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Differential expression of intestinal membrane transporters in cholera patients

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Abstract ***Vibrio cholerae*** **causes the cholera disease through secretion of cholera toxin (CT), resulting in severe diarrhoea by modulation of membrane transporters in the intestinal epithelium. Genes encoding membrane-spanning transporters identified as being differentially expressed during cholera disease in a microarray screening were studied by real-time PCR, immunohistochemistry and in a CaCo-2 cell model. Two amino acid transporters, SLC7A11 and SLC6A14, were upregulated in acute cholera patients compared to convalescence. Five other transporters were downregulated; aquaporin 10, SLC6A4, TRPM6, SLC23A1 and SLC30A4, which have specificity for water, serotonin (5-HT), magnesium, vitamin C and zinc, respectively. The majority of these changes appear to be attempts of the host to counteract the secretory response. Our results also support the concept that epithelial cells are involved in 5-HT signalling during acute cholera.**

Keywords: *Vibrio cholerae*; Cholera toxin; CaCo-2 cells; Microarray; Membrane transporters; Human

1. Introduction

Cholera has spread from the endemic region in South Asia in repeated epidemics and is still causing a large number of deaths in Asia, Africa, and to a lesser extent in Latin America. *Vibrio cholerae* O1 and O139 are the causative agents of cholera. *V. cholerae* colonises the small intestine, and after an incubation period between 1 and 2 days, there is an abrupt onset of the disease with vomiting and electrolyte-rich watery diarrhoea. The fluid loss is often so rapid and massive that patients can die if left untreated [1]. The treatment is based on the simple concept of replacing water and electrolytes as fast as they are being lost. The patients are given an electrolyte solution intravenously followed by an oral rehydration solution (ORS). The ORS is supplemented with carbohydrates for more effective reabsorption of electrolytes, as it is known that sugars are cotransported with Na^+ over the apical membrane of the enterocytes [2].

The major virulence factor responsible for the dehydrating features observed during cholera is the cholera toxin (CT), which is secreted by *V. cholerae* into the small intestine. The severe diarrhoea evoked by CT is in part due to a direct effect on the epithelial cells, caused by an increase in the intracellular levels of cyclic adenosine monophosphate (cAMP). CT binds to the epithelial cells via its five identical B-subunits and is taken up by retrograde transport [3]. Thereafter, its enzymatically active A-subunit ADP-ribosylates $G_{s\alpha}$, leading to the activation of adenylate cyclase and production of cAMP. There is also a substantial amount of evidence to suggest the importance of the local nerve reflexes, elicited by the CT-mediated release of serotonin (5-HT) from enterochromaffin cells, for the secretory response [4–7]. The 5-HT released stimulates a nerve reflex that activates secretomotor neurons to release their transmitters onto the epithelial cells [8,9]. Thus, the direct and 5-HT-mediated effects on the epithelial cells converge to induce a cAMP-dependent secretion of Cl^- and HCO_3^-, and together with the accompanying inhibition of uptake of Na^+ and Cl^-, they result in a massive loss of electrolytes and water during cholera.

Previous studies in various intestinal models, by us and others, have shown that CT and cAMP also modulate numerous additional transporters, including aquaporins (AQPs) and sodium-dependent carriers [10–12]. We have recently performed a whole-genome microarray screening of seven cholera patients during the acute and convalescent phase of the disease [13]. In this paper, we have focused on the genes known to encode membrane-spanning transporters. The membrane transporters expressed differentially during the acute and convalescent phase in intestinal mucosal biopsies from cholera patients have been confirmed and analysed further using a CaCo-2 cell model.

