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Extracellular pressure stimulates colon cancer cell proliferation via a mechanism requiring PKC and tyrosine kinase signals

M. F. Walsh, R. K.-Y. Woo, R. Gomez and M. D. Basson

Wayne State University School of Medicine, John D. Dingell VAMC, Detroit, MI, USA

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Abstract. Pressure in colonic tumours may increase during constipation, obstruction or peri-operatively. Pressure enhances colonocyte adhesion by a c-Src- and actin-cytoskeleton-dependent PKC-independent pathway. We hypothesized that pressure activates mitogenic signals.

Methods. Malignant colonocytes on a collagen I matrix were subjected to 15 mmHg pressure. ERK, p38, c-Src and Akt phosphorylation and PKCα redistribution were assessed by western blot after 30 min and PKC activation by ELISA. Cells were counted after 24 h and after inhibition of each signal, tyrosine phosphorylation or actin depolymerization.

Results. Pressure time-dependently increased SW620 and HCT-116 cell counts on collagen or fibronectin ($P < 0.01$). Pressure increased the SW620 S-phase fraction from 28 ± 1 to $47 \pm 1\%$ ($P = 0.0002$). Pressure activated p38, ERK, and c-Src ($P < 0.05$ each) but not Akt/PKB. Pressure decreased cytosolic PKC activity, and translocated PKCα to a membrane fraction. Blockade of p38, ERK, c-Src or PI-3-K or actin depolymerization did not inhibit pressure-stimulated proliferation. However, global tyrosine kinase blockade (genistein) and PKC blockade (calphostin C) negated pressure-induced proliferation.

Conclusions. Extracellular pressure stimulates cell proliferation and activates several signals. However, the mitogenic effect of pressure requires only tyrosine kinase and PKCα activation. Pressure may modulate colon cancer growth and implantation by two distinct pathways, one stimulating proliferation and the other promoting adhesion.

INTRODUCTION

Physical forces, either within tissues, or applied externally, impact all tissues of the body and have been studied in some detail (Duncan & Turner 1995; Osol 1995; Chien *et al.* 1998; Sai *et al.* 1999). Mechanotransduction converts biophysical forces into cellular responses that influence gene expression, protein synthesis, proliferation and morphogenesis. The mechanisms coupling mechanical signals with biochemical events remain poorly understood. Responses to stretch, shear and deformation have been investigated in bone, vascular cells, cardiac fibroblasts

Correspondence: Marc D. Basson, Chief, Surgical Service, John D. Dingell VAMC, 4646 John R. Street, Detroit, MI 48201–1932, USA. Tel.: 313-576-3598; Fax: 313-576-1002; E-mail: marc.basson@med.va.gov

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and myocytes and mesangial cells, but less is known about the effects of pressure, and the distinction is important because different physical forces may have different effects (Han *et al.* 1998b; MacKenna *et al.* 1998; Ingram *et al.* 1999; Han *et al.* 2001; Moalli *et al.* 2001; Basson & Coppola 2002; Hosokawa *et al.* 2002; Zhang *et al.* 2003).

The normal gut experiences complex patterns of contractility and motility and changes in pressure during normal peristalsis. These internally generated pressures may help maintain normal gut cytoarchitecture. However, pressures elevated by diet or illness may adversely impact gut physiology (Brodribb *et al.* 1979). Luminal jejunal pressure may reach 50 mmHg in irritable bowel syndrome (Kellow & Phillips 1987). Abdominal pressures of 15 mmHg are common during insufflation for laparoscopy and surgical manipulation yields pressures exceeding 1500 mmHg (Dregelid & Svendsen 1988). Intra-abdominal pressure also increases after surgery as a result of tissue oedema (Granger & Barrowman 1983). Such pressures may affect cancer dissemination (Basson *et al.* 2000; Thamilselvan & Basson 2004) and the function of intra-abdominal organs (Walker & Criddle 2003).

Early concerns about cancer recurrence at surgical wounds after laparoscopic surgery (Pahlman 1997) prompted studies of the role of pressure in cancer cell adhesion and proliferation (Jacobi *et al.* 1998; Hewett *et al.* 1999; Jacobi *et al.* 2002; Dahn *et al.* 2003). Animal studies suggest that pressure increases may contribute to tumour growth after laparoscopic procedures (Bouvy *et al.* 1998). In mice, pneumoperitoneum enhanced growth of GW39 human colon cancer cells (Wu *et al.* 1997). Pressure (15 mmHg) applied to colon 26 cells increased liver metastasis regardless of insufflation gas (Gutt *et al.* 2000; Ishida *et al.* 2001), suggesting that even this modest pressure increase might influence malignant cell proliferation. The incidence of wound metastasis after laparoscopic and open surgery today is similar (Silecchia *et al.* 2002; Patankar *et al.* 2003), perhaps offset by immune depression after open surgery (Kawasaki *et al.* 2001), or application of substantial pressure to tumours during open surgical dissection (Dregelid & Svendsen 1988).

