RESEARCH PAPER

Endothelin-1 and its receptors on haemorrhoidal tissue: a potential site for therapeutic intervention

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BACKGROUND AND PURPOSE
Haemorrhoids is a common anorectal condition affecting millions worldwide. We have studied the effect of endothelin-1 (ET-1) and the role of endothelin ET_A and ET_B receptors in haemorrhoid tissue.

EXPERIMENTAL APPROACH
Protein expression of ET-1, ET_A and ET_B receptors were compared between haemorrhoids and normal rectal submucosa using Western blot analysis, with the localization of proteins determined by autoradiography and immunohistochemistry. Effects of ET-1 and sarafotoxin 6a on human colonic and rectal arteries and veins was assessed by wire myography and the involvement of receptor subtypes established by selective antagonists.

KEY RESULTS
Dense binding of [^{125}I]-ET-1 to haemorrhoidal sections was reduced by selective receptor antagonists. A higher density of ET_B than ET_A receptors was found in haemorrhoidal, than in control rectal tissue and confirmed by Western blot analysis. ET_A and ET_B receptors were localized to smooth muscle of haemorrhoidal arteries and veins, with ET_B receptors on the endothelium. Human colonic and rectal arteries and veins were similarly sensitive to ET-1 and affected by the ET_A selective antagonist, but sarafotoxin S6a-induced contractions were more pronounced in veins and antagonized by a selective ET_B receptor antagonist.

CONCLUSIONS AND IMPLICATIONS
ET_A and ET_B receptors are present in human haemorrhoids with ET_B receptors predominating. ET_A receptors are activated by ET-1 to mediate a contraction in arteries and veins, but the latter are selectively activated by sarafotoxin S6a – a response that involves ET_B receptors at low concentrations. Selective ET_B agonists may have therapeutic potential to reduce congestion of the haemorrhoidal venous sinusoids.

Abbreviations
CR, concentration ratio; CRC, concentration–response curve; ET-1, endothelin-1; HMA, human mesenteric artery; HMV, human mesenteric vein; NSB, non-specific binding

Tables of Links

TARGETS	
GPCRs	
ET$_A$ receptor	
ET$_B$ receptor	

LIGANDS	
[125I]-ET-1	ET-1, endothelin-1
BQ123	PD-156707
BQ788	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

Haemorrhoids is a common anorectal condition defined as the symptomatic enlargement and distal displacement of the normal anal cushion that affects millions worldwide and represents a major medical and socio-economic problem (Loder *et al.*, 1994; Lohsiriwat, 2012; Jacobs, 2014). The prevalence of haemorrhoids has been reported to be as high as 30–39% in the adult general population, (Loder *et al.*, 1994; Acheson and Scholefield, 2008) with the most common symptom being rectal bleeding upon bowel movement. Pain is a symptom generally associated with advanced internal haemorrhoids, once they become strangulated or exhibit signs of thrombosis, and can occur with external haemorrhoids (Kaidar-Person *et al.*, 2007; Acheson and Scholefield, 2008). Surgical approaches are often used for the worst grades of haemorrhoids, but are generally avoided because of the potential for severe post-operative pain and long-term complications (Kaidar-Person *et al.*, 2007; Acheson and Scholefield, 2008) with pharmacological intervention preferred instead (Acheson and Scholefield, 2008).

A characteristic feature of haemorrhoids is abnormal venodilatation together with destructive changes in the supporting connective tissue within the anal cushion (Loder *et al.*, 1994; Lohsiriwat, 2012). Furthermore, there is evidence of dysregulation of vascular tone and vascular hyperplasia associated with the enlargement and development of haemorrhoidal tissues (Loder *et al.*, 1994; Lohsiriwat, 2012). For example, Aigner and colleagues (Aigner *et al.*, 2006) have reported that blood flow in the terminal branches of the superior rectal artery of patients with haemorrhoids is two to threefold greater than that found in healthy subjects. Moreover, these changes correlated with the calibre of the vessel and the Goligher's grade of internal haemorrhoids. It is well established that the arterial supply and venous drainage of the anal cushions and haemorrhoids are part of the splanchnic (mesenteric) circulation and haemorrhoidal arteries connect to an extensive arteriovenous plexus within the anorectal submucosa without a capillary bed (Aigner *et al.*, 2009). However, microcasts of haemorrhoidal tissue have revealed that the capacitance side of the arteriovenous plexus accounts for practically the whole volume of this vascular bed (Aigner *et al.*, 2009).

The existing creams used to treat the symptoms of haemorrhoids generally consist of an anti-inflammatory glucocorticoid, an 'astringent' claimed to influence vascular permeability, a local anaesthetic and, in some instances, a vasoconstrictor to limit blood flow ((Acheson and Scholefield, 2008; Lohsiriwat, 2012). In the case of Preparation H®, for example, phenylephrine is incorporated as a vasoconstrictor agent and is generally known to exert a preferential effect on the arterial side of the circulation (Lohsiriwat *et al.*, 2011).

As far as we are aware, there is little information regarding the distribution and function of receptors on the arterial and venous side of anal cushions and their role in controlling overall tissue volume. Part of the problem may relate to the difficulty in studying small calibre blood vessels in haemorrhoidal tissue *in vitro*. In a recent study, however, we demonstrated that vasoconstrictor endothelin ET$_A$ and ET$_B$ receptors are present on sheep rectal arteries and veins (vessels that supply and drain anal cushions) with the latter selectively activated by the snake venom peptide sarafotoxin S6a (Lohsiriwat *et al.*, 2011). Endothelin-1 (ET-1) is the most potent vasoconstrictor currently known acting via the two distinct ET$_A$ and ET$_B$ receptors (Barton and Yanagisawa, 2008). Although the endothelium is a major source of ET-1, other cells are capable of generating the peptide such as macrophages, vascular smooth muscle and myocardial cells (Masaki, 1993; Luscher and Barton, 2000; Fagan *et al.*, 2001). The vasoconstrictor action of ET-1 involves both ET$_A$ and ET$_B$ receptors located on vascular smooth muscle and ET-1-mediated vasodilatation via endothelial ET$_B$ receptors. While no selective ET$_A$ receptor agonists are currently available, sarafotoxin S6a and S6c are peptide-based toxins from *Atractaspis engaddensis* (McMahon *et al.*, 1991; Minkes *et al.*, 1992; Alexander *et al.*, 2015) that are thought to preferentially activate ET$_B$ receptors

In this study we have (i) determined the density and distribution of ET-1, ET$_A$ and ET$_B$ receptor binding sites in haemorrhoidal tissue using *in vitro* receptor autoradiography and immunohistochemistry; (ii) compared the level of protein expression of ET$_A$ and ET$_B$ receptors in haemorrhoids and normal rectal submucosa using Western blot analysis; and (iv) using wire myography, pharmacologically examined the receptors activated by ET-1 and sarafotoxin S6a in segments of human colonic and rectal arteries and veins.

