

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 $\times 100$.

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 PGE₂ 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 PGE₂ content was assayed using a commercial radioimmunoassay kit (Amersham, Chicago, IL). Total protein concentration per well was estimated colorimetrically with BCA protein assay kits (Pierce Chemicals, Rockford, IL).

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

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 . Cells were then loaded with Fluo-3 for 50 minutes at 25°C in an atmosphere of 5% CO₂ 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 CYT-OFLUOR 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:

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

where $F_{\text{min}} = 1.25 F_{\text{MnCl}_2} - 0.25 F_{\text{max}}$ 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_{MnCl_2} using 2 mM MnCl₂ 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, PGE₂, 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 \pm 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 PGE₂ 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

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