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Cytoprotective Effect of Curcumin in Human Proximal Tubule Epithelial Cells Exposed to Shiga Toxin

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We conducted the following experiments to determine whether curcumin, an antioxidant compound extracted from the spice tumeric, inhibits cell death induced by Shiga toxin (Stx) 1 and 2 in HK-2 cells, a human proximal tubule cell line. Cells were incubated for 24–48 h with Stx1 or Stx2, 0–100 ng/ml. Test media contained either no further additives or 10–50 μM curcumin. Exposure to Stx1 and Stx2, 100 ng/ml, reduced cell viability to approximately 25% of control values after 24 h and 20 μM curcumin restored viability to nearly 75% of control. Cell staining confirmed that Stx1 and Stx2-induced damage in HK-2 cells involved a combination of apoptosis and necrosis. Thus, Stx1 caused apoptosis and necrosis in 12.2 ± 2.2 and 12.7 ± 0.9% of HK-2 cells, respectively. Similarly, Stx2 caused apoptosis and necrosis in 13.4 ± 2.1 and 9.0 ± 0.5% of HK-2 cells, respectively. Addition of 20 μM curcumin decreased the extent of apoptosis and necrosis to 2.9 ± 2.0 and 3.8 ± 0.2%, respectively in the presence of Stx1 and to 3.0 ± 2.1 and 3.9 ± 0.3%, respectively, for Stx2 ($P < 0.01$). Stx-induced apoptosis and its inhibition by curcumin were confirmed by DNA gel electrophoresis and by an assay for fragmentation. The protective effect of curcumin against Stx1 and Stx2-induced injury to HK-2 was not related to its antioxidant properties. Instead, curcumin enhanced expression of heat shock protein 70 (HSP70) in HK-2 cells under control conditions and after exposure to Stx1 or Stx2. No injury was detectable after incubation of LLC-PK$_1$ or OK cells, non-human proximal tubule cell lines, with Stx1 or Stx2. Thus, curcumin inhibits Stx-induced apoptosis and necrosis in HK-2 cells *in vitro*. The cytoprotective effect of curcumin against Stx-induced injury in cultured human proximal tubule epithelial cells may be a consequence of increased expression of HSP70.

Key Words: **Shiga toxin; proximal tubule epithelial cells; curcumin; apoptosis necrosis; hemolytic uremic syndrome.**

Diarrheal-associated hemolytic uremic syndrome (D + HUS) occurs in 5–10% of children who experience a prodromal gastrointestinal infection with Shiga toxin (Stx)-producing strains of *Escherichia coli* (STEC) (1). These microorganisms elaborate Stx1 and Stx2 that initially bind to the glycolipid globotriasylceramide (Gb_3) receptor on the surface of endothelial cells. Internalization of the toxin inhibits protein synthesis resulting in diffuse vascular injury, cell necrosis, and organ damage, involving primarily the kidney (1, 2). Recent findings indicate that renal dysfunction may also be the consequence of Stx-mediated apoptosis of tubular epithelial cells (3, 4).

Curcumin, [1,6-heptadiene, 3-5-dione, 1,7-bis (4-hydroxy-3-methoxyphenyl) ($C_{21}H_{20}O_6$)], a yellow–orange dye derived from the spice tumeric, is cytoprotective and exerts antioxidant and anti-inflammatory actions *in vitro* and *in vivo* (5). It is a bioflavanoid compound that protects bovine aortic endothelial cells and renal tubular epithelial cells (LLC-PK$_1$) against oxidant-induced injury (6, 7). The structurally related molecule, quercetin, inhibits lipid peroxidation and damage to cultured renal tubular epithelial cell (8). Excessive protection of oxygen free radicals may be pivotal in promoting apoptosis, programmed cell death, in renal tubular cells (9, 10). Quercetin exerts a cytoprotective action against hydrogen peroxide-induced apoptosis in mesangial cells (11). Therefore, we conducted the following experiments to determine whether curcumin could reduce Stx-triggered apoptosis and necrosis in HK-2 cells, a human proximal tubule cell line.

