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ORIGINAL ARTICLE

Altered ryanodine receptor gene expression in Hirschsprung's disease

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Abstract

Aim of the study Ryanodine receptors are the largest of all ion channels, named after their exogenous ligand, ryanodine. The ryanodine receptor calcium release channel is central to cytoplasmic Ca^{2+} signalling in skeletal muscle, the heart, and many other tissues, playing a vital role in muscular contraction. Three ryanodine receptors exist, Ryr1, Ryr2 and Ryr3. The ryanodine receptor, Ryr3, is encoded by the *Ryr3* gene, which has been reported to be highly specific to colonic smooth muscle cells in mice. We designed this study to investigate *Ryr1, Ryr2* and *Ryr3* gene expression in the normal human colon and in Hirschsprung's disease (HSCR).

Methods HSCR tissue specimens ($n=6$) were collected at the time of pull-through surgery, while control samples were obtained at the time of colostomy closure in patients with imperforate anus ($n=6$). qRT-PCR analysis was undertaken to quantify *Ryr1, Ryr2* and *Ryr3* gene expression, and immunolabelling of Ryr1, Ryr2 and Ryr3 proteins was visualised using confocal microscopy.

Main results qRT-PCR analysis revealed a significant downregulation of the *Ryr1* and *Ryr3* genes in both aganglionic and ganglionic HSCR specimens compared to controls ($p<0.05$). Confocal microscopy revealed Ryr1, Ryr2 and Ryr3 protein expressions within the smooth muscle, with a reduction in aganglionic and ganglionic HSCR colon compared to controls.

Conclusions *Ryr1* and *Ryr3* gene expression is significantly downregulated in HSCR colon, suggesting a role for these genes in colonic smooth-muscle motility. *Ryr1* and *Ryr3* downregulations within ganglionic specimens highlight the physiologically abnormal nature of this segment which may explain the occurrence of persistent bowel symptoms in some HSCR patients following a properly performed pull-through operation.

Keywords Hirschsprung's disease · Aganglionosis · Ryanodine receptors · Colonic smooth muscle

Introduction

Normal peristalsis of the gastrointestinal tract is achieved through rhythmic contraction and relaxation of the smooth-muscle layers. This mechanism is dependent on the flow of Ca^{2+} across cell membranes. The release of calcium ions from intracellular membrane-bound stores is a key step in a wide variety of biological functions [1]. The release of calcium is predominantly mediated by two related calcium release channel families: the ryanodine receptors and inositol 1,4,5-triphosphate receptors [1].

Ryanodine receptors (Ryr) are the largest of all ion channels. Ryr receptors are tetramers composed of four homogenous subunits with a molecular weight of 556 kDa, which form together as a cation-selective channel, with a four-leaf clover shape [2]. The Ryr family comprises Ryr1–Ryr3, encoded by the Ryr1–Ryr3 genes. The Ryr Ca^{2+} release channel is central to cytoplasmic Ca^{2+} signalling in skeletal muscle, the heart, and many other tissues, playing a vital role in muscular contraction. One distinct feature of ryanodine receptors, which distinguishes them from other Ca^{2+} channels, is their modification by the plant alkaloid ryanodine, from which their name derives [1]. Lee et al. have previously undertaken a major transcriptome study to identify serum response factor-regulated genes specifically expressed in murine jejunum and colonic smooth-muscle cells (SMCs) [3]. They found that the *Ryr3* gene was one of the most specifically expressed genes in murine colonic SMCs. *Ryr3* encodes a ryanodine receptor that functions to release calcium from intracellular stores and, therefore, plays an essential role in triggering muscular contraction.

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We designed this study to investigate *Ryr1*, *Ryr2* and *Ryr3* gene expression in the normal human colon and in HSCR.

Hirschsprung's disease (HSCR) is a congenital condition, affecting 1:5000 live births, which is characterised by the absence of ganglia in the distal colon. The extent of the aganglionic segment varies between patients from a short segment to total colonic aganglionosis. This condition is present from birth, with patients presenting with failure to pass meconium, constipation, abdominal pain and bilious vomiting. Treatment involves the surgical removal of the affected aganglionic segment.