2. Materials and methods

2.1. Study group

The study included 7 adult male patients, described previously [13], with cholera caused by *V. cholerae* O1 El Tor and treated at the ICDDR, B hospital in Dhaka, Bangladesh. Briefly, the patients were

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Abbreviations: CT, cholera toxin; ORS, oral rehydration solution; RT-PCR, real-time PCR; SLC, solute carrier; AQP, aquaporin; TRPM6, transient receptor potential cation channel subfamily M member 6; SERT, serotonin transporter; SVCT1, sodium-dependent vitamin C transporter 1; ZnT4, zinc transporter 4

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Table 1
Primers used for RT-PCR

Transcript	Forward primer	Reverse primer
AQP10	5′-gggtcaagctccccatttacatc-3′	5′-tgtatagttctgtagggcatcatggtaga-3′
AQP10v	5′-ggacagtgttctttctccaagtcatattc-3′	5′-gcatgcctaagaacacaacctctaaat-3′
SLC6A4	5′-caattacttctccgaggacaacatca-3′	5′-ccccttagaccggtggatctg-3′
SLC6A14	5′-gctgcttggttttgtttctccttggtc-3′	5′-gcaattaaaatgccccatccagcac-3′
SLC7A11	5′-caaatgcagtggcagtgacctt-3′	5′-accgttcatggagccaaagc-3′
SLC23A1	5′-tcaatacaggcattcttgaagtggat-3′	5′-gcactgtgttgtcaagtatgaaagca-3′
SLC30A4	5′-tgttaactgacctaagcgccatca-3′	5′-cctctaagcgatgaaatccaaagg-3′
TRPM6	5′-gtggccattggcctgttttc-3′	5′-caggagccgtgagaaccagaa-3′
GAPDH	5′-gagcaccaggtggtctcctctgacttc-3′	5′-gccaaattcgttgtcataccaggaaatg-3′

severely dehydrated, as assessed by a physician. For microbiological diagnosis, stool samples were analysed by dark-field microscopy and for reactivity with serogroup-specific antibodies. To confirm the causative agent of the disease, stools were then plated and suspected vibrio colonies were identified by slide agglutination. The stools were also screened for other common enteric pathogens, including both bacteria and parasites, of which none were detected in any of the patients. All patients received intravenous rehydration, oral rehydration solution, and antibiotics (five patients were given doxycycline, and two patients were given ciprofloxacin) on the day of admission, which was 1 day before biopsies were first collected. The compositions of the rehydration solutions have been described in detail before [1]. This study was approved by the ethics review committee of ICDDR, B and by the human research board in Gothenburg.

2.2. Sample collection

Mucosal punch biopsies were collected from the second part of the duodenum on the second day of hospitalization. This was considered to be approximately two days after onset of diarrhoea (day 2) and is referred to as the acute phase. Samples were also collected at day 30, which is referred to as convalescence. Biopsy specimens were immediately put into RNAlater solution (Ambion) and kept at −70°C, or fixed in buffered formaldehyde and stored at 4°C until used for isolation of RNA and immunohistochemistry, respectively.

2.3. Cell culture and challenge with CT

The human intestinal epithelial cell line CaCo-2 was used for experiments involving stimulation with CT as described previously [13]. Cells were grown in 6-well plates in Dulbecco's modified Eagle medium with non-essential amino acids (Gibco), 3 ml/well, supplemented with 10% foetal calf serum (FCS), glutamine, β-mercaptoethanol and gentamycin under a 5% CO_2 atmosphere at 37°C. The cells were grown for 9 days after confluence before addition of CT (1 μg/ml) to the culture medium. FCS was not present in the medium during CT challenge and during the 12-h period preceding stimulation. For the controls, the medium was just changed to a serum-free medium without the addition of CT. The cells were challenged for 18 h before RNA was isolated.

2.4. RNA isolation

Total RNA was isolated from RNAlater-preserved biopsies and CaCo-2 cells using the GenElute Mammalian Total RNA Kit (Sigma) according to the manufacturer's instructions. The RNA concentration was measured spectrophotometrically and the quality was checked with an Agilent 2100 Bioanalyzer. Total RNA was used for real-time PCR (RT-PCR) and microarray analyses.

