Protection against ethanol injury by prostaglandin in a human intestinal cell line: role of microtubules

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Banan, A., G. S. Smith, C. L. Rieckenberg, E. R. Kokoska, and T. A. Miller. Protection against ethanol injury by prostaglandin in a human intestinal cell line: role of microtubules. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G111–G121, 1998.—Prostaglandins have been shown to protect the gastrointestinal (GI) epithelium from injury induced by various luminal insults independent of their known acid-inhibitory effects, a process termed “cytoprotection.” The mechanism of this protective action remains unknown. The present investigation determined the role of microtubules (a major cytoskeletal component) in GI injury induced by ethanol (EtOH) and its prevention by 16,16-dimethylprostaglandin E$_2$ (dmPGE$_2$) using cells from a human colonic cell line known as Caco-2 cells. These cells were preincubated in Eagle’s minimum essential medium with and without dmPGE$_2$ (2.6 µM) for 15 min and subsequently incubated in media containing 1, 2.5, 5, 7.5, and 10% EtOH. The effects on cell viability and tubulin (the major protein backbone of microtubules) were then determined. EtOH concentrations ≥2.5% extensively disrupted the microtubules as demonstrated by fragmentation, kinking, and perturbation of the microtubule organizer center. EtOH treatment also led to a significant decrease in the S2 (polymerized) fraction and an increase in the S1 (monomeric) pool of tubulin. Concomitant with these effects were marked decreases in cellular viability. DmPGE$_2$ pretreatment abolished the disruption of microtubules, significantly increased the S2 fraction of tubulin, and increased cellular viability in cultures exposed to EtOH. Furthermore, pretreatment with colchicine, an inhibitor of microtubule assembly, prevented the cytoprotective action of dmPGE$_2$. Taxol, a microtubule stabilizing agent, mimicked the effects of dmPGE$_2$ by also enhancing microtubule integrity and increasing cellular viability in cells exposed to EtOH. Our data indicate that organization and stabilization of microtubules may play an essential role in the mechanism of prostaglandin-induced protection.

16,16-dimethylprostaglandin E$_2$; cytoskeleton; Taxol; colchicine; Caco-2 cells; tubulin; quantitative Western immunoblot.
Four hours after colchicine incubation, cells were effective in disrupting microtubules under in vitro conditions. MEM was added to cultured cells that were incubated at 37°C in Eagle's minimum essential medium (MEM), containing 20% fetal bovine serum, 1% nonessential amino acids, penicillin (50 µU/ml), streptomycin (50 µg/ml), and amphotericin B (50 µg/ml), in an atmosphere of 5% CO2 and 100% relative humidity. Cells were split at a ratio of 1:6 on reaching confluency every 6 days and were set up in either 6- or 24-well plates for experiments on T-175 flasks for the maintenance of stocks. Medium was changed every two days. All experiments were performed in triplicate at least three times (n = 9).

Experimental design. The first experiment evaluated the effect of graded concentrations of ethanol (vol/vol) or MEM on microtubule stability and cell viability. For these studies, 1, 2.5, 5, 7.5, and 10% ethanol or MEM were added to monolayers of Caco-2 cells for 5 min. Slides were processed for immunofluorescent labeling of microtubules, and the percentage of cells with normal and/or disrupted microtubules was determined as described. Using the same experimental protocol, we assessed viability by trypan blue dye exclusion.

In a second experiment, the effect of 16.16-dimethylprostaglandin E2 (dmPGE2) (Sigma Chemical, St. Louis, MO) on ethanol-induced cell injury and microtubule stability was assessed. This experiment was conducted in a similar fashion as the first experiment, except that dmPGE2 was added to the culture medium 15 min before cells were exposed to graded concentrations of ethanol. Again, slides from each well were processed for microtubule staining. In addition, slides were processed for immunofluorescent staining of microtubules and quantitation of the percentage of cells with normal or disrupted microtubules. In a corollary experiment, dmPGE2 alone at a final concentration of 2.6 µM was added to the cell culture medium to investigate its direct effect on microtubule stability and cell viability. The concentration of dmPGE2 used in these studies had previously been shown to have cytoprotective properties in a primary gastric cell culture (44).

