

# Cholera toxin induces expression of ion channels and carriers in rat small intestinal mucosa

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**Abstract** Cholera toxin causes cyclic adenosine monophosphate (cAMP)-induced electrolyte and water secretion in the small intestine. The toxin-induced change in gene expression in rat small intestine was evaluated with microarray technique and the results were confirmed by semiquantitative polymerase chain reaction (PCR). The transporter CNT2 for nucleosides was upregulated between 6 and 18 h after challenge, whereas the level of GLUT1 transporter for glucose became elevated at 6 h. Both changes probably facilitate uptake of these nutrients in the gut. At 18 h, the major chloride channel in the villus, CIC2, was upregulated. Aquaporin 8 was downregulated at 6 h and two mucin-producing genes were upregulated 18 h after toxin challenge. The expression was back to normal after 72 h, which is the turnover time for intestinal epithelial cells.

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**Key words:** Microarray; Cholera toxin; Gene expression; Membrane transporter; Mucin

## 1. Introduction

*Vibrio cholerae* causes disease by colonising the small intestine and producing cholera toxin (CT), a potent enterotoxin that affects the epithelial cells, inducing massive salt and water secretion which results in severe diarrhoea. The toxin consists of five B-subunits which bind to the ganglioside GM1, present on most mammalian cells, and one enzymatically active A-subunit [1]. After binding to GM1 at the apical side of the epithelial brush border, the toxin is transported retrogradely to the endoplasmic reticulum [2]. In this compartment the A1 chain of the A-subunit is released and transported to the cytosol [3] where it catalyses the adenosine diphosphate (ADP) ribosylation of the  $G_{s\alpha}$  protein. This leads to an activation of adenylate cyclase and cyclic adenosine monophosphate (cAMP)-dependent net secretion of electrolytes and water from the serosal to the mucosal side of the epithelium.

CT also stimulates release of the secretagogue serotonin (5-HT) from enterochromaffin cells [4–6] as well as a rapid secretion of mucin from goblet cells [7,8] which, together with the water secretion and increased motility of the bowel, will help to clear the mucosa from the *V. cholerae* bacteria. The CT-induced net secretion of ions depends mainly on two events: the active secretion of chloride ions and the simultaneous inhibition of absorption of  $Na^+$  and  $Cl^-$  ions in the epithelium. The classical model of intestinal secretion postulates that  $Cl^-$  secretion occurs primarily from the crypts [9]. However, the chloride channel 2 (CIC2), predominantly expressed in the villi, has been suggested to contribute to the native  $Cl^-$  secretion [10].

The coupled absorption of  $Na^+$  and  $Cl^-$  in the intestinal mucosa occurs by the dual operation of  $Na^+/H^+$  and  $Cl^-/HCO_3^-$  exchangers. These channels are blocked in villi cells by the CT-induced cAMP and 5-HT, respectively [11,12]. Under normal conditions, however,  $Na^+$  is also absorbed together with other substances present in the diet. In the intestinal epithelium there are a number of transporters that take advantage of the  $Na^+$  gradient, generated by the  $Na^+/K^+$  adenosine triphosphatase (ATPase) at the basolateral membrane, to cotransport sugars, amino acids and nucleosides. Hence, it has been known for many years that oral treatment with a glucose-based electrolyte solution augments the  $Na^+$  reabsorption, and thus moderates the water loss during the cholera disease [13]. In this case,  $Na^+$ -dependent glucose transporters (SGLTs) are responsible for cotransport of  $Na^+$  and glucose over the apical membrane. The absorbed sugars are then transported over the basolateral membrane by facilitative sugar transporters (GLUTs), whereas the reabsorbed  $Na^+$  can be transported to the serosal side by an active  $Na^+/K^+$  ATPase [14]. In a similar manner,  $Na^+$  is reabsorbed together with nucleosides via the concentrative nucleoside transporter 2 (CNT2) [15].

Over the last 10 years, the role of aquaporins (AQPs) in water transport has been widely discussed. The direction of water flux through these channels is determined by the osmotic gradient [16,17]. However, the transport of water by this route can be regulated by altered expression, membrane insertion or gating of the AQPs [16]. Further, CT has been shown to affect the water permeability of AQPs [18].

The aim of the present work was to screen for changes in gene expression, induced by treatment with CT, of a great number of transporters in the intestinal mucosa. Rats were challenged with CT and the transcript levels were assayed by the microarray technique.

