An Interventional Approach to Block Brain Damage Caused by Shiga Toxin–Producing Escherichia coli Infection, by Use of a Combination of Phosphodiesterase Inhibitors

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We tested the combination of phosphodiesterase (PDE) 3 and PDE4 inhibitors as an interventional approach to prevent the development of brain damage after Shiga toxin (Stx)–producing Escherichia coli (STEC) infection, using mice with protein calorie malnutrition. The combination consisted of pentoxifylline and rolipram; the dose of each inhibitor was 7.5 mg/kg. Treatment with this combination, which was administered intraperitoneally twice daily at 12-h intervals, increased serum concentrations of each inhibitor to >2 μg/mL and afforded significant levels of protection when it was continued for 3 days, starting on day 2 (95% survival rate; P < .001) or day 3 (63% survival rate; P < .01) of infection. The treatment reduced plasma levels of Stx2; consequently, immunoreactions of Stx2 were not found in the brain, and survivors did not show neurologic symptoms. Protection was associated with decreased levels of tumor necrosis factor (TNF)–α and increased production of interleukin-10 in serum, the brain, and the cecum. Although the combination at doses >2 μg/mL reduced Gb3 content of and Stx2 binding to Caco-2 cells, its ability to suppress production of TNF-α seemed to be more important for the decrease in cell-bound Stx2 in intestinal epithelial cells. Therefore, the combination of PDE3 and PDE4 inhibitors might be used as an interventional approach to prevent brain damage caused by STEC infection.

Shiga toxin (Stx)–producing Escherichia coli (STEC) infection often causes hemolytic uremic syndrome [1], which manifests as systemic vascular damage characterized by thrombotic microangiopathy in affected organs, such as the kidneys and the brain [2]. Such vascular damage may result from the synergistic action of Stx and proinflammatory cytokines [3]. Among the cytokines, tumor necrosis factor (TNF)–α and interleukin (IL)–1β are considered to play an important role in damaging vascular endothelial cells [4]. TNF-α mediates or enhances cytotoxicity of microglia (MG) or macrophages to oligodendrocytes in the brain [5, 6], and it causes neuronal cell death in glial cells [7]. Thus, regulation of production of TNF-α might reduce brain damage after STEC infection.

Inhibition of phosphodiesterase (PDE) effectively reduces the production of TNF-α, IL-1β, and IL-6 by MG and macrophages [8]. PDE inhibitors, therefore, are likely to reduce the brain damage after STEC infection, since the inhibitors can pass through the blood-brain barrier. For the clinical use of PDE inhibitors, high concentrations of PDE inhibitors inevitably elicit deleterious effects, particularly in patients with renal disorder [9]. It has been reported, however, that a combination of PDE inhibitors reduced the dose of individual drugs needed to suppress production of cyto-
kines [10]. We therefore attempted to use the combination of PDE3 and PDE4 inhibitors as an interventional approach to prevent STEC infection–associated encephalopathy, since the mononuclear cell lineage predominantly expresses these 2 PDE isoforms [11].

In the present study, we tested the combination of PDE3 and PDE4 inhibitors for its therapeutic effect, using mice with protein calorie malnutrition (PCM) that developed encephalopathy after STEC infection [12]. In mice with PCM in infected with STEC, we confirmed the therapeutic efficacy of the combination during the postinfection window [13].

**MATERIALS AND METHODS**

**PDE inhibitors.** Pentoxifylline (3,7-dimethyl-1-[5-oxohexyl] xanthine) (PDE3 inhibitor) and rolipram (racemate of 4-[[3'-cyclopentyloxy-4'-methoxyphenyl-2- pyrrolidone]] (PDE4 inhibitor) were purchased from Sigma Chemicals. Fresh solutions of individual drugs were prepared for each experiment by use of DMSO (Sigma) diluted 1:1000 with culture medium. All combinations consisted of an equal dose of PDE3 and PDE4 inhibitors; the dose for each combination was expressed as either an administration dose (milligrams per kilogram) or a final concentration in culture system (micrograms per milliliter) of a single inhibitor.