EXPERIMENTAL METHODS

Renal tubular epithelial cell lines. HK-2 (CRL-2190), OK, and LLC-PK$_1$ cells were initially obtained from American Type Culture Collection (Rockville, MD). All three epithelial cell lines originate from the proximal tubule. HK-2 cells are derived from human kidney tissue, OK from opossum kidney, and LLC-PK$_1$ from porcine tissue. Cells were plated in 24-well plates, $1.0–2.5 \times 10^4$ cells per well, for fluorescence staining with Hoechst (H)-33342 and propidium iodide to determine apoptotic versus necrotic cell death and in 96-well

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plates, 2.5×10^4 cells/well, for assessment of cell viability with MTT. Cells were grown in 75-cm^2 flasks for the DNA fragmentation assay and for DNA isolation to demonstrate internucleosomal fragmentation by agarose gel electrophoresis and in 25-cm^2 flasks for Western immunoblot studies. LLC-PK_1 and OK cells were maintained in Dulbecco's modified Eagle (DME) medium that was supplemented with 100 μg/ml streptomycin, 100 μg/ml penicillin and 10% fetal bovine serum (FBS). HK-2 cells were grown in keratinocyte serum free (KSF) medium that was supplemented with epidermal growth factor (EGF), 5 ng/ml, and bovine pituitary extract (BPE) 40 μg/ml. Media were changed every 2–3 days and cells were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air (10% CO_2–90% air for LLC-PK_1 and OK cells).

Experimental conditions. After reaching confluence, HK-2 cells were growth-arrested for 24 h by maintaining them in KSF media containing EGF, 1 ng/ml, and BPE, 8 μg/ml. LLC-PK_1 and OK cells were serum deprived by reducing the FBS concentration to 1%. Cells were then exposed to one of the following test conditions for 24 h: (1) Control: media with no additives; (2) curcumin: Media containing curcumin, 10–50 μM; (3) Shiga toxin 1 (Stx1): Media containing Stx1, 1–100 ng/ml; (4) Stx1 + curcumin: Media containing the combination of Stx1 and curcumin described in conditions 2 and 3; (5) Shiga toxin 2 (Stx2): Media containing Stx2, 1–100 ng/ml; and (6) Stx2 + curcumin: Media containing the combination of Stx2 and curcumin described in conditions 2 and 5.

Measurement of cell viability. Renal tubular epithelial cell viability was determined with a colorimetric method using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS).

Fluorescence cell staining. At the end of the incubation period, aliquots of methanol containing H-33342, 1 μg/ml, were added to each well and cells were incubated for 10 min at 37°C. Subsequently, without washing, the plates were placed on ice and propidium iodide, 1 μg/ml, was added to each well. Cells were incubated with the dye for 10 min on ice, protected from light, and then examined under ultraviolet light using a Hoechst filter (Nikon, Garden City, NY). The percentage of live, apoptotic, and necrotic cells were counted in eight random fields distributed throughout the well.

Agarose gel visualization of DNA fragmentation. After 24 h exposure to the experimental conditions, both adherent and nonadherent cells were transferred to microtubes and spun at 13,000*g* for 1 h. Pellets were resuspended in 15–30 μl of DNA lysis buffer containing 100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA and 1% *N*-laurylsarcosine. For every 15 μl of lysis buffer, 4 μl of a solution containing proteinase K, 10 mg/ml, was added and incubated at 45°C for 2 h. Following this, 2 μl of a solution containing RNase, 10 mg/ml, was added and samples were incubated at room temperature for 1 h. Samples were then loaded onto 1.5% agarose gels and run at 50 V for approximately 1–2 h in TBE buffer. Gels were soaked overnight in a shaker bath and stained with SYBR Green nucleic acid reagent (Molecular Probes Inc., Eugene, OR) to enhance the visibility of the DNA bands.

DNA fragmentation assay. At the completion of the experimental period, the cells were scraped into a lysis buffer containing 10 mM Tris–HCl, 10 mM EDTA and 0.5% Triton X-100. After a 30-min incubation at 4°C, samples were spun at 13,000*g* for 15 min. Pellets were resuspended in 0.25 ml of lysis buffer and 0.25 ml 1 M perchloric acid, and then heated at 70°C for 20 min. To each supernatant, pellet, and blank sample, 1 ml of a 3% diphenylamine solution (dissolved in glacial acetic acid and concentrated sulfuric acid) was added and incubated for 16 h at 30°C in a water bath. Samples were spun for 30 s and absorbance was measured at 600 nm. %DNA fragmentation was determined in accordance with the following equation: OD_{600} supernatant/[OD_{600} supernatant + OD_{600} pellet].

