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Cytotoxic effect of Shiga toxin-1 on human glomerular epithelial cells[1]

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Cytotoxic effect of Shiga toxin-1 on human glomerular epithelial cells.

Background. Shiga toxin-1 (Stx-1) has been implicated in the pathogenesis of postdiarrheal hemolytic-uremic syndrome (Stx HUS). Endothelial cells had been felt to be the primary renal target of Stx-1; however, recent studies suggest that renal epithelial cells may also be responsive. To further examine this issue, we evaluated the responsiveness of human glomerular epithelial cells (GECs) to the cytotoxic effects of Stx-1.

Methods. Cultured GECs were exposed to Stx-1 in the presence and absence of a variety of inflammatory factors likely to be elevated in the kidney or serum of patients with Stx HUS. Cell survival, protein synthesis, total cell Gb3 levels and synthesis, and Stx-1 binding were measured.

Results. GECs were sensitive to Stx-1, with an LD_{50} of approximately 10^{-7} g/L (1.4 pmol/L). Interleukin-1 (IL-1), lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), and butyrate increased Stx-1 cytotoxicity and total cell Gb3 levels. These agents, with the exception of TNF-α, also increased Stx-1 binding to GECs. IL-6 failed to alter Stx-1 toxicity, binding, or Gb3 content.

Conclusions. These studies indicate that GECs are sensitive to the cytotoxic effects of Stx-1 and that inflammatory factors can increase toxin responsiveness. GECs may be a target of Stx-1 action in Stx HUS.

Postdiarrheal hemolytic-uremic syndrome [Shiga toxin (Stx) HUS] is the leading cause of acute renal failure in children [1, 2]. Until recently, renal damage in Stx HUS had been generally thought, on the basis of histologic studies, to primarily involve endothelial cells. Support for endothelial cell targeting in Stx HUS was lent by observations that the putative pathogenic toxin, Stx, had a relatively potent cytotoxic effect on cultured renal microvascular endothelial cells as compared with nonrenal endothelial cell types [3–5]. This paradigm of renal damage in Stx HUS was changed, however, by studies implicating renal epithelial cells as targets of Stx action. Of these epithelial cells, proximal tubules have been the first to be clearly identified. Stx-1 (an isoform of Stx involved in Stx HUS) binds to renal cortical tubular epithelial cells [4, 6, 7]. Furthermore, cultured human proximal tubule cells contain extremely high levels of Gb3 (galactose-α-1,4, galactose-β-1,4, glucose-ceramide), the cognate binding site of Stx, and are highly sensitive to Stx-1 cytotoxicity [6]. Thus, proximal tubules may be an important early target of Stx-1 and could contribute to Stx HUS-associated acute renal failure. The finding that renal tubular epithelial cells were responsive to Stx-1 raised the possibility that other renal epithelial cell types could also be important targets of the toxin. Of these, glomerular epithelial cells (GECs) are of great interest. Stx modulation of GEC function could clearly have a significant impact on glomerular function through alterations in glomerular filtration rate, vasoactive factor production, basement membrane integrity, or other parameters. Indeed, recent studies raise the possibility that GECs may be a site of Stx action. Adult human podocytes in vivo can bind Stx-1, albeit relatively weakly [8]. Furthermore, primary cultures of human GECs have been reported to be sensitive to the cytotoxic effect of Stx-1, although the magnitude of Stx-1 binding, Gb3 expression and synthesis, or factors affecting Stx-1 toxicity were not fully assessed [9]. Consequently, the first part of the present study was undertaken to more fully evaluate the cytotoxic effect of Stx-1 on GECs.

Numerous studies have indicated that Stx cytotoxicity can be potentiated by inflammatory factors in which production may be enhanced in Stx HUS. Lipopolysaccharide (LPS), interleukin-1 (IL-1), and tumor necrosis factor (TNF-α) increase the cytotoxic effect of Stx on human endothelial cells [10, 11]. Butyrate, which may be elevated in the circulation of patients with Stx HUS because of disruption of colonic epithelium, sensitizes human umbilical vein endothelial cells (HUVECs) to Stx toxicity [12]. Inflammatory factors may also up-regulate

[1]**See Editorial by Kaplan and Meyers, p. 2650**

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epithelial cell sensitivity to Stx-1 cytotoxicity: IL-1, LPS, and butyrate increase proximal tubule responsiveness to Stx-1 [6]. Hence, it would be important to clarify the role of inflammatory factors that are likely to be elevated in Stx HUS on GEC responsiveness to Stx-1. Accordingly, the second goal of the current study was to examine the effect of inflammatory factors on the cytotoxic effect of Stx-1 on GECs.

METHODS

Reagents

Human kidneys were obtained from 10 different patients undergoing nephrectomies for renal cell carcinoma or Wilm's tumor, aged 7 months to 65 years, from various hospitals in the Salt Lake Valley according to University of Utah Institutional Review Board-approved protocols. Vero cells (African Green Monkey kidney) were obtained from American Type Tissue Collection (ATCC #CRL-81; Rockville, MD, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Glacial acetic acid was from Mallinckrodt (Paris, KY, USA). Ethanol was from Quantum Chemical Co. (Anaheim, CA, USA). Trypsin/ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin, L-glutamine, Dulbecco's modified Eagle's medium (DMEM):F12, and M199 were purchased from GIBCO/BRL (Gaithersburg, MD, USA), and polyisobutylmethacrylate was from Aldrich Chemical Corp. (Milwaukee, WI, USA). All tissue culture flasks, dishes, and multiwell plates were Falcon Brand (Becton Dickinson, Lincoln Park, NJ, USA). ^{3}H-leucine and ^{125}I-goat anti-mouse IgG were from DuPont NEN (Boston, MA, USA). Na ^{125}I was from ICN (Costa Mesa, CA, USA), and UDP-^{14}C galactose was from Amersham (Arlington Heights, IL, USA). Recombinant IL-1β (IL-1), IL-6, and TNF-α (TNF-α) were from R&D Systems (Minneapolis, MN, USA). Lactosylceramide and globotriaosylceramide (Gb3) were from Matreya (Pleasant Gap, PA, USA). Stx-1 was isolated and purified as previously described [6]. A mouse monoclonal antibody to the B-subunit of Stx-1 [13] was purified from a hybridoma cell line, 13C4 (ATCC), as previously described [6].