A third experiment determined the effect of colchicine (Sigma Chemical) on ethanol-induced injury and microtubule stabilization. Colchicine at a final concentration of 50 µM in MEM was added to cultured cells that were incubated at 37°C. This concentration of colchicine has been shown to be effective in disrupting microtubules under in vitro conditions (33). Four hours after colchicine incubation, cells were exposed for 15 min to dmPGE2 at a concentration of 2.6 µM. After dmPGE2 treatment, ethanol in a range of concentrations from 1 to 10% was added to the medium. After 5 min of incubation in ethanol, slides were processed for microtubule staining. Cellular viability was determined in each case as described below. In a corollary experiment, colchicine alone at the final concentration of 50 µM was added to the cell culture medium to investigate its direct effect on microtubule integrity and cell viability and the relationship between microtubule disassembly and cellular disruption. It should be noted that microtubule depolymerization was achieved by incubating cells in cold medium containing 50 µM of colchicine for 30 min at 4°C and then warming them to 37°C in the presence of colchicine for an additional 3.5 h as previously described (33).

In a fourth experiment, Taxol (Sigma Chemical) at a final concentration of 50 µM in MEM was added to cultured cells and incubated at 37°C for 1 h. This concentration of Taxol has been shown to be effective in stabilizing microtubules under in vitro conditions (50). After 1 h of Taxol exposure, cells were incubated in ethanol at concentrations between 1 and 10% for 5 min. Cellular viability and microtubule stability were again assessed. Similar to the studies with colchicine, Taxol alone at a final concentration of 50 µM (1-h incubation at 37°C) was added to the medium to investigate its effects on microtubule integrity and cell viability.

In a fifth experiment, ethanol at concentrations of 1, 2.5, 5, 7.5, and 10% or MEM was added to the monolayers of Caco-2 cells for 5 min, and monomeric and polymerized fractions of tubulin were separated as described below. The resultant tubulin fractions were processed for quantitative immunoblotting. Additional studies to determine and quantitate any effects that dmPGE2 might have on ethanol-induced changes in microtubule polymerization or depolymerization were carried out using dmPGE2 at a concentration of 2.6 µM for 15 min before exposing cell cultures to identical concentrations of ethanol. Control studies utilized saline in place of dmPGE2. Finally, dmPGE2 alone (2.6 µM) was added to cell cultures to investigate any effects it might have on polymerization or depolymerization of tubulin by itself.

Immunofluorescence of the microtubule cytoskeleton. Cells were fixed in cytoskeletal stabilization buffer [1 part 3.7% paraformaldehyde plus 9 parts PEM buffer plus 0.2% Triton X-100; PEM buffer consisted of 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA), and 2 mM MgCl2, pH 6.8] for 15 min followed by a rinse in Dulbecco's phosphate-buffered saline (DPBS) solution. They were then postfixed in 95% ethanol at −20°C and subsequently blocked and rehydrated in 1% bovine serum albumin (BSA) fraction V; Sigma Chemical) in DPBS solution at room temperature for 30 min and then incubated with a primary antibody [monoclonal anti-β-tubulin or mouse immunoglobulin G1 (IgG1); Sigma Chemical] at a 1:200 dilution for 1 h at 37°C temperature. Slides were washed three times in DPBS and then incubated with a secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or FITC anti-mouse IgG; Sigma Chemical] at a 1:50 dilution for 1 h at room temperature, washed three times in DPBS and once with deionized H2O, and subsequently mounted in Aqua Mount. All antibodies were diluted with DPBS containing 0.1% BSA. Samples were stored in the dark at 4°C and examined within 72 h of preparation (long-term storage at −70°C) (2).

