Hydrogen Sulfide and Substance P in Acute Pancreatitis

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Abstract

The pancreas produces hormones that are secreted into the blood which regulate metabolism and it produces digestive enzymes that are secreted into the duodenum. During pancreatitis these digestive enzymes are activated and released into the pancreas, which results in the digestion of the organ itself causing extensive tissue damage. This leads to severe local inflammation that can develop into a pathological systemic inflammatory state. Hydrogen sulfide (H$_2$S) and substance P (SP) have recently been discovered to play a significant role in the pathological development of acute pancreatitis (AP). H$_2$S is a gaseous molecule previously considered to be an inconsequential byproduct of reactions in the body. However, recently it has been discovered that the enzymes involved in the endogenous production of H$_2$S are upregulated during inflammatory conditions and that inhibition of these enzymes reduces inflammation. As a result of these and other studies, H$_2$S is now thought of as gaseous signalling molecule involved in mediating inflammation and plays a crucial role in the pathophysiology of AP. SP is a tachykinin that is produced from primary nerve terminals and has been found to be a key signalling molecule in the neurogenic inflammatory response. SP activates neurokinin receptors, particularly neurokinin-1 receptor (NK$_1$R). Activation of NK$_1$R causes inflammatory symptoms such as vasodilation, cytokine release and leukocyte activation. Animal studies have shown that SP levels increase 24-fold during the first 12 hours of AP. Furthermore, mice lacking a gene necessary for SP production had significantly less severe pancreatitis symptoms. Interestingly, several studies have shown that H$_2$S levels significantly effect SP production and NK$_1$R expression. This research suggests that H$_2$S and SP have significant roles in the signalling of inflammation during AP and these signalling pathways are interdependent. Therefore, both pathways are excellent candidates for pharmacological intervention as a treatment for AP.
Key Words
Pancreatitis, inflammation, substance P, Hydrogen Sulfide, cytokines, immune cells, signalling pathways, neurokinin receptor, tachykinin, gaseous signalling molecule, acini, digestion, leukocyte, L-cysteine, cystathionine-γ-lyase, CSE, cystathionine-β-synthase, CBS, vanilloid, preprotachykinin-A.
**Introduction**

The pancreas is a vital organ responsible for regulating energy homeostasis. It is composed of two different cellular groups, endocrine and exocrine, with quite differing functionalities. The endocrine pancreatic islet cells produce and secrete hormones required for energy and glucose homeostasis including insulin, glucagon, somatostatin, and pancreatic polypeptide [1]. The exocrine acinar cells are responsible for the synthesis and secretion of hydrolytic enzymes that act in the small intestine to aid in food digestion. The three main categories of digestive enzymes secreted by the acini are α-amylase; to digest carbohydrates, lipases; to hydrolyse fatty acids, and proteases; to digest protein in the diet. The digestive enzymes are stored in secretory granules near the apical portion of the acini and in response to food ingestion they are transported through a series of ducts of increasing size to the duodenum. The proteases are stored in the acini as inactive zymogen precursors and once in the duodenum they are then activated by the enterocyte-associated enzyme enterokinase [2]. This activation mechanism prevents pathological autodigestion of the gland and related gastrointestinal structures. Other mechanisms to prevent premature activation of these zymogens include low intracellular Ca$^{2+}$, low pH and the presence of protease inhibitors within the zymogen storage granules [2]. However, in some disease states, such as acute pancreatitis (AP), these zymogens are activated within the pancreas and this results in the digestion of functional cellular proteins and ultimately widespread cellular death and tissue damage.

AP is an inflammatory disease of the pancreas characterised by pancreatic tissue oedema, acinar cell necrosis, haemorrhage and inflammation of the damaged pancreas [1]. In developed countries, the main causes are alcohol abuse (36%) and obstruction of the bile duct by gallstones (38%) [3]. Other rare and more controversial causes include pancreas divisum; a congenital
morphological aberration of the pancreatic duct system, an intraduct papillary mucinous tumour, and hypercalcaemia [3]. The pathophysiology of AP begins with the premature intra-acinar activation of zymogens and the autodigestion of the proteins within the organ [4,5]. The resulting pancreatic damage leads secretion of inflammatory signalling molecules and a local inflammatory response, which can then be followed by a systemic inflammatory condition referred to as the systemic inflammatory response syndrome (SIRS) [6]. SIRS is a systemic response to a local inflammatory assault and can include fever, tachycardia, respiratory insufficiency and multiple organ dysfunction syndrome (MODS) [6,7]. The overall mortality of AP is approximately 5%, and this is higher in patients with necrotizing pancreatitis (17%). This increase in mortality is due to the heightened risk of organ failure, as opposed to those with interstitial pancreatitis (3%) where little necrosis occurs [5].

