Anisodamine Inhibits Shiga Toxin Type 2-Mediated Tumor Necrosis Factor-α Production *in Vitro* and *in Vivo*[1]

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Cytokines, in particular tumor necrosis factor (TNF), appear to be necessary to develop the pathological process of Shiga toxin-producing *Escherichia coli* (STEC) infection. In this study we examined the effect of anisodamine, a vasoactive drug, on TNF-α production in Shiga toxin type 2 (Stx2)-stimulated human monocytic cells *in vitro* and in Stx2-injected mice sera *in vivo*. Human monocytes and THP-1 cells were stimulated by Stx2 (1–100 ng/ml) with or without anisodamine addition (1–400 μg/ml). For *in vivo* evaluations, C57BL/6 mice were given a single intraperitoneal injection of anisodamine (6–50 mg/kg) or saline after intraperitoneal injection of Stx2 (50 ng/kg). The results showed that anisodamine suppressed Stx2-induced TNF-α production in a dose- and time-dependent manner. Anisodamine also suppressed Stx2-induced TNF-α mRNA expression. Further study showed that endogenous prostaglandin E2 may be involved in this inhibitory effect. In contrast to TNF-α mRNA, anisodamine at concentrations as high as 400 μg/ml did not decrease Stx2-induced IL-1β and IL-8 mRNA levels. In addition, anisodamine (>50 μg/ml) increased Stx2-stimulated THP-1 cell viability. Levels of TNF-α in anisodamine-treated mice sera were significantly lower than those in the saline-treated group 1.5 and 24 hr after Stx2 injection. Anisodamine induced a lower percentage of death in Stx2-injected mice. Taken together, our results indicate that anisodamine has an important regulatory effect on Stx2-induced TNF-α production *in vitro* and *in vivo*. The present study suggested that this drug should be further investigated for its effects on Stx2-mediated diseases in humans. [Exp Biol Med Vol. 226(6):597–604, 2001]

Key words: anisodamine; Stx2; TNF-α

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Haemolytic uraemic syndrome (HUS) is a clinical syndrome consisting of haemolytic anaemia, thrombocytopenia, and acute renal insufficiency (1). The syndrome is often associated with infection by Shiga toxin-producing *Escherichia coli* (STEC) (2). A number of observations indicate that inflammatory mediators, in particular tumor necrosis factor (TNF) α, contribute to this pathologic process in HUS (3–5). TNF-α has been shown to upregulate the expression of the Stx receptor on endothelial cells. This resulted in endothelial cells becoming more sensitive to the toxic effect. However, the direct cytotoxic effect of Stx on human vascular endothelial cells *in vitro* was minimal in the absence of TNF-α (4, 6). In addition, TNF-α increased endothelial cell procoagulant activity (7). TNF-α also initiated the release of interleukin (IL)-6 and IL-8, followed by an increase in the levels of circulating markers of thrombin and fibrin generation (8). In the current concept of coagulation, thrombin generation is induced by the assembly of tissue factor (TF) VIIa complex, and TNF-α induces the TF expression on monocytes and endothelial cells (8).

The role of antibiotics in the prevention and amelioration of HUS remains controversial and an optimal treatment for STEC infection has not been established (9, 10). Anisodamine, an alkaloid extracted from a Chinese herb, is a vasoactive drug that appears efficacious in clinical and experimental bacteremic shock, and is well known for its dramatic therapeutic effect on acute disseminated intravascular coagulation (DIC) (11–14). Earlier studies demonstrated that it had anti-platelet-aggregating, microcirculation-facilitating, thromboxane-B2-inhibiting, malondialdehyde-inhibiting, and 6-keto-PGF1 alpha-sparing effects (11, 12, 15). However, whether anisodamine is efficacious in STEC infection has not been investigated.

To clarify this possibility, based on the fact that TNF-α is a key molecule in the pathogenesis of HUS, we investigated whether anisodamine could modulate Stx2-induced TNF-α production *in vitro* and *in vivo*.

Materials and Methods

Reagents. Purified Stx2 (0.24 mg of protein/ml) was kindly provided by Fumio Gondaira (Denka Seiken Co., Tokyo, Japan). The Stx2 preparations contained undetectable endotoxin (less than 0.05 endotoxin units/ml) contamination according to a limulus amebocyte lysate assay. The clinical preparation of anisodamine (raceanisodamine hydrochloride; $C_{17}H_{24}NO_4$; 10 mg/ml in saline) was purchased from Hangzhou Drug Co. (Hangzhou, China). The chemical structure of anisodamine has been described previously (11, 27). Indomathacin was obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant TNF-α (rTNF-α) was obtained from R & D Systems, Europe (Abingdon, Oxon, England) and prostaglandin E_2 (PGE_2) was obtained from Amersham Life Science (Buckinghamshire, UK).

