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Survivin is a Key Factor in the Differential Susceptibility of Gastric Endothelial and Epithelial Cells to Alcohol-Induced Injury

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Summary

We previously demonstrated that the anti-apoptosis protein, survivin, plays a protective role against alcohol-induced gastric injury. Since the endothelium is a primary target of alcohol-induced gastric damage, we investigated whether survivin expression is a key factor in the greater susceptibility of gastric endothelial vs. epithelial cells to alcohol-induced injury. Here, we demonstrate that rat gastric epithelial cells (RGM1 cells, an epithelial cell line derived from normal rat gastric mucosa) expressed 7.5-fold greater survivin protein levels vs. rat gastric endothelial cells. Survivin expression correlated with resistance of gastric epithelial vs. endothelial cells to both alcohol-induced cell damage and alcohol-induced apoptosis. Suppression of survivin protein expression levels using siRNA rendered the gastric epithelial cells as susceptible to both alcohol-induced cell damage and apoptosis as the gastric endothelial cells. Conversely, forced overexpression of survivin by transient transfection rendered gastric endothelial cells as resistant to both alcohol-induced cell damage and apoptosis as mock-transfected gastric epithelial cells. Moreover, overexpression of a threonine-34 to glutamate phosphorylation mimic mutant survivin construct rendered gastric endothelial cells significantly more resistant to alcohol-induced damage and apoptosis vs. mock-transfected gastric epithelial cells. These findings indicate that disparate survivin expression levels can explain the discrepancy between gastric epithelial and endothelial cell susceptibility to alcohol-induced injury; and, that a negative charge at amino acid residue 34 on survivin, such as that which naturally occurs by phosphorylation of threonine-34, enhances its property in conferring gastric mucosal protection.

Keywords

Apoptosis; Cell death; In vitro Mutagenesis; Overexpression; Phosphorylation mimic; si RNA Knockdown; Transfection

Introduction

The gastric mucosa is especially susceptible to injury induced by noxious agents as it is a first-order contact organ. Ingestion of alcohol causes gastric mucosal damage within five minutes and further extends to within 6 to 12 hours before the processes of healing initiate. Endothelial cells lining gastric mucosal microvessels are major and early targets of acute injury by ethanol (1–3). Injury of the microvascular endothelial cells by ethanol leads to the microvascular stasis and ischemia that result in focal deep necroses, e.g., mucosal erosions (1–5). The repair of such injury requires not only restoration of the surface epithelium, glandular epithelial cells, and connective tissue but also, most importantly, a reestablishment of the microvascular network,

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crucial for delivery of oxygen and nutrients to the area (6). The finding that microvascular damage occurs in areas where glandular and surface epithelial cells are not severely damaged emphasizes the likelihood that the microvascular epithelium is the major target of alcohol-induced gastric mucosal damage (3). Moreover, the importance of this is underscored by the fact that although restoration of damaged surface epithelium is a rapid process, healing of injured gastric microvasculature is more complex and thus microvascular injury can lead to necrotic damage of deeper mucosal layers (3).

Survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family. It also differs from other members of this family in that it regulates not only cell survival but also cell division (7–9); and, unlike other IAP members, survivin deficiency, e.g. homozygous deletion of the survivin gene, during development causes embryonic lethality in mice (10). Although survivin expression is absent in most non-cancerous adult differentiated tissues, some normal adult cell types do express survivin including thymocytes, CD34-expressing bone marrow stem cells, and basal colonic and gastric mucosal epithelial cells (11–14).

We previously demonstrated that survivin is a mediator of epithelial cytoprotection against alcohol-induced gastric mucosal damage (15). Whether survivin does or can mediate protection to gastric endothelial cells against alcohol-induced damage has not been explored. Nevertheless, we have previously demonstrated that survivin expression in normal, non-cancerous stomach is primarily localized to surface epithelial cells (14) implicating survivin may provide a selective survival advantage to gastric epithelial cells against at least some noxious agents that is not available to gastric endothelial cells. The present study was undertaken to determine whether disparate expression of survivin could account, in part, for the increased susceptibility of the gastric mucosal endothelium to the injurious effects of alcohol relative to the gastric mucosal epithelium.

Materials and Methods

Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committee of the VA Long Beach Healthcare System. Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing 225–250 g were used and were fasted for 18 hours prior to the studies. To investigate potential systemic effects of ethanol that may be relevant to gastric cytoprotection and the involvement of survivin, a study comparing the extent of gastric mucosal cytoprotection produced by intravenous (i.v.) administration of ethanol vs. intragastric (i.g.) ethanol administration was performed. Rats (n=8 per group) were pretreated with either: a) 2 ml sterile water i.g. (vehicle controls); b) 2 ml 21% ethanol i.g.; c) 74 mg/dl (total blood volume) ethanol i.v. via the tail vein. The amount of ethanol administered i.v. was chosen based on a study that measured blood alcohol concentrations (BAC) in fasted rats following i.g. administration of 21% ethanol (~2 ml) and reported that this BAC was attained within 30 minutes and remained relatively unchanged for approximately 3 hours (16). Total blood volumes for the rats were calculated based on body weight (17). Rats then received 2 ml 50% ethanol i.g. 30 minutes after the respective pretreatments (corresponding controls received water). The extent of macroscopic gastric mucosal damaged was determined at 3 hours and gastric tissue was used to determine gastric mucosal survivin levels by immunoblot analysis and immunofluorescence staining (see below). To assess comparative survivin protein expression levels between primary gastric epithelial cells and RGM1 cells, primary gastric epithelial cells were partially purified by combination and modification of three published gastric epithelial cell enrichment/isolation protocols (18–20). Briefly, the stomachs of 5 fasted and anesthetized (ketamine/xylazine) male Sprague-Dawley rats (~250g) were removed following laparotomy and opened along the lesser curvature to expose the mucosa. The antrum and forestomach were discarded and the fundic stomach was washed several times in changes

of cold phosphate buffered saline containing 1mM $CaCl_2$ (PBS-Ca). The stomach surface was then gently wiped with sterile gauze to remove the mucus gel layer and the mucosa was separated from the muscle layers and stored in a 50 ml centrifuge tube containing oxygenated modified Eagle's medium (MEM; Invitrogen, Carlsbad, CA) + 20mM HEPES buffer (pH 7.4), 0.2% BSA and 1% gentamycin until all 5 stomachs were harvested and processed. The mucosal preparations were then centrifuged at 15×g twice at 4°C, the supernatants were discarded and the mucosal preparations were placed in small erlenmeyer flasks with 10 ml of PBS-Ca containing 0.2% w/v type I collagenase (Sigma Chemical Co., St. Louis, MO), 0.2% w/v bovine serum albumin (Sigma) and 2mM EDTA. The preparations were incubated at 37°C in a shaking incubator with a shaking speed of 155 rpm for 30 minutes. During this incubation time, the digestion and cell dispersal was intermittently aided by pipetting with a Pasteur pipette. The cells were then filtered through a 105-μm mesh (Spectrum Laboratories, Inc., Rancho Dominguez, CA) into a 50 ml centrifuge tube, centrifuged at 250×g for 3 minutes at 4°C and resuspended in cold PBS. An enrichment procedure by one-step density gradient centrifugation (18) produced a fraction that was >80% surface epithelial cells and was used to determine relative survivin protein expression levels (denoted partially purified primary gastric epithelial cells). Nevertheless, this partially purified fraction was contaminated with roughly 10–15% parietal cells, 2–3% chief cells and 1% other cell types including mesenchymal cells as identified by immunofluorescence staining (see below). It is primarily for this reason that we chose to use an epithelial cell line for the present studies.

Cell culture

RGM1, an epithelial cell line derived from normal rat gastric mucosa, was obtained from Riken Cell Bank (Tsukuba, Japan) and was used at passages 11–20. The cells were maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 20% FBS and 2 mM of L-glutamine at 37°C with 5% CO_2 and 95 % air in a humidified incubator. Isolation of the rat gastric microvascular endothelial cells has been described previously (21). These cells were maintained in endothelial SF medium (Invitrogen) supplemented with 20% FBS, 100 μg/ml heparin sulfate, endothelial cell growth supplement (Sigma), and antibiotics/antimycotics. Cells were plated in 100 mm dishes at a density of 4.0×10^6 cells/dish, or in 6-well plates at a density of 4.0×10^5 cells/well and incubated until ~80% confluent. Cells were serum starved for 24 hours prior to experiments. Serum-free conditions were employed to avoid possible confounding effects of protective factors contained in the serum and because serum resulted in interference with the LDH assay used to assess cell damage (see below). The cells were then incubated in serum-free medium containing the indicated concentration of ethanol or equal volume of water (control). Cells were lysed or medium was collected following the indicated incubation times.

Immunoblot analysis

RGM1 and RGMEC cells were lysed in a lysis buffer containing 62.5 mM ethylenediamine-tetraacetic acid; 50 mM Tris-HCl, pH 8.0; 0.4% deoxycholic acid; 1% Nonidet P-40; 0.5 μg/ml leupeptin; 0.5μg/ml pepstatin; 0.5 μg/ml aprotinin; 0.2 mM phenylmethylsulfonyl fluoride; 0.05 mM aminoethyl benzene sulfonyl fluoride 0.1mM sodium vanadate following the indicated treatment(s). The cell lysates were then centrifuged (14,000 rpm for 10 minutes at 4°C). The protein content of the lysates was determined by the bicinchoninic acid protein assay using a commercial kit (BCA Protein Assay Reagent, Pierce Chemical Company, Rockford, IL.) or by modified Bradford method using a commercial Bradford protein assay kit (Biorad, Hercules, CA). Total survivin, pro-caspase 9, cleaved caspase 9, pro-caspase 3, cleaved caspase 3, Hsp90 and Hsp27 protein levels were determined from equal amounts of total tissue homogenate or cell lysate protein. The total survivin signal was detected with rabbit polyclonal anti-survivin antibody (Novus Biologicals, Littleton, CO). Expression of the survivin HA-tagged fusion protein was detected with rabbit polyclonal anti-HA antibody (Sigma Chemical

Co., St. Louis, MO). Pro- and cleaved caspase 9, and pro- and cleaved caspase 3, signals were detected with rabbit polyclonal antibodies that recognize both the pro- and cleaved forms of the respective caspase (BioVision, Inc., Mountain View, CA). The Hsp90 and Hsp27 signals were detected with rabbit polyclonal antibodies specific for the respective heat shock protein (Assay Designs, Inc., Ann Arbor, MI). All membranes were also stripped and reprobed with mouse monoclonal anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO) to control for protein loading and membrane transfer. Signals were visualized by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). Quantification of the data was performed using a video image analysis system (Image-1/FL; Universal Imaging, Westchester, PA) after normalizing for the corresponding total protein and/or β-actin signal.