The severity of AP can differ widely, however, the cellular immune response and processes are very similar regardless of etiology and involves both the local and systemic overproduction of inflammatory mediators. One of the primary focuses of research into AP has been inflammatory cytokines. Increased concentrations of the inflammatory cytokines interleukin-1 (IL-1), IL-6, IL-8 and tumour necrosis factor-α (TNF-α) have been associated with greater disease severity [7]. Intervention studies have shown that these inflammatory signalling molecules are crucial to the development of pancreatitis [8,9]. The damaged acinar cells release chemotactic factors facilitating the recruitment of inflammatory cells, leading to the release of these inflammatory cytokines and other key inflammatory mediators including platelet-activating factor (PAF) and bradykinin [10]. Recently, substance P (SP) and hydrogen sulfide (H₂S) have been identified as signalling molecules in novel pathways of inflammation and the pathogenesis
of AP. This chapter will discuss the roles of these compounds in AP outlining the key research that elucidated the involvement of SP and H₂S in inflammation signalling [10].

**Hydrogen Sulfide**

H₂S is a foul smelling gas associated with swamps and rotten eggs where it is formed as a byproduct of bacterial anaerobic digestion [11]. Historically it has been viewed as a toxicant responsible for many industrial deaths, as it is also generated as a byproduct in petrol refineries, paper mills and tanneries [11]. However, this ubiquitous molecule has more recently been found to be produced by the body and is involved in the regulation of many endogenous processes. The physiological concentrations of H₂S have been a contentious issue with reported plasma concentrations ranging from sub-micromolar levels to 300 µM [12]. Despite these issues, the majority of groups in the field agree that H₂S is produced by the body and this production is upregulated during several pathologies [13-16]. Furthermore, inhibiting this production has been shown to be beneficial during some of these pathologies [17]. Exogenous H₂S exhibits a steep dose-response curve, with obvious odour at 3-10 ppm, respiratory tract irritation at 50-100 ppm and dizziness, unconsciousness and death occurring at levels of 500 ppm and above [18]. As a broad-spectrum toxicant it affects many organ systems including lung, brain, kidney and gastrointestinal tract. H₂S is weakly acidic and exists in plasma as the un-dissociated gas or in one of two dissociation states, the hydrosulfide anion (HS⁻) and the sulfide anion (S²⁻). At physiological pH of 7.4, the total sulfide pool is comprised of 18.5% un-dissociated H₂S and 81.5% as HS⁻ [19]. Due to the current limitations in H₂S measurement, it is difficult to confirm whether the physiological actions of H₂S are mediated by H₂S directly or by the dissociated form.
**H₂S Synthesis**

The amino acid L-cysteine is the predominate sulfide donor molecule in the endogenous production of H₂S. L-cysteine can be obtained from the diet, synthesised from L-methionine or ‘liberated’ from endogenous proteins [20]. In mammalian tissues, H₂S is produced from the enzymatic desulfhydration of cysteine by at least three separate endogenous pathways [11]. The key enzymes in the two primary pathways are cystathionine-γ-lyase (CSE, EC 4.4.1.1) or cystathionine-β-synthase (CBS, EC 4.2.1.22) (Fig.1) [21]. A third minor pathway involves the enzymes 3-mercapto-sulfurtransferase (3-MST, EC 2.8.1.2) and cysteine aminotransferase (CAT, EC 2.6.1.3) (Fig.1) [21]. Both CSE and CBS are pyridoxal phosphate (PLP)-dependent enzymes and govern H₂S synthesis in quite separate organ systems.

CBS is the largest contributor to endogenous H₂S in the central nervous system with roles in both neuroinflammation and cognition [22,23]. It also has a minor role in H₂S production in the periphery including the liver, kidney and ileum [24]. CBS activity is enhanced by the allosteric activator S-adenosyl-L-methionine (SAM) as well as by binding of Ca²⁺/Calmodulin, L-glutamate and also electrical stimulation [23]. At this time, two H₂S-generating pathways have been described for CBS. The first and best characterised involves the condensation of homocysteine and serine to form cystathione, with the other pathway involving hydrolysation of L-cysteine as the substrate to form L-serine and H₂S (Fig.1) [24].