Cell Cultures and Cytokine Production. Human peripheral blood monocytes were derived from heparinized venous blood collected from healthy volunteers. Mononuclear cells were separated by Ficoll-Hypaque (ICN Biomedicals, Aurora, OH) gradient centrifugation, and plastic nonadherent cells were removed after 1 hr of incubation at 37°C. Monocytes were identified by staining for nonspecific esterase and viability was higher than 96%. A human monocytic cell line, THP-1 (16), was purchased from ATCC (Rockville, MD). All monocytic cells were cultured in RPMI-1640 (ICN Biomedicals) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), and 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY) at 37°C in humidified 5% CO_2. Human monocytes (1×10^5 cells/ml) and THP-1 cells (1×10^6 cells/ml) were either incubated under basal conditions or stimulated by different concentrations of Stx2 (1–100 ng/ml) with or without anisodamine addition (1–400 μg/ml) for various periods of time. To determine the effect of indomethacin on TNF-α production, THP-1 cells were pretreated with or without 1 μM indomethacin for 2 hr, and were then incubated with 5 ng/ml Stx2 plus 50 μg/ml anisodamine for 24 h. To confirm the inhibitory effect of PGE_2 on TNF-α production, THP-1 cells were also stimulated by Stx2 (5 ng/ml) with or without a PGE_2 (10 ng/ml) addition for 24 h.

Measurement of Cytokines. After incubation, the cells were procured and centrifuged. The supernatants were collected and stored at −20°C until they were assayed for the presence of cytokines. TNF-α, IL-1β, IL-8, and IL-10 in the supernatants were determined with an enzyme-linked immunosorbent assay (ELISA) using commercial kits (Genzyme-THCHNE, Minneapolis, MN; ENDOGEN, Inc. Woburn, MA). Concentrations of mouse TNF-α in serum were also assessed with an ELISA kit (Genzyme-THCHNE) with a sensitivity of 23 pg/ml.

Detection of mRNA. After incubation, THP-1 cells were pelleted via centrifugation and total cellular RNA was extracted using acid guanidinium thiocyanate/phenol/chloroform. The amount of RNA was assessed with a RiboGreen RNA quantitation Kit (Molecular Probes, The Netherlands). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously (17). Briefly, cDNA was synthesized in 10 μl of reaction volumes containing 0.5 μg of total RNA, 0.25 μg of oligo $(dT)_{12-18}$, 10 mM dithiothreitol, 1.0 mM dNTPs, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL/Life Technologies, Rockville, MD). After a 10-min incubation at 25°C followed by a 50-min incubation at 42°C, samples were heated at 90°C for 5 min and were then quickly chilled on ice. DNA amplification was performed with 5 μl of the RT reaction mixture in a 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin) supplemented with 25 pmol each of 5′- and 3′ specific primers for cytokines and 0.625 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer/Roche Molecular Systems, Branchburg, NJ) in a final volume of 25 μl. The initial denaturation step consisted of 9 min at 95°C followed by amplification over 20 to 45 segmental cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec (GeneAmp PCR system 2400; Perkin-Elmer, Norwalk, CT). Primers for TNF-α, IL-1β, IL-8, and GAPDH (Table I) were kindly provided by Dr. Yasuhiro Natori (International medical center of Japan, Tokyo, Japan). To determine the linear range of amplification, the number of cycles run for amplification of cytokine mRNA was checked (Table I). Five microliters of the reaction mixture was electrophoresed in a 2% agarose gel (FMC Bioproducts, Rockland, ME) and stained with ethidium bromide to visualize the amplification products. Negative con-

Table I. Primers and Conditions for RT-PCR

	5′ primers/3′ primers (5′–3′)	PCR cycles[a]	Size of PCR product (basepairs)
TNF-α	GGACGTGGAGCTGGCCGAGGAG CACCAGCTGGTTATCTCTCAGCTC	35–45	352
IL-1β	AAACAGATGAAGTGCTCCTTCCAGG TGGAGAACACCACTTGTTGCTCCA	30–45	391
IL-8	ATGACTTCCAAGCTGGCCGTGGCT TCTCAGCCCTCTTCAAAAACTTCTC	35–45	292
GAPDH	GGGAGCCAAAAGGGTCATCATCTC CCATGCCAGTGAGCTTCCCGTTC	20–45	353

[a] Cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min.

trols were performed by omitting RNA from cDNA synthesis and specific PCR amplification.

The specificity of the RT-PCR products was confirmed by restriction enzyme digestion. Restriction profiles were analyzed using gel electrophoresis. IL-1β cDNA generated 309- and 82-bp fragments using Hind III restriction endonuclease, while TNF-α cDNA generated 298- and 54-bp fragments using Msp I restriction endonuclease. IL-8 cDNA generated 205- and 87-bp fragments using Hind III restriction endonuclease, and GAPDH cDNA generated 234- and 119-bp fragments using Msp I restriction endonuclease.

Measurements of Cellular Lactate Dehydrogenase (LDH). The determination of the amount of the enzyme LDH release from lysed target cells was a sensitive and precise measure of cellular cytotoxicity (18, 19). The LDH activity of cellular culture supernatants was determined as NADH oxidation/INT reduction using a LDH cytotoxicity detection kit (TaKaRa Biomedicals, Tokyo, Japan). Reaction products were then assayed using a microplate reader at A_{490}. Cell viability (%) was calculated from A_{490} measurements as follows: $100 - 100 \times (A_{490}$ of sample $- A_{490}$ of background$)/(A_{490}$ of total $- A_{490}$ of background).

Measurement of Intracellular PGE_2. After incubation, the cells were pelleted via centrifugation, and intracellular PGE_2 was extracted and measured using a PGE_2 enzyme immunoassay system (Amersham) according to the manufacturer's instructions.