2.5. Microarray experiments

The present study is based on a recent microarray screening [13]. Briefly, the RNA prepared from the cholera patients was converted into labelled target cRNA and hybridised to Affymetrix human GeneChip U133 plus 2.0 containing 54000 probe sets representing 47000 different transcripts. The hybridisation procedure was followed by washing, staining and scanning, according to the GeneChip Expression Analysis manual (Affymetrix). The resulting images were analysed with Affymetrix GeneChip Operating Software. All probe sets were used for scaling and normalisation. Log_2 ratios for all transcripts between the acute phase and the convalescence sample were calculated for each patient, generating a total of seven values for each transcript. A higher or lower degree of expression in the acute phase compared to the convalescent phase was referred to as up-regulation or down-regulation, respectively. Transcripts for which the mean $\log_2$ acute phase to convalescence ratio was above 1.0 or below −1.0 were identified. To minimise the effect of individual outliers, transcripts were only selected if an effect corresponding to a $\log_2$ ratio above 0.5 for up-regulations or below −0.5 for down-regulations was observed in at least five of the seven patients.

2.6. Relative quantification by RT-PCR

Two micrograms of total RNA for each sample was reverse transcribed into cDNA. Oligonucleotide primers (Table 1) purchased from TAG Copenhagen A/S were used for the relative quantification of the selected transcripts using Applied Biosystems' 7500 Real Time PCR System according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene in all experiments. The relative levels of transcripts as $\log_2$ ratios between the acute and convalescence samples for each patient were calculated. For the CaCo-2 experiments a $\log_2$ ratio between the target gene and the reference gene was derived for each sample. These relative expression values were adjusted so that the mean in the control group was set to 0.

2.7. Immunohistochemistry

Paraffin-embedded duodenal sections from the cholera patients were studied by immunohistochemistry to detect the SLC6A14 protein and serotonin transporter (SERT) in the mucosa. A peptide corresponding to the C-terminus of human SLC6A14 (CADHEIPTVSGSRKPE) [14] was synthesised and used to raise a rabbit polyclonal antiserum (Innovagen AB, Lund, Sweden). For detection of SERT a monoclonal antibody (Abcam) was used. Alkaline phosphatase-conjugated antibodies (Jackson ImmunoResearch) were used as secondary reagent and the sections were developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrate.

2.8. Statistical analysis

Student's *t*-test was used to evaluate differences in gene expression between CT-stimulated and non-stimulated CaCo-2 cells. Correlation between gene expression data generated by the microarray technique and RT-PCR was determined with Pearson's test.

3. Results

3.1. Microarray experiments

Recently, we have applied whole-genome microarrays to screen for differentially expressed genes during acute cholera compared to convalescence [13]. A total of 29 up-regulated and 33 down-regulated transcripts were identified. Since, we had described the differential expression of a number of membrane transporter genes in the rat small intestine after CT challenge in an earlier study [10], we focused on this group of transcripts in the current study. Among the differentially expressed transcripts in acute cholera, seven are known to encode

membrane transporter proteins (Table 2). Two amino acid transporters, SLC7A11 (xCT) and SLC6A14 (B0+), were up-regulated, and five transporters; the water channel AQP10, the Mg^{2+} channel transient receptor potential cation channel subfamily M member 6 (TRPM6), the serotonin transporter SLC6A4 (SERT), the vitamin C transporter SLC23A1 (SVCT1), and the zinc transporter SLC30A4 (ZnT4), were down-regulated during the acute phase in cholera patients compared to convalescence.

3.2. RT-PCR

All the transcripts listed were subjected to confirmatory RT-PCR analysis. Since two splice variants have been reported for AQP10, designated AQP10v and AQP10, a primer pair specific for AQP10v was used in addition to the primer pair recognising the core region [15,16]. The differential expression was confirmed for all the transcripts, including AQP10v (Fig. 1). At the level of gene expression in individual patients, the results generated by the microarray and PCR techniques correlated well by showing the same trend in 44 of 49 analyses, and they showed opposite results only once (a SLC30A4 measurement). In addition, when applying Pearson's test we also found a close correlation between the results ($P < 0.0001$; $r = 0.92$).