Methods

Tissue preparation

After obtaining approval from the Nottingham Research Ethics Committee (reference number: 05/Q2403/171) and patients' informed written consent, human haemorrhoid tissue was obtained from patients with grade III (Figure 1) or grade IV haemorrhoids undergoing haemorrhoidectomy at the Division of Gastrointestinal Surgery, Queen's Medical Centre, University of Nottingham, UK, between March and December 2008. For comparison, rectal mucosa and submucosa were obtained from rectal cancer patients who underwent anterior resection or abdominoperineal resection without previous pelvic radiotherapy. The required anorectal tissues were dissected from the whole surgical specimen immediately after removal from the patient. Samples were frozen on CO_2 pellets and stored at $-80°C$ until use. For autoradiography and immunohistochemistry, frozen 6 μm sections were prepared at $-25°C$ and mounted on polylysine-coated slides (VWR International bvba, Germany), and stored at $-80°C$.

Autoradiography of ET-1 and its receptors

The autoradiography protocol used for ET-1 and ET_A/ET_B receptor binding was that previously described (Dashwood *et al.,* 1993; Ali *et al.,* 2000; Tsui *et al.,* 2004; Hoosein *et al.,* 2007). Frozen sections of three haemorrhoid specimens were incubated in buffer containing 150 pM [^{125}I]-ET-1 (specific activity 74 TBq·mmol^{-1}, GE Healthcare, UK) to identify radioligand binding sites with non-specific binding (NSB) being determined by incubating adjacent sections in the presence of 1 μM unlabelled ET-1 (Bachem Fine Chemicals, Switzerland). ET_A and ET_B receptor binding sites were studied in corresponding sections that were incubated in the presence of the selective ET_A receptor antagonist, BQ123, or the selective ET_B receptor antagonist, BQ788 (both 1 μM; Tocris, UK). For quantitative assessment of binding, ^{125}I

Figure 1

Under light microscopy, haematoxylin and eosin-stained sagittal sections of haemorrhoids showed marked dilatation of vascular spaces, particularly venous channels, together with a loose and fragmented connective tissue stroma. Scale bar = 5 mm.

scales were prepared using serial dilutions of radioligand that were spotted onto filter paper and attached to microscope slides that were co-exposed to radiation-sensitive film along with the slide-mounted sections.

'Low-resolution' autoradiography. After incubation, slide-mounted sections were placed in X-ray cassettes, exposed to Hyperfilm™ MP (GE Healthcare, UK) for 14 days and then processed according to the manufacturer's instructions. Densitometric analysis of autoradiographs was performed using a Biospectrum AC Imaging System (Ultraviolet Products, Cambridge, UK) and presented as radioactivity mm^{-2} tissue (dpm $\times 10^3$ mm^{-2}) as previously described (Ali *et al.,* 2000; Tsui *et al.,* 2004; Hoosein *et al.,* 2007). Specific binding was determined by subtracting NSB from total binding.

'High-resolution' autoradiography. After exposure to film, selected slide-mounted sections were dipped in molten K2 emulsion (Ilford, UK) for microscopic localization of radioligand binding. Emulsion was processed according to the manufacturer's instructions and sections stained with haematoxylin and eosin for histological examination as previously described (Ali *et al.,* 2000; Hoosein *et al.,* 2007). Autoradiographs were viewed under dark-field illumination and stained tissue under bright-field illumination.

Immunohistochemistry

Standard immunohistochemistry was performed on slide-mounted tissue sections (from 14 haemorrhoids and 6 rectal samples) using the Avidin-Biotin Complex Alkaline Phosphatase method (ABC-Alkaline Phosphase kit, Vector Laboratories Inc, USA) following the manufacturer's instructions. Vector® red substrate was used as the chromogen, and sections were counterstained with Haematoxylin. The following antibodies were used: mouse anti-human monoclonal antibody against endothelial cells (CD31, clone JC70A, 1:100, DakoCytomation, Denmark); rabbit anti-human monoclonal antibodies against ET_A and ET_B receptor (both 1:200, Alomone Labs, Israel), mouse anti-human monoclonal antibody against smooth muscle actin (clone 1A4, 1:200, DakoCytomation, Denmark).

Western blotting

Standard Western blot analysis was used. De-epithelialised haemorrhoid specimens ($n = 13$) and rectal submucosal tissue ($n = 6$), where the arteriovenous plexus is located, was used to prepare protein samples. Sample mixtures were loaded onto the SDS-PAGE and human microvascular endothelial cell solution was used as a positive control. Proteins in the gel were transferred to a nitrocellulose membrane (Hybond ECL, Amersham, UK), and 5% powdered milk in PBS-Tween solution was used to block non-specific protein binding. Membranes were incubated with primary antibody at a suitable dilution and, as a loading control, the membrane was also subjected to a mouse monoclonal IgG to GAPDH (1:5000, Santa Cruz Biotechnology Inc, USA). Membranes were then processed with ECL™ Western Blot detection reagent and film was developed using Hyperfilm ECL (both
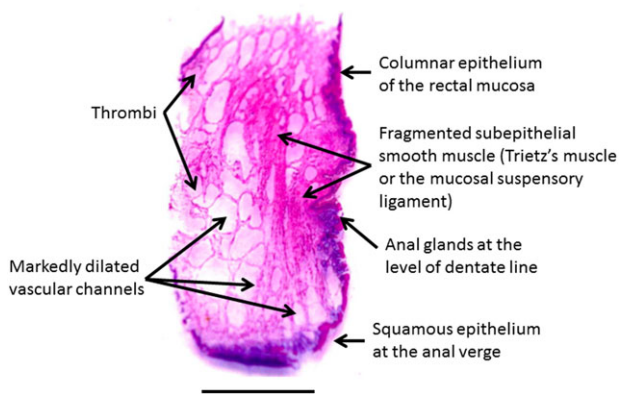
GE Healthcare, UK), and bands were analysed and quantified densitometrically using Biospectrum AC Imaging System (Ultraviolet Products, Cambridge, UK). Protein was standardized to the level of GAPDH and reported as a percentage of GAPDH protein level. Rabbit anti-human monoclonal antibody against ET_A and ET_B receptors (both 1:200, Alomone Labs, Israel) were used.

