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Shiga Toxin-Associated Hemolytic Uremic Syndrome: Effect of Sodium Butyrate on Sensitivity of Human Umbilical Vein Endothelial Cells to Shiga Toxin

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***Escherichia coli* O157:H7-related vascular damage such as hemolytic uremic syndrome is believed to require the Shiga-like toxins. This study demonstrated that sodium butyrate sensitized human umbilical vein endothelial cells to Shiga toxin and increased the expression of Shiga toxin receptor, globotriaosylceramide (Gb_3), on human umbilical vein endothelial cells.**

Shiga toxin-producing *Shigella dysenteriae* 1 and *Escherichia coli* O157:H7 are associated with the development of hemorrhagic colitis and hemolytic uremic syndrome (5, 7, 8). Intoxication of cells by Shiga toxin involves binding of the toxin to a cell surface receptor followed by internalization and transport to the trans-Golgi network (for a review, see reference 17). Retrograde transport of toxin from the Golgi to the endoplasmic reticulum may also be required for the action of Shiga toxin toward cells (23, 25). Shiga toxin acts on eukaryotic ribosomes to inhibit protein synthesis (17). The protein synthesis-inhibitory action of Shiga toxin is believed to be responsible for its cytotoxicity toward a number of different cell types.

All Shiga toxin-sensitive cells express the receptor for Shiga toxin, globotriaosylceramide (Gb_3) (10, 12, 32). Some Shiga toxin-insensitive cells do not express Gb_3, whereas other insensitive cell types do (9, 23–25). In both cases, the cells are sensitized to Shiga toxin by incubation with sodium butyrate (9, 23–25). Butyrate is a naturally occurring compound in the colon and in the peripheral circulation (1, 4) and may play an important role in sensitizing cells to Shiga toxin during the pathogenesis of Shiga toxin-related diseases. Under normal conditions, the concentration of butyrate in the colon is in the millimolar range, whereas in the systemic circulation it is present in micromolar amounts (4). The concentration of butyrate in the circulation of patients with hemorrhagic colitis and/or hemolytic uremic syndrome is not known but may be elevated because of the disruption of the colonic epithelium and endothelium during the course of infection with *E. coli* O157 or *S. dysenteriae* 1.

Vascular endothelial cells are the putative target of Shiga toxin during the pathogenesis of hemorrhagic colitis and hemolytic uremic syndrome (5, 11, 20–22). For this reason, the ability of sodium butyrate to sensitize human umbilical vein endothelial cells (HUVEC) to Shiga toxin was examined. Further, the results were compared with those obtained with tumor necrosis factor alpha (TNF), which is known to sensitize HUVEC to Shiga toxin (14, 18, 28, 30, 31). Additionally, since TNF induces expression of the Shiga toxin receptor Gb_3 (18, 31), activation of the transcription factor NF-κB (15), and expression of the procoagulant molecule tissue factor (16) in HUVEC, the ability of butyrate to induce these activities in HUVEC was examined.

HUVEC were obtained, passaged, and plated as described previously (14). Cells were treated with sodium butyrate (Sigma Chemical Co., St. Louis, Mo.), TNF (yeast recombinant; Boehringer-Mannheim, Indianapolis, Ind.), and/or Shiga toxin (gift of J. E. Brown), as described in each figure legend. The number of viable cells was determined as the uptake of the dye neutral red, as reported previously (14). For binding studies, Shiga-like toxin 1 (SLT-1; purified as described in reference 6) was radiolabeled with Iodobeads (Pierce, Rockford, Ill.) for 5 min at 25°C to a specific activity of 293,000 cpm/pmol. SLT-1 was used in place of Shiga toxin because of its ease of availability; it differs from Shiga toxin by only a single amino acid in a nonbinding region of the protein (17, 26). HUVEC were incubated with 5 nM [^{125}I]SLT-1 for 5 h at 4°C. This incubation period was sufficient to achieve binding equilibrium (unpublished data). Cells were then rinsed twice and were solubilized in 0.5 N NaOH for 30 min at 37°C. Counts per minute (cpm) of the solubilized cell solution were determined in a gamma counter. Nonspecific binding was determined as the amount of [^{125}I]SLT-1 bound in the presence of 200 nM unlabeled SLT-1, a concentration of unlabeled toxin which has previously been shown to compete effectively with [^{125}I]SLT-1 for binding to HUVEC (13). This value was subtracted from the total binding to obtain the specific binding values reported in Fig. 2.

