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FORUM REVIEW ARTICLE

The Renin–Angiotensin System and Reactive Oxygen Species: Implications in Pancreatitis

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Abstract

Significance: The renin–angiotensin system (RAS) is a circulating hormonal system involved in the regulation of blood pressure and circulating fluid electrolytes. Recent findings have revealed that locally generated angiotensin (Ang) II plays a pivotal role in normal physiology as well as pathophysiology in various tissues and organs, including the pancreas. This review article summarizes current progress that has been made in elucidating the putative roles of Ang II in both acute and chronic pancreatitis. ***Recent Advances:*** A convergence of evidence suggests that the underlying mechanism may involve reactive oxygen species (ROS)-generating systems, such as nicotinamide adenine dinucleotide phosphate oxidase, and subsequent elevation of proinflammatory and profibrogenic gene expression as well as protein activity. More importantly, Ang II-induced ROS interacts with other ROS-generating systems to positively feed-forward the ROS-induced signaling. ***Critical Issues and Future Directions:*** Advances in basic research indicate that RAS blockers may provide potential therapeutic role for the management of pancreatic inflammation and, more importantly, pancreatitis-associated complications. Genetic alterations resulting from a malfunction in the epigenetic control of pancreatic RAS could be a causative factor in the development of pancreatitis. *Antioxid. Redox Signal.* 15, 2743–2755.

Introduction

Pancreatic inflammation, known clinically as pancreatitis, represents one of the most catastrophic disorders in the upper abdomen. In general, pancreatitis can be classified as acute pancreatitis (AP) and chronic pancreatitis (CP). In the United States, costs related to AP hospitalization are nearly 2 billion dollars a year (32), whereas the annual total cost for managing CP has reached ~63.8 million dollars a year (113). This substantial medical burden is because pancreatitis can be accompanied by life-threatening complications, including systemic inflammatory syndromes, diabetes, and malnutrition, which require intensive care and long-term follow-up treatments. Epidemiological studies reveal that the morbidity of AP depends on the regions studied, ranging from 50 to 800 per 1,000,000 population per year. The incident rate of AP is 700 to 800 and 150 to 420 per million/year in United States and United Kingdom, respectively (4, 107), whereas it lies between 106 and 205 per million/year in Japan (98). Surprisingly, the incidence rate of CP is relatively low ranging from 81 to 86 per million in United States and United Kingdom, respectively (53). There is an increasing trend of pancreatitis incidence over decades, probably due to change in life style, enhanced exposure to the risk factors, or improvement in diagnostic technology (53, 98).

AP and CP

Although being similar, AP and CP are discrete in their cellular pathology, clinical diagnosis, treatment courses, and complications. AP is generally characterized by parenchymal edema, tissue necrosis (sometimes with pseudocysts and abscesses), hemorrhage, and inflammatory cell infiltration. Meanwhile, CP is associated with pancreatic atrophy, fibrosis, calcification, and exocrine and endocrine dysfunction (71). The major cell type responsible for AP is the pancreatic acinar cell, which is responsible for secretion of digestive enzymes into the gastrointestinal tract. It is believed that direct insults exerted on acinar cells, such as hyperstimulation, autoimmunity, toxins, and acute ischemia, trigger initiation of AP. Those stimuli favor intrapancreatic activation of trypsinogen in a lysosomal hydrolases-dependent fashion (116). The activated trypsin would in turn activate trypsinogen in zymogen, thus giving rise to a series of protease activation within the pancreas (15). The protease would degrade a number of cellular proteins, eventually leading to collapse and malfunction of acini, thus resulting in pancreatic lesion. On the other hand, excessive injury would positively reinforce more acinar cells to undergo necrotic cell death and to produce an overwhelming amount of proinflammatory cytokines (71). This

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self-sustained cascade favors a continuous inflammatory reaction in the pancreas and explains why AP can quickly develop into a systemic inflammatory response syndrome. Pancreatic stellate cells (PSCs) are key players in the fibrogenic phenotype of CP. Its development requires a continuous, sub-lethal stimulation of PSCs, resulting in their differentiation into a myofibroblast-like phenotype, in which they secrete profibrogenic and tissue-remodeling factors. Replacement of functional units (mostly acinar cells) by fibrotic tissue ensues and eventually leads to development of pancreatic fibrosis and pancreatic exocrine insufficiency. In some cases, destruction of pancreatic islets occurs, leading to endocrine dysfunction and subsequent pancreatic diabetes. Depending on the etiology, AP has a wide disease spectrum, ranging from mild edematous to severe necrotic, hemorrhagic pancreatitis. Most patients who develop mild self-limiting AP require no special treatments and can be discharged from the hospital within days. However, AP becomes severe in around 20% of patients, with a concomitance of potentially lethal complications which brings the mortality rate in this group up to 20%–25%, compared with 1%–3% in the patients with mild disease (43). For CP, the mortality rate is unclear since it is usually associated with several complications such as diabetes mellitus and pancreatic carcinoma.

Repeated bouts of AP can gradually develop into CP, a phenomenon referred to as the necrosis-fibrosis theory. It is believed that necrotic acinar cells in AP induce activation of PSCs and trigger transition to a profibrogenic phenotype, which subsequently leads to pancreatic fibrosis (71). It is of paramount importance to emphasize that some CP patients were reported to experience a history of repeated AP relapse (2). The rate of progression from AP to CP correlates with the number of relapse and severity of AP (53). Understanding the underlying mechanism would allow for the development of potential therapies to prevent the onset of CP in those patients who suffer from continual AP relapse.

Over several decades, there have been extensive investigations concerning the pathophysiology of pancreatitis. Yet, promising and effective therapy has not been deduced for these complicated diseases. Accumulating evidence has revealed that a local renin–angiotensin system (RAS) plays a vital role in the pathogenesis of pancreatitis (14–17, 52, 71, 110, 111), thus providing a new avenue in the treatment of this disorder. The details of the relevant studies are discussed in the following sections, with particular focus on the signaling pathways relating RAS to reactive oxygen species (ROS).

