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Adaptive Cytoprotection Induced by Ethanol in Human Intestinal Cells

Role of Prostaglandins and Calcium Homeostasis

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Objective

To determine whether adaptive cytoprotection exists in human intestinal cells under *in vitro* conditions and what role, if any, endogenous prostaglandins or calcium may play in mediating this protective response.

Summary Background Data

Adaptive cytoprotection can be defined as that process whereby the administration of a low concentration of a damaging agent, termed a "mild irritant," which by itself is not injurious, can attenuate gastrointestinal mucosal injury subsequently induced by the application of higher concentrations of the same or other necrotizing agents. Despite substantial investigation, the mediator or mediators of adaptive cytoprotection remain poorly understood.

Methods

Postconfluent Caco-2 cells were used in all experiments. Cellular death was quantitated using a dual-component fluorescent assay. Changes in intracellular calcium concentration were quantitated by measuring fluorescent signal changes of the single wavelength calcium indicator (Fluo-3). Finally, prostaglandin E_2 release into the media was quantitated by radioimmunoassay.

Results

Pretreatment of Caco-2 cells with low concentrations of ethanol (mild irritant) significantly attenuated injury induced by higher damaging concentrations of ethanol. The protection conferred by the mild irritant was directly dependent on both the concentration of the irritant used and the duration of exposure and was abrogated when cells were pretreated with an endogenous prostaglandin inhibitor (indomethacin) or if the mild irritant was administered in calcium-free media. Cells exposed to ethanol had a significant and concentration-dependent increase in intracellular calcium concentration, an effect that was highly related to cellular injury. Pretreatment with a mild irritant significantly decreased intracellular calcium increases induced by not only ethanol but also by a calcium ionophore (A23187). Cells treated with low concentrations of ethanol demonstrated no significant elevation in prostaglandin E_2 release.

Conclusions

Adaptive cytoprotection induced by ethanol exists in human colonocytes under *in vitro* conditions independent of mucosal blood flow, neural innervation, or circulating humoral factors. The authors' data suggest that this response does not require endogenous prostaglandin synthesis but may involve processes whereby intracellular calcium accumulation is prevented.

The concept of gastroduodenal cytoprotection originated from the work of Robert in the late 1970s.[1] Direct cytoprotection was defined as the ability of exogenous prostaglandins (PGs), independent of their effects on acid secretion, to protect gastrointestinal (GI) mucosae against injury induced by a wide variety of damaging agents.[2] Several years later, he observed another response that he termed adaptive cytoprotection, which can be defined as that process whereby the administration of a low concentration of a damaging agent, termed a "mild irritant," which by itself is not injurious, can attenuate GI mucosal injury subsequently induced by the application of higher concentrations of the same or other necrotizing agents.[3] Robert and Chandhury[4] demonstrated

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that pretreatment of the gastric mucosa with varying mild irritants (bile salts, ethanol, HCl) significantly attenuated injury induced by damaging concentrations of taurocholate or other agents. In addition, because this response was partially reversed with indomethacin (a reversible cyclooxygenase inhibitor) pretreatment, they proposed that endogenous PG synthesis played a critical role in mediating this protective response.[3–5]

Several investigators subsequently confirmed Robert's original hypothesis that mild irritant pretreatment protected gastroduodenal mucosae against injury induced by various necrotizing agents and, furthermore, that indomethacin blocked this protective response.[6,7] To date, however, a definitive role for endogenous PGs as mediators of adaptive cytoprotection remains controversial.[8–10] More recently, additional mediators of adaptive cytoprotection have been proposed, including nitric oxide, glutathione, dopamine, the internal enteric reflex, mucus secretion, salivary secretions, or the formation of a protective covering of surface debris.[10–16] Nonetheless, despite substantial investigation, the mechanism or mechanisms of adaptive cytoprotection remain poorly understood.