We have shown that a 30-min exposure of non-adherent primary human colon cancer and SW620 cells to 15 mmHg of extracellular pressure increases cell adhesion to both collagen I and endothelial cells via a FAK/c-Src dependent mechanism that is also blocked by phalloidin treatment (Basson *et al.* 1992; Thamilselvan & Basson 2004). In the present study, we sought to determine whether longer exposure to increases of this magnitude affects proliferation in adherent cells. Previous *in vitro* studies of pressure-mediated proliferation in other cells have considered much higher pressures. In mesangial cells, hydrostatic pressure induces proliferation in an intensity-dependent fashion (Mattana & Singhal 1995; Kawata *et al.* 1998a). In the same cells, 40–80 mmHg pressures stimulated proliferation in a time- and pressure-dependent fashion (Kato *et al.* 1999). Pressures of 105 mmHg increase proliferation in vascular smooth muscle (VSMC) and endothelial cells (Sumpio *et al.* 1994; Watase *et al.* 1997). In VSMC, pressure-induced proliferation is inhibited by PKC, p38 and ERK inhibition as well as by the tyrosine kinase inhibitor, genistein (Tsuda *et al.* 2002). In fibroblasts, a mechanical load elicits both MAPK and NF-κB activation (Chiquet *et al.* 2003). Li and Xu postulated that stretch of the VSMC membrane alters tyrosine receptor or G-protein conformation, initiating signalling pathways normally used by growth factors (Hu *et al.* 1998; Li & Xu 2000) that stimulate PKC, MAPK, c-fos and other gene expression (Li *et al.* 1999). Similarly, in rodent mesenteric arteries, increased pressure enhances c-fos expression, an effect inhibited by genistein (Miriel *et al.* 1999), and requiring both c-Src and ERK (Wesselman *et al.* 2001; Rice *et al.* 2002). Other investigators suggest that integrin aggregation per se may activate tyrosine kinase receptors in the absence of ligand (Miyamoto *et al.* 1996; Esposito *et al.* 2003), another possible mechanism for the transduction of external physical forces such as pressure.

We hypothesized that longer exposures to a physiologically relevant increase of 15 mmHg in pressure might stimulate colon cancer cell proliferation. We delineated the time course of this effect in human SW620 colon cancer cells and examined whether it could be duplicated in human HCT-116 colon cancer cells. We further investigated whether pressure activated several intracellular signals known to be involved in proliferation in response to other forces or growth factors, particularly c-Src, PI-3-K, MAPK, and PKC. We then assessed proliferation in the presence of specific signal inhibition, or the global tyrosine kinase inhibitor genistein or phalloidin, to block actin re-organization.

MATERIALS AND METHODS

Cell culture

SW620 cells were cultured at 37 °C in 5% CO_2 in an equal mixture of Dulbecco's minimal essential medium (DMEM) and RPMI 1540 containing 5% foetal bovine serum (FBS, Gibco Life Technologies Inc., Rockville, MD, USA), 20 mM glutamine, 1 μM sodium pyruvate, 10 mM Hepes, 100 U/ml penicillin G, 100 μg/ml streptomycin (Sigma, St Louis, MO, USA) and 0.525 μg/ml transferrin (Roche Diagnostics, Indianapolis, IN, USA). The HCT-116 cells were maintained in DMEM supplemented with 10% FBS. Both human colon cancer cell lines were obtained from ATCC (American Tissue Culture, Collection, Rockville, MD, USA).

Matrix pre-coating

Six-well plates were pre-coated with collagen I or plasma fibronectin (12.5 μg/ml, Sigma) as previously described (Basson *et al.* 1992) and the wells were rinsed with sterile phosphate-buffered saline prior to cell seeding.

Pressure application

Pressure was applied by placing the six-well plates and cells within an airtight box equipped with inlet and outlet valves and a pressure gauge as previously described (Basson *et al.* 2000; Vouyouka *et al.* 2003; Thamilselvan & Basson 2004). The box was pre-warmed to 37 °C; the temperature did not fluctuate more than ±2 °C for the duration of the experiment. The internal pressure was set at 15 mmHg and maintained within ±1.5 mmHg.

Proliferation assays

Uniform cell aliquots were dispensed into the pre-coated wells and allowed to reach 70% confluence before being subjected to increased pressure (15 mmHg) for 24 h. At that time, the cells were detached with trypsin/EDTA, the trypsin was neutralized with medium containing 5% FBS and cell aliquots were counted in a Coulter Counter (Coulter Electronics, Hialeah, FL, USA). Control cells maintained in the same incubator but outside of the pressure box were treated similarly. When used, the following inhibitors, PD98059 (20 μM), SB203580 (20 μM), PP2 (20 μM), LY294002 (10 μM), genistein (10 μM), phalloidin (10 μM), or calphostin C (100 nM, light-activated for 1 h) (all Calbiochem, La Jolla, CA, USA), were added to the cells 30 min before the application of pressure. Appropriate volumes of the dimethylsulfoxide (DMSO) or ethanol vehicle were added to the corresponding controls at the same time. Assays were normally performed on cells in DMEM/RPMI supplemented with 5% FBS. Basic proliferation assays in response to pressure were duplicated under serum-free conditions to corroborate results obtained with flow cytometry.