Immunofluorescence staining

RGM1 and RGMEC grown on sterile microscope slides in 100 mm culture dishes were serum-starved and treated with the indicated concentrations of ethanol in serum-free culture medium for the indicated time increment(s). The cells were then gently washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and then in acetone for 5 min at −20°C. Non-specific staining was blocked by incubation in serum-free protein block solution (Dako, Carpenteria, CA). The slides were incubated with rabbit polyclonal anti-survivin antibody (Novus Biologicals, Littleton, CO) at a dilution of 1:100 for 16 hours at 4° C. The slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit secondary antibody (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:100 for one hour. The slides were counterstained with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) to confirm cell nuclei. To assess cellular contamination of the partially-purified primary gastric epithelial cell fraction, aliquots of a cell suspension were allowed to air-dry on microscope slides that were further fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and then in acetone for 5 min at −20°C as above. Gastric parietal cells were identified by staining with a dolichos biflorus agglutinin (DBA)- FITC conjugate (Sigma) or staining for the hydrogen potassium ATPase beta subunit using a specific mouse monoclonal antibody (Abcam Inc., Cambridge, MA). Gastric chief cells were identified by staining for pepsinogen using a specific sheep polyclonal antibody (Abcam Inc.). Mesenchymal cells were identified by staining for vimentin using a specific mouse monoclonal antibody (Millipore Corp., Billerica, MA). Myofibroblasts were identified by staining for -smooth muscle actinusing a specific mouse monoclonal antibody (Sigma). For analysis of survivin expression in gastric tissue, formalin-fixed, paraffin-embedded rat gastric tissue sections (from the “Animal studies” described above) were used. The sections were incubated with rabbit polyclonal anti-survivin antibody (Novus Biologicals, Littleton, CO) for either 1 hour at room temperature or 16 hours at 4°C. The sections were then stained with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit secondary antibody (Sigma Chemical Co., St. Louis, MO) for one hour. Staining was visualized using a Nikon Optiphot epifluoresence microscope (Nikon, Inc.) with Omega FITC/Texas Red filter. Specificity of staining was confirmed by omitting the primary antibody and using an appropriate blocking peptide.

TUNEL assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed using an Apop Tag in situ detection kit (Serologicals Corporation, Norcross, GA) according to the manufacturer’s instructions. For assessing apoptosis *in vitro* by TUNEL assay, RGM1 and RGMEC cells, or RGM1 and RGMEC transfected or control (e.g. mock-transfected) cells, grown on sterile microscope coverslips in 6 well culture dishes were serum-starved and treated with the indicated concentrations of ethanol in serum-free culture medium for the indicated time increment(s). The cells were then gently washed with PBS and fixed with 1% paraformaldehyde in PBS for 20 minutes at room temperature. Percent apoptosis was determined by counting the number of TUNEL-positive

cells in 5 random fields and dividing by the total number of cells in the same field under 100X magnification. Images were captured using a camera attached to a Nikon Optiphot microscope (Nikon, Inc., Melville, NY).

Survivin suppression using siRNA

Double stranded short interfering RNA (siRNA) oligonucleotides were designed with a software program (www.qiagen.com) to be specific for rat survivin. The double stranded siRNA was composed of the following oligonucleotides: r(UGAGCCUGAUUUGGCCCAG) d(TT) and r(CUGGGCCAAAUCAGGCUCA)d(TT) (Qiagen-Xeragon, Germantown, MD). A fluorescein labeled double stranded siRNA having no known homology with mammalian genes (Qiagen-Xeragon) was used to control for nonspecific silencing effects and to assess efficiency of transfection. RGM1 and RGMEC cells were seeded in 6-well plates (or on sterile microscope coverslips in 6-well plates) at 10^5 cells per well 24 hours prior to transfection. The cells were transfected with 200nM double-stranded survivin siRNA (or equimolar control RNA) complexed with 3 μl of Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA), according to manufacturers protocol, in 1 ml of Opti-MEM I reduced serum medium (Invitrogen). Control cells were also treated with the oligofectamine reagent in the absence of RNA to control for possible effects of the reagent. The growth medium was replaced 24 hours post transfection with serum-free medium and the cells were incubated an additional 24 hours. For determining the extent of suppressed survivin expression levels, cells were lysed on ice and survivin protein levels were assessed by immunoblot analysis as described above. For determination of resistance to ethanol-induced cell damage (see below) and apoptosis (TUNEL), serum-starved transfected cells were incubated in serum-free medium containing the indicated concentrations of ethanol for the indicated times.

Survivin overexpression studies

Total RNA from normal (non-injured) rat gastric tissue was reverse transcribed and used to amplify a cDNA fragment encoding the complete wild type rat survivin sequence with the following primers: 5'-*GAATTC*GATCATGGGTGCTACGGC-3' (sense) and 5'-*GGATCC*GCGTAAGGCAGCTGCTCAATG-3' (antisense). The added restriction sites for cloning (*Eco*RI and *BamHI*, respectively) are italicized and the corresponding specific rat survivin sequences are underlined. The resulting PCR product was directionally ligated into the phCMV3 mammalian expression vector (Genlantis, San Diego, CA) to create a C-terminal HA tag. The resulting construct was subsequently sequenced to ensure proper insertion with respect to the HA-tag sequence and/or plasmid promoter as well as fidelity of the PCR reaction (Davis Sequencing, Inc., Davis CA). The pcDNA3 plasmid construct harboring the threonine-34-to-glutamate (T34E) HA tagged survivin mutant was purchased from Health Research, Inc. (Buffalo, NY). RGMEC cells seeded at 5×10^5 in 60 mm culture dishes were transfected with 2 μg each of the survivin-HA phCMV3 plasmid, T34E mutant survivin-HA pcDNA3 plasmid or 2 μg of the respective "empty" phCMV3 and pcDNA3 (Invitrogen) plasmids (to obtain corresponding control transfectants) complexed with Primefect™ reagent (Lonza, Walkersville, MD) according to manufacturer's instructions. RGM1 cells were transfected likewise with 2 μg "empty" phCMV3 plasmid to obtain "mock" control transfectants. Following the transfection, cells were allowed to recover for 24 hours until confluent. HA-tagged survivin and HA-tagged mutant survivin protein expression levels were assessed by immunoblot analysis as described above. For determination of resistance to ethanol-induced cell damage (see below) and apoptosis (TUNEL), serum-starved transfected cells were incubated in serum-free medium containing the indicated concentrations of ethanol for the indicated times.

Determination of cell damage

Culture medium, collected following cell incubation under the indicated conditions, was assayed for lactate dehydrogenase (LDH) release (an indicator of cellular damage) using a colorimetric assay kit (Genotech, St. Louis, MO) according to the manufacturer's instructions. Optical density (OD) was measured at 490 nm using a DU640B spectrophotometer (Beckman Coulter, Fullerton, CA). Percent cell damage was quantified as:

$$\%\text{Cell Damage} = (\text{experimental OD}_{490} - \text{spontaneous OD}_{490}) \div \text{maximum OD}_{490} \times 100$$

Experimental OD was determined from serum-free culture medium of cells cultured under the indicated condition (e.g. ethanol concentration and incubation time). Spontaneous OD was determined from serum-free culture medium of like cells (e.g. RGM1 and RGMEC). Maximum OD was determined by sonication disruption of an equal number of like cells in an equal volume of serum-free culture medium (22).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Student's 2-tailed *t* test was used to determine statistical significance between control and experimental groups. A P value of <0.05 was considered statistically significant. Comparisons of data between multiple groups were performed with analysis of variance (ANOVA).

Results

We recently demonstrated that exposure of gastric epithelial cells to a sub-cytotoxic concentration of ethanol results in accumulation of survivin protein and that this accumulation reduces susceptibility of the cells to both damage and apoptosis caused by subsequent exposure to cytotoxic concentrations of ethanol (15). We hypothesized that differential survivin expression levels might also regulate the degrees of injury susceptibility between different cell types of the gastric mucosa. Since alcohol-induced injury to the gastric microvasculature was previously shown to precede damage to glandular and epithelial cells *in vivo* (3), we examined the potential differential susceptibility of isolated gastric mucosal endothelial cells vs. gastric mucosal epithelial cells to ethanol-induced damage *in vitro*. *In vitro* studies were used to control for possible confounding effects of fibrin deposition, platelet thrombi formation or neutrophil infiltration/adherence etc.; and, because of inherent difficulties in manipulating survivin expression levels in the gastric mucosa *in vivo* (e.g. the embryonic lethality of homozygous survivin gene deletion and the lack of gastric mucosal-specific Cre mouse models to enable gastric surface epithelial cell-specific survivin deficiency). As shown in Figure 1A, gastric endothelial cells sustained a greater than 3-fold ($P<0.005$) extent in damage vs. RGM1 gastric epithelial cells (an epithelial cell line derived from normal rat gastric mucosa) upon exposure to 5% ethanol for 3 hours. This difference in susceptibility to ethanol-induced cell damage was further underscored by the finding that the gastric endothelial cells sustained moderate but significant damage when exposed to an ethanol concentration of 2%, which failed to induce any significant injury in the gastric epithelial cells exposed for the same time period (Figure 1A). Because the assay we used to evaluate overall ethanol-induced cell damage (lactate dehydrogenase release) does not distinguish between necrotic and apoptotic mechanisms, we examined the extent of ethanol-induced apoptosis more directly by TUNEL assay. For this determination, we found it necessary to lower the concentration of alcohol from 5% to 3% in order to discern the apoptotic vs. necrotic (general) causation of cellular damage/cell death. As shown in Figure 1B, gastric endothelial cells sustained a greater than 2.5-fold ($P<0.001$) increase in apoptosis vs. gastric epithelial cells upon exposure to 3% ethanol for 3 hours.

We next examined total survivin protein expression levels. As shown in Figure 2A, gastric epithelial cells expressed 7.5-fold ($P<0.0001$) greater survivin levels vs. the gastric endothelial cells. Moreover, as shown in Figure 2B, ethanol treatment affected intracellular survivin localization in the gastric epithelial cells, in which survivin was present in both the nucleus and cytoplasm of epithelial cells exposed to ethanol whereas in control cells not treated with ethanol, survivin was primarily localized to the nucleus. In contrast, ethanol exposure failed to appreciably affect a redistribution of survivin in the gastric endothelial cells and survivin remained primarily, although not exclusively, in the nuclei of both ethanol-treated endothelial cells and non-treated control cells (Figure 2B). We fully recognize that a phenotypic comparison between a cell line (RGM1) and isolated primary cells (RGMEC) presents limitations with regard to outcome interpretation; and, as such, caution in over-extrapolating our presented results is warranted. Nevertheless, a comparison between survivin protein expression levels of an extract of the RGM1 cells and a protein extract of partially-purified primary gastric mucosal epithelial cells demonstrated that the differential survivin expression between RGM1 and RGMEC is not likely to be a predominant result of cell line establishment (Figure 2C). In addition, although clearly the RGMEC cells are more susceptible to both total cellular damage (as evidenced by LDH release) and apoptosis (as evidenced by TUNEL assay) compared to the RGM1 cells even in the absence of alcohol exposure, the cell culture conditions imposed were not shown to create an a priori increase in two prominent apoptosis effectors, caspase-9 and caspase-3, in the RGMEC vs. RGM1 cells (Figure 2D). However, following exposure to 3% ethanol, there was demonstrably greater cleavage (an indicator of activation) of caspase-9, and an even more substantive cleavage of caspase-3, in the RGMEC vs. RGM1 cells (Figure 2D). It is our contention that the differential survivin expression levels between the RGM1 and RGMEC cells accounts, at least in part, for this finding.

To determine whether the difference in degree of susceptibility to alcohol-induced damage and apoptosis of the gastric epithelial and endothelial cells is a resultant of the differential survivin expression levels, we first examined how injury susceptibility of the two cell types would compare following expression knockdown of survivin in the gastric epithelial cells. As shown in Figure 3A, using transient transfection of survivin-specific siRNA oligonucleotides, we were able to achieve a 72% ($P<0.0002$) reduction in survivin protein expression levels 48 hours post-transfection in the gastric epithelial cells, which approximated very closely the survivin expression levels of control RNA oligonucleotide-transfected gastric endothelial cells ($P=0.09$). Transfection with control oligonucleotides used to control for transfection efficiency and non-specific expression effects did not affect survivin expression levels (Figure 3A). Gastric epithelial cells transfected either with the survivin-specific siRNA oligonucleotides or control oligonucleotides, together with control RNA oligonucleotide-transfected gastric endothelial cells, were then treated 48 hours post-transfection with 5% ethanol for 3 hours and the extent of overall cell damage was compared. As shown in Figure 3B, gastric epithelial cells treated with survivin-specific siRNA oligonucleotides sustained overall cell damage by exposure to 5% ethanol for 3 hours, which was similar within significance to the damage sustained by the control RNA oligonucleotide-transfected gastric endothelial cells treated with 5% ethanol for 3 hours (51% ± 9% vs. 67% ± 8% for siRNA-treated epithelial vs. control RNA-treated endothelial cells, $P=0.07$). We then examined the extent of ethanol-induced apoptosis by TUNEL assay. We, again, lowered the concentration of alcohol from 5% to 3% in order to discern the apoptotic vs. necrotic (general) causation of cellular damage/cell death. As shown in Figure 3C, gastric epithelial cells treated with survivin-specific siRNA oligonucleotides also had increased apoptosis vs. epithelial cells treated with the control oligonucleotides upon exposure to 3% ethanol for 3 hours, which was similar within significance to the gastric endothelial cells transfected with control RNA (27 ± 4% vs. 31 ± 5% for siRNA-treated epithelial vs. control RNA-treated endothelial cells, $P=0.06$).

