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Endothelin-1 and -3 induce choleresis in the rat through ET_B receptors coupled to nitric oxide and vagovagal reflexes

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Abstract

We have reported previously that centrally applied ET (endothelin)-1 and ET-3 induce either choleresis or cholestasis depending on the dose. In the present study, we sought to establish the role of these endothelins in the short-term peripheral regulation of bile secretion in the rat. Intravenously infused endothelins induced significant choleresis in a dose-dependent fashion, ET-1 being more potent than ET-3. Endothelins (with the exception of a higher dose of ET-1) did not affect BP (blood pressure), portal venous pressure or portal blood flow. ET-1 and ET-3 augmented the biliary excretion of bile salts, glutathione and electrolytes, suggesting enhanced bile acid-dependent and -independent bile flows. ET-induced choleresis was mediated by ET_B receptors coupled to NO and inhibited by truncal vagotomy, atropine administration and capsaicin perivagal application, supporting the participation of vagovagal reflexes. RT (reverse transcription)–PCR and Western blot analysis revealed ET_A and ET_B receptor expression in the vagus nerve. Endothelins, through ET_B receptors, augmented the hepatocyte plasma membrane expression of Ntcp (Na^+/taurocholate co-transporting polypeptide; Slc10a1), Bsep (bile-salt export pump; Abcb11), Mrp2 (multidrug resistance protein-2; Abcc2) and Aqp8 (aquaporin 8). Endothelins also increased the mRNAs of these transporters. ET-1 and ET-3 induced choleresis mediated by ET_B receptors coupled to NO release and vagovagal reflexes without involving haemodynamic changes. Endothelin-induced choleresis seems to be caused by increased plasma membrane translocation and transcriptional expression of key bile transporters. These findings indicate that endothelins are able to elicit haemodynamic-independent biological effects in the liver and suggest that these peptides may play a beneficial role in pathophysiological situations where bile secretion is impaired.

Key words: bile salt, bile secretion, endothelin receptor, hepatic transporter

INTRODUCTION

Endothelins are a family of 21-amino-acid-related peptides encoded by distinct genes that bind to specific receptors widely expressed in numerous tissues and cell types. The family, comprising ET (endothelin)-1, ET-2 and ET-3, exerts diverse biological effects mainly in an autocrine and/or paracrine manner [1,2]. Endothelins whether centrally or peripherally applied play a relevant role in BP (blood pressure) regulation acting synergistically with AngII (angiotensin II) and catecholamines [2–4].

Two pharmacologically characterized GPCRs (G-protein-coupled receptors), ET_A and ET_B, receptors mediate the biological effects of endothelins [2]. The ET_A receptor exhibits higher affinity for ET-1 and ET-2 than for ET-3, whereas the ET_B receptor binds the three isopeptides with similar affinity [2]. However, atypical responses, where biological effects mediated by

Abbreviations: AQP8, aquaporin 8; BP, blood pressure; Bsep, bile-salt export pump; ET, endothelin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L-NAME, N^G-nitro-L-arginine methyl ester; Mrp2, multidrug resistance protein-2; Ntcp, Na^+/taurocholate co-transporting polypeptide; NOS, nitric oxide synthase; qPCR, quantitative real-time PCR; PBF, portal blood flow; RT, reverse transcription; SNS, sympathetic nervous system.

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Table 1 Primer nucleotide sequences and conditions used in RT–PCR and qPCR

Gene	Primer sequence (5′→3′)	Denaturation	Annealing	Elongation
ET_A receptor		94 °C (5 min, 30 s)	59.5 °C (50 s)	72 °C (45 s)
Forward	GTTTCCTCCAGCCGAGACTG			
Reverse	CACACCTTTCCTTCCCCTTAGA			
ET_B receptor		94 °C (5 min, 30 s)	59.5 °C (50 s)	72 °C (45s)
Forward	CAAAGACTGGTGGCTGTTCAGTT			
Reverse	TCAAGGCAATCTGCATACCACTT			
Ntcp		94 °C (15 s)	58 °C (45 s)	72 °C (20 s)
Forward	CCTCCCTGATGCCCTTCTCT			
Reverse	GAATCCTGTTTCCATGCTGATG			
Bsep		94 °C (15 s)	58 °C (45 s)	72 °C (20 s)
Forward	TCTCCACCACTATCGCAGAAA			
Reverse	ATTAGCATCCTTGGCAGCTTG			
AQP8		94 °C (15 s)	50 °C (45 s)	72 °C (20 s)
Forward	AAGACCATGCTGCTAATTCC			
Reverse	TCCACAATGACAGAGAAACC			
Mrp2		95 °C (15 s)	60 °C (45 s)	72 °C (30 s)
Forward	ACCTTCCACGTAGTGATCCT			
Reverse	ACCTGCTAAGATGGACGGTC			
β-Actin		94 °C (15 s)	58 °C (45 s)	72 °C (20 s)
Forward	TTCTGTGTGGATTGGTGGCTCTA			
Reverse	CTGCTTGCTGATCCACATCTG			
GAPDH		94 °C (15 s)	58 °C (45 s)	72 °C (20 s)
Forward	CCTGCACCACCAACTGCTTAGC			
Reverse	GCCAGTGAGCTTCCCGTTCAGC			

endothelins fail to be mimicked or inhibited by selective agonists or antagonists, suggest the existence of additional endothelin receptor subtypes. ET_A and ET_B receptors are coupled to multiple signalling pathways and are expressed in several tissues and cells types including hepatocytes [1].

Various studies have shown that endothelins produce a sustained pressor response of hepatic circulation behaving as cholestatic agents [5–8]. However, it was also reported that in isolated perfused livers ET-1, through ET_A receptors, dose-dependently raises portal venous pressure, resulting in either choleresis or cholestasis despite pressure elevation [9]. Extensive work has been reported regarding the role of endothelins in the regulation of portal venous pressure and in pathophysiological conditions such as cirrhosis or portal hypertension, but only a few have addressed the role of these peptides in normal liver function [7–10].

Thus it was shown that ET-1 inhibits secretin-stimulated ductal bile secretion by interacting with ET_A receptors on cholangiocytes [11].

We have reported previously that ET-1 and ET-3 applied to the brain induce opposite dose-dependent regulatory effects on bile secretion despite vascular changes [12,13].

Lower doses of endothelins (1 fM) increased bile flow and bicarbonate excretion, whereas higher doses (1 nM) decreased bile flow and bile acid output. Both the choleretic and the cholestatic effects of ET-1 were mediated through vagal pathways by presumably distinct ET_A receptors, whereas the effects of ET-3 were dependent on brain NO and were independent of the autonomic nervous system or haemodynamic variations [12,13].

In the present study, we sought to establish the effect of peripherally infused ET-1 and ET-3 on bile secretion and to characterize the receptors, pathways and mechanisms involved.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats (School of Pharmacy and Biochemistry, University of Buenos Aires) weighing between 250 and 270 g were used. Animals were housed in steel cages and maintained at a temperature between 22 and 24 °C in a controlled room with a 12 h light/dark cycle (light from 07.00 to 19.00 h). All experiments were performed following the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication N85-23, 1985, revised 1996). The experimental protocols were approved by the Animal Care Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires. Unless stated, drugs or reagents of analytical or molecular biology quality were obtained from standard sources.