We have recently investigated adaptive cytoprotection under *in vitro* conditions in human gastric cells. This work used deoxycholate as both the mild irritant and damaging agent and indicated the existence of adaptive cytoprotection despite the absence of intact blood flow, neural innervation, or circulating humoral mediators. Our findings also indicated that stimulation of endogenous PG synthesis was not a prerequisite in mediating this protective response.[17,18] Furthermore, subsequent investigation suggested that adaptive cytoprotection induced by deoxycholate exposure involved the regulation of calcium homeostasis.[19,20]

Given these results, we questioned whether adaptive cytoprotection under *in vitro* conditions was specific to either the mild irritant (deoxycholate) or the cell line (human gastric cells) used. Therefore, the objectives of the current study were to determine whether adaptive cytoprotection induced by ethanol existed under *in vitro* conditions in human intestinal cells and what role, if any, endogenous PGs play in this protective response. A portion of this work has been previously presented in abstract form.[21]

MATERIALS AND METHODS

Cells

The human colonic carcinoma cell line Caco-2 was obtained from American Type Culture Collection (Rockville, MD) at passage 15. These cells, when grown to postconfluency, possess the unique ability to polarize, differentiate, and develop morphologic characteristics of normal enterocytes.[22,23] Cells were maintained at 37°C in an atmosphere of 5% CO_2 and 100% relative humidity. Cells were split on a weekly basis at a ratio of 1:6 on reaching confluency. Cells were detached using 0.5 g porcine trypsin and 0.2 g EDTA tetrasodium per liter of Hank's balanced salt solution and then plated into either 24- or 48-well plates (Costar, Cambridge, MA) for experiments or into 150-cm^2 flasks for propagation. All experiments were performed at 2 to 4 days postconfluence. Cell passage was maintained between 50 and 65 and media was changed every 2 to 3 days. Caco-2 media consisted of Eagle's minimum essential medium supplemented with 20% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

Solutions

Before all experiments, the medium was aspirated from the plates and replaced with Hank's balanced salt solution plus 10 mM HEPES (HBSS; H 8264, Sigma, St. Louis, MO: 137 mM NaCl, 5.7 mM $NaHCO_3$, 5.3 mM KCl, 1.26 mM $CaCl_2$, and 0.8 mM $MgSO_4$). Experiments involving calcium-free buffer used Hank's balanced salt solution plus 10 mM HEPES (HBSS[−Ca]); H 6648, Sigma: 137 mM NaCl, 5.7 mM $NaHCO_3$, and 5.3 mM KCl). All test compounds were dissolved in either HBSS or HBSS(−Ca). Unless otherwise specified, cells were pretreated with low concentrations of ethanol for 20 minutes and subsequently exposed for 20 minutes to either higher concentrations of ethanol or the calcium ionophore A23187 (4-bromo A23187; Molecular Probes, Eugene, OR). A23187 was stored at −80°C as a stock solution of 1 mg in 500 μl DMSO. Experiments were performed at 37°C in a humidified incubator.

Indomethacin (Sigma) was added to a solution of sodium bicarbonate (50 mM) in normal saline to make a 5 mM solution. Experiments with indomethacin involved a 60-minute preincubation followed by the addition of indomethacin to all subsequent solutions within treatment groups. 16,16-dimethyl prostaglandin E_2 (PGE_2; Sigma) was maintained at −20°C as a stock solution of 1 mg/1 ml in ethanol, and cells were pretreated for 20 minutes at a final concentration of 2.6 μM.

Cell Death

Death was assessed using a fluorescent cellular assay that simultaneously determined living and dead cells by measuring two parameters of cell viability: intracellular esterase activity and plasma membrane integrity (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes). The first component, calcein AM (2 μM), is converted by intracellular esterases to a fluorescent stain, which is retained within living cells and produces an intense uniform green fluorescence (at 530 nm). Ethidium homodimer-1 (4 μM), the second component, enters cells through damaged membranes and undergoes enhanced fluorescence on binding to nucleic acids. The fluorescent probe produces a bright-red fluorescence (at 600 nm) in dead cells.[24,25] In accordance with the respective experimental protocols, cells were loaded with the two fluorescent probes for 20 minutes at 37°C and

evaluated using a Nikon diaphot inverted fluorescent microscope, using the appropriate filter cubes. Only cells in the central portion of the well were evaluated, and the numbers of live and dead cells within an eyepiece grid were counted (>250 cells/well). The mortality index (%) was defined as the number of dead cells divided by the number of total cells × 100.