Conjugated diene assay. At the end of the experimental period, 3 ml PBS containing 0.01 M EDTA, pH 7.4 along with 3 ml PBS containing 0.01 M EDTA and 5% trichloroacetic acid (TCA), pH 7.4 was added to each plate and incubated on ice for 15 min. Cells were scraped and the plates were rinsed a second time. The combined rinses were homogenized and an aliquot was removed for protein determination. Samples were then homogenized twice in 3 ml chloroform:methanol (2:1). 2 ml of water was added to the combined 6 ml supernatant, vortexed, and the upper aqueous phase was removed. The remainder was dried under N_2 at 40°C in a water bath and resuspended in 1.2 ml acetonitrile. The absorbance of the solution was measured at 235 nm and compared to acetonitrile blank. Conjugated diene content was expressed as absorbance per milligram protein (11).

Western analysis and immunoblotting. Cells here harvested after the 24 h experimental period and the homogenates were boiled in the presence of 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol in 60 mM Tris–HCl buffer, pH 7.5, for 6–8 min. They were subjected to 7.5% SDS–PAGE and electroblotted onto nitrocellulose membranes. Protein transfer was performed in 25 mM Tris–HCl buffer containing 150 mM glycine and 20% (v/v) methanol at 4°C with a current of 350 mA for 1 h. Membranes were incubated with a peroxidase-labeled primary antibody to HSP70 (Transduction Laboratories, Lexington, KY) and developed using a peroxidase substrate. The intensity of the protein bands was quantitated by scanning the gels (IS-1000 Digital imaging system, Alpha Innotech Corp., San Leandro, CA).

Chemical and reagents. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Purified Stx1 and Stx2 were kindly provided by David Acheson, M.D. (Department of Infectious Diseases and Geographic Medicine, Tufts University School of Medicine, Boston, MA). Cell culture media and supplements were obtained from Gibco Laboratories (Grand Island, NY). Sterile tissue culture plasticware was from Falcon Plastics (Cockeysville, MD).

Statistical methods. The experimental data are reported as mean ± SEM. Inter-group comparisons were made using an analysis of variance and the t-test and differences were considered significant if the *P* value was less than 0.05.

RESULTS

Cell viability. Preliminary studies indicated that incubation of HK-2 cells with 1–10 ng/ml of either Stx did not alter viability. In contrast, addition of 100 ng/ml of Stx1 or Stx2 significantly inhibited HK-2 cell proliferation by approximately 75% after 24 h and by 90% after 48 h (Fig. 1). 10 μM curcumin did not alter the viability of HK-2 cells exposed to Stx1 or Stx2 (data not shown). However, 20 μM curcumin increased HK-2 cell viability to 76.3 ± 5.2% of the control level for Stx1-exposed cells and to 72.5 ± 3.8% for Stx2-exposed cells after 24 h ($P < 0.001$) (Fig. 2A). Similarly, after 48 h, addition of 20 μM curcumin increased HK-2 cell viability to 24.3 ± 1.7 and 22.8 ± 1.7% of control levels following incubation with Stx1 and Stx2, respectively ($P < 0.001$) (Fig. 2B). The 50 μM concentration of curcumin interfered with optical density measurements due to the brilliant orange color and, therefore, its effect on HK-2 cell viability was not tested.

Apoptosis and necrosis. The type of cell death was determined after 24 h exposure to Stx1 or Stx2, the time point when the greatest beneficial effect of HK-2 cell viability was achieved with curcumin. H-33342 staining confirmed that Stx1 and Stx2-associated in-

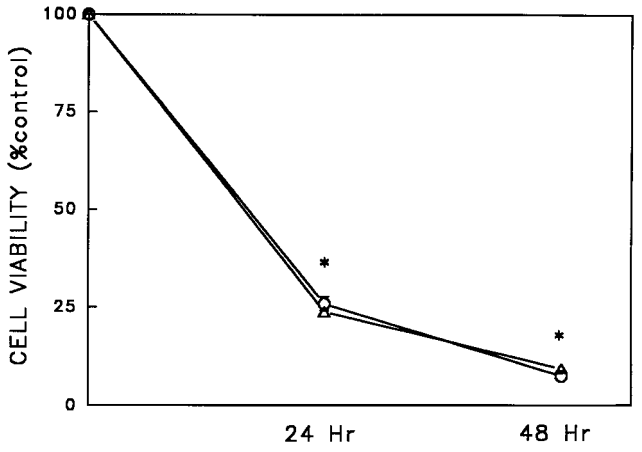

FIG. 1. HK-2 cell viability assessed using the MTT reagent after exposure to Stx1 (open circles) or Stx2 (open triangles) ($n = 4$) for 24 or 48 h. *$P < 0.001$ versus control condition.