The rats were given water and commercial chow *ad libitum*. The food, but not the water, was withheld 14 h before the experiments to avoid the release of hormones and/or gastrointestinal peptides that may eventually affect bile secretion. Fasted rats were anaesthetized with urethane (1.2 g/kg of body weight, intraperitoneal; Sigma) and the left jugular vein cannulated with

a polyethylene catheter (PC-40 PL; Rivero & Cia) for the infusion of saline (control) or endothelins (experimental groups). Animals were prepared with bile duct cannulation as described previously [12,13]. Rats remained anaesthetized during bile collection which was performed between 09.00 and 11.00 h to avoid circadian variations [14]. Body temperature was kept at 37°C with a heating pad.

Secretory studies

Bile secretory experiments were performed as detailed previously [12,13]. Briefly, after a stabilization period of bile flow (15 min) ET-1 (0.5, 1 and 5 ng/kg of body weight per min), ET-3 (0.5, 1 and 5 ng/kg of body weight per min) (American Peptide) or saline (0.1 ml/min) was intravenously infused. Bile samples were collected every 30 min for 120 min in ice-cold microcentrifuge tubes. ET-1 and ET-3 at approximately 1 and 5 ng/kg of body weight per min respectively were used to identify the receptors and mechanisms involved given that they induced similar response in bile flow. To study stimulated-bile secretion rats were prepared as detailed above and following the stabilization period animals were infused with sodium taurocholate (1 μmol/min per 100 g of body weight) (Sigma) for 10 min, followed by ET-1 or ET-3 infusion for 20 min [15].

The concentration of bile acids was assessed by the 3α-hydroxysteroid dehydrogenase assay [16]. Total glutathione was measured using glutathione reductase and DTNB (Ellman's reagent) [5,5′-dithiobis-(2-nitrobenzoic acid)] [17]. Bicarbonate and chloride were measured by capillary electrophoresis (Quanta 400; Waters) [18], whereas biliary sodium and potassium were measured by the selective ion method (AVL-OMNI), and total proteins measured according to Lowry [19]. Biliary excretion rate was calculated from bile flow (μl/min per 100 g of body weight) and biliary concentration values.

To identify endothelin receptors, animals were pre-treated with BQ-610 (0.15 μmol/kg of body weight) or BQ-788 (0.5 μmol/kg of body weight), selective ET_A and ET_B receptor antagonists respectively [20] (American Peptide). Both antagonists were dissolved in saline and administered *in bolus* 10 min before endothelins infusion.

The role of the parasympathetic nervous system was evaluated by truncal vagotomy, atropine administration and capsaicin perivagal application. Bilateral truncal vagotomy was induced by sectioning both branches of the vagus and vagal afferents at the level of the lower oesophagus 2 h before bile secretion experiments [21]. Cervical vagotomy was avoided to prevent haemodynamic changes resulting from the cardiac vagal branches section. Atropine sulfate was administered *in bolus* (100 μg/kg of body weight, intravenous) 30 min before saline or endothelins and followed by infusion (100 μg/kg of body weight per h) during the experimental period. Capsaicin was prepared in 10% (v/v) ethanol, 10% (v/v) Tween 80 and saline. Following anaesthesia the abdominal vagal trunks were exposed and a small piece of gauze soaked in 1% (w/v) capsaicin was left around the vagal trunks for 30 min, whereas gauze soaked in vehicle was applied to control rats. Biliary secretory experiments were performed 5 days following the perivagal application of capsaicin [21]. The contribution of the SNS (sympathetic nervous system) was evaluated as previously reported [22]. The role of nitric oxide was investigated by the administration of L-NAME (N^G-nitro-L arginine methyl

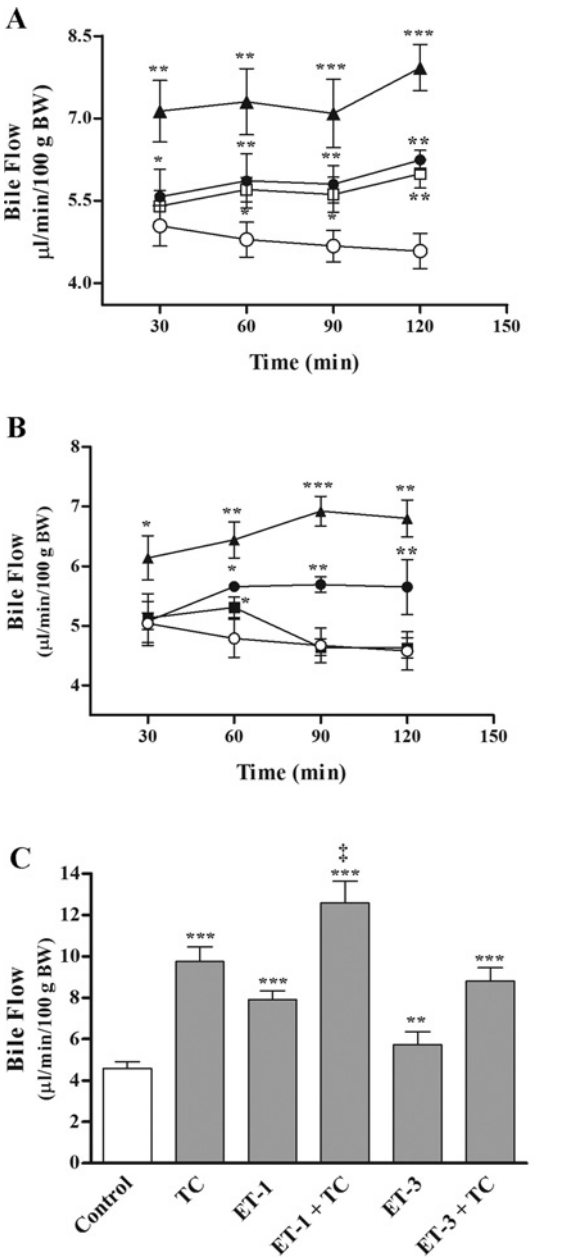


Figure 1 Effect of ET-1 and ET-3 on basal (A and B) and stimulated (C) bile flow

(**A**) ET-1 was infused for 120 min and bile samples collected every 30 min as detailed in the Materials and methods section. (○) Control, (■) 0.5 ng/kg of body weight per min, (▲) 1 ng/kg of body weight per min, and (●) 5 ng/kg of body weight per min. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control. $n = 10$–12. (**B**) ET-3 was infused for 120 min and bile samples collected every 30 min as detailed in the Materials and methods section. (○) Control, (■) 0.5 ng/kg of body weight per min, (●) 1 ng/kg of body weight per min, and (▲) 5 ng/kg of body weight per min. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control. $n = 10$–12. (**C**) Bile flow was stimulated by sodium taurocholate (TC) infusion, followed by the administration of ET-1 and ET-3 at 1 and 5 ng/kg of body weight per min respectively. $**P < 0.01$ and $***P < 0.001$ compared with control; ‡$P < 0.05$ compared with ET-1. $n = 6$–8 experiments. BW, body weight.