Epifluorescence and confocal microscopy. Cells were observed with both confocal and epifluorescence microscopy using a 63-µm immersion plan-apochromat objective (NA 1.4; Zeiss, Oberkochen, Germany) in a confocal laser-scanning microscope (Zeiss). Fields were selected randomly; the only criterion for cell selection was that cells were not in contact with other cells. An argon laser (wave length, 488 nm) was used to photograph FITC-labeled cells, and the cytoskeletal elements were examined for their overall morphology and orientation. The microtubule cytoskeleton in Caco-2 cells was considered injured based on one or more of the following criteria: fragmentation, kinking, disruption of microtubule organizer center (MTOC), or detachment from anchoring proteins. We examined 100–150 cells per slide (well) by fluorescence microscopy, and the percentage of cells displaying normal or disorganized microtubules was determined. To avoid bias, slides were examined in a blinded fashion; the slides were coded so that the examiner had no knowledge of
the experimental protocols. The slides were only decoded and the data analyzed after the examinations were complete.

Tubulin fractionation. Polymerized and monomeric fractions of tubulin were isolated as described previously by Black and Keyser (8). Caco-2 cells were pelleted with centrifugation at low speed (700 rpm for 7 min at 4°C) and subsequently resuspended in microtubule stabilization extraction buffer (MSBE; containing 0.1 M PIPES, pH 6.9, 30% glycerol, 5% dimethyl sulfoxide, 1 mM MgSO4, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM EGTA, and 1% Triton X-100) at room temperature for 20 min. Cell lysates were centrifuged at 105,000 g for 45 min at room temperature. The supernatant containing the soluble monomeric pool of tubulin (S1) was gently removed. The polymerized tubulin fraction (P1) was then resuspended in 0.3 M calcium-containing depolymerization buffer (0.1 M PIPES, pH 6.9, 1 mM MgSO4, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 10 mM CaCl2) and incubated on ice for 60 min. Subsequently, the samples were centrifuged at 48,000 g for 15 min at 4°C, and the supernatant (S2; cold/calcium soluble fraction) was removed. The remaining pellet was treated with the calcium-containing depolymerization buffer twice by resuspension and centrifugation. Microtubules were recovered by incubating (at 37°C for 30 min) the S1 and S2 fractions with Taxol and GTP at 20 μM and 1 mM, respectively, in microtubule stabilization buffer (MSB; containing 0.1 mM PIPES, 1 mM MgCl2, 1 mM EGTA, 1 mM GTP, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor) to promote polymerization. Microtubules were recovered by centrifugation and resuspended in the MSB buffer. All samples were flash frozen in liquid nitrogen and then stored at −70°C until immunoblotting studies were carried out.

Quantitative Western blotting of tubulin. Samples were placed in SDS sample buffer (250 mM Tris-HCl, pH 6.8, 2% glycerol, 5% mercaptoethanol), boiled for 5 min, and then subjected to electrophoresis on 7.5% polyacrylamide gels. Each lane was loaded with 30 μg protein. All procedures for Western immunoblotting were performed at room temperature as previously described (8). Briefly, after the transfer of proteins to nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 10× PBS-Tween 20 (PBST: 15 mM NaH2PO4, 1.5 M NaCl, pH 7.5, and 0.5% [vol/vol] Tween 20). Immunologic evaluation was then performed for 1 h in 1% BSA and PBST buffer containing 0.5 μg/ml mouse monoclonal β-tubulin antibody conjugated to peroxidase by protein cross-linking with 0.2% glutaraldehyde. After extensive washing with 1× PBST, the immunocomplexes on filters were reacted with enhanced chemiluminescence reagent (Du Pont-NEN). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s. The protein content of all the samples was estimated by the Bradford method (10). To quantify the relative levels of tubulin, the optical density of the autoradiographic bands corresponding to immunoradiolabeled tubulin on blots was measured with an SI laser densitometer. The methods employed to isolate these S1 and S2 pools of tubulin contain a variety of components to minimize artifacts associated with spontaneous depolymerization of microtubules under these conditions. Thus the MSBE utilized in this essay contains pepstatin, leupeptin, and soybean trypsin inhibitor, which are potent protease inhibitors utilized to minimize the spontaneous degradation of tubulin. This buffer also contains MgSO4 (in mM), which is a compound known to help prevent the spontaneous depolymerization of microtubules. Additionally, because microtubules are prone to degradation in the presence of calcium, we employed the calcium chelator EGTA (in mM) in the initial isolation steps to minimize the adverse influence of calcium on microtubule stability. Lastly, it has been demonstrated that microtubule stability can be adversely affected by the pH of the assay system. Because it has been demonstrated that microtubule integrity is most stable in a slightly acidic pH (6.8–6.9), this is the pH range in which we ran these experiments.