jury to HK-2 cells involved apoptosis. Stx1 and Stx2 induced apoptosis in 12.2 ± 2.2% and 13.4 ± 2.1% of HK-2 cells after 24 h, respectively. Under these conditions, 20 μM curcumin decreased apoptosis to 2.9 ± 2.0% for Stx1 and 3.0 ± 2.1% for Stx2 ($P < 0.001$) (Fig. 3). The nearly 85% protective effect of curcumin against apoptosis paralleled the improvement in cell viability.

Stx1 and Stx2 also caused HK-2 cell necrosis. Thus, after a 24 h exposure, Stx1 and Stx2 increased the number of necrotic cells to 12.7 ± 0.9 and 9.0 ± 0.5%, respectively, compared to 1.5 ± 0.3% in control cells ($P < 0.001$). Curcumin alone caused a modest increase in the number of necrotic cells to 4.8 ± 0.6% in the absence of Stx1 or Stx2 ($P < 0.05$). However, addition of curcumin in the presence of Stx1 or Stx2 significantly lowered the number of necrotic HK-2 cells to 3.8 ± 0.2% ($P < 0.001$) and 3.9 ± 0.3% ($P < 0.01$), respectively (Fig. 4).

DNA oligonucleosome laddering. Stx-induced apoptosis and inhibition by curcumin were confirmed by DNA fragmentation experiments. DNA laddering, indicative of apoptosis, was observed in lanes 6 and 8 (Stx 1 and 2) but not in lanes 7 and 9 (Stx + curcumin) (Fig. 5).

DNA fragmentation. Stx1 and Stx2-induced apoptosis was confirmed using an assay of DNA fragmentation. Thus, 24 h exposure to Stx1 and Stx2 increased DNA fragmentation in HK-2 cells to 32.6 ± 0.3 and 31.2 ± 0.7%, respectively, compared to 3.4 ± 0.3% in control cells ($P < 0.001$). In the absence of Stx1 or Stx2, curcumin did not alter the amount of DNA fragmentation in HK-2 cells, i.e., 4.0 ± 0.2%. However, exposure of HK-2 cells to Stx1 or Stx2 in the presence of curcumin lowered DNA fragmentation to normal, 5.0 ± 0.4 and 4.3 ± 0.3% ($P < 0.001$), respectively (Fig. 6).

Conjugated dienes. Exposure to Stx1 or Stx2 was not associated with an increase in the conjugated diene content (a marker of oxidant stress) of lipids extracted from the HK-2 cells. Moreover, addition of curcumin had no significant effect on the amount of conjugated dienes under control conditions or after incubation with Stx1 or Stx2 (Table 1).

Western immunoblot studies. Exposure of HK-2 cells to 20 μM curcumin under control conditions or together with Stx1 or Stx2 resulted in increased expression of HSP70 protein (Fig. 7). The approximately 30% increase in HSP protein was confirmed by scanning densitometry.

OK and LLC-PK$_1$ cells. The viability of nonhuman proximal tubule epithelial cells was unaffected by exposure to Stx1 or Stx2. Thus, there was no apoptosis or necrosis under any of the test conditions (data not shown). Interestingly, exposure of LLC-PK1 and OK cells to 20 μM curcumin in the absence or pres-