3.3. Stimulation of CaCo-2 cells

The transporters were examined further in CaCo-2 cells, a human intestinal cell line known to spontaneously differentiate into cells resembling mature enterocytes when grown in culture [17]. Five of the seven genes investigated, SLC7A11, SLC6A14, TRPM6, SLC6A4 and SLC23A1, showed the same expression pattern in CaCo-2 cells after challenge with CT for 18 h as observed during acute cholera. No significant change in the levels of AQP10 and SLC30A4 transcripts were detected between the control and the CT-stimulated group in the CaCo-2 model (Fig. 2).

3.4. Immunohistochemistry

Immunohistochemical analyses could detect the expression of the SLC6A14 protein and SERT in the intestinal epithelium of cholera patients. In three of the patients, the analyses showed higher expression of the SLC6A14 protein in the brush-border membrane of the epithelium during the acute stage of cholera than during convalescence, whereas the expression of SERT in the epithelial cells was reduced

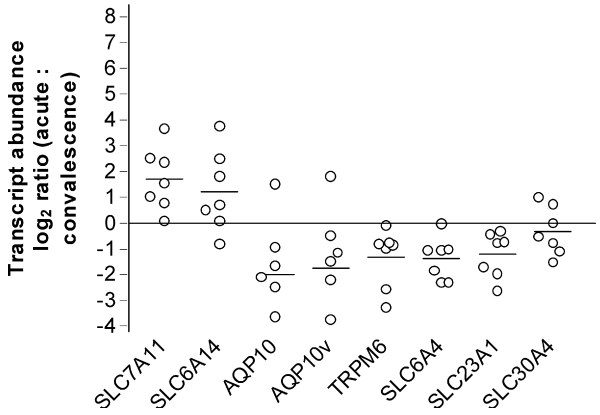

Fig. 1. Differentially expressed transporters during acute cholera as compared to convalescence shown as $\log_2$ ratios measured by RT-PCR. Horizontal lines indicate mean values.

(Fig. 3). However, although these proteins were detected to some extent in all patients, no differences in the expression could be established in four of the patients using this approach.

4. Discussion

In this study, we have concentrated on seven genes known to encode membrane-spanning transport proteins shown to be differentially expressed, two up-regulated and five down-regulated, during acute cholera in comparison to convalescence in a previous whole-genome microarray screening [13]. Each of these changes was confirmed by RT-PCR. The differential expression of five of the seven genes was also detected in CaCo-2 cells after challenge with CT, indicating direct effects of CT on the epithelial cells in these cases. This also implies that CaCo-2 cells are a good model for study of the epithelial response to CT during acute cholera, which affords the opportunity to differentiate the direct effects of CT on the epithelial cells from the nerve-mediated effects also present in the in vivo situation.

The two up-regulated genes encode the amino acid transporters SLC6A14 and SLC7A11, the former being dependent on Na^+ and having specificity for neutral and positively

Table 2
Differentially expressed membrane transporters during acute cholera identified by the microarray technique