Animals and Treatments. Male C57BL/6 mice aged 5 to 6 weeks and weighing 15 to 20 g were used throughout the experiments. They were purchased from Seiwa Experiment Animals (Fukuoka, Japan) and were fed a standard diet and water. To evaluate the *in vivo* effect of anisodamine, C57BL/6 mice were intraperitoneally injected with Stx2. In preliminary experiments we evaluated the *in vivo* lethality by serial dilutions in pyrogen-free saline. We chose a dose of 50 ng/kg of Stx2 (approximately 1 ng/mouse), which induced a mortality of >50% between 3 and 4 days after injection. Thirty minutes after injection of the toxin, the groups of anisodamine-treated mice were then intraperitoneally injected with 0.125 to 1 mg of anisodamine (approximately 6–50 mg/kg) and the group of untreated mice was injected with the same volume of saline concurrently. Mice intraperitoneally injected with saline only were used as controls. Then, 1.5 and 24 hr after injection, whole blood samples were obtained and stored. The death of the animals followed up to 7 days after Stx2 injections. To investigate whether murine survival is in fact directly due to changes in TNF-levels, an additional group of mice was previously injected with 1 ng of Stx2, and 30 min later was intraperitoneally injected with 1 mg of anisodamine plus 10 ng of rTNF-α under the same conditions.

Statistical Analysis. Data from the *in vitro* study were evaluated using the Student's *t* test and from the *in vivo* study by Wilcoxon's signed-ranks test. The statistical significance of the survival rate between treated and untreated groups was evaluated by Fisher's exact probability test. *P* values <0.05 were considered significant.

Results

Effects of Anisodamine on Stx2-Induced Cytokine Production. As shown in Figure 1, treatment with anisodamine resulted in a marked decrease in Stx2-induced TNF-α production in human monocytic cells. Figure 1A shows the time course of TNF-α production inhibition by anisodamine in human monocytes. Anisodamine at 50 μg/ml suppressed TNF-α production after 24 hr. Compared with Stx2-stimulated monocytes, TNF-α production by anisodamine-treated cells was suppressed by 47%, 52%, 56%, and 52% after 6, 12, 24, and 48 hr of incubation, respectively. Anisodamine at concentrations as 400 μg/ml inhib-

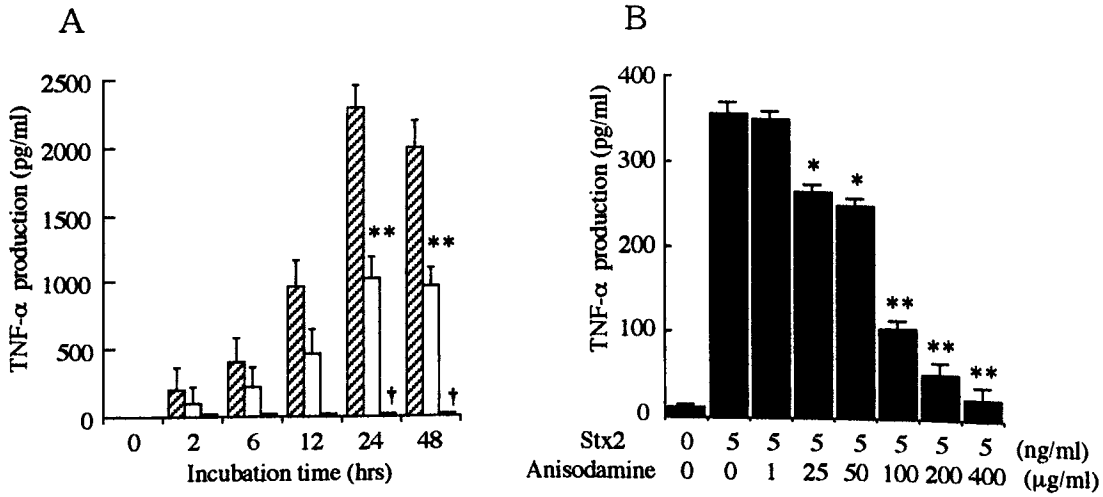

Figure 1. Suppression of Stx2-induced TNF-α production by anisodamine. (A) Time course of anisodamine-induced suppression of TNF-α production. Human monocytes were stimulated with 5 ng/ml Stx2 (striped bars) and were treated with anisodamine at 50 (white bars) or 400 μg/ml (black bars) for 2, 6, 12, 24, and 48 hr. (B) Dose dependence of anisodamine-induced suppression of TNF-α production. THP-1 cells were stimulated with or without 5 ng/ml Stx2 and were treated with anisodamine at the concentrations indicated for 24 hr. TNF-α levels in the culture supernatant were determined by ELISA. The results are presented as means ± SD of triplicate experiments ($n = 6$). *, $P < 0.05$; **, $P < 0.01$; †, $P < 0.001$ compared with the levels of TNF-α produced by Stx2-stimulated cells, evaluated by Student's *t* test.

ited Stx2-induced TNF-α production completely ($P < 0.001$). Figure 1B shows the dose dependence of anisodamine-induced suppression of TNF-α production. THP-1 cells were stimulated with or without 5 ng/ml Stx2, and were treated with anisodamine for 24 hr. Increasing the amount of anisodamine from 25 to 400 μg/ml suppressed TNF-α production in a dose-dependent manner. This inhibiting effect was also observed when the concentrations of Stx2 were increased to 100 ng/ml or decreased to 1 ng/ml in all monocytic cells (data not shown).