Renin–angiotensin system

The RAS is a circulating hormonal system that is crucial for blood pressure regulation and homeostasis. Its key components include the precursor angiotensinogen, the critical enzyme renin, angiotensin-converting enzyme (ACE), and several bioactive peptides, most notably the various forms of angiotensin (Ang), as well as their respective receptors. Figure 1 summarizes the major components of RAS and their respective biological functions. Angiotensinogen, an α-2-globulin with a molecular weight of $\sim$60 kDa, is the ultimate precursor protein for Ang peptides. It is converted to Ang I by renin, which catalyzes the hydrolysis of a peptide bond between leucine and valine residues on angiotensinogen. In response to low intrarenal pressure or low sodium delivery,

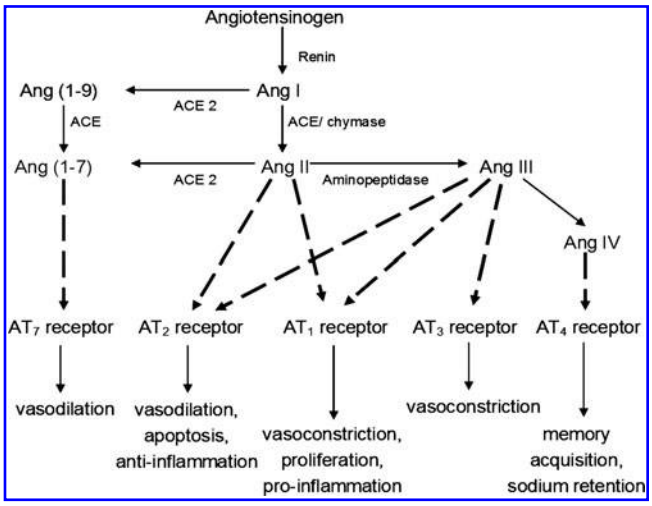

FIG. 1. Schematic diagram showing major components of RAS and their interactions. In response to low sodium and blood pressure, angiotensinogen is first processed by renin to form Ang I, which has limited biological function. Ang I is then converted by ACE into Ang II, which elicits biological actions *via* binding of AT_1 and AT_2 receptors. Ang II can be degraded by aminopeptidase and ACE2 to generate Ang III and Ang (1-7), respectively. Ang III interacts with the AT_3 receptor to produce effects similar to AT_1 receptor activation. Ang (1-7) serves as a ligand for the AT_7 receptor to counterbalance AT_1 receptor activity. Ang III is further metabolized into Ang IV, which is abundant in the central nervous system. RAS, renin–angiotensin system; ACE, angiotensin-converting enzyme; Ang, angiotensin; AT_1, angiotensin II type 1; AT_2, angiotensin II type 2.

renin is released from renal juxtaglomerular cells into the circulation to elevate the synthesis of Ang peptides for maintenance of systemic pressure and sodium retention (28). Ang I is a decapeptide with limited biological effect, but is converted into Ang II by removal of histidine and leucine residues in the C-terminal. This reaction is catalyzed by the transmembrane enzyme ACE, which is most abundant on the endothelial surface of pulmonary blood vessels (68). Different isoforms of ACE, other than somatic ACE in endothelial cells, have been described in humans, including testicular and soluble isoforms, thought their functions have yet to be delineated (28). Ang II is an octapeptide that elicits its biological actions *via* binding to Ang II type 1 (AT_1) receptor and Ang II type 2 (AT_2) receptor. Most, but not all, of the actions of Ang II are mediated through AT_1 receptors. It is generally believed that stimulation of AT_1 receptors results in vasoconstriction, proliferative effects, and proinflammatory actions, whereas AT_2 receptor activation results in vasodilatation, apoptosis, and anti-inflammatory effects (28, 68). Ang III, the heptapeptide formed from the N-terminal cleavage of Ang II by aminopeptidase A, acts similarly to Ang II through its interactions with a variety of Ang receptors, including AT_1, AT_2, and AT_3 receptors (69). Ang III activity enhances aldosterone release and renal blood flow, and to a lesser extent modulates vasopressor activity. Ang IV, previously regarded as a fragment of Ang II, appears to be associated with memory acquisition and recall, as well as inhibition of blood flow and sodium retention, *via* its interaction with AT_4 receptors (28).

In 2000, an ACE homologue known as ACE 2 was identified (29). It catalyzes the hydrolysis of the carboxy-terminal leucine in Ang I to form Ang (1-9) or that in Ang II to form Ang (1-7), with a higher affinity toward the latter reaction. However, it does not catalyze the hydrolysis of bradykinin (29). Ang (1-7), *via* binding of the AT_7 receptor, exerts opposite effects of Ang II, thereby negatively regulating blood pressure (56). A low dose of Ang (1-7) was shown to improve cardiac output and antagonize Ang II-induced vasoconstriction, thus fine-tuning the ultimate RAS signal (56, 95).

Pancreatic RAS: Its Role in Pancreatitis

Unlike circulating RAS, a local RAS appears to function independently and does not rely on the bioavailability of circulating hormones in the blood stream. In 1997, Leung *et al.* first reported the existence of a local RAS in the pancreas (69). AT_1 and AT_2 receptor expression was detected predominantly in the endothelia of blood vessels and the epithelia of the pancreatic ductal system, and at a weaker intensity in acini. The same group later reported the presence of angiotensinogen and renin in the rodent pancreas, as demonstrated by immunohistochemistry and real-time polymerase chain reaction (70). The local RAS was also more localized to acinar cells, PSCs, and islet cells (68, 72). Moreover, expression of angiotensinogen and of AT_1 and AT_2 receptors has been detected in isolated pancreatic acinar cells and the AR42J pancreatic acinar cell line (18, 110). Quiescent and activated PSCs express both AT_1 and AT_2 receptors (47, 96). Ang II-positive PSCs have also been identified after stimulation with glucose (59). Taken together, all these findings support the notion that a complete local RAS is present in acinar cells and PSCs; such a local RAS could play a role in physiological as well as pathophysiolgical conditions (72).

Physiological role of pancreatic RAS

The first functional study of Ang II in pancreatic acinar cells was carried out in 1999 (21). Exogenous Ang II treatment caused accumulation of inositol 1,3,4-trisphosphate and dose dependently increased amylase secretion by AR42J cells. Specific AT_1 receptor blockade with losartan, but not AT_2 antagonism with CGP42112, abolished Ang II-provoked α-amylase secretion, indicating that the AT_1 receptor mediates digestive enzyme secretion (21). More recently, *in vitro* stimulation of isolated pancreatic acini with Ang II elevated α-amylase and lipase secretion in a dose-dependent fashion. Again, these effects were reversed by blockade of AT_1, but not AT_2 receptors (110). The above phenomenon could be explained by the involvement of intracellular calcium in Ang II actions. Actually, Ang II can induce a rapid increase in intracellular calcium in AR42J cells in an AT_1 receptor-dependent manner (19). Given that exocytotic release of digestive enzymes depends on calcium and related processes, it is likely that intracellular calcium is involved in Ang II-induced enzyme secretion in pancreatic acinar cells. It should be mentioned that systemic infusion of Ang II failed to alter digestive enzyme secretion *in vivo* (18), further indicating that local RAS and circulating RAS are discrete entities in terms of function and source of effective mediator.