**Measurement of plasma PDE inhibitors.** Concentrations of pentoxifylline in plasma were determined by reverse-phase high-performance liquid chromatography after chiral derivatization of the metabolite, exactly following the method of Nicklasson et al. [14]. In brief, the sample was extracted from plasma by use of dichloromethane, and the organic phase that had been separated by centrifugation was evaporated to dryness before chiral derivatization was performed. The mean extraction yield of pentoxifylline from plasma was ∼98% in this assay system when determined by use of the admixture of pentoxifylline and normal mouse plasma. The minimum detectable concentration of pentoxifylline was 2.5 ng/mL.

After the sample was extracted from plasma by use of solid-phase extraction cartridges (Varian) and eluted with methylene chloride/isopropanol:ammonium hydroxide (80:20:2), concentrations of rolipram were determined according to methods described elsewhere [15]. The mean extraction yield of rolipram from plasma was ∼98% in this assay system when determined by use of the admixture of rolipram and normal mouse plasma. The minimum detectable concentration of rolipram was 3.4 ng/mL.

**Mouse infection protocol.** Specific pathogen–free 3-week-old female C57BL/6 mice (Charles River Breeding Laboratories) were fed a low-protein calorie diet (5% [wt/wt] casein) (Oriental BioService) for 2 weeks, to achieve PCM [12]. At 5 weeks of age, the mice with PCM were infected intragastrically with 2 × 10⁷ cfu of STEC O157:H7 strain N-9; this strain produced greater quantities of Stx2, compared with Stx1, and was resistant to 100 μg/mL streptomycin (SM). Establishment of infection was confirmed by detection of O157 antigens and Stx2 in stool specimens by use of EIA kits (Meridian Diagnostic) [12]. These tests were performed on day 2 of infection, and only mice positive for the O157 antigens and Stx2 in stool specimens were used in subsequent experiments. A total of 52 mice that were positive for these 2 antigens were randomly allocated to 4 groups. Groups of 13 infected mice were injected intraperitoneally (ip) with the combination of PDE3 and PDE4 inhibitors, in accordance with indicated schedules. Control mice received ip injections of 0.1 mL of diluted DMSO. The treatment was performed at 8:00 am and 8:00 pm and continued for 3 days. On day 3 of infection, we randomly selected 3 mice from each group and extracted blood, to measure serum levels of Stx2 by EIA, to ensure the systemic progression of infection [12]. On day 4, the same experiments were repeated twice. Infected mice with negative STEC cultures were excluded from the final analysis. The Committee of Animal Ethics of Nara Medical University approved all animal experiments in the present study, and, in the performance of all animal experiments, we followed the animal-experimentation guidelines of our university.

**Measurement of cytokines.** Cytokines in the brain, the cecum, serum, and culture supernatants were quantified by use of EIA kits (Genzyme). Homogenates of the brain and cecum, for EIA tests, were prepared as described elsewhere [16]. A standard curve for each cytokine was constructed with the appropriate recombinant mouse cytokine (Genzyme) incorporated into serum or tissue extracts from healthy mice [16]; the limits of detection for IL-1β, IL-6, IL-10, and TNF-α in serum and culture supernatants were 14, 20, 25, and 18 pg/mL, respectively. The limit of detection for cytokines in brain extracts was 30 pg/mg tissue protein, and that for intestinal extracts was 45 pg/mg tissue protein, irrespective of the cytokine measured. Serum samples were diluted 2:1 before measurement. The protein content of each tissue extract was determined by the dye-binding assay, by use of a DC protein assay kit (Bio-Rad Laboratories).

**Detection of Stx2 in the brain and cecum.** For histochemical detection of Stx2 in the brain, sections were pretreated with normal goat serum and stained with a monoclonal antibody (MAB) to Stx2 (IgG class; Toxin Technology), which was followed by treatment with biotinylated anti–mouse IgG F(ab')₂ (Organon Teknika) and a streptavidin-peroxidase complex (Histofine SAB-PO kit; Nichirei) [12]. Diaminobenzidine (DAB)–H₂O₂ was used for colorization. The sections were counterstained with hematoxylin.