Cell culture

Renal cortical slices were minced, pushed, and rinsed through a stainless steel screen (60 mesh, Fisher, Houston, TX, USA) and filtered through sieves with pore sizes of 100 mesh for adults or 140 mesh for children. The glomeruli, which were retained by the 100 or 140 mesh sieves, were centrifuged at 2000 × *g* for three minutes and suspended in phosphate-buffered saline (PBS). Glomeruli were then incubated at 37°C for 2 to 15 minutes in 1 mg/mL collagenase (type IV; Sigma, St. Louis, MO, USA) in M199. When the glomerular size was reduced by about 50 to 75%, the tube was placed on ice and GEC media (M199 + 20% FBS + 100 μg/mL endothelial growth supplement (PerImmune) + 100 U/mL penicillin/streptomycin added. Glomerular remnants were centrifuged at 2000 × *g*, resuspended in GEC medium, and plated onto 10 cm tissue culture dishes. Primary cultures and subcultures were maintained at 37°C in a 5% CO_2 environment. Plates were left undisturbed for the next five to seven days to facilitate primary outgrowth of cells from glomeruli. When the plates were approximately 40% confluent (greater degrees of confluence were associated with mesangial cell outgrowth), cells were trypsinized and passaged. All cells were studied after no more than three passages.

Cell purity was assessed by immunofluorescent staining. Primary antihuman antibodies used were mouse antifactor VIII-related antigen (Boehringer Mannheim, Indianapolis, IN, USA), mouse anti-PECAM (R&D Systems), mouse anti–E-selectin (R&D Systems), mouse anticytokeratin-18 (Sigma), mouse anticytokeratin-19 (Sigma), mouse antipan-cytokeratin (reacts poorly with cytokeratin 18 and not with 19; Sigma), rabbit anti–WT-1 (Santa Cruz, Santa Cruz, CA, USA), mouse antivimentin (R&D Systems), and mouse antimyosin (Accurate, Westbury, NY, USA). Secondary antibodies were mouse antirabbit IgG (Sigma) and rabbit antimouse IgG (Boehringer). Positive controls were used for all staining experiments: primary cultures of HUVECs (kindly provided by Dr. Tom McIntyre, University of Utah, Salt Lake City, USA), human mesangial cells P3-P5 (Clonetics, Palo Alto, CA, USA), and human proximal tubular cells P2-P3 (Clonetics). Freshly isolated human glomeruli also served as positive controls.

All GECs were studied under growth-arrested conditions. Barely confluent GECs were placed in DMEM:F12 containing 2 mmol/L L-glutamine and 100 U/mL penicillin/streptomycin for 24 hours prior to any experimental maneuvers.

Vero cells were grown and studied in M199 containing 2.2 g/L sodium bicarbonate, 25 mmol/L HEPES, 100 U/mL penicillin/streptomycin, and 5% FBS.

Neutral red cytotoxicity assay

The neutral red cytotoxicity assay was adapted from previously described protocols [14, 15]. Confluent GECs in 96-well plates were exposed to 10^{-5} to 10^{-11} g/L Stx-1 or 0.1 to 100 μmol/L cycloheximide (CHX) for 0 to 96 hours. Two hundred microliters of neutral red media (50 μg/mL neutral red in M199 containing 5% FBS) were then added to each well and incubated for two to three hours at 37°C in a 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 was read at 450 nm on a Molecular Devices ThermoMax Microplate reader (Menlo Park, CA, USA).

For studies examining the effect of inflammatory fac-

tors on Stx-1 or CHX toxicity, GECs were incubated with varying concentrations of LPS, butyrate, IL-1, IL-6, or TNF for 4 to 48 hours in growth-arrest media before the addition of the cytotoxic agent. Subsequently, Stx-1 (10^{-8} g/L) or CHX (10 μmol/L) were added to the media already on the cells and incubated for an additional 72 hours, followed by analysis of neutral red uptake.

^{3}H-leucine incorporation

Glomerular epithelial cells were grown and assayed in 24-well plates. Cells were exposed to 10^{-5} to 10^{-11} g/L Stx-1 or 0.1 to 100 μmol/L CHX for 24 to 96 hours. The media were then removed, and 250 μL of HBSS containing 1 μCi/mL ^{3}H-leucine were added for 20 minutes. Cells were then rinsed with ice-cold HBSS and solubilized in 500 μL 0.1% sodium dodecyl sulfate (SDS). A 200 μL aliquot was mixed with 25 μL of 200 mg/mL bovine serum albumin (BSA) and 2 mL 10% tricarboxylic acid (TCA). The precipitate was incubated on ice for 60 minutes and collected on prewetted GF/C filters (Whatman, Kent, UK) through a vacuum manifold, and the filters were washed with 10% ice-cold TCA followed by ice-cold 95% ethanol and air dried. Filters were added to scintillation vials with 5 mL Ready Safe scintillation cocktail (Beckmann, Fullerton, CA, USA), and the cpm were determined with a Packard 2200CA β counter (Downers Grove, IL, USA).

Isolation and quantitation of Gb3

Glomerular epithelial cells and Vero cells were grown in six-well plates and growth arrested at confluence. GECs were stimulated with inflammatory factors for 24 hours followed by extraction of total cellular lipids as previously described [6]. Cells were washed with ice-cold PBS, scraped off the plates, and centrifuged at 1500 r.p.m. The cell pellet was extracted three times in 1 mL of 5:10:3 chloroform:methanol:water and dried under vacuum. The dried total lipid product was suspended in 2:1 chloroform:methanol and separated on high-performance thin layer chromatography-silica plates (Mallinckrodt Baker Inc., Paris, KY, USA) by ascending chromatography in 50:40:10 chloroform:methanol:water containing 0.025% $CaCl_2$. Gb3 standards were run on each plate to permit Gb3 quantitation. Total neutral lipid content was determined by running duplicate plates and visualizing lipids with Orcinol spray.

For immunostaining, these plates were dried, immersed in 0.5% polyisobutylmethacrylate in acetone for one minute, and dried. Plates were then incubated in Tris-buffered saline Tween 20 (TBST; 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% Tween 20) containing 10% goat serum for 30 minutes, washed in 2% goat serum/TBST, incubated with 1.5 μg/mL Stx-1 in 2% goat serum/TBST for two hours, washed, incubated with 5 μg/mL anti–Stx-1 monoclonal antibody (13C4; discussed later in this article) in 2% goat serum/TBST for one hour, washed, and incubated with 0.1 μg/mL ^{125}I-goat antimouse IgG in 2% goat serum/TBST for one hour. Plates were then washed and air dried, and specific binding was visualized by autoradiography. Gb3 concentrations were calculated by densitometry using Gaussian density distribution with Eagle Eye II ONE-Dscan software (Stratagene, La Jolla, CA, USA).