To further verify that survivin is likely a key factor in the differential susceptibility to alcohol-induced injury of the gastric epithelial vs. endothelial cells, we transiently transfected the gastric endothelial cells with an expression plasmid harboring cDNA encoding full-length (142 amino acids) or "wild-type" survivin. As shown in Figure 4A, gastric endothelial cells transfected with the survivin plasmid construct showed similar survivin protein expression levels, 48 hours post-transfection, to gastric epithelial cells transiently transfected with an "empty vector" control plasmid not harboring survivin cDNA. Gastric endothelial cells transiently transfected with the empty vector control also did not have significantly increased or altered survivin protein expression levels (Figure 4A). To test the effect of increased survivin expression levels on the susceptibility of the transfected gastric endothelial cells to alcohol-induced damage, 48 hours post-transfection we exposed endothelial cells transfected with either the survivin expression plasmid or control plasmid, together with control plasmid-transfected gastric epithelial cells, to 5% ethanol and assessed damage by LDH release at 3 hours. As shown in Figure 4B, gastric endothelial cells transfected with the survivin expression plasmid had a similar extent of damage vs. gastric epithelial cells transfected with the control plasmid, which were both significantly less than the extent of damage sustained by the endothelial cells transfected with the control plasmid (36% ±5% vs. 25% ± 7%, P=0.08 for endothelial cells transfected with the survivin expression plasmid vs. control plasmid-transfected epithelial cells; 52% ± 8% for control plasmid-transfected gastric endothelial cells, P<0.04 vs. survivin expression plasmid-transfected endothelial cells). We also examined the effect of increased survivin expression levels on the susceptibility of the transfected gastric endothelial cells to alcohol-induced apoptosis. As shown in Figure 4C, following exposure to 3% ethanol for 3 hours, to discern the apoptosis from more general necrosis, gastric endothelial cells transfected with the control plasmid showed significantly increased apoptosis vs. endothelial cells transfected with the survivin expression plasmid, which was not significantly greater than the apoptosis of the gastric epithelial cells transfected with the control plasmid (23% ± 10% vs. 32% ± 5%, P<0.05 for endothelial cells transfected with the survivin expression plasmid vs. control plasmid-transfected endothelial cells; 15% ± 7% for control plasmid-transfected gastric epithelial cells, P=0.06 vs. survivin expression plasmid-transfected endothelial cells). It should be noted, however, that while the apoptosis indices were statistically comparable between the survivin-transfected RGMEC cells and the control plasmid-transfected RGM1 cells, the remaining high level of apoptosis observed in the transfected RGMEC cells under the serum-free conditions imposed by the study design indicates that survivin expression alone cannot completely confer gastric cellular protection.

We previously demonstrated that gastric epithelial cells transfected with a threonine-34 to glutamate-34 (T34E) mutant survivin expression plasmid construct were significantly more resistant to alcohol-induced cell damage compared to epithelial cells transfected with the wild-type survivin expression plasmid (15). We, therefore, examined whether gastric endothelial cells overexpressing the T34E mutant form of survivin would be more resistant to alcohol-induced damage compared to gastric epithelial cells transfected with the control plasmid. As shown in Figure 4A, gastric endothelial cells transiently transfected with the T34E mutant survivin plasmid construct showed moderate to high expression levels of the tagged T34E mutant form of survivin at 48 hours post-transfection. To test the effect of the T34E mutant form of survivin on susceptibility of the gastric endothelial cells to alcohol-induced damage, we exposed endothelial cells transfected with either the T34E survivin expression plasmid or control plasmid, together with control plasmid-transfected gastric epithelial cells, to 5% ethanol and assessed damage following 3 hours. As shown in Figure 4B, gastric endothelial cells expressing the T34E mutant form of survivin were moderately but significantly more resistant to overall alcohol-induced cell damage vs. the control plasmid-transfected gastric epithelial cells (14% ± 2% vs. 25% ± 7% P<0.02 for endothelial cells transfected with the T34E mutant survivin expression plasmid vs. control plasmid-transfected epithelial cells; 52% ± 8% for control plasmid-transfected gastric endothelial cells, P<0.001 vs. T34E mutant survivin

expression plasmid-transfected endothelial cells). Additionally, we examined the effect of the T34E mutant form of survivin on the susceptibility of gastric endothelial cells to alcohol-induced apoptosis. As shown in Figure 4C, following exposure to 3% ethanol (to discern apoptosis vs. necrosis) for 3 hours, gastric endothelial cells transfected with the T34E survivin expression plasmid showed significantly reduced apoptosis vs. both the gastric epithelial cells transfected with the control plasmid and the control plasmid-transfected endothelial cells (11% ± 2% vs. 15% ± 7%, $P<0.02$ for endothelial cells transfected with the T34E survivin expression plasmid vs. control plasmid-transfected epithelial cells; 32% ± 5% for control plasmid-transfected gastric endothelial cells, $P=0.0001$ vs. T34E survivin expression plasmid-transfected endothelial cells).

Finally, in light of our recent findings regarding the role of survivin in gastric epithelial cell cytoprotection (15), and the present findings indicating that a similar cytoprotective role of survivin is not available to gastric endothelial cells, we investigated the extent of gastric mucosal cytoprotection produced by intravenous vs. intragastric ethanol administration. As shown in Figure 5A, while intragastric administration of mild-irritant (21% v/v) ethanol provided greater than 90% ($P<0.0001$) cytoprotection against subsequent challenge with concentrated (50% v/v) ethanol, intravenous pretreatment with ethanol, corresponding to the same intragastric mild irritant concentration, was without affect against subsequent concentrated ethanol challenge. Moreover, pretreatment with low-dose ethanol i.g. alone induced a significant 1.5-fold ($P<0.005$) increase in gastric mucosal survivin levels whereas pretreatment with ethanol i.v. did not (Figure 5B). It should be noted that gastric survivin levels significantly increased within 3 hours of 50% ethanol administration i.g. regardless of the pretreatment given (Figure 5B). It should also be noted, however, that we avoided taking necrotic tissue areas for the homogenates used to assess survivin protein expression levels by immunoblot analysis. This likely contributed to our lack of obtaining statistical significance in survivin expression levels following 50% ethanol administration between control pretreatment and intravenous and intragastric mild-irritant ethanol pretreatment. To address the apparent discrepancy regarding cause and effect of survivin expression levels between intragastric and intravenous ethanol pretreatment and survivin levels at 3 hours post 50% ethanol administration, we examined survivin expression immunohistochemically. As shown in Figure 5C, survivin expression was demonstrably increased within 30 minutes of intragastric administration of 21% (v/v) ethanol whereas intravenous administration of ethanol, corresponding to the same intragastric mild irritant concentration, did not result in a demonstrable alteration in survivin expression levels at 30 minutes compared to controls. However, in agreement with the immunoblot analysis (Figure 5B), at 3 hours following 50% ethanol administration, survivin levels were increased above baseline regardless of pretreatment (Figure 5C). Importantly with respect to cytoprotection, gastric injury was already evident while survivin levels were not demonstrably altered compared to controls at 30 minutes following 50% ethanol administration in contrast to 30 minutes following intragastric administration of 21% ethanol (Figure 5C). Insofar as much has been already established regarding the mechanisms of mild-irritant-induced gastric cytoprotection including: increased endogenous prostaglandin synthesis, nitric oxide release, vagal innervation, sensory nerves, blood flow, Ca^{2+} influx, heat shock proteins and a physical barrier resulting from mucosal surface exfoliation (23–31), we examined heat shock protein expression in the gastric mucosa following intragastric administration of 21% ethanol in vivo. As shown in Figure 5D, intragastric administration of 21% ethanol failed to affect a significant alteration in the expression levels of either Hsp90 or Hsp27 compared to baseline controls.

Discussion

The gastric mucosa is particularly susceptible to injury, which requires a rapid cellular turnover rate second only to the skin (32). The gastric epithelium creates a barrier responsible for

protecting underlying structures, notably the gastric mucosal microvasculature, from noxious agents including alcohol. Nevertheless, the endothelial cells of the gastric mucosal microvessels are a primary target of alcohol-induced gastric damage, which results in deep hemorrhagic necrosis (3). Survivin plays a dual function during development as both a regulator of cellular proliferation and apoptosis (33). Although highly expressed in most, if not all, forms of human cancer, survivin ceases to be appreciably expressed in the majority of normal differentiated tissues making it an attractive target for therapeutic intervention (34). However in organ tissues such as the gastric mucosa, survivin may play an important protective regulatory role that extends beyond development and helps to maintain tissue integrity and to promote restitution following injury. We previously demonstrated that survivin plays a role in mediating gastric epithelial cell cytoprotection against alcohol-induced injury (15). Interestingly, however, although survivin is strongly expressed during development in endothelial cells of the vasculature and microvasculature, endothelial cells of the differentiated gastric mucosa do not appreciably express survivin (14).

In the present study, we have quantified the differential expression levels of survivin between gastric mucosal epithelial and endothelial cells. The observed partial redistribution of survivin from the nucleus to the cytoplasm in the gastric epithelial cells exposed to ethanol may be of importance since studies have shown that nuclear export is necessary for the cytoprotective function of survivin (35,36). We further established that the significantly greater expression level of survivin in gastric epithelial vs. endothelial cells likely plays an important role in the increased resistance to both alcohol-induced damage and apoptosis of the gastric epithelial cells. The evidence for this is three-fold: 1) knockdown of survivin expression in gastric epithelial cells via siRNA increased susceptibility to alcohol-induced damage and apoptosis to a statistically comparable extent as that obtained from control RNA-treated gastric endothelial cells; 2) forced overexpression of wild-type survivin in gastric endothelial cells via transient transfection increased resistance of the transfected endothelial cells against alcohol-induced damage and apoptosis, which were both statistically comparable to mock-transfected gastric epithelial cells; 3) forced overexpression of a T34E phosphorylation mimic survivin mutant in gastric endothelial cells significantly increased resistance of the transfected endothelial cells against alcohol-induced damage and apoptosis that was greater than the resistances obtained from mock-transfected gastric epithelial cells. The latter findings are in agreement with both our previous report and an additional recent report indicating that phosphorylation of threonine 34 (or mimicking phosphorylation at this residue by substitution with a negatively charged amino acid such as glutamate) increases the cytoprotective function of survivin (15,37). It is noteworthy that, although the T34E mutant form of survivin was found to increase the cytoprotective function, expression of T34E survivin led to reduced cellular proliferation when compared to cells expressing the non-phosphorylatable T34A mutant form of survivin (37). We have observed that phosphorylation of threonine-34, leading to accumulation of gastric survivin expression levels, is an early event following administration of “mild” alcohol concentrations (15). Taken together, these findings indicate that survivin likely plays dual roles in protecting against gastric injury, or at least regulating the extent of injury, and in promoting gastric mucosal injury healing, which may be governed, respectively, by the phosphorylation state of threonine-34. The findings presented here also suggest, however, that these roles of survivin do not apply to the endothelial cells of the gastric mucosal microvasculature.