Determination of cell integrity. Trypan blue dye exclusion was used to assess cell viability in various treatments as described previously (35). At the end of each incubation, 0.1 ml trypan blue (0.4% wt/vol solution; Sigma Chemical) was added directly to the incubate and mixed. The number of stained dead cells that took up the dye and nonstained living cells that excluded the dye was counted in a microscope at ×200 magnification within 10 min. All experiments were performed by counting 100–200 cells in a randomized fashion from each slide. Cell viability was expressed as follows: percentage viability = nonstained cells/stained cells + nonstained cells × 100.

Statistical analysis. Data are presented as means ± SE. All experiments were performed in triplicate at least three times. Statistical analysis was carried out using analysis of variance followed by Dunnett's multiple-range test (18). P < 0.05 was deemed to represent statistical significance.

RESULTS

Control colonic intestinal cells (Fig. 1A) showed a radial and stellar distribution of microtubules with an intact MTOC. Incubation of Caco-2 cells with increasing concentrations of ethanol dose dependently produced microtubule disruption and/or fragmentation as shown by the immunofluorescent staining (Fig. 1, B and C, only 2.5 and 10% ethanol treatment shown, respectively). The lowest ethanol concentration noted to induce disruption and/or disorganization of microtubules was 2.5% (Fig. 1B). Additional details on the percentage of Caco-2 cells displaying disrupted microtubules with the varying concentrations of ethanol studied are shown in Table 1.

Incubating Caco-2 cells for 5 min with increasing ethanol concentrations resulted in a significant reduction in viability as assessed by trypan blue dye exclusion, which first became statistically significant at a concentration of 2.5% (Fig. 2). Pretreatment with 2.6 μM dmPGE2 stabilized and decreased the extent of damage to the microtubule cytoskeleton in all ethanol-treated groups that elicited microtubule disruption otherwise. The protection afforded by dmPGE2 to the microtubule cytoskeleton is demonstrated in Fig. 3. In addition, trypan blue exclusion demonstrated that preincubation with dmPGE2 (2.6 μM) before exposure to 2.5–10% ethanol significantly increased cellular viability compared with that of ethanol-treated groups alone (Fig. 4).

The results of quantitative Western immunoblotting of the polymerized pool of tubulin (S2 fraction) and monomeric pool of tubulin (S1 fraction) in response to various treatments are shown in Fig. 5. Increasing ethanol concentrations (2.5–10%) caused a significant reduction in the S2 polymerized pool and an increase in the S1 monomeric pool of tubulin compared with control cells. dmPGE2 pretreatment significantly in-
creased the polymerized tubulin fraction (S2) and decreased the monomeric tubulin fraction (S1) in cells subsequently exposed to damaging concentrations of ethanol (Fig. 5).

Colchicine pretreatment of Caco-2 cells followed by dmPGE₂ before exposure to ethanol significantly prevented the protective action of dmPGE₂ as determined by trypan blue exclusion (Fig. 6). Colchicine alone did not have any adverse effect on cellular viability after a 4-h incubation as shown. However, by 8–10 h after colchicine treatment alone significant reductions (75–80%) in cell viability were observed (data not shown). Immunofluorescent staining of microtubules indicated extensive depolymerization of microtubules in cells treated alone with colchicine (Fig. 7B) and ethanol-treated cells (Fig. 7C, only 10% ethanol group is shown) after colchicine pretreatment compared with that of control cells in which an intact microtubule cytoskeleton was demonstrated (Fig. 7A). Furthermore, colchi-