The results of the Gb3 quantitation were standardized for cell number by cell counting and total protein determination. Prior to centrifugation and lipid extraction, a small aliquot of cells was solubilized in 0.1 N NaOH and mixed with Bradford reagent (Bio-Rad, Richmond, CA, USA), and the protein concentration was determined by measuring absorbance at 590 nm [16]. Quantitative comparisons between Gb3 in different cell types or under different experimental conditions did not differ whether the data were expressed per total cell protein or cell number; for simplicity, all data are expressed per cell number.

UDP-galactose lactosylceramide galactosyltransferase assay

Glomerular epithelial cells were grown in 10 cm dishes and growth arrested at confluence. Cells were then stimulated with inflammatory factors for 24 hours and were scraped and centrifuged at 1500 r.p.m. The pellet was resuspended in 500 μL 50 mmol/L [N-morpholino]ethanesulfonic acid (MES), pH 6.5. Cells were disrupted with a five-second pulse from an ultrasonic tip processor (amplitude 30 at 25 W; Cole Parmer Instrument Co., Chicago, IL, USA) and assayed for total protein using the Bradford method. GalT6 activity was assayed using a modification of the method by Mobassaleh, Mishra, and Keusch [17] and as previously described [6]. Lactosylceramide (LacCer, 25 nmol) was aliquoted in 2:1 chloroform:methanol and dried. Sodium cholate in water (250 μg) was added to the dried LacCer and dried under vacuum, and the dried mixture was incubated for 60 minutes at 4°C. A total volume of 100 μL 50 mmol/L MES, pH 6.5, containing 10 mmol/L $MnCl_2$, 100 μmol/L 5′-adenylimidodiphosphate [p(NH)ppA], 250 μmol/L cold UDP-galactose, 44 μmol/L UDP-^{14}C galactose (150,000 to 400,000 cpm), and 125 μg total cellular protein was added to the dried LacCer/sodium cholate. The samples were vortexed briefly and incubated in a slowly agitating 37° water bath for 60 minutes, and the reaction was stopped by adding 1 mL 2:1 chloroform:methanol. A Folch partition was established by adding 200 μL 0.1 mol/L KCl and the upper phase re-extracted by adding 500 μL 2:1 chloroform:methanol. The lower phase was re-extracted by adding 500 μL 1:1 methanol:0.1 mol/L KCl. The lower phases were combined, dried, and chromatographed as described previously in this article for Gb3.

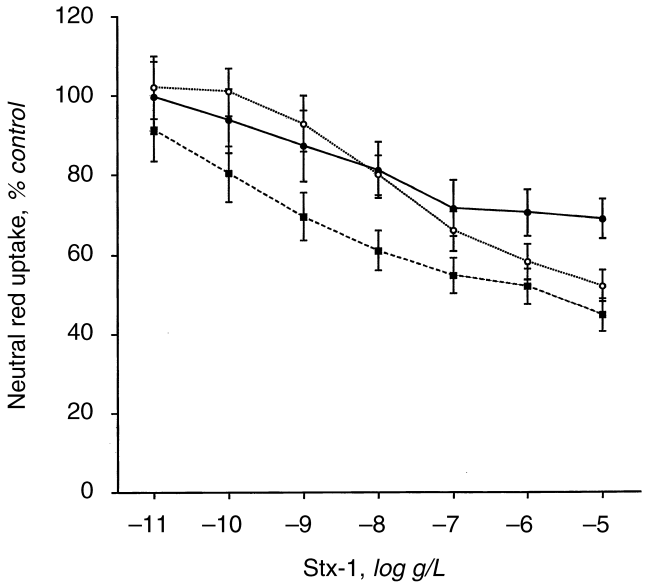

Fig. 1. Cytotoxic effect of Shiga toxin-1 (Stx-1) on glomerular epithelial cells (GECs; N = 12 each data point). Cells were incubated with Stx-1 for (●) 24, (○) 48 and (■) 72 hours, followed by determination of neutral red uptake.

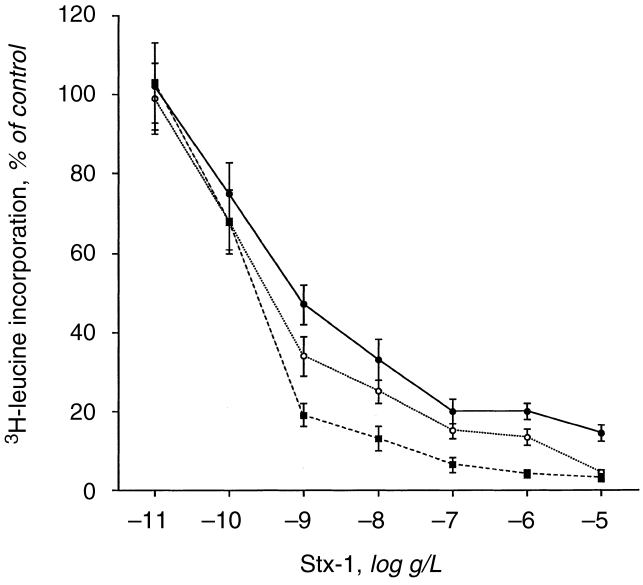

Fig. 2. Effect of Stx-1 on ^{3}H-leucine incorporation by GECs (N = 3 each data point). Cells were incubated with Stx-1 for (●) 24, (○) 48 and (■) 72 hours, followed by an addition of 1 μCi/mL ^{3}H-leucine for 20 minutes.

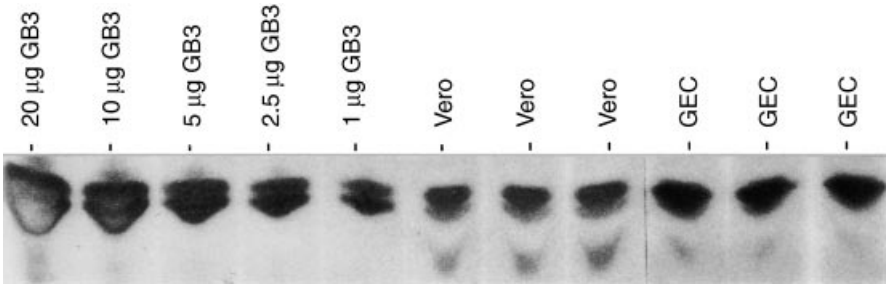

Fig. 3. Total globotriaosylceramide (Gb3) content in GEC and Vero cells. A standard curve using purified Gb3 is shown on the left side of the autoradiogram for comparison. Each experimental lane represents a different cell culture preparation (**Methods** section).