Wire myography

After obtaining approval from the local research ethics committee and written patients' consent, human mesenteric (colonic or rectal) vessels were collected from patients undergoing colectomy and/or proctectomy at the Division of Gastrointestinal Surgery, Queen's Medical Centre, University of Nottingham, UK, between August 2009 and March 2010. Patients with a history of previous abdominal/pelvic radiotherapy, and patients with endocrine tumours or concomitant intra-abdominal infection were excluded from this study. The mesentery containing the required segment of blood vessels was dissected from the whole surgical specimen immediately after removal and stored in pre-oxygenated Krebs–Henseleit buffer solution maintained at 4°C until use. Human mesenteric (colonic/rectal) vessels were divided into 5 mm ring segments that were suspended between two 200 μm wire supports in an organ bath containing 20 mL Krebs–Henseleit buffer solution. After a 20 min equilibration period, initial resting tension of 8 g wt (arteries) or 1 g wt (veins) was gradually applied to the ring segments and left to relax for 30–45 min. The final resting tension varied between 1.5–3 g wt for human mesenteric artery (HMA), and between 0.1 and 0.35 g wt for human mesenteric vein (HMV). Once the vascular segments were fully equilibrated, they were tested for contractile activity with 60 mM KCl on three occasions until consistent responses were obtained. After a further period of 60 min, a single agonist concentration–response curve (CRC) was constructed in either the absence or presence of 0.1 μM PD 156707 (selective for ET_A receptors; Maguire *et al.,* 1997), 0.1 μM BQ788 (selective for ET_B receptors; Russell and Davenport, 1996), or a combination of the two antagonists. The maximum contraction produced by the third exposure to 60 mM KCl was used as a reference contraction for all subsequent responses. ET-1 and sarafotoxin S6a were the endothelin receptor agonists used and in some experiments responses were compared to those elicited by noradrenaline.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). The level of expression of each protein was reported as a percentage of the GAPDH protein level. Results are expressed as mean ± SEM of *n* observations, where *n* is the number of tissues from different patients. In the wire myograph studies, a curve-fitting programme (KaleidaGraph, Synergy Software, Reading, PA) was used to construct a CRC that had the best fit to a series of data points, and to determine maximum responses (E_{max}) and agonist potency (pEC_{50}: the negative logarithm of the
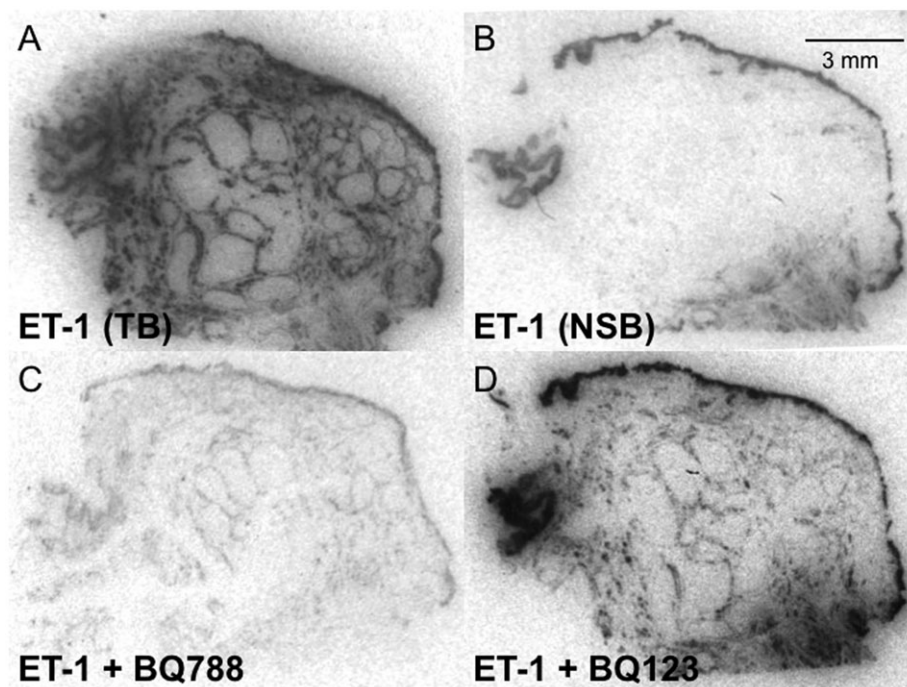
Figure 2
Low-resolution autoradiographs of [^{125}I]-ET-1 binding in haemorrhoid sections; (A) total [^{125}I]-ET-1 binding (TB), (B) NSB in the presence of 1 μM unlabelled ET-1, (C) in the presence of 1 μM selective ET_B receptor antagonist BQ788 and (D) in the presence of 1 μM selective ET_A receptor antagonist BQ123: identifying ET_A and ET_B receptor binding sites respectively.
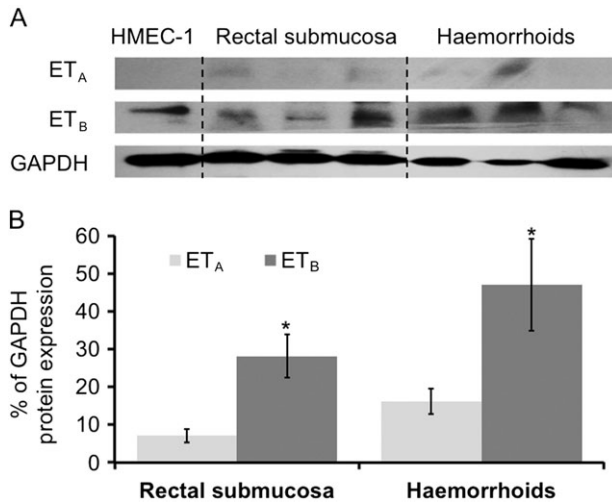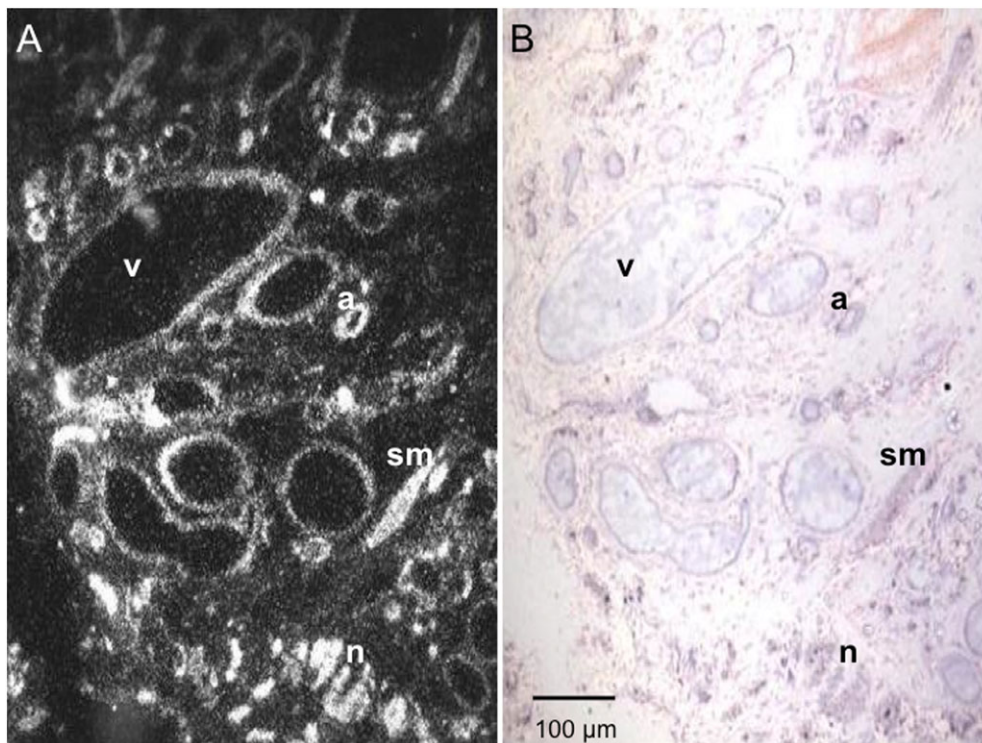
Figure 3