Table 1. Percentage of Caco-2 cells with disrupted microtubules

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%Disrupted</th>
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<tbody>
<tr>
<td>Control</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>1% EtOH</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>2.5% EtOH</td>
<td>32 ± 4*</td>
</tr>
<tr>
<td>5% EtOH</td>
<td>76 ± 8*</td>
</tr>
<tr>
<td>7.5% EtOH</td>
<td>79 ± 7*</td>
</tr>
<tr>
<td>10% EtOH</td>
<td>84 ± 7*</td>
</tr>
<tr>
<td>PGE alone</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>PGE + 2.5% EtOH</td>
<td>15 ± 4†</td>
</tr>
<tr>
<td>PGE + 5% EtOH</td>
<td>25 ± 8†</td>
</tr>
<tr>
<td>PGE + 7.5% EtOH</td>
<td>22 ± 4†</td>
</tr>
<tr>
<td>PGE + 10% EtOH</td>
<td>28 ± 5†</td>
</tr>
<tr>
<td>Taxol alone</td>
<td>2 ± 2†</td>
</tr>
<tr>
<td>Taxol + 2.5% EtOH</td>
<td>3 ± 3†</td>
</tr>
<tr>
<td>Taxol + 5% EtOH</td>
<td>5 ± 4†</td>
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<tr>
<td>Taxol + 7.5% EtOH</td>
<td>6 ± 5†</td>
</tr>
<tr>
<td>Taxol + 10% EtOH</td>
<td>2 ± 2†</td>
</tr>
<tr>
<td>Colchicine alone</td>
<td>100*</td>
</tr>
<tr>
<td>Colchicine + PG + 5% EtOH</td>
<td>100*</td>
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<tr>
<td>Colchicine + PG + 10% EtOH</td>
<td>100*</td>
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Values are means ± SE after treatments for Caco-2 cells with disrupted microtubules after various treatment modalities. Cells were incubated in various ethanol (EtOH) concentrations alone for 5 min or were preincubated with either 2.6 µM 16,16-dimethylprosta-glandin E₂ (dmPGE₂) for 15 min or 50 µM Taxol for 1 h before EtOH exposure for 5 min. Colchicine at a final concentration of 50 µM was added to media 4 h before subsequent incubation in dmPGE₂ for 15 min followed immediately by a 5-min EtOH treatment. Controls were incubated in MEM. We examined 4 different fields by counting a total of 100–150 cells in a blinded fashion by epifluorescence microscopy and then determined the % cells demonstrating disrupted microtubules. PGE, dmPGE₂. *P < 0.05 compared with control. †P < 0.05 compared with 2.5, 5, 7.5, and 10% EtOH-alone treatments and colchicine-pretreated groups (n = 9 per group).
cine incubation of Caco-2 cells alone or in combination with subsequent incubation in dmPGE2 followed immediately by 10% ethanol, significantly decreased the S2 polymerized pool to ~0.8% as determined by quantitative immunoblotting (Fig. 8).

Taxol pretreatment followed by 2.5-10% ethanol, in the absence of dmPGE2, stabilized and protected the microtubule skeleton (Fig. 9, B–E) to an extent that was morphologically similar to control cells (Fig. 9A). Taxol pretreatment of ethanol-exposed cultures in the absence of dmPGE2 also significantly increased cellular integrity to a level comparable to that of controls and dmPGE2-pretreated ethanol groups, as assessed by trypan blue exclusion (Fig. 10). Finally, Taxol treatment of Caco-2 cells alone, or in combination with subsequent exposure to 10% ethanol, also increased the S2 polymerized fraction of microtubules and significantly decreased the S1 monomer pool of tubulin (Fig. 8).