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Flow cytometry

To document proliferation, cells exposed to pressure for 24 h were detached with trypsin/EDTA and fixed in 70% ice-cold ethanol. The cells were then stained with propidium iodide (5 μg/ml PBS, pH 7.4 containing 200 μg/ml DNase-free Rnase A and 0.1% Triton X-100). The cells were then analysed for transition to S-phase by the Wayne State University School of Medicine Flow Cytometry Core Facility. Assays were performed on cells maintained in 5% FBS and repeated in cells rendered quiescent by serum starvation for 24 h prior to pressure exposure.

Signal activation assays

For signal activation studies, cells were exposed to 15 mmHg pressure for 30 min. The cells were then lysed on ice for 30 min in buffer containing 150 mM NaCl, 10 mM Tris, 1% TritonX-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.4. After centrifugation, supernatant protein concentrations were measured by the BCA protein method (Pierce, Rockford, IL, USA). Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) followed by transfer to Hybond ECL nitrocellulose and incubation with antibodies specific for the active (phosphorylated) forms of ERK (p44/42), p38, c-Src, and Akt/PKB (Cell Signalling, Beverly, MA, USA; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Transduction Laboratories, San Diego, CA, USA). Membranes were then stripped and re-probed with antibodies to the total form of each molecule. After exposure to the appropriate second antibody coupled to horseradish peroxidase, bands were detected with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and analysed with a Kodak Image Station 440CF (Perkin Elmer, Boston, MA, USA).

PKC activity and translocation

PKC activity was assayed in cytosolic fractions utilizing a synthetic PKC pseudosubstrate following the manufacturer's directions (Calbiochem). Briefly, control and test cells exposed to 15 mmHg for 30 min were lysed, sonicated on ice and centrifuged at 100 000 ***g*** for 60 min. Supernatant protein concentrations were equalized prior to assay for PKC activity. Cells treated with PMA (1 μM) served as a positive control. Calcium- and lipid-sensitive PKC activity was quantified using a colourimetric ELISA based upon a monoclonal antibody to the phosphorylated form of the pseudosubstrate. For the translocation studies, soluble and particulate cell fractions were prepared according to the methods of Bissonnette (Bissonnette *et al.* 1994). After solubilization in extraction buffer (20 mM HEPES, pH 7.6, supplemented with 5 mM EGTA, 5 mM Na pyrophosphate, 1 mM $MgCl_2$, 1 mM PMSF and 10 μM leupeptin), the samples were centrifuged at 100 000 *g* for 60 min and the soluble fraction was removed. The particulate fraction was re-suspended in 20 mM HEPES, pH 7.6 containing 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100. Equal protein aliquots of soluble and particulate fractions were separated via SDS–PAGE and transferred to nitrocellulose. Blots were probed with both a pan-PKC antibody that recognizes all PKC isoforms and an antibody specific for PKCα (Calbiochem and Chemicon, Temecula, CA, USA). Protein loading was controlled for with GAPDH (Biodesign International, Saco, ME, USA, soluble fraction) and Na/K ATPase (Sigma, particulate fraction).

Statistical analysis

Results are expressed as mean ± SEM and differences between groups were evaluated using both unpaired and paired Student's *t*-tests as warranted with statistical significance being set a priori at $P < 0.05$.

RESULTS

15 mmHg pressure significantly increases cell proliferation

SW620 cell proliferation on collagen I is increased after 6 h of pressure. Using the pressure apparatus described above, we first determined the effects of increasing pressure by 15 mmHg over ambient pressure for 24 h on SW620 cells. As illustrated in Fig. 1(a), exposure to pressure increased cell number by 51 ± 7% ($n = 14$, $P = 0.0001$). Data shown represent absolute cell counts normalized to time = 0 (cell counts at the onset of the study) and are expressed as mean ± SEM. A time course study was performed to further delineate the pressure effect on proliferation. Cells were exposed to pressure for 0, 3, 4.5, 6, 12 and 24 h and then maintained under ambient pressure for the balance of a 24-h period prior to trypsinization and cell counting. As shown in Figs 1(b), 3 h of pressure did not result in a subsequent significant increase in cell number (3.4 ± 3.0%). However, after 6 h of pressure, cell numbers at 24 h were significantly enhanced compared with cells exposed to ambient pressure. Furthermore, the magnitude of this increase was similar to that observed after either 12 or 24 h exposure to pressure. Figure 1(b) represents absolute cell counts two-point normalized between $t = 0$ and $t = 24$ to adjust for differences in proliferation between studies. Thus, pressure-mediated increases in cell number appeared duration-dependent, requiring 6 h of exposure to reach maximal stimulation. Apoptosis, both early and late, accounted for only 1% of cells in either condition (data not shown). To document that the result of exposure of SW620 cells to pressure of 15 mmHg over ambient pressure cells for 24 h was proliferation, cells were assessed for S-phase transition via flow cytometry following propidium iodide staining. When the cells were maintained in complete medium with 5% FBS, conditions for the original proliferation experiments, the percentage of cells in S-phase increased from 39.5 ± 1.2 in control cells to 42.9 ± 2.2 in cells under pressure ($P = 0.03$, $n = 6$). When cells were rendered quiescent by serum-starvation for 24 h prior to pressure, the difference was magnified. As shown in Fig. 1(c), 27.8 ± 1.0% of serum-starved cells at ambient pressure were found to be in S-phase compared with 46.6 ± 0.8% of serum-starved cells subjected to pressure ($P = 0.0002$, $n = 6$). Cell count studies repeated in cells rendered quiescent showed proliferation of a similar magnitude to that observed in non-quiescent cells (data not shown).