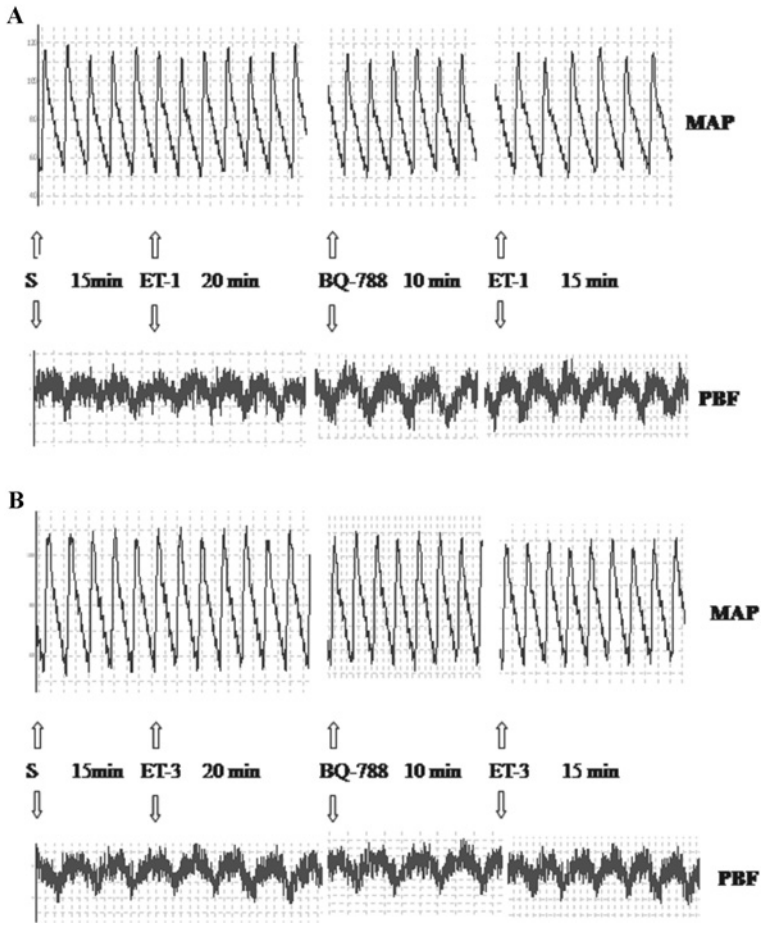


Figure 2 Effect of ET-1 and ET-3 on mean arterial BP and PBF
Blood flow was assessed as detailed in the Materials and methods section before and following infusion with ET-1 (1 ng/kg of body weight per min) (**A**) and ET-3 (5 ng/kg of body weight per min) (**B**) in the absence or presence of BQ-788. MAP, mean arterial pressure. $n = 5$–8 experiments. S, start.

ester; 10 mg/kg of body weight, intravenous; Sigma), a NOS (nitric oxide synthase) inhibitor, for 15 min before endothelin infusion.

RT (reverse transcription)–PCR and qPCR (quantitative real-time PCR) studies

The mRNA expression of ET_A and ET_B receptors in the vagus nerve was assessed by RT–PCR. Total RNA was isolated from the liver and vagus nerve by the MasterPure RNA Purification Kit (Epicenter Biotechnologies). RNA samples were treated with RQ1 RNase-free DNase (Promega) to eliminate genomic DNA and the RNA quality and quantity was assessed by 1% (w/v) agarose gel electrophoresis and UV spectrometry, respectively. RT was performed by adding M-MuLV reverse transcriptase (Fermentas Life Science) and oligodT15 primer (Biodynamics), followed by incubation for 60 min at 42 °C in M-MuLV buffer. RT was terminated by enzyme heat-inactivation at 70 °C for 10 min. The PCR was performed in the same reaction mixture containing forward and reverse primers and Go Taq Green master mix (Promega), cDNA liver (positive control) or cDNA vagus. The products were then submitted to electrophoresis on 1% (w/v) ethidium bromide-stained agarose gel. Primer sequences for the endothelin receptors and conditions are summarized in Table 1 [23].

Table 2 Effect of ET-1 and ET-3 on mean arterial BP and PBF
Blood flow was assessed as detailed in the Materials and methods section before and following infusion of of ET-1 (1 ng/kg of body weight per min) and ET-3 (5 ng/kg of body weight per min) in the absence or presence of BQ-788. MAP, mean arterial BP.

Endothelin	MAP (mmHg)	PBF (ml/min)
ET-1		
Control	116 ± 5	5.33 ± 0.25
ET-1	119 ± 6	5.45 ± 0.30
BQ-788	118 ± 4	5.40 ± 0.41
BQ-788 + ET-1	117 ± 6	5.37 ± 0.43
ET-3		
Control	120 ± 9	5.52 ± 0.32
ET-3	121 ± 4	5.53 ± 0.41
BQ-788	119 ± 7	5.55 ± 0.33
BQ-788 + ET-3	121 ± 5	5.58 ± 0.23

Possible endothelin-induced changes in the mRNA expression of Ntcp (Na^+/taurocholate co-transporting polypeptide;

Table 3 Effect of ET-1 and ET-3 on portal venous pressure
Portal venous pressure was recorded as detailed in the Materials and methods section before and during the infusion of ET-1 (1 ng/kg of body weight per min) and ET-3 (5 ng/kg of body weight per min).

	Portal venous pressure (mmHg)	
Time	**ET-1**	**ET-3**
0	4.1 ± 0.7	4.1 ± 0.7
10 s	4.9 ± 1.5	4.5 ± 1.1
30 s	5.0 ± 1.5	4.9 ± 0.8
1 min	4.5 ± 1.1	4.3 ± 1.0
2 min	4.4 ± 1.2	4.5 ± 0.8
3 min	5.1 ± 1.1	4.8 ± 1.0
5 min	4.7 ± 1.0	4.4 ± 1.0
10 min	4.3 ± 0.9	4.7 ± 0.9

Slc10a1), Bsep (bile salt export pump; Abcb11), Mrp2 (multidrug resistance protein-2; Abcc2) and AQP8 (aquaporin 8) were evaluated by qPCR. The RNA from livers of rats infused with ET-1 and ET-3 for 2 h was isolated and the cDNA reversed-transcribed as detailed for RT–PCR. The specific mRNA in each sample was measured in the ROTOR GENE-Q termocycler (Qiagen). Each reaction was performed with Real Mix containing Eva-green fluorocrome (Biodynamics), specific primers and RT product. Fluorescence signals were monitored sequentially for each sample once per cycle at the end of extension. All samples were assessed in duplicate. An external standard RNA concentration curve for each primer pair was generated using pooled RNA samples and verified by agarose gel electrophoresis. The specificity of PCR products was confirmed by melting curves analysis showing the presence of a single sDNA product per primer pair, and by agarose gel electrophoresis revealing a single band of the predicted size for each product. To correct for minor variability among samples, Ntcp, Bsep and Mrp2 expression was normalized to β-actin expression, whereas AQP8 was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Relative expression quantification was performed by the standard curve method. Sequences of primer pairs [24–26] and conditions are summarized in Table 1.