**DISCUSSION**

Although the preponderance of studies exploring the cytoprotective action of prostaglandins have utilized in vivo preparations, a number of reports (5, 6, 12, 25, 44, 47) have clearly indicated that this protective action can also be demonstrated under in vitro conditions. In a rodent gastric mucosal cell preparation, prostaglandins were shown to prevent gastric injury against diverse damaging agents such as indomethacin and ethanol when this damage was assessed using biochemical markers (5, 12, 25, 44). Studies in other species have demonstrated similar findings. dmPGE2 was shown to protect isolated guinea pig gastric glands or cells from injury induced by ethanol exposure. These in vitro studies demonstrating the cytoprotective actions of prostaglandins indicate that the mechanism(s) responsible for this activity are independent of an intact blood supply, neural innervation, and circulating humoral agents. Despite nearly 20 years of intensive investigation to unravel the mechanism(s) of cytoprotection, the precise mediators have continued to remain elusive.

The crucial role that the cytoskeleton might play in maintaining cellular integrity has been demonstrated in a variety of cell types (1, 3, 29, 39). Microtubules are an important component of the normal cytoskeleton. This component plays many vital roles in maintaining the normal physiology of the cell, includ-
importance of this cytoskeletal component in normal cellular physiology, the role of microtubules in prostaglandin-induced cytoprotection is unknown. Preliminary studies from our laboratory have shown that prostaglandins can significantly prevent the disruption of microtubules in both a rat duodenal-derived intestinal crypt cell line (43) and a rat primary gastric epithelial cell culture (42). To further explore the role that microtubules might play in cytoprotection, we undertook the present study, utilizing a transformed colonocyte cell line known as Caco-2 cells.

This cell line was chosen for our studies because Caco-2 cells form monolayers that can be studied for weeks, rather than just days, as is typical of most in vitro preparations. Furthermore, Caco-2 cells closely resemble small intestinal cells in that they have defined apical brush borders, form tight junctions, and exhibit a highly organized microtubule network on differentiation (9, 17, 34). These cells also express small intestinal hydrolases such as sucrase-isomaltase and alkaline phosphatase (19, 34, 40). In addition, these cells are similar to small intestinal absorptive cells in that they have receptors for prostaglandins, insulin-like growth factor I, vasoactive intestinal polypeptide, low-density lipoprotein, insulin, and specific substrates such as dipeptides, fructose, glucose, hexoses, and vitamin B₁₂ (13–15, 20, 22, 27, 30, 31, 54). Because prostaglandin cytoprotection has been demonstrated throughout the gastrointestinal tract to include the small intestine and colon as well as the stomach (16, 32, 33, 35, 36, 37).
36, 37, 52), this cell line provides a unique in vitro model for cytoprotection studies, hence its use in the present investigation.

A major focus of our study was to explore the effects of ethanol-induced injury on both the shape and structure of microtubules. To accomplish this goal, not only did we examine evidence for damage or modification of microtubules, but we also specifically measured alterations in tubulin, the major protein component of microtubules. It was our belief that a damaging agent, such as ethanol, should disassemble microtubules and prostaglandin treatment should prevent this disassembly if this cytoskeletal component was indeed intimately involved in cytoprotection.

Our studies demonstrated that ethanol, in a dose-dependent fashion, significantly damaged and disintegrated the microtubule cytoskeleton by producing fragmentation, kinking, and detachment from the MTOC. Preincubation of Caco-2 cells with dmPGE2 not only stabilized microtubules but also protected these cells from damage induced by ethanol under in vitro conditions. Increased cellular viability as determined by trypan blue exclusion supports this latter contention. Trypan blue has been shown to be a reliable index of cell membrane integrity in cells throughout the gastrointestinal tract when challenged by various noxious insults (45, 46, 51).

Ethanol treatment in concentrations ranging from 2.5 to 10% significantly decreased the amount of polymerized microtubules as supported by quantitative immunoblotting. Both extensive disruption, fragmentation (indicated by immunofluorescence), and depolymerization of microtubules (supported by Western blotting) occurred after ethanol insult on cultured colonocytes. Our most noteworthy finding was that pretreatment with dmPGE2 significantly increased the polymerized pool of tubulin (S2) to a level comparable to control cells and decreased the cytoplasmic monomeric fraction (S1). To our knowledge, this is the first demonstration that prostaglandins can influence the dynamics of microtubule assembly under in vitro conditions, and these data indicate the importance of prostaglandins in the promotion of microtubule organization and remodeling under such conditions.

