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Original article

Cytotoxic effect of Shiga toxin-2 holotoxin and its B subunit on human renal tubular epithelial cells

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Abstract

Shiga toxin-producing *Escherichia coli* produces watery diarrhea, hemorrhagic colitis and hemolytic–uremic syndrome (HUS). In Argentina, HUS is the most common cause of acute renal failure in children. The purpose of the present study was to examine the cytotoxicity of Stx type 2 (Stx2 holotoxin) and its B subunit (Stx2 B subunit) on human renal tubular epithelial cells (HRTEC), in the presence and absence of inflammatory factors. Cell morphology, cell viability, protein synthesis and apoptosis were measured. HRTEC are sensitive to both Stx2 holotoxin and Stx2 B subunit in a dose- and time-dependent manner. IL-1, LPS and butyrate but not TNF, IL-6 and IL-8, increased the Stx mediated cytotoxicity. The effects of Stx2 B subunit appear at doses higher than those used for Stx2 holotoxin. Although the physiological importance of these effects is not clear, it is important to be aware of any potentially toxic activity in the B subunit, given that it has been proposed for use in a vaccine.
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Keywords: Proximal tubule; Hemolytic uremic syndrome; Acute renal failure; Cytotoxicity; Shiga toxin; Inflammatory factors

1. Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) colonizes the distal small intestine and colon causing a spectrum of disorders, including watery diarrhea, hemorrhagic colitis, and hemolytic–uremic syndrome (HUS) [1]. It has been estimated that HUS is the most common cause of acute renal failure in infants of Argentina. *E. coli* serogroup O157 bacteria expressing both Stx1 and Stx2 are the microorganisms most frequently isolated from children with HUS, although strains that express only Stx2 are highly prevalent in Argentina [2,3]. In spite of the fact that the detailed pathogenesis of HUS still remains unknown, Stx1 and Stx2 have been unambiguously associated with the disease [4]. Stx is a 71 kDa complex comprised of one A subunit (32 kDa) and five B subunits (7.7 kDa). The holotoxin binds via a B subunit pentamer to the globotriaosylceramide (Gb3) receptor located on the plasma membrane of target cells. Binding of Stx to Gb3 is the primary determinant of its cytotoxic effects and results in toxin internalization and cell killing by inactivation of 60S host cell ribosomes [5,6]. Histopathological studies in the animals orally infected with STEC or intravenously injected with Stxs showed swollen and detached endothelial cells, increased fibrinogen deposition in the glomeruli, organelle disintegration and necrosis of renal tubules [7]. Therefore, the glomerular endothelium and also the tubular epithelium may contribute to the renal failure observed in HUS. The finding that human renal tubular epithelial cells (HRTEC) express high levels of Gb3 as compared to epithelial cells found in other organs and that they are responsive to Stx1 and Stx2 raised the possibility that the toxin could have a significant impact on tubular cells. While the inhibition of protein synthesis by the active A subunit is considered responsible for these events, other biological actions appear to contribute to the mechanism by which Stx damages or kills target cells [8]. Although the inflammatory cytokine response to HUS has not been extensively characterized, it is well known that Stx may act in concert with inflammatory cytokines to elicit cellular dysfunction. Stx can induce the production of tumor necrosis factor (TNF) and interleukins-1 (IL-1) and -6 (IL-6), by macrophages [9] rendering the endothelial

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cells more sensitive to the toxin [10]. Butyrate, which may be elevated in the circulation of patients with HUS because of disruption of colonic epithelium, and lipopolysaccharide (LPS), may be absorbed from the inflamed gastrointestinal tract [11,12]. Interestingly, urinary excretion of TNF, IL-6 and IL-8 is high during the acute phase of HUS and gradually decreases until recovery correlating well with the degree of renal injury [13,14]. In addition, renal proximal tubular cells are sensitive to the cytotoxic effects of Stx, and release cytokines that may up-regulate epithelial cell sensitivity to Stx [15]. Therefore, a crucial role of the inflammation system has been suggested in HUS. Nevertheless, attempts to prove that patients with acute episodes of HUS have elevated systemic proinflammatory cytokine levels in comparison to those in children with infectious diarrhea have failed [16]. An alteration in the balance between proinflammatory and anti-inflammatory cytokines seems not to be a residual effect of the infection but a preexisting characteristic of the patient. Experiments that can evaluate how Stx is enhanced by inflammatory cytokines in the kidney will help to understand the role of renal cytokines in children with typical HUS. Stx induces apoptosis in many cell types [17,18]. Apoptosis of renal cortical cells has been shown in renal tissues obtained from HUS cases and in vitro [19,20], suggesting that the toxin may trigger programmed cell death by different mechanisms [21]. It has recently been reported that newly expressed genes are important to the induction of apoptosis [22]. The A subunit from Stx2 but not from Stx1 induces apoptosis by a sequence capable of binding anti-apoptotic proteins, which may provide a second mechanism for Stx2-induced cell death [23].

The pentamer B subunit can also induce apoptosis but at a concentration much higher than that at which holotoxin can. This has been shown in Burkitt's lymphoma cells by the B pentamer from Stxs [24]. Recently, we have reported an inhibition of water absorption in human colonic mucosa in vitro and fluid accumulation in rat colon loops ex vivo caused by the cloned Stx2 B subunit [25]. These effects could occur if Stx2 B subunit modulates specifically the transport mechanisms involved in water absorption in the human colon and/or affects the general mechanisms related with epithelial cell viability.

The purpose of the present study was, therefore, to examine the cytotoxic effects of Stx2 holotoxin and the cloned Stx2 B subunit on primary cultures of HRTEC and to determine if this response can be enhanced by inflammatory factors. These studies will add considerably to understanding the pathogenesis of acute renal injury in Stx HUS.