Affymetrix ID	#1[a]	#2	#3	#4	#5	#6	#7	$\log_2$ ratio mean [b]	Gene name	Gene product
217678_at 209921_at	1.4	0.9	0.9	1.2	−0.6	3.1	2.6	1.36	SLC7A11	Solute carrier family 7, member 11 (Amino acid transporter xCT)
219795_at	3.0	1.2	−0.2	0.9	−0.7	2.6	1.9	1.24	SLC6A14	Solute carrier family 6, member 14 (Amino acid transporter B0+)
1555338_s_at	−2.8	−0.9	−1.3	−1.7	1.2	−1.8	−2.5	−1.40	AQP10	Aquaporin 10
224412_s_at	−1.7	−0.4	−0.6	−0.7	−0.3	−0.8	−4.0	−1.21	TRPM6	Transient receptor potential cation channel subfamily M member 6
207519_at	−1.6	−0.8	−0.7	−0.5	−0.8	−1.8	−1.9	−1.16	SLC6A4	Solute carrier family 6, member 4 (Serotonin transporter, SERT)
223732_at	−1.8	−0.3	−0.7	−0.8	−0.8	−1.3	−2.2	−1.13	SLC23A1	Solute carrier family 23, member 1 (Sodium-dependent vitamin C transporter 1, SVCT1)
207362_at	−1.0	0.2	−0.8	0.7	−1.1	−2.6	−2.7	−1.04	SLC30A4	Solute carrier family 30, member 4 (Zinc transporter 4, ZnT4)

[a]Columns designated #1–#7 represent individual $\log_2$ ratios for the seven patients included in the study.
[b]The $\log_2$ ratios represent the ratios for acute/convalescence phase.