(A) Representative Western blots of ET_A and ET_B receptors from three de-epithelialised haemorrhoids, three submucosal tissues of the distal rectum and human microvascular endothelial cell (HMEC-1). GAPDH was used for standardization of protein expression of each sample. (B) Histograms of protein expression of ET_A and ET_B receptors in haemorrhoids and rectal submucosa (expressed as a percentage of GAPDH expression). Histograms represent mean ± SEM of 13 de-epithelialised haemorrhoid tissues and 6 normal rectal submucosal tissues. *$P < 0.05$, significantly different from values for ET_A receptors.

concentration required to produce 50% of the maximum response). The E_{max} values are expressed as a percentage of the third 60 mM KCl response. Results are expressed as mean ± SEM of *n* observations, where *n* is the number of studies in tissue from different patients. The logarithm of dissociation constant of the antagonist (pK_B) was estimated based on the agonist concentration ratio (CR) in the presence and absence of the antagonist using the equation $pK_B = \log$ [Antagonist] − log (CR-1).

All data were prepared and compiled using the SPSS® software (version 15.0 for Windows, Illinois, USA). The Kolmogorov–Smirnov test was used to test for the pattern of data distribution. Unpaired or paired Student's *t*-test was used to compare data between two groups when the data were in a normal distribution pattern. The Mann–Whitney *U*-test or Wilcoxon Signed Rank test was used to compare data between two groups when the data were in a non-normal distribution. If there were more than two groups being analysed, the ANOVA with an appropriate *post hoc* test would be used. A *P*-value <0.05 was considered statistically significant.

Materials

The drugs used were: PD 156707 (sodium 2-benzo(1,3ioxol-5-yl-4-(4-methoxy-pheny l)-4-oxo-3-(3,4,5-trimethoxyben-zyl)-but-2-enoate), BQ123 (D-tryptamine-D-aspartic acid-L-proline-D-valine-L-leucine), BQ788 (N-cis-2,6-dimethylpi-peridinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyl-tryptophanyl-D-norleucine), ET-1, sarafotoxin S6a (Tocris Bioscience, UK) and noradrenaline bitartrate
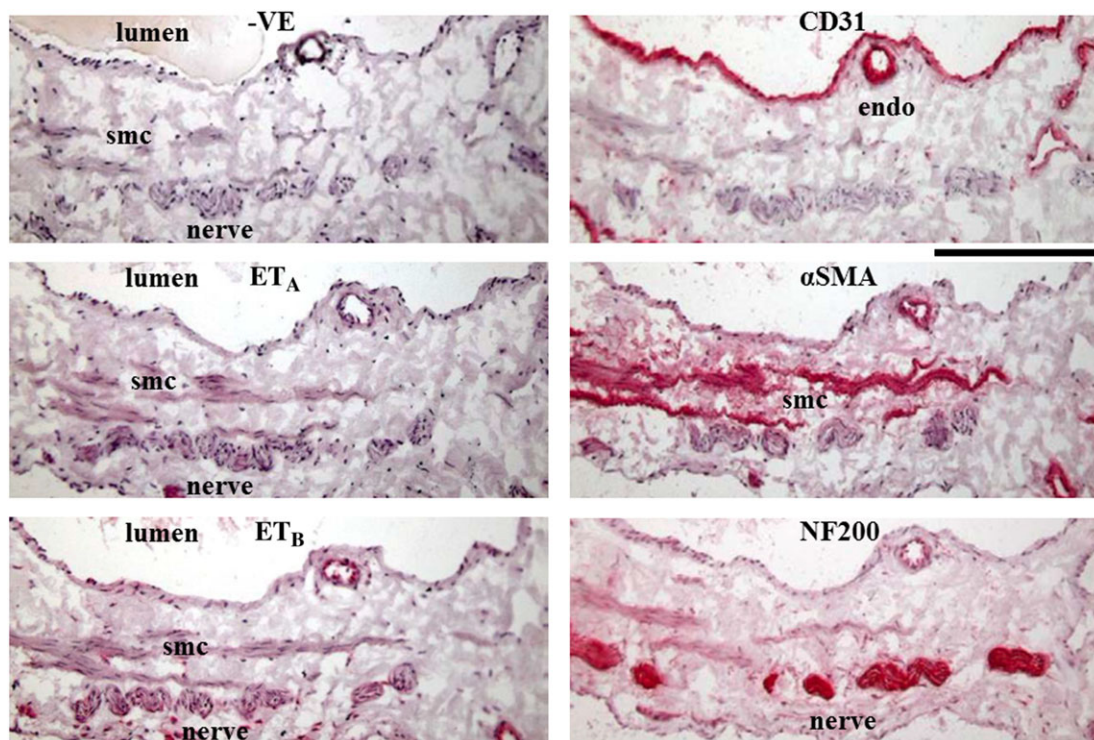

Figure 4

High-resolution autoradiograph of [125I]-ET-1 binding in a haemorrhoid section. (A) Under dark-field illumination [125I]-ET-1 binding is evident as white grains on a black background. [125I]-ET-1 binding is associated with blood vessels (a, artery; v, vein), smooth muscle (sm) and nerve (n), shown on the histology of the haematoxylin and eosin-stained underlying tissue (B).