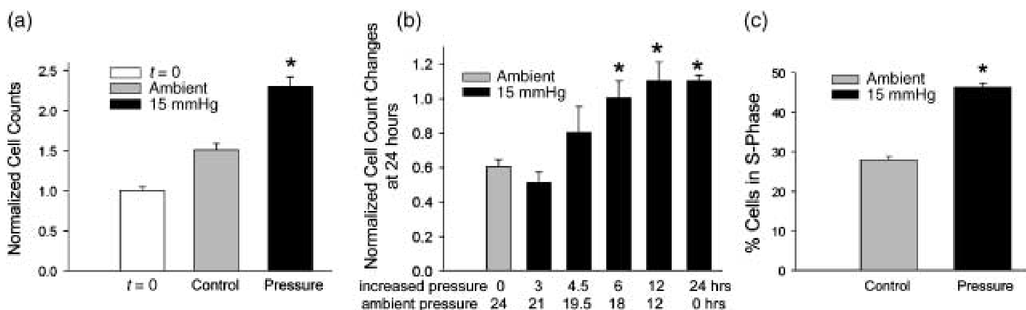


Figure 1. Pressure stimulates time-dependent SW620 cell proliferation. (a) 15 mmHg pressure application for 24 h results in a 51 ± 7% increase in cell number compared with the ambient pressure control at 24 h ($n = 14$, $P = 0.0001$). (b) Time course of the pressure effect. Cells were exposed to pressure for 0, 3, 4.5, 6, 12 or 24 h and then maintained at ambient pressure for the balance of the 24-h period. Cell numbers are significantly ($P < 0.05$) increased over control values after 6 h of exposure (data shown are two-point normalized between $t = 0$ and 24 h of exposure to pressure) and remain elevated for 24 h. (c) Flow cytometry in cells rendered quiescent by serum deprivation. The ratio of cells in S-phase after 24 h of pressure was 46.6 ± 0.8% compared with 27.8 ± 0.1% in cells maintained at ambient pressure ($P = 0.0002$, $n = 6$). All data, mean ± SEM; $t = 0$, initial cell count; control, ambient pressure; pressure, 15 mmHg.

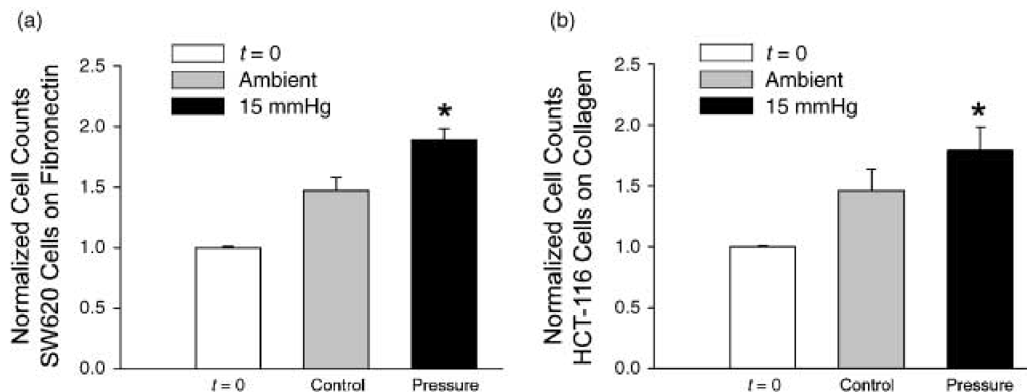


Figure 2. Pressure-stimulated colon cancer cell proliferation is independent of matrix and cell line. (a) In SW620 cells on a fibronectin matrix, 24 h of exposure to 15 mmHg increased pressure increased cell number by 29 ± 6% over ambient pressure controls at 24 h ($P = 0.0013$, $n = 6$). (b) Similarly, in HCT116 cells plated on collagen I, pressure enhanced cell number by 27 ± 8% ($P = 0.009$, $n = 6$). All data, mean ± SEM normalized to cell counts at $t = 0$.

