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Supraphysiologic extracellular pressure inhibits intestinal epithelial wound healing independently of luminal nutrient flow

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Abstract

Introduction—Luminal pressure may injure the gut mucosa in obstruction, ileus, or IBD.

Methods—We formed Roux-en-Y anastomoses in 19 mice, creating proximal and defunctionalized partially obstructed limbs and a distal limb to vary luminal pressure and flow. We induced mucosal ulcers by serosal acetic acid, and **assessed** proliferation (PCNA) and ERK (immunoblotting). Parallel studies compared Caco-2 enterocyte migration and proliferation after pressure and/or ERK blockade.

Results—At 3 days, anastomoses were probe-patent, proximal and distal limbs contained chyme, and defunctionalized limbs were empty. The proximal and defunctionalized limbs exhibited increased pressure and slower healing despite increased proliferation, ERK protein and ERK activation. *In vitro*, pressure decreased Caco-2 migration across collagen or fibronectin, stimulated proliferation, and activated ERK. However, ERK blockade did not prevent pressure effects.

Conclusions—Luminal pressure during obstruction or ileus may impair mucosal healing independently of luminal flow despite increased mitosis and ERK activation.

Keywords

Force; mechanotransduction; migration; pressure; proliferation; Roux-en-y

Introduction

The intestinal mucosa experiences diverse forces in normal and diseased states. The small intestine mixes and propels the chyme by peristaltic and segmental contractions, pendular movements (1), and villous motility (2). The liquid luminal contents are largely non-compressible (3) and interact repetitively with the gut mucosa in complex ways as they pass along the villi (4). These forces all alter intra-luminal pressure, which along with other physiologic forces may support the normal gut mucosal cytoarchitecture (5).

However, supraphysiologic forces, such as elevated pressure, caused by diet or illness, may adversely impact gut physiology and mucosal healing (6). Luminal jejunal pressure reaches 50 mm Hg in irritable bowel syndrome (7). Intra-abdominal pressure may also increase after surgery due to tissue edema (8). Inflammation and injury increase luminal pressure in chronic

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inflammatory states such as Crohn's disease or ulcerative colitis (9–11). Such increases in pressure could affect wound healing (5,12).

The gut mucosa is constantly subjected to injuries that it must heal to maintain normal function (13) Biophysical forces in the gut stimulate intestinal epithelial proliferation and modulate intestinal epithelial differentiation in vitro (14), and activate mucosal tyrosine kinases in vivo (15) Mucosal repair is required for recovery from pathologic injury such as chronic ulceration and inflammation in inflammatory bowel disease, and is likely deficient when the mucosal barrier deteriorates in sepsis. (16,17) Mucosal healing is affected in many pathophysiologic states that exhibit altered luminal pressure. Sepsis, ileus, fasting, and inflammatory bowel disease may be associated with altered contractile rhythms, villous motility, and mucosal deformation from luminal contents with consequent changes in luminal pressure. Ulcerative colitis, for example, exhibits decreased contractility, increased low-amplitude propagating contractions, and variable transit times (18). Anastomoses rupture at lower pressures in a rat inflammatory bowel disease model, suggesting impaired healing (19).

In vitro, repetitive deformation promotes intestinal epithelial proliferation and differentiation when the enterocytes are cultured on collagen or laminin substrates (20), but inhibits proliferation and promotes epithelial sheet migration on fibronectin substrates (21). However, the effects of physical forces on the biology of the intestinal mucosa in vivo are less clear, although repetitive deformation stimulates mucosal tyrosine kinase activity in anesthetized rats (15). Moreover, pressure may affect intestinal epithelial cells differently than repetitive deformation (12). It therefore becomes important to understand how increased pressure might modulate mucosal healing during altered intestinal homeostasis. We created a murine model of partial bowel obstruction in a defunctionalized jejunal Roux-en-Y limb in which the effects of luminal pressure were dissociated from luminal contents, and compared mucosal healing, proliferation, and ERK signaling among the proximal partially obstructed bowel, the defunctionalized partially obstructed bowel, and the bowel distal to the partial obstruction. We studied ERK because it critically mediates the effects of repetitive deformation on intestinal epithelial cells in vitro. (21) We validated our observations in vitro by studying the effects of extracellular pressure on human Caco-2 intestinal epithelial migration, proliferation, and ERK activation, and blocked ERK with the MEK inhibitor PD98059. Caco-2 cells are a common model for human intestinal epithelial biology. (22)