Iodination and binding of Stx-1

Shiga toxin-1 was iodinated according to the Iodobead manufacturer's protocol (Pierce, Rockford, IL, USA) and as previously described [6]. For ^{125}I-Stx-1 binding assays, GECs in 96-well plates were preincubated in growth-arrest media for 24 hours with or without inflammatory cytokines. At the time of study, media were removed, and 17,000 cpm of ^{125}I-Stx-1 in 100 μL M199 containing 5% FBS and 25 mmol/L HEPES plus varying concentrations of unlabeled Stx-1 were added for 24 hours at 4°C. Cells were then rinsed with ice-cold HBSS and solubilized in 0.1 N NaOH, and cpm were determined on a Packard 5000 γ counter.

Statistics

All data were analyzed by one-way analysis of variance (ANOVA). Results are expressed as mean ± SEM. P values < 0.05 were taken as significant. Wherever the data are expressed as a percentage of control, the sample size (N) refers to the number of experimental and control samples used for that particular data point.

RESULTS

Human glomerular epithelial cell culture

Originally, our aim was to isolate and culture human glomerular endothelial cells. It was ultimately determined that this was best achieved by using a cell-sorting strategy using antibody-coated magnetic beads; however, in the process, it was determined that GECs could readily be cultured. Initial outgrowths of glomerular remnants contained about 95% GECs and 5% endothelial cells, as determined by immunofluorescent staining. About 5% of cells from initial outgrowths (HUVEC and freshly isolated glomeruli served as positive controls and proximal tubule cells as negative controls) stained positive with anti-PECAM, anti–E-selectin (after 10-min exposure to 100 U/mL TNF-α), and antifactor VIII-related

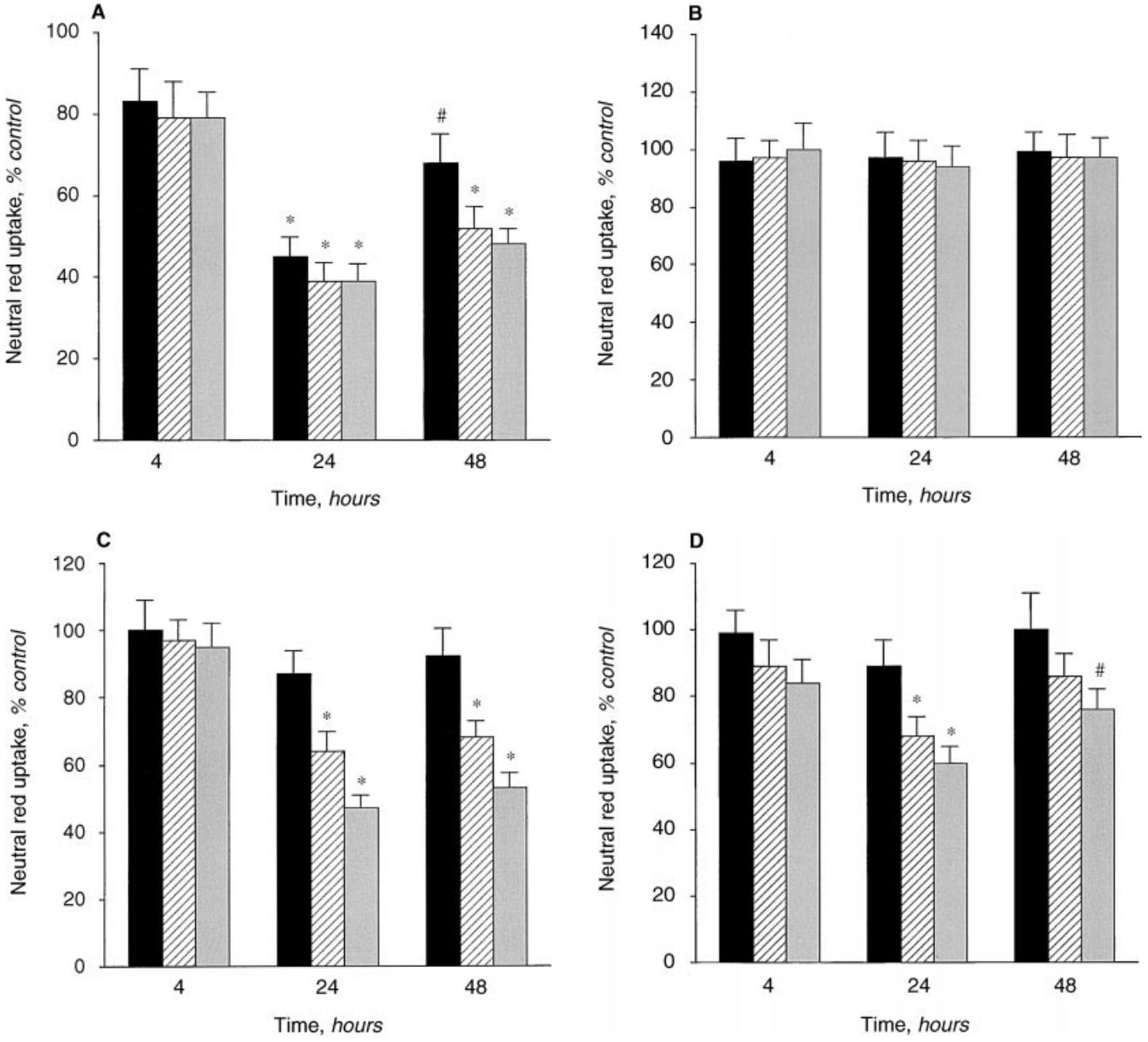

Fig. 4. Effect of IL-1 (*A*), IL-6 (*B*), TNF-α (*C*), LPS (*D*), or butyrate (mmol/L, *E*) on Stx-1 (10^{-8} g/L or 140 fmol/L) cytotoxicity on GECs (N = 8 each data point). $^{*}P < 0.001$; $^{\#}P < 0.01$, both vs. Stx-1 alone. Cells were preincubated with inflammatory factors for 24 hours prior to exposure to Stx-1 for 72 hours and subsequent determination of neutral red uptake. Symbols in A are: (■) 10 U/mL IL-1; (▨) 100 U/mL IL-1; (▩) 1000 IL-1. Symbols in B are: (■) 0.5 ng/mL IL-6; (▨) 2 ng/mL IL-6; (▩) 5 ng/mL IL-6. Symbols in C are: (■) 10 U/mL TNF-α; (▨) 100 U/mL TNF-α; (▩) 1000 U/mL TNF-α. Symbols in D are: (■) 0.1 μg/mL LPS; (▨) 1 μg/mL LPS; (▩) 10 μg/mL LPS. Symbols in E are: (■) 0.5 mmol/L butyrate; (▨) 2 mmol/L butyrate; (▩) 5 mmol/L butyrate.

antigen antibodies. These presumed endothelial cells were negative for all cytokeratins and for WT-1. About 95% of cells from initial outgrowths stained positive with antibodies to pan-cytokeratin, cytokeratins 18 or 19, and vimentin, but were negative for myosin or any of the endothelial cell markers described previously in this article. Initial outgrowths did not stain positive for WT-1, although cells within glomerular remnants were positive for WT-1.