We found that anisodamine also inhibited Stx2-induced IL-1β and IL-8 production. However, in contrast with TNF-α, anisodamine at a concentration of 50 μg/ml did not suppress IL-1β and IL-8 production ($P > 0.05$). However, anisodamine at 100 μg/ml significantly decreased IL-1β and IL-8 production by 39% and 42% ($P < 0.01$) after 24 hr of incubation, compared with the levels of IL-1β and IL-8 produced by Stx2-stimulated monocytes, respectively. When anisodamine was increased to 400 μg/ml, it inhibited Stx2-induced IL-1β and IL-8 production by 58.3% and 61.7% ($P < 0.01$) after 24 hr of incubation, respectively. In comparison with the effect on TNF-α, anisodamine could not completely inhibit IL-1β and IL-8 production.

Finally, we determined the effect of anisodamine on IL-10 production in monocytes. Stx2 did not induce IL-10 production and anisodamine was not able to enhance IL-10 production in response to Stx2 stimulation (data not shown). In addition, anisodamine alone did not induce cytokine production.

Effects of Anisodamine on Stx2-Induced Cytokine Gene Expression. Total cellular RNA was extracted after a 24-hr incubation period from THP-1 cells and was then analyzed by RT-PCR. To determine the linear ranges of amplification, the number of cycles run for amplification of TNF-α, IL-1β, and IL-8 mRNA was varied (30–45 cycles). Figure 2A shows that only weak bands were observed in nonstimulated THP-1 cells (lane 1) after 35, 40, and 45 cycles, whereas marked increases in the TNF-α PCR signal became visible in Stx2-stimulated cells (lane 2). Compared with mRNA levels in Stx2-stimulated cells, anisodamine treatment drastically decreased Stx2-induced TNF-α mRNA levels after 35 and 40 cycles, and a weaker band was observed after 45 cycles (lane 3). The housekeeping gene GAPDH was simultaneously amplified in parallel tubes (Fig. 2A; right panel), indicating the equivalent loading of the samples, and anisodamine did not cause a general degradation of mRNA. Corresponding reactions lacking RT did not show any RT-PCR products (lane C). In addition, anisodamine alone did not induce TNF-α mRNA expression (data not shown). In contrast to TNF-α mRNA, anisodamine at concentrations as high as 400 μg/ml did not decrease Stx2-induced IL-1β and IL-8 mRNA levels (Fig. 2B, lanes 3 and 4). We confirmed that anisodamine did not decrease Stx2-induced IL-1β and IL-8 mRNA levels with any PCR cycles.

Effect of Anisodamine on Cell Viability. The effect of anisodamine on Stx2-induced THP-1 cell death was also investigated by determination of the activity of the LDH enzyme released from lysed cells in culture supernatants. Compared with the Stx2-stimulated cells, there was a reduction in specific cell lysis after addition with anisodamine. Figure 3 shows that incubation of THP-1 cells with Stx2 (5 ng/ml) in the presence of anisodamine (>50 μg/ml) increased cell viability after 24 hr. In contrast, cells exposed to Stx2 alone showed loss of cell viability.

The Effect of Endogenous PGE_2 on TNF-α Production. THP-1 cells constitutively synthesized very low levels of PGE_2 without stimulation and increased amounts of PGE_2 after incubation with Stx2 (5 ng/ml) for 24 hr (Fig. 4). However, treatment with anisodamine (50 μg/ml) significantly enhanced PGE_2 production in response to Stx2 stimulation compared with cells only stimulated with Stx2 (Fig. 4A). The cells incubated with anisodamine alone did not produce higher than basal levels of PGE_2.

Furthermore, we determined whether the inhibition of PGE_2 biosynthesis with indomethacin had any effect on