Western blot studies

Protein expression of Ntcp, Bsep, Mrp2 and AQP8 was assessed by Western blotting in plasma and intracellular (vesicular) membrane fractions obtained from livers of rats infused with endothelin. Hepatic membrane fractions were prepared as detailed previously [27,28]. Their purity was assessed by enzyme markers and results were comparable with those reported previously [27] and similar between treated and control rats. Endothelin receptors were also evaluated by immunoblotting in the vagus nerve. Experimental procedures for Western blot were as detailed in previous studies. Membranes were exposed to anti-ET_A (1:1000 dilution; Sigma), anti-ET_B (1:1000 dilution; Sigma), anti-AQP8 (1:1000 dilution; Alpha Diagnostics), anti-Ntcp (1:500 dilution; Santa Cruz Biotechnologies), anti-Bsep (1:500 dilution; Santa Cruz Biotechnologies) or anti-Mrp2 (1:1000 dilution; Alexis Biochemicals) antibodies overnight at 4 °C, followed by HRP (horseradish peroxidase)-conjugated anti-rabbit or anti-goat antibodies (1:5000 dilution; Santa Cruz Biotechenologies) for 1 h at room temperature (22–24 °C). Membranes were developed by Super Signal West Femto kit (Pierce). Loading control was controlled by β-actin.

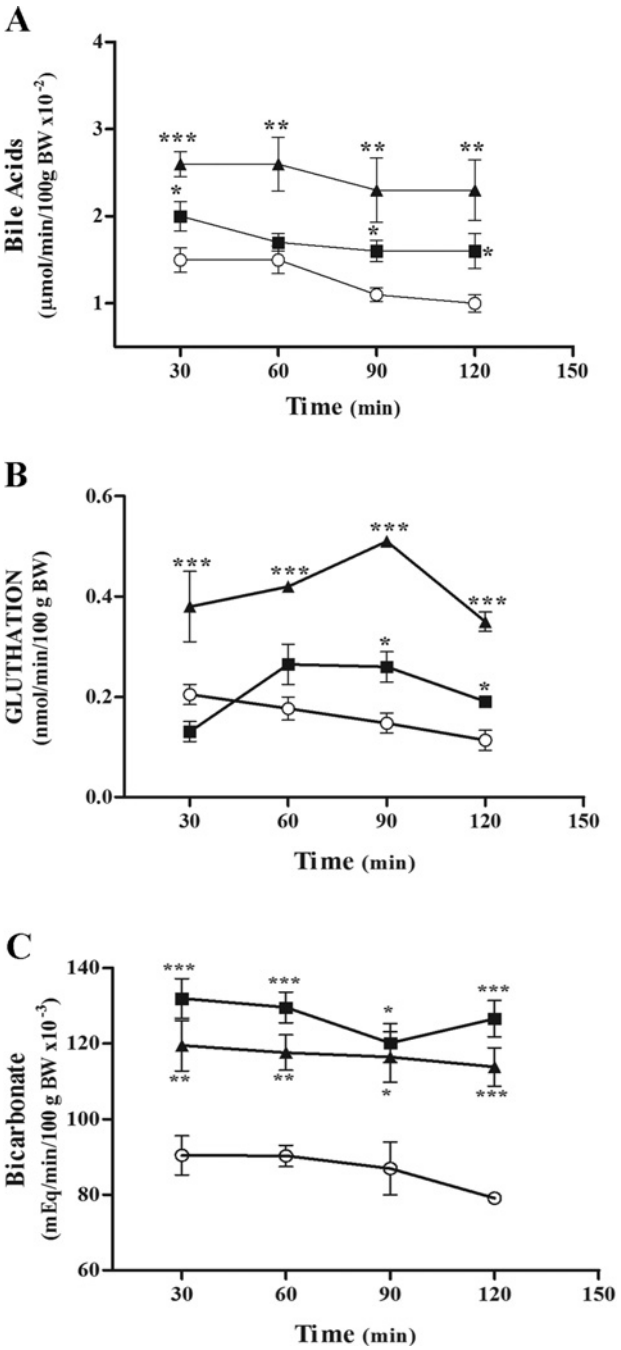


Figure 3 Excretion of biliary constituents in the presence of endothelins
ET-1 (1 ng/kg of body weight per min) and ET-3 (5 ng/kg of body weight per min) were infused for 120 min and bile samples collected every 30 min. Bile acids (**A**), glutathione (**B**) and bicarbonate (**C**) were measured in bile samples. (○) Control, (▲) ET-1, and (■) ET-3. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control. $n = 8$–10 experiments. BW, body weight.

