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Reduction of hydrogen sulfide synthesis enzymes cystathionine-β-synthase and cystathionine-γ-lyase in the colon of patients with Hirschsprungs disease

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ABSTRACT

Purpose: Hirschsprung associated enterocolitis (HAEC) is the most common cause of morbidity and mortality in Hirschsprung Disease (HSCR). The pathogenesis of HAEC is poorly understood. In recent years, there is increasing evidence that a compromised intestinal barrier function plays a major role in the pathogenesis of HAEC. Hydrogen sulfide, synthesized from L-cysteine by two key enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lysase (CSE) is reported to play a key role in regulating gastrointestinal motility and promoting resolution of inflammation. We designed this study to test the hypothesis that CBS and CSE expression is altered in the colon of patients with HSCR.

Methods: We investigated CBS and CSE protein expression in both the aganglionic and ganglionic regions of HSCR patients ($n = 10$) versus healthy control colon ($n = 10$). Protein distribution was assessed by using immunofluorescence and confocal microscopy. Gene and protein expression was quantified using quantitative real-time polymerase chain reaction (qPCR), Western blot analysis, and densitometry.

Main results: qPCR and Western blot analysis revealed that CBS and CSE are expressed in the normal human colon. CBS and CSE expression was significantly decreased ($p < 0.003$) in the ganglionic and aganglionic bowel in HSCR compared to controls. Confocal microscopy revealed that CBS and CSE expression in smooth muscles, interstitial cells of Cajal, platelet-derived growth factor-alpha receptor-positive cells, enteric neurons and colonic epithelium was markedly decreased in HSCR specimens compared to controls.

Conclusion: We demonstrate for the first time the expression and distribution of CBS/CSE in patients with HSCR. The observed decreased expression of CBS and CSE may affect mucosal integrity and colonic contractility and thus render HSCR patients more susceptible to develop HAEC.

Hirschsprung associated enterocolitis (HAEC) is the most serious complication of Hirschsprung Disease (HSCR) and can occur pre- and postoperatively in up to 40% of patients [1]. Although the clinical course of HAEC with abdominal distension, vomiting, and bloody diarrhea is well acknowledged, its pathogenesis is poorly understood. In recent years, numerous theories have been put forward to understand the underlying basis of HAEC [2–4] including intestinal epithelial barrier dysfunction and altered gastrointestinal motility patterns [5]. It is well recognized that intestinal epithelial barrier dysfunction may allow the passage of macromolecular antigens or harmful substances through the barrier to reach subepithelial regions, coming in contact with immune cells and initiating altered immune responses, which in turn trigger the development of intestinal inflammation [6].

Hydrogen sulfide (H_2S) is an important regulator of gastrointestinal motility and mucosal homeostasis. H_2S is endogenously synthesized from L-Cysteine by the enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). Also, H_2S can be produced by sulfate-reducing bacteria [7]. However, the role of bacterial H_2S still has to be established. The function of H_2S has been implicated in smooth muscle relaxation, increased colonic secretion and the protection of the intestines from ischemia–reperfusion injury [8–11]. Interestingly, suppression of colonic H_2S synthesis in mice with colitis is reported to impair mucosal integrity and alter intestinal barrier function [8]. Since in recent years, damaged intestinal barrier function is being increasingly

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implicated in the pathogenesis of HAEC, we designed this study to test the hypothesis that the H_2S synthesis enzymes CBS/CSE are lacking or reduced in the colonic specimens from patients with HSCR.

1. Material and methods

1.1. Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital (Ref GEN.292/12) and tissue samples were obtained with informed parenteral consent. HSCR specimens from 10 patients (7 male, three female, 3–14 months, median 5.5 months) who underwent pull-through surgery were studied (Table 1). Delphi criteria for HAEC diagnosis were applied [12]. These specimens were divided into aganglionic and ganglionic samples. Ganglionic samples were taken from the most proximal margin of the pull-through specimen while aganglionic samples were taken from the most distal margin of the pull through specimens. Normal control samples included ten specimens from patients who underwent sigmoid colostomy closure following anorectoplasty for imperforate anus (6 male, four female, 8–19 months, median 8.5 months). Tissue specimens were either snap-frozen in liquid nitrogen and stored at −80 °C for protein extraction or embedded in OCT Mounting Compound (VWR International, Leuven, Belgium) for immunofluorescence and stored at −80 °C until use.

1.2. RNA isolation from HSCR specimens

Isolate II RNA Mini Kit (Roche Diagnostics, West Sussex, UK) was used for the extraction method to isolate total RNA from aganglionic and ganglionic HSCR as well as controls (n = 10 for each group) according to the manufacturer's protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Scientific Fisher, Wilmington, DE). The RNA solution was stored at −80 °C until further use.