MATERIALS AND METHODS

In Vivo Studies

Roux-en-Y anastomosis—We created defunctionalized Roux limbs in C57 Black mice by an approved protocol. We divided the jejunum 1 cm from the ligament of Treitz with 5-0 silk, anastomosed the proximal jejunum side to side to the distal gut 2 cm distal to the original transection with 9-0 vicryl, and closed the abdomen with running 5-0 silk.

Pressure Determination within the murine small bowel—We assessed luminal pressures with a Stryker 295-1 Pressure Monitor (Kalamazoo, MI) before and three days after defunctionalized limb creation. The pressures measured in the animal model using the Stryker needle were on the order of 0–6 mmHg. Preliminary calibration demonstrated the accuracy of the needle and pressure monitor, measured against a clinical ICU pressure transducer (not shown). These pressures were reproducible, but notably lower than those reported in human unobstructed or obstructed bowel. (9–11) These differences may reflect species differences or the use of the Stryker needle technique in which the fluid is injected and the pressure peaks briefly before the fluid runs away into the measured space. However, the constraints generated by the closed and intact abdominal wall may also contribute substantially to measurements of bowel pressures in intact humans, and were absent when we measured bowel pressure in the

mouse at necropsy. We therefore chose to apply a pressure of 80 mmHg to the human Caco-2 intestinal epithelial cells based upon literature reports of such pressures within the lumen of obstructed human bowel.

Mucosal ulcers—Paper disks pretreated with 70% acetic acid were applied to the small bowel serosa for 15 seconds to create circumscribed ischemic mucosal ulcers of predictable size, by a modification of a method for gastric ulcers (23). Ulcers were photographed and measured on a Kodak Image Station (Perkin Elmer, Boston, MA). Initial studies showed that this routinely produces jejunal mucosal ulcers of 3.1+/−0.3 mm^2 in normal jejunum at ulcer induction.

Immunohistochemical studies of proliferation—Tissue from the proximal, distal and Roux limbs was fixed in 10 % formalin for 24 hours and embedded in paraffin. Five micron sections were stained for Proliferating Cell Nuclear Antigen (PCNA) (Zymed, CA), haematoxylin-counterstained for orientation, and visualized and photographed on a Nikon Microphot-FXA. These results were confirmed utilizing antibody against KI 67 (not shown).

Western Blotting—Samples were immediately immersed in 4^{o}C lysis buffer, agitated by a Tissue Tearor (Biospec Products, Bartlesville, OK) at 10,000–15,000g, centrifuged at 12,000g for 30 min at 2°C, assayed for protein, subjected to Western blot for phospho-ERK Thr 202, Tyr204 or total ERK, and visualized using enhanced chemiluminescence as previously described (21). Membranes were re-probed for GAPDH and appropriate secondary antibody as a loading controls. All exposures were within the linear range.

In Vitro Studies

Cell culture—We studied Caco-2BBE intestinal epithelial cells, a subclone of the original Caco-2 cell line selected for its ability to differentiate in culture (24). Caco-2 cells were cultured as previously described.(25)

Pressure regulation—Pressure was controlled using a prewarmed airtight box with an inlet valve for gassing and an outlet connected to a manometer (5). Temperature was maintained within 2°C, and pressure within 1.5 mmHg.

Matrix and medium modulation—6-well dishes were pre-coated with 12.5 ug/mL collagen I or tissue fibronectin (Sigma, St Louis, MO) at saturating concentrations. Cells were seeded 50,000/well for proliferation studies and serum-starved for 24 hours. Cells seeded at 500,000/well were grown to confluence for migration assays. ERK was blocked by 20 mM MEK antagonist PD98059 (Calbiochem, La Jolla, CA) for 30 minutes before application of pressure to assess wound closure with inhibited ERK. Control cells were treated with a 0.1% DMSO vehicle control.