2. Materials and methods

2.1. Reagents

Purified Stx2 holotoxin was purchased from Denka Seiken Co., Ltd. (*Chuo-Ku*, Tokyo, Japan). *E. coli* O111:B4 LPS, tumor necrosis factor alpha (TNF-α) and human interleukins-1beta (IL-1β), -6 (IL-6), -8 (IL-8) were from R&D System (Minneapolis, MN, USA). RPMI was from GIBCO (Grand Island, NY, USA). Unless stated otherwise, all other reagents were obtained from *Sigma Chemical Co.* (St. Louis, MO, USA). All tissue culture flasks, dishes, and multiwell plates were from Falcon (*Orange Scientific*, Graignette Business Park, Belgium).

2.2. Stx2 B subunit purification

Stx2 B subunit was purified by metal-affinity chromatography under native conditions as described in our previous article [25]. Briefly, recombinant *E. coli* containing the *stx2b* gene fused in frame with the 6XHis tag gene from pQE-70 vector (*QIAGEN*, Hilden, Germany) were cultured in LB supplemented with 100 μg/ml ampicilin until an OD_{600} of 0.6. The bacterial pellet obtained after centrifugation at 6500 rpm for 10 min was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl) and incubated on ice for 30 min. The suspension was then sonicated and the supernatant was mixed with a 50% Ni-NTA. The lysate/Ni-NTA mixture was washed and eluted four times with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8).

To determine the Stx2 B subunit purity, all fractions were diluted in loading buffer, separated on a 12.5% SDS polyacrylamide gel, electrotransferred to nitrocellulose membrane (Hybond ECL, *Amersham Pharmacia Biotech*) and immunoblotted with the monoclonal anti-verotoxin II (slt-2b) (*Biodesign International Maine*, USA) directed against the B subunit of Stx2. The third eluted fraction showed a band of approximately 7.5 kDa corresponding to the monomeric conformation of Stx2 B subunit. The quantity of this protein bands was determined on the nitrocellulose membrane by densitometry using β-actin as a reference standard. Stx2 B subunit was then dialyzed against 10 mM phosphate buffered saline (PBS, 145 mM NaCl/10 mM NaH_2PO_4, pH 7.4) to remove imidazole and applied to a HPLC (C4 colonne) using PBS as the elution buffer. A single peak of purified Stx2 B subunit was observed. This purification procedure routinely resulted in approximately 150 μg Stx2 B subunit purified to at least 97% homogeneity from a 1-l culture.

2.3. Cell culture

HRTEC were isolated from kidneys removed from different adult patients undergoing nephrectomies for renal cell carcinoma from the Unidad de Urología, Hospital de Clínicas "José de San Martín", Buenos Aires, Argentina. The removal of part of the renal tissue for research purposes was approved by the ethics committee of the University of Buenos Aires. The cortex was dissected from the renal medulla and the primary culture of the HRTEC was performed according to the methods described previously [26]. Briefly, the cortical fragments were incubated for 1 h at 37 °C in a buffer containing 0.1% collagenase type I (*Sigma Chemical Co*). Cells were

washed, centrifuged and resuspended in RPMI 1640 (*HyClone*) medium supplemented with 5% fetal calf serum (*GIBCO*), 2 mM L-glutamine (*GIBCO*) and 100 U/ml penicillin/streptomycin (both from *GIBCO*). Cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2 and grown to confluence. The cell isolates were trypsinized, concentrated in fetal calf serum containing 5% dimethyl sulfoxide (*Sigma Chemical Co*) and stored in liquid nitrogen for subsequent use. Cells were cultured in flasks or in 96-well or 24-well plates in medium with supplements and 1% endothelial cell growth factor (*Sigma Chemical Co*) and used between three and five passages. By light microscopy, more than 95% of the cells had similar morphologies. These cells were confirmed as epithelial cells by positive staining for cytokeratins (*Sigma Chemical Co*). Less than 10% of cells were positive with the antibody against an epithelial membrane antigen (EMA, *Dako*, Glostrup, Denmark) present in the distal tubular epithelial cells. The presence of fibroblasts was ruled out by lack of reactivity with an antibody directed to human fibroblast common antigen (*Dako*). Furthermore, the cells were also negative for the endothelial cell antibody PECAM CD31 (*Dako*). All samples were developed by immunoperoxidase by using RTU Vectastain Kit (*Vector*, Burlingame, USA). All HRTEC were studied under growth-arrested conditions (with serum free-medium). Barely confluent cells were placed in RPMI 1640 containing 2 mM L-glutamine and 100 U/ml penicillin–streptomycin for 24 h prior to any experimental maneuvers.

2.4. Cell morphology

To study cell morphology, HRTEC were fixed for 2 h at room temperature in fixative solution containing 10% formol in PBS and then stained with hematoxylin–eosin (H and E) and observed in light microscopy (*Zeiss Axiophot*, Germany). Cells count was performed on four fields of × 200 magnification each. Area cell values were obtained by using NIH image software.

2.5. Neutral red cytotoxicity assay

The neutral red cytotoxicity assay was adapted from previously described protocols [19]. HRTEC were plated in 96-well plates and grown to confluence in complete RPMI medium. The cells were then washed in PBS and exposed to 0.001 to 100 ng/ml Stx2 holotoxin or 1 to 10,000 ng/ml Stx2 B subunits in growth-arrested conditions for 24–72 h. Two hundred microliters of freshly diluted neutral red in PBS was added to a final concentration of 50 µg/ml and cells were incubated for additional 3 h at 37 °C in 5% CO_2 incubator. Cells were then washed with 200 µl 1% $CaCl_2$ + 1% formaldehyde and solubilized in 200 µl 1% acetic acid in 50% ethanol. Absorption in each well was read in an automated plate spectrophotometer at 540 nm. Results were expressed as neutral red uptake percent, and 100% represents cells incubated under identical conditions but without toxin treatment. For studies examining the effect of inflammatory factors on Stx2, HRTC were incubated with LPS (1 µg/ml), IL-1β (0.1 ng/ml), butyrate (2 mM), TNF- (1 ng/ml), IL-6 (10 ng/ml) or IL-8 (10 ng/ml) for 24 h in growth-arrested conditions before the addition of the toxin. The cells were then washed in PBS and incubated with 0.001–10 ng/ml Stx2 holotoxin for additional 72 h, followed by analysis of neutral red uptake. To study the effect of inflammatory factors on Stx2 B subunit, a similar protocol was followed using LPS (1 µg/ml), IL-1β (0.1 ng/ml) or butyrate (2 mM) and 10–1000 ng/ml Stx2 B subunit.