Prostaglandin Synthesis

Newly synthesized PGs are not stored intracellularly but are released into the extracellular space.[26] For this reason, we used the following protocol to quantitate PGE_2 production by Caco-2 cells. After 20 minutes of exposure of Caco-2 cells to ethanol, the buffer was immediately transferred to microcentrifuge tubes and stored at −80°C, and plates containing cells were immediately frozen at −20°C for subsequent protein determinations. Samples were then thawed and PGE_2 content was assayed using a commercial radioimmunoassay kit (Amersham, Chicago, IL). Total protein concentration per well was estimated colormetrically with BCA protein assay kits (Pierce Chemicals, Rockford, IL).

Measurement of $[Ca^{++}]_i$

Changes in intracellular calcium concentration were quantitated using the single wavelength calcium indicator Fluo-3 (Molecular Probes). Fluo-3 was chosen because it exhibits a large fluorescent enhancement on calcium binding (40-fold), has a lower overall affinity for calcium binding than other probes, and exhibits an enhanced resistance to autobleaching.[27]

Before Fluo-3 loading, cells were washed twice with HBSS. Fluo-3 was initially dissolved in Pluronic F-127 (20% solution in DMSO; Molecular Probes) to make a 1 mM working solution, and was subsequently added to HBSS plus 1% fetal bovine serum for a final loading concentration of 4 μM.28 Cells were then loaded with Fluo-3 for 50 minutes at 25°C in an atmosphere of 5% CO_2 and 100% relative humidity. Loading at a lower temperature significantly decreases indicator compartmentalization into the endoplasmic reticulum or mitochondria.[28]

Caco-2 cells were then washed three times to ensure removal of all unloaded Fluo-3, and control and test solutions were then added to the respective wells. Continuous fluorescent signals were quantitated over time using a CYTOFLUOR II Fluorescent Multi-well Plate Reader (PerSeptive Biosystems, Framingham, MA), using 485 nm and 530 nm as the excitation and emission spectra, respectively. At each time point, intracellular calcium concentration was calculated using the following equation:

$$[Ca^{++}]_i\ (nM) = K_d \frac{(F - F_{min})}{(F_{max} - F)}$$

where $F_{min} = 1.25\ F_{MnCl2} - 0.25\ F_{max}$ and $K_d = 400$ nM.[29]

The maximal Fluo-3 signal (F_{max}) was determined by permeabilizing Caco-2 cells with 50 μM digitonin (Sigma). The Fluo-3 signal was quenched to obtain F_{MnCl2} using 2 mM $MnCl_2$ and 50 μM digitonin in HBSS(−Ca). Tetrakis (2-pyridylmethyl) ethylenediamine 50 μM (Molecular Probes) was used in all solutions as a heavy metal scavenger.[28]

Experimental Design

The first experiment investigated the effect of graded concentrations of ethanol on cell death in Caco-2 cells. The second experiment involved pretreatment of cells with HBSS, PGE_2, or low concentrations of ethanol (mild irritant) followed by subsequent exposure to varying higher concentrations of ethanol. The next two experiments were designed to determine the optimal mild irritant concentration as well as the duration required to elicit protection. The fifth experiment quantitated the changes in intracellular calcium concentration induced by graded concentrations of ethanol. The sixth experiment was designed to determine the effect of various pretreatments (including the mild irritant, the mild irritant in a calcium-free buffer, and the mild irritant in the presence of indomethacin) on both cell death and intracellular calcium changes induced by a damaging concentration of ethanol (12%). PG synthesis was then measured in response to varying concentrations of ethanol. Finally, we investigated the effect of mild irritant pretreatment on cell death and intracellular calcium changes induced by a calcium ionophore (A23187; 10 μM).

Statistics

Statistical evaluation was performed by analysis of variance with a Scheffe *post hoc* test. Data (n = 6/group minimum) are reported as mean ± standard error of the mean. $P < 0.05$ was taken to represent statistical significance.

RESULTS

Cell Damage by Ethanol

Caco-2 cells exposed to graded concentrations of ethanol for 20 minutes demonstrated concentration-dependent increases in cell death. The lowest concentration of ethanol required to elicit injury under these conditions was noted to be 5% (Fig. 1). However, when cells were pretreated for 20 minutes with either 2.6 μM PGE_2 or 1.5% ethanol (mild irritant) and subsequently exposed to damaging concentrations of ethanol for 20 minutes, cellular death was significantly attenuated when compared with control cells pretreated with buffered saline (Fig. 2). These data demonstrate that both direct and adaptive cytoprotection against ethanol challenge exist under *in vitro* conditions in human-derived enterocytes.