Motility measurement—Caco-2 monolayers on 6 well matrix-coated dishes were subjected to 0–80 mmHg increased pressure for 24 hours after induction of small uniform circular wounds as described. (26) We measured wound areas at 0 and 24 hours on a Kodak Image Station (Perkin Elmer, Boston, MA), and calculated percent closure.

Proliferation—Subconfluent (30–40%) cells were serum-starved for 24 hours. We reserved one 6-well plate for a time 0 measurement, and incubated the remaining serum-starved cells in normal growth medium under ambient or pressure conditions for 24 hours before counting cells by a crystal violet absorbance assay over the linear assay range, with interpolation against a standard curve, as described (27).

Western blotting—Following pressure, Caco-2 cells were lysed in buffer with protease inhibitors, centrifuged at 10,000*g* for 15 minutes at 4°C, and resolved by SDS-PAGE as described (21). Membranes were blotted for phospho-ERK Thr 202, Tyr204 or total ERK, re-probed with antibodies specific for GAPDH and appropriate secondary antibody as a loading control, and detected by enhanced chemiluminescence as described (28).. All exposures used for densitometric analysis were within the linear range.

Data analysis—All data are expressed as mean±SE. Statistical analysis was performed using paired or unpaired *t* tests or analysis of variance as appropriate. A *P* value less than 0.05 was considered significant.

Results

Murine Roux limb formation affects pressure within small bowel lumen, as well as mucosal healing and proliferation—Creation of a defunctionalized roux limb in mice (Figure 1a) yielded probe-patent but partially obstructed small bowel. We induced ulcers on the roux, proximal and distal gut (Figure 1b). At 72 hours, the roux and proximal limb pressures were 3.3+/−0.1 and 5.0+/−0.1 fold normal respectively (n=10,p<0.0001). Ulcer size was measured after 72 hours, at necropsy. Ulcers in proximal and defunctionalized limbs were 224 +/−14.9% and 55.7+/−7.8% larger than those in the distal limbs respectively (Figure 1c, n=15,p<0.0001 each). Proliferation was assessed using a PCNA stain for proliferating cells (Figure 2a). PCNA immunoreactivity within the mucosa of the proximal and defunctionalized limbs was 186+/−15.8% and 256 +/−9.9% greater than in the distal limb respectively (n=4,p<0.05 each, Figure 2b). We obtained similar results using Ki67 staining (not shown).

Pressure increases phosphorylated and total ERK in vivo—Phosphorylated ERK was increased in the proximal and Roux limbs compared to the distal limb (Figure 3, top blot, 171.0+/−37.5% and 140.6+/−39%, n=19, p<0.05). Total ERK was also increased in the Roux and proximal limbs compared to the distal limb (Figure 3, middle blot, 97.9+/−33.7% and 60.9 +/−30.9%, n=19, p<0.05). To ensure that the increase in phosphorylated ERK was not an artifact of the increase in total ERK, we calculated the phosphorylated to total ERK ratio (Figure 3a). The ratio of activated to total ERK in the proximal and Roux limb was also increased in the proximal and defunctionalized limbs by 184.6+/−83.6% and 243.3+/−127.5% respectively compared to the distal limb (n=19, p<0.05).

Pressure activates ERK in Caco-2 cells—ERK is activated in Caco-2 cells by repetitive strain (22). We asked whether pressure also activates ERK *in vitro* in parallel to ERK activation in the partially obstructed mucosa *in vivo*. When confluent Caco-2 cells on collagen substrates were subjected to 30 minutes of 80 mm Hg increased pressure on collagen, pressure increased ERK activation by 35.5+/−6.6% (Figure 3b, n=18, p<0.05).