2.6. Protein synthesis inhibition assay

HRTEC were grown to confluence in 96-well plates and incubated for 24 and 48 h in growth-arrested conditions either with Stx2 holotoxin or with Stx2 B subunit at the same concentrations as those used for the cytotoxicity assays. At the end of the incubation, plates were washed twice with PBS to remove the toxin and incubated with PBS containing 10 µCi/ml of $[^{35}S]$-methionine for 90 min at 37 °C in 5% CO_2 incubator. The medium was then removed and the cells were washed three times in PBS and three times in 10% trichloroacetic acid on ice for 10 min each. Precipitated proteins were solubilized in 0.5% SDS and 0.1 N NaOH and counted in a liquid scintillation counter. Results were expressed as percent of protein synthesis in toxin-treated cells compared to non-treated control cells.

2.7. DNA fragmentation assay

Since fragmentation of cellular DNA into low-molecular weight oligomers is characteristic of apoptosis, HRTC cells grown to confluence were incubated with different concentrations of either Stx2 holotoxin or Stx2 B subunit for 48 h. The cells were then washed with PBS and lysed with ice-chilled TE (10 mM Tris, pH 7.4; 10 mM EDTA) buffer containing NaCl 5 M and 0.6% SDS. After 30 min of incubation in lysis buffer, the samples were centrifuged at 13,000 × *g* for 20 min. Fragmented DNA remaining in the resulting supernatant solutions was treated with 2 µg of RNAse (*GIBCO*) per ml for 1 h at 37 °C and 0.2 mg of proteinase K (*GIBCO*) adding 10 vol. of 95% ethanol and 1 vol. of 3 M sodium acetate. After 30 min of incubation, the precipitated DNA was collected by centrifugation at 13,000 × *g* for 15 min. The DNA fragments were washed with 70% ethanol, resuspended in 20 µl of TE buffer and electrophoresed on a 1.2% agarose gel electrophoresis. The gel was subsequently stained with ethidium bromine for 30 min. DNA was visualized under ultraviolet light.

2.8. Flow cytometry for cell cycle

To analyze cell cycles, flow cytometry was performed on propidium iodide (PI)-stained cells (10^6) as usually described. Briefly, HRTEC were incubated with different concentrations of either Stx2 or Stx2 B subunit for 72 h and collected

by centrifugation at 750 × g for 5 min. HRTEC were then washed with PBS and fixed in 70% ethanol. After fixation, cells were treated with RNAase (100 µg/ml) and stained with PI (40 µg/ml). The mixed cells were incubated in the dark at room temperature for 30 min, and analyzed with a flow cytometer (Coulter XL). Data were analyzed by using WinMDI 2.8 program.

2.9. Statistical analysis

Data shown are mean ± S.E.M. The statistical significance between two mean values obtained for two different experimental conditions was calculated by the Student' s t-test. All data from the curves were analyzed by one-way analysis of variance (ANOVA). P values < 0.05 were considered significant.

3. Results

Cytotoxicity of Stx2 holotoxin and Stx2 B subunit on HRTEC was assessed by a combination of cell morphology, cell viability, protein synthesis and apoptosis.

3.1. Cell morphology

Fig. 1 shows HRTEC incubated under growth-arrested conditions either with (B) or without (A) Stx2 holotoxin (10 ng/ml) for 24 h or with Stx2 B subunit (100 ng/ml) for 24 and 72 h (C, D). Treatment of HRTEC with Stx2 holotoxin resulted in a clear reduction in viable attached cells (Fig. 1F) and the remaining cells showed a significant increase of cell area when compared to controls (Fig. 1E). These morphological alterations were not observed after 24 h of Stx2 B subunit even at concentrations 10-fold higher than that used