No endothelial or mesangial cells could be detected in cultures after the first passage. Passaged cells that had just attained confluence stained positive for vimentin and with all cytokeratin antibodies, but were negative for all endothelial makers. The passaged cells uniformly exhibited cobblestone morphology typical of epithelial cell cultures (glomerular endothelial cell cultures also exhibit cobblestone morphology; however, the individual cells tend to be larger than epithelial cells). Approxi-

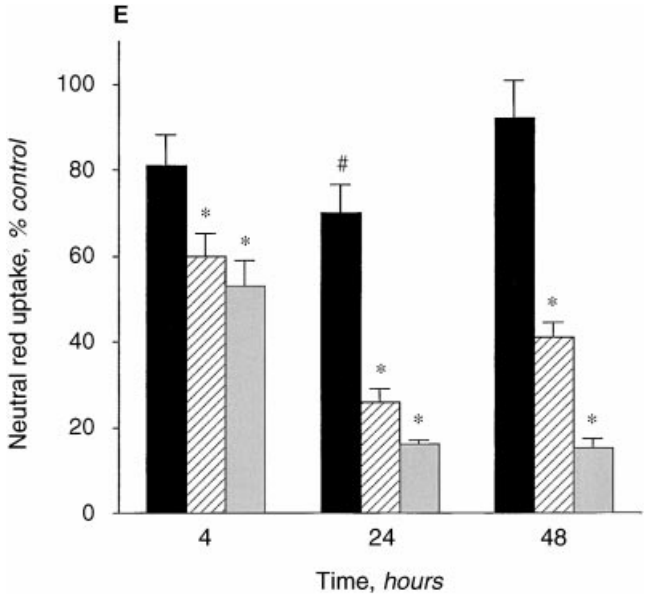

Fig. 4. (Continued).

mately 10% of cells stained positive for WT-1. While it has been reported that maintaining confluent GECs in culture for several days or even weeks leads to morphologic changes and increased WT-1 expression [18], we found that, in our hands, GECs exhibited increased granularity, flattened out, and began detaching when maintained as confluent cultures for greater than one week.

Stx-1 cytotoxicity and protein synthesis inhibition

Cytotoxicity was assessed by a combination of neutral red uptake, total cell protein, cell number, and cell morphology. As described previously, neutral red uptake has been determined to correlate well with changes in cell morphology and be preferable to using cell protein or cell number as an indicator of Stx-1 cytotoxicity [6]. Stx-1 dose dependently killed cultured GECs (Fig. 1). The toxic effect of Stx-1 was detectable at the highest concentrations by 24 hours; the LD_{50} of Stx-1 at 72 hours was approximately 10^{-7} g/L (1.4 pmol/L).

Shiga toxin-1 also dose dependently inhibited protein synthesis by GECs, as assessed by ^{3}H-leucine incorporation (Fig. 2). After 24 hours of exposure, 10^{-7} g/L (1.4 pmol/L) Stx-1 reduced protein synthesis by about 80%, while this degree of protein synthesis inhibition was evident after 72 hours with 10^{-9} g/L (14 fmol/L) Stx-1. Cell death lagged markedly behind protein synthesis inhibition, with approximately a 500-fold higher concentration of Stx-1 being required to kill, as compared with inhibit protein synthesis, in 50% of the cells after 72 hours of exposure to the toxin.

Gb3 was readily detectable in GECs (Fig. 3). By way of comparison, GECs contained more total cellular Gb3 (1.41 ± 0.31 μg/10^7 cells) than did Vero cells (0.64 ± 0.086 μg/10^7, $N = 3$, $P < 0.025$).

Effect of inflammatory factors on Stx-1 cytotoxicity

The effect of IL-1, IL-6, TNF-α, LPS, and butyrate on the cytotoxic effect of Stx-1 on GECs was examined. None of these agents alone significantly affected neutral red uptake; however, GECs exposed to the highest concentration (5 μmol/L) of butyrate exhibited increased granularity and spreading, signs typically associated with early toxicity. Of the inflammatory cytokines, IL-1 and TNF-α (Fig. 4 A, C), but not IL-6 (Fig. 4B), enhanced Stx-1 sensitivity (Stx-1 dose chosen that caused approximately a 50% reduction in cell viability at 72 h). The inhibitory effect of IL-1 and TNF-α was evident after 24 and 48 hours, but not 4 hours, of preincubation with the cytokines before the addition of Stx-1.

Lipopolysaccharide and butyrate also augmented Stx-1 cytotoxicity in GECs (Fig. 4 D, E). Similar to the inflammatory cytokines, LPS up-regulation of Stx-1 cytotoxicity occurred after 24 and 48 hours, but not 4 hours, of preincubation with endotoxin before addition of Stx-1. Butyrate, particularly at the highest concentration (5 μmol/L), markedly increased GEC Stx-1 sensitivity. As noted earlier in this article, although butyrate alone did not affect neutral red uptake, it did alter GEC morphology in a manner suggestive of early toxicity.

Mechanism of inflammatory factor up-regulation of Stx-1 cytotoxicity

Modulation of cycloheximide cytotoxicity. To test the specificity of inflammatory factor modulation of Stx-1 action, the effect of these agents on CHX, a protein synthesis inhibitor and cytotoxic agent, was examined. It was first necessary to determine a CHX concentration that, like the Stx-1 concentration used in the studies mentioned earlier in this article, caused approximately a 50% reduction in cell viability. A dose (0.1 to 100 μmol/L)- and time (24 to 72 h)-response curve revealed that 10 μmol/L CHX killed approximately 40% of GECs after 72 hours ($N = 12$ each data point, data not shown); these conditions were used for subsequent studies.