Pressure inhibits Caco-2 epithelial sheet migration—Basal migration was more rapid on collagen I than on fibronectin, consistent with previous findings (25). On collagen substrates, migration was reduced at 20–80 mmHg increased pressure (n=18, p<0.001 each). (Figure 4a). Caco-2 motility was similarly inhibited by 20 and 80 mHg pressures across fibronectin (n=12, p<0.001 each) (Figure 4b). It was thought that Caco-2 migration may be influenced by ERK (21). However, blocking ERK with the MEK inhibitor PD98059 did not prevent the inhibition of migration by pressure at either 20 or 80 mm Hg (n=6 each, p<0.05 each) (Figure 4c). Additional studies conducted in parallel confirmed that pressure inhibits IEC-6 cell motility similarly to its effects on Caco-2 cells (not shown).

Pressure stimulates Caco-2 cell proliferation—Caco-2 cells exposed to pressure at 20–80 mm Hg, over 24 hours, exhibited increased cell numbers compared with control cells at

ambient pressures. Blocking ERK with PD98059 did not prevent the mitogenic effect of increased pressure. (Fig. 5, n=32, $p<0.05$ for all)

Discussion

Peristalsis and the interactions of the luminal contents with the mucosa expose the small intestinal epithelium to a wide range of luminal pressures. Nocturnal luminal pressure manometry in healthy volunteers (29) demonstrated a mean average pressure of 15–20 mm Hg during phase II of the migrating motor complex (MMC). Luminal pressures fall to almost zero during phase I of the MMC, before phase II restarts. Thus, the gut mucosa normally experiences changes in luminal pressure ranging from 0–30 mm Hg over 85–110 minutes with average pressure of 15–21 mm Hg. We studied 0–80 mm Hg pressures to approximate the normal variance of the intra-luminal pressure and the high supra-physiologic pressures in pathologic states. Our results suggest that supraphysiologic pressure inhibits mucosal wound healing in vivo and in vitro independently of both extracellular matrix regulation and intracellular ERK activation. The effects on wound healing are likely to reflect true motility rather than effects on proliferation, because proliferation is actually stimulated by pressure, an effect that was also ERK-independent.

In vivo wound healing was significantly decreased in the defunctionalized and proximal bowel as compared to the distal gut. Ulcer healing was slowest in the proximal limb. The magnitude of inhibition of restitution correlated with luminal pressure. The presence or absence of luminal chyme and its own chemical and physical interactions with the mucosa is an additional variable *in vivo*. However, luminal chyme was present within the proximal limb while the roux limb was empty and had no significant intra-luminal contents at necropsy, being distended only by gas. Since both limbs generally exhibited the same tendencies to decreased wound healing, increased proliferation, and ERK activation, and since the *in vitro* model reproduced this effect simply by manipulating extracellular pressure, our results suggest that the effects of pathologic luminal pressure dominates any effect of the chyme itself. The murine ulcers were created simultaneously with the Roux-en-Y preparation. It is conceivable that the edema of the anastomosis contributed to the proximal and Roux obstruction. If the anastomosis had been allowed to heal for a period of weeks, it is possible that the obstruction would have cleared, intraluminal pressure would have normalized, and the epithelial migration and mucosal healing in the defunctionalized and proximal limbs would no longer have demonstrated a pressure effect. However, this awaits further study beyond the scope of the current manuscript.

The magnitude of wound closure in these studies appears modest, but is highly statistically significant. We (14,30) and others (31–34) have described similar magnitude changes in intestinal epithelial migration, proliferation, or signaling due to other stimuli. For instance, strain induces an average 22% change in proliferation in Caco-2 cells plated on various matrices (14). However, the effects of physiologic repetitive strain on intestinal epithelial cells are matrix-dependent while those of pathologic pressures seem to escape regulation by the extracellular matrix just as they are independent of luminal chyme. Repetitive strain accelerates Caco-2 or IEC-6 monolayer wound closure but inhibits proliferation on fibronectin while deformation inhibits wound closure but stimulates proliferation on collagen (14,21). In contrast, increased pressure inhibited wound closure *in vitro* on either collagen or fibronectin.