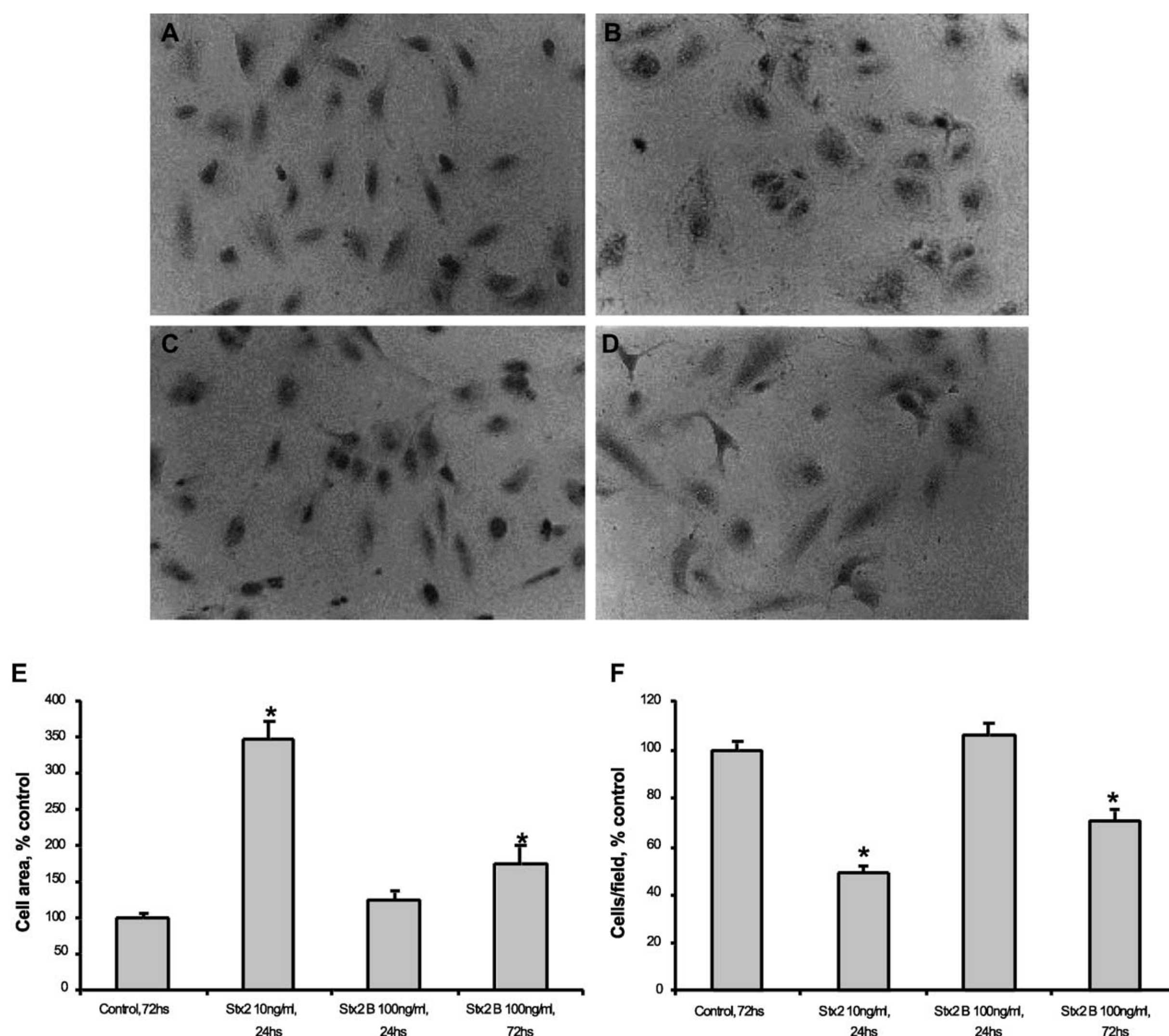

Fig. 1. Effect of Stx2 holotoxin and Stx2 B subunit on the morphology of HRTEC.
Cells were kept in growth-arrested conditions either with (**B**) or without (**A**) 10 ng/ml Stx2 holotoxin for 24 h, or with 100 ng/ml Stx2 B subunit for 24 h (**C**) and 72 h (**D**). H and E, ×200. HRTEC areas (**E**) were measured by using NIH image software and number of HRTEC (**F**) was counted by light microscopy. Data are shown as mean ± S.E.M. of three experiments performed in sextuple and expressed as percent. 100% represent values of cells control. * P < 0.05 was significant at this level.

for Stx2 holotoxin (Fig. 1C, E). There were minimal changes in cell morphology and a few cells were detached when compared to controls (Fig. 1A). After 72 h of exposure to Stx2 B subunit, there was a reduction in the number of cells attached (Fig. 1D, F), which showed an elongated shape with significant increase of cell area when compared to controls (Fig. 1E).

3.2. Inhibition of cytotoxicity and protein synthesis by Stx2 holotoxin and Stx2 B subunits

Alteration in cell morphology generally agreed with neutral red uptake data. As illustrated in Fig. 2, Stx2 holotoxin and Stx2 B subunit caused a reduction of HRTEC viability in a dose- and time-dependent manner. Incubation with 0.001–100 ng/ml Stx2 holotoxin for 24, 48 and 72 h resulted in marked cytotoxic effects (Fig. 2A). These data are consistent with morphological alterations seen above (Fig. 1B). In contrast, 24 and 48 h but not 72 h exposure to 1–100 ng/ml of Stx2 B subunit had no detectable effect on HRTEC viability

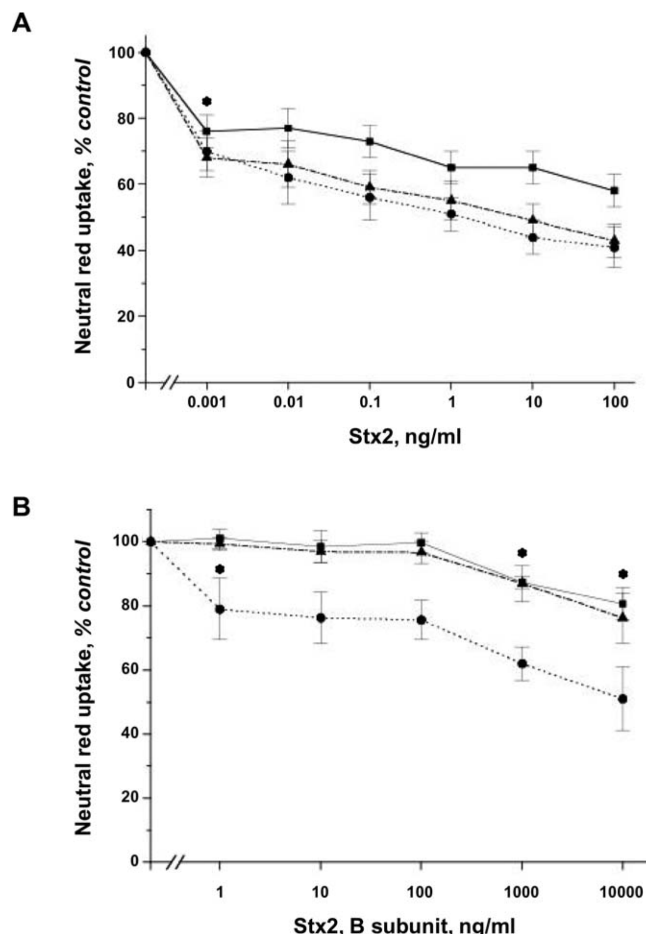

Fig. 2. Cytotoxic effect of Stx2 and Stx2 B subunit on HRTEC.
Cells were incubated under growth-arrested conditions in the presence of Stx2 holotoxin (**A**) or Stx2 B subunit (**B**) for various times, followed by determination of the uptake of neutral red, a measure of cell viability. Symbols are: (■) 24 h, (▲) 48 h, (●) 72 h. Data are shown as mean ± S.E.M. of five to ten experiments performed in sextuple and expressed as percent. Hundred percent represent cells incubated under identical conditions without toxin treatment. * $P < 0.05$ was significant at this level.