The subsequent two experiments investigated the optimal

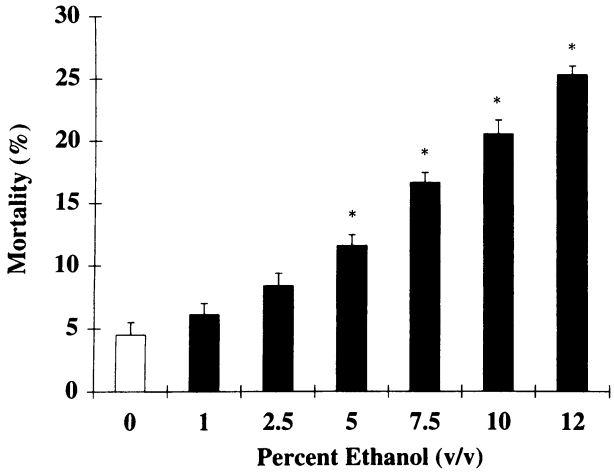

Figure 1. Cell death in Caco-2 cells exposed to graded concentrations of ethanol for 20 minutes. *p < 0.01 *vs.* control; n = 6/group.

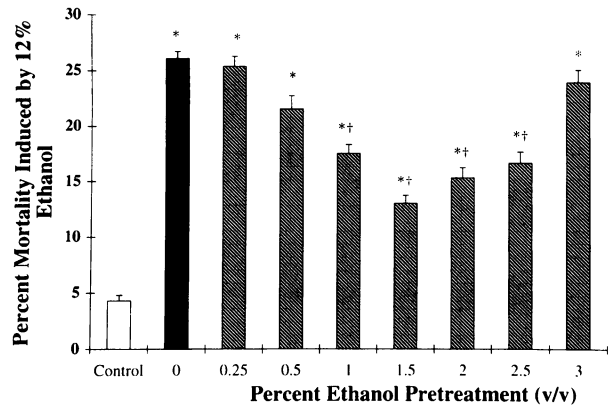

Figure 3. The effect of mild irritant concentration (20 minutes of pretreatment) on cell death induced by 12% ethanol (20 minutes of posttreatment). *p < 0.01 *vs.* control; †p < 0.01 *vs.* control injury; n = 6/group.

mild irritant duration and concentration necessary to achieve protection against injury induced by damaging concentrations of ethanol. Figure 3 depicts the effect of mild irritant concentration on cell death induced by subsequent exposure to 12% ethanol. Ethanol concentrations ranging from 1% to 2.5% attenuated injury induced by 12% ethanol, with the greatest protection evident with 1.5% ethanol pretreatment. Figure 4 demonstrates the effect of pretreatment duration with 1.5% ethanol on cell mortality induced by a damaging concentration of ethanol. Pretreatment durations between 10 and 40 minutes significantly decreased injury induced by 12% ethanol, whereas shorter or longer pretreatment times conferred no protection. Because pretreatment of Caco-2 cells with 1.5% ethanol for 20 minutes appeared to provide the greatest degree of protection, this mild irritant concentration and pretreatment duration was used for all subsequent experiments.

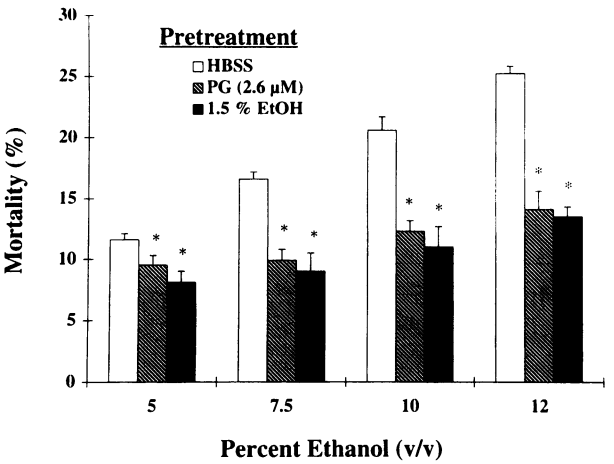

Figure 2. The effect of various pretreatments for 20 minutes on cell death induced by 20 minutes posttreatment with damaging concentrations of ethanol. HBSS, buffered saline; PG, 16,16-dimethyl PGE_2. *p < 0.01 *vs.* control injury; n = 6/group.