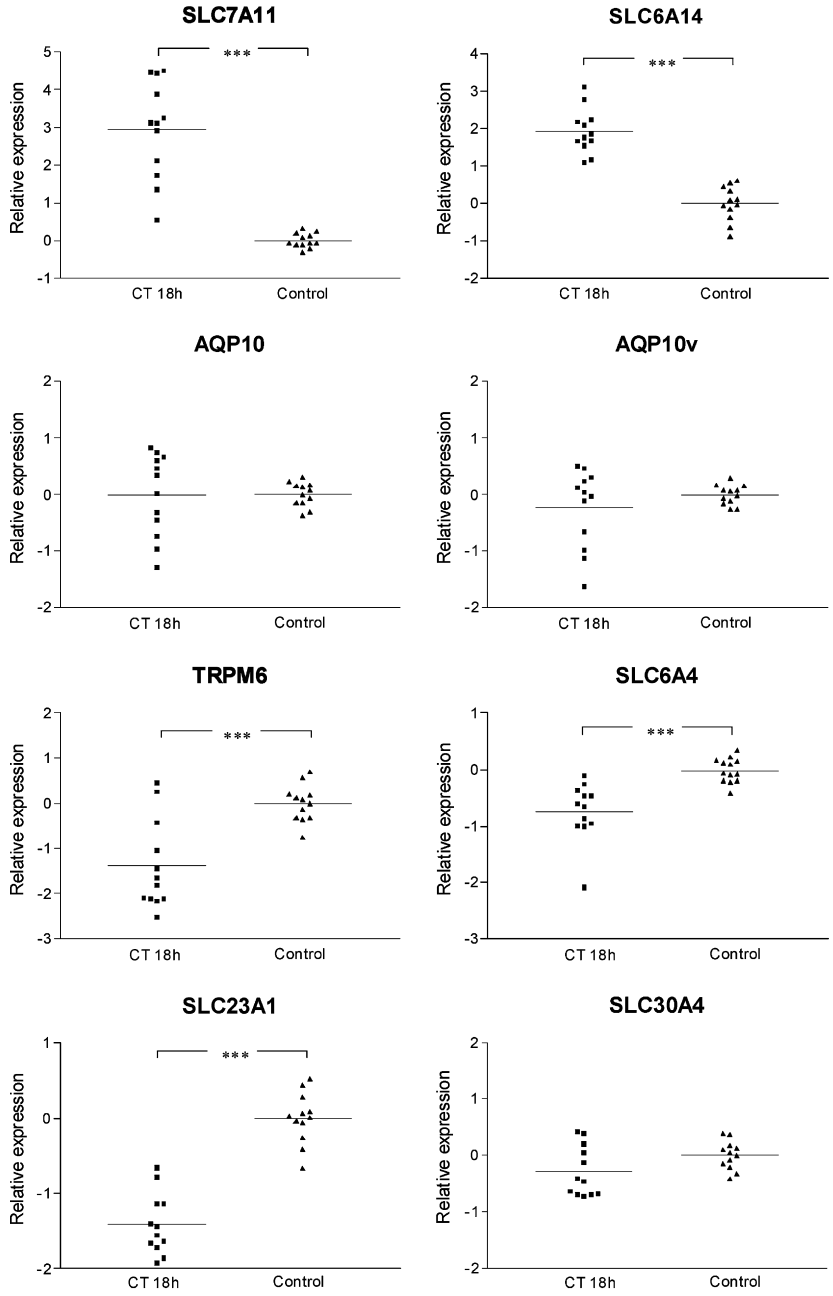

Fig. 2. Expression of transporters in CaCo-2 cells stimulated with CT for 18 h. The relative expression values are adjusted transcript abundance $\log_2$ ratios, target gene:GAPDH, measured by RT-PCR. Mean values are indicated by horizontal bars. A significant difference between the CT-challenged group and the control is denoted by ** ($P < 0.01$) and *** ($P < 0.001$). The data are based on three independent experiments (four cell cultures per group and experiment).

charged amino acids, whereas the latter is dependent on Cl^- and highly specific for negatively charged amino acids, which suggests a broad uptake of amino acids during cholera. The results also suggest that the addition of amino acids to the ORS used to treat cholera patients should be reconsidered. In accordance with the up-regulation of SLC7A11, it has been shown that CT and cAMP can induce the expression of this transporter in astrocytes and a neuronal cell line [18,19]. The up-regulation of SLC7A11 may protect against oxidative stress, as the transported cystine is the precursor for the antioxidant glutathione. Regarding the up-regulation of SLC6A14, we have found in an earlier study that CT induces the expression of a Na^+-coupled transporter for another major nutrient (concentrative nucleoside transporter 2) in the rat small intestine, a host response that possibly counteracts the secretory process [10]. That the SLC6A14 protein was found to be localised to the brush-border membrane by immunohistochemistry further emphasizes its role in the absorptive process.

SLC6A4, which encodes the serotonin transporter SERT, was down-regulated during the acute stage of cholera. SERT has earlier been shown, and was confirmed here, to be expressed in the intestinal epithelial cells [20,21], and has been identified as the transporter responsible for turning off the 5-HT signalling [22,23]. This, together with the fact that the