(Fig. 2B). Higher concentrations of Stx2 B subunit up to 72 h caused a significant reduction in cell survival (Fig. 2B). These data are also consistent with the light microscopy studies (Fig. 1C, D).

The decrease of viability caused by 1 μg/ml of Stx2 B subunit after 24 h of incubation (85 ± 3% vs. control, $P < 0.05$) was neutralized when Stx2 B subunit was preincubated with a specific anti-Stx2 B antibody (101 ± 2% vs. control, $P > 0.05$).

A comparison of the data obtained indicates that an approximately 1000-fold higher dose of Stx2 B subunit was required to reduce cell survival to the same level as Stx2 holotoxin.

To further evaluate the possibility that these effects could be associated with reduced protein synthesis, ^{35}S-methionine uptakes were studied in HRTEC incubated with Stx2 and Stx2 B subunit at concentrations and time-courses indicated in Fig. 3. Stx2 holotoxin produced a protein synthesis inhibition of approximately 40% at concentrations as low as 10 pg/ml and an incubation time of 1 h (Fig. 3A), while Stx2 B subunit caused an inhibition of protein synthesis of approximately 20% (Fig. 3B) with as much as 100 ng/ml and an incubation time of 12 h.

Together with the cytotoxicity studies described above, these results show a good correlation in cell survival and protein synthesis for HRTEC incubated with Stx2 B subunit. In contrast, Stx2 holotoxin effects on cell viability do not correlate well with protein synthesis. Cell death lagged markedly behind protein synthesis inhibition as has already been reported for Stx1 holotoxin [15].

3.3. Effect of inflammatory factors on HRTEC viability

As illustrated in Fig. 4, the effects of LPS, IL-1ß and butyrate on the cytotoxicity of Stx2 holotoxin and its B subunit on HRTEC were examined. Preincubation of HRTEC with LPS (1 μg/ml), IL-1ß (0.1 ng/ml) or butyrate (2 mM) for 24 hours prior exposure to 0.001, 0.1 and 10 ng/ml Stx2 holotoxin for 72 hours markedly increased the cytotoxic effect of the toxin measured by neutral red uptake (Fig. 4A). TNF-α(1 ng/ml), IL-6 (10 ng/ml) and IL-8 (10 ng/ml) did not alter Stx2 cytotoxicity at the same toxin concentrations and incubation times (Fig. 4A). Under the same experimental conditions, LPS, IL-1ß and butyrate increased the cytotoxicity of Stx2 B-subunit at 10 and 1000 ng/ml (Fig. 4B).

3.4. Apoptogenic activity of Stx2 holotoxin and Stx2 B subunits

To determine whether the cytotoxic effects of Stx2 holotoxin and Stx2 B subunit were, at least in part, due to apoptosis, HRTEC exposed to the toxins were evaluated by DNA analysis (Fig. 5).

HRTEC incubated for 48 h with 0.1 and 10 ng/ml Stx2 holotoxin displayed a characteristic DNA fragmentation pattern when examined by agarose gel electrophoresis (Fig. 5A).