Pathophysiolgical role of pancreatic RAS

Given that Ang II serves as a key player in inflammatory responses and plays a crucial role in pancreatic physiology, it is not surprising that pancreatic Ang II should participate in the inflammatory response during pancreatitis (Fig. 2). The major components of pancreatic RAS, namely, the AT_1 receptor, the AT_2 receptor, and angiotensinogen, were upregulated in caerulein-induced AP (110). Similarly, upregulation of angiotensinogen has been observed in obstructive AP (16, 17). Prophylatic treatment with the nonspecific Ang II receptor blocker (ARB) saralasin inhibited the onset of pancreatitis, as evidenced by suppression in serum α-amlyase and histological deterioration (52, 112). Another independent study revealed beneficial effects of the ACE inhibitor captopril on serum amylase, trypsinogen activation peptide, and vascular permeability in taurocholate-induced AP rats (20). The specific AT_1 receptor antagonist losartan was able to abolish the pancreatic injury brought by hyperstimulation of caerulein (111). These results were further supported by the finding that losartan abrogated pancreatic neutrophil infiltration and histological deterioration in obstructive AP (16) and taurocholate-induced AP (89). On the other hand, lisinopril, an ACE inhibitor, attenuated pancreatic atrophy, granulocyte infiltration, collagen deposition, and fibrous tissue ratio in Wistar Bonn/Kobori spontaneous CP rats (60). Similar studies using specific AT_1 receptor blockers, candesartan (122) and losartan (74), also documented an inhibition of inflammatory infiltration and fibrous tissue in experimental CP animals. Moreover, a combination therapy with an ACE inhibitor and an AT_1 receptor antagonist synergistically alleviated pancreatic chronic inflammation and fibrosis (123). These protective effects might be due to the induction of apoptosis given that losartan can dose dependently trigger spontaneous apoptosis in human PSCs (73). Interestingly, the

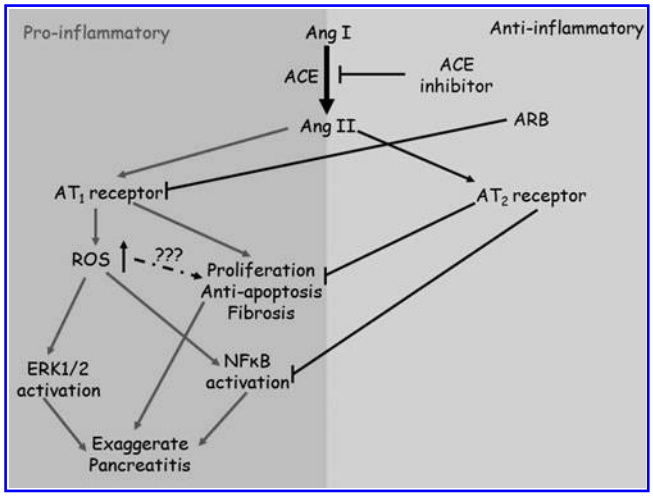

FIG. 2. Dual effects of pancreatic Ang II. Ang II exerts proinflammatory actions in an AT_1 receptor-mediated manner, involving generation of ROS, activation of proinflammatory mediators (NFκB and ERK1/2), and prosurvival of PSCs. On the other hand, AT_2 receptor activation results in NFκB inactivation and amelioration of the fibrogenic response. ARBs could protect pancreatitis by blocking AT_1 receptor activation and, at the same time, allowing more Ang II to interact with AT_2 receptors and thus exert anti-inflammatory actions. ROS, reactive oxygen species; PSC, pancreatic stellate cell; NFκB, nuclear factor kappa B; ARB, angiotensin II receptor blocker; ERK, extracellular-regulated kinase.

proapoptotic effect seems to be specific to PSCs since elimination of PSCs, but not acinar cells, was observed in RAS inhibitor-treated CP animals (74). Mice with genetic deficiency of the AT_1 receptor exhibited attenuated fibrogenic response and ameliorated PSC activation upon repeated hyperstimulation, further reinforcing the concept that pancreatic Ang II exerts detrimental effects *via* AT_1 receptor during CP pathogenesis (84). More recently, it has been shown that the AT_2 receptor, which counteracts the AT_1 receptor, also plays a role in the pathogenesis of pancreatitis. Relative to wild-type mice, AT_2 receptor-deficient mice showed more pronounced parenchymal atrophy, fibrosis, and exaggerated PSC activation upon chronic repeated hyperstimulation (115). Our unpublished data also suggest that AT_2 receptor antagonism activates proinflammatory signaling nuclear factor kappa B (NFκB) in Ang II-stimulated pancreatic acinar cells (Chan and Leung, *unpublished observations*). In obstructive pancreatitis, AT_2 receptor expression was upregulated nearly two-fold (Chan and Leung, unpublished observations), which further implicates a local RAS in pancreatitis. Considering the aforementioned lines of evidence, we hypothesize that, during the pathogenesis of pancreatitis, Ang II has dual effects; it acts as both a proinflammatory and anti-inflammatory mediator, depending on the receptor with which it interacts. AT_1 receptor blockade could exert its beneficial effects in two ways: (i) by directly inhibiting proinflammatory signaling from AT_1 receptors, and (ii) by allowing Ang II to activate AT_2 receptors and thereby exert anti-inflammatory effects.

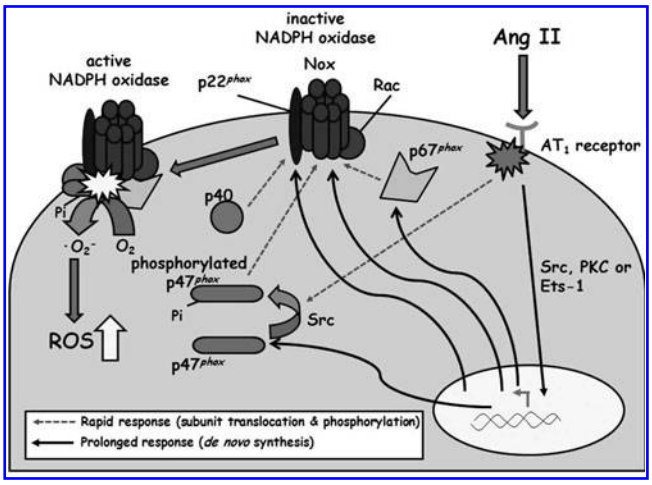

FIG. 3. AT_1 receptor-dependent activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase by Ang II. Upon binding to the AT_1 receptor, Ang II triggers phosphorylation of the $p47^{phox}$ subunit and cytosol-to-membrane translocation. Prolonged Ang II stimulation leads to *de novo* synthesis of NADPH oxidase subunits, namely, Nox, $p22^{phox}$ $p67^{phox}$, and $p47^{phox}$. Both instantaneous and sustained activation lead to elevation in conversion of molecular oxygen to superoxide free radicals.