ERK stimulates proliferation and migration in the absence of physical forces (35,36) and mediates the mitogenic effects of repetitive deformation in intestinal epithelial cells on collagen and the motogenic effects of repetitive deformation in intestinal epithelial cells on fibronectin (21,22). It was therefore somewhat surprising that pressure activates ERK while inhibiting motility in vivo and that blocking ERK did not prevent inhibition of motility by pressure in vitro. However, not all physical force effects on intestinal epithelial cells involve ERK. Repetitive deformation inhibits intestinal epithelial cell motility across collagen substrates

independently of ERK (21). Moreover, extracellular pressure stimulates colon cancer cell adhesion independently of ERK (12). We hypothesize that pressure may inhibit intestinal epithelial motility by an ERK-independent signal mechanism similar to that responsible for the inhibition of intestinal epithelial migration across collagen. The precise signals that mediate these effects await further study. Moreover, it seems likely that the activation of intestinal epithelial ERK by pressure in vivo and in vitro reflects some other yet to be identified ERK-mediated effect of pressure on the gut mucosa.

Intestinal mucosal healing is a complex process involving lamellipodial extension of epithelial cells, migration, and proliferation. Physical forces due to intestinal contraction as in peristalsis, villous motility, and interactions with luminal contents likely interact with the effects of cell-matrix interactions and soluble factor signaling to influence these processes. These effects are interesting because the intestinal lumen experiences greatly increased pressure during diverse pathologic states, including the exacerbation of inflammatory bowel disease, post-operative ileus, and partial or complete bowel obstruction (37–39). In some pathologic situations that increase pressure within the bowel, this pressure may itself have a deleterious effect that further worsens the patient's condition. When the normal homeostasis of the small bowel is disturbed by disease, the normal physical forces of the gut are altered and the response to these forces also changes. In particular, increased pressures within the gut may impair mucosal healing in intestinal disease or injury.

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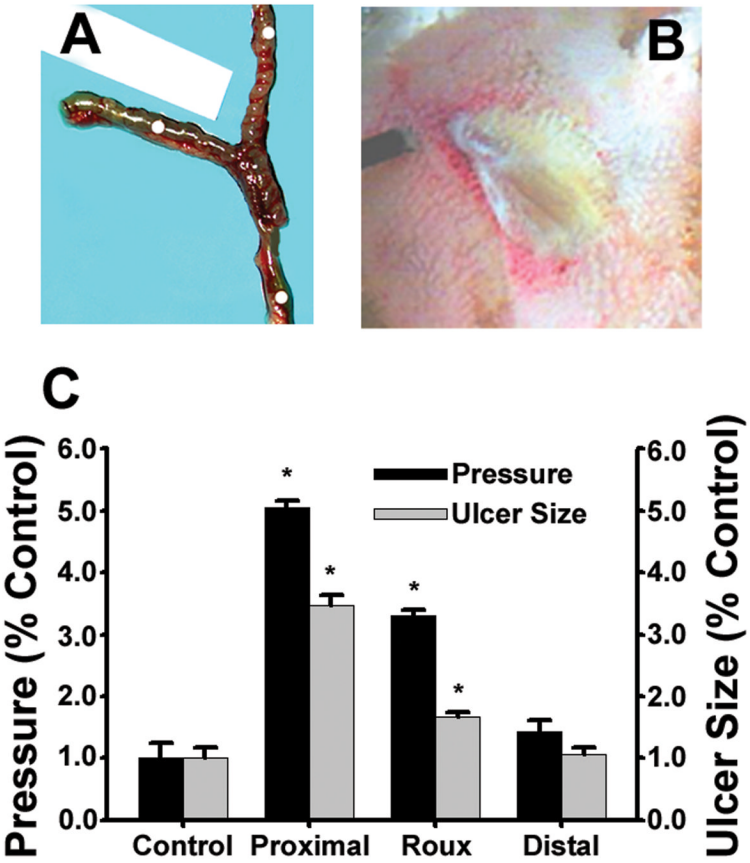

Figure 1.