CSE is the enzyme primarily involved in peripheral H₂S synthesis, including muscle tissues, vascular systems, digestive tract, liver, pancreas and kidneys [24]. Hydrolysis of L-cysteine by CSE produces H₂S, pyruvate and ammonia (NH₃), and CSE can also catalyse the β-
disulfide elimination reaction of cystine to thiocysteine, which is then hydrolysed by CSE to cysteine and H$_2$S (Fig. 1) [21]. CSE activity is irreversibly inhibited by D,L-propargylglycine (PAG) and reversibly inhibited by β-cyano-L-alanine (BCA) [25].

3-MST and CAT are non-PLP dependent enzymes located both in the mitochondria and cytosol of the vascular endothelium and brain. CAT catalyses the reaction of L-cysteine with keto-acids forming 3-mercaptopyruvate which is then desulfurated in a zinc-dependent reaction by 3-MST, yielding H$_2$S and pyruvate (Fig. 1) [26]. This pathway is of particular interest in the brain where it produces H$_2$S more efficiently than the pathway catalysed by CBS [27]. H$_2$S can also be formed non-enzymatically via reduction of thiols and thiol-containing molecules as a way of liberating H$_2$S from intracellular sulfur stores [19].

H$_2$S is rapidly oxidised in the mitochondria yielding thiosulfate (S$_2$O$_3^{2-}$), which is then converted to sulfite (SO$_3^{2-}$) by rhodanese (EC 2.8.1.1), followed by oxidation by sulfate oxidase to sulfate (SO$_4^{2-}$) and renal excretion [20]. It is interesting to note that the oxidation of sulfide takes preference over the oxidation of other carbon-based substrates in the mitochondria, ensuring that the intracellular levels of H$_2$S remain constitutively low [12].
Figure 1: Potential pathways for H\textsubscript{2}S production. CAT, cysteine aminotransferase; CBS, cystathionine-\(\beta\)-synthase; CSE, cystathionine-\(\gamma\)-lyase; DHLA, Dihydro lipoid acid; 3-MP, 3-mercaptopyruvate; 3-MST, 3-mercapto-sulfurtransferase; R-SH, thiol; Trx, thioredoxin.

Pathological Effects of H\textsubscript{2}S

The main pathological effect of H\textsubscript{2}S is by direct inhibition of mitochondrial cytochrome c oxidase, hindering oxidative phosphorylation [28]. This is thought to be the primary mechanism of H\textsubscript{2}S-related toxicity and deaths. It has been found that H\textsubscript{2}S is a more potent inhibitor of mitochondrial activity than cyanide [18].
H₂S is also a potent mediator of inflammation in many pathological conditions. H₂S levels were shown to be markedly increased in rat septic and endotoxic shock models, and to correlate negatively with blood pressure and cardiac function [29]. H₂S was also found to be a crucial inflammatory mediator in a lipopolysaccharide-induced mouse model of septic shock [30]. The deleterious haemodynamic effects of H₂S are due to the interaction of H₂S with K_ATP channels of vascular smooth muscle cells, causing hyperpolarisation of the cell membrane [31]. This effect also increases vascular permeability and promotes oedema by allowing neutrophil and leukocyte infiltration [32]. H₂S contributes to inflammation by a variety of other routes. H₂S promotes transient receptor potential vanilloid-1 (TRPV1)-mediated neurogenic inflammation in sepsis [33]. Activation of TRPV1 by H₂S in sepsis evoked lung injury causes the upregulation of COX-2 and PGE-2 metabolites, which are implicated in the augmentation of inflammation in both sepsis and respiratory disease [14]. In polymicrobial sepsis H₂S activation of TRPV1 resulted in release of the inflammatory tachykinin SP culminating in an increase in ERK₁/₂ and IκB-α phosphorylation [13]. H₂S acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in the mouse by increasing levels of inflammatory cytokines including IL-1β, IL-6, TNF-α and monocyte chemotactic protein-1 (MCP-1) via nuclear factor-κB (NFκB) activation [34]. Furthermore, H₂S inhibition decreases severity of both local and systemic inflammation [34]. Therefore, not only does H₂S cause deleterious hypotensive haemodynamic effects due to its vasorelaxation properties, it also promotes the development and progression of inflammation and multi-organ damage that is associated with sepsis and other inflammatory conditions.
**H$_2$S in Acute Pancreatitis**