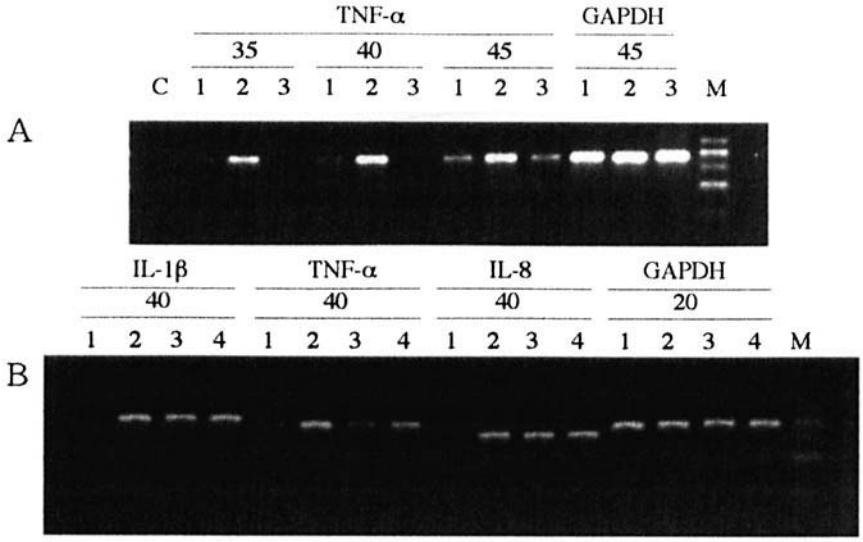

Figure 2. Suppression of Stx2-induced cytokine mRNA expression by anisodamine. (A) The effect of anisodamine on TNF-α mRNA expression. After 24 hr of incubation, THP-1 cells were procured and total cellular RNA was extracted. Messenger RNA was reverse transcripted and amplified in 45 (GAPDH) and 35, 40, and 45 segmental cycles (TNF-α). Lane C, no RT control; Lane 1, medium; Lane 2, stimulated with 5 ng/ml Stx2; Lane 3, stimulated with 5 ng/ml Stx2 and treated with 100 μg/ml anisodamine. (B) The effect of anisodamine on IL-1β and IL-8 mRNA expression. After 24 hr of incubation, total cellular RNA was extracted from THP-1 cells. Messenger RNA was reverse transcripted and amplified in 20 (GAPDH) and 40 (IL-1β, TNF-α, and IL-8) cycles. Lane 1, medium; Lane 2, stimulated with 5 ng/ml Stx2; Lane 3, stimulated with 5 ng/ml Stx2 and treated with 400 μg/ml anisodamine; Lane 4, stimulated with 5 ng/ml Stx2 and treated with 100 μg/ml anisodamine. A representative experiment of three identical experiments is shown.

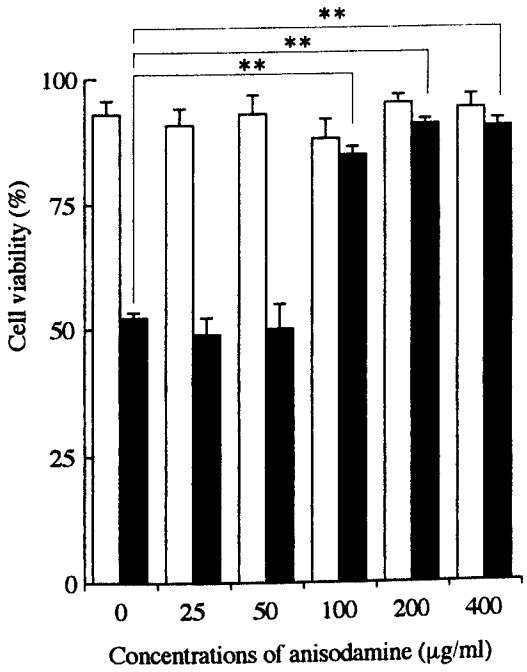

Figure 3. Effect of anisodamine on cell viability. Cell viability was determined by measuring the activity of the enzyme LDH released from lysed cells in culture supernatants after 24 hr of incubation. Cells were treated with anisodamine only (white bars) or 5 ng/ml Stx2 plus different concentrations of anisodamine (black bars). The results are presented as means ± SD of triplicate experiments (n = 6). **, $P < 0.01$ compared with the Stx2-stimulated cells, evaluated by Student's t test.

anisodamine-induced suppression of cytokine production by THP-1 cells. The results in Figure 4B show that pretreatment of cells with indomethacin significantly reversed anisodamine-suppressed TNF-α production. On the other hand, the inhibitory effect of PGE_2 (10 ng/ml) on Stx2-induced TNF-α production was also observed. These data suggested that endogenous PGE_2 was involved in the down-regulation of TNF-α production induced by anisodamine.

The *In Vivo* Effect of Anisodamine. As shown in Table II, concentrations of TNF-α in serum were detected in mice with or without anisodamine treatment after Stx2 injection. Increasing the dose of anisodamine from 0.125 to 1 mg per mouse (6–50 mg/kg) suppressed TNF-α production in a dose-dependent manner. Levels of TNF-α in the anisodamine-treated group (50 mg/kg) were significantly lower than those in the saline-treated group 1.5 and 24 hr after toxin injection. Anisodamine induced a lower percentage of death in Stx2-injected mice. However, giving recombinant TNF-α to anisodamine-treated mice did not diminish the effect of the drug completely.

Discussion

The present study investigated the effects of anisodamine on TNF-α production with *in vitro* and *in vivo* studies. Our results showed that anisodamine significantly suppressed Stx2-induced TNF-α production and mRNA expression. Anisodamine treatment decreased the levels of TNF-α in mice sera. Further study showed that alterations in endogenous PGE_2 levels may be involved in the suppression of TNF-α by anisodamine.

For the *in vitro* study, we investigated the effects of anisodamine on TNF-α production using human monocytic cells (THP-1 cells and human monocytes) because the mononuclear phagocytic system is the major source of this cytokine and because monocytes/macrophages may be relevant targets for Stx action in STEC infection (20). In addition, the influx of monocytes into the glomeruli may be an