The Roux limb ex-vivo is shown in (A) with the defunctionalized Roux limb to the left, the proximal limb at the top, and the distal limb at the bottom. (B) illustrates the gross appearance of an acetic acid induced jejunal mucosal ulcer after 3 days. At 3 days (C), the proximal limb exhibits the highest luminal pressure and the slowest ulcer healing (n=10 and 15 respectively, $P<0.05$ for each), compared to the distal limb. The Roux limb also exhibits higher pressure and slower healing (n=10 and 15 respectively, $P<0.05$ for each) than the distal limb.

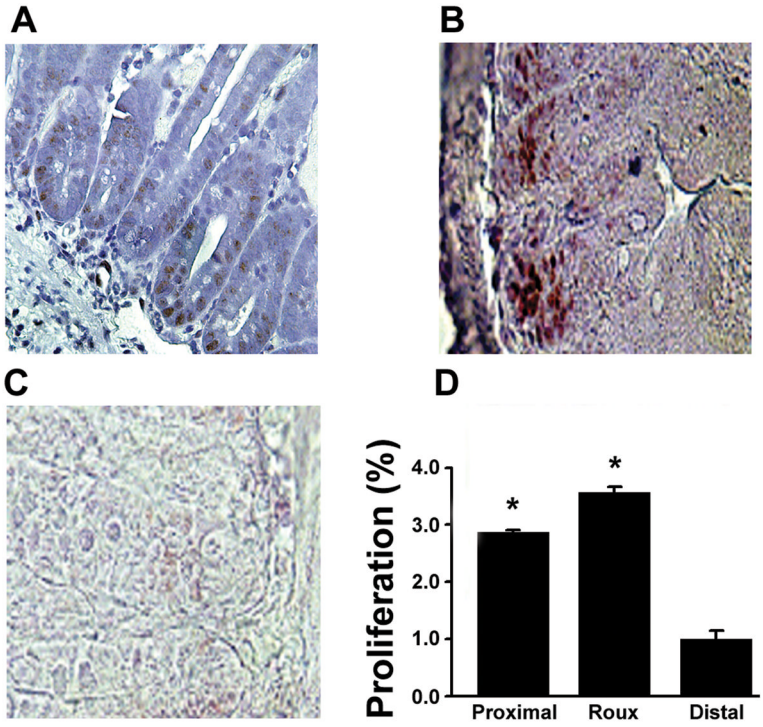

Figure 2.

Mucosal PCNA staining. Typical images are shown of PCNA staining of the mucosa from the proximal limb (A), Roux limb (B), and distal limb (C). Areas of brown stain indicate proliferating cells. (D) The proliferative index is increased in Roux and proximal limb mucosa compared to the distal limb (n=4, $p<0.001$ for each).

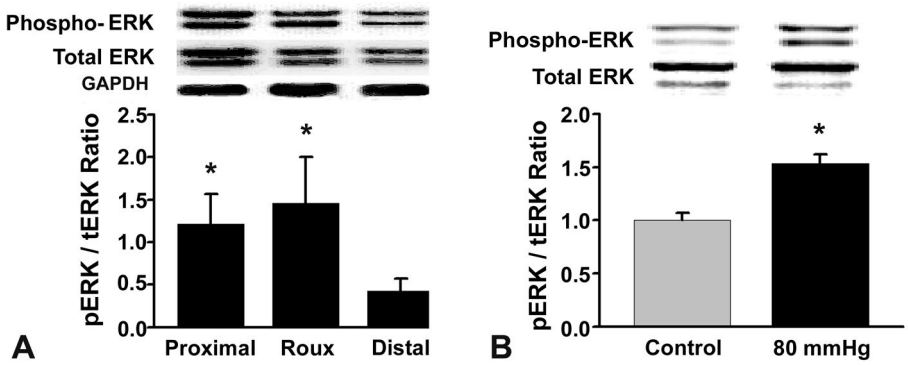

Figure 3.