Moreover, in the present study we have also demonstrated that “adaptive cytoprotection” is only manifested by direct exposure of the gastric mucosa to mild irritant alcohol concentrations. Intravenous administration of the theoretically equal mild irritant ethanol concentration failed to elicit cytoprotection against subsequent intragastric challenge with concentrated ethanol. Nor was intravenous administration of mild irritant ethanol accompanied by increased gastric survivin expression levels within 30 minutes as was found to be the case for intragastric mild

irritant ethanol administration. Furthermore, as with intravenous administration of mild irritant ethanol, survivin levels remained unaltered within 30 minutes of intragastric administration of concentrated (50%) alcohol. By contrast, administration of concentrated ethanol intragastrically resulted in significantly increased survivin levels by 3 hours irrespective of the pretreatment condition. In this regard, we previously reported that administration of 50% ethanol in the absence of cytoprotective ethanol pre-administration resulted in significantly increased total survivin protein expression levels in the gastric mucosa immediately bordering necrotic lesions at 3 hours and speculated that increased survivin may also play an important general role in regulating the extent of gastric injury, or may possibly play a role in gastric injury healing, in addition to its role in mediating cytoprotection against gastric injury (15). We are currently investigating these latter possibilities. Nevertheless, such roles, if found, will likely be distinct from the role of survivin in gastric cytoprotection since significant cytoprotection was only obtained 30 minutes following intragastric administration of mild irritant alcohol, which was also the only pretreatment or treatment condition accompanied by an early increase in survivin protein expression levels. Although it is fully acknowledged that many other factors have been shown to participate in gastric cytoprotection (as mentioned), under the cytoprotection pretreatment condition employed in the present study a generalized alteration in protein expression levels was not obtained as evidenced by the lack of altered expression of either Hsp90 or Hsp27. While this finding neither diminishes the importance of factors found previously to be important for gastric cytoprotection nor supports an absolute required role for survivin in gastric cytoprotection, the findings of our present study, taken together with those of our previous study (15), do lend further credence to survivin playing *an* important role in gastric cytoprotection.

Whether the lack of survivin-based endothelial cell protection is particular to the gastric mucosal microvasculature is, at present, uncertain. Survivin has, for example, been shown to play a cytoprotective role in endothelial cells of the brain, skin and myocardium following ischemic preconditioning (38–40). Nevertheless, the differential expression levels of survivin demonstrated in the present study for gastric epithelial vs. endothelial cells, together with the demonstrated corresponding relationships between survivin expression levels and resistance/susceptibility to injury and apoptosis, can explain, in part, why gastric endothelial cells are a primary target of alcohol-induced gastric damage.

The roles of survivin in development and tumorigenesis have been, and continue to be, extensively studied. In addition to our present and previous studies, however, other investigations have uncovered plausible roles for survivin in regulating the homeostasis of at least some organ tissues (41–43). It is our contention that, although highly promising, the development of survivin-targeted therapeutic modalities for treating cancer and other diseases should include consideration of possible deleterious effects that universal targeting may have on the physiological roles of survivin.

Acknowledgments

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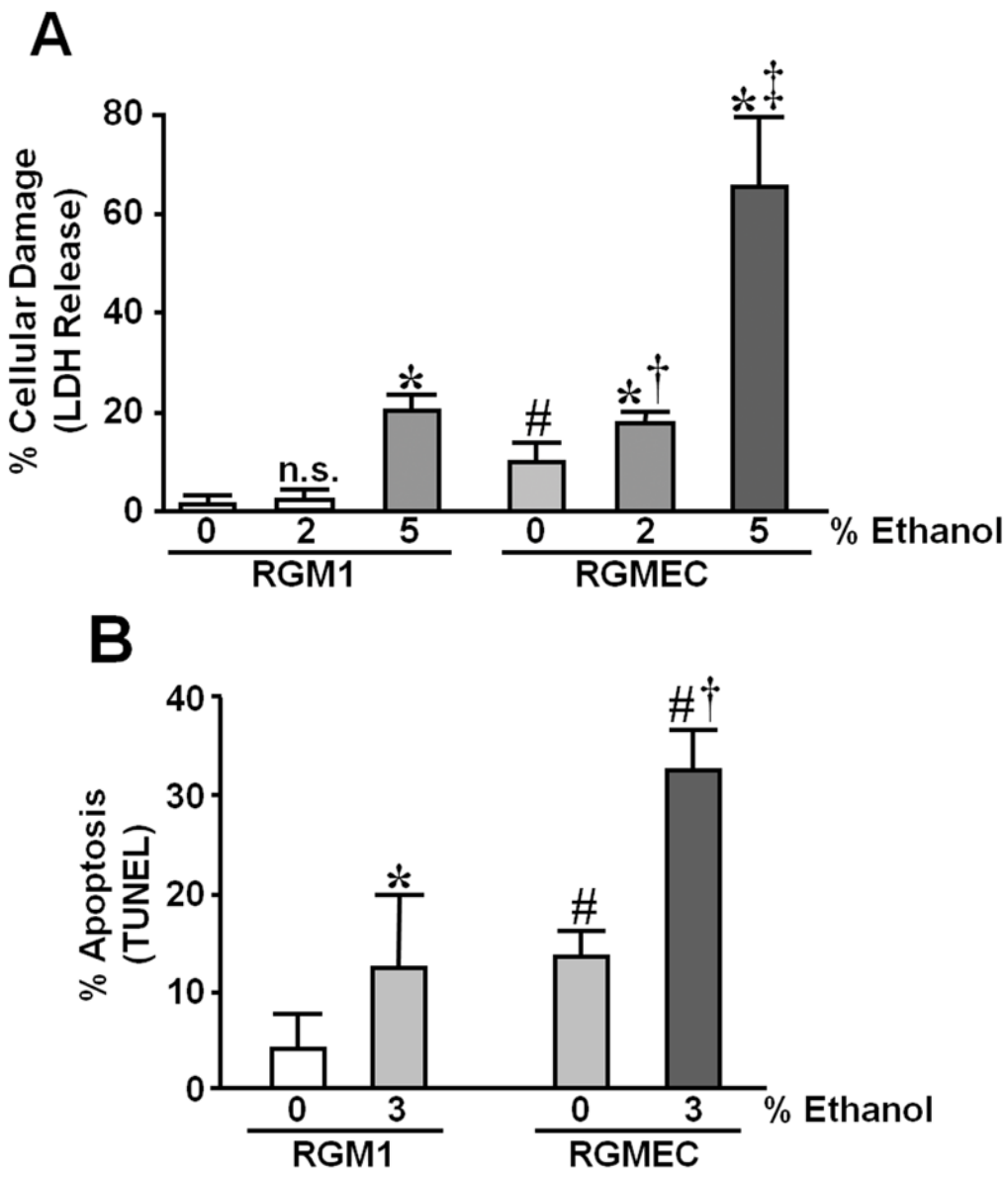
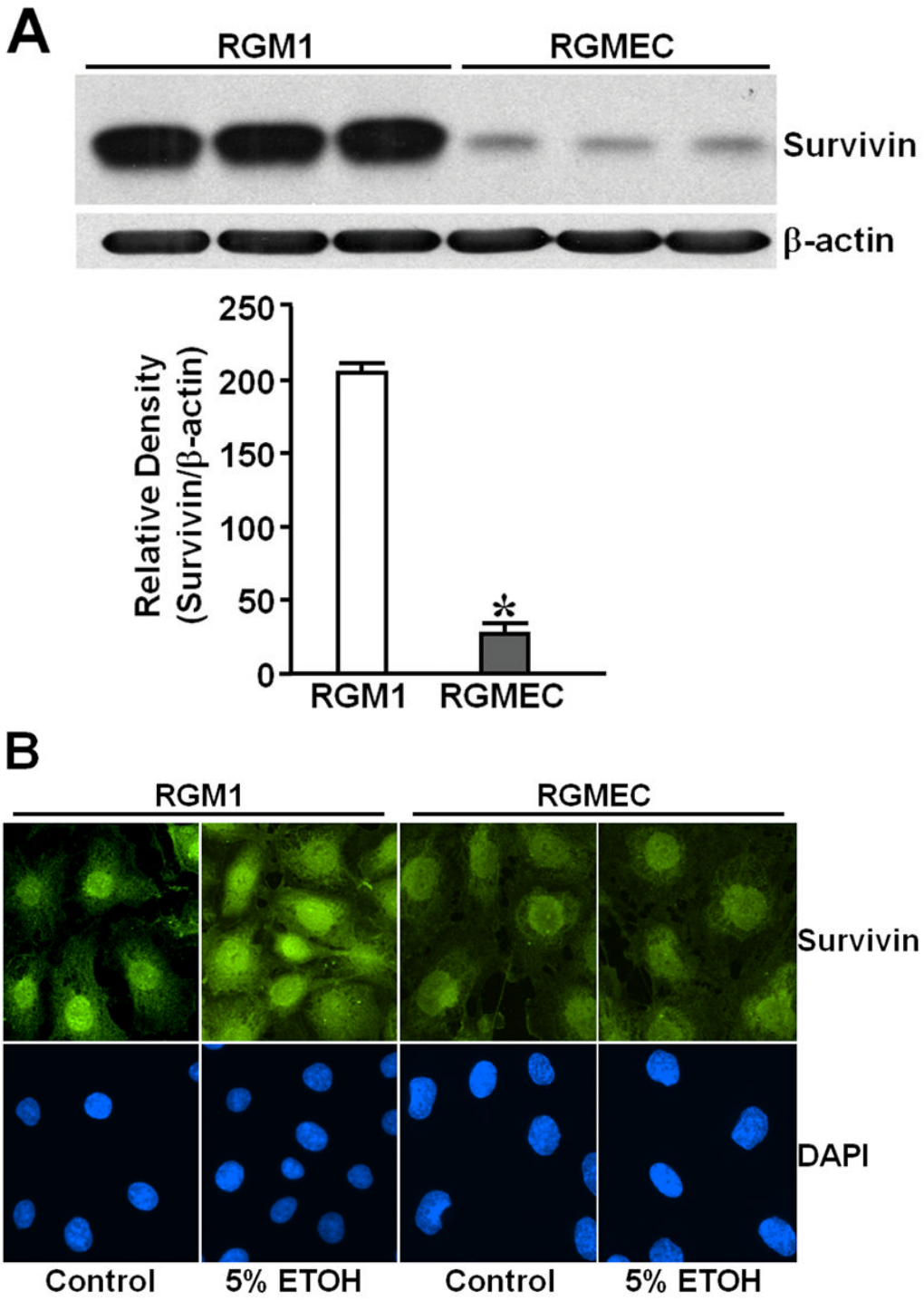
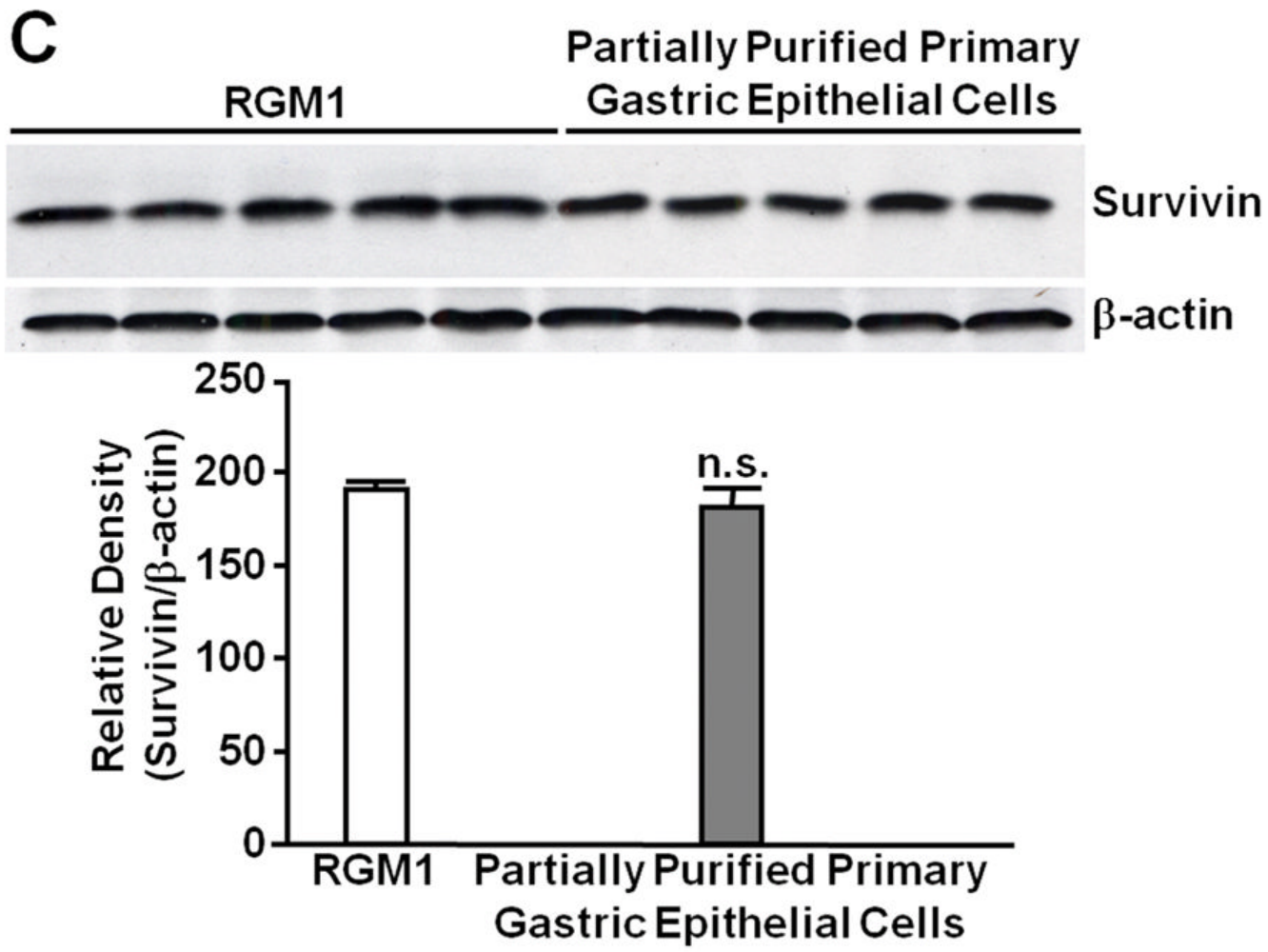