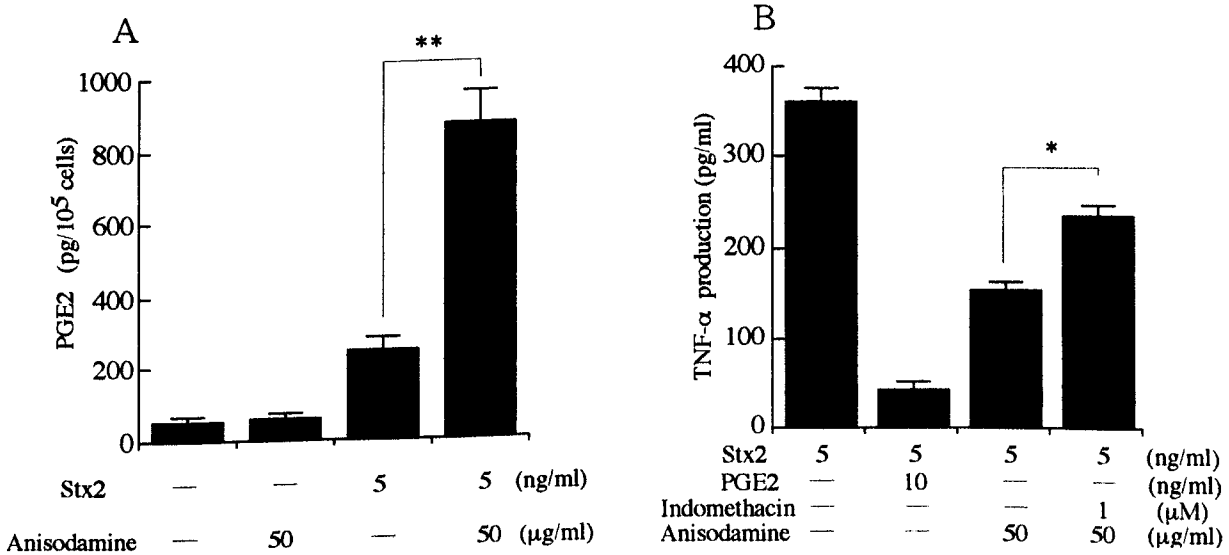

Figure 4. (A) Effect of anisodamine on intracellular PGE_2 synthesis in response to Stx2. THP-1 cells (1×10^6 cells/ml) were stimulated with or without 5 ng/ml Stx2 and treated with or without 50 μg/ml anisodamine for 24 hr. Intracellular PGE_2 was extracted and assayed with a PGE_2 enzyme immunoassay system. (B) The effect of endogenous PGE_2 on cytokine production. THP-1 cells were pretreated with or without 1 μM indomethacin for 2 hr and were then incubated with 5 ng/ml Stx2 plus 50 μg/ml anisodamine for 24 hr. THP-1 cells were also stimulated by Stx2 (5 ng/ml) with or without PGE_2 (10 ng/ml) addition for 24 hr. The levels of TNF-α released from the cells were determined by ELISA. The results are presented as means ± SD of triplicate experiments. (n = 6; *, $P < 0.05$; **, $P < 0.01$, evaluated by Student's t test).

Table II. TNF-α Levels in Mice Sera

Groups[a]	Dose of agents administered intraperitoneally[b]			TNF-α (pg/ml)		No. of survivors	% of protection
	Stx2	Ani	rTNF-α	1.5 hr	24 hr		
Controls (4)	0	0	0	<23	<23	4	100
Stx2 + saline (8)	50	0	0	146 ± 25	119 ± 49	0	0
Ani-1 (10)	50	6	0	152 ± 31	111 ± 26	0	0
Ani-2 (10)	50	13	0	139 ± 22	108 ± 37	2	20
Ani-3 (10)	50	25	0	97 ± 33	88 ± 28	4	40
Ani-4 (8)	50	50	0	55 ± 45[c]	38 ± 37[d]	5	63[e]
Ani + rTNF-α (8)	50	50	10	155 ± 16	94 ± 30	2	25

Note. Data are expressed as means ±SD. The death of animals occurred up to 7 days after Stx2 injection. There were no additional mortalities to those shown in the table.

[a] No. of mice examined in parentheses; Ani, Anisodamine.

[b] Stx2, ng/kg; Ani, mg/kg; rTNF-α, ng/mouse.

[c,d] Significant difference ([c]$P < 0.01$, [d]$P < 0.05$) compared with saline-treated mice at the same times, evaluated by Wilcoxon's signed-ranks test.

[e] $P < 0.05$ compared with saline-treated mice, evaluated by Fisher's exact probability test.

important event in the initiation, prolongation, and progression of glomerular endothelial cell damage in HUS patients (21). Our results showed that anisodamine significantly suppressed Stx2-induced TNF-α production in both types of monocytic cells. The concentrations of anisodamine we used in the *in vitro* study may appear high. In clinical use, doses of anisodamine as high as 30 to 50 mg/kg may be given intravenously several times a day. Thus, despite a short plasma half-life (estimated at 40 min), peak plasma levels approaching 1 mg/ml can be seen (22). We believe that the concentrations used are consistent with clinical situations.

Sakiri *et al.* (23) previously demonstrated that Stx1 causes increased TNF-α production through transcriptional activation. They showed that the dose-dependent production of soluble TNF correlated with increased levels of TNF-α mRNA transcripts isolated from Stx1-treated THP-1 cells, and that increased levels of TNF-α mRNA are preceded by the nuclear translocational activators nuclear factor-κB and activator protein-1. To determine whether anisodamine suppressed TNF-α production by altering the levels of TNF-α mRNA transcripts, we evaluated the effect of anisodamine on TNF-α mRNA expression in THP-1 cells using RT-PCR analysis. Our results showed that anisodamine suppressed Stx2-induced TNF-α production, which was closely correlated with the anisodamine-inhibited gene expression of this cytokine. Anisodamine at concentrations as high as 400 μg/ml did not decrease Stx2-induced IL-1β and IL-8 mRNA levels, suggesting anisodamine downregulated TNF-α mRNA specifically.