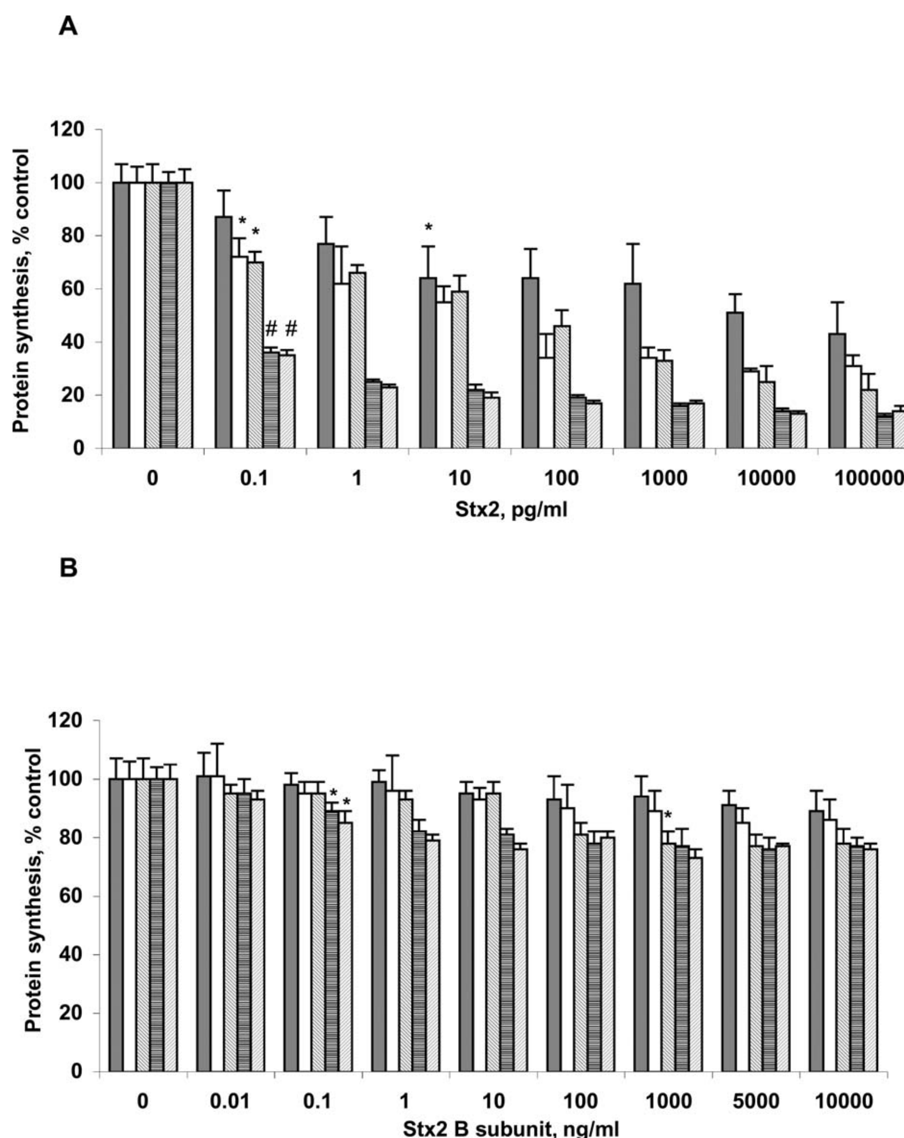

Fig. 3. Inhibitory effect of Stx2 and Stx2 B subunit on protein synthesis in HRTEC.
Cells were incubated under growth-arrested conditions with Stx2 holotoxin (**A**) or Stx2 B subunit (**B**) for 1 h (■), 6 h (□), 12 h (□), 24 h (■) and 48 h (□). Protein synthesis was measured by the incorporation of [^{35}S]-methionine into total cellular protein for 90 min. Data are shown as mean ± S.E.M. of two to four experiments performed in sextuple and expressed as percent. Hundred percent represent values of cells control. *$P < 0.05$; #$P < 0.001$ both were significant at this level.

This pattern appears to be greater than that observed with 1000 ng/ml Stx2 B subunit for 48 h. There was no evidence of DNA fragmentation in control cells until 48 h.

Flow cytometric DNA analysis was performed to quantify diminished stainability (Fig. 5B). The hypodiploid DNA peak of apoptotic cells can be distinguished from the normal diploid DNA peak on the fluorescence profiles of PI-stained cells. DNA content histograms were analyzed after PI-stained-HRTEC were exposed to either Stx2 holotoxin or Stx2 B subunit for 72 h. Control cells with no toxin added (I) exhibited normal cellular distribution in G1 and G2/M phases. Cells exposed to Stx2, 10 ng/ml (II) and Stx2B subunit, 1000 ng/ml (III) lost the normal distribution profile of the cells and exhibited the reduced G1 peak with appearance of a distinct hypodiploid sub-G1 peak (A).

The induction of apoptosis was studied by calculating the percentages of normal cell population (G1 and G2/M) and apoptotic cells (A). The results are shown in Fig. 5C. Stx2 holotoxin significantly increased apoptosis at the different concentrations used (0.001, 0.1 and 10 ng), whereas Stx2B subunit also increased the number of apoptotic cells but at concentrations higher than 1000 ng/ml.

4. Discussion

Stx has been associated with renal tubular injury in the pathogenesis of HUS, although the mechanism by which the toxin causes the disease is still unknown. Recent studies have shown that renal tubular epithelial cells express Gb3 and that