Ang and ROS

As mentioned in the previous section, most of the actions of Ang II are mediated through AT_1 receptors. AT_1 receptor is a G-protein coupled receptor that elicits actions *via* activation of phospholipase A, phospholipase C, phospholipase D, or protein kinase C (PKC), as extensively reviewed elsewhere (37, 39). Of note, ROS generation represents a crucial mechanism in transducing the Ang II signal from the extracellular to the intracellular space. ROS accumulation occurs when there is an imbalance between the activities of ROS-generating enzymes and antioxidant enzymes (71). Ang II can directly or indirectly activate ROS-generating enzymes, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (37, 39) and xanthine oxidase (XOD) (62, 63). Further, ROS-induced ROS release (RIRR) has recently been demonstrated in certain pathological conditions (127), illustrating a possible self-feed forward mechanism in Ang II-associated disorders such as pancreatitis. Application of an ARB attenuates ROS generation and subsequent oxidative stress, making it a good candidate for oxidative stress management in a wide variety of diseases.

NADPH oxidase

NADPH oxidase is the transmembrane enzyme that catalyzes the reduction of molecular oxygen to the superoxide free radical (Fig. 3). It was first characterized in phagocytes and later found to be expressed in variety of cells, including endothelial cells, vascular smooth muscle cells (VSMCs), hepatic stellate cells, PSCs, and pancreatic acinar cells. Phagocytic NADPH oxidase was first characterized as consisting of Rac, $gp91^{phox}$, $p67^{phox}$, $p47^{phox}$, and $p22^{phox}$ (39). Additional gene family members were determined, namely, Nox, Nox activator (Noxa), and Nox organizer (Noxo) (71). Ang II-dependent activation of NADPH oxidase includes a transient rapid activation as well as a prolonged sustained activation. The rapid activation takes place within minutes after stimulation (66, 99). The mechanism of rapid ROS generation depends on cytosol-to-membrane translocation of $p47^{phox}$ (or Noxo). In an unstimulated state, $p47^{phox}$ is localized in the cytosolic compartment. Upon activation, $p47^{phox}$ docks at the membrane and interacts with membrane-bound complexes to elicit enzymatic activity. Ang II can phosphorylate $p47^{phox}$ in a Src-dependent manner (109). It is believed that phosphorylated $p47^{phox}$ associates with the cytoskeleton protein cortactin, which is crucial for proper assembly of NADPH oxidase subunits to elicit their full oxidase activity (108).

Prolonged Ang II exposure not only facilitates the cytosol-to-membrane translocation of NADPH oxidase subunits, but also enhances *de novo* synthesis of the subunits. Protein expression of $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ was elevated after a 6-h and 24-h Ang II treatment (109). Other studies revealed that sustained ROS generation by Ang II is attributable to upregulation of Nox 1 (64, 120) and $p22^{phox}$ (36). The underlying molecular mechanism may involve Ang II-induced activation of PKC (64) and Src activation (109). Recently, it has been shown that the $p47^{phox}$ promoter contains putative binding sites for the v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets-1) transcription factor (85). Ang II induced Ets-1 and $p47^{phox}$ protein expression, which was abrogated by Ets-1 knockdown. Ang II-induced $p47^{phox}$ expression and superoxide generation were compromised in Ets-1 knockout mice, as well as in wild-type mice after infusion of Ets-1 blocking peptide (85). These data directly demonstrate that Ets-1 serves as a crucial transcription factor in inducing NADPH oxidase synthesis *de novo*. However, the precise molecular mechanism involved and the corresponding transcription factors causing the expression of other

NADPH oxidase subunits such as Nox, $p22^{phox}$ and $p67^{phox}$ remain to be elucidated.

Self-amplifying signal

Ang II-induced ROS production not only depends on NADPH oxidase but also relies on other ROS-generating systems to amplify and sustain the pro-oxidative state (Fig. 4). XOD is a player involved in sustaining generation of ROS from Ang II. This enzyme, with a molecular weight of 150 kDa, is widely distributed in different tissues such as blood vessels, liver, kidney, intestine, and pancreas (83, 125). Under physiological conditions, xanthine oxidoreductase (XOR) exists in the form of xanthine dehydrogenase (XDH) with low ROS-generating activity (118). Upon stimulation, XOR undergoes a conformational change to exhibit a high affinity toward molecular oxygen, forming XOD and producing hydrogen peroxide (118). XOD is reversibly converted by sulfhydryl reduction to XDH, which has a high affinity for NAD^+ instead of molecular oxygen (118). A correlation between Ang II and XOD has been demonstrated in the vascular system. Ang II induced protein expression of XOD, but not XDH, in bovine aortic endothelial cells (BAECs) (63). Concomitantly, Ang II-induced superoxide production in BAECs was attenuated by the specific XOD inhibitors oxypurinol and tungsten, indicating that Ang II-induced XOD activation is a contributing source of overall superoxide generation. Similarly, patients suffering from coronary artery disease (CAD) exhibited elevated circulating Ang II together with augmented XOD activity and protein expression, but no alteration in XDH levels (44). Treatment with losartan or the XOD inhibitor allopurinol significantly blunted endothelium-bound XOD activity in the CAD patients, indicating that Ang II induced XOD activity *via* AT_1 receptors (63). Ang II-induced XOD activation appears to be due to NADPH oxidase-triggered ROS. This hypothesis is supported by the finding that NADPH oxidase inhibition results in attenuation in Ang II-induced XOD activity. Apocynin, a potent NADPH oxidase inhibitor, or siRNA against $p47^{phox}$ reversed Ang II-induced XOD protein expression, and subsequent superoxide generation (63). Aortic endothelial cells isolated from $p47^{phox}$ knockout mice ($p47^{phox-/-}$) showed reduced XOD protein levels compared with their wide-type littermates (79). Interestingly, XDH protein levels remained intact in both types of animals, indicating that NADPH oxidase activity is a crucial component of XOD activation, but not its overall expression. Moreover, hydrogen peroxide alone was sufficient to raise the XOD to XDH ratio (80). The aforementioned data support the hypothesis that Ang II-induced NADPH oxidase activation induces XOD activity in a superoxide-dependent manner, sustaining ROS signaling during Ang II stimulation.