The Gb_3 contents of untreated and butyrate-treated HUVEC were determined by high-performance liquid chromatography (HPLC) of whole-cell extracts as described previously (18). Tissue factor activity was determined by cleavage of the chromogenic substrate S2222 (KABI Pharmacia, Franklin, Ohio) in the presence of coagulation factors VII, IX, X, and II (proplex T; Baxter Healthcare, Glendale, Calif.) as described previously (27). NF-κB-binding activity was determined by electrophoretic mobility shift assays with HUVEC nuclear extracts prepared by the method of Marui et al. (15). The protein concentration of these extracts was determined by the Bio-Rad protein assay kit with bovine serum albumin as the standard, such that 3 μg of protein was added to each binding reaction. Binding-reaction mixtures (20 μl) consisted of 10 mM Tris-Cl (pH 7.5), 60 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA,

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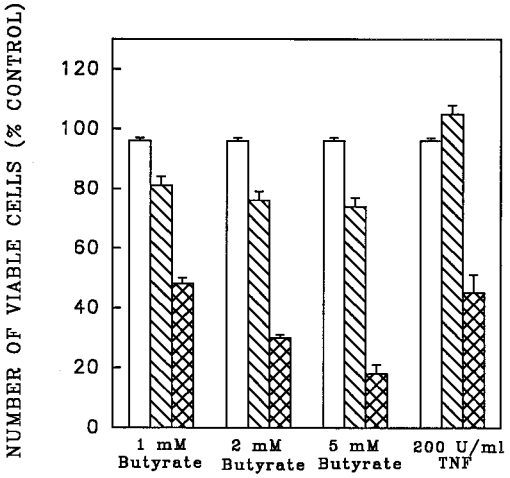

FIG. 1. Butyrate increases HUVEC sensitivity to Shiga toxin. HUVEC were plated into 96-well plates at a density of 12,000 cells per well and allowed to attach overnight. Medium was removed, and cells were treated with medium alone, medium plus the indicated concentrations of butyrate, or medium plus 200 U of TNF per ml. Following a 24-h incubation at 37°C, medium was changed and cells were treated for an additional 24 h at 37°C with the same experimental agents as described above. The cells were then treated with 1 nM Shiga toxin for 24 h at 37°C. Cell viability was determined by the neutral red assay. Open bars, treatment with Shiga toxin only (no butyrate or TNF pretreatment); hatched bars, pretreatment with butyrate or TNF only (no subsequent Shiga toxin treatment); cross-hatched bars, pretreatment with butyrate or TNF followed by treatment with Shiga toxin. Control untreated cells represent 100% viability.

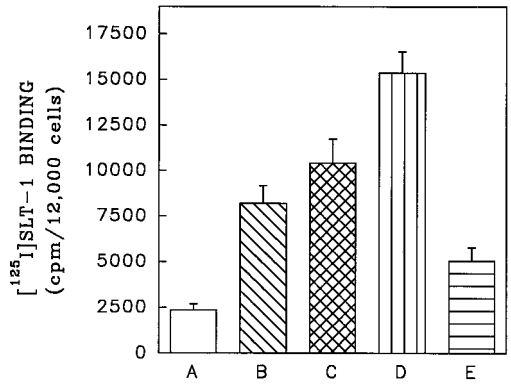

FIG. 2. Butyrate increases [^{125}I]SLT-1 binding to HUVEC. HUVEC were plated into 96-well plates at a density of 12,000 cells per well. After overnight attachment, medium was changed and cells were treated with control medium (A), 1 mM butyrate (B), 2 mM butyrate (C), 5 mM butyrate (D), or 200 U of TNF per ml (E) for 24 h at 37°C. Medium was replaced with fresh medium containing the substances listed above, and the cells were incubated for an additional 24 h at 37°C. Medium was removed, cold (4°C) medium was added to the wells, and the cells were incubated for 0.5 h at 4°C. SLT-1 was then added to the wells (final incubation volume, 30 μl), and the amount of binding was determined as described in the text. Results are expressed as specific binding per 12,000 cells.