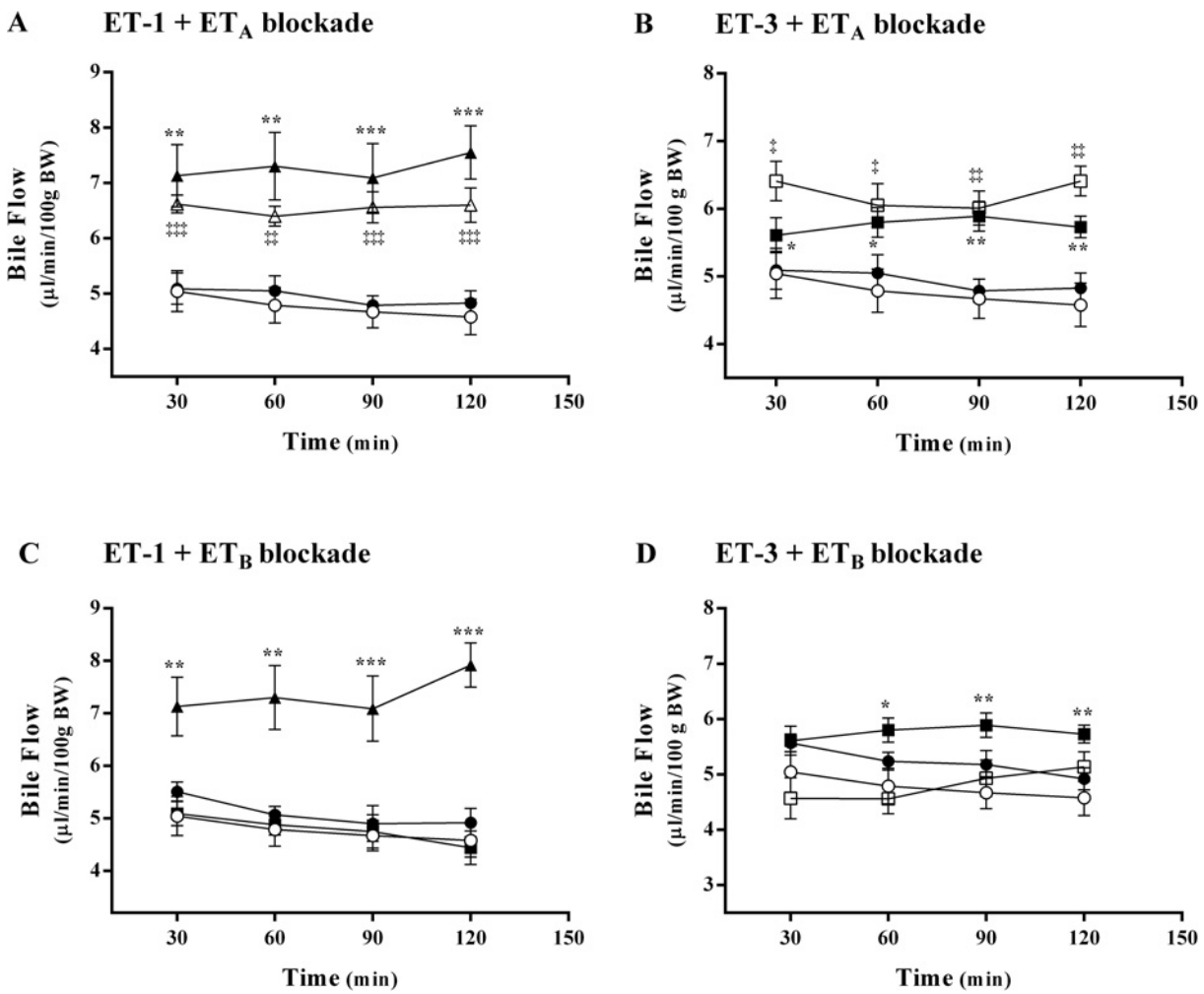


Figure 4 Effect of ET_A and ET_B receptor blockade on endothelin-induced choleresis

Rats were pre-treated with BQ-610 (an ET_A receptor selective antagonist) (**A** and **B**) or BQ-788 (an ET_B receptor selective antagonist) (**C** and **D**), followed by infusion with ET-1 (**A** and **C**) or ET-3 (**B** and **D**) for 120 min. (○) Control, (●) BQ-610 (**A** and **B**) or BQ-788 (**C** and **D**), (▲) ET-1 (**A** and **C**), (■) ET-3 (**B** and **D**), (△) BQ-610 + ET-1 (**A**) or BQ-788 + ET-1 (**C**), and (□) BQ-610 + ET-3 (**B**) or BQ-788 + ET-3 (**D**). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control; ‡$P < 0.05$, ‡‡$P < 0.01$ and ‡‡‡$P < 0.001$ compared with BQ-610. $n = 6$–8 experiments. BW, body weight.

Haemodynamic studies

PBF (portal blood flow) was assessed by a Flow Meter (Transition System) in the presence of ET-1 and ET-3. Rats were prepared with jugular vein and carotid artery cannulation to infuse endothelins and to record BP respectively. A specific flow probe MA2PSB (Transition System) was inserted in the portal vein and values were simultaneously registered for PBF and BP before and after infusion with endothelins for 30 min. The effect of BQ-788 was also assessed. Portal venous pressure was measured as detailed previously [12,13].

Statistical analysis

The statistical analysis was performed using ANOVA followed by a Student's *t* test modified by Bonferroni. Results are expressed as the means ± S.E.M. *P* values of 0.05 or less were considered statistically significant.

RESULTS

Both ET-1 and ET-3 induced choleresis at all doses and times studied, with ET-1 stimulating a higher response than that of ET-3 at equimolar doses (Figures 1A and 1B). As expected, TC infusion significantly increased only bile flow, and ET-1, but not ET-3, enhanced it further (Figure 1C).

The doses of endothelins used in the present study, except for ET-1 at 5 ng/kg of body weight per min, did not induce haemodynamic changes. BP, portal venous pressure or PBF were not affected following the infusion of ET-1 at 0.5 and 1 ng/kg of body weight per min, ET-3 at 0.5, 1 and 5 ng/kg of body weight per min or BQ-788 (an ET_B receptor antagonist). Results for ET-1 at 1 ng/kg of body weight per min and ET3 at 5 ng/kg of body weight per min are shown in Figure 2 and Tables 2 and 3. These doses of ET-1 and ET-3 elicited similar choleretic responses so