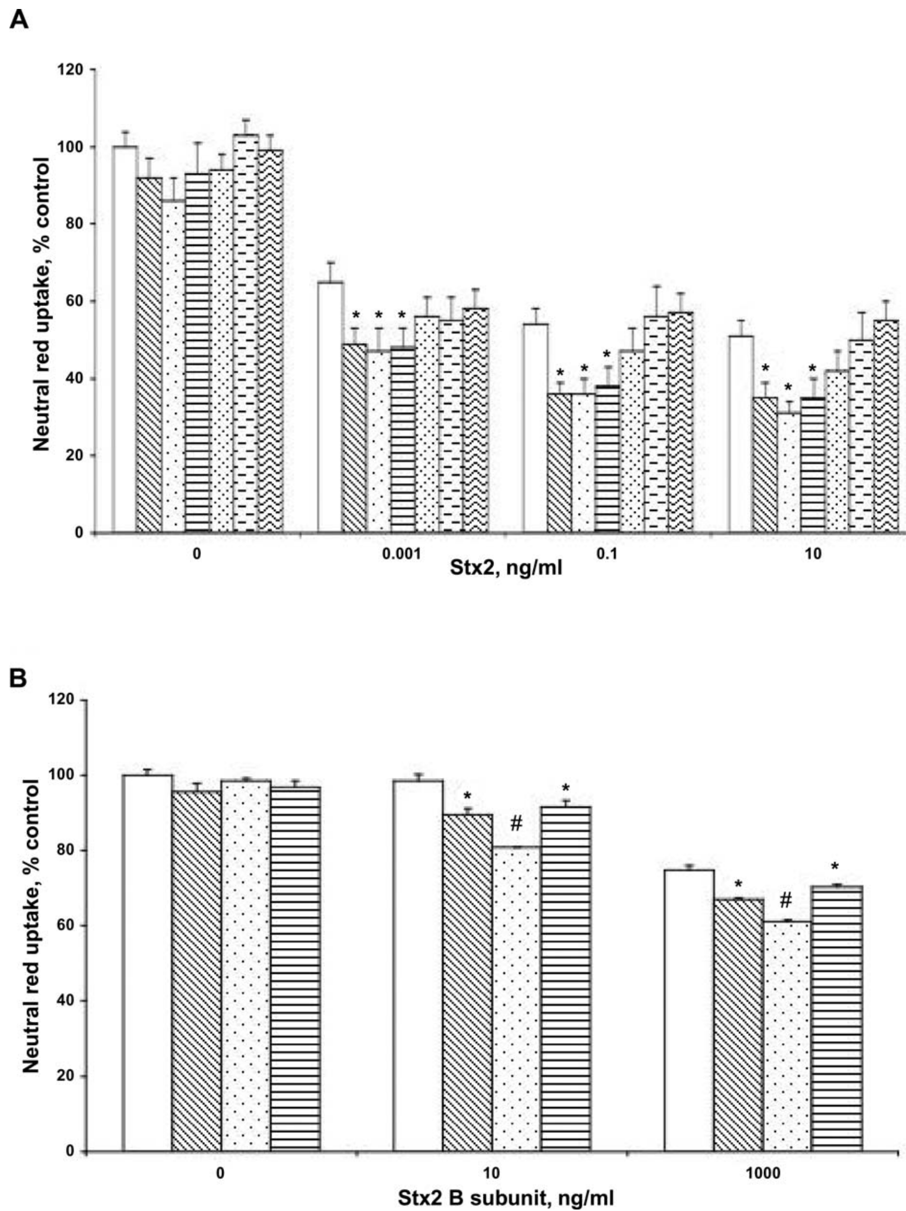

Fig. 4. Effect of inflammatory factors on Stx2 holotoxin and Stx2 B subunit cytotoxicity.
For the studies shown in **A** ($N = 11$) experiments performed in sextuple. HRTEC were preincubated for 24 h with 1 µg/ml LPS, 0.1 ng/ml IL-1β, 2 mM butyrate, 1 ng/ml TNF-α, 10 ng/ml IL-6 or 10 ng/ml IL-8 prior to exposure to 0.001–10 ng/ml Stx2 holotoxin for 72 h and subsequent determination of neutral red uptake. For the studies shown in **B** ($N = 3$) experiments performed in sextuple), HRTEC were preincubated with 1 µg/ml LPS, 0.1 ng/ml IL-1β or 2 mM butyrate for 24 h and then exposed to 10 and 1000 ng/ml Stx2 B subunit. Symbols are: (▧) LPS, (⚄) IL-1β, (▤) butyrate, (⁙) TNF, (▤) IL-6, (▨) IL-8. *$P < 0.05$; #$P < 0.001$, both vs. Stx2 holotoxin or Stx2 B subunit.

Stx indeed can bind to those cells in vivo [26–28] and in vitro [15,19] inducing apoptosis and/or necrosis.

The current study examines the ability of Stx2 holotoxin and the Stx2 B subunit to induce changes in cell morphology, viability, protein synthesis and apoptosis in HRTEC in vitro. Our results indicate that HRTEC are sensitive to both Stx2 holotoxin and its B subunit in a dose- and time-dependent manner. The 50% cytotoxic dose (CD_{50}), corresponding to the dilution required to decrease 50% the viability of HRTEC after 72 h of exposure, was about 1 ng/ml for Stx2 holotoxin and 10,000 ng/ml for Stx2 B subunit. The sensitivity of Vero cells to Stx2 holotoxin has been found to be 1000-fold higher (CD_{50} ~ 1 pg/ml), while no toxic effects have been reported with Stx2 B subunit [25]. The differences in Stx-sensitivity can be attributed to the cell origin since Vero cells constitute a cell line derived from monkey kidney and HRTEC are primary culture cells obtained from human kidney. Previous reports have shown, however, a high sensitivity of proximal tubular cells to Stx1 similar to that seen with Vero cells [15]. Taking into account that Stx1 and Stx2 have the same binding pattern in the kidney [28], differences in Stx-sensitivity may be due to different intracellular responses to both toxins.

Two major intracellular mechanisms for Stx action have been proposed: a) cell necrosis as a result of protein synthe-