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**Abbreviations:** CT, cholera toxin; CIC2, chloride channel 2; GLUT, glucose transporter; CNT2, concentrative nucleoside transporter 2; AQP, aquaporin; MUC2, mucin 2; MUC3, mucin 3

Table 1  
Primers and MgCl<sub>2</sub> concentrations used for RT-PCR

Gene	Forward primer	Reverse primer	[MgCl <sub>2</sub> ] (mM)
AQP8	5'-cccctagccccattctccattgg-3'	5'-ggaagtcccagtagccagccatcac-3'	4
CNT2	5'-catagggatcacactgggaggttgac-3'	5'-cactgatgaaggaaacacaggcacctg-3'	4
GLUT1	5'-cccgtctctctgctcatcaatcg-3'	5'-tgaccttctctctccgcacatctg-3'	4
CIC2	5'-gcaaaagctaaggaaagcccagatgtc-3'	5'-cctgagtcctctgtgttcacctggaag-3'	5
MUC2	5'-tcctaccagtggcaatggtggtgac-3'	5'-cccagctctgtccagtcgagtttg-3'	4
MUC3	5'-tgacacggatctgtggagctgaagac-3'	5'-tccagcatcgtctctctcgtatccattc-3'	5
GAPDH	5'-gagaaggctggggctcacctgaag-3'	5'-gcattgctgacaatcttgaggaggtt-3'	5

## 2. Materials and methods

### 2.1. Animals and challenge

Male Sprague–Dawley strain rats (ALAB, Sollentuna, Sweden) 8–12 weeks of age were used in all experiments. The experiments were performed after obtaining approval from the local research ethical committee. The animals were housed in an environmental room at 22°C and 58–65% relative humidity, with a controlled 12 h light–dark cycle, and with free access to a standard pellet diet and tap water. For at least 7 days before the experiments, the rats were kept in cages containing up to five animals and deprived of food for the 24-h period preceding the experiment.

Rats were given, intragastrically by use of a baby-feeding catheter (outer diameter = 1.7 mm) and under Isoflurane<sup>®</sup> anaesthesia, 100 µg CT (List Biological Laboratories) dissolved in 5 ml phosphate-buffered saline (PBS) containing 6% NaHCO<sub>3</sub> and 0.5% albumin as described previously [19]. As a positive control osmotic secretion was induced by 4% sorbitol in PBS [20]. As a negative control vehicle was given alone intragastrically.

### 2.2. RNA isolation

The rats were killed 2, 6, 18 or 72 h after intragastric administration and the small intestine was excised. The jejunum was opened longitudinally and the proximal part was scraped with a knife, with the start-point some 40 cm from the pylorus, to cut off the upper parts of the villi [21]. The tissue samples were immediately homogenised in lysis buffer from the GenElute Mammalian Total RNA Kit (Sigma) and total RNA was isolated according to the manufacturer's instructions. The RNA concentration was measured spectrophotometrically and the quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was used for real-time polymerase chain reaction (RT-PCR) analysis and in some cases for microarray analysis.

### 2.3. Microarray experiments

The microarray experiments were performed in two rounds. Groups of three rats were used. In the first experiment, RNA from negative control rats and from rats challenged with CT for 2 h was used. In the second round, RNA from three groups was used; negative control, 6 h CT challenge and 18 h CT challenge. RGU34A GeneChips (Affymetrix) were used in all experiments. The RNA was converted into labelled target cRNA and hybridised to the GeneChip followed by washing, staining and scanning according to the GeneChip Expression Analysis manual (Affymetrix).

The resulting images were analysed with Affymetrix Microarray Suite 5.0 software. All probe sets were used for scaling and normalisation. Default values were used during the analysis. All CT-challenged samples were compared with all control samples run on the same occasion, which gave a total of nine comparisons for each group. A mean value was calculated from the signal log<sub>2</sub> ratios for each gene and group. A 1.75-fold change, i.e. an average log<sub>2</sub> ratio of above 0.8 or below −0.8 for at least one timepoint, was used as a threshold value during the screening process.

### 2.4. Relative quantification by RT-PCR

5 µg of total RNA for each sample was converted into cDNA in a volume of 20 µl. The reaction consisted of 1× first-strand buffer, 10 mM dithiothreitol (DTT), 1 mM advantage ultrapure deoxynucleotide mix, 1 µl PowerScript reverse transcriptase (all purchased from Clontech Laboratories) and 3.75 µM random hexamer primers (Applied Biosystems). The RNA was reverse transcribed for 1 h at 42°C and

the reactions were then terminated by 15 min incubation at 70°C. The cDNA was diluted 1:50 in ribonuclease-free water. The diluted cDNA was used for relative quantification by RT-PCR.