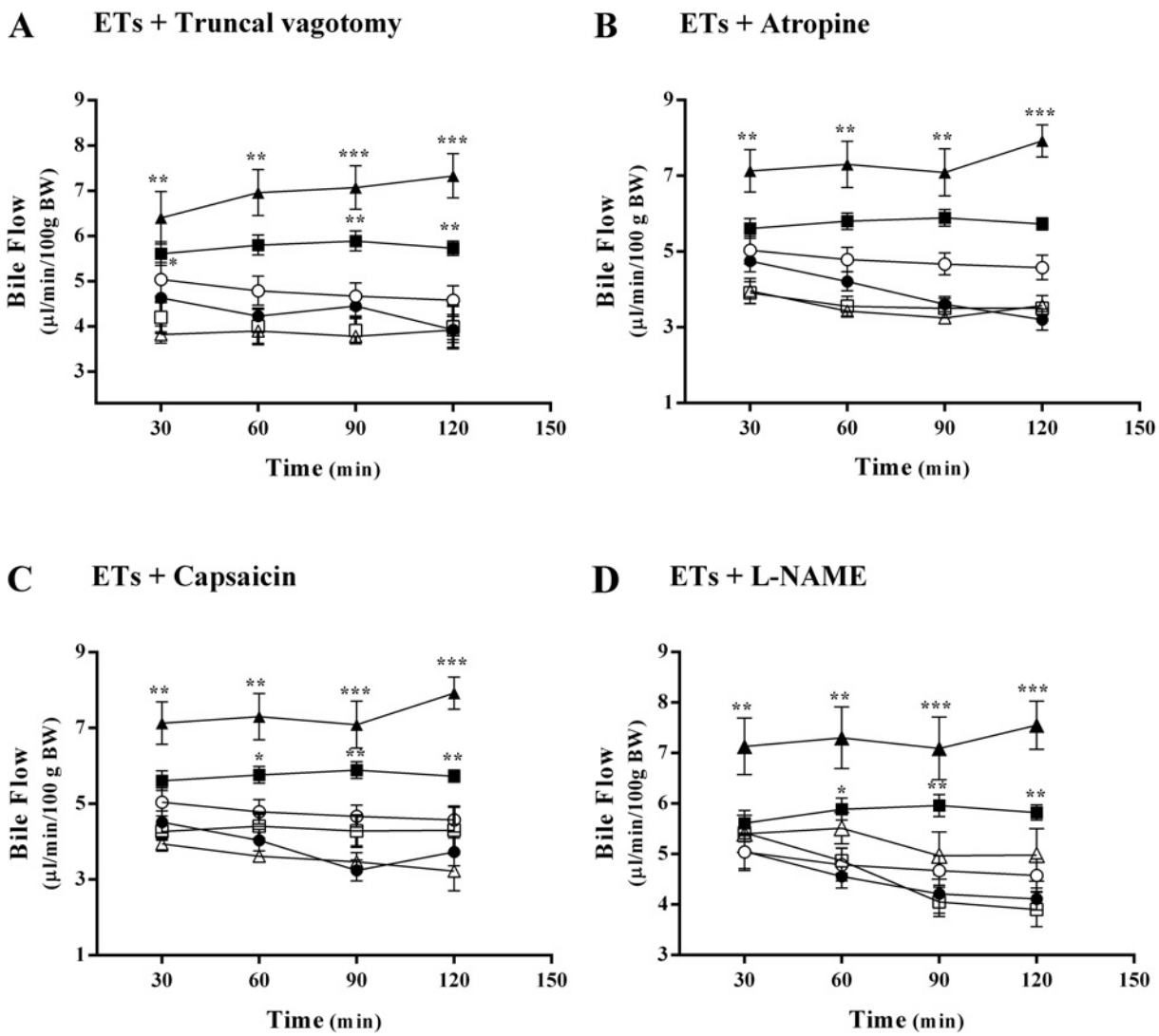


Figure 5 **Role of afferent and efferent parasympathetic pathways and NO in endothelin-induced choleresis**
ET-1 and ET-3 were infused for 120 min in rats with truncal vagotomy (**A**), treated with atropine (**B**), with perivagal application of capsaisin (**C**) or pre-treated with L-NAME (**D**). Bile flow was expressed as μl/min per 100 g of body weight. (○) Control, (●) vagotomy (**A**), atropine (**B**), capsaicin (**C**) or L-NAME (**D**), (▲) ET-1 at 1 ng/kg of body weight per min; (■) ET-3 at 5 ng/kg of body weight per min, (△) ET-1 + vagotomy (**A**), + atropine (**B**), + capsaicin (**C**) or L-NAME (**D**), (□) ET-3 + vagotomy (**A**), + atropine (**B**), + capsaicin (**C**) or L-NAME (**D**). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control. $n = 6$–8 experiments. BW, body weight.

they were used in further studies to determine the underlying mechanisms.

Biliary constituents were measured to unveil the underlying mechanisms of endothelins-induced choleresis. Bile acid output (Figure 3A), glutathione (Figure 3B) and bicarbonate (Figure 3C) were increased by ET-1 and ET-3. Furthermore, the biliary excretion of sodium, potassium and chloride, but not proteins, were also augmented (results not shown). These findings suggest that endothelins enhanced both the bile acid-dependent and -independent bile flows.

The identification of the ET receptor involved in choleretic response by endothelins was assessed by pre-treatment with selective ET receptor antagonists. Neither BQ-610 nor BQ-788 alone modified bile flow. ET_A receptor blockade failed to affect ET-1 or ET-3 response (Figures 4A and 4B) but ET_B receptor blockade abolished the endothelin-induced choleresis, supporting that the response was mediated by ET_B receptors (Figures 4C and 4D). Furthermore, endothelin-induced bile salt and glutathione excretion were also inhibited by BQ-788 (results not shown).

Bilateral truncal vagotomy and atropine administration inhibited endothelin-induced choleresis, strongly supporting the involvement of the vagus nerve and muscarinic receptors (Figures 5A and 5B). Perivagal application of capsaicin also inhibited the endothelin-induced increase in bile secretion, suggesting the contribution of vagal afferent pathways (Figure 5C). Adrenergic blockade altered neither basal flow nor endothelins response (results not shown). Blockade of NOS by L-NAME did not affect basal bile flow, but it inhibited endothelin-mediated

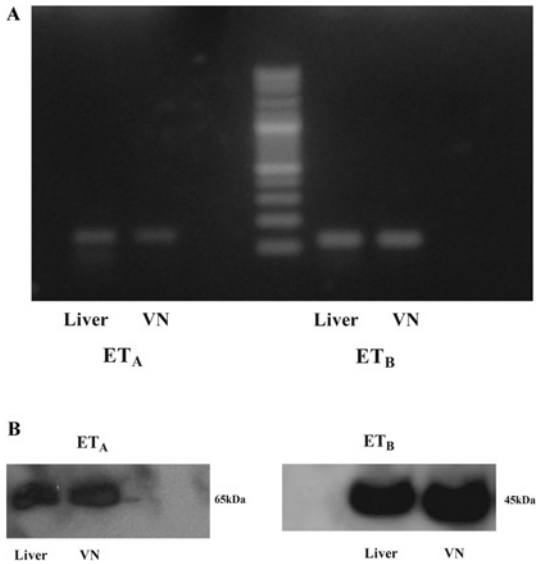


Figure 6 Expression of endothelin receptors in the vagus nerve
(**A**) ET_A and ET_B receptors mRNAs were assessed by RT–PCR as detailed in the Materials and methods section. (**B**) Protein expression of ET_A and ET_B receptors was assessed by Western blotting and the immunoblots shown are representative of four independent experiments. VN, vagus nerve.

choleresis, suggesting release of NO on ET_B receptor activation (Figure 5D). The inactive enantiomer D-NAME failed to induce the same response.

RT–PCR and Western blot analysis confirmed the presence of ET_A and ET_B receptors in the liver and revealed their expression in the vagus nerve (Figures 6A and 6B). ET_A and ET_B receptor primers yielded products of the expected size (133 and 119 bp respectively).

Both ET-1 and ET-3 caused a significant increase in plasma and intracellular membranes expressions of Ntcp, Bsep, Mrp2 and AQP8, although the effect of ET-3 was not statistically significant for Mrp2 (Figure 7). Endothelin-induced changes in hepatic transporters were blocked by pre-treatment with BQ-788 (Figure 7).

Both ET-1 and ET-3 also increased the mRNAs of the hepatic transporters as revealed by qPCR (Figure 8). Ntcp, Bsep, Mrp2 and AQP8 primers yielded products of the expected size (109, 89, 450 and 275 bp respectively).

DISCUSSION

The major finding of the present study was that ET-1 and ET-3 induced choleresis mediated by ET_B receptor activation coupled to nitric oxide and vagovagal reflexes without involving haemodynamic changes.