The use of high doses of the peptide secretagogue cholecystokinin (CCK) or its analogue caerulein has been well validated as an experimental model of AP [35]. This model has identified H$_2$S as an important mediator in the pathogenesis of AP. Pancreatic acinar cells express both the genes for CSE and CBS, however, only CSE mRNA expression is upregulated in response to administration of caerulein. This suggests that CSE is responsible for the increase in H$_2$S concentrations during AP and therefore, is the most suitable target for pharmacological intervention in AP [36]. Furthermore, the upregulation of CSE corresponds to a significant increase in H$_2$S production by isolated acinar cells, an effect that was abated with the pre-administration of 3 mM of the CSE inhibitor DL-proplyargylglycine (PAG) [36]. Mice with caerulein-induced AP (50 µg/kg i.p. per hour for 10 hours) showed a 27% increase in plasma H$_2$S levels compared to saline controls, as well as increased myeloperoxidase (MPO) levels indicating neutrophil infiltration [37]. Both prophylactic and therapeutic treatment with PAG significantly decreased these levels as well as decreasing amylase secretion and the degree of pancreatic injury. Furthermore, there is evidence that H$_2$S is involved in AP-related pain and hyperalgesia through interaction with T-type Ca$^{2+}$ channels [16].

A predominant mechanism of H$_2$S-induced inflammation in AP is through the activation of pro-inflammatory CC type chemokines. *In vitro*, caerulein hyperstimulation augmented the pancreatic production and secretion of the CC chemokines MCP-1, MIP-2 and MIP-1α [17]. This was also shown to occur *in vivo*, with both the pancreas and the lungs showing increased mRNA expression of these chemokines, implicating them in the development of both local and systemic inflammation in AP. As PAG pre-treatment decreased this chemokine expression, it is thought that the pro-inflammatory effects of H$_2$S in AP are at least partly mediated by these CC
chemokines [17]. Brady et al. [38], found in an in vivo model of AP that CXC type chemokines may also be involved with increased concentrations of the CXC chemokine CINC, in caerulein-hyperstimulated mice. Furthermore, Tamizhelvi et al. [39], found that H$_2$S causes neutrophil attraction and attachment through intra-cellular adhesion molecule-1 (ICAM-1) activation, mediated via NFκB and SFKs. Therefore, H$_2$S seems to mediate the activation of CC and CXC chemokines as well as other inflammatory cytokines in AP.

**Substance P**

SP belongs to the tachykinin family of neuropeptides. Tachykinins are one of the largest families of neuropeptides and are characterised by a conserved C-terminal sequence of Phe-X-Gly-Leu-Met-NH$_2$, in which X encodes an aromatic (Phe, Tyr) or aliphatic (Val, Ile) amino acid [40]. The integrity of this C-terminal sequence is important for mediating their biological effects. SP is encoded by the preprotachykinin-A (PPT-A) gene, which also encodes another tachykinin, Neurokinin A (NKA). Alternative splicing of the PPTA genes results in three primary mRNAs: α-, β-, and γ-PPT-A. The α-PPT-A encodes SP solely in the CNS, while β-, and γ-PPT-A code for the synthesis of both NKA and SP [40]. SP is released primarily from neuronal cells in peripheral endings of widely dispersed capsaicin-sensitive primary afferent neurons as well as enteric neurons of the submucosal and myenteric plexuses in the gut [41]. SP synthesis and release from primary nerve terminals is increased remarkably in both the CNS and PNS during inflammation, in a phenomenon known as ‘neurogenic inflammation’ [41,42]. SP has been shown to be a major mediator of neurogenic inflammation in a range of tissues including skin, cardiovascular tissue, and cephalic structures, as well as in the respiratory and gastrointestinal tract [10].
The biological actions of SP are mediated primarily through the NK₁R, though at high concentrations SP can also activate NK₂ and NK₃ receptors in a number of tissues [42]. Neurokinin receptors are G-protein coupled receptors (GPCRs), and activation of NK₁R leads to phospholipase activation, inositol-1,4,5-triphosphate (IP₃) turnover and increased levels of intracellular calcium [42]. In neurogenic inflammation, the binding of SP to NK₁Rs leads to activation of a variety of immune and non-immune cells [41]. Activation of NK₁R on endothelial cells of blood vessels results in increased vascular permeability, vasodilation and plasma extravasation of neutrophils and monocytes [41]. Macrophage NK₁R activation results in release of inflammatory cytokines and additional SP, which causes release of histamine and bradykinin from mast cells and contributes to vascular permeability and oedema [41].