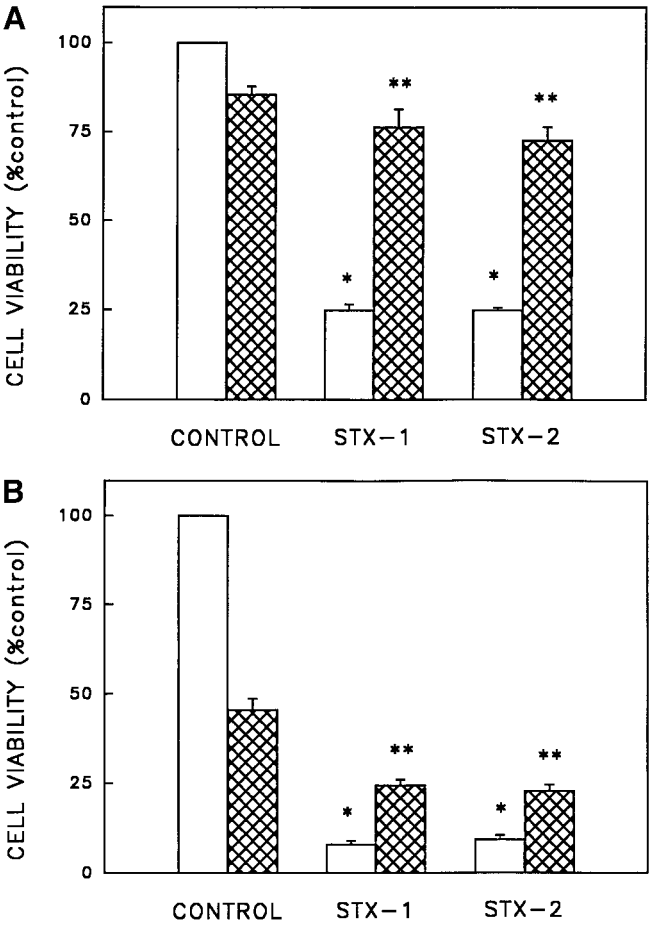

FIG. 2. HK-2 cell viability, assessed using the MTT reagent, after exposure to Stx1 or Stx2 without any further additives (open bars) or in the presence of 20 μM curcumin (cross-hatched bars) ($n = 4$). (A) 24 h and (B) 48 h. *$P < 0.001$ versus control condition; **$P < 0.001$ versus Stx1 or Stx2 alone.

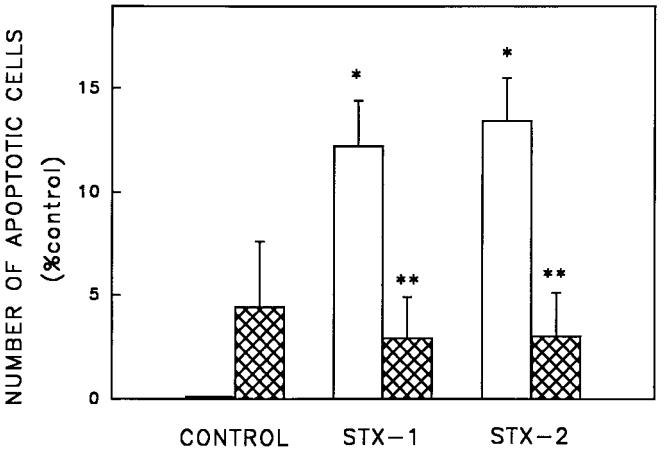

FIG. 3. HK-2 cell apoptosis, assessed using the H-33342 staining, after exposure to Stx1 or Stx2 for 24 h without any further additives (open bars) or in the presence of 20 μM curcumin (cross-hatched bars) ($n = 8$). *$P < 0.001$ versus control condition; **$P < 0.001$ versus Stx1 or Stx2 alone.

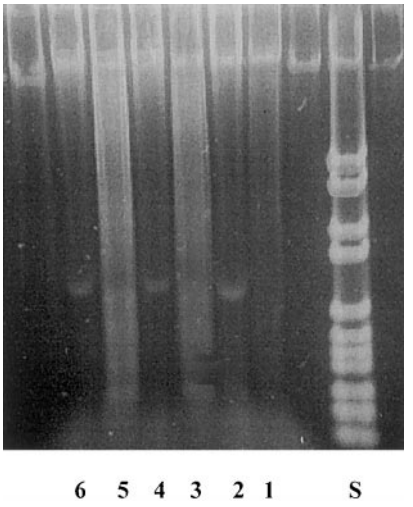

FIG. 5. DNA gel electrophoresis demonstrating apoptosis in HK-2 cells after exposure to Stx1 or Stx2 (100 ng/ml) for 24 h and the effect of 20 μM curcumin. The lanes are labeled in the following manner: S, standard DNA ladder; lane1, control; lane 2, control + curcumin; lane 3, Stx1; lane 4, Stx1 + curcumin; lane 5, Stx2; lane 6, Stx2 + curcumin.

ence of Stx1 or Stx2 provoked a comparable increase in HSP70 expression to that observed in HK-2 cells (Fig. 7).