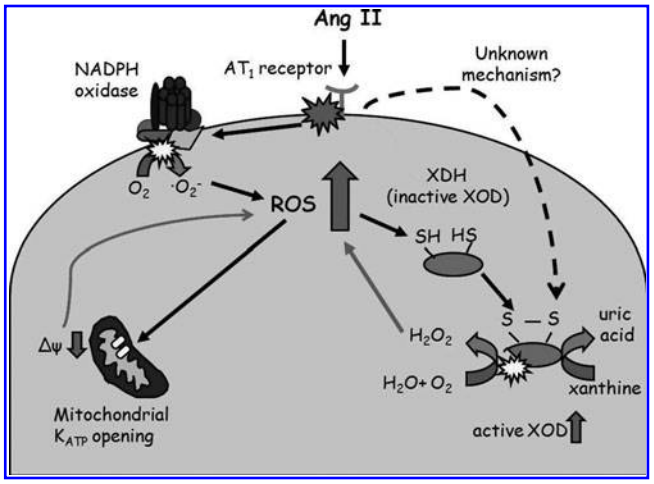

FIG. 4. ROS-induced ROS release in Ang II signaling. Ang II induces NADPH oxidase activation to trigger ROS production. ROS oxidize the disulfur linkage of xanthine dehydrogenase (XDH), forming xanthine oxidase (XOD), which preferentially produces H_2O_2 using xanthine and oxygen as substrates. NADPH oxidase-dependent ROS, on the other hand, induce mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) opening, depolarizing the inner membrane. These signaling pathways converge to produce more ROS, producing a feed-forward effect that sustains ROS signaling.

Apart from XOD, mitochondrial ROS serve as a major player in the self-amplifying loop of Ang II-NADPH oxidase signaling. Mitochondria have clearly been demonstrated to serve as a major source of ROS in eukaryotic cells. Univalent reduction of molecular oxygen to superoxide free radical was demonstrated in both complex I and complex III of the mitochondrial electron transport chain (12, 114). The rate of mitochondrial ROS production depends on the mitochondrial inner membrane potential ($\Delta\psi$), which is modulated by the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) (48). Opening of mitoK_{ATP} results in depolarization of the mitochondrial inner membrane (loss of $\Delta\psi$), and subsequently leads to production of ROS (38). In BAECs and VSMCs, Ang II treatment induced $\Delta\psi$ depolarization in a mitoK_{ATP}-dependent manner (30, 57). Ang II-induced mitochondrial ROS production was reversed by either 5-hydroxydecanoate, a specific inhibitor of mitoK_{ATP} (30), or rotenone, a mitochondrial respiratory complex I inhibitor (87). Interestingly, treatment with either apocynin or siRNA against $p22^{phox}$ suppressed Ang II-induced $\Delta\psi$ depolarization and mitochondrial ROS production (30), directly indicating that NADPH oxidase-derived superoxide is involved in Ang II-dependent mitochondrial ROS release. Of note, sulfhydryl alkylating agent blocked superoxide-induced $\Delta\psi$ depolarization, suggesting the possibility that sulhydryl group oxidation contributes to mitoK_{ATP} channel opening in response to oxidative stress (126). Taken together, these data consolidate the concept that RIRR following Ang II stimulation may contribute to a sustained oxidative environment in Ang II-related disorders.

Ang II-ROS axis in pancreatitis

Similar to the RAS in the cardiovascular system, pancreatic RAS relays its intracellular signals *via* ROS generation (Fig. 5). During AP and CP, overwhelming Ang II-induced increases in ROS levels are believed to exert oxidative insult upon the gland, leading to extensive necrotic injury (71). Leung and colleague have shown that nonselective blockade of Ang II reduced oxidative damage in caerulein-induced AP animals, as evidenced by glutathione restoration, suppression of lipid peroxidation, and inhibition of protein carbonyl formation (52). Additionally, selective AT_1 receptor blockade attenuated superoxide generation and oxidative damage in obstructive

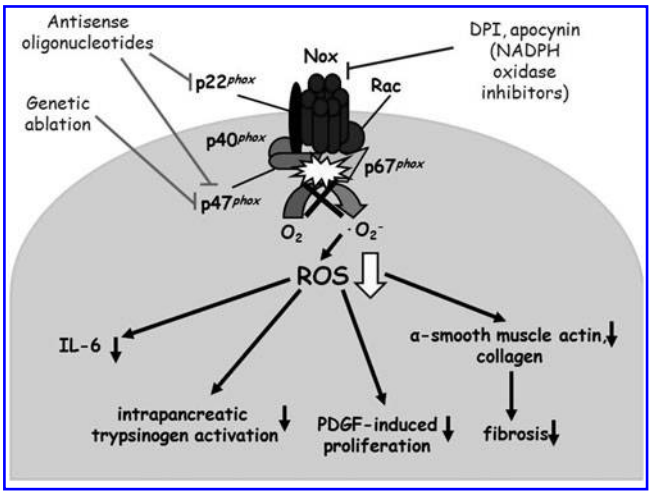

FIG. 5. A variety of approaches, including pharmacological inhibitors, antisense oligonucleotides, and genetic ablation, have been employed to demonstrate the role of NADPH oxidase in the pathogenesis of pancreatitis. NADPH oxidase-induced ROS have been shown to be associated with production of the proinflammatory cytokine IL-6, intrapancreatic trypsinogen activation, PDGF-induced proliferation, and profibrogenic response, thus regulating the resultant pathological phenotype. PDGF, platelet-derived growth factor; IL, interleukin.

pancreatitis (16, 17). These findings are in line with *in vitro* analysis showing that exposure of pancreatic acinar cells (17) and PSCs (77) to Ang II-induced ROS production, which was reversed by DPI, an NADPH oxidase inhibitor. Further, a single, high-dose administration of losartan has been shown to suppress pancreatic expression of $p67^{phox}$ and $p22^{phox}$, as well as NADPH oxidase enzymatic activity in experimental AP models (16, 111). Indeed, inhibition of NADPH oxidase alone is sufficient to reduce proinflammatory and profibrogenic signaling, subsequently alleviating pancreatic injury (Fig. 5). Pharmacological inhibition of NADPH oxidase abrogated caerulein-induced (124) or Ang II-induced (17) interleukin-6 (IL-6) expression in pancreatic acinar cells. Antisense oligonucleotides against $p47^{phox}$ and $p22^{phox}$ abolished secretagogue-induced IL-6 expression and NFκB activation in AR42J cells (124). Similar results were observed in PSCs showing that fibrogenic activity is subject to regulation by NADPH oxidase. PSCs isolated from $p47^{phox-/-}$ mice did not proliferate in response to platelet-derived growth factor (PDGF) (49). Apocynin reversed PDGF-induced proliferation as well as IL-1β-induced chemokine production and expression of α-smooth muscle actin and collagen in PSCs (77). These data were corroborated by *in vivo* analysis demonstrating a crucial role of NADPH oxidase in pancreatitis pathology. $p47^{phox-/-}$ mice exhibited an attenuated pancreatic injury in caerulein-induced AP, as evidenced by inhibition of intrapancreatic trypsinogen activation and hyperamylasemia (41). Administration of DPI suppressed histological deterioration and fibrosis in Wistar Bonn/Kobori spontaneous CP rats and DBTC-induced CP animals (77). Ang II-induced NADPH oxidase activation switches on proinflammatory signaling such as NFκB and extracellular-regulated kinase (ERK1/2) (16, 17), subsequently leading to transcription of an array of proinflammatory genes, thus conferring its proinflammatory actions. It should be emphasized that the aforementioned *in vivo* studies examined the systemic response, and thus the effect of NADPH oxidase inhibitor or deficiency on other relavent cells such as neutrophils needs to be considered.