Ethanol exposure increased intracellular calcium concentration in Caco-2 cells in a concentration-dependent fashion. A large increase in intracellular calcium content was first observed 2 minutes after exposure to 12% ethanol. These elevations in intracellular calcium decreased over time but still remained significantly higher than control values throughout the remaining treatment period. Of note, however, cells exposed to the mild irritant (1.5% ethanol) demonstrated an initial increase in intracellular calcium that decreased to levels comparable to control values after 10 minutes of exposure. These results are summarized in Figure 5.

We then investigated the effect of various pretreatments on either cell death (Fig. 6A) or intracellular calcium accumulation (Fig. 6B) induced by 12% ethanol. Pretreatment with the mild irritant (1.5% ethanol), indomethacin (100 μM), indomethacin plus the mild irritant, or the mild irritant in a calcium-free buffer followed by treatment with buffered saline did not adversely influence either cell death or intracellular calcium content when compared with control cells. Cells pretreated with buffered saline and subsequently ex-

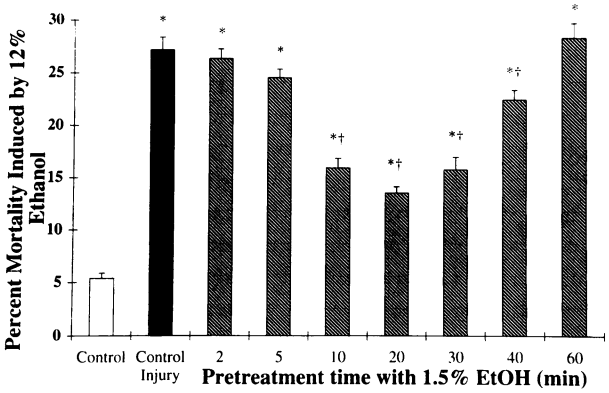

Figure 4. The effect of pretreatment duration with 1.5% ethanol on cell death induced by 12% ethanol (20 minutes of posttreatment). *p < 0.01 *vs.* control; †p < 0.01 *vs.* control injury; n = 6/group.

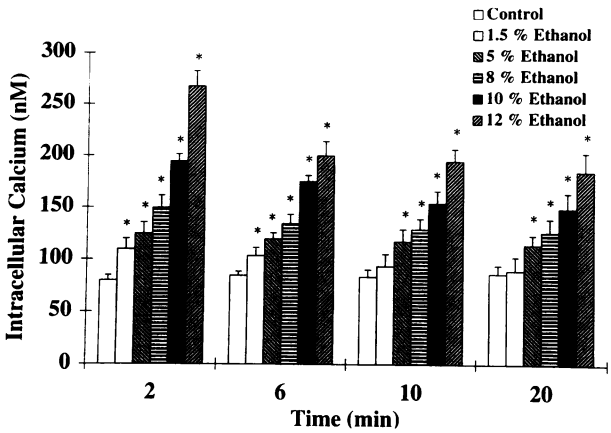

Figure 5. Changes in intracellular calcium concentration in Caco-2 cells induced by graded concentrations of ethanol over a 20-minute treatment period. Intracellular calcium content in control cells was stable over the entire experimental period. Ethanol, in a concentration-dependent manner, elicited a large increase in intracellular calcium first evident at the earliest time. It decreased but remained elevated during the remaining treatment period. *$p < 0.01$ *vs.* control; n = 6/group.

posed to 12% ethanol, however, demonstrated significant increases in both cell death and intracellular calcium concentration; this effect was significantly attenuated when cells were pretreated with the mild irritant. These protective effects induced by the mild irritant were abrogated when mild irritant exposure was preceded by indomethacin pretreatment. Finally, although pretreatment of cells with the mild irritant in a calcium-free medium partially attenuated intracellular calcium accumulation induced by 12% ethanol in calcium-containing buffer, cell death was unaffected.