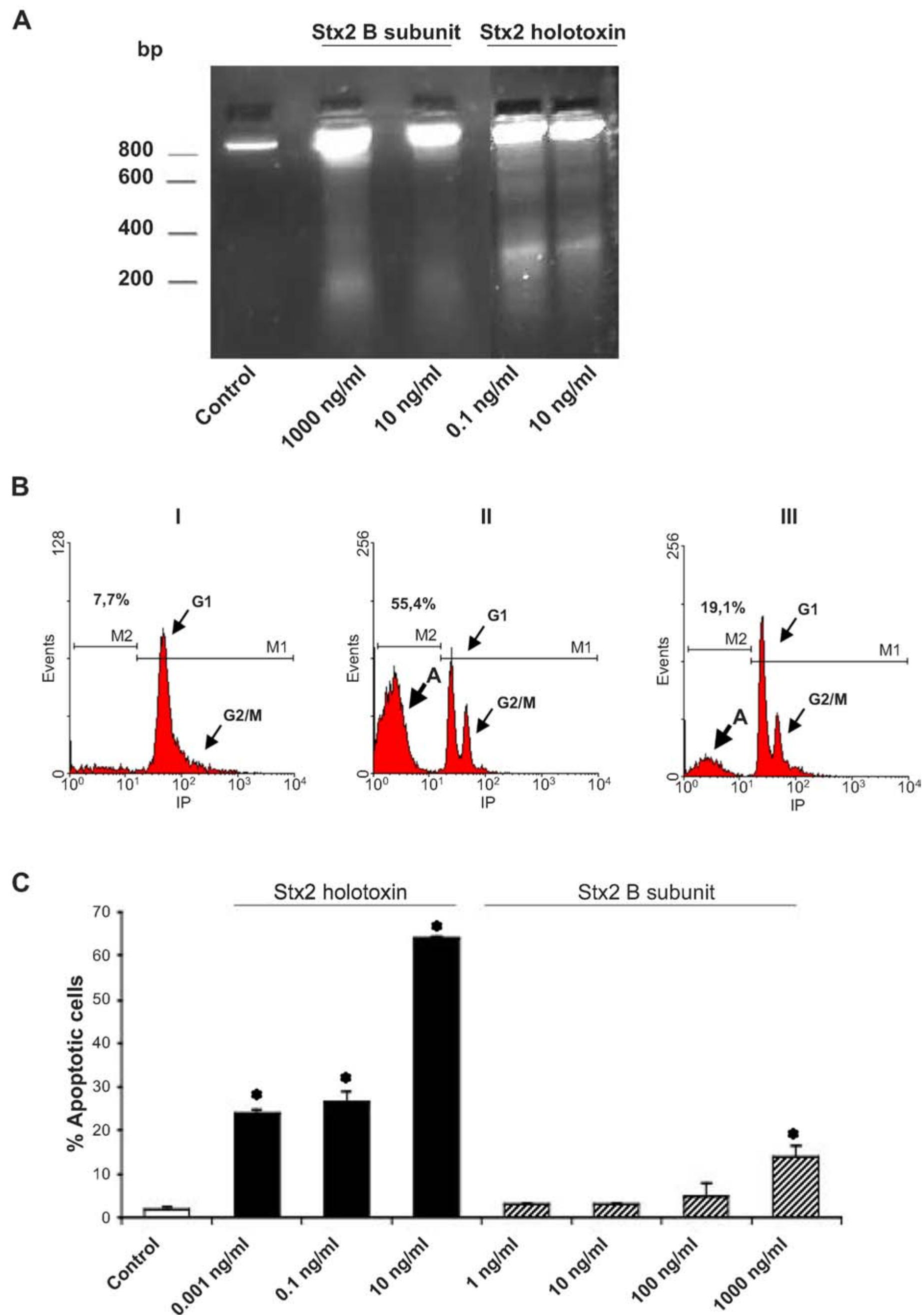

Fig. 5. Induction of apoptosis in HRTEC by Stx2 holotoxin and Stx2 B subunit. Cells were incubated under growth-arrested conditions up to 72 hours with the indicated concentrations of Stx2 holotoxin and Stx2 B subunit. Apoptotic activity was then detected by agarose gel electrophoretic separation of DNA fragmentation (A) or by flow cytometry (B, C). Histograms derived from analysis of HRTEC exposed for 72 hours to either medium alone (I), Stx2 holotoxin (10 ng/ml) (II) or Stx2 B-subunits (1000 ng/ml) (III). Cell with subdiploid DNA content (indicated as A) represent apoptotic cells. *$P < 0.05$, Stx2 holotoxin or Stx2 B subunit vs control.

sis inhibition, and b) apoptosis. We have clearly demonstrated in this study that both Stx2 holotoxin and Stx2 B subunit inhibited protein synthesis and were also able to induce apoptosis in HRTEC.

The mechanism(s) by which Stx2 holotoxin initiates the apoptotic process is not clear. The kinetics of apoptosis as shown by flow cytometry resembles that obtained when using cytotoxic assays with neutral red, but greatly differs from that seen when using protein synthesis inhibition assays. Both apoptosis and reduction of cell viability significantly increased about 50% in HRTEC after 72 h of incubation with 1 ng/ml of Stx2 holotoxin, whereas protein synthesis was inhibited more than 80% at concentrations as low as 0.1 pg/ml of Stx2 and for an incubation time of 24 h. The apoptotic response clearly followed the protein synthesis inhibition as it has been previously reported in human proximal tubules using Stx1 [15]. Therefore, it is possible that the protein synthesis inhibition that occurs in HRTEC exposed to Stx2 holotoxin is, in itself, not lethal to cells, since it could be affecting the production of the inhibitory signal that leads to apoptosis

and cell death. Further studies are required to clarify the inductive processes and signals mediated by Stx2 holotoxin that are a requisite for apoptosis in HRTEC.

Stx2 B subunit induced apoptosis in HRTEC at a concentration of 1000 ng/ml, thus decreasing cell viability. These findings are supported by previous reports showing that the B subunit of Stx1 and Stx2 is able to trigger apoptosis via the cellular receptor Gb3 [21,29]. The mechanism could be different from ribosome inactivation caused by the Stx2 A subunit. While the effects on protein synthesis caused by Stx2 A subunit seem to be highly related to the apoptotic process, this same relationship between protein synthesis and apoptosis is not so clear with the Stx2 B subunit. Further experiments will be necessary to clarify this point.

We also investigated the effect of inflammatory factors on Stx2 holotoxin in HRTEC in order to know their toxin sensitivity in the context of inflammatory factors likely to be elevated either locally in the kidney or in the serum during the course of HUS. Several studies have reported that culture renal proximal tubule cells express Gb3 and are more sensitive to Stx-injury when they are pre-exposed to inflammatory cytokines [15,19].

Our results demonstrate that the sensitivity of HRTEC to the cytotoxic effect of Stx2 holotoxin was enhanced by IL-1β, LPS and butyrate but not by TNF-, IL-6 and IL-8. This could be explained by increased cell surface Gb3 expression due to increased Gb3 translocation to, or reduced removal from, the plasma membrane as has been discussed by Hughes et al. [15]. Pre-stimulation of cells with 1 ng/ml TNF-α did not increase Stx2 toxicity in HRTEC. An effect with TNF has been reported in renal cortical cells [19], although using a 40-fold higher concentration than in the present work. This difference may be due, at least in part, to a dose–response modulation of Stx2 sensitivity by inflammatory factors.

Further evaluation revealed that the viability effect on HRTEC caused by Stx2 B subunits can also be up-regulated by IL-1β, LPS and butyrate. Since the apoptogenic activity of the B subunit is via the cellular receptor Gb3, it is likely that these inflammatory factors increase the susceptibility of HRTEC to Stx2 B subunit by increasing Gb3 expression.

In summary, our results demonstrate that HRTEC is sensitive to Stx2 holotoxin and that factors likely to be elevated in the kidney of patients with HUS augment proximal tubule responsiveness to Stx2. HRTEC is also sensitive to Stx2 B subunit but at higher concentrations than those of Stx2 holotoxin. Although the physiological importance of the effects of Stx2 B subunit seen with these high doses is not clear, it is important to be aware of any potentially toxic activity of the B subunit, given that it has been proposed as a component in an acellular STEC vaccine [30] or as a vector in DNA [31] and cancer vaccines [32].

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