Interleukin-1, IL-6, TNF-α, and LPS preincubation for 4 to 48 hours, at the maximal concentrations used for studies on regulation of Stx-1 cytotoxicity, had no effect on CHX cytotoxicity ($N = 6$ each data point; Fig. 5). Butyrate increased CHX cytotoxicity, albeit at the highest concentration (5 μmol/L) or the longest time point (48 h). Notably, the butyrate effect was much less pronounced on CHX toxicity as compared with that with Stx-1.

Modulation of Gb3 expression, GalT6 activity, and SLT-1 binding. To test whether cell surface expression of Gb3, or some other Stx-1 receptor, could be increased by inflammatory factors, GECs were incubated with the

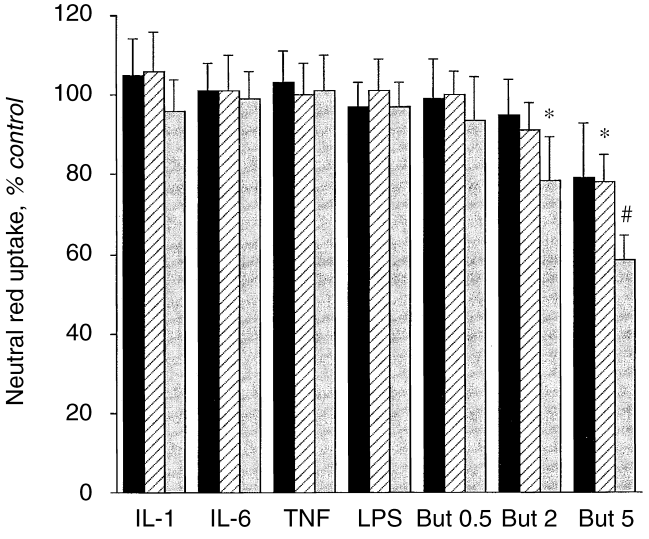

Fig. 5. Effect of 4 (■), (▨) 24, and (▩) 48 hours of preincubation with 100 U/mL IL-1, 5 ng/mL IL-6, 100 U/mL TNF-α, 1 μg/mL LPS, or 0.5 to 5 mmol/L butyrate on CHX (10 μmol/L) cytotoxicity on GECs (N = 8 each data point). $^*P < 0.05$; $\#P < 0.01$, both vs. control.

various agents for 24 hours followed by the determination of ^{125}I-Stx-1 binding in the presence of increasing concentrations of unlabeled Stx-1. While a K_d and B_{max} could potentially be estimated from these curves, such calculations were not made since it was extremely difficult to know the precise molar concentration (and hence specific activity) of ^{125}I-Stx-1 due to difficulties in measuring very small concentrations of Stx-1 recovered from the iodination process. Nonetheless, it appears that IL-1, LPS, and butyrate increased total Stx-1 binding (Fig. 6). IL-6, which had no effect on Stx-1 toxicity, did not alter ^{125}I-Stx-1 binding. Finally, TNF-α, which did increase Stx-1 cytotoxicity, did not change toxin binding.

Since up-regulation of Stx-1 binding has been associated with increased cellular Gb3 content, the effect of inflammatory factors on GEC Gb3 levels was assessed. An incubation time of 24 hours and the maximal stimulatory (of Stx-1 toxicity) concentration of inflammatory substance were chosen. As shown in Figure 7, all agents that enhanced Stx-1 cytotoxicity (IL-1, TNF-α, LPS, and butyrate) increased the total Gb3 content in GEC. Note that IL-1, LPS, and butyrate stimulation of cell Gb3 levels correlated with their up-regulation of Stx-1 binding; however, TNF-α enhancement of Gb3 content was not associated with altered Stx-1 binding. Finally, IL-6 had no effect on Gb3 levels; this correlated with the failure of IL-6 to alter Stx-1 binding.

Since an increase in total cellular Gb3 content could be due to increased Gb3 synthesis, the effect of the inflammatory factors on GalT6 activity was determined. No factor increased total GalT6 activity (Fig. 8). IL-1 tended to give the highest enzyme activity, but it did not achieve statistical significance.

DISCUSSION

The current study demonstrates that GECs bind Stx-1, synthesize and express Stx-1 binding sites (Gb3), and are sensitive to the cytotoxic effect of Stx-1. The LD_{50} of Stx-1 after 72 hours of exposure to GECs was about 10^{-7} g/L (1.4 pmol/L). This compares with an LD_{50} of Stx-1 (after 72 h of exposure) of 10^{-9} g/L (14 fmol/L) for human proximal tubule cells and $10^{-9.5}$ g/L (4 fmol/L) for Vero cells [6], 10^{-5} g/L (140 pmol/L) for human mesangial cells (albeit after 48 h) [19], less than 6% of human glomerular endothelial cells being killed after 24 hours of exposure to 10^{-4} g/L (1.4 nmol/L) Stx-1, and virtually no effect of Stx-1 on noncytokine primed HUVECs [11]. Thus, the cytotoxic effect of Stx-1 on GECs appears to occur at toxin concentrations in between the most (proximal tubule) and least (mesangial) Stx-1–sensitive renal cells examined to date. Admittedly, such comparisons of Stx-1 toxicity are fraught with confounding variables, including different degrees of cell confluence, different periods of Stx-1 exposure, and different proliferative states. With regard to proliferative state, it is important to note that all GEC studies were conducted under growth-arrested conditions. The proliferative state can alter Stx sensitivity: Actively growing cells are more susceptible to the toxin than are quiescent cells [20]. We also examined the effect of Stx-1 on proliferating GECs and found that Stx-1 sensitivity was increased approximately 50- to 100-fold (data not shown). Despite this heightened sensitivity, growth-arrested conditions were used since this presumably more closely mimics the natural state of GECs. In addition, it avoids differences in Stx-1 sensitivity caused by fluctuations in the proliferative state of the cells.