10% glycerol, 1 μg of poly(dI-dC), 1 μl (approximately 100,000 cpm) of double-stranded ^{32}P-labeled oligonucleotide containing the NF-κB site from the murine immunoglobulin kappa enhancer (5′-AGCTTAGAGGGGACTTTCCGAGAGGA-3′, labeled with 25 μCi of [α-^{32}P]dATP and the Klenow fragment of *E. coli* DNA polymerase I), and 3 μg of nuclear extract. Binding-reaction mixtures were incubated at room temperature for 25 min. The reaction mixtures were then loaded onto 4% polyacrylamide gels and were electrophoresed for 1.5 h at 32 mA, as specified for the high-ionic-strength conditions described by Ausubel et al. (2). The gels were dried and autoradiographed for 18 h.

Error bars in each figure represent the mean and standard deviation of triplicate samples. Each figure is representative of several experiments.

Treatment of HUVEC with sodium butyrate sensitized the HUVEC to the cytotoxic action of Shiga toxin (Fig. 1). This sensitization was dependent on the dose of butyrate (Fig. 1) and was greater after 48 h than after shorter incubation times (unpublished observations). The 48 h required for induction of HUVEC sensitivity to Shiga toxin was similar to that described by Sandvig et al. for A431 cells and may reflect a requirement for de novo gene transcription and/or protein synthesis (25).

To determine whether the greater sensitivity of butyrate-treated HUVEC was due to a larger number of Shiga toxin receptors on the HUVEC cell surface, the ability of butyrate to increase the binding of [^{125}I]SLT-1 to HUVEC was examined. Treatment of HUVEC with 5 mM butyrate caused a sixfold increase in the amount of [^{125}I]SLT-1 bound to HUVEC (Fig. 2). Lower concentrations of butyrate induced [^{125}I]SLT-1 binding to a lesser extent (Fig. 2). The same concentrations of butyrate which induced [^{125}I]SLT-1 binding to HUVEC also increased the Gb_3 content of HUVEC (Fig. 3). These effects of butyrate on Gb_3 expression occurred despite no apparent change in cellular glucosylceramide content or in cell size (unpublished observations). These results suggest that the sensitization of HUVEC to Shiga toxin by butyrate may be due to an increased number of Shiga toxin receptors on HUVEC.

Since these effects of butyrate qualitatively resembled those of the cytokine TNF on HUVEC (13, 14, 18, 28, 30, 31), the actions of TNF were directly compared with those of butyrate toward HUVEC. Taking into account the inhibitory effect of butyrate alone on HUVEC cell number, TNF (200 U/ml) and butyrate (5 mM) sensitized HUVEC to Shiga toxin to approximately the same degree (Fig. 1). That is, pretreatment of HUVEC with 5 mM butyrate followed by treatment with 1 nM Shiga toxin reduced the number of viable cells to 18% of that in untreated controls. Pretreatment of HUVEC with 200 U of TNF per ml followed by treatment with 1 nM Shiga toxin reduced the HUVEC cell number to 45% of that in untreated controls. The difference between butyrate- and TNF-treated