Further evidence that the cytoprotective action of dmPGE2 is associated with microtubule integrity is derived from our colchicine studies. Colchicine, an agent known to disrupt microtubule assembly, should abolish cytoprotection if indeed this component of the cytoskeleton plays a role in the protective action of prostaglandins. Interestingly, pretreatment with colchicine prevented the cytoprotective effects of dmPGE2 in
Caco-2 cells when subsequently exposed to ethanol at all concentrations tested. Although colchicine alone (4-h incubation) did not affect viability in Caco-2 cells, paralleling previous findings by Arakawa and colleagues (4, 5) in cultured gastric cells, it did induce injury to colonic cells exposed to 1% ethanol (that did not occur in the absence of colchicine), which was not overcome by prostaglandin pretreatment. Of additional note, colchicine pretreatment alone did cause significant cell death after treatment times exceeding 8 h. Stated another way, microtubule disassembly by colchicine occurred much earlier than the cell death that was subsequently noted in Caco-2 cells on treatment with this agent. These findings indicate that colchicine potentiates ethanol-induced injury, suggesting that the microtubule cytoskeleton plays a critical role in the maintenance of cellular integrity. In support of this interpretation, colchicine pretreatment led to the complete depolymerization and disappearance of the microtubule network as indicated by immunocytochemistry and quantitative immunoblotting (Figs. 7 and 8). After 4 h of colchicine exposure alone, the only remaining polymerized tubulin was detected as punctate “nuclei” in the cytoplasm, confirming previous observations by Gilbert et al. (17). These findings suggest that the effect of colchicine on the prevention of cytoprotection by dexamethasone (dMPE) is caused by the disruption of tubulin skeleton in vitro and lends additional support to the contention

![Microtubule immunocytochemistry from control Caco-2 cells (A) or those pretreated with Taxol before exposure to 2.5% (B), 5% (C), 7.5% (D), or 10% EtOH (E). A: control cell displays normal stellate appearance of microtubules coursing through cytosol. B: similar findings are demonstrated in 2 Caco-2 cells pretreated with Taxol before exposure to 2.5% EtOH. Virtually identical findings can be observed in cells in C (2 cells), D (4 cells), or E (2 cells), which were pretreated with Taxol before exposure to higher concentrations of EtOH (5, 7.5, and 10%, respectively). Thus these micrographs demonstrate that Taxol pretreatment confers microtubule stability in cells exposed to graded concentrations of EtOH. Bar, 25 µm.](image)

![%Cellular viability in Caco-2 cells as determined by trypan blue dye exclusion after pretreatment with and without Taxol and subsequent incubation in EtOH. *P < 0.05 compared with control. †P < 0.05 compared with 2.5–10% EtOH groups without Taxol (n = 9 per group).](chart)

![Fig. 10. %Cellular viability in Caco-2 cells as determined by trypan blue dye exclusion after pretreatment with and without Taxol and subsequent incubation in EtOH. *P < 0.05 compared with control. †P < 0.05 compared with 2.5–10% EtOH groups without Taxol (n = 9 per group).](chart)
that microtubules are necessary for prostaglandin cytoprotection to occur.