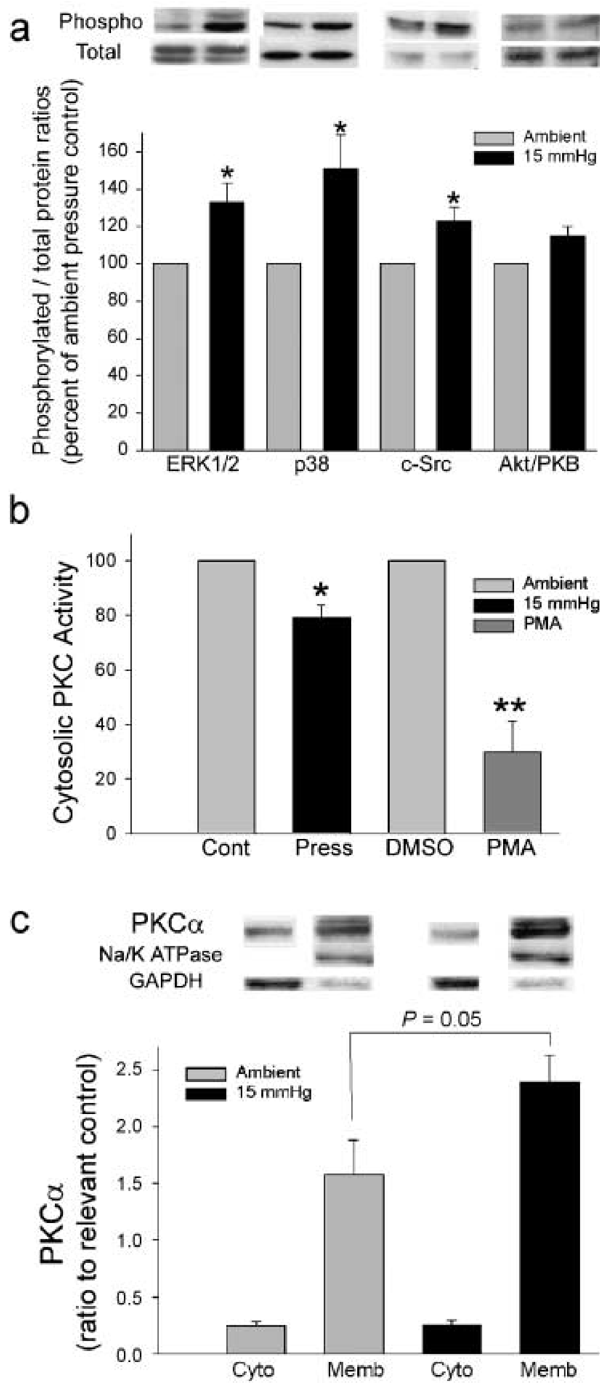
Pressure enhances proliferation on a different matrix and in a different cell line

We previously have reported that another physical force, cyclic strain, is mitogenic to cells on collagen but not to those fibronectin and that fibronectin inhibits strain-stimulated signals (Zhang *et al.* 2003). To determine whether the pressure effect on proliferation was similarly inhibited by fibronectin, the experiments were repeated in SW620 cells plated on to plasma fibronectin. As seen in Fig. 2(a), a 24-h exposure to 15 mmHg increased cell number by 29 ± 6% ($P = 0.0013$, $n = 6$). Results are normalized to cell counts at time = 0. Similar results were obtained in HCT-116 colon cancer cells plated on to collagen I, in which cell number was increased by 27 ± 8% by pressure ($P = 0.009$, $n = 6$) (Fig. 2b). These results suggest that pressure-mediated colon cancer cell proliferation is neither limited to a single cell line nor inhibited by fibronectin.

15 mmHg pressure activates intracellular signals after 30 min

ERK, p38 and c-Src are activated, but not Akt. We next examined several intracellular signals that have been implicated in either cellular proliferation or physical force effects in colonocytes or other cell systems. To determine whether activation of these signals preceded the observed proliferation, SW620 cells plated on collagen I were exposed to 15 mmHg pressure for 30 min. Cell lysates of control and experimental cells were analysed by western blot for their activated, phosphorylated forms. Results in the bar graphs in Fig. 3a were calculated as the ratio of phosphorylated to total band intensity for each signal and expressed as per cent of ambient control. Statistics were calculated on the initial raw data ratios before normalization to control values. Representative western immunoblots for the phosphorylated and total molecule are shown above

Figure 3. Pressure induces early (30 min) signal activation. (a) Western blot assays of ERK, p38, c-Src and Akt/PKB phosphorylation presented as the ratio of phosphorylated to total forms. Bars depict the results of densitometric analysis (mean ± SEM, $n = 3–7$, $P < 0.05$). Representative western blots are shown above the bars. ERK, p38, and c-Src but not Akt/PKB, were activated by 30 min of pressure. (b) Cytosolic PKC activity, expressed as percentage of control, is significantly decreased by pressure ($P = 0.026$, $n = 3$). Activation by 1 µg/ml PMA was used as a positive control. DMSO-treated cells were assayed as a vehicle control for the PMA. (c) Western blot analysis of PKCα subcellular distribution in response to pressure. Bars represent densitometric ratios of PKCα to GAPDH for the cytosolic fraction (Cyto) and to Na/K ATPase for the membrane fraction (Memb) (*$P = 0.05$ membrane pressure vs. membrane control, $n = 3$). A representative western blot is shown above the bars. Control, ambient; pressure, 15 mmHg.


