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H_2S is a key antisecretory molecule against cholera toxin-induced diarrhoea in mice: Evidence for non-involvement of the AC/cAMP/PKA pathway and AMPK

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ABSTRACT

Hydrogen sulphide (H_2S) is a gasotransmitter that participates in various physiological and pathophysiological processes within the gastrointestinal tract. We studied the effects and possible mechanism of action of H_2S in secretory diarrhoea caused by cholera toxin (CT). The possible mechanisms of action of H_2S were investigated using an intestinal fluid secretion model in isolated intestinal loops on anaesthetized mice treated with CT. NaHS and Lawesson's reagent and L-cysteine showed antisecretory activity through reduction of intestinal fluid secretion and loss of Cl^- induced by CT. Pretreatment with an inhibitor of cystathionine-γ-lyase (CSE), DL-propargylglycine (PAG), reversed the effect of L-cysteine and caused severe intestinal secretion. Co-treatment with PAG and a submaximal dose of CT increased intestinal fluid secretion, thus supporting the role of H_2S in the pathophysiology of cholera. CT increased the expression of CSE and the production of H_2S. Pretreatment with PAG did not reverse the effect of SQ 22536 (an AC inhibitor), bupivacaine (inhibitor of cAMP production), KT-5720 (a PKA inhibitor), and AICAR (an AMPK activator). The treatment with Forskolin does not reverse the effects of the H_2S donors. Co-treatment with either NaHS or Lawesson's reagent and dorsomorphin (an AMPK inhibitor) did not reverse the effect of the H_2S donors. H_2S has antisecretory activity and is an essential molecule for protection against the intestinal secretion induced by CT. Thus, H_2S donor drugs are promising candidates for cholera therapy. However, more studies are needed to elucidate the possible mechanism of action.

1. Introduction

Cholera, a disease characterised by acute secretory diarrhoea, is caused by intestinal infection from the gram-negative bacterium *Vibrio cholerae* serogroups O1 and O139 [1]. This disease causes large epidemics worldwide and is a serious threat to public health, particularly in developing countries [2]. The main virulence factor responsible for the dehydration observed during cholera is cholera toxin (CT), which is secreted by *V. cholerae* into the small intestine [3].

CT causes severe diarrhoea through a direct effect on intestinal epithelial cells. More specifically, CT binds to intestinal enterocytes via interaction of five identical B-subunits with the GM1 ganglioside receptor, which is then internalized through retrograde endocytosis [4]. Within the cell, the A subunit causes constitutive

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Abbreviations

AMPK	AMP-activated protein kinase
AC	Adenylyl cyclase
cAMP	Cyclic adenosine monophosphate
CBS	Cystathionine-β-synthetase
CFTR	Cystic fibrosis transmembrane conductance regulator
CSE	Cystathionine-γ-lyase
CT	Cholera toxin
GM1	ganglioside receptor
GSH	Glutathione reduced
H_2S	Hydrogen sulphide
NaHS	Sodium hydrosulfide
ORS	Oral rehydration solution
PAG	L-propargylglycine
PKA	Protein kinase A

activation of Adenylyl cyclase (AC) by inactivation of the stimulatory G protein Gsα, resulting in elevated levels of intracellular cAMP [5]. The increase in intracellular cAMP results in activation of protein kinase A (PKA) and subsequent Cl^- channel opening [cystic fibrosis transmembrane conductance regulator (CFTR)], causing an excessive secretion of Cl^- accompanied by the osmotic movement of a large quantity of water into the intestinal lumen [6]. The fluid loss is often so rapid and large that patients can die if left untreated [7].

The treatment of cholera involves replacing water and lost electrolytes, and consists of the administration of oral rehydration solution (ORS). Although this solution is effective for hydration and reduces mortality, ORS neither inhibits cholera toxin-mediated excessive secretion nor eliminates the infection from *V. cholera* [8]. Consequently, ORS does not decrease diarrhoea in the short term. Because of these factors, currently there are no pharmacological approaches to treat cholera; treatment could be achieved by investigation of signalling molecules that can inhibit the increase of secretion induced by cholera toxin. Among the molecules that play many important physiological and pathophysiological roles in human health, gaseous mediators such as hydrogen sulphide (H_2S) are of particular interest.