Figure 1.

Gastric epithelial cells are significantly more resistant to both alcohol-induced cellular damage and alcohol-induced apoptosis compared to gastric endothelial cells. **(A)** Serum-starved rat gastric mucosal epithelial cells (RGM1) and primary rat gastric endothelial cells (RGMEC) were exposed to the indicated concentration of ethanol in serum-free medium for 3 hours. Cellular damage was determined by lactate dehydrogenase (LDH) release and quantified as described in Materials and Methods. Values are expressed as mean percent ± SD. **P*<0.005 vs. control RGM1 cells not exposed to alcohol; $^{\#}P$<0.02 vs. control RGM1 cells not exposed to alcohol; $^{\dagger}P$<0.05 vs. control RGMEC cells not exposed to alcohol; $^{\ddagger}P$<0.005 vs. RGMEC cells exposed to 2% alcohol; n.s. not significant. **(B)** Serum-starved RGM1 and RGMEC cells grown on sterile glass microscope slides were exposed to the indicated concentration of ethanol in serum-free medium for 3 hours. Extent of apoptosis was determined by TUNEL assay and quantified as described in Materials and Methods. Values are expressed as mean percent ± SD. **P*<0.05 vs. control RGM1 cells not exposed to alcohol; $^{\#}P$<0.0005 vs. control RGM1 cells

not exposed to alcohol; †$P<0.0001$ vs. RGM1 cells exposed to 3% ethanol and control RGMEC cells not exposed to alcohol. All experiments were performed three independent times; and, for each experiment, the incubations were performed in triplicate.





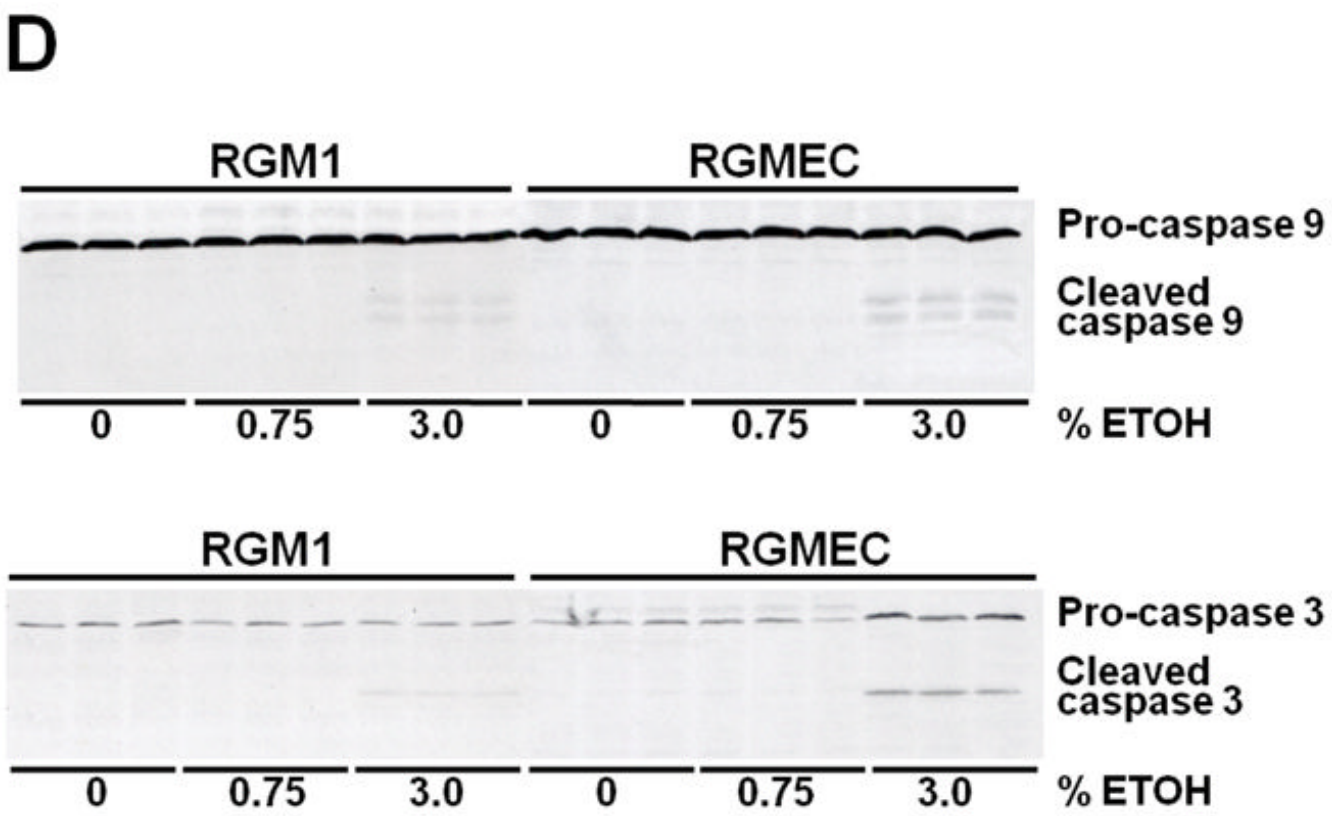
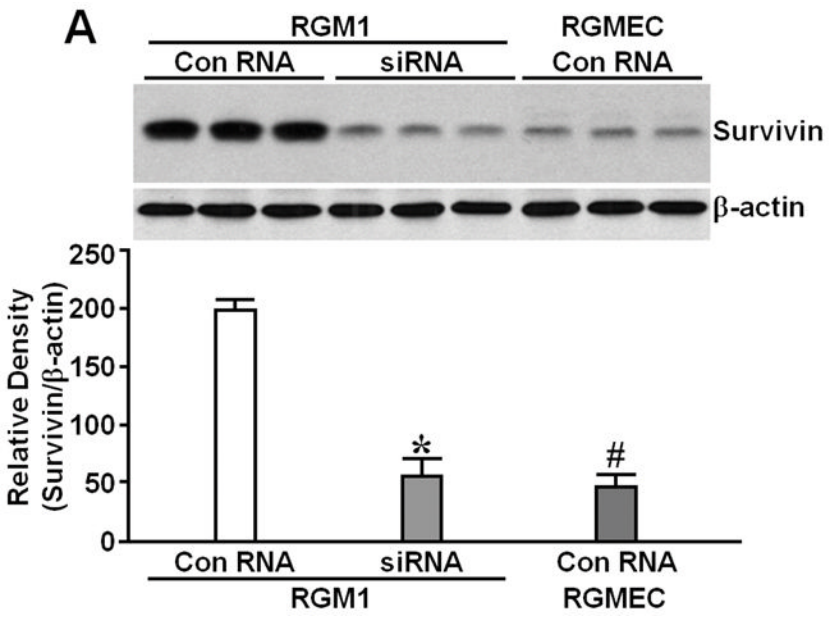
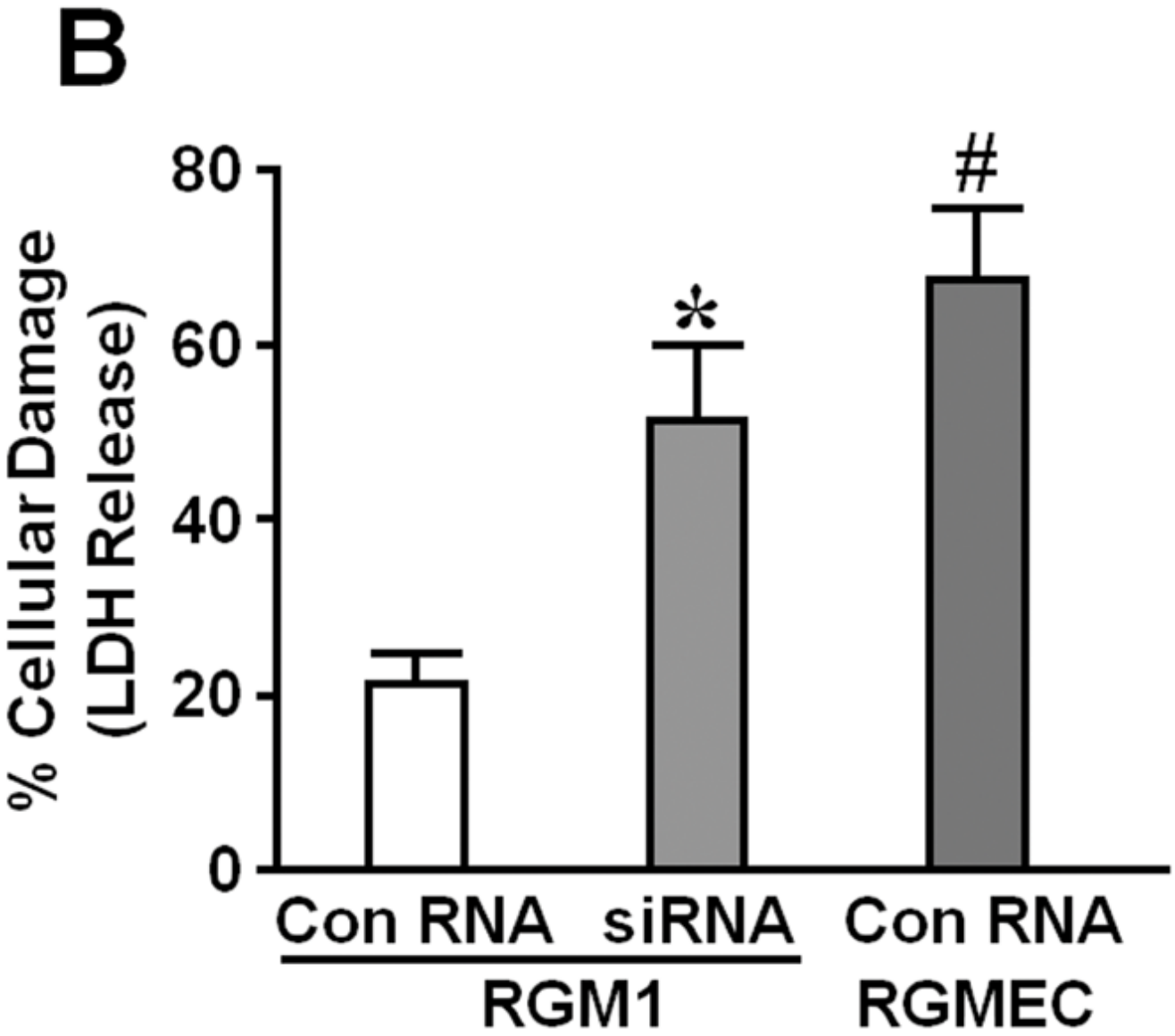