a
Phospho
Total
Phosphorylated / total protein ratios (percent of ambient pressure control)
0
20
40
60
80
100
120
140
160
Ambient
15 mmHg
ERK1/2
p38
c-Src
Akt/PKB
b
Cytosolic PKC Activity
0
20
40
60
80
100
Ambient
15 mmHg
PMA
Cont
Press
DMSO
PMA
c
PKCα
Na/K ATPase
GAPDH
P = 0.05
PKCα (ratio to relevant control)
0.0
0.5
1.0
1.5
2.0
2.5
Ambient
15 mmHg
Cyto
Memb
Cyto
Memb

the bars. After 30 min of pressure, ERK (p44/42) activity was higher than that of control by 33 ± 10% ($P = 0.0035$, $n = 7$), p38 activation was increased by 51 ± 18% ($P = 0.0005$, $n = 5$), and c-Src phosphorylation was enhanced by 23 ± 7% ($P = 0.05$, $n = 3$). Akt, however, was not significantly activated by pressure (15 ± 5%, $n = 6$).

PKC activity is lower in the cytosolic fraction while PKCα is increased in the membrane fraction of cells exposed to pressure

Activation of the various forms of PKC usually involves translocation of the enzyme from the cytosol to the membrane (Sylvester *et al.* 2001). We therefore measured calcium- and lipid-dependent PKC activity in whole cell lysates and cytosolic fractions of control cells and cells subjected to 15 mmHg pressure for 30 min. Activity was compared with that obtained after exposure of the cells to 1 μg/ml PMA for 30 min. Data presented in Fig. 3(b) show per cent of control cell activity for both the pressure and PMA experiments. Pressure induced a 21 ± 5% decrease in overall PKC activity, smaller than the 70% decrease seen with PMA but nevertheless significant ($P = 0.026$, $n = 3$). To further demonstrate PKC activation, both the soluble (cytosolic) and particulate (membrane) fractions of cells exposed to pressure and their controls were assayed for PKC content. Western blotting with an anti-PKC antibody that cross reacts with all PKC isozymes demonstrated significant pressure-induced PKC translocation to the membrane fraction (data not shown). However, we chose to focus on PKCα because it is a calcium-and lipid-dependent isoform of the enzyme and previous work in our laboratory documented its activation in response to another physical force, repetitive deformation, in the Caco-2 intestinal epithelial cell line (Han *et al.* 1998b). GAPDH and Na/K ATPase were used as controls for protein loading in the cytosolic and membrane fractions, respectively, because GAPDH is known to be a cytosolic enzyme and Na/K ATPase is a traditional membrane marker. Cytosolic PKCα content was not measurably changed by pressure, perhaps because it was much lower than that of the membrane fraction even in unstimulated cells (Fig. 3c) and any change would have been difficult to discern. However, membrane PKCα, calculated as the ratio of PKC to Na/K ATPase, was significantly higher in the membrane fraction of cells exposed to pressure (2.39 ± 0.24 vs. 1.58 ± 0.30, $P = 0.05$, $n = 3$), corroborating the PKC activity data and indicating that at least the α isoform of the PKC enzyme is translocated by pressure.

Pressure-induced proliferation is inhibited by tyrosine kinase and PKC inhibition

We next sought to determine which of the signals shown to be activated by pressure might play a role in pressure-stimulated SW620 cell proliferation. We used the following inhibitors: SB203580 (20 μM) for p38, PD98059 (20 μM) for p44/42, PP2 (20 μM) for c-Src, LY294002 (10 μM) for PI-3 kinase, and light-activated calphostin C (100 nM) for PKC. We have previously demonstrated signal inhibition in SW620 by the SB, PD, LY and PP2 compounds at these doses (Walsh *et al.* 2003; Thamilselvan & Basson 2004; Thamilselvan *et al.* 2004) but inhibition of the requisite signal was further documented by western blot analysis in the current study (data not shown). As mechanical forces have been suggested to act directly on the cytoskeleton, we also used phalloidin (10 μM) to stabilize the actin cytoskeleton. In addition, we examined the effects of the less specific tyrosine kinase inhibitor, genistein (10 μM). Preliminary data indicated that the DMSO and ethanol vehicles, at the volumes used, did not affect cell proliferation. (The increase in pressure-induced proliferation in these experiments, expressed as a percentage of respective control values was 44 ± 9% in untreated cells, 52 ± 11% in cells exposed to 0.1% DMSO, and 40 ± 10% in cells in 0.1% ethanol.) Therefore, results were not normalized and are presented as the number of cells exposed to the inhibitor both under ambient (control) pressure and 15 mmHg for 24 h. Of the inhibitors used, those that did not affect pressure-induced cell