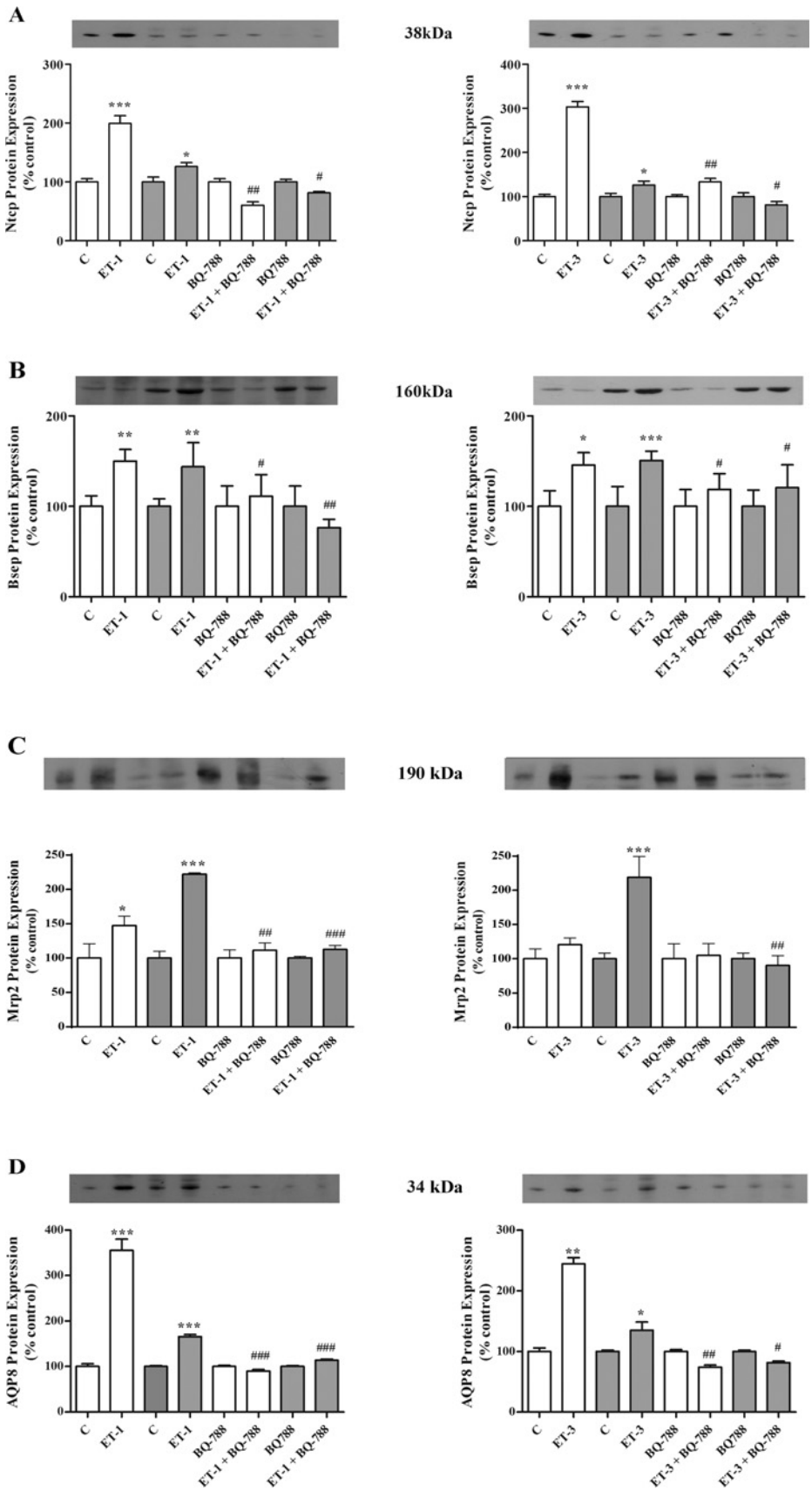
Endothelins are potent vasoconstrictor peptides, which play a well-characterized physiological role in the regulation of BP [2]. Endothelins enhance not only peripheral vascular resistance but also sympathetic output leading to BP elevation. The effect of endothelins in the liver has been studied extensively, but most reports address their participation in pathophysiological conditions such as pre-hepatic portal hypertension or cirrhosis [29,30]. It has been shown that ET-1 increases portal venous pressure and induces cholestasis through ET_A receptor activation [31]. However, studies in isolated perfused livers show that, although ET-1 dose-dependently increases portal pressure, it stimulates or reduces bile secretion depending on the dose [9]. In the present study, endothelins enhanced bile flow at all doses and times studied, with ET-1 having a higher choleretic response than ET-3. Furthermore, ET-1 increased basal as well as bile-salt-stimulated bile flow without involving haemodynamic changes. The highest choleretic response was achieved by doses of endothelins that did not induce changes in portal venous pressure and PBF, supporting that these peptides are also able to elicit haemodynamic-independent biological effects in the liver. A previous *in vivo* study in rats showed that ET-1 induced only a modest, non-significant increase in basal bile flow [11]. The reason for this discrepancy is unclear, although differences in rat strains and ET-1 doses as well as possible variations in hepatic haemodynamic parameters may be involved.

Both endothelin receptors are expressed in different cell types in the rat and human liver including hepatocytes, stellate cells, cholangiocytes and sinusoidal endothelial cells [1,11]. ET_A receptors seem to mediate vascular effects [20] and the ET-1 inhibition of secretin stimulated ductal bile secretion [11]. In our study, we provide evidence for an ET-induced choleresis mediated by ET_B receptors.

The activation of ET_B receptors suggests the involvement of nitric oxide since in many tissues this receptor subtype is coupled to NOS activation [22,32]. In the present study, NOS blockade by L-NAME abolished the response of ET-1 and ET-3, suggesting that NO is released upon ET_B receptor activation. These findings correlate with previous studies in isolated rat hepatocyte couplets or perfused liver showing that NO is a potent stimulator of bile flow [33,34].Interestingly it has been shown that ET_B receptor expression is enhanced in cirrhosis and it was suggested that it would stress the vascular properties of endothelins [35]. However, in the light of present findings it is possible to consider that it may represent a regulatory mechanism attempting to counterbalance further deleterious effects of endothelins through ET_A receptors.

We next identified the fraction of bile flow stimulated by endothelins. Since bile acids, glutathione and bicarbonate are known to act as the main osmotic driving forces in bile formation, their biliary excretion was assessed. Both peptides stimulated bile acid-dependent flow as supported by the enhanced excretion rate of bile acids. The effect of ET-1 was more pronounced than that of ET-3 in accordance with its higher choleretic response. In addition, the endothelin-induced total glutathione and bicarbonate excretion suggests that endothelins also stimulated bile acid-independent flow. These findings support that endothelin-induced choleresis involved stimulation of both bile acid-dependent and -independent bile flow fractions and suggest increased activity of the hepatic transporters responsible for bile formation. Increased canalicular solute excretion was mediated by ET_B receptors and presumably achieved by short-term mechanisms, such as rapid translocation of hepatic transporters to the plasma membrane from vesicular (endosomal) compartments.

Under physiological conditions most bile salts are removed from the sinusoidal blood by the sodium-dependent transport


A
38kDa
Ntcp Protein Expression (% control)
C
ET-1
ET-3
BQ-788
ET-1 + BQ-788
ET-3 + BQ-788
BQ788
B
160kDa
Bsep Protein Expression (% control)
C
Mrp2 Protein Expression (% control)
190 kDa
D
34 kDa
AQP8 Protein Expression (% control)