(Sigma-Aldrich, UK). All drugs used were of analytical grade. PD156707 and BQ788 were dissolved in DMSO (Sigma-Aldrich, UK). ET-1 and sarafotoxin S6a were prepared in distilled water. Noradrenaline was dissolved in aqueous solution containing 20 μM EDTA. The drug concentrations reported are the final concentrations in the organ bath solution.

Results

Patient demographics

The mean age (range) of patients with haemorrhoids and those with rectal cancer was 59 (46–76), and 69 (57–79) respectively. Male patients accounted for 57% of the haemorrhoid group and 67% of the rectal cancer group. There

Figure 5

Positive immunoreactivity of ET_A receptors (middle left) and ET_B receptors (lower left), endothelium (CD31, upper right), smooth muscle actin (middle right) and nerves (NF200, lower right) in tissue sections from haemorrhoids: Positive immunostaining is red. The negative control panel (Top left) shows no evidence immunostaining. The horizontal scale bar represents 100 μm.
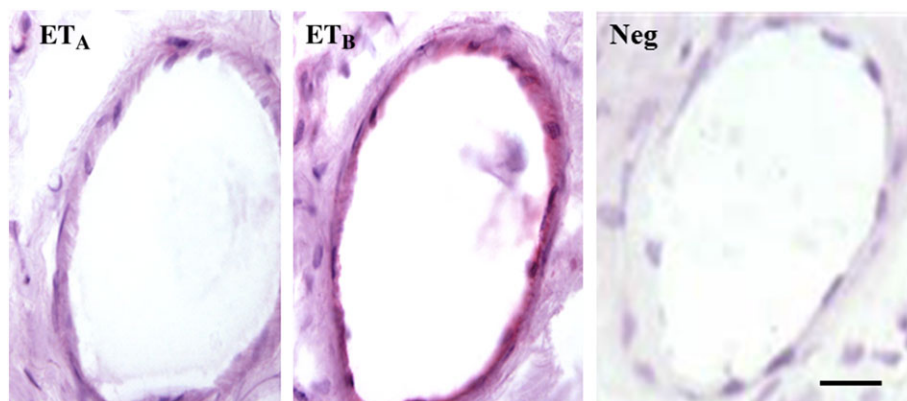
Figure 6

Positive immunoreactivity of ET_A receptors (left) and ET_B receptors (middle) in venous sinusoids from haemorrhoids at high magnification: positive immunostaining is red. There is stronger vascular smooth muscle staining for ET_B than for ET_A receptors. The control slide (Neg, right) showed no evidence of positive staining. The horizontal scale bar represents 25 μm.

was no significant difference in age and gender between the two groups. Of 14 haemorrhoid specimens, 12 (86%) were grade III and two (14%) were grade IV haemorrhoids.

Endothelin receptor binding sites and Western blots

There was dense [125I]-ET-1 binding to various areas of haemorrhoidal specimens, including both vascular channels and interstitial tissue (Figure 2A). NSB was low and accounted for 10–15% of total binding with a higher degree of binding mainly located in the mucosa and the area of vascular thrombosis which was evident in the right lower quadrant of Figure 2B. The density of specific [125I]-ET-1 binding in haemorrhoids, excluding the mucosal area, was 18.2 ± 0.6 dpm $\times 10^3$ mm^{-2} ($n = 3$). In the presence of the selective ET$_B$ receptor antagonist BQ788 and the selective ET$_A$ receptor antagonist BQ123, [125I]-ET-1 binding was reduced but remained detectable. [125I]-ET-1 binding was stronger in the presence of 1 μM BQ123 than in the presence of 1 μM BQ788, indicating binding to haemorrhoids was predominantly to ET$_B$ sites. Although the density of non-mucosal ET$_B$ receptor binding (Figure 2D) was higher than that of ET$_A$ receptor binding (Figure 2C) (12.7 ± 3.0 vs. 4.4 ± 2.0 dpm $\times 10^3$ mm^{-2}), this did not attain statistical significance ($P > 0.05$, $n = 3$).

Protein expression of ET$_A$ and ET$_B$ receptors in de-epithelialised haemorrhoid specimens ($n = 13$) and submucosal tissue of the distal rectum ($n = 6$) was confirmed by Western blot analysis (Figure 3A). In general, haemorrhoids had a non-significant higher protein expression of both ET$_A$ and ET$_B$ receptors compared to the underlying rectal tissue (Figure 3B). Moreover, in haemorrhoidal tissue, the protein level of ET$_B$ receptors was significantly threefold higher than that of ET$_A$ receptors ($P < 0.05$). Rectal submucosa also had about four times higher protein expression of ET$_B$ receptors than ET$_A$ receptors ($P < 0.05$)

Immunohistochemistry revealed the wall of dilated veins that contained barely any vascular smooth muscle, while the endothelium was relatively well preserved. Nerve fibres, subepithelial smooth muscle (so called Trietz's muscle or the mucosal suspensory ligament) and other fibro-elastic tissues were located between dilated vascular spaces (not shown).