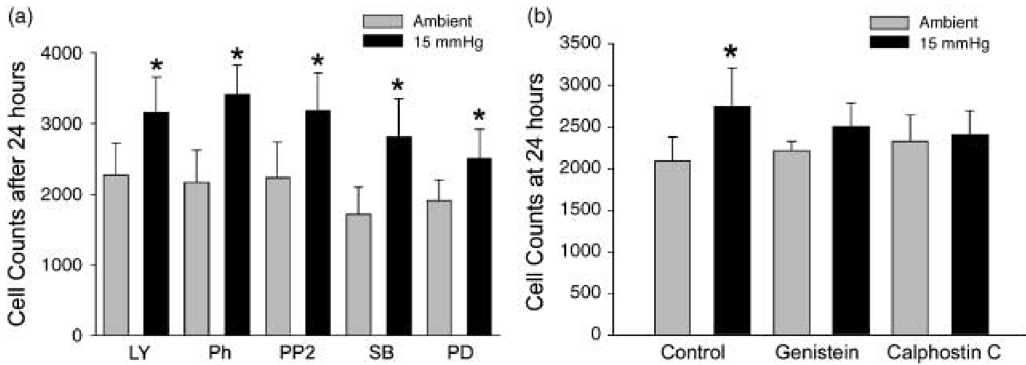


Figure 4. Tyrosine kinase and PKC inhibition block pressure-induced proliferation. (a) Pharmacological blockade of PI-3-kinase with 20 μM LY294002 (LY), actin depolymerization with 10 μM phalloidin (Ph), c-Src blockade with 20 μM PP2 (PP2), p38 inhibition with 20 μM SB203580 (SB) and MEK inhibition with 20 μM PD98059 (PD) all failed to negate the pressure effect on proliferation. *$P < 0.05$ vs. respective control (ambient). Appropriate vehicle controls yielded similar pressure mediated increases in cell number. (b) Genistein (10 μM) and calphostin C (100 nM) abrogate pressure-stimulated cell proliferation, limiting increases in cell number to 11 ± 9% with genistein and 5 ± 3% with calphostin C ($n = 5$, each).

proliferation are shown in Fig. 4(a). Pressure increased cell number by 44 ± 11% in LY-treated cells ($P = 0.003$), 75 ± 17% in phalloidin-treated cells ($P = 0.0003$), 55 ± 22% in PP2-treated cells ($P = 0.04$), 73 ± 19% in SB-treated cells ($P = 0.015$) and 32 ± 8% in PD-treated cells ($n = 4$–7 for each). Thus, none of these agents blocked pressure-stimulated SW620 cell proliferation. However, as shown in Fig. 4(b), exposure to either genistein or calphostin C prevented pressure-induced cell proliferation. Whereas pressure enhanced cell number by 30 ± 9% in control cells ($P = 0.04$), the increase was limited to 11 ± 9% by genistein and 5 ± 3.5% by calphostin C ($n = 5$ each), not significantly different from control in either case, suggesting that pressure stimulates proliferation in SW620 cells by both PKC- and tyrosine-kinase-dependent mechanisms.

DISCUSSION

We have previously reported that a 15 mmHg increase over ambient pressure increases colon cancer cell adhesion by a mechanism requiring FAK, c-Src, and an intact actin-cytoskeleton but is independent of PKC or ERK (Basson *et al.* 2000; Thamilselvan & Basson 2004). We now demonstrate that exposure to a 15 mmHg elevation in pressure stimulates colon cancer proliferation in a time-dependent but matrix-independent manner in adherent cells from two different colon cancer cell lines. Changes in proliferation (assessed by S-phase fraction) is likely to explain this increase in cell number. Proliferation was preceded by pressure activation of p38, ERK, c-Src and PKCα. However, only PKC blockade or global tyrosine kinase inhibition negated the pressure effect on proliferation.

Pressure enhancement of proliferation has been reported in mesangial (Mattana & Singhal 1995; Kawata *et al.* 1998a,b; Kato *et al.* 1999), vascular smooth muscle (Hishikawa *et al.* 1994; Watase *et al.* 1997; Tsuda *et al.* 2002) and endothelial cells (Sumpio *et al.* 1994). Because interest in these cells is related to hypertension, most of those studies were performed at the 40–120

mmHg pressures found in the vascular system, The same high pressures enhanced proliferation and DNA synthesis in a rat intestinal epithelial cell line (Hirokawa *et al.* 2001). In contrast, published studies have focused on the effects of pressures within the 15 mmHg range on colon cancer cells *in vivo.* A pneumoperitoneum yielding 15 mmHg pressures promoted the occurrence of liver metastasis in mice implanted with colon 26 cells (Ishida *et al.* 2001; Gutt *et al.* 2003). The mechanisms of this result, however, are unclear, and certainly may include the effects of pneumoperitoneal pressures on adhesion (Basson *et al.* 2000; Thamilselvan & Basson 2004), in addition to the outcome on proliferation suggested by our current observations. Pressures between 6 and 12 mmHg enhanced the proliferation of CX-2 colon adenocarcinoma *in vitro* after 5 days but, unlike our results, proliferation was suppressed by pressure at earlier time points (Gutt *et al.* 2000). The disparity between the prior results and ours may reflect the somewhat higher pressure used in our study, an idiosyncracy of the CX-2 cell line, or the effect of a more physiologic matrix protein substrate. The CX-2 cells were cultured on tissue culture plastic, without a matrix substrate, perhaps eliminating the contribution of integrin engagement to the transduction of the pressure signals (Miyamoto *et al.* 1996; Esposito *et al.* 2003).