The effect of anisodamine on cell viability was also determined. Compared to Stx2-stimulated cells, anisodamine treatment (>50 μg/ml) increased cell viability. The mechanism behind this effect remains to be clarified. The data suggested that anisodamine has no cytotoxicity towards monocytic cells, and that low cytokine levels are not due to the decrease in cell viability that could be induced by anisodamine.

IL-10 and PGE_2 are known to be potent inhibitory molecules of TNF-α production in monocytes (24, 25). To further determine the underlying mechanism by which anisodamine downregulated TNF-α production, we evaluated whether IL-10 or endogenous PGE_2 was involved in the modulation. Our results showed that anisodamine was able to enhance PGE_2 production in response to Stx2 stimulation. Pretreatment with indomethacin, a cyclooxygenase inhibitor, reversed anisodamine-inhibited TNF-α production. However, we could not confirm that anisodamine was able to enhance IL-10 production in response to Stx2 stimulation. It seems that downregulation of TNF-α is not associated with IL-10 production, but that endogenous PGE_2 may be involved. PGE_2 has previously been shown to have two different, dose-dependent effects: low PGE_2 concentrations stimulate, whereas higher concentrations suppress, TNF-α release (24). In our study, the stimulated or suppressed effect of PGE_2 on TNF-α production was also observed. Low intracellular PGE_2 levels (<500 pg $PGE_2/10^5$ cells) induced TNF-α production, but higher PGE_2 levels inhibited it. There seems to be a threshold level of PGE_2 that must be reached in order to inhibit TNF-α production.

It has been reported that mice slightly infected with 200 CFU of STEC O157:H7 and intraperitoneally injected with recombinant TNF-α developed severe neurotoxic symptoms and had a higher frequency of systemic symptoms and glomerular pathology; however, mice infected with the same number of bacteria without TNF-α injection showed no histological changes, similar to the findings in control mice (26). Therefore, TNF-α is thought to be important in modifying diseases caused by STEC infection. In our *in vivo* experiments, a significant difference was found between the level of TNF-α in Stx2-injected animals compared with anisodamine-treated animals. We also found marked protection against Stx2 toxicity due to anisodamine treatment. The results are in agreement with a previous study reported by Palermo *et al.* (20). They reported that the depletion of hepatic and splenic macrophages significantly reduced the

level of TNF-α in serum and decreased lethality in mice injected with Stx2 (20).

Anisodamine was originally extracted from *Anisodus tanguticus*, a variety of Datura (27). It was first used in an attempt to improve microvascular perfusion in bacteremic shock, especially in DIC, in 1965 in China (13). It has been credited with contributing to an improved survival rate in the treatment of acute epidemic meningococcal meningitis; the mortality from meningococcemia fell from 67% to 12.4% after anisodamine treatment (13). Based on results that treatment with anisodamine improves the survival of mice intraperitoneally injected with Stx2, it is possible that anisodamine may also be efficacious in STEC infection. Although further studies are necessary to investigate the mechanism of this effect, our study indicated that anisodamine, at least in part, exerts a protective effect against Stx2 toxicity by inhibition of TNF-α production. Giving recombinant TNF-α to anisodamine-treated mice did not diminish the effect of the drug completely. The data suggested that anisodamine may have multifunctional effects on Stx2-injected mice. Anisodamine is also an inhibitor of platelet aggregation, granulocyte aggregation, and thromboxane synthesis (11, 12). Other data indicate that anisodamine is a potent vasodilator of preglomerular renal vessels (28). HUS is a disease characterized by the deposition of fibrin thrombi within glomeruli (1, 2). A previous study suggested an important local role for monocytes in the process of glomerular endothelial cell damage. Chemokines such as monocyte chemoattractant protein-1 may be implicated in the pathogenesis of HUS through the recruitment and activation of monocytes (21). To develop a rational treatment for STEC infection with anisodamine, we are currently investigating its efficacy using a renal tubular epithelial cell line.

In conclusion, our results indicate that anisodamine has an important regulatory effect on Stx2-induced TNF-α production *in vitro* and *in vivo*. Endogenous PGE_2 may be involved in this inhibitory effect of anisodamine on TNF-α. Treatment with anisodamine increased the survival rate of mice. The present study suggested that anisodamine should be further investigated for its effects on Stx2-mediated diseases in humans.