Stx2 binding to cecal epithelial cells was quantified by use of a method described elsewhere [17]. In brief, a whole cecum was
Interventional Approach to STEC infection

Figure 1. Dose-dependent suppression of production of tumor necrosis factor (TNF-α) by microglia (MG), mesangial cells (MCs), and macrophages (J774A.1), by phosphodiesterase (PDE) inhibitors. Cells, at a density of 2 × 10⁶ cells/500 µL/well, were stimulated with 20 pg/mL Shiga toxin 2 (0.2 CD₅₀ for MG and 0.04 CD₅₀ for MCs and J774) and 10 ng/mL lipopolysaccharide, for 18 h, in the presence of pentoxifylline (○), rolipram (●), or the combination (△), and culture supernatants were subjected to TNF-α EIA. At the end of incubation, >98% of the initial input remained viable, irrespective of cell types and additives. Concentrations for the combination represent doses of a single inhibitor—that is, the 0.5-µg/mL combination consisted of 0.5 µg/mL pentoxifylline and 0.5 µg/mL rolipram in culture. Each point represents the mean value (n = 6), and, at each point, the SE was within 17.5% of each mean value. A, MG; B, MCs; C, J774.

Minced into small pieces, after the lumen had been completely washed with saline containing 50 µg/mL gentamycin. A single-cell suspension was made following the method of Stern [18], by use of citrate in Ca²⁺- and Mg²⁺-free balanced salt solution (CMFS) containing 1.5 mmol/L EDTA but no proteases or other enzymes; the method isolated only epithelial cells [18]. Cell suspensions (5 × 10⁶ cells/mL in CMFS containing 0.1% Triton-X) were sonicated; extracts were centrifuged at 15,000 g for 20 min at 4°C. Stx2 in clear supernatants was measured by EIA and expressed as picograms per 5 × 10⁶ cells.

Preparation of Stx2 and lipopolysaccharide (LPS). Recombinant Stx2 was prepared as described elsewhere [19]. LPS (smooth type) was extracted from E. coli O113 (Stx₁ and Stx₂), an isolate from the stool specimen from a healthy male mouse, by use of the method of phenol-water extraction followed by gel filtration.

Figure 2. Plasma concentrations of pentoxifylline and rolipram. Mice received one of the combinations (2.5, 5, 7.5, or 10 mg/kg) intraperitoneally (ip). Each combination contained equivalent doses of individual inhibitors; for example, the 2.5-mg/kg combination consisted of 2.5 mg/kg pentoxifylline and 2.5 mg/kg rolipram. ip injections were performed at 12-h intervals, and plasma concentrations were determined 4 h after every injection. Data are expressed as mean ± SE (n = 6). Bars represent SE values. ○, Pentoxifylline; ●, rolipram.
Figure 3. Protection of mice against Shiga toxin–producing Escherichia coli (STEC) infection, by phosphodiesterase inhibitors. Mice were infected intragastrically with cfu of STEC O157:H7 strain N-972 and kept for 22 days, to determine survival. Groups of mice received the 7.5-mg/kg combination intraperitoneally, starting on day 2 (D2), day 3 (D3), or day 4 (D4) of infection and continuing for 3 days. Control mice (C) received vehicle treatment for 3 days, starting on day 2 of infection.

Cell culture. Primary mixed glial cells were prepared from newborn BALB/c mice [20], and MG were isolated from cultures of the mixed glial cells by use of the shaking-off method [20]. MG were cultured in Dulbecco’s modified Eagle medium (DMEM) (GIBCO) with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO), 100 U/mL penicillin (Pc), 50 µg/mL SM, 5 µg/mL bovine insulin, and 0.2% glucose. Isolated cells consisted of a cell population in which >98% of cells stained positively with F4/80 MAb (CI; A3-1) (Serotec) and <1% of cells stained positively with antibodies specific to the astrocyte marker, glial fibrillary acid protein (Sigma); these glial cells were defined as MG.