Oligonucleotide primers were designed using the MacVector 4.1 software to obtain primer pairs with optimal annealing temperature of approximately 58°C (Table 1). The primers were purchased from Scandinavian Gene Synthesis (Köping, Sweden). The Lightcycler-FastStart DNA Master SYBR Green I Kit (Roche) was used for all reactions according to the manufacturer's instructions. The MgCl<sub>2</sub> concentration was optimised for each primer pair to obtain a single-peak melting curve. After an initial 10 min preincubation at 95°C, the mixtures were subjected to 42 cycles of a three-step PCR, comprising 15 s of denaturation at 95°C, a 4-s annealing phase at 58°C and a 6-s elongation phase at 72°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene in all experiments. Due to interexperimental variation, only samples processed on the same day were compared with each other. The relative levels of transcripts as ratios between negative control and challenged rats were calculated for each target gene by the formula described by Pfaffl [22]. The ratios were converted into log<sub>2</sub> values.

### 2.5. Histochemical staining

Rats were killed 30 min and 2 h after CT challenge. Samples from the jejunum were fixed in 4% buffered formaldehyde, embedded in paraffin and sectioned at 4 µm. To visualise the mucus in the goblet cells, the sections were stained with the PAS method (oxidation in periodic acid followed by staining with Schiff's reagent), counterstained with haematoxylin, dehydrated and mounted.

### 2.6. Statistical analysis

Statistical analysis of the data expressed as log<sub>2</sub> ratios was performed by using One sample *t*-test with the hypothetical value set to zero.

## 3. Results

To elucidate the effects of CT on gene expression *in vivo*, we challenged rats perorally with CT for 2, 6 or 18 h. During this period a net fluid secretion takes place in the jejunum of the CT-treated animals [19,23]. Alterations in expression of genes associated with secretion and transport over membranes in the jejunal villi were screened for by the microarray technique. Four transcripts known to encode transporters did reach the threshold value. CNT2, GLUT1 and CIC2 were upregulated, whereas AQP8 was found to be downregulated in the CT-treated animals (Fig. 1). The most pronounced effects were seen 6 h after CT challenge for AQP8 and GLUT1 and after 18 h for CNT2 and CIC2. Of the four differently expressed transcripts detected only two, AQP8 and CNT2, showed a change greater than 2-fold at any timepoint. A number of transporter transcripts were present in the intestinal villi epithelium but were not changed after challenge with CT, these included three Cl<sup>−</sup> channels, five K<sup>+</sup> channels, one Na<sup>+</sup> channel, three different Na<sup>+</sup>/K<sup>+</sup> ATPase subunits, three Na<sup>+</sup>/H<sup>+</sup> exchangers, two Cl<sup>−</sup>/HCO<sub>3</sub><sup>−</sup> exchangers, six Na<sup>+</sup> cotransporters, three GLUTs and four AQPs.

The altered expression patterns after challenge with CT

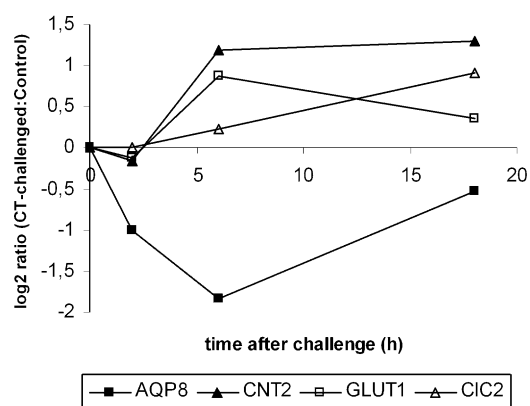


Fig. 1. Differently expressed transporters after peroral challenge with 100  $\mu$ g CT. Transcript abundance ratios, challenged:control, plotted over time were measured by the microarray technique. Each value represents the mean of a group of three animals. The different transcripts correspond to the following probe sets on the RGU34A array: AB005547\_at (AQP8), U66723\_s\_at (CNT2), S68135\_s\_at (GLUT1) and AF005720mRNA#3\_s\_at (CIC2).

were confirmed by RT-PCR (Fig. 2). A larger number of animals were used in each group compared to the microarray analysis. Thus, somewhat different expression patterns were detected by RT-PCR analysis. However, the tendency was the same for all genes examined, irrespective of whether microarray or RT-PCR was used. The level of AQP8 transcripts was shown to be significantly decreased in the CT-treated rats at 6 h ( $P < 0.001$ ), whereas the level of CNT2 transcripts was increased at 2 ( $P < 0.01$ ), 6 ( $P < 0.01$ ) and 18 h ( $P < 0.001$ ). In agreement with the microarray analysis, the most pronounced increase in the levels of GLUT1 and CIC2 transcripts was seen at 6 ( $P < 0.05$ ) and 18 h ( $P < 0.01$ ), respectively. The level of the examined transcripts in the CT-challenged rats had reverted to the same level, as seen in control animals, after 72 h (Fig. 2).