With high-resolution autoradiography, the degree of NSB for [125I]-ET-1 was low and similar to that in the low-resolution study: about 10–20% of total binding. [125I]-ET-1 binding was associated with vascular and non-vascular structures (nerve fibres, myofibroblasts and epithelium) in haemorrhoid specimens (Figure 4A). Regarding the vascular structures, [125I]-ET-1 binding sites were evident on vascular smooth muscle, particularly the tunica media of both arteries and veins (Figure 4B).
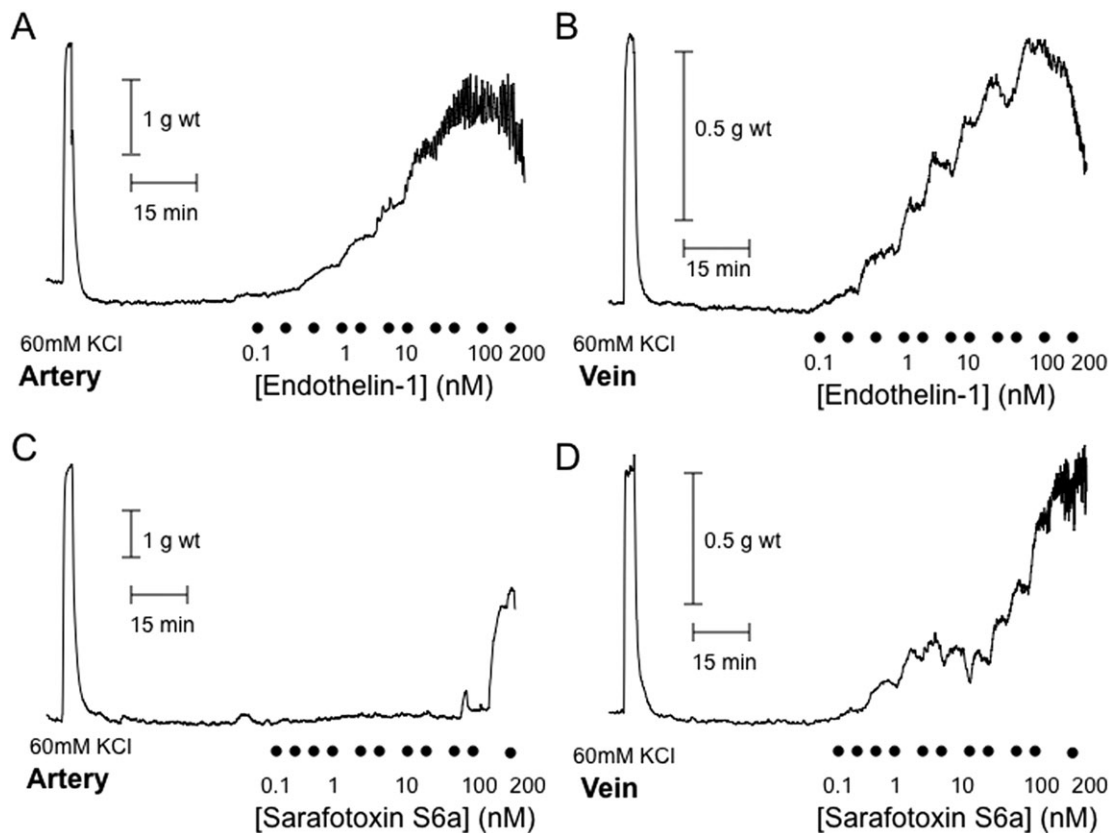
Figure 7
Representative traces of the contractile response to ET-1 and sarafotoxin S6a in the human isolated mesenteric artery (A,C) and vein (B,D). Increments of two and fivefold agonist concentration were conducted but only log increments (10-fold) are labelled.

Immunhistochemical studies revealed the presence of ET_A (Figure 5 middle left) and ET_B (Figure 5 lower left) receptors in haemorrhiodal tissues. Both receptors are associated with the smooth muscle actin (Figure 5 middle right) and located between venous sinusoids, while ET_B receptors were also localized to the endothelium, as confirmed by the endothelial marker, CD31 (top right). Figure 5 (lower right) also shows the presence of nerves, as determined by NF200 staining. Figure 6 shows greater immunohistochemical staining for ET_B (right) and ET_A (left) receptors in venous sinusoids at high magnification. ET_B receptor immunoreactivity was also observed in all rectal tissues (6/6; not shown).

Wire myography

Exposure to 60 mM KCl caused a contraction of both HMA and HMV. The mean contraction to 60 mM KCl in the HMA was 3.78 ± 0.21 g wt ($n = 29$), and that in the HMV was 1.10 ± 0.08 g wt ($n = 39$). In preliminarily experiments, we tested the viability of the vascular endothelium in three HMA after storage for 8–10 h, by exposing them to the endothelium-dependent vasodilators acetylcholine (1 μM) and bradykinin (100 nM) after contraction with ET-1 (approximately 60% of response to 60 mM KCl) and found that the maximum relaxations were greater than 40 and 80% of constrictor tone respectively.

Noradrenaline (data not shown) and ET-1 (Figure 7A,B) caused sustained, concentration-dependent contractions in both blood vessels, with similar maximum contractions, relative to the responses to 60 mM KCl (80 to 130%). However, ET-1 was approximately 100-fold more potent than noradrenaline in the HMA (pEC_{50} 8.50 ± 0.12, $n = 5$ compared to pEC_{50} 6.21 ± 0.08, $n = 5$) and HMV (pEC_{50} 8.40 ± 0.17, $n = 5$ compared to pEC_{50} 6.18 ± 0.21, $n = 5$). As shown in Figure 7C,D, sarafotoxin S6a also elicited concentration-dependent contractions in the HMA and HMV, but was markedly less potent and efficacious than ET-1 in the HMA with only concentrations greater than 0.1 μM eliciting a response (Figure 8A). As shown in Figure 7D, in some preparations, a biphasic CRC to sarafotoxin S6a could be observed, with contractions equivalent to 20% of maximum response elicited with concentrations as low as 1 nM. Although the maximum response to sarafotoxin S6a was comparable to that of ET-1 in HMV (Figure 8B), the potency of sarafotoxin S6a was approximately 1/5th of that of ET-1.