A considerable number of studies support the view that, beyond NADPH oxidase, the down-stream ROS generating systems in the self-amplifying loop (XOD and mitochondrial ROS) are also involved in the pathophysiology of pancreatitis. XOD levels are elevated in taurocholate-induced AP animals (35, 92), choline-deficient ethionine-supplement diet-induced AP mice (86), and Wistar Bonn/Kobori spontaneous CP rats (125). Mitochondrial dysfunction and $\Delta\psi$ depolarization were observed in ROS-treated pancreatic acinar cells (31) as well as biopsies from CP patients (104). However, concrete data concerning RIRR, as well as the involvement of Ang II, were not demonstrated in these studies. Further investigation is required to clarify whether pancreatic RAS is correlated with XOD/mitochondrial ROS production and possible participation in RIRR during the pathogenesis of pancreatitis.

Although ROS is believed to serve as the major intracellular transducer in Ang II signaling, the octapeptide may also elicit ROS-independent effects to exacerbate pancreatic damage during pancreatitis. Ang II, *via* the AT_1 receptor, can transactivate and phosphorylate epidermal growth factor receptor in PSCs, leading to stimulation of DNA synthesis in an ERK1/2-dependent manner (47). Further, Ang II can limit PSC growth arrest by direct inhibition of transforming growth factor-associated Smad3/4 nuclear translocation (46). It has been shown that Ang II induces PKC activation, thereby rapidly activating Smad 7 and thus antagonizing Smad3/4 activity. Overexpression of Smad 7 alone was able to mimic Ang II-induced PSC proliferation (46).

Perspectives of RAS in Pancreatitis

Therapeutic implications: ARB in oxidative stress management in pancreatitis

In the clinical setting, the current standards of AP treatment require enteral or parenteral nutrition support (1). Nutritional support is given to reduce the disease burden without stimulating pancreatic secretion (78). In severe AP, negative nitrogen balance is usually associated with poor clinical outcome (81) and nutritional support has been shown to greatly improve this situation (42). When pancreatic necrosis with infection ensues, surgical drainage (necrosectomy) is crucial for patient survival (82). Patients suffering from CP usually experience severe abdominal pain, and conventional treatment generally fails to achieve satisfactory analgesia (58). Surgical intervention becomes critical when CP is associated with pseudocysts. In some cases, decompression, denervation, and resection of the pancreatic head are recommended to achieve pain relief and improve clinical outcome (3). In current practice, pancreatitis management seems to focus more on complications than the disease itself. A recent cohort clinical study indicated that Ang II blocker or ARB might reduce AP incidence (105). All of the patients enrolled in the study were diagnosed as hypertensive and had been prescribed an ARB, ACE inhibitor, calcium channel blocker, β-adrenergic antagonist, or diuretics as an antihypertensive treatment. In this population-based, case–control study, ARB users exhibited a 37% reduced risk of AP development, a

difference that was encouraging though it did not reach statistical significance. None of the other antihypertensive treatments appeared to reduce AP incidence, suggesting that specific blockage of AT_1 receptor might be beneficial in pancreatitis.

Oxidative stress management has long been a pre-eminent strategy for tackling pancreatic inflammation, and a number of clinical trials have examined antioxidant therapy for pancreatic inflammation (71). However, those studies yielded marginal clinical outcomes. Some of the studies, using single- or cocktail-antioxidant treatments, even failed to exert any prophylactic benefit on pancreatitis (postendoscopic retrograde cholangiopancreatography-induced AP) (71). It should be emphasized that antioxidants temporarily quench oxidative damage, but continuous ROS generation would gradually exceed the scavenging ability, eventually leading to a collapse in the defense mechanism. This relentless ROS generation might explain the failure of antioxidant regimes to achieve clinical benefits in these studies. As mentioned in preceding section, Ang II is a master molecule, controlling a number of ROS-generating systems *via* the AT_1 receptor. Thus, therapies targeting AT_1 receptors directly could exert much more powerful effects by tackling a number of ROS generating systems, instead of neutralizing particular ROS molecules. If so, a low dose of ARB would be expected to create an ROS-modulating effect comparable to a much more substantial application of antioxidant drugs. Given the wide applicability of ROS quenching across several diseases in the clinical setting (51, 54, 61, 63), ARB seems to be an ideal candidate for antagonizing ROS signaling and achieving oxidative stress management in patients suffering from pancreatitis.

Cross-talk between pancreatitis and associated pancreatic disorders: killing two birds with one stone using ARB

The systemic inflammatory response is a major complication of AP patients. Leung and colleague have reported that ARB could not only relieve local pancreatic injury, but also exerted protective effects on AP-associated pulmonary injury (14). Systemic administration of the ARB losartan significantly suppressed AP-induced pulmonary neutrophil infiltration, microvascular leakage, and morphological deterioration. Further, ongoing experiments suggest that the protective effects of ARB on AP-induced distant organ injury are consistent across animal models (Chan and Leung, unpublished observations). Thus, ARB might serve as a universal therapeutic strategy for treating or preventing AP-associated complications, regardless of etiology. Importantly, there is evidence indicating that ARB treatment exerts anti-inflammatory effects on sepsis and systemic inflammatory response (45, 102). ARB may relieve AP-associated distant organ injury *via* two mechanisms: (i) attenuation of pancreatitis secondary to systemic inflammation and (ii) direct inhibition of RAS in distant organs.

Pancreatic diabetes, which is discrete from classical type I and II diabetes, is a serious complication of pancreatitis. Approximately 50% of AP patients present with transient hyperglycemia, whereas 30%–80% of CP patients develop pancreatic diabetes (27). The pathogenesis of CP-associated diabetes is likely attributable to islet fibrosis. Indeed, islet RAS has been associated with the pathogenesis of diabetes (25). Exogenous Ang II treatment impaired glucose oxidation and glucose-induced proinsulin biosynthesis in isolated murine islets in an AT_1 receptor-mediated manner (65). Major components of RAS were found to be upregulated in type II diabetic islets (23), and administration of the ARB losartan significantly improved islet function and blood glucose levels (23, 55). Another study revealed that insulin-secreting pancreatic β-cells treated with Ang II produced monocyte-chemoattractant protein-1, which is a crucial factor in the recruitment of inflammatory cells (22). Similar to Ang II involvement in pancreatitis, Ang II appears to elicit its detrimental effects on diabetic islets mostly *via* NADPH oxidase-dependent oxidative stress and mitochondrial dysfunction (24). If ARB treatment by itself is able to improve islet function and correct blood glucose levels, one could speculate that individuals having CP superimposed on diabetes would exhibit an improved hyperglycemic condition when given an ARB. Moreover, administration of ARB could inhibit the onset of CP-associated diabetes *via* interactions with PSCs and islet RAS. Further investigations are needed to evaluate the actual therapeutic efficacy of ARBs in treating CP-induced diabetes.