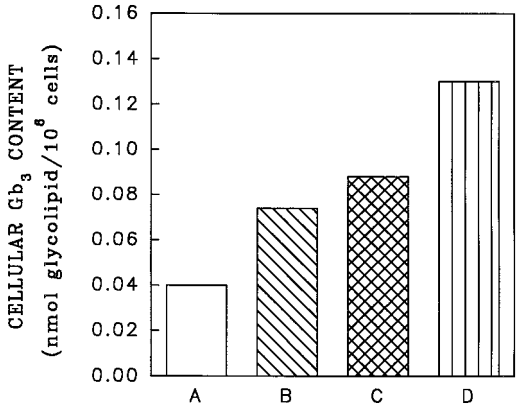

FIG. 3. Butyrate increases the Gb_3 content of HUVEC. HUVEC were grown to confluency in T-75 flasks. The medium was then changed, and the cells were either not treated (A) or treated with 1.2 mM (B), 2.5 mM (C), or 5 mM (D) butyrate for 24 h at 37°C. The medium was changed again, and the cells were treated with the same concentrations of butyrate for an additional 24 h at 37°C. The cells were then removed from the flasks, and the Gb_3 content of the cell pellets was assayed.

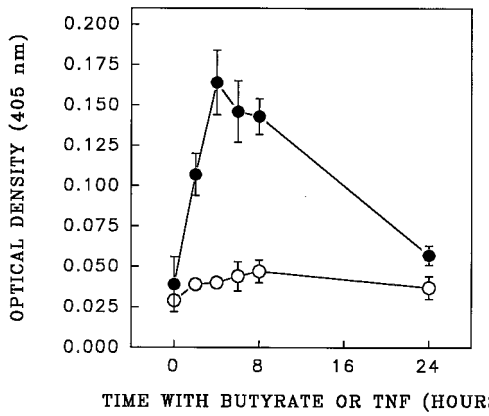

FIG. 4. Butyrate does not induce tissue factor expression in HUVEC. HUVEC were plated into 96-well plates at a density of 12,000 cells per well. Following overnight attachment, medium was removed and cells were treated either with 5 mM butyrate (open circles) or with 200 U of TNF per ml (solid circles) at 37°C for the times indicated in the figure. Medium was removed, and the cells were rinsed twice. S2222 and proplex T (final concentrations of 250 μg/ml and 0.5 factor VII U/ml, respectively) were then added to the cells, which were incubated for 2 h at 37°C. Then optical density units were determined at a wavelength of 405 nm.

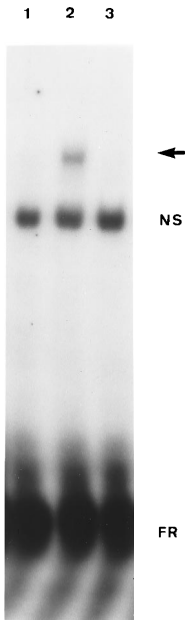

FIG. 5. Butyrate does not induce NF-κB binding activity in HUVEC nuclear extracts. HUVEC were split into T-75 flasks and grown to confluence. The medium was changed, and HUVEC were stimulated with no agent (lane 1), with 200 U of TNF per ml (lane 2), or with 5 mM sodium butyrate (lane 3). Following a 1-h incubation at 37°C, cells were removed from the flasks and nuclear extracts were prepared; the binding activity of these extracts to an oligonucleotide containing the NF-κB site of the murine immunoglobulin kappa enhancer was determined by electrophoretic mobility shift assay. Arrow, specific NF-κB-binding activity which was inhibited by a 100-fold excess of unlabeled oligonucleotide; NS, nonspecific binding of extract which was not inhibited by a 100-fold excess of unlabeled oligonucleotide; FR, free (unbound) oligonucleotide.

HUVEC was likely to be due to the effect of butyrate itself on HUVEC cell number. That is, treatment of HUVEC with 5 mM butyrate, in the absence of subsequent Shiga toxin treatment, resulted in 74% viable cells compared with untreated controls (Fig. 1). In contrast, 200 U of TNF per ml did not affect HUVEC cell number in the absence of subsequent treatment with Shiga toxin (105% viable cells). Since butyrate is known to inhibit the proliferation of HUVEC in culture (29), the smaller number of butyrate-treated HUVEC than of TNF-treated HUVEC upon subsequent treatment with Shiga toxin reflected this effect of butyrate itself on HUVEC cell number rather than a greater relative ability of butyrate to sensitize HUVEC to Shiga toxin.