Figure 2.

Survivin protein expression levels are significantly greater in gastric epithelial cells compared to gastric endothelial cells and survivin protein undergoes intracellular redistribution in gastric epithelial cells upon exposure to ethanol. **(A)** Total survivin protein expression levels were determined by immunoblot analysis of whole cell lysates from serum-starved RGM1 and RGMEC cells as described in Materials and Methods. Upper panel shows survivin in a representative blot of three independent lysates from RGM1 and three independent lysates from RGMEC. Lower panel shows reprobing of the same blot for β-actin as an internal control for protein loading and transfer. Bottom graph shows densitometric quantification of the survivin-to-β-actin signal ratio for each lane. Values are expressed as mean ratio ± SD. **P*<0.0001 vs. survivin-to-β-actin signal ratios obtained for RGM1. **(B)** Serum-starved RGM1 and RGMEC cells were exposed for 30 minutes to serum-free medium containing either 0% (as control) or 5% ethanol prior to paraformaldehyde fixation and immunofluorescence staining for survivin as described in Materials and Methods. DAPI counterstaining was used to visualize/localize cell nuclei. Upper panels show survivin expression/localization in representative cells both in the absence of alcohol exposure and following alcohol exposure. Each lower panel shows the nuclei of the same cells in the immediately adjacent upper panel. Magnification: 400x. **(C)** A partially purified fraction of primary gastric surface epithelial cells was obtained as described in Materials and Methods and whole cell lysates were used to assess relative expression levels of the primary cells compared to that of the RGM1 gastric mucosal epithelial cell line by immunoblot analysis as in (A). Upper panel shows survivin in a representative blot of five independent lysates from RGM1 and the lysates of five independent partially purified primary epithelial cell fractions obtained from five different stomachs. Lower panel shows reprobing of the same blot for β-actin as an internal control for protein loading and transfer. Bottom graph shows densitometric quantification of the survivin-to-β-actin signal ratio for each lane. Values are expressed as mean ratio ± SD. n.s. not significantly different

from RGM1 survivin expression levels (P=0.07). **(D)** A comparison of expression levels and cleavage (a measure of activation state) of two prominent apoptosis effectors, caspase 9 and caspase 3, between RGM1 cells and RGMEC cultured under normal growth conditions (control) or in the presence of “mild irritant” 0.75% ethanol (ETOH) or 3% ETOH used to assess apoptosis susceptibility was determined by immunoblot analysis as described in Materials and Methods. All determinations were performed in triplicate, each from a minimum of three independent experiments.





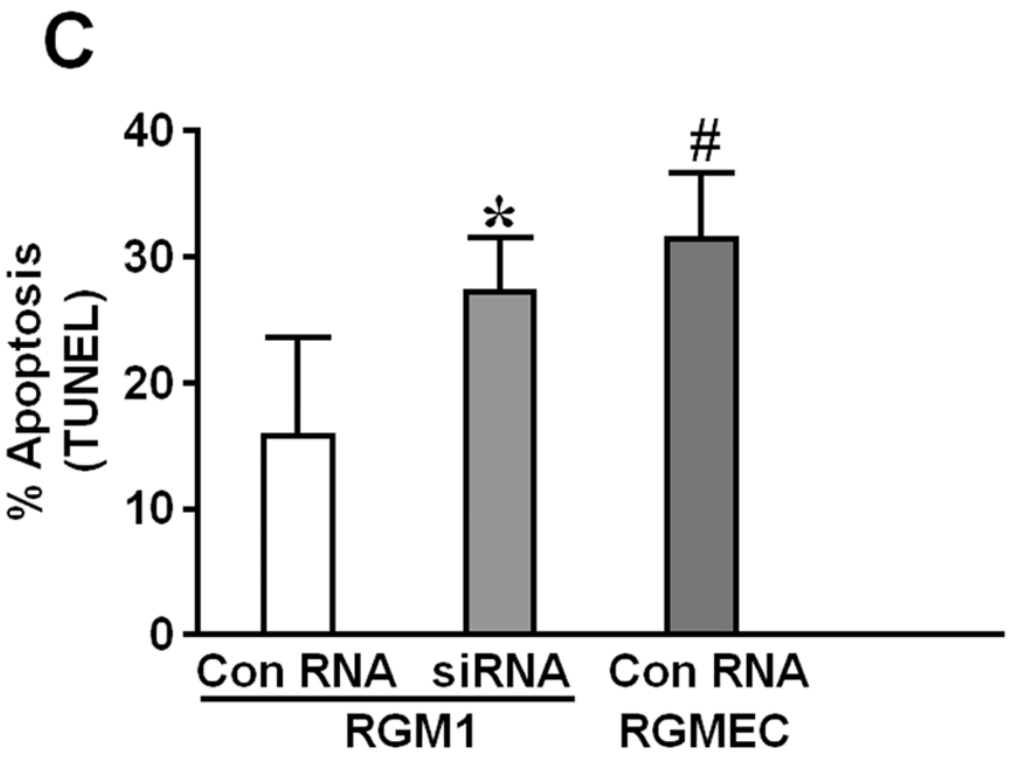
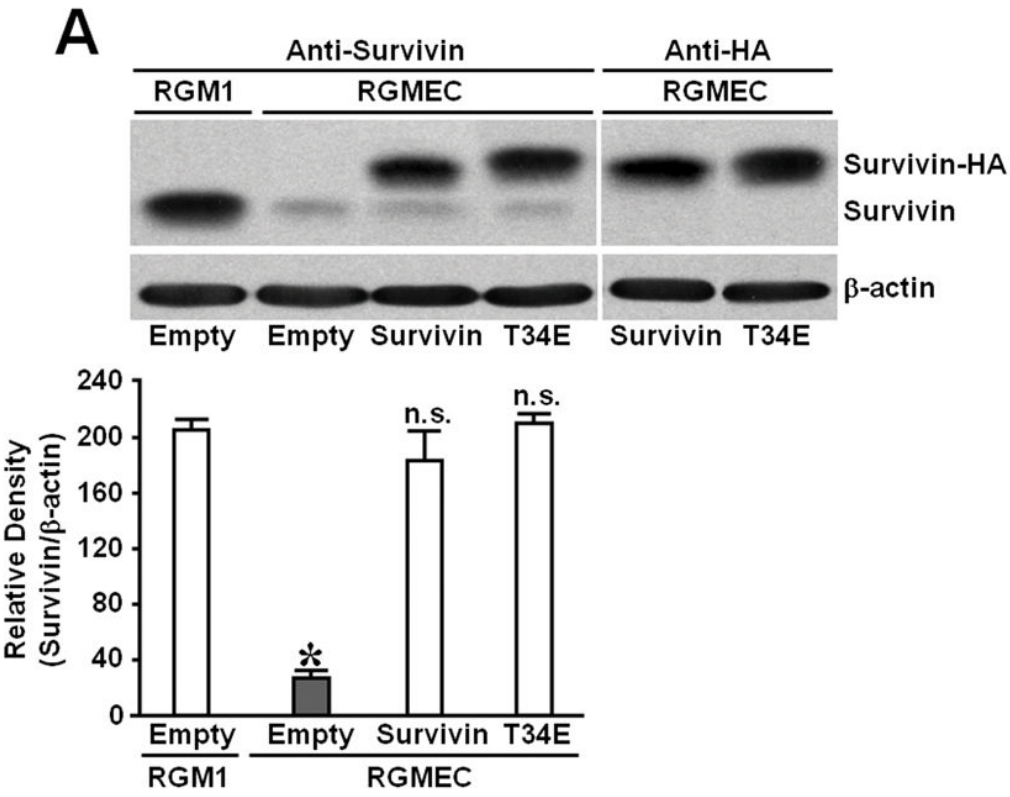


Figure 3.

Suppression of survivin protein expression levels renders gastric epithelial cells as susceptible to both alcohol-induced cell damage and apoptosis as gastric endothelial cells. **(A)** RGM1 and RGMEC cells were transfected with control RNA oligonucleotides or survivin specific-siRNA oligonucleotides followed by recovery for 24 hours in complete growth medium. The medium was replaced with serum-free medium and the transfected cells were serum-starved for 24 hours. Total survivin protein expression levels were determined by immunoblot analysis of whole cell lysates as in Figure 2A. Upper panel shows survivin in a representative blot of three independent lysates from RGM1 transfected with control RNA oligonucleotides (Con RNA); three independent lysates from RGM1 transfected with survivin-specific siRNA oligonucleotides (siRNA); and, three independent lysates from RGMEC transfected with control RNA oligonucleotides (Con RNA). Lower panel shows reprobing of the same blot for β-actin as an internal control for protein loading and transfer. Bottom graph shows densitometric quantification of the survivin-to-β-actin signal ratio for each lane. Values are expressed as mean ratio ± SD. *P<0.0002 vs. survivin-to-β-actin signal ratios obtained for RGM1 transfected with control RNA oligonucleotides; $^{\#}P$=0.09 vs. survivin-to β-actin ratios obtained for RGM1 transfected with survivin-specific siRNA oligonucleotides. **(B)** Serum-starved RGM1 cells, transfected with control RNA oligonucleotides (Con RNA) or survivin-specific siRNA oligonucleotides (siRNA), and serum-starved RGMEC transfected with control RNA oligonucleotides (Con RNA), were exposed to 5% ethanol in serum-free medium for 3 hours. Cellular damage was determined by lactate dehydrogenase (LDH) release and quantified as described in Materials and Methods. Values are expressed as mean percent ± SD. *P<0.01 vs. RGM1 transfected with control RNA oligonucleotides; $^{\#}P$=0.07 vs. RGM1 transfected with survivin-specific siRNA oligonucleotides. **(C)** Serum-starved RGM1 cells, transfected with control RNA oligonucleotides (Con RNA) or survivin-specific siRNA oligonucleotides (siRNA), and serum-starved RGMEC transfected with control RNA oligonucleotides (Con RNA), were exposed to 3% ethanol in serum-free medium for 3 hours. Extent of apoptosis was determined by TUNEL assay and quantified as described in Materials

and Methods. Values are expressed as mean percent ± SD. *P<0.002 vs. RGM1 transfected with control RNA oligonucleotides; $^{\#}P$=0.06 vs. RGM1 transfected with survivin-specific siRNA oligonucleotides.