Contractions of the HMA and HMV to ET-1 were displaced in a parallel manner by the selective ET_A receptor antagonist 0.1 μM PD 156707 (Table 1), with an estimated pK_B of 7.89 ± 0.26 ($n = 4$) and 7.37 ± 0.27 ($n = 6$) respectively. The presence of the selective ET_B receptor antagonist BQ788 (0.1 μM) failed to affect responses to ET-1 in the HMA and HMV, either in the presence or absence of 0.1 μM PD 156707 (Table 1). In contrast, 0.1 μM BQ788 caused a non-parallel rightward shift of the CRC to sarafotoxin S6a in the HMV (Figure 8C), with the lower part of the CRC more affected by the antagonist. The pEC_{50} value of sarafotoxin S6a in the absence of BQ788 was 7.68 ± 0.08 ($n = 6$) whereas that in the presence of 0.1 μM BQ788 was 7.20 ± 0.07 ($n = 6$). The estimated pK_B for BQ788 sarafotoxin S6a was 7.30 ± 0.07 ($n = 6$).
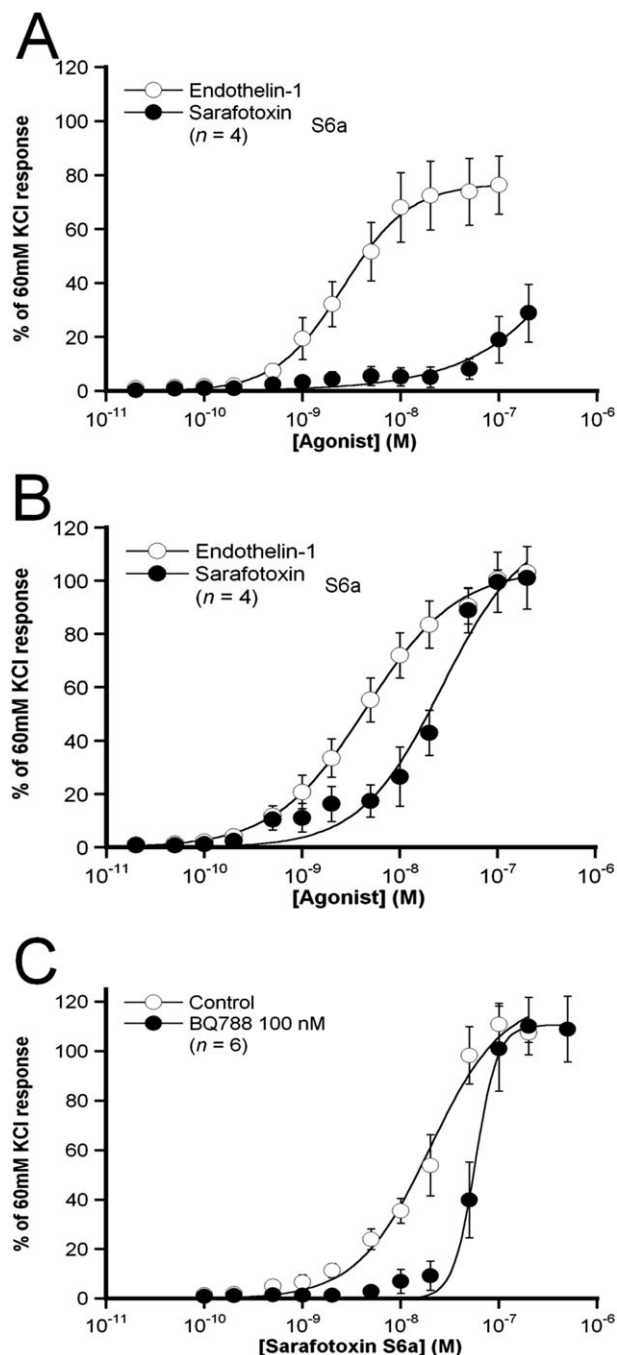
Figure 8

CRC of ET-1 and sarafotoxin S6a in the human isolated mesenteric artery (A) and vein (B). Responses have been expressed as a percentage of the contraction to 60 mM KCl. (C) The effect of 100 nM BQ788 on contractions induced by sarafotoxin S6a in the human isolated mesenteric vein. All points represent the mean of five to six observations, and the vertical lines indicate the SEM.

Discussion and conclusions

Endothelin-1 and endothelin receptors

We have identified dense binding sites for $[^{125}I]$-ET-1 in both vascular and non-vascular haemorrhoidal tissue. Based on

Table 1

The effect of ET receptor antagonists on the mean potency (pD_2) and maximum response to ET-1 in human mesenteric (colonic/rectal) blood vessels

	Human Mesenteric Artery ($n = 4$)		Human Mesenteric Vein ($n = 5$)	
	pD_2	% of max[a]	pD_2	% of max[a]
Control	8.46 ± 0.13	113.0 ± 25.5	8.66 ± 0.21	129.0 ± 13.4
0.1 μM PD 156707	7.41 ± 0.12*	102.7 ± 16.0	8.05 ± 0.17*	130.1 ± 17.0
0.1 μM BQ788	8.53 ± 0.12	122.4 ± 13.1	8.81 ± 0.22	131.7 ± 13.0
0.1 μM PD 156707 and 0.1 μM BQ788	7.50 ± 0.12*	97.8 ± 17.2	8.24 ± 0.18	119.1 ± 15.3

[a]maximum response expressed as a percentage of the response to 60 mM KCl

*$P < 0.05$, significantly different from control; ANOVA with *post hoc* Dunnett's test

low-resolution autoradiography and the use of selective antagonists for endothelin receptors, the density of ET_B receptor binding was generally higher than that of ET_A receptor binding, but was variable and the near threefold mean difference did not reach statistical significance. This trend was largely confirmed by Western blotting studies on haemorrhoidal tissue that also revealed the level of ET_B receptor protein significantly threefold higher than that of the ET_A receptor protein. These novel findings are consistent with the known distribution of endothelin receptors on large blood vessels in the rest of the circulation (Masaki, 1993; Luscher and Barton, 2000).

Positive immunostaining of ET_B receptors was uniformly observed in blood vessels of all haemorrhoidal and rectal tissues. Apart from an association with smooth muscle actin-staining, ET_B receptor immunoreactivity was also localized to the endothelium. These studies also revealed observations consistent with the report indicating that the arteriovenous plexus comprise large venous sinusoids located alongside small supply arteries (Aigner et al., 2009). High-resolution autoradiographic studies provided further insight into the distribution of [^{125}I]-ET-1 binding sites in haemorrhoids, which is largely similar to that described by Egidy and colleagues (Egidy et al., 2000) for normal human colon. In submucosal tissue, [^{125}I]-ET-1 bound to both vascular structures (endothelium and smooth muscle cells) as well as to non-vascular structures, including neurons, ganglion cells, myofibroblasts, macrophages and crypt epithelial cells. It should be noted, however, that these studies also revealed areas of the sections where ET_A and ET_B receptor binding sites in the intravascular region occur at similar density.

As the endothelin system is an important regulator of angiogenesis and blood perfusion, any change in ET-1, or receptor expression, could be associated with vascular hyperplasia and high blood flow in haemorrhoids (Loder et al., 1994). It is conceivable that mechanical injury to the endothelium, caused by shearing forces during defaecation, could increase ET-1 secretion, but it would be necessary to compare ET-1 levels in normal anal cushions with haemorrhoidal tissues. If this is the case, the available evidence suggests that ET_A receptors, rather than ET_B receptors, are likely to be involved in any endothelin-mediated hyperplasia (Ali et al., 2000; Maguire et al., 2002; Kitada et al., 2009).