Materials and methods

Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital, Dublin, Ireland (Ref. GEN/292/12) and tissue samples were obtained with informed parental consent. HSCR specimens from six patients who underwent pull-through surgery were studied. These specimens were divided into aganglionic and ganglionic specimens. We compared the most distal aganglionic segments with the most proximal ganglionic segments. HSCR patients were 6 ± 3 months old. No additional health issues existed in these patients. Colonic control samples included six specimens from patients who underwent colostomy closure following surgical correction of imperforate anus. Control samples were taken from patients who were 11 ± 4 months old. None of the imperforate anus patients had HSCR. 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.

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® Plus slides (VWR International, Leuven, Belgium), and fixed with 10% buffered formalin for 5 min. Sections underwent cell membrane permeabilization with 1% TritonX-100 for 20 min at room temperature. After blocking with 10% normal goat serum (Sigma-Aldrich Ltd, Arklow, Ireland) for 30 min, to avoid non-specific absorption, sections were incubated with primary antibodies; rabbit anti-ryanodine-1 (Abcam, UK), rabbit anti-ryanodine-3 (Abcam, UK), mouse anti-smooth muscle actin (Sigma-Aldrich, Ireland), all used at a dilution of 1:100 in PBS + 0.05% TritonX-100, overnight at 4 °C. Sections were then washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies; goat anti-rabbit Alexa Fluor® 488, dilution 1:200 and goat anti-mouse Alexa Fluor® 594, dilution 1:200, Abcam, UK, for 1 h at room temperature. After washing, sections were counterstained with DAPI antibody, dilution 1:1000 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed, mounted and coverslipped with Fluorescent Mounting Medium (DAKO Ltd, Cambridgeshire, UK). All sections were independently evaluated by two investigators with an LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

qRT-PCR

TRIzol reagent (Invitrogen) was used for the acid guanidinium–thiocyanate–phenol–chloroform extraction method to isolate total RNA from HSCR and control tissues ($n = 6$ 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, USA). The RNA solution was stored at − 20 °C until further use. cDNA synthesis and quantitative polymerase chain reaction–reverse transcription of total RNA were carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing) and at 92 °C for 10 min (reverse transcriptase inactivation) using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instructions. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRTPCR) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 20 µl per well. The following gene-specific primer pairs were used: Human Ryr1 (Eurofins) sense primer 5′ TCA CAT GTA CGT GGG TGT CC and Human Ryr1 (Eurofins) antisense primer 5′ CAA CAG GAT GAC GAT GAC GA, Human Ryr2 (Eurofins) sense primer 5′ GCG AAG ACG AGA TCC AGT TC and Human Ryr2 (Eurofins) antisense primer 5′ CCT GCA CAC ACT TGG TGA TG, Human Ryr3 (Eurofins) antisense primer 5′ CTG GGA CAC ACT TCG AAG CT and Human Ryr3 (Eurofins) sense primer 5′ GCG AGA GTC CCT GAA AAC CA. For normalisation purposes, real-time RT-PCR was performed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH sense primer 5′ ACA TCG CTG AGA CAC CAT GG and GAPDH antisense primer 5′ GAC GGT GCC ATG GAA TTT GC were 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) and the relative changes in gene expression level of interest were normalised against the level of GAPDH gene expression in each sample (DDCT method). Experiments were carried out in duplicate for each sample and primer.

Statistical analyses

A one-way ANOVA was conducted to determine a statistically significant difference between aganglionic, ganglionic and healthy controls ($p < 0.05$). Data are presented as mean ± standard error. Specimens were classified into three groups: aganglionic ($n = 6$), ganglionic ($n = 6$) and normal controls ($n = 6$).

Results

Immunofluorescence staining and confocal microscopy

Immunofluorescence in conjunction with confocal microscopy revealed Ryr1, Ryr2 and Ryr3 protein expressions within the smooth muscle layers, with a reduction in aganglionic and ganglionic HSCR colon compared to controls (Fig. 1). Ryr1 and Ryr3 were found to be co-localised to smooth muscle actin-labelled SMCs.

qRT-PCR

The relative mRNA expression levels of the *Ryr1* and *Ryr3* genes were significantly decreased in both aganglionic and ganglionic HSCR specimens compared to normal controls ($p < 0.05$) (Fig. 2).