The pancreas is a mixed gland, with exocrine and endocrine constituents. Although they mediate different functions, pancreatic cells are anatomically arranged in a specialized manner that allows them to communicate with one another. Most of the islets are surrounded by acinar cells, and a portion of the islets are in contact with the ductal system. Islets are usually connected with centroacinar cells in the small intralobular ducts (67), though some are connected to the pancreatic duct, establishing extensive contact between β-cells and exocrine cells (5). Individual endocrine cells, including α-cells, β-cells, δ-cells, and PP-cells, are also scattered throughout the ductal system (6, 91). A portion of those endocrine cells is subject to abrupt changes in diabetic animals, indicating that endocrine function might be an important regulator of the residence of these scattered cells (91). Given the close physical proximity among pancreatic cells, their special anatomical arrangement, as well as the crucial role of RAS in these cells, we hypothesize that islet RAS could be fused by PSC/acinar RAS in a dual paracrine fashion. We posit that RASs thus communicate with each other and share the overall pool of the bioactive peptides during normal physiological as well as pathophysiological conditions. In our view, pancreatic cellular RASs should not be regarded as individual units, but rather as an integrated system which functions in an orchestrated manner to support the physiology and biology of the pancreas, and probably to dictate cell fate and responses during the pathological conditions such as pancreatitis. Figure 6 summarizes our hypothesis of cross-talk between acinar, PSC, and islet RASs in the control of their physiological functions as well as pathological outcomes.

Epigenetic control of RAS: possible role of microRNAs in pancreatitis

Studies correlating genetic polymorphisms with inflammatory disorders have drawn substantial attention in recent decades. Certain genetic aberrations, such as in cationic trypsinogen gene (*PRSS1*), serine protease inhibitor, Kazal type 1 (*SPINK1*), and cystic fibrosis transmembrane conductance regulator, have been shown to be associated with the

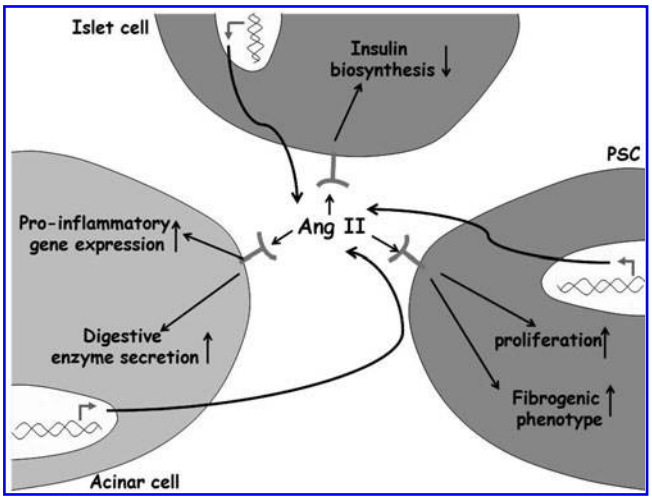

FIG. 6. Possible cross-talk among pancreatic cell RASs. Different pancreatic cells have their own intrinsic RASs. Owing to their proximity and the pancreas' special anatomic structure, acinar cells, PSCs, and islet cells could share the overall pool of Ang II in a paracrine/autocrine manner, thus allowing their RASs to communicate with one another and thus sustain the physiological function of the pancreas, as well as certain pathological conditions such as pancreatitis-associated diabetes.

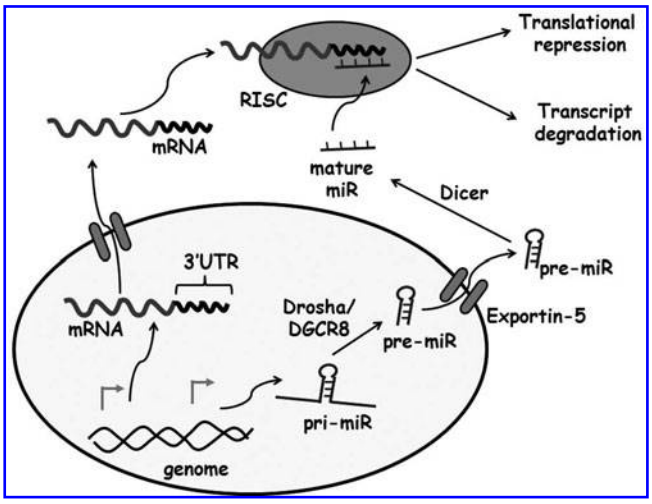

FIG. 7. Mechanism of miR regulation of gene expression. Pri-miRs are derived from the genome by RNA polymerase and truncated by miR biogenesis complex (Drosha/DGCR8) to form premature miRs (pre-miRs), which are transported out of the nucleus *via* exportin-5 and further processed by Dicer to form mature miRs. Mature miRs may be loaded into the RISC complex and allowed to bind to the 3′-UTR of its target mRNA to achieve gene silencing, either *via* translational repression or transcript degradation. miR, microRNA; pri-miR, primary microRNA; 3′-UTR, 3′ untranslated region.

incidence of CP (26, 101, 119, 121). On the other hand, AP patients with biliary origin disease and idiopathic CP patients have been genotyped as carriers of genetic alterations in GSH S-transferase theta-1 (*GSTT-1*) (93, 94). It has been shown that a polymorphism in the intron 16 region of the *ACE* gene is associated with elevated plasma ACE enzymatic activity (97). A deletion (DD) genotype appears more frequently in patients suffering from myocardial infarction than does an insertion (II or I/D) genotype (13). Studies examining a correlation between ACE polymorphism and pancreatitis incidence have been reported recently. In 2006, Bhaskar *et al.* reported that neither the I/D genotype nor the DD genotype of the ACE gene correlated with the presence of tropical calcific pancreatitis or fibro-calculous pancreatic diabetes (7). The ACE I/D polymorphism does not appear to be a crucial factor in determining susceptibility to AP and CP (50, 88). Moreover, the DD polymorphism in the *ACE* gene does not affect disease severity in AP patients (90). These findings appear to be in conflict with basic research observations. Nevertheless, we should keep in mind that ACE is not the only player in pancreatic RAS. Actually, certain ACE-like enzymes, such as chymase, which is abundant in the pancreas, could actively convert angiotensinogen into Ang I, and further into Ang II (117). Further, expression of a gene is not solely influenced by polymorphisms in the exonic or intronic region; genetic alterations in the flanking regions or in other regulatory sequence such as microRNAs (miRs) can also substantially influence a gene expression (Fig. 7).