Despite the approximately equal ability of butyrate and TNF to sensitize HUVEC to Shiga toxin at the dosages used, butyrate caused a much larger increase in [^{125}I]SLT-1 binding to HUVEC than did TNF (Fig. 2). This result suggests that butyrate may induce a subclass of SLT-1-binding sites which are not functional receptors for Shiga toxin in HUVEC. Treatment of HUVEC with butyrate resulted in the appearance of a second peak in the elution profile of Gb_3 by HPLC (unpublished observations). This result suggests that the fatty acid composition of Gb_3 may in fact differ in butyrate-treated and untreated HUVEC. Since the lipid moiety of Gb_3 profoundly affects its ability to serve as a functional receptor for Shiga toxin (3, 10, 12, 19), any change in the type(s) of Gb_3 could be an important factor in the sensitivity of cells to Shiga toxin. Butyrate is known to change the fatty acid composition of Gb_3 expressed in A431 cells (25). The implications of these observations in HUVEC and A431 cells remain to be determined.

Nevertheless, our binding studies suggest a distinct difference between the actions of butyrate and TNF toward HUVEC. This applies to other HUVEC parameters as well. For instance, treatment of HUVEC with TNF results in the transient expression of a procoagulant molecule, tissue factor, on the endothelial cell surface (Fig. 4) (16). In contrast, butyrate did not induce tissue factor expression in HUVEC at any time point examined (Fig. 4). Further, butyrate differed from TNF in that butyrate did not induce transcription factor NF-κB binding activity in nuclear extracts of HUVEC, whereas TNF did (Fig. 5). Thus, while some of the actions of butyrate toward HUVEC are similar to those of TNF (Fig. 1), some of the mechanisms by which butyrate acts on HUVEC almost certainly differ from those used by TNF. We do not yet know whether the activation of NF-κB is necessary for the sensitization of HUVEC to Shiga toxin by TNF. Nevertheless, these data suggest that the action of butyrate toward HUVEC does not require NF-κB.

Butyrate may influence intracellular trafficking in HUVEC, as well as increasing Shiga toxin binding to the cell surface. That is, the cytotoxic action of Shiga toxin requires transport of the toxin to the Golgi apparatus and possibly to the endoplasmic reticulum as well (23–25). These processes are stimulated by butyrate in A431 cells (23–25). However, it is also possible that toxin transport is not affected by butyrate in HUVEC, since there are several distinct differences in the regulation of Shiga toxin sensitivity in A431 cells and HUVEC. For instance, elevation of cyclic AMP (cAMP) levels in A431 cells, either with cholera toxin or with 8-bromo-cAMP, sensitized A431 cells to Shiga toxin (25). In contrast, treatment of HUVEC for 24 h with the cAMP-elevating agents forskolin (1 to 100 μM), dibutyryl cAMP (1 to 10 μM), or 3-isobutyl-1-methylxanthine (100 to 3,000 μM) failed to sensitize HUVEC to Shiga toxin (unpublished data). Thus, cAMP does not appear to play a role in the regulation of HUVEC sensitivity to Shiga toxin, although we did not confirm that the addition of these agents elevated intracellular cAMP levels in the HUVEC used for those experiments. Nevertheless, the action of TNF also differed in A431 cells and HUVEC, since TNF sensitized HUVEC but not A431 cells to Shiga toxin (Fig. 1) (14, 25). These results indicate that the factors which govern the sensi-

tivity of different cells to Shiga toxin, as well as their responses to different pharmacologic agents, may depend on the cell type being examined.

An understanding of the effects of butyrate on different cell types may lead to a greater understanding of Shiga toxin binding, internalization, intracellular transport, and activation in eukaryotic cells. These studies may also lead to a greater appreciation of the mechanisms which determine the sensitivity or resistance of different cell types to Shiga toxin. The ability of Shiga toxin to damage different cells is likely to influence the severity of Shiga toxin-related disease. Thus, a more comprehensive knowledge of the differences between Shiga toxin-sensitive and insensitive cells will be important to our understanding of the vascular and nonvascular complications of Shiga toxin-associated infections.

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