Under control conditions, PGE_2 concentration in the medium was determined to be 63 ± 4 pg/mg protein. Caco-2 cells incubated with 20 μM A23187 demonstrated a significant increase in endogenous PGE_2 production (99 ± 6 pg/mg protein, n = 6/group, $p < 0.01$). Indomethacin (100 μM) pretreatment significantly reduced both basal and stimulated PGE_2 synthesis (data not shown). Interestingly, in separate experiments (n = 9), Caco-2 cells exposed to graded concentrations of ethanol did not demonstrate significant endogenous PGE_2 release until ethanol concentrations approached 25% to 30%. Thus, the concentrations used for the mild irritant elicited no enhanced endogenous PGE_2 release (data not shown).

To determine whether the protection conferred by the mild irritant was specific to damage induced by ethanol, the effect of mild irritant pretreatment on cellular death and intracellular calcium accumulation induced by a calcium ionophore (A23187; 10 μM) was then investigated (Figs. 7A and 7B, respectively). Cells treated for 20 minutes with A23187 demonstrated a significant increase in both cell death and intracellular calcium content. These effects elicited by the ionophore were reversed when cells were first pretreated with the mild irritant. Data from Figures 6 and 7 are plotted in Figure 8 to display the relation between intracellular calcium accumulation and cell death. The correlation between these two factors was highly significant ($p < 0.01$).

DISCUSSION

Despite significant investigation, the mechanism or mechanisms underlying adaptive cytoprotection remain elusive. Although Robert and others have suggested that endogenous PGs play a major role in mediating this protective response,[3–5] this opinion has not been shared by all investigators.[9,14] Further, multiple additional mediators of adaptive cytoprotection have been purported from studies that in large part used *in vivo* models.[10–16]

The current study suggests that adaptive cytoprotection induced by ethanol exists under *in vitro* conditions in human enterocytes. These results are similar to those of prior work in our laboratory investigating adaptive cytoprotection induced by deoxycholate in human gastric cells.[17–20] Thus, as we observed with deoxycholate, the protective effect of a low concentration of ethanol appeared to be directly related to both the concentration used and the duration of exposure. Although indomethacin pretreatment reversed the protective effects of both mild irritants, neither ethanol in a human

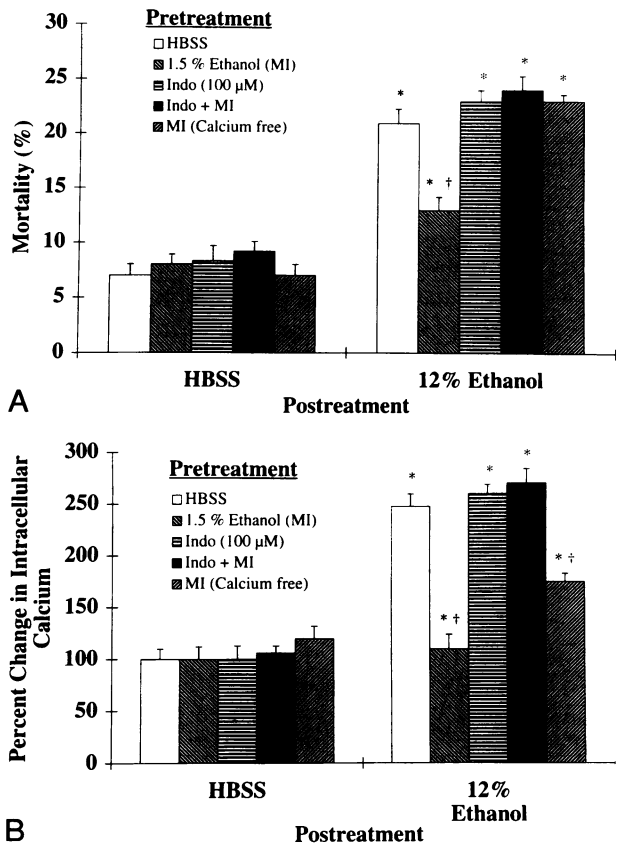

Figure 6. The effect of various pretreatments (20 minutes) on cell death (A) and intracellular calcium accumulation (B) in Caco-2 cells subsequently exposed to either buffered saline (HBSS) or 12% ethanol (20 minutes of posttreatment). MI, mild irritant; Indo, indomethacin. *$p < 0.01$ *vs.* control; †$p < 0.01$ *vs.* control; n = 6/group.