Discussion

The complexity of calcium signalling is based on the diversity of Ca^{2+} channels. The release of sarcoplasmic reticulum (SR)-stored Ca^{2+} is principally performed by inositol 1,4,5-triphosphate receptors and Ryr receptors, and is necessary for several muscular functions such as contraction, relaxation, proliferation and differentiation [4]. Ryrs are the largest known ion channels (> 2MDa) and exist as three mammalian isoforms (Ryr 1–Ryr3), all of which are homotetrameric proteins that interact with and are regulated by phosphorylation, redox modifications, and a variety of small proteins and ions [5]. Most Ryr channel modulators interact with the large cytoplasmic domain, whereas the carboxy-terminal portion of the protein forms the ion-conducting pore. Mutations in the *Ryr2* gene are associated with human disorders such as catecholaminergic polymorphic ventricular tachycardia, whereas mutations in the *Ryr1* gene underlie diseases such as central core disease and malignant hyperthermia [5].

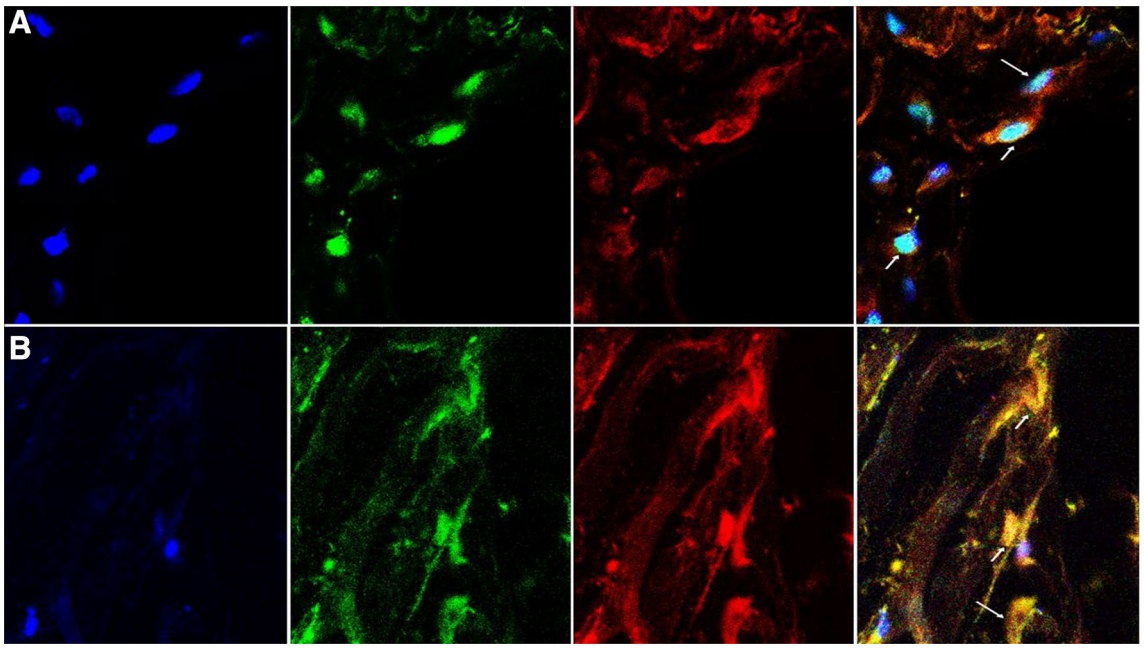

Fig. 1 Immunofluorescent staining of **a** Ryr1-positive cells (green) co-localised with smooth muscle actin-labelled SMCs (red) and **b** Ryr3-positive cells (green) co-localised to smooth muscle actin-labelled SMCs (red). Nuclei were stained with DAPI (blue). Arrows show co-localisation