**Substance P in Acute Pancreatitis**

The first indication of the role of SP in AP was from a study by Bhatia et al. [43] that induced AP in mice using supramaximally-stimulating doses of caerulein, and found a time-dependent increase in pancreatic SP levels [43]. This culminated in a 24-fold increase in SP levels over control after 12 hours and was correlated with an increase in NK₁R density in the pancreas. Genetic deletion of NK₁Rs (NK₁R<sup>−/−</sup> mice) markedly reduced the extent of pancreatic injury as well as lung injury in response to caerulein compared to the wild type controls. This study also showed no effect of either NK₁R or PPT-A gene deletion on acinar amylase secretion, indicating that acinar cell responsiveness to caerulein was not altered and that SP mediates more downstream inflammatory effects [43]. This indicates SP, through the NK₁R, plays a significant role in the pathophysiology of AP and ensuing lung injury. The use of the specific NK₁R inhibitor CP-96345 also reduced the extent of caerulein-induced AP and lung injury in mice [44].
Neutral Endopeptidase (NEP) is an enzyme that hydrolyses tachykinins, including SP thereby terminating their effects. NEP knock out mice (NEP\(^{-/-}\)) were found to have more severe pancreatitis symptoms (increased serum amylase, neutrophil sequestration and acinar cell necrosis) and a greater degree of lung injury after 4 and 8 hours of caerulein administration compared to wild type [45]. However after 12 hours the level of pancreatic injury was similar between NEP\(^{-/-}\) and NEP\(^{+/+}\) mice, indicating NEP only plays a role in the early stages of AP.

Several in vitro studies have probed the mechanisms by which SP contributes to AP. Hyperstimulation with caerulein increased SP levels, as well as those of its receptor, NK\(_1\)R, in isolated pancreatic acini [36]. This inflammatory upregulation of SP is mediated by the kinases extracellular signal-regulated kinase (ERK1/2), and c-Jun N-terminal kinase (JNK) as well as the transcription factors NF\(_{κB}\) and activator protein-1 (AP-1) [46]. SP caused a dose-dependent upregulation of the pro-inflammatory chemokines MCP-1, MIP-1\(α\) and MIP-2 in pancreatic acinar cells via an NF\(_{κB}\)-dependent pathway [47]. Further studies found that SP-induced NF\(_{κB}\) activation of these inflammatory chemokines is mediated through several distinct pathways all involving kinase activation. The first pathway involves the activation of the Ca\(^{2+}\)-independent kinase protein kinase-\(δ\) (PKC-\(δ\)) (Fig. 2). SP phosphorylation of PKC-\(δ\) resulted in increased activation of MAPKKK, MEKK1, and MAPK (ERK and JNK), leading to transcription factor NF\(_{κB}\) and AP-1 activation and chemokine synthesis (Fig. 2) [48]. SP also activates phospholipase C (PLC) resulting in increased intracellular Ca\(^{2+}\) levels and PKC-\(α\) phosphorylation, also culminating in ERK, JNK, NF\(_{κB}\) and AP-1 activation (Fig. 2) [49]. Src family kinase (SFK) phosphorylation (Tyr416) is also increased in response to SP-NK\(_1\)R interaction as well as ERK and JNK and activation of the STAT3, NF-κB and AP-1 transcription factors (Fig. 2) [49].
**Figure 2: Proposed pathways of Substance P-induced chemokine synthesis.**

**H₂S and SP interplay in AP**

Both H₂S and SP have been shown to mediate inflammation in AP independently, but there is increasing evidence that some of the inflammatory effects described above may be due to direct H₂S and SP interaction. Treatment of pancreatic acinar cells with the H₂S donor NaHS caused a significant increase in SP levels along with upregulation of PPT-A and NK₁R expression [36]. Furthermore, administration of the CSE inhibitor PAG to caerulein-treated acinar cells, decreased the levels of SP, PPT-A and NK₁R expression compared to the control acinar cells. This was also seen in the *in vivo* model with PAG administration significantly
attenuating the upregulation of SP and NK₁R expression in the plasma, pancreas and lung tissue in response to caerulein administration [50]. This indicates that the elevated concentration of H₂S in AP stimulates expression of the PPT-A and NK₁R genes in the pancreas and lungs leading to increased SP production and activity in these tissues. The inflammation and pancreatic injury attributed to the action of H₂S may be partially, or completely, attributed to SP mediated mechanisms, though further elucidation of the mechanism(s) of this interplay is required to fully understand the relationship between SP and H₂S in AP.

Summary

During AP the digestive enzymes produced by the pancreas are activated and cause autodigestion of the organ. The subsequent inflammatory response causes further damage to the pancreas and can develop into a systemic inflammatory condition. H₂S and SP have recently been discovered to be integral signalling molecules in the inflammatory response of AP. Inhibition of either of these pathways has been shown to reduce the severity of the pathology. Interestingly, these pathways appear to be interdependent. More research is needed to completely elucidate the mechanisms by which H₂S and SP contribute to inflammation during AP. Furthermore, research into the development of novel and specific compounds that inhibit the production of H₂S and SP is needed as it may lead to new treatment strategies for AP in the clinic.
References