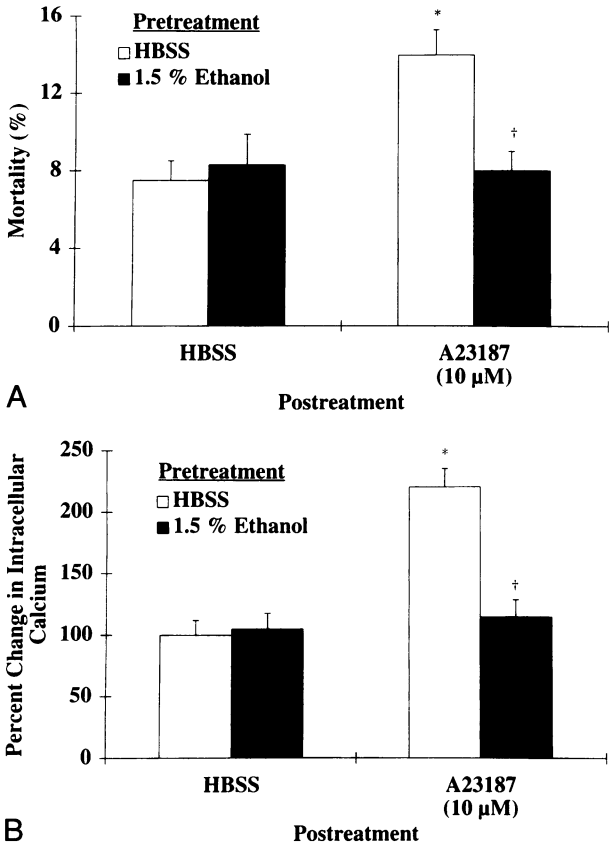

Figure 7. The effect of mild irritant pretreatment for 20 minutes on cellular death (A) and intracellular calcium accumulation (B) induced by 20 minutes of exposure to either buffered saline (HBSS) or a calcium ionophore (A23187; 10 μM). *p < 0.01 *vs.* control; †p < 0.01 *vs.* control; n = 6/group.

enterocyte cell line nor deoxycholate in a human gastric cell line, at concentrations noted to induce protection, elicited significant PGE_2 synthesis. Finally, mild irritant pretreatment significantly decreased intracellular calcium accumulation induced by not only higher concentrations of the respective damaging agents but also the calcium ionophore A23187, an effect that correlated with enhanced cellular viability. Thus, adaptive cytoprotection under *in vitro* conditions does not appear to be specific to the mild irritant, the damaging agent, or the human GI cell line used.

Most studies investigating adaptive cytoprotection have involved the assessment of mucosal damage in the stomach.[4,5,8,9,11,12,16] However, several investigators have observed similar protective responses in the proximal small intestine. Foschi et al.[30] reported in an endoscopic study involving human volunteers that pretreatment of the duodenal mucosa with 20% ethanol (a noninjurious concentration) significantly attenuated injury induced by subsequent exposure to topical 40% ethanol. Further, this protective effect was noted to be abolished with acetylsalicylic acid pretreatment. Lugea et al.[31] demonstrated that low concentrations of acid reversed injury induced by damaging concentrations of acid in the rat duodenum, an effect that was negated with indomethacin pretreatment. Subsequent work in their laboratory suggested that adaptive cytoprotection of the duodenal mucosa was independent of changes in mucosal flow.[15]

Cepinskas et al.[32] also investigated adaptive cytoprotection induced by ethanol in Caco-2 cells. They did not observe significant protection against ethanol injury when using mild irritant concentrations ranging from 3% to 6% ethanol. Our work confirms these results. We found that ethanol pretreatment (1% to 2.5%) significantly attenuated injury induced by damaging concentrations of ethanol, and higher concentrations of ethanol were not only nonprotective (3%) but also damaging (5%). Protection was conferred by the mild irritant depending on concentration and also duration of exposure. These results suggest that cellular exposure to mild irritant concentrations too low (<1% ethanol) or of too short a duration (<10 minutes) does not provide sufficient stimulation for production of the mediator(s) responsible for this protective response, whereas pretreatment with concentrations too high (>3% ethanol) or for too long a duration (>30 minutes) may cause excessive cellular injury, leading to a loss of the capability to induce protection. This is consistent with prior observations from our laboratory as well as those of others.[16,33]