DISCUSSION

Direct evidence of Stx-induced cytotoxicity in the kidney has been confirmed in several non-renal and kidney cell culture systems (3, 13–5) and *in vivo* in mice and humans (13, 16–18). The necrosis of tubular epithelial cells, glomerular endothelial cells and mesangial cells that occurs after exposure to Stx has been attributed to internalization of toxin and inhibition of protein synthesis in the ribosome (19). However, an alternative type of cytotoxicity, apoptosis, has been documented in human renal tubular epithelial cell, a major site of the renal pathology early in the course of HUS (3, 4).

Several cell types, including renal tubular epithelial cells, express the Gb3 (CD77) receptor and are susceptible to induction of apoptosis by Stx (3, 4). It is not known whether this effect requires internalization of the toxin; however, it is likely to involve significant disturbances in normal protein synthesis, including activation of the caspase cascade (20). Our finding that Stx1 and Stx2 were cytotoxic only in HK-2 cells is

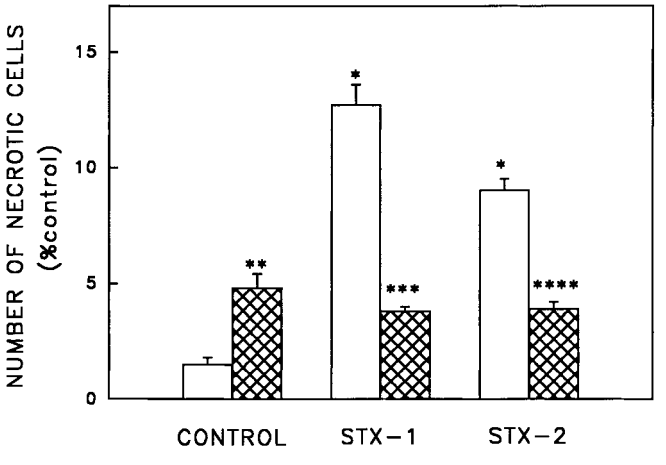

FIG. 4. HK-2 cell necrosis, assessed using propidium iodide staining, after exposure to Stx1 or Stx2 for 24 h without any further additives (open bars) or in the presence of 20 μM curcumin (cross-hatched bars) ($n = 4$). *$P < 0.001$ versus control condition; **$P < 0.05$ versus control condition; ***$P < 0.001$ versus Stx1 alone; ****$P < 0.01$ versus Stx2 alone.

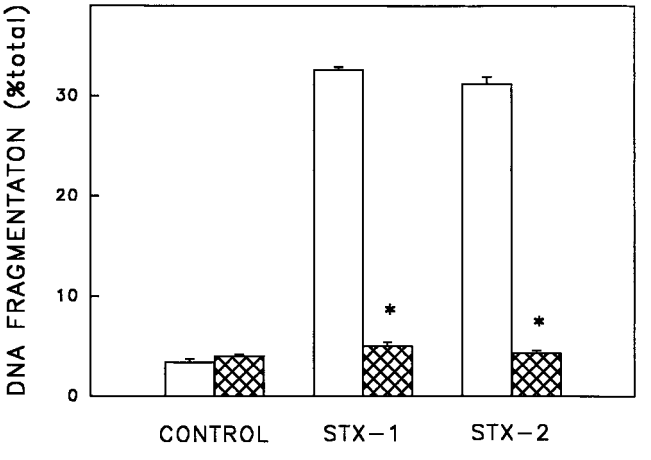

FIG. 6. DNA fragmentation in HK-2 cells after exposure to Stx1 or Stx2 (100 ng/ml) for 24 h without any further additives (open bars) or in the presence of 20 μM curcumin (cross-hatched bars) ($n = 4$). *$P < 0.001$ versus Stx1 or Stx2 alone.

consistent with data that primate (human and baboon) proximal tubule epithelial cells express the membrane receptor for Stx (21, 22). The absence of Gb3 in the LLC-PK_1 and OK cells, which are derived from the pig and opossum, respectively, accounts for absence of an adverse effect in these cells following exposure to Stx. It is interesting, in this regard, that the nonpathogenicity of STEC in cattle has also been attributed to the lack of Gb3 in the gastrointestinal epithelial cell membrane (23).