Mesangial cells (MCs) were isolated, by use of the method of Deocharan et al. [21], from the outer cortices of kidneys of 6-week-old BALB/c mice. MCs were cultured in RPMI 1640 medium supplemented with 20% FBS, 5 µg/mL bovine insulin, Pc/SM, and 2 mmol/L L-glutamine. D-valine (20 µg/mL), instead of L-valine, was added to the culture to inhibit the growth of fibroblasts. Isolated MCs consisted of a cell population in which 97% of cells stained positively with antibodies specific to fibronectin and desmin (Sigma) and <1% of cells stained positively with anti-cytokeratin or anti-Ia antibody (Serotec). J774A.1 macrophages (TIB-67; American Type Culture Collection) were cultured in DMEM with 10% heat-inactivated FBS. These cells, at a density of cells/500 µL/well, were stimulated with 20 pg/mL Stx2 and 10 ng/mL LPS for 18 h in the presence of pentoxifylline, rolipram, or the combination of PDE3 and PDE4 inhibitors; this dose of Stx2 was equivalent to 0.2 CD50 for MG and 0.04 CD50 for MCs and J774 cells. Diluted DMSO was used as a vehicle control for stimulation.

Caco-2 cells (human colonic adenocarcinoma; HTB-37; American Type Culture Collection) were cultured in DMEM containing 26 mmol/L NaHCO3 and supplemented with 0.1 mmol/L nonessential amino acids, 10% FBS, and Pc/SM [pH 7.4] in a 5% CO2 atmosphere at 37°C. Cells were grown to confluence and harvested 7 days after reaching confluence. Harvested cells were distributed into Eppendorf tubes (1.5 mL) at a density of 2 × 105 cells/tube, in 200 µL of the medium (FBS was reduced to 2% [vol/vol]), and recombinant human TNF-α (20 U/mL; R&D Systems) or medium alone (50 µL) was added. The tubes were horizontally rotated at 10 rpm for 24 h at 37°C in a 5% CO2 incubator; the combination (50 µL) was added to each tube 18 h before the end of incubation. After incubation, cells were washed and used for Gb3 extraction. For the Stx2-binding assay, cells were distributed into 96-well plates (2 × 103 cells/well) and stained positively with antibodies specific to the astrocyte marker, glial fibrillary acid protein (Sigma); these glial cells were defined as MG.

Gb3 content and Stx2 binding. Gb3 content was determined as described elsewhere [22]. Caco-2 cells were extracted in chloroform-methanol-water and separated on high-performance thin-layer chromatography silica plates (0.2 mm thick; Merck AG) [16]. Purified Gb3 (Biocarb Chemical) was also separated...
on the same plate as a positive control. The plates were immersed in 0.5% polyisobutylmethacrylate in acetone, transferred to polyvinylidene difluoride (PVDF) membranes, and sequentially incubated with anti-Pk MAb (mouse IgM; Accurate Chemical & Scientific) and peroxidase-conjugated goat anti-mouse IgM F(ab')2 (Organon Teknika) [16]. The color reaction was performed with DAB-H2O2. Gb3 concentrations were calculated by densitometry and standardized to the total protein level, which was measured with a DC protein assay kit.

For the Stx2-binding assay, cells grown in 96-well plates (100 μL) were incubated for 24 h with 20 U/mL recombinant human TNF-α (R&D Systems), and varying doses of the combination (10 μL) were added for the final 18 h. Stx2 was radiolabeled with Na125I by use of the method of Fraker and Speck [23, 24]. The Na125I-labeled Stx2-binding assay was performed following a method reported elsewhere [25]. Na125I-labeled Stx2 (16,000–18,000 cpm) in 100 μL of the medium (5% FBS) was added to wells, and cells were incubated for 1 h at 4°C. After extensive washing, cells were dissolved in lysis solution (0.1 N NaOH and 0.5% SDS). Recovered radioactivity was measured by use of a γ-counter. Specific binding of the radiolabeled Stx2 was completely inhibited by the presence of excess unlabeled Stx2.

Statistics. The significance of the difference observed was assessed by use of Fisher’s exact test for 2 × 2, when comparing survival time, and by use of the Kruskal-Wallis 1-way analysis of variance, when comparing cytokine-specific mRNA levels, EIA, Gb3 content, and Stx2-binding values. P < .05 was considered to be significant.