H_2S is a gaseous signalling molecule endogenously generated mainly from L-cysteine through the activity of the enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS), although there are alternative sources (e.g., cysteine aminotransferase and/or 3-mercaptosulfotransferase [9]. Studies have shown that H_2S has important biological effects on the intestinal epithelium, local microcirculation, and inflammatory processes, and promotes changes in gastrointestinal smooth muscle [10–12]. In addition, recent data have shown that H_2S inhibits the AC/cAMP pathway [13,14] and promotes the activation of AMP-activated protein kinase (AMPK) [15], an important kinase that phosphorylates the CFTR channel and inhibits the secretion of chloride ions in intestinal epithelial cells [16]. These effects indicate that H_2S may have beneficial effects on the pathophysiology of cholera, which involves increased activation of AC. Therefore, based on this background information and owing to the absence of reports about the role of H_2S in the secretory diarrhoea induced by cholera toxin, we evaluated the potential antisecretory effect of H_2S in secretory diarrhoea induced by the enterotoxin of *V. cholerae* and the possible mechanisms involved in this effect.

2. Materials and methods

2.1. Chemicals and drugs

All drugs were purchased from Sigma Chemical Company, St. Louis, MO, USA. 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, (acadesine; N1-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide; AICAR), 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (9-THF-Ade; SQ 22536), Forskolin and bupivacaine HCl were dissolved in 0.9% saline and administered directly on the loops at concentrations of 1 mM, 0.01 M, 20 μM and 100 μM, respectively. (9S,10S, 12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-*kl*]pyrrolo[3,4-*i*] [1,6] benzodiazocine-10-carboxylic acid hexyl ester (KT 5720) and 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo [1,5-a]pyrimidine (dorsomorphin; compound C) were dissolved in PBS and administered directly to the loops at 1 μg and 30 μM, respectively. These doses were selected from published studies and the previous work of our research group [17–20]. L-cysteine (H_2S precursor), DL-propargylglycine (PAG; inhibitor of CSE) and NaHS (H_2S donor) were dissolved in 0.9% saline and were administered by gavage [21]. Lawesson's reagent (H_2S donor) was suspended in 1% carboxymethylcellulose and also administered orally. Cholera toxin was dissolved in PBS and administered directly to the loops (1 μg). All other chemicals and reagents were of analytical grade and obtained from standard commercial suppliers.

The pH of the studied solutions are in the range of 4–5. More specifically the pH of NaHS was 4.67, L-cysteíne 5.38 and Lawesson's reagent 4.12, which was adjusted to pH 3. The pH adjust of the Lawesson's reagent solution was necessary in view of the fact that the literature shows that GYY4137, or morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate, a Lawesson's reagent derivative, has a greater release at acidic pH like pH 3.0 and very slowly release under physiological conditions (pH 7.4) [22,23].

2.2. Animals

All in vivo work was subject to internal ethical review and conducted in accordance with Home Office requirements under the Animals Scientific Procedures Act (1986), with the approval of the Local Ethical Committee (No. 079/2015, Ethics Committee in Research of the Federal University of Piauí, Brazil). Swiss mice of both sexes (weight: 25–30 g) were used in this study. They were maintained in cages under laboratory conditions at a temperature of (23 ± 1) °C under a 12 h light/dark cycle with free access to a standard pellet diet and drinking tap water ad libitum. Mice were randomly assigned into groups of six to eight. They were deprived of food for 24 h before the experiments, but still allowed free access to water.

2.3. Cholera toxin-induced fluid secretion in closed intestinal loops

The antisecretory effect of H_2S was measured by assay of the intestinal fluid secretion induced by cholera toxin inoculation, as previously described by Ref. [24], with some modifications. Mice were treated by gavage with L-cysteine (10 or 50 mg kg^{-1}), NaHS (3, 9 or 27 μmol kg^{-1}), Lawesson's reagent (3, 9 or 27 μmol kg^{-1}), or saline (2.5 mL kg^{-1}). Another group received DL-propargylglycine (PAG) 100 mg kg^{-1} by gavage, an inhibitor of CSE, 30 min before administration of L-cysteine (50 mg kg^{-1} by gavage). After 30 min, the mice were intraperitoneally anaesthetized with a combination of xylazine hydrochloride (8 mg kg^{-1}) and ketamine (40 mg kg^{-1}), and a median laparotomy was performed to expose the small intestine. A portion of the jejunum was isolated and closed with double ties to form an intestinal loop measuring approximately

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