(A) Phosphorylated and total ERK are each increased significantly in the proximal and Roux limbs compared to the distal limbs of the anastomoses. The phospho ERK/total ERK ratio demonstrates a true increase in the proportion of activated ERK in both proximal and Roux limbs compared to the distal gut (n=19, $p<0.05$ for each comparison of phosphorylated and total ERK and for the ratio) (B) Phosphorylated ERK is increased in pressurized Caco-2 cells compared to controls. (n=18, $p<0.05$)

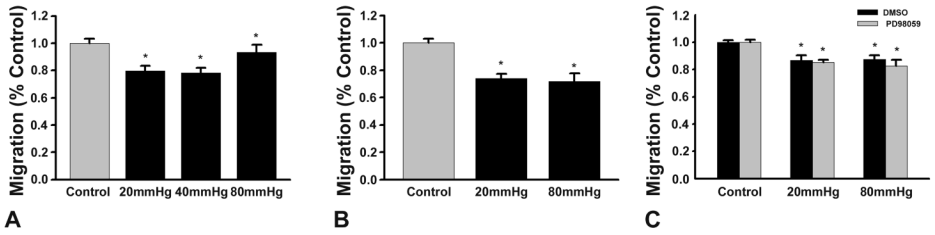

Figure 4.

(A) Wound closure on a collagen substrate is reduced when cells are exposed to 20–80 mmHg increased pressure vs. ambient pressure control (n=36 each, P<0.005 for each comparision). (B) 20 and 80 mm Hg pressures reduce migration across fibronectin vs. ambient pressure control (n=24 each, P<0.005 each). (C) Pressure decreases wound closure on a collagen substrate similarly after treatment with a DMSO vehicle control or after ERK blockade by the upstream MEK inhibitor PD 98059 vs. ambient pressure control (n=36 each, P<0.005 each).

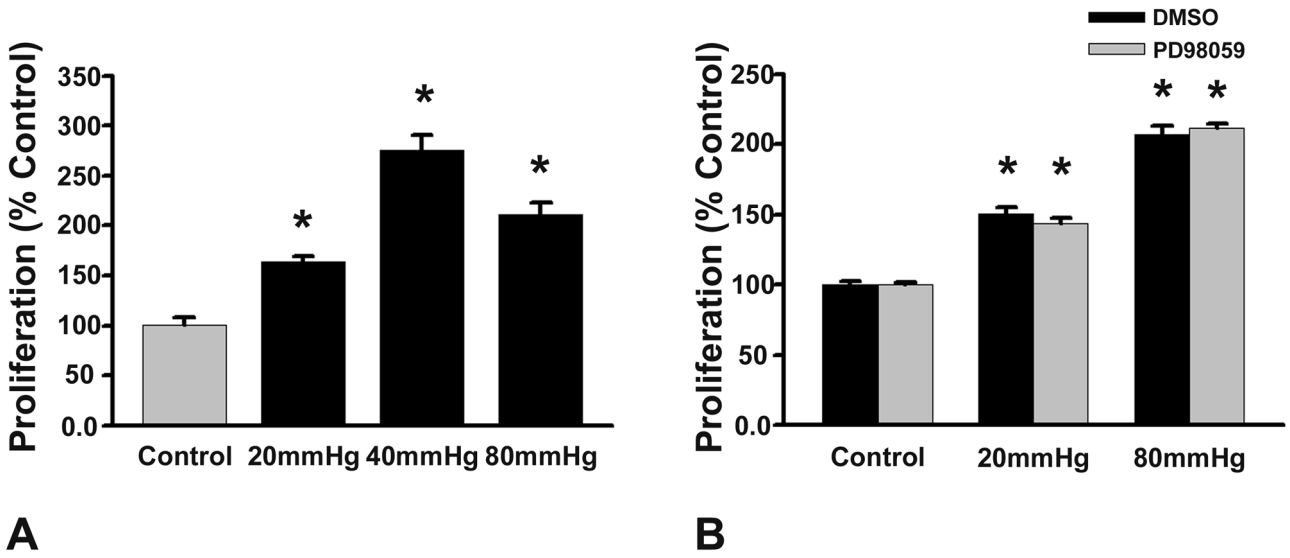

Figure 5.
Proliferation is increased on collagen by a 20 or 80 mm Hg increase in pressure (black bars). This effect is not prevented by ERK blockade by PD98059 (grey bars) compared to ambient controls (n=22, $p<0.05$ for each)