Robert and others have reported that adaptive cytoprotection is at least partially reversed by indomethacin pretreatment,[3,4,5,8,30,31] suggesting that the mild irritant may stimulate endogenous PG synthesis, which in turn protects the mucosa against subsequent injury. These results are consistent with those of the current study. In addition, pretreatment of Caco-2 cells with exogenous PGE_2 conferred protection against injury induced by ethanol, suggesting a common, shared mechanism between direct and adaptive cytoprotection. However, we were unable to demonstrate significant endogenous PGE_2 synthesis in response to mild irritant exposure, even though indomethacin, an established PG synthesis inhibitor, obviated the protective action of the mild irritant. The explanation for these seemingly contradictory findings is not immediately obvious and will require further investigation. One possibility is

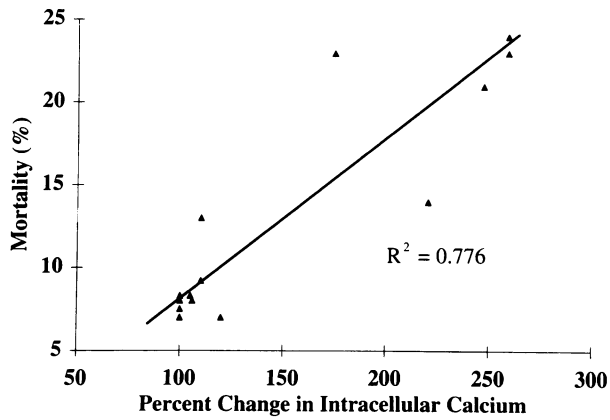

Figure 8. The relation between changes in intracellular calcium accumulation and cell death in Caco-2 cells. p < 0.01.

that adaptive cytoprotection requires the presence of intact basal PG levels to express itself, even though enhanced synthesis is not a prerequisite. Alternatively, indomethacin may have pharmacologic actions in addition to cyclooxygenase inhibition that ultimately result in an increased mucosal susceptibility to injury.

It has been suggested that calcium homeostasis is critical for the maintenance of GI mucosal integrity.[34] Further, it has been reported that calcium plays a major role in promoting mucosal injury induced by agents such as indomethacin, ethanol, or excessive nitric oxide.[35–37] Proposed mechanisms by which sustained elevations in intracellular calcium cause cellular toxicity include disruption of the cytoskeleton, phospholipid hydrolysis, and protease and endonuclease activation.[38,39] The current study supports a strong association between ethanol exposure and intracellular calcium accumulation. Furthermore, increases in intracellular calcium content appeared to be highly related to cellular injury.

Mild irritant pretreatment significantly decreased elevations in intracellular calcium accumulation induced by damaging concentrations of ethanol, and this effect was associated with cellular protection against injury, suggesting that the protective effect of the mild irritant may be related to the maintenance of normal calcium homeostasis. Several additional observations also lend credence to this concept. The optimal concentration of the mild irritant (1.5% ethanol) elicited a small increase in intracellular calcium, which returned to basal values within 10 minutes and did not result in cellular death. We also noted that the protective effect of the mild irritant was lost when calcium was removed from the experimental solution. Finally, mild irritant exposure not only prevented injury and changes in intracellular calcium induced by higher concentrations of ethanol but also those induced by the calcium ionophore A23187.

Given these results, we hypothesize that the mild irritant, in combination with extracellular calcium, may cause a small initial disruption in intracellular calcium concentration, which then leads to mild disruption of normal calcium homeostasis and/or the stimulation of calcium-dependent second messengers. This perturbation then elicits a cellular response involving active calcium efflux (by ATPases) and/or active sequestration into intracellular organelles (mitochondria and/or endoplasmic reticulum), resulting in protection against subsequent injury induced by damaging agents, which probably mobilize calcium.

In summary, we presented an experimental model of adaptive cytoprotection induced by ethanol in postconfluent Caco-2 cells. Under *in vitro* conditions, we demonstrated that this protective response exists on a cellular level and remains independent of mucosal blood flow, neural innervation, and circulating humoral factors. Our data suggest that stimulation of endogenous PG synthesis is not the major mediator of adaptive cytoprotection under these conditions, and that this process may involve mechanisms whereby intracellular calcium accumulation is prevented.

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