RESULTS

Dose-finding studies. A single inhibitor, either pentoxifylline or rolipram, required doses >7 μg/mL to decrease production of TNF-α by Stx2 plus LPS–stimulated MG, MCs, and J774 cells to 50% of respective control levels, whereas the 1.0-μg/mL combination achieved the same degree of suppression in culture of the stimulated cells (figure 1). We attempted to maintain plasma concentrations of each inhibitor at levels >2.0 μg/mL.

Combinations of PDE3 and PDE4 inhibitors were prepared at 8 different doses (0.05, 0.5, 1.0, 2.5, 5, 7.5, 10, and 20 mg/kg). Each combination contained equivalent doses of pentoxifylline and rolipram; for example, the 5-mg/kg combination consisted of 5 mg/kg pentoxifylline and 5 mg/kg rolipram. Each combination was administered ip at 12-h intervals, and plasma concentrations were measured 4 h after every injection. Combinations at doses <5 mg/kg did not achieve effective plasma concentrations of each inhibitor (figure 2A and 2B); 4 h after the sixth injection, maximal mean ± SE plasma concentrations achieved by injecting the 5-mg/kg combination were 1.52 ± 0.31 μg/mL for pentoxifylline and 1.27 ± 0.40 μg/mL for rolipram. The 7.5-mg/kg combination achieved effective concentrations for both drugs 4 h after every injection (mean ± SE range: pentoxifylline, 3.52 ± 0.55–4.02 ± 0.29 μg/mL; rolipram, 2.58 ± 0.36–3.51 ± 0.33 μg/mL [over the course of 6 measurements]) (figure 2C) and did not induce any unfavorable effects. Injection of either the 10-μg/mL combination or the 20-mg/kg combination achieved sufficient plasma concentrations for both inhibitors (minimal mean ± SE level: pentoxifylline, 3.52 ± 0.55–4.02 ± 0.29 μg/mL; rolipram, 2.58 ± 0.36–3.51 ± 0.33 μg/mL [over the course of 6 measurements]).

Table 1. Quantity of fecal and cecal epithelial cell–bound Shiga toxin (Stx) 2 in Stx-producing Escherichia coli–infected mice treated with protocol 1 or vehicle.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stx2 in stool specimens, pg/mg of stool</th>
<th>Cell-bound Stx2, pg/5 × 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 6</td>
</tr>
<tr>
<td>Vehicle</td>
<td>42.3 ± 6.4</td>
<td>54.8 ± 7.3</td>
</tr>
<tr>
<td>Protocol 1</td>
<td>38.8 ± 7.1</td>
<td>49.8 ± 5.2</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SE. Samples of individual mice (n = 3) were tested in triplicate. Results were obtained from 3 separate experiments. Cell-bound Stx2 represents the quantity of Stx2 extracted from cecal epithelial cells.

* P < .05.

† P < .01.
9.62 ± 0.78 μg/mL; rolipram, 7.81 ± 0.84 μg/mL [4 h after the first injection of the 20-mg/kg combination]). However, these 2 doses made mice behave ill after the third injection. The 7.5-mg/kg combination was therefore selected for the treatment.

**Therapeutic effects of the combination.** We started a 3-day treatment with the 7-mg/kg combination on day 2 (D2 group), day 3 (D3 group), or day 4 (D4 group) of infection. The vehicle control group was set only for the D2 group. In respective trials, 3 mice selected on day 3 from each of the groups were positive for serum Stx2. STEC was recovered on day 4 from stool specimens from all mice in the D2 group, whereas the pathogen was recovered from stool specimens from 19 mice in the vehicle control, D3, and D4 groups.