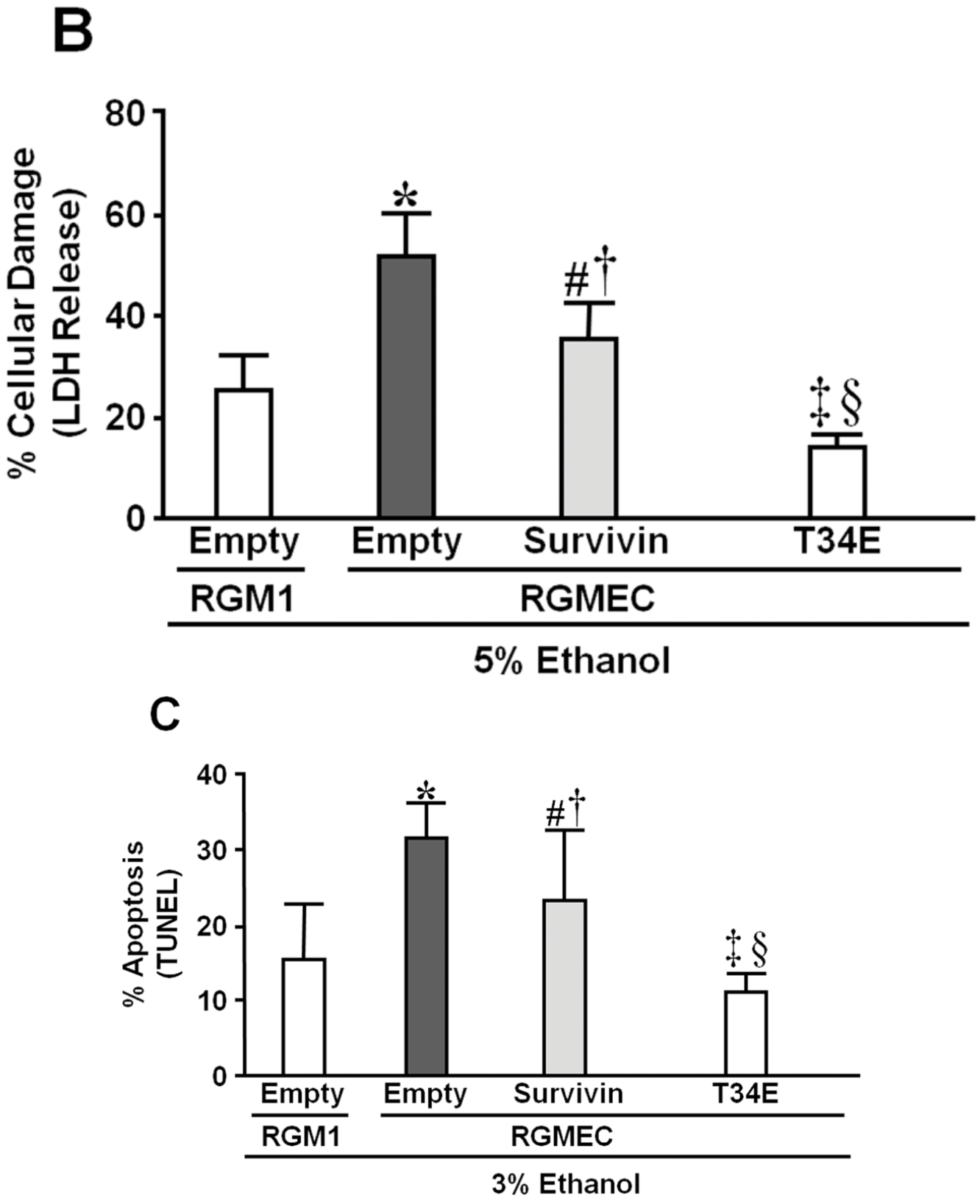
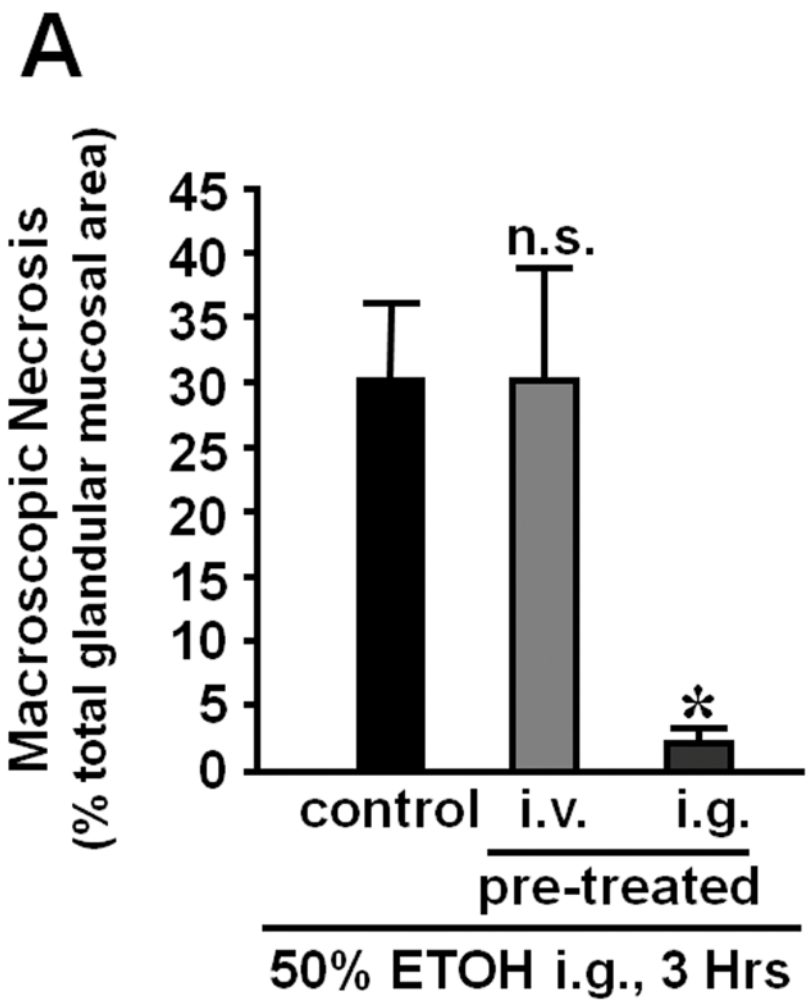
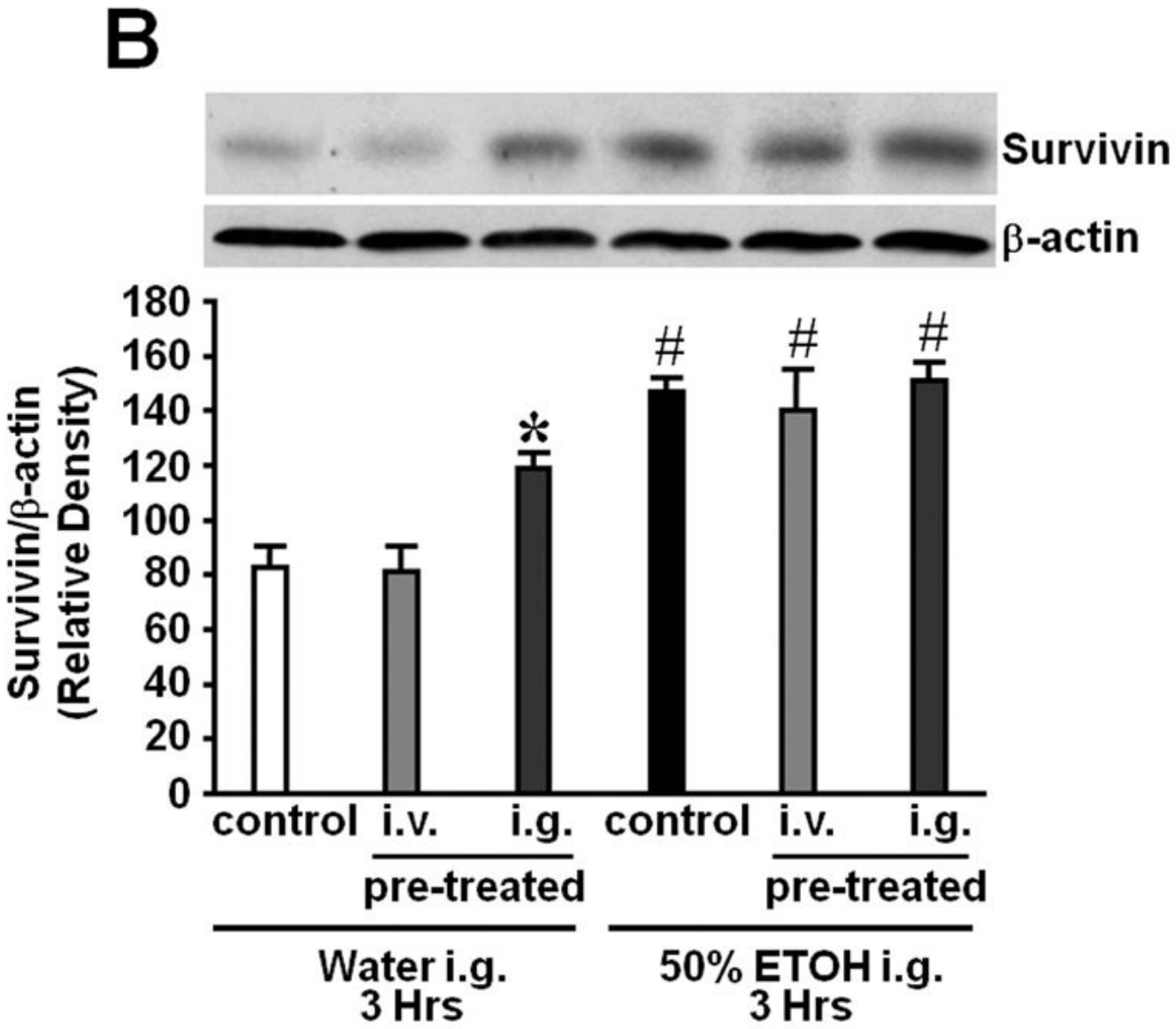
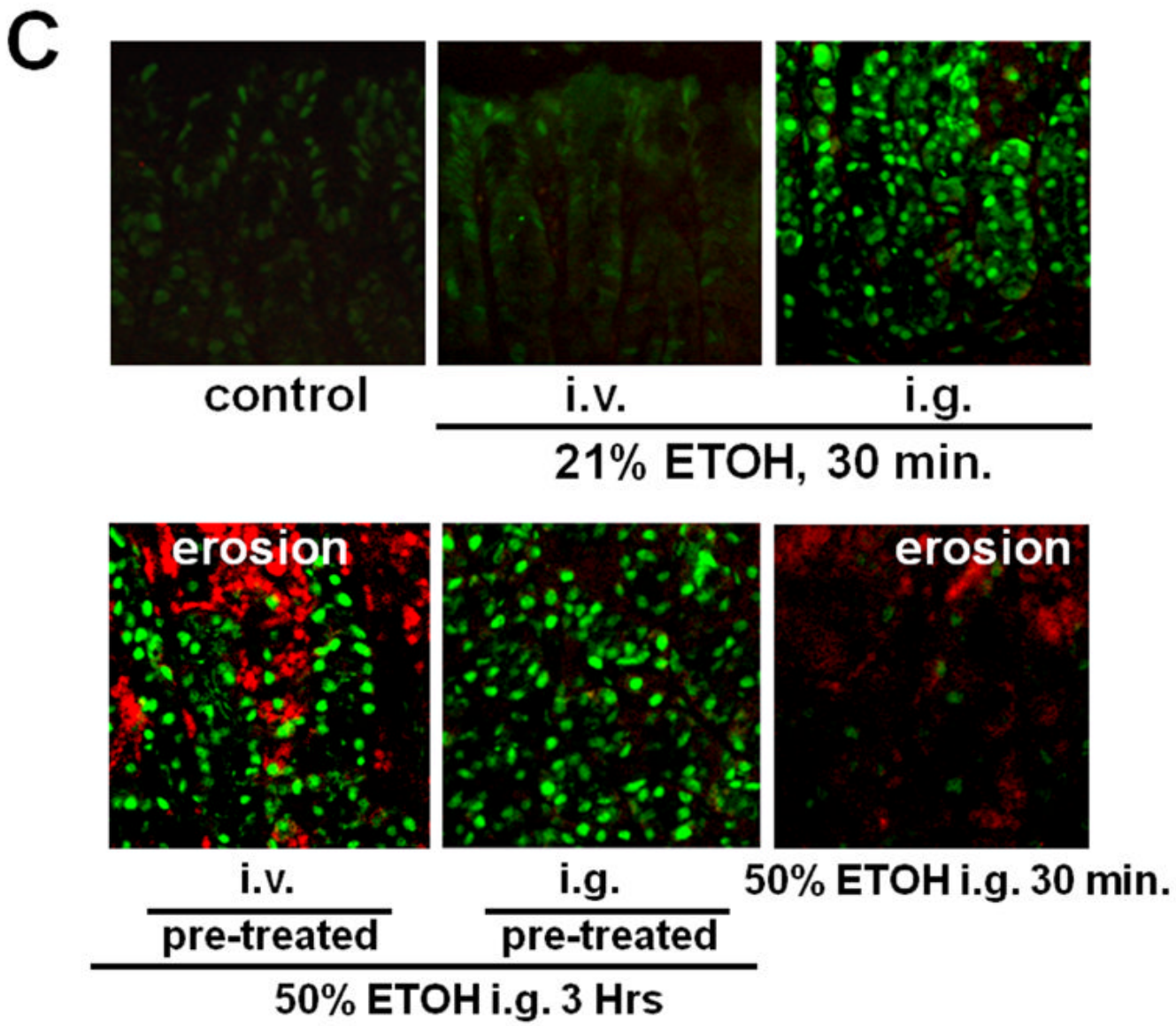