Stx1 and Stx2 caused both necrosis and apoptosis in susceptible HK-2 cell. Both mechanisms of cell death have been demonstrated *in vivo* in kidney specimens obtained from children with D + HUS (13, 17). It is likely that Stx trigger a range of perturbations in HK-2 cells that independently culminate in apoptosis and necrosis because these two forms of cell death differ markedly in their enzymatic requirements, dependence upon cytosolic ATP stores, and consequences for the local environment. The observation that curcumin diminished both forms of cell death suggests that this compound activates a process that is shared by both apoptosis and necrosis. Curcumin was equally effective against both forms of Stx, implying that it should be active against all STEC strains, despite variable expression and production of Stx1 and Stx2.

Previous work has focused primarily on the antioxidant effects of curcumin to explain its cytoprotective effects in various injury states (5, 7, 24). However, our data indicate that this is not the underlying mechanism of action whereby curcumin reduces Stx-induced HK-2 cell death. Instead, we observed that curcumin stimulated the expression of the molecular chaperone HSP70 in HK-2 cells, under normal conditions and following exposure to Stx1 or Stx2. This finding is especially noteworthy because the detrimental effects of Stx on ribosomal function would be expected to result in a broad inhibition of protein synthesis. We detected no independent effect of Stx on HSP70 expression. The induction of HSP70 by curcumin is likely to represent an intrinsic property of the compound because it was noted to occur in the other two cell lines (LLC-PK_1 and OK cells) that were not susceptible to Stx-mediated injury. Non-specific induction of HSP70 by curcumin may have counteracted the cell stress provoked by exposure to Stx and reduced the amount of apoptosis and necrosis (25). There is precedent for this action of curcumin in colorectal carcinoma cells (26). Moreover, addition of quercetin to endothelial cells incubated in a hypoxic environment for 18 h improved viability and increased expression of heme oxygenase-1, also known as HSP32 (6).

TABLE 1

Conjugated Dienes in Stx-Exposed HK-2 Cells

	Control	Stx1	Stx2
No additives	100	76 ± 10	77 ± 10
Control + 20 μM curcumin	116 ± 13	89 ± 11	119 ± 16

Note. Conjugated dienes determinations are measured as absorbance units per mg protein. The data are reported as percentage of cells in control media no additives. Each experimental condition was studied in quadruplicate. Results are provided as means ± SEM.

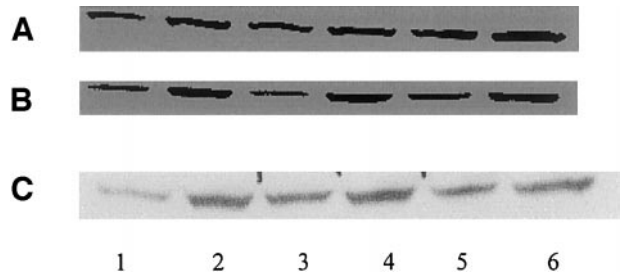

FIG. 7. Western immunoblot of HSP70. Row A represents HK-2, row B represents LLC-PK_1, and row C, represents OK cells. The lanes are labeled in the following manner: C, HSP70 standard; lane 1, control; lane 2, control + curcumin; lane 3, Stx1; lane 4, Stx1 + curcumin; lane 5, Stx2; lane 6, Stx2 + curcumin.

The attack rate of D + HUS in children following STEC infection is 5–10% (1, 2). There is currently is no adequate explanation for the occurrence of this serious complication in only a small subset of at-risk children with STEC-related hemorrhagic colitis. Our findings raise the possibility that, during the prodromal phase, variable dietary intake of nutritional components such as curcumin that exert a non-specific protective effect against Stx-induced injury to renal tubular epithelial cells may account, in part, for the observed differences in the incidence of D + HUS after STEC infection in defined patient populations.

In conclusion, we have demonstrated that curcumin reduces Stx-induced apoptosis and necrosis in HK-2 cells *in vitro*. The beneficial effect of curcumin against Stx-induced damage to cultured human proximal tubule epithelial cells may be a consequence of increased expression of HSP70. The clinical utility of these findings deserves further study in children with D + HUS.

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