In the epigenetic era, post-transcriptional modification, which affects mRNA stability and translational initiation, is regarded as a major regulatory mechanism of gene expression in biological systems. The discovery of miRs, which are small noncoding RNAs, holds great promise. miRs are ~22 nucleotide base-pair RNAs derived mainly from noncoding regions and transcribed as primary miRs *via* RNA polymerase. After several maturation processes and nucleus export *via* Drosha/DGCR8, Dicer and exportin-5 [for details, please refer to reference (103)], miRs are incorporated into a RISC complex to elucidate their gene regulatory effects. In general, the core sequence of miR responsible for the miR-mRNA complex, which is known as the seed sequence, imperfectly complements the 3′ untranslated region (3′-UTR) of a gene. This interaction leads to inhibition of translational initiation or deadenylation of mRNA, thus triggering mRNA degradation (Fig. 7). In this regard, any genetic alterations in sequences that express miRs or polymorphisms in the flanking region that affect normal miR binding would lead to dysregulation of a given gene. These mechanisms have been shown in RAS gene regulation for miR-155. Briefly, miR-155 binds to the 3′-UTR region of the *AGTR1* gene (encoding the AT_1 receptor), thus attenuating its expression (75). An A/C transversion at position 1166 in the 3′-UTR of the human *AGTR1* gene abrogates miR-155 binding, subsequently abolishing miR-dependent regulation of AT_1 receptor expression (76, 100). This inhibition leads to upregulation of AT_1 receptor-Ang II binding, thus rendering the hypertensive phenotype associated with this polymorphism. Another independent study revealed that, in addition to the AT_1 receptor, ACE is also regulated by miR. Boettger *et al.* reported that mice with genetic ablation of the miR-143/145 cluster exhibited overexpression of ACE in their aortae (10). Interestingly, miR-155 and miR-143/145 are both present in the pancreas and are aberrantly expressed under tumorigenic conditions (9, 106). Whether these miRs play a role in the pathogenesis of pancreatitis is not yet clear. However, these findings give us an important clue: genetic alteration in the binding sites of miRs (or in genetic loci encoding the miRs) may lead to aberrant expression of pancreatic RAS genes, potentially affecting the

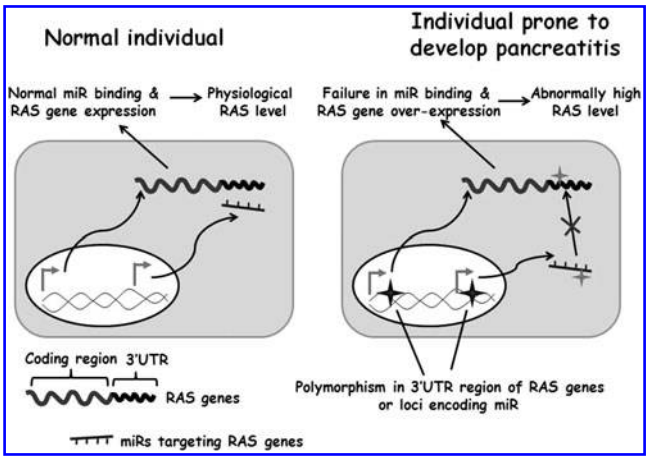

FIG. 8. Polymorphisms in miR binding sites of RAS genes or genetic loci transcribing miR targeting RAS genes could be a crucial factor differentiating normal and pancreatitis-prone individuals. Such genetic alterations could lead to an inability for miRs to selectively control RAS expression, thus leading to supra-physiological expression of RAS and thereby possibly enhancing susceptibility to pancreatitis development. It should be emphasized that such polymorphisms have not been experimentally validated in the setting of pancreatitis.

normal regulation of pancreatic RAS and subsequently increasing risk of pancreatitis development (Fig. 8). Identification of such polymorphisms would provide critical information about the epigenetic regulation of pancreatic RAS, which could then enable us to screen for genetic alterations that lead to greater susceptibility to pancreatitis.

Conclusions

Pancreatic RAS is a crucial mediator in the pathogenesis of AP and CP. Ang II elucidates its proinflammatory actions *via* ROS generation in an NADPH oxidase-dependent manner. RAS blockade, such as by an ARB, has been shown to exert protective effects against pancreatitis in a wide range of experimental AP and CP models. The clinical translation of RAS blockade in pancreatitis is still at a very early stage. The availability of ARB treatment for patients with pancreatic inflammation may be hampered by a number of conflicting reports concerning RAS blocker-induced pancreatitis (8, 11, 33, 34). Nevertheless, it should be emphasized that confounding factors, such as smoking, use of multiple prescribed drugs, and environmental toxin exposure, were not controlled for in prior studies. Another concern is the adverse effects of ARB in certain critical situations such as hypotension, which is often associated with severe AP. Affected patients are reported to exhibit elevated serum active renin activity, which regressed significantly during recovery (40). Elevated circulating Ang II might be crucial in supporting blood pressure during this critical condition. Care should be taken if an ARB is chosen as an anti-inflammatory strategy. At the same time, genetic alterations should be screened for in individuals who are prone to developing pancreatitis to facilitate delivery of preventive measures. With the discovery of miRs and other epigenetic control mechanisms, it is possible that RAS gene polymorphisms that affect miR binding (or genetic alternation in loci that transcribe miRs targeting RAS genes) could be of crucial factors in determining individual patients' susceptibility to development of pancreatitis. To the best of our knowledge, only one clinical trial has examined the beneficial effects of an ARB on pancreatitis. Large-scale, placebo-controlled clinical trials should be conducted to investigate the true therapeutic value of ARBs in pancreatitis, as well as in pancreatitis-associated complications.

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Abbreviations Used

ACE = angiotensin-converting enzyme
Ang = angiotensin
AP = acute pancreatitis
ARB = angiotensin II receptor blocker
AT_1 = angiotensin II type 1
AT_2 = angiotensin II type 2
BAEC = bovine aortic endothelial cell
CAD = coronary artery disease
CP = chronic pancreatitis
ERK = extracellular-regulated kinase
IL = interleukin
miR = microRNA
$mitoK_{ATP}$ = mitochondrial ATP-sensitive potassium channel
NADPH = nicotinamide adenine dinucleotide phosphate
NFκB = nuclear factor kappa B
PDGF = platelet-derived growth factor
PKC = protein kinase C
pre-miR = premature microRNA
pri-miR = primary microRNA
PSCs = pancreatic stellate cells
RAS = renin–angiotensin system
RIRR = ROS-induced ROS release
ROS = reactive oxygen species
UTR = untranslated region
VSMC = vascular smooth muscle cells
XDH = xanthine dehydrogenase
XOD = xanthine oxidase
XOR = xanthine oxidoreductase