Glomerular epithelial cells were exquisitely sensitive to Stx-1 inhibition of protein synthesis. The ED_{50} of Stx-1 (24-h exposure) for inhibition of protein synthesis was about 10^{-9} g/L (14 fmol/L); this value is similar to that observed for proliferating human GECs [9] and compares with an ED_{50} at 24 hours of $10^{-9.5}$ g/L (4 fmol/L) for growth-arrested human proximal tubule cells and proliferating vero cells [6], an ED_{50} at 24 hours of about 10^{-6} g/L (14 pmol/L) for growth-arrested human mesangial cells [19], and an ED_{50} at 24 hours of about 10^{-5} g/L (140 pmol/L) for proliferating human glomerular endothelial cells [3]. Again, comparison of cell sensitivities must be interpreted cautiously; however, it would appear that GEC sensitivity to Stx-1 protein synthesis inhibition approaches that of the most sensitive renal cells. This conclusion is supported by the finding that GECs and human cortical tubular epithelial cells (albeit both cell types were proliferating) had similar sensitivi-

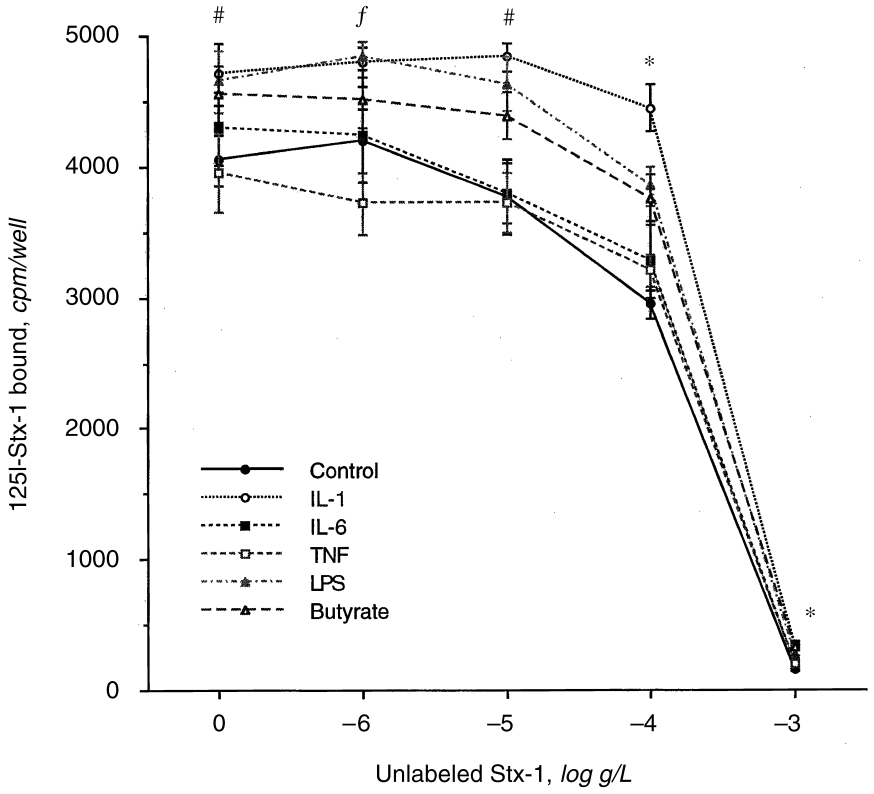

Fig. 6. Effect of 24 hours of incubation with (●) control, (○) 100 U/mL IL-1, (■) 5 ng/mL IL-6, (□) 100 U/mL TNF-α, (▲) 1 μg/mL LPS, or (△) 0.5 mmol/L butyrate on binding of ^{125}I-Stx-1 to GECs (N = 6 each data point). Approximately 17,000 cpm of ^{125}I-Stx-1 were added per well for 24 hours at 4°C. Cells were washed and solubilized, and bound cpm were determined. *$P < 0.001$ for IL-1 and $P < 0.005$ for LPS and $P < 0.001$ for butyrate, all vs. control; #$P < 0.005$ for IL-1 and $P < 0.005$ for LPS and $P < 0.025$ for butyrate, all vs. control; $fP < 0.005$ for IL-1 and $P < 0.005$ for LPS, both vs. control.

ties to Stx-1 inhibition of protein synthesis [9]. It is striking that Stx-1 concentrations that almost completely blocked protein synthesis in GECs were 100- to 500-fold less than concentrations that caused only a 50% reduction in cell survival. Such a phenomenon has been previously observed with Stx-1: The toxin caused a 75% reduction in mesangial cell protein synthesis, but had minimal effect on cell viability [21]. This toxin effect on mesangial cells was due to the A-subunit, since the B-subunit given alone had no effect on protein synthesis. Therefore, it would appear that GECs can survive relatively marked reductions in protein synthesis under the culture conditions employed. It may be, however, that such degrees of protein synthesis inhibition in vivo, where concurrent ischemic or other injurious conditions could exist in Stx HUS, could lead to greater GEC death than realized in the current study. Finally, it should be noted that, while beyond the scope of the present study, Stx-1 might kill GECs via induction of apoptosis and not solely through inhibition of protein synthesis. Such a possibility is supported by observations that Stx-1 causes apoptosis in Burkitt lymphoma cells [22]; however, recent studies in proliferating GECs have detected no effect of Stx-1 on DNA fragmentation [9].

Glomerular epithelial cells contain relatively abundant Gb3 levels; in the current study, they exceeded the Gb3 content of Vero cells. Gb3 content generally parallels Stx sensitivity [11, 23], although exceptions to this relationship have been described [24]. Our study did not directly assess cell surface Gb3 expression, nor did it evaluate whether Stx-1 binds to receptors other than glycosphingolipids with a galα1-4gal linkage. Nonetheless, it seems likely that the high sensitivity of GECs to the protein synthesis inhibitory effect of Stx-1 relates, at least partially, to abundant cell surface Gb3 expression.

As discussed in the introduction, a number of inflammatory factors are elevated either locally in the kidney or in the serum and may be capable of modulating cell Stx-1 sensitivity. To investigate this, growth-arrested GECs were preincubated with IL-1, IL-6, TNF-α, LPS, or butyrate at varying doses for up to 48 hours prior to the addition of Stx-1 for an additional 72 hours. All of these factors, with the exception of IL-6, increased GEC sensitivity to Stx-1. First, IL-1 and LPS had similar effects on Stx-1 toxicity. Both agents increased GEC sensitivity to the cytotoxic effects of Stx-1. This up-regulation was associated with augmented Stx-1 binding and increased total cell Gb3 content. The observation that none of these factors altered GalT6 activity suggests several possibilities. It is conceivable that translocation of Gb3 to the cell membrane was enhanced and/or removal of Gb3 from the cell membrane was reduced. Alternatively, it is possible that while total GalT6 activity was unchanged, these inflammatory factors may have altered the form