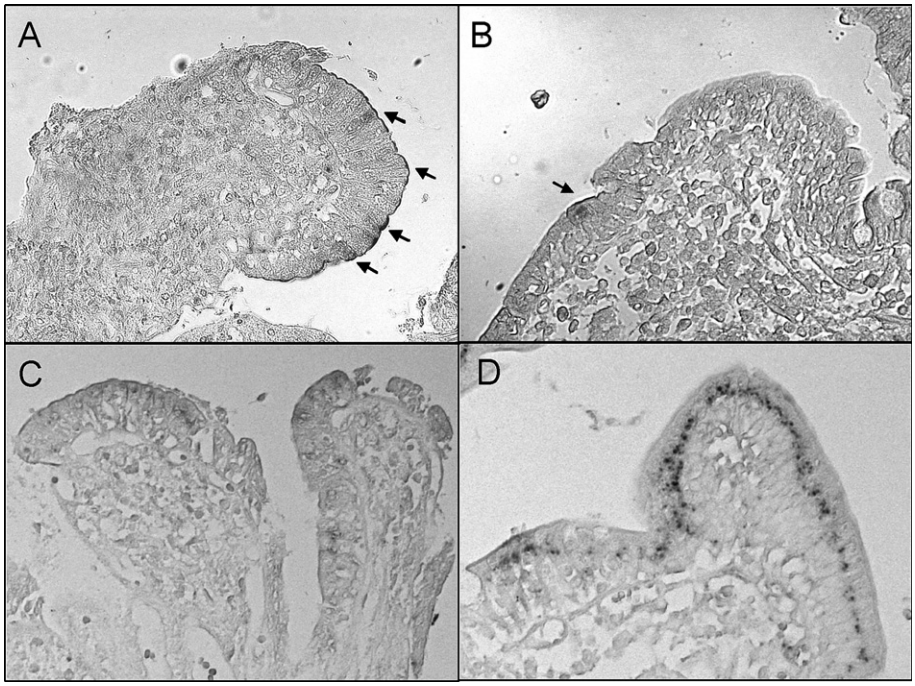

Fig. 3. Immunolocalization of SLC6A14 (A,B) and SERT (C,D) during the acute (A,C) and convalescent (B,D) stage of cholera. Expression of SLC6A14 protein in the brush-border membrane of the duodenal epithelium is indicated by arrows.

up-regulated SLC6A14 transports tryptophan, the precursor for 5-HT, would potentiate the 5-HT-mediated secretion observed during cholera. The finding that CT regulates these transporters in the same manner in CaCo-2 cells further suggests that epithelial cells are involved in the 5-HT signalling in the gut mucosa. Indeed, the epithelial cells are the most likely candidates to terminate the 5-HT signalling through SERT-mediated uptake [20,23]. Furthermore, transgenic mice lacking SERT show abnormal gastrointestinal motility associated with increased volume of water in their stool [24].

The down-regulated AQP10 was initially shown to be expressed in villous epithelial cells of the human small intestine [15]. This would be in good agreement with the location proposed for rodent AQP8 [25,26], the AQP shown to be down-regulated after CT challenge in the rat intestine [10]. However, it has been reported recently that the splice variant AQP10v is located in capillary endothelial cells, whereas AQP10 is located in endocrine cells of the small intestine [27]. Our results show that AQP10v is down-regulated during acute cholera. In addition, a reduced level of AQP10 transcripts was also detected using a primer pair that catches both of the AQP10 splice forms. The sustained effect using the AQP10 primer pair is accompanied by a significantly higher expression signal than when using the AQP10v primer pair, indicating that both splice forms are affected. In accordance with the suggested localization of the AQP10 proteins, we detected no significant change in the expression in CT-challenged enterocytes in vitro, although the AQP10 transcripts were detected in these cells, suggesting that the effects observed during cholera are restricted to other cell types. In any case, a reduced expression of AQPs should reduce the water permeability of the cell membranes and thus limit the secretory response.

Three of the down-regulated transporters are involved in the intestinal uptake of vitamins and minerals: SLC23A1 (vitamin C), SLC30A4 (zinc) and TRPM6 (magnesium) [28–30]. Of these, the down-regulation of SLC23A1 and SLC30A4 may be of particular interest, since vitamin C has been shown to increase the "open" probability and thus the Cl^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) [31], the main target for the modulatory action of CT on Cl^- secretion [32]. Also, there are significant losses of zinc during diarrhoea and zinc replenishment has been reported to reduce the recovery time in children with persistent diarrhoea [33]. Zinc has also been shown to inhibit CT-induced secretion in a CaCo-2 cell model [34]. In addition, SLC30A2, another zinc transporter, which like SLC30A4 is believed to function in the sequestration of zinc by intracellular vesicles, is down-regulated upon severe zinc depletion in a rat model [35]. Thus, the down-regulation of SLC30A4 during acute cholera is probably an effect of intestinal zinc losses and not a result of a direct effect on the epithelial cells, which is supported by the results that CT did not affect the gene expression in the CaCo-2 cell model. The down-regulation of the magnesium channel TRPM6 is probably an attempt from the host to limit the magnesium losses that are known to occur during cholera [36].

In conclusion, we have determined the differential transcription of seven membrane transporters during acute cholera compared to convalescence. The majority of these changes seems to be due to the direct effect of CT on epithelial cells. These results may add new clues to help in elucidation of the secretory response observed during cholera, and how it is counteracted by the host. They may also suggest new therapeutic targets.

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