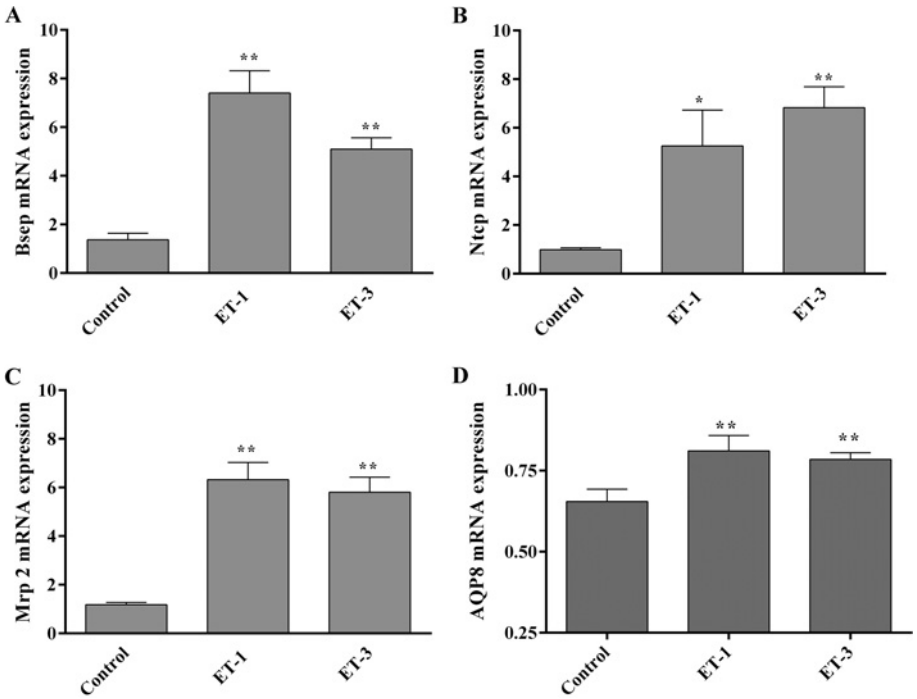


Figure 8 Expression of Ntcp (A), Bsep (B), Mrp2 (C) and AQP8 (D) mRNAs
The expression of the transporters was assessed by qPCR in livers from rats infused with endothelins for 120 min, as detailed in the Materials and methods section. Results were normalized to β-actin (Ntcp, Bsep and Mrp2) or GAPDH (AQP8). $*P < 0.05$ and $**P < 0.01$ compared with control.

system Ntcp located on the basolateral membrane [36]. Bile salts are then secreted at the canalicular membrane through Bsep, which is a member of the ABC family (ATP-binding cassette family) [37]. Both transporters are also located in vesicular (endosomal) compartments allowing their rapid insertion in the plasma membrane on demand [38]. Present findings show that both endothelins through ET_B receptors induced the short-term increase of Ntcp and Bsep expression in plasma membranes, suggesting a rapid vesicular translocation of these transporters. Nevertheless, Ntcp and Bsep expression in intracellular membranes did not decrease as expected from a vesicular redistribution of intracellular transporters to the cell surface [38]. In contrast, Ntcp and Bsep increased their intracellular expression, which is in line with the endothelin-induced mRNA expression of these transporters. Thus endothelins may up-regulate the plasma membrane of transporters by vesicle translocation as well as by transcriptional expression. Similar results were observed for Mrp2, which transports lipophilic substances conjugated with glutathione, glucuronate, sulfate and glutathione into bile. Mrp2 is considered critical to the generation of bile salt-independent flow and its function is also regulated by the dynamic retrieval and exocytic insertion between the canalicular membrane and an intracellular pool of vesicles [38,39]. Thus enhanced plasma membrane expression of Ntcp, Bsep and Mrp2 correlated well with stimulated canalicular solute excretion.

Endothelin-stimulated biliary excretion of solutes through the canalicular membrane should be associated with enhanced osmotically driven canalicular secretion of water which, in hepatocytes, is mediated by AQP8 water channels [40]. Rat hepatocytes contain an intracellular pool of AQP8 vesicles that upon choleretic stimuli shuttle to the canalicular membrane to increase membrane water permeability, a process that would facilitate osmotic water transport during bile formation [41,42]. Both endothelins, through ET_B receptor activation, enhanced the expression of AQP8 in plasma membrane and intracellular hepatic fractions. Furthermore the endothelins also increased AQP8 mRNA expression, consistent with increased AQP8 content in intracellular membranes.

The parasympathetic nervous system plays a relevant role in the regulation of digestive secretions. The dorsal vagal complex

Figure 7 (A) Expression of Ntcp (A), Bsep (B), Mrp2 (C) and AQP8 (D) in hepatic membranes following infusion of endothelins
Rats were infused with ET-1 (left-hand panels) and ET-3 (right-hand panels) for 120 min and the expression of the hepatic transporters assessed by immunoblotting in plasma membrane (open bars) and intracellular (vesicular) membranes (close bars), as detailed in the Materials and methods section. Representative immunoblots are shown with the corresponding densitometric analysis of four independent experiments. Each lane was loaded with 20 μg of protein. Equal protein loading was controlled by β-actin antibody (results not shown) and results are expressed as a percentage of controls. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with the respective controls; $\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$ compared with ET-1 or ET-3.

located in the brain stem represents the major brain site involved in the regulation of the gastrointestinal function. The dorsal motor vagal and ambiguous nuclei supply efferent preganglionic parasympathetic nerves to the liver, which constitute the hepatic branch of the vagal nerve [43]. In addition, the liver receives adrenergic innervation via the splanchnic nerve and has also a vast supply of autonomic nerves originated in the brainstem. Bilateral truncal vagotomy, atropine administration and capsaicin perivagal application evoked no statistically significant changes in basal bile flow but abolished endothelin-induced choleresis, supporting that vagal efferent and afferent pathways mediated the response of the endothelins. These findings suggest the involvement of a vagovagal reflex in endothelin-induced choleresis. Although vagovagal reflexes regulate gastrointestinal motility and pancreatic secretion, their participation in the control of liver function is elusive. This is to our knowledge the first report to show that vagal reflexes contribute to increase bile flow. Although endothelin-induced choleresis was vagally mediated, we investigated any contribution of the SNS based on previous studies showing that endothelins enhance sympathetic outflow [3]. We found that adrenergic blockade failed to modify the increase in bile flow induced by endothelins.

Our findings show that endothelins induced choleresis through ET_B receptors coupled with NO and vago-vagal reflexes stimulated at the level of vagal afferent fibres. ET_B receptors coupled with NOS activation are expressed in the liver, but in the present study we show by RT–PCR and Western blot assays that ET_A and ET_B receptors were also expressed in the vagus nerve. Therefore it is possible to assume that endothelins may also directly activate vagal reflexes by stimulating ET_B receptors located on the vagus.

The present findings show that endothelins induced choleresis by activating ET_B receptors at doses that failed to modify BP, portal venous pressure and PBF, supporting that these peptides display biological responses other than the regulation of BP and blood flow in the liver. Both endothelins induced choleresis independently of haemodynamic changes through an increase in bile acid-dependent and -independent bile flows.

CLINICAL PERSPECTIVES

- It is well known that cholestasis is characterized by reduced expression of Mrp2, Bsep, Ntcp and AQP8 water channels in the hepatocyte plasma membrane.
- In the present study, we found that endothelins acting through ET_B receptors induced choleresis by increasing plasma membrane translocation and transcriptional expression of the main transporters involved in bile genesis, suggesting that these peptides may have a potential beneficial role in pathophysiological conditions where bile secretion is impaired.
- The present findings further suggest that ET_B receptors may represent a promising therapeutic target in pathophysiological situations like hepatocellular cholestasis.

AUTHOR CONTRIBUTION

Myrian Rodriguez, Leandro Soria, María Ventimiglia, Ana Najenson, Adrián di María, Paula Dabas and Andrea Fellet performed the experiments and analysed the data. Raúl Marinelli, Marcelo Vatta and Liliana Bianciotti conceived and designed the research.

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