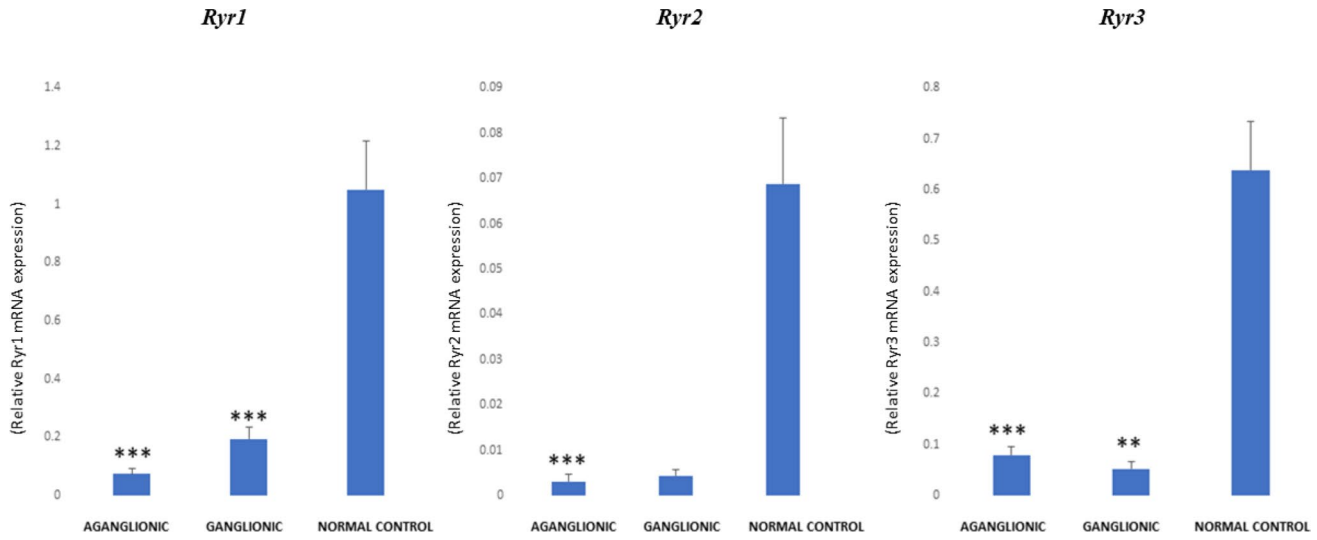

Fig. 2 qRT-PCR revealed significantly decreased relative mRNA expression levels of *Ryr1* and *Ryr3* in both the aganglionic and ganglionic HSCR specimens ($n = 6$) compared to normal control tissue ($n = 6$). Results are presented as mean ± SEM (**$p < 0.01$, ***$p < 0.001$)

Zhang et al. investigated the molecular mechanisms underlying the basal tone of smooth muscle sphincters. They found that the pharmacological regulation of Ryr receptors, among others, significantly changes global cytosolic calcium concentration and the tone [1]. The authors suggested that targeting these types of receptors may lead to new treatments for conditions such as faecal incontinence.

In our current study, we revealed that *Ryr1* and *Ryr3* gene expression is significantly downregulated in both aganglionic and ganglionic HSCR colon, suggesting a role for these genes in colonic smooth muscle motility. Co-localisation of Ryr1 and Ryr3 proteins with ICCs and SMCs of the human colon was evident in both normal controls and HSCR colon. These results suggest that the ganglionic specimen in HSCR patients, despite being normally ganglionated, is abnormal.

A disruption of neural crest cell migration during the early stages of embryonic development is thought to be the main cause of HSCR, with neural crest cells failing to complete their cranio-caudal colonisation of the gastrointestinal tract. In addition to a lack of ganglia, many studies have documented deficiencies in smooth-muscle proteins, extracellular matrix molecules, ion channels and various other important molecules in HSCR colon [6–9]. The goal of surgical treatment for HSCR is to enable the affected child to have regular spontaneous bowel motions without soiling. Advances in the management of HSCR afford most patients a satisfactory outcome following a properly performed pull-through operation. However, a substantial cohort of patients continues to have persistent bowel dysfunction despite adequate resection of the aganglionic bowel segment. The postoperative bowel dysfunction includes enterocolitis, constipation and incontinence [10–12]. Postoperative enterocolitis has been reported in 6–20% of HSCR patients and its incidence is unrelated to the timing of definitive surgery. Constipation and soiling have been reported to occur in 11–35% of patients after pull-through operations [10]. While a proportion of these patients is found to have treatable pathology such as strictures, residual aganglionosis or transition zone, the majority have no identifiable cause for their ongoing bowel dysfunction. Persistent bowel problems in these patients after a properly performed pull-through operation have led to the increasing realisation that within the pulled-through segment of bowel, the presence of normal ganglion cells is not sufficient as an indicator of satisfactory outcome. Our current results add to mounting evidence that the ganglionic segment in HSCR patients is abnormal.

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