1.3. cDNA synthesis and quantitative polymerase chain reaction

Reverse transcription of total RNA was carried out at 25 °C for 10 min, at 37 °C for 120 min and 85 °C for 5 min using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instruction. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 25 μl per well. The following gene-specific primers were used: Human CBS (Eurofins) sense primer 5'TGCCAGAGAAGATGAGCTCC and Human CBS antisense primer 5'GGGATTTCGTTCTTCAGCCG as well Human CSE (Eurofins) sense primer 5'TGACATTGAAGGCTGTGCAC and Human CSE antisense primer 5'GACACCAGGCCCATTACAAC. For normalization purposes, real-time RT-PCR was performed for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH sense primer 5'AACGGTTCATGTTGTAATG and GAPDH antisense primer 5′ ACACAGGTTGTGGAGTAACA has been used. After 5 min of initial denaturation at 95 °C, 55 cycles of amplification for each primer were carried out. Each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics, Mannheim, Germany). The relative changes in gene expression levels of CBS/CSE were normalized against the level of GAPDH gene expression in each sample (ΔΔCT-method). Experiments were carried out in triplicate for each sample and primer.

1.4. Protein extraction and Western blot

Specimens of HSCR colon and control colon were homogenized in RIPA buffer (Radio-Immunoprecipitation Assay, Sigma-Aldrich Ltd., Wicklow, Ireland) containing 1% protease inhibitor cocktail (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). Protein concentrations were determined using a Bradford assay (Sigma-Aldrich Ireland Ltd., Wicklow Ireland). A total volume of 40 μl Laemmli Sample Buffer (Sigma-Aldrich, Ireland Ltd., Wicklow, Ireland) containing 10 μg Protein was loaded in the 10% SDS-PAGE gel (NuPAGE Novex Bis-Tris gels, Invitrogen, Carlsbad, USA) for electrophoretic separation. The electrophoresis was performed in MES SDS (2-(N-morpholino) ethane sulfonic acid, sodium dodecyl sulfate) running buffer (Invitrogen, Carlsbad, USA). Proteins were then transferred to 0.45 μm nitrocellulose membrane (Millipore Corporation, Billerica, USA) by Western blotting. Following Western blotting, the membranes were blocked with 3% skimmed milk for 60 min before antibody detection. The primary antibodies; rabbit anti-CBS (Abcam, Cambridge, UK, ab140600, GR103934) dilution 1:1000 and rabbit anti-CSE (Abcam, Cambridge, UK, ab136604, GR188522) were used, and incubation was performed overnight at 4 °C. Following extensive washing (four times in PBS (Phosphate-buffered saline)–0.05% Tween) the membranes were incubated with goat antirabbit IgG HRP-linked secondary Antibody (dilution 1:10,000, Abcam, Cambridge, UK) followed by washing (four times in PBS-0.05% Tween). Detection was performed with the ECL Plus chemiluminescence kit (Thermo, Fisher Scientific, Dublin, Ireland). We used GAPDH (mouse anti-GAPDH, dilution 1:1000, Abcam, Cambridge, UK) as an additional loading control.

Quantification of Western blot has been conducted with ImageJ software.

(U.S. National Institutes of Health, Bethesda, Maryland, USA) by measuring the density of each single band.

1.5. Immunofluorescence staining and confocal microscopy

Frozen blocks of HSCR colon and normal control samples were sectioned transversely at a thickness of 10 μm, mounted on Superfrost®

Table 1
Clinical data of patients with Hirschsprung disease.

	Age at surgery (month)	Gender	Associated anomalies	Extent Aganglionosis	Colostomy prior to pullthrough	Enterocolitis	
						Preoperative	Post pullthrough
1	10	Female	Trisomy 21, ASD, PDA	Long segment	–	yes	–
2	8	Female	Trisomy 21, ASD	Rectosigmoid	yes	–	yes
3	3	Male	ASD	Rectosigmoid	–	–	–
4	14	Female	ASD	Rectosigmoid	–	–	–
5	5	Male	–	Rectosigmoid	–	–	–
6	7	Male	–	Subtotal colonic	Yes	Yes	–
7	4	Male	–	Rectosigmoid	–	–	–
8	5	Male	–	Rectosigmoid	–	–	–
9	5	Male	–	Rectosigmoid	Yes	Yes	–
10	5	Male	PDA Ligation	Rectosigmoid	–	–	–