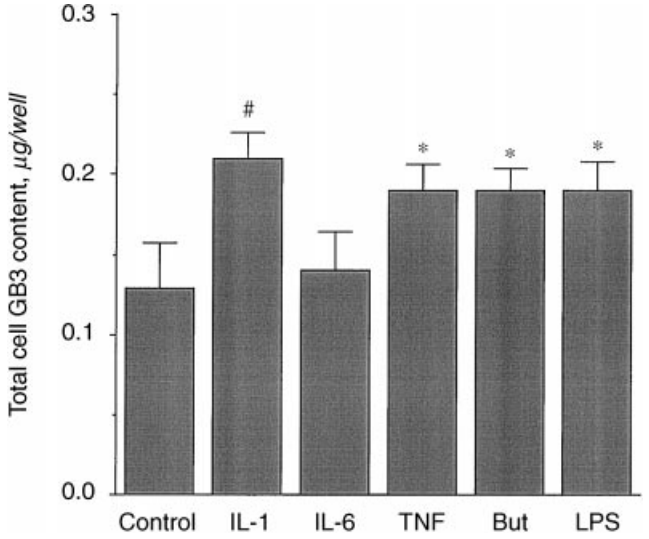

Fig. 7. Effect of 24-hour incubation with 100 U/mL IL-1, 5 ng/mL IL-6, 1000 U/mL TNF, 1 μg/mL LPS, or 0.5 mmol/L butyrate on total Gb3 content in GECs ($N = 6$ each data point). $^*P < 0.025$; $\#P < 0.01$, both vs. control. Lanes were loaded with an equal weight of total cellular lipids (**Methods** section).

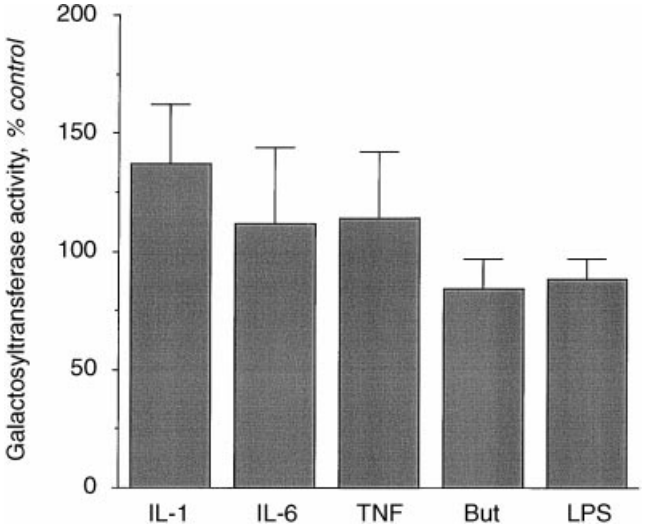

Fig. 8. Effect of 24-hour incubation with 100 U/mL IL-1, 5 ng/mL IL-6, 100 U/mL TNF, 1 μg/mL LPS, or 0.5 mmol/L butyrate on GalT6 activity in GECs ($N = 3$ each data point). Control GalT6 activity was 0.36 ± 0.04 densitometry units (**Methods** section).

of Gb3 that was synthesized. The Gb3 assay employed in the current study detects Gb3 by Stx-1 binding to the chromatography plate. In contrast, the GalT6 assay measures incorporation of ^{14}C-galactose into Gb3, but does not assess the ability of newly synthesized Gb3 to bind Stx-1 (the assay is not this sensitive). Since galactose could be added in a β1-3 linkage (does not bind Stx-1) or an α1-4 linkage (binds Stx-1), it is possible that IL-1 and LPS caused a relative shift to α1-4 linkages without an overall increase in GalT6 activity. This possibility might be tested by quantitating GalT6 activity in the presence and absence of α- or β-galactosidases; however, we have attempted such studies (data not shown) and found them to be relatively nonquantitative. Taken together, these considerations suggest that IL-1 and LPS increase Stx-1 cytotoxicity, at least in part, by increasing toxin binding to the cell membrane. This does not, however, preclude other mechanisms by which these inflammatory factors might increase Stx-1 sensitivity. For example, LPS increases proximal tubule sensitivity to Stx-1 cytotoxicity; however, it does not alter Stx-1 binding or Gb3 content in these cells [6], suggesting that LPS has the potential to augment Stx-1 action through mechanisms independent of Stx-1 binding. This observation also underscores the finding that inflammatory factor up-regulation of Stx-1 sensitivity is not necessarily similar in all responsive cell types.

Butyrate increased GEC responsiveness to Stx-1; this effect was associated with increases in Gb3 content and Stx-1 binding, but no change in GalT6 activity, in a manner similar to that seen with IL-1 and LPS. In contrast, butyrate (at the doses used) appeared to have a direct toxic effect. Furthermore, butyrate was the only agent that enhanced CHX cytotoxicity, supporting the notion that, in addition to modulation of Stx-1 binding, butyrate nonspecifically enhances cell toxicity associated with inhibition of protein synthesis. It should be noted that relatively similar results were seen with butyrate treatment of human proximal tubular cells [6].

Tumor necrosis factor-α also augmented Stx-1 cytotoxicity in GECs, increased total Gb3 content, and did not change GalT6 activity. Unlike the other factors, TNF-α did not change Stx-1 binding to GECs. This finding was surprising but not unprecedented. In a previous study, our group found that butyrate elevated proximal tubule cell Gb3 content and Stx-1 cytotoxicity, but did not alter Stx-1 binding [6]. Therefore, it is evident that the total Gb3 content does not necessarily reflect cell surface Gb3 expression, although the mechanisms responsible for this are unclear. In addition, TNF-α increased proximal tubule cell Stx-1 sensitivity, but did not change Gb3 content or Stx-1 binding [6]. Thus, it appears that TNF-α can increase Stx-1 sensitivity in both GECs and human proximal tubule cells through mechanisms distal to Stx-1 binding.

At this point, the significance of Stx-1 inhibition of GEC protein synthesis or induction of cytotoxicity is unknown. GEC injury has not been described as a prominent feature of Stx HUS; however, it is difficult to identify GEC damage morphologically other than by nonspecific fusion of foot processes. Proteinuria is variable, while hematuria and red cell casts are commonly observed, suggesting some damage to podocytes [2]. Severe acute renal insufficiency has been described in the absence of histologically apparent endothelial cell damage

[2], suggesting that other renal cells types could be involved. Conceivably, GECs could be early targets of Stx-1 with resultant stimulation of inflammatory cytokine, procoagulant, chemotactic, extracellular matrix-degrading enzyme, or vasoactive factor release. Regardless of how GEC injury contributes to renal dysfunction in Stx HUS, it is evident that this cell type is highly sensitive to at least the protein synthesis inhibitory effects of Stx-1 and that inflammatory factors likely to be elevated in the kidney of patients with Stx HUS augment GEC responsiveness to Stx-1. Clearly, further studies are needed to clarify the effects of Stx-1 on GECs.

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