Figure 4.

Forced overexpression of survivin renders gastric endothelial cells as resistant to both alcohol-induced cell damage and apoptosis as gastric epithelial cells, while forced overexpression of T34E mutant survivin renders gastric endothelial cells significantly more resistant to both alcohol-induced cell damage and apoptosis compared to gastric epithelial cells. **(A)** RGMEC cells were transiently transfected with expression plasmids encoding either full-length wild-type survivin (Survivin) or mutant survivin in which threonine-34 was substituted with glutamate (T34E). Both RGMEC and RGM1 were also transiently transfected with plasmid lacking a coding sequence (Empty) to serve as controls. The cells were allowed to recover for 24 hours in complete growth medium followed by incubation in serum-free medium for an additional 24 hours. Endogenous and exogenous survivin protein expression levels were determined by immunoblot analysis of whole cell lysates as in Figures 2A and 3A. Upper panel shows endogenous survivin expression levels, as assessed by the same antibody used in Figures 2 and 3, as well as survivin expression levels from the transgenes, as assessed by an anti-HA antibody. Lower panel shows reprobing of the same blots for β-actin as an internal control for protein loading and transfer. Bottom graph shows densitometric quantification of the survivin-to-β-actin signal ratio for each lane. Values are expressed as mean ratio ± SD. $^{*}P<0.0001$ vs. RGM1 transfected with control "empty" plasmid; n.s. not significant vs. RGM1 transfected with control "empty" plasmid. **(B)** Serum-starved RGM1 and RGMEC cells, transfected with control "empty" plasmid, and serum-starved RGMEC transfected with wild-type or mutant survivin expression plasmids, were exposed to 5% ethanol in serum-free medium for 3 hours. Cellular damage was determined by lactate dehydrogenase (LDH) release and quantified as described in Materials and Methods. Values are expressed as mean percent ± SD. $^{*}P<0.005$ vs. RGM1 transfected with control "empty" plasmid; $^{\#}P=0.08$ vs. RGM1 transfected with control "empty" plasmid; $^{\dagger}P<0.04$ vs. RGMEC transfected with control "empty" plasmid; $^{\ddagger}P<0.02$ vs. RGM1 transfected with control "empty" plasmid; $^{\S}P<0.001$ vs. RGMEC transfected with control "empty" plasmid. **(C)** Serum-starved RGM1 and RGMEC cells, transfected with control "empty" plasmid, and serum-starved RGMEC transfected with wild-type or mutant survivin expression plasmids, were exposed to 3% ethanol in serum-free medium for 3 hours. Extent of apoptosis was determined by TUNEL assay and quantified as described in Materials and Methods. Values are expressed as mean percent ± SD. $^{*}P<0.001$ vs. RGM1 transfected with control "empty" plasmid; $^{\#}P=0.06$ vs. RGM1 transfected with control "empty" plasmid; $^{\dagger}P<0.05$ vs. RGMEC transfected with control "empty" plasmid; $^{\ddagger}P<0.02$ vs. RGM1 transfected with control "empty" plasmid; $^{\S}P<0.0001$ vs. RGMEC transfected with control "empty" plasmid.







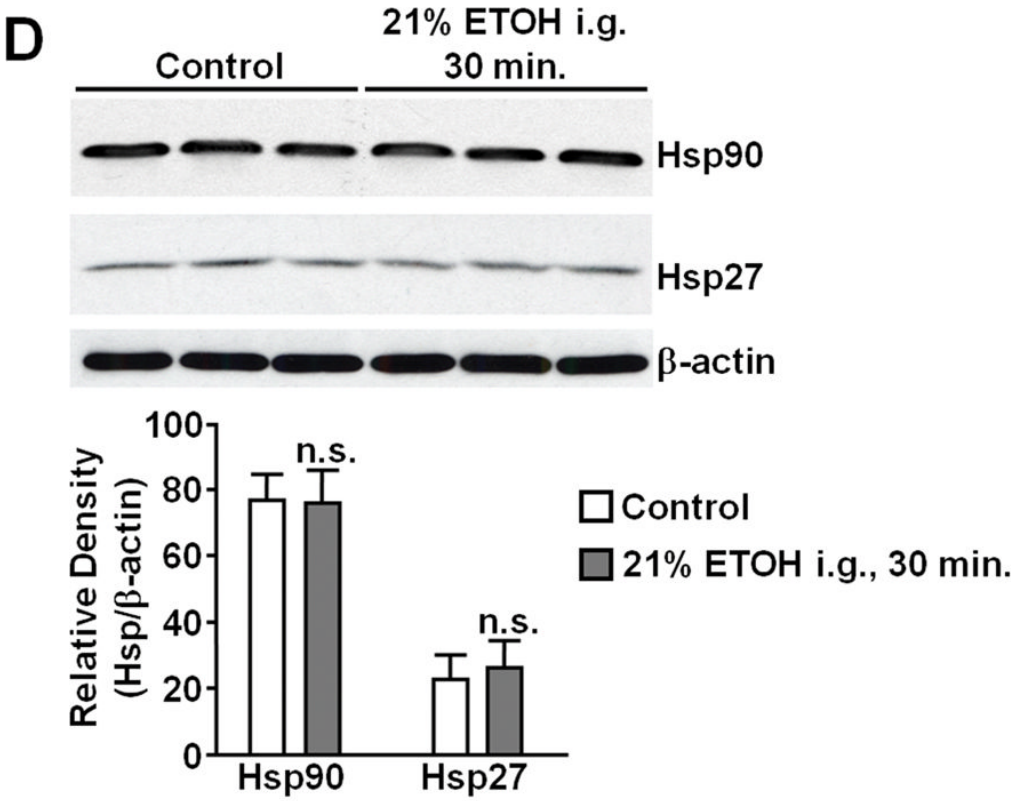


Figure 5.

Intragastric administration, but not systemic intravenous administration, of mild-irritant alcohol elicits significant cytoprotection against subsequent exposure to concentrated alcohol. **(A)** Rats (n=8/group) were administered water (as a control for both the possible protective physical effect provided by mere gastric "filling;" and, the possible injurious effect of the gavaging method of alcohol administration), 21% ethanol intragastrically or 74 mg/dl (total blood volume) ethanol intravenously. Thirty minutes later all rats were administered 50% ethanol intragastrically. Stomachs were harvested 3 hours later and assessed for macroscopic necrosis as described in Materials and Methods. Results are expressed as mean percent of total glandular mucosal area ± SD. **P*<0.0001 vs. macroscopic necrosis of rats pre-administered water prior to administration of 50% ethanol; n.s. not significantly different from macroscopic necrosis of rats pre-administered water prior to administration of 50% ethanol. **(B)** Total survivin protein expression levels from gastric mucosal tissue homogenates obtained from stomachs harvested as described in (A) were assessed by immunoblot analysis. Upper panel shows survivin expression in a representative blot of gastric mucosal homogenates from stomachs harvested following the indicated pre-treatment and treatment conditions. Lower panel shows reprobing of the same blot for β-actin as an internal control for protein loading and transfer. Bottom graph shows densitometric quantification of the survivin-to-β-actin signal ratio for each lane. Values are expressed as mean ratio ± SD. **P*<0.001 vs. survivin-to-β-actin signal ratios obtained for gastric tissue homogenates from rats pre-administered water intragastrically (as a control for the possible protective physical effect provided by mere gastric "filling") 30 minutes prior to intragastric administration of water (as an additional control for the possible physical/mechanical injurious effect of the gavaging method of alcohol administration); $^{\#}P$<0.0001 vs. survivin-to-β-actin signal ratios obtained for gastric tissue homogenates from rats pre-administered water intragastrically 30 minutes prior to intragastric administration of water (control). **(C)** Immunofluorescence staining of formalin-fixed,

paraffin-embedded gastric tissue sections from rats administered: (upper left) water by intragastric gavage 30 minutes prior to stomach harvest (control); (upper middle) 21% ethanol (ETOH) by intravenous injection 30 minutes prior to stomach harvest; (upper right) 21% ETOH by intragastric gavage 30 minutes prior to stomach harvest; (lower left) 21% ETOH by intravenous injection 30 minutes prior to 50% ETOH administration by intragastric gavage followed by stomach harvest 3 hours later; (lower middle) 21% ETOH by intragastric gavage 30 minutes prior to 50% ETOH administration by intragastric gavage followed by stomach harvest 3 hours later; (lower right) 50% ETOH administration by intragastric gavage 30 minutes prior to stomach harvest. **(D)** Relative protein expression levels of two heat shock proteins (Hsp90 and Hsp27) associated with gastric cytoprotection and/or resistance to apoptosis. Rats (n=9/group) were administered water by intragastric gavage (control) or 21% ethanol by intragastric gavage 30 minutes prior to stomach harvest as described in Materials and Methods. Representative immunoblot of: (upper panel) Hsp90; (middle panel) Hsp27. Lower panel shows reprobing of the same blot for β-actin as an internal control for protein loading and transfer. Bottom graph shows densitometric quantification of the Hsp90- or Hsp27-to-β-actin signal ratio for each lane. Values are expressed as mean Hsp90- or Hsp27-to-β-actin signal ratios ± SD. n.s. not significantly different from Hsp90 (P=0.81) and Hsp27 (P=0.34) expression levels obtained from controls. Although the representative immunoblot depicts analyses of 3 rats/group, as a result of space constraints, the bottom graph includes quantification of all 9 rats/group examined.