The effect of endothelin receptor agonists and antagonists on the HMA and HMV

Blood supply to the arteriovenous plexus of haemorrhoidal tissue is provided by the rectal (mesenteric) artery and drains into the rectal (mesenteric) vein. As it is not possible at the moment to directly determine the effect of ET-1 on haemorrhoidal vessels, we examined the effect of the peptide on the connecting arteries and veins. In the present study, ET-1 elicited large contractile responses in both the HMA and HMV but (relative to the response to KCl) with a slightly higher maximal response in the vein. The potency of ET-1 was approximately 100-fold greater than noradrenaline, which is consistent with that observed in numerous other human blood vessels. Pharmacological examination of the responses to ET-1 using selective receptor antagonists revealed the involvement of ET_A receptors in both preparations. Although the pK_B for PD 165707 (7.3–7.7) is lower than that reported for the interaction at ET_A receptors (Maguire et al., 1997), the selective ET_B receptor antagonist BQ 788 failed to modify responses either in the absence or presence of PD 165707. These observations with ET-1 in the mesenteric arteries are also consistent with similar observations in both sheep and man (Ferrero et al., 2008; Lohsiriwat et al., 2011).

The selective ET_B receptor agonist sarafotoxin S6a (McMahon et al., 1991; Minkes et al., 1992) was more potent in the vein than in the artery, with an almost fourfold greater maximum response in the former. In some venous preparations, responses to sarafotoxin 6a was observed at concentrations as low as 1 nM (100-fold lower than that seen in arterial preparations) and there was a biphasic concentration response relationship in terms of contractile response. Interestingly, the selective ET_B receptor antagonist BQ788 inhibited sarafotoxin S6a-induced contractions in the HMV, with an estimated pK_B of 7.3, close to that reported for ET_B receptors (Russell and Davenport, 1996). However, this effect of BQ-788 was associated with a change in the slope of the CRC (steepening) to sarafotoxin 6a, suggesting that both ET_A and ET_B receptors are activated by the toxin in this vessel, albeit at different concentrations. Although ET_B receptors are present on the endothelium and mediate vasodilatation, those on vascular smooth muscle produces vasoconstriction. In the present study, only contractile responses were observed when the vessels were activated by ET-1 and sarafotoxin S6a. A similar finding has been reported in several studies of

endothelium-intact human vessels including the internal mammary artery and vein (Seo *et al.,* 1994), pulmonary artery (McCulloch *et al.,* 1996), coronary artery (Holm and Franco-Cereceda, 1996), umbilical vein (Mildenberger *et al.,* 2008) and saphenous vein (White *et al.,* 1994).

Interestingly, sarafotoxin S6a has been reported to possess similar pharmacological properties to sarafotoxin S6c (McMahon *et al.,* 1991), a snake toxin that has been evaluated in man. Sarafotoxin S6c infused intra-arterially in healthy volunteers (5–10 pmol·min^{-1} for up to 90 min) is known to cause a sustained reduction in forearm blood flow without any systemic side effects (Ferro *et al.,* 2002). Similarly, intravenous infusion of sarafotoxin S6c caused a sustained reduction in dorsal hand vein blood flow (Strachan *et al.,* 2000). As sarafotoxin S6a exerts a venoselective constrictor action, the possibility exists that a similar action on the arteriovenous plexus of haemorrhoids could pharmacologically reduce the volume of the tissue, the likelihood of prolapse and also control rectal bleeding. While pain is another key symptom of haemorrhoids (Loder *et al.,* 1994; Kaidar-Person *et al.,* 2007; Lohsiriwat, 2012) and local application of ET-1 is known to induce nociception in rats (Piovezan *et al.,* 2000), it is noteworthy that this action does not appear to involve ET_B receptors. Thus, sarafotoxin S6a could offer a distinct advantage over existing treatments that incorporate a vasoconstrictor agent (e.g. phenylephrine) that appears to selectively target the arterial (in flow) side of the vascular bed. An important step in verifying whether this is true for sarafotoxin 6a would be to establish whether it is possible to adapt for haemorrhoidal tissue (obtained from haemorroidectomies) the tissue slice technique (Sanderson, 2011) developed for simultaneously viewing changes in small arteries and veins *in situ* in lung slices. This approach has successfully been used to demonstrate that selective α_2-adrenoceptor agonists with decongestant activity can reduce the volume of venous sinusoids in slices of porcine nasal mucosa, without affecting arterial diameter in the same preparation (see Corboz *et al.,* 2008).

The idea of using a snake toxin, or analogues, to possibly alleviate the vascular symptoms associated with haemorrhoids clearly represents an unconventional approach and is not without major toxicological considerations and the need to develop a suitable formulation. It should be noted, however, that botulinum toxin, from the microorganism *Clostridium botulinum,* is currently used in an injectable form to treat anal fissures (Nelson *et al.,* 2012) and toxins in general represent a rich area of therapeutic possibilities (Lewis and Garcia, 2003). However, before considering the use of sarafotoxins as a novel therapeutic strategy for haemorrhoids, it will be necessary to establish whether the functional properties of ET_A and ET_B receptors within the haemorrhoidal vascular-plexus are similar to those noted in colonic and rectal vessels in this study and other human tissue (Maguire *et al.,* 2012).

In conclusion, we have established that the ET_B receptors are present on haemorrhoids at greater density than ET_A receptors, yet the latter is the principal receptor activated by ET-1 to induce contraction of human mesenteric arteries and veins. In contrast, the snake toxin sarafotoxin S6a exerts a selective venoconstrictor effect involving ET_B receptors. The latter finding raises the intriguing possibility that topical application of sarafotoxin S6a may be useful to reduce both bleeding and swelling associated with low grade haemorrhoids.

Author contributions

J.H.S., M.R.D. and V.G.W. conceived the study. V.L., J.H.S., V.G.W. and M.R.D. designed the protocol. J.H.S. was the principal investigator for the ethically approved protocol. V.L. and J.H.S. managed patient recruitment. V.L. and M.R.D. performed endothelin receptor binding studies and Western blot analyses. V.L. and V.G.W. performed contractile studies. V.L. and V.G.W. collected and analysed data. V.L., V.G.W. and M.R.D. wrote the manuscript. J.H.S. critically reviewed the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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