Although it might be argued that microtubule disassembly by itself is not associated with cell death since colchicine-treated cells demonstrated profound microtubule perturbations at least 4 h before the demonstration of alterations in cellular viability, the fact remains that such cells did ultimately die. Furthermore, the observation that 1% ethanol was damaging in colchicine-treated cells, which was not the case without such treatment, underscores the linkage between microtubule disruption and predisposition to injury. Although the exact sequence of events between microtubule disassembly and ultimate cellular death has yet to be defined, a conceptual framework follows. Microtubules contribute to the overall stability and shape of the plasma membrane. When disrupted by an agent such as colchicine, the cells are rendered more susceptible to injury, because this cytoskeletal component is no longer able to contribute to the maintenance of cellular structure and transport functions. Thus, when ethanol is added to colchicine-treated cultures, it likely intercalates into membranes that already have been structurally weakened, increasing their fluidity, and thereby more likely allowing for the intracellular accumulation of cytotoxic concentrations of calcium, which is known to lead to cell death. Several experimental observations support this scenario. First, elevations in intracellular calcium have been shown to disassemble established microtubules, inhibit the assembly of new microtubules, and induce cellular injury in the context of these alterations (21, 23, 24). Second, microtubules have been demonstrated to play a role in calcium flux in dispersed parietal cells, suggesting that they help modulate calcium homeostasis (49). Third, calcium-induced disruption of microtubules has been observed in monkey epithelial cells that correlated with significant reductions in cell viability (41). Fourth, exposure of gastrointestinal epithelial cells to ethanol elicited the influx of calcium through lanthanum-sensitive channels, thus leading to injury (26). Conceivably, enhancement of membrane fluidity by an agent such as colchicine through disassembly of microtubules could further perturb calcium homeostasis. Finally, colchicine did not potentiate ethanol-induced damage in isolated rat gastric cells in calcium-free medium even though such potentiation was evident when calcium was present, suggesting that microtubules may play a role in the maintenance of cellular integrity via regulation of calcium flux (4). Thus it is likely that both the disruption of microtubules and corresponding changes in calcium influx eventuating in cytotoxic concentrations create conditions leading to cellular injury. Of equal likelihood, prostaglandins evoke their protective actions by reversal of these processes.

Our Taxol experiments add further support to the hypothesis that microtubules are linked to prostaglandin-induced protection. Taxol, a drug known to stabilize microtubules, was also noted to be protective against alcohol injury in Caco-2 cells, demonstrating effects not dissimilar to those with dmPGE2 pretreatment. Taxol pretreatment not only prevented the disruption of microtubules, despite exposure to a range of damaging concentrations of ethanol, but also significantly increased cellular viability as indicated by trypan blue exclusion. In addition, Taxol stabilization of microtubules was further demonstrated by the presence of a larger S2 polymerized tubulin pool, confirming observations of intact microtubules observed by immunofluorescence microscopy. These findings provide additional evidence that the maintenance of microtubule integrity plays an important role in the prevention of ethanol-induced injury.

Increased tubulin polymerization by prostaglandins, as our data indicate, may help to stabilize the microtubule cytoskeleton by the addition of monomers (GTP-tubulin) to the ends of unstable (GDP-tubulin) or fragmented microtubules (3, 29). It may additionally help anchor the microtubules via increased polymerization at the MTJ and/or by regulating the formation of nucleation sites (3, 29). Such modifications could allow stabilized microtubules to reinforce cell shape, to maintain membrane support, and to participate in structure and transport functions (1–3, 7, 11, 29). These functions are based on the capacity of tubulin to polymerize and on the stability of preassembled microtubules. Microtubules are clearly required for many important activities, and their continued study is certain to reveal further details on structure-function integration within eukaryotic cells. These functions are essential to the ability of cells to cope with damaging insults such as ethanol. Furthermore, the ability of prostaglandins to increase tubulin polymerization may be the means by which stabilization of the cytoskeleton occurs, allowing microtubules to carry out their normal functions and thus maintain cellular integrity in the face of potentially injurious challenges.

In summary, the present study demonstrates that microtubules are extensively disrupted after exposure of Caco-2 cells to injurious concentrations of ethanol, coincident with significant reductions in cell viability. Prostaglandin pretreatment obviated these effects. Colchicine, a potent microtubule disruptive drug, not only depolymerized microtubules, but also prevented cytoprotection induced by dmPGE2 and made the cells more susceptible to ethanol-induced injury. On the other hand, Taxol, a tubulin-stabilizing drug, mimicked the effects of dmPGE2 on microtubule stability and cell viability by preventing alcohol-induced damage. Taken together, these observations suggest that microtubule organization and stability play important roles in preventing cell injury by ethanol and may be essential to the mechanism of prostaglandin-induced cytoprotection.

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