP_3R blockade, 2) inhibition of Ca^{2+} entry through Orai1 blockade and 3) prevention of MPTP formation. Clinical trials of selective drugs, such as CM_128, which prevent mitochondrial dysfunction induced by Ca^{2+} overload of pancreatic acinar cells, are imminent and will hopefully provide significant patient benefit for a disease that currently lacks specific therapy.

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