Rats were challenged with 4% sorbitol in order to investigate whether altered expression of AQP8 and CNT2 was due to the physiological conditions seen in the CT-treated animals, or to a more CT-specific response. Peroral challenge with 4% sorbitol induces a rapid and severe hypersecretory response, thus making it a good control. No significant difference could be seen in the levels of AQP8 and CNT2 transcripts between challenged and unchallenged rats at any timepoint (Fig. 3).

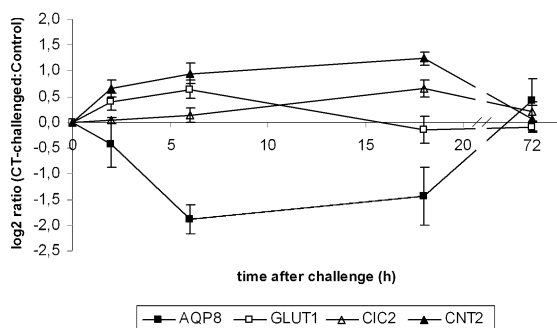


Fig. 2. Expression of transporters after peroral challenge with 100  $\mu$ g CT. Transcript abundance ratios, challenged:control, plotted over time were measured by RT-PCR. Each value represents the mean  $\pm$  S.E.M. of a group of four to nine animals.

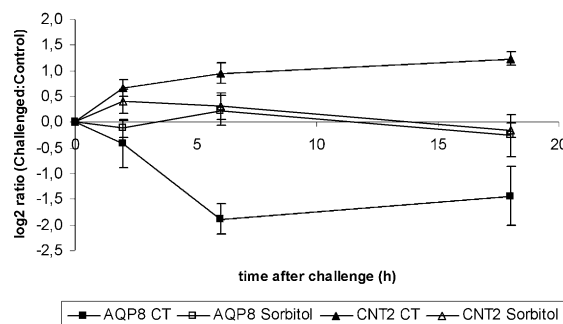


Fig. 3. Expression of transporters after peroral challenge with either 100  $\mu$ g CT or 4% sorbitol. Transcript abundance ratios, challenged:control, plotted over time were measured by RT-PCR. Each value represents the mean  $\pm$  S.E.M. of a group of four to nine animals.

A well-known intestinal effect of CT is the secretion of mucin from goblet cells, which was confirmed by staining jejunal sections from rats challenged with CT for 30 min and 2 h with the PAS method (Fig. 4A). It was interesting, therefore, to notice that microarray analysis revealed increased levels of transcripts encoded by two different mucin genes, mucin 2 (MUC2) and mucin 3 (MUC3), 18 h after CT challenge with no change at 2 and 6 h (data not shown). The increased level of MUC2 and MUC3 transcripts was confirmed by RT-PCR ( $P < 0.0001$  and  $P < 0.05$ , respectively) (Fig. 4B).

#### 4. Discussion

The changes in gene expression induced by CT involve both carriers and channels. Three of the six differently expressed transcripts detected probably counteract the secretory process: the decrease in AQP8 should reduce the fluid transport across the epithelium, whereas the upregulation of the CNT2 and the GLUT1 probably leads to increased absorption of saline and water. In contrast, upregulation of the CIC2 should enhance the fluid secretion. Finally, the increased synthesis of mucins contributes to restoring the destroyed mucus layer in the gut. Due to replacement of the epithelial cells after 2–3 days, the expression of the genes had normalised 72 h after the challenge with CT.

Of the transporters known to be involved in sodium reabsorption and giving present calls in the microarray analysis, two changed above the threshold value: GLUT1 and CNT2. The upregulation of GLUT1 may be of great importance in light of the use of glucose in oral rehydration solutions. GLUT1 is localised to the basolateral membrane of the epithelial cells and transports glucose to the serosal side. This transport accompanies the coupled absorption of sodium and glucose which takes place over the apical membrane. An increased expression of GLUT1 suggests a more efficient absorption of sodium during treatment with glucose-based rehydration solutions. The second induced transporter with a putative role in sodium reabsorption is CNT2. This  $\text{Na}^+$ /nucleoside cotransporter has been cloned recently and only a few studies have been reported. However, a role for CNT2 has been suggested in sodium reabsorption over epididymal epithelium [15]. The upregulation of CNT2 is probably of importance for the uptake of nucleosides and sodium during cholera and related diarrhoeal diseases. Thus, addition of nucleoside to oral rehydration solutions should be considered.