All of the mice in the vehicle control group developed neurologic symptoms, including hind-leg paralysis and jerky rhythmic motion, and died by day 10 of infection (figure 3). Throughout 2 trials, only 1 mouse in the D2 group died, on day 8, after developing neurologic symptoms; the others survived without neurologic disorder (mean survival rate, 95%; P < .001). In the D3 group, 7 of 19 mice with neurologic symptoms died between days 16 and 18 of infection (mean survival rate, 63%; P < .01). All mice (n = 19) in the D4 group developed neurologic symptoms and died by day 16. Therefore, the early initiation of the combination was effective in prevention of fatal combined diseases.

**Assessment of therapeutic effects.** Next, we characterized the therapeutic effects of the 7.5-mg/kg combination in mice in the D2 group. Serum levels of TNF-α, IL-1β, and IL-6 decreased in treated mice between days 3 and 6 of infection (P < .05), compared with those in vehicle control mice, whereas serum levels of IL-10 increased between days 4 and 6 of infection (P < .05) (figure 4). Such differential regulation of inflammatory cytokines, especially TNF-α and IL-10, by the combination (figures 4 and 5) is consistent with the in vitro findings reported by other investigators [10, 26]; also, this ability appears to be common to agents that increase intracellular levels of cAMP [27–32]. With regard to STEC infection, several investigators have implicated IL-10 as a host-defending cytokine, since it suppresses Stx-induced apoptosis [16] and LPS-induced production of TNF-α [33].

Unexpectedly, serum levels of Stx2 decreased to undetectable levels after 2 injections of the combination (figure 4). The combination reduced amounts of cell-associated Stx2 in cecal epithelial cells to a significant extent (P < .05, for day 4; P < .01, for day 6) (table 1), although it did not affect the growth of STEC in the intestine (data not shown). Because this cytokine
DISCUSSION

Recently, PDE4 inhibitors have been shown to promote cholesterol efflux from cells by enhancing the expression of ATP cassette binding protein 1, which controls the apolipoprotein-mediated cholesterol efflux pathway [36]. Thus, it might be speculated that enhanced cholesterol efflux alters Gb3 clustering in lipid rafts of Caco-2 plasma membranes. However, it has been shown that disruption of lipid rafts by cholesterol depletion does not inhibit the binding of Stx1-B but does affect Stx1-B internalization and translocation across the intestinal epithelia [37]. Since the expression of Gb3 in mouse intestinal epithelia is very low, the findings of these studies suggest that the combination reduces the quantity of Stx2 in cecal epithelial cells by suppressing internalization of Stx2 in synergy with its ability to suppress production of TNF-α, rather than by affecting Stx2 binding to the cells. In fact, differences between the D2 group and the vehicle control group, in the quantity of cell-bound Stx2 in the cecum on day 6 (table 1), appeared to be greater than expected from the results of the Stx2-binding assay. Further investigation is necessary to elucidate how the combination reduces the quantity of Stx2 in intestinal epithelial cells.

In the brains of moribund vehicle control mice, immunoreactions of Stx2 were noticed in the cortex of the temporal lobe (figure 7A). In contrast, we did not find immunoreactions at this area in mice in the D2 group (figure 7B). In addition to the differential regulation of TNF-α and IL-10 in the brain by the combination (figure 5), rolipram is reported to have a stabilizing effect on the blood-brain barrier [38]; this effect might reduce the inflow of TNF-α and Stx into the brain from the circulation.

Finally, pentoxifylline is known to be an antithrombotic agent: it increases erythrocyte flexibility, reduces blood viscosity, and decreases platelet aggregation [39, 40]. Therefore, these effects might be expected to reduce the risk of microangiopathy. In addition, rolipram has been shown to control MG function, the enhancement of which may promote the inflammatory pathological processes that are either neurotropic or neurotoxic for neurons in the brain [41]. It is also known that, at high concentrations, pentoxifylline produces cardiovascular adverse effects and rolipram induces nausea and vomiting [9]. However, the 7.5-mg/kg combination did not increase plasma concentrations of these inhibitors to such an extent that deleterious effects were elicited in treated mice.

In conclusion, this combination therapy might be used clinically with less limitation of timing for its initiation, since there are longer periods for a postinfection window in clinical cases than in infected mice. Therefore, this combination therapy is one of the promising interventional approaches for prevention of development of STEC infection–induced brain damage.

References