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1. Fong JS, De Chadarevian JP, Kaplan BS. Hemolytic uremic syndrome: Current concepts and management. Pediatr Clin North Am **29:**835–856, 1982.
2. Karmali MA, Petric M, Lim C, Fleming DC, Arbus GS, Lior H. The association between idiopathic hemolytic syndrome and infection by verocytotoxin producing *Escherichia coli*. J Infec Dis **151:**775–782, 1985.
3. Harel Y, Silva M, Giroir B, Weinberg A, Cleary TB, Beutler B. A reporter transgene indicates renal-specific induction of tumor necrosis factor (TNF) by Shiga-like toxin: Possible involvement of TNF in hemolytic uremic syndrome. J Clin Invest **92:**2110–2116, 1993.
4. van Setten PA, Monnens LA, Verstraten RG, van den Heuvel LP, van Hinsbergh VW. Effects of verocytotoxin-1 on nonadherent human monocytes: Binding characteristics, protein synthesis, and induction of cytokine release. Blood **88:**174–183, 1996.
5. Tesh VL, Samuel JE, Perera LP, Sharefkin JB, O'Brien AD. Evaluation of the role of Shiga and Shiga-like toxins in mediating direct damage to human vascular endothelial cells. J Infect Dis **164:**344–352, 1991.
6. van de Kar NC, Kooistra T, Vermeer M, Lesslauer W, Monnens LA, van Hinsbergh VM. Tumor necrosis factor alpha induces endothelial galactosyl transferase activity and verocytotoxin receptors: Role of specific tumor necrosis factor receptors and protein kinase C. Blood **85:**734–743, 1995.
7. Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotra RS, Gimbrone MA. Recombinant tumor necrosis factor produces pro-coagulant activity in cultured human vascular endothelium. Proc Natl Acad Sci U S A **83:**4533–4537, 1986.
8. van Gorp ECM, Suharti C, ten Cate H, Dolmans WMV, van der Meer JWM, ten Cate JW, Brandjes DPM. Review: Infectious diseases and coagulation disorders. J Infect Dis **180:**176–186, 1999.
9. Trachtman H, Christen E. Pathogenesis, treatment, and therapeutic trials in hemolytic uremic syndrome. Curr Opin Pediatr **11:**162–168, 1999.
10. Izumikawa K, Hirakata Y, Yamaguchi T, Takemura H, Maesaki S, Tomono K, Igimi S, Kaku M, Yamada Y, Kohno S, Kamihira S. *Escherichia coli* O157 interaction with human intestinal Caco-2 cells and the influence of fosfomycin. J Antimicrob Chemother **42:**341–347, 1998.
11. Xiu RJ, Hammerschmidt DE, Coppo PA, Jacob HS. Anisodamine inhibits thromboxane synthesis, granulocyte aggregation, and platelet aggregation. A possible mechanism for its efficacy in bacteremic shock. J Am Med Assoc **247:**1458–1460, 1982.
12. Zhang S, Chang AM, Li CF, Li ZJ, Yin ZJ, Zhao X, Liang SL. Mechanism of the therapeutic effect of anisodamine in disseminated intravascular coagulation: Study of platelet adhesion and aggregation, malondialdehyde, thromboxane B2, 6-keto-prostaglandin F1 alpha, and microcirculation. Exp Hematol **15:**65–71, 1987.
13. Xiu RJ. Studies on microcirculation in institute of basic medical sciences, Chinese academy of medical sciences. Microvasc Res **20:**371–373, 1980.
14. Su JY, Wu L, Tang C. Experimental study in rabbits of the antishock effect of anisodamine (654-2), and its mechanism of action. Resuscitation **10:**173–184, 1983.
15. Xu SH, Zhu GJ, Wu QX, Chen HC. A preliminary study of the inhibiting mechanism of anisodamine on rabbit platelets activated by *E. coli* endotoxin. Chin Med J (Engl) **102:**879–884, 1989.
16. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer **26:**171–176, 1980.
17. Yamasaki C, Natori Y, Zeng XT, Ohmura M, Yamasaki S, Takeda Y, Natori Y. Induction of cytokines in a human colon epithelial cell line by Shiga toxin 1 (Stx1) and Stx2 but not by non-toxic mutant Stx1 which lacks N-glycosidase activity. FEBS Lett **442:**231–234, 1999.
18. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods **64:**313–320, 1983.
19. Decker T, Lohmann Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods **115:**61–69, 1988.
20. Palermo MS, Alves Rosa MF, Van Rooijen N, Isturiz MA. Depletion of liver and splenic macrophages reduces the lethality of Shiga toxin-2 in a mouse model. Clin Exp Immunol **116:**462–467, 1999.
21. van Setten PA, van Hinsbergh VW, van den Heuvel LP, Preyers F, Dijkman HB, Assmann KJ, van der Velden TJ, Monnens LA. Monocyte chemoattractant protein-1 and interleukin-8 levels in urine and

serum of patients with hemolytic uremic syndrome. Pediatr Res **43:**759–767, 1998.

22. Anonymous, from the Chinese Academy of Medical Sciences and Beijing Friendship Hospital. Adsorption, distribution and excretion of anisodamine. Chin Med J **1:**274–278, 1973.
23. Sakiri R, Ramegowda B, Tesh VL. Shiga toxin type 1 activates tumor necrosis factor-α gene transcription and nuclear translocation of the transcriptional activators nuclear factor-κB and activator protein-1. Blood **92:**558–566, 1998.
24. Spenger RN, Spenger ML, Strieter RM, Remick DG, Larrick JW, Kunkel SL. Modulation of tumor necrosis factor-alpha gene expression: Desensitization of prostaglandin E2-induced suppression. J Immunol **142:**4346–4350, 1989.
25. De Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. J Exp Med **174:**1209–1220, 1991.
26. Isogai E, Isogai H, Kimura K, Hayashi S, Kubota T, Fujii N, Takashi K. Role of tumor necrosis factor alpha in gnotobiotic mice infected with an *Escherichia coli* O157:H7 strain. Infect Immunol **66:**197–202, 1998.
27. Anonymous, from the Chinese Academy of Medical Sciences and Beijing Friendship Hospital. Pharmacologic effects of anisodamine. Chin Med J **1:**133–138, 1975.
28. Zou AP, Parekh N, Steinhausen M. Effect of anisodamine on the microcirculation of the hydronephrotic kidney of rats. Int J Microcirc Clin Exp **9:**285–296, 1990.