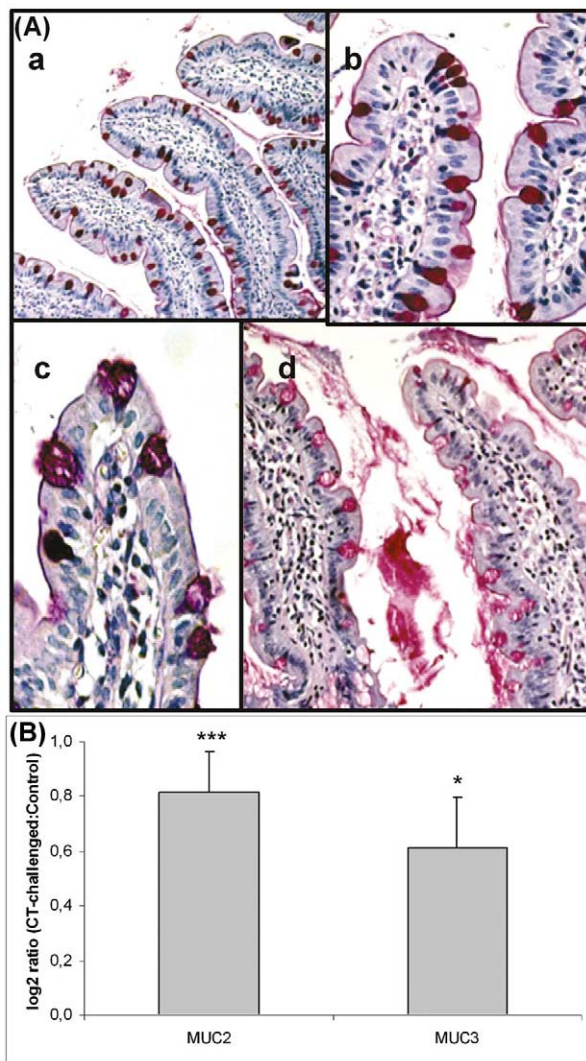


Fig. 4. Secretion and resynthesis of mucus after peroral challenge with 100 µg CT. A: PAS-stained jejunal sections after CT challenge show the secretion of mucins into the lumen by goblet cells. The tissues were taken from rats given vehicle only (a and b) and from rats challenged with CT for 30 min (c) and 2 h (d). The magnification was  $\times 20$  for a and d, and  $\times 40$  for b and c. B: Transcript abundance ratios, challenged:control, for MUC2 and MUC3 were measured by RT-PCR 18 h after challenge with CT. Each value represents the mean of a group of nine animals. Error bars represent S.E.M. A significant increase compared to the control is denoted by \* ( $P < 0.05$ ) and \*\*\* ( $P < 0.001$ ).

The decrease in AQP8 after CT challenge was probably triggered by the elevated cAMP level, which has been shown previously to influence the insertion of AQP8 into the plasma membrane [24]. In accordance with this, under normal conditions AQP8 is predominantly located in intracellular vesicles in the small intestine [25,26]. AQP8 has been suggested to be a player in a proposed model for water transport during canalicular bile secretion [27]. In addition, CT has been shown to have an effect on the permeability of AQPs for water [18]. Thus, CT affects AQPs at different levels: transcription, cellular localisation and transport capacity. The downregulation of AQP8 may therefore play a considerable role in diminishing water transport during cholera.

The CIC2 is mainly expressed in epithelial cells of the jeju-

nal villus [28,29]. Recently, it was shown that they contribute to native chloride currents in Caco-2 intestocytes measured by patch clamp electrophysiology [10]. The quantitative contribution of this channel to the total chloride flux remains to be evaluated. The present results have revealed that CIC2 was upregulated 18 h after CT administration, which would probably increase the fluid response.

The increase in MUC2 and MUC3 expression is probably a consequence of the release of the mucin content from the goblet cells triggered by CT [7,8]. This agrees with earlier studies showing that CT induces secretion of both preformed and newly synthesised mucin in cultured goblet cells [30,31]. It is of great importance for the host to re-establish a functional mucus layer since it provides a physical barrier to larger-sized molecules, including bacterial toxins. The upregulation of intestinal mucins enhances this process.

In conclusion, CT-induced effects on the gene expression of four transporters and two mucin genes were detected in our microarray experiments. The observed changes were confirmed by semiquantitative PCR. This shows that the microarray technique was reproducible in our *in vivo* experiments with rat intestinal mucosa.

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