Of the signals studied, p38, ERK, c-Src, and PKC were activated after 30 min of exposure to 15 mmHg pressure, but Akt/PKB was not. Many of these signals are activated in some, but not all, cells in response to pressure or other mitogenic stimuli such as growth factors (Hishikawa *et al.* 1994; Tsuda *et al.* 2002) (Kawata *et al.* 1998a,b; Kato *et al.* 1999). Tyrosine kinase receptors activate PKC and PI-3-K in SW480 and Caco-2 human colon cancer cells (Graness *et al.* 1998; Jasleen *et al.* 2000). In HT-29 colon cancer cells, growth factors stimulate c-Src, PI-3-K, Akt/PKB, ERK, and proliferation (Kim *et al.* 2002; Golubovskaya *et al.* 2003). Proliferative activity in five human colon cancer cell lines has been linked to c-Src (Sekharam *et al.* 2003) but our previous results (Thamilselvan & Basson 2004) and those of others (Haier *et al.* 2002; Jones *et al.* 2002) suggest that c-Src may be more important for pressure stimulation of cell adhesion than proliferation. In this study, c-Src inhibition did not prevent the mitogenic effects of pressure. Similarly, although phalloidin blocks the stimulation of SW620 or primary human colon cancer cell adhesion by pressure (Thamilselvan & Basson 2004), phalloidin did not inhibit pressure-induced proliferation, suggesting that the mitogenic effects of pressure in adherent cells may not require cytoskeletal input.

In non-adherent SW620 cells, pressure activates p38 but not ERK (Thamilselvan & Basson 2004). Pressure stimulates both ERK and p38 in adherent cells, but inhibition of these MAPK components did not block proliferation. ERK activation has been associated with proliferation (Shapiro *et al.* 1996). However, ERK activity may need to exceed a specific threshold to stimulate proliferation (Kuwada & Li 2000). In addition, and consistent with our results, MAPK activation is not necessarily related to proliferation in other cells (Xu *et al.* 2000). Strain stimulates endothelial ERK and PKC, but only PKC inhibition prevents proliferation (Ikeda *et al.* 1999). Similarly, collagen expression in mesangial cells required both ERK and PKC. Interestingly, the PKC-δ, -ε and -ζ effects were ERK-dependent but those mediated via PKC-α and -β were ERK-independent (Hua *et al.* 2001). We found similar dissociation between ERK and PKC-α effects.

Although pressure activated MAPK, c-Src, and PKC, only genistein and calphostin C negated the mitogenic effect of pressure. The genistein data suggest that some receptor or intracellular tyrosine kinase is necessary. Genistein blocks pressure-stimulated smooth muscle and mesangial cell proliferation and the mitogenic effects of GLP-2 and strain in Caco-2 cells (Han *et al.* 1998b; Kawata *et al.* 1998a,b; Tsuda *et al.* 2002). However, in VSMC both ERK and p38 are required (Tsuda *et al.* 2002), whereas Caco-2 mitogenicity is sensitive to PI-3-K and MAPK blockade (Han *et al.* 1998b; Jasleen *et al.* 2000). In contrast, the genistein-sensitive mitogenic pressure effect in SW620 cells was independent of c-Src or the MAPK. Genistein may be inhibiting

tyrosine kinase activation of a number of growth factor receptors, such as those of EGF or IGF-1, intermediary or docking proteins such as Shc and Grb2, or the JAK/STAT pathway, among others. Each of these has been implicated in cell proliferation but their role(s) in the mitogenic effect of pressure awaits further study.

Here, exposure to 15 mmHg pressure for 30 min stimulated PKC-α translocation to the membrane and PKC inhibition by calphostin C blocked the ensuing proliferation. Early mitogenic signals are commonly associated with delayed effects, because either the signals themselves or their downstream consequences persist. Proliferation and PKC activation have been closely linked in colonocytes, and we have previously reported that a rapid biphasic PKC activation over 1–5 min was associated with increased Caco-2 cell number 24 h later in response to cyclic strain (Cadoret *et al.* 1998; Han *et al.* 1998a,b; Davidson *et al.* 2000; Cerda *et al.* 2001). The PKC family of serine/threonine kinases includes conventional lipid- and calcium-sensitive PKCs (α, β and γ), novel lipid-sensitive calcium-independent PKCs (δ, ε, η and θ), and atypical PKCs (Mellor & Parker 1998; Toker 1998). PKC isoform abundance varies with proliferation, differentiation and apoptosis in normal colon (Cesaro *et al.* 2001). Human, mouse and rat colonocytes, both normal and malignant, express the α, β, δ, ε and ζ isoforms, with lower expression in tumour cells (Kahl-Rainer *et al.* 1994; Perletti *et al.* 1996; Han *et al.* 1998a,b; Verstovsek *et al.* 1998). Each isoform may subserve a different role in colon cancer proliferation (Perletti *et al.* 1998, 1999; Weller *et al.* 1999) although their exact function is controversial (Scaglione-Sewell *et al.* 1998). PKC-α supports proliferation, a role also documented for PKC-ε and -β in HT-29 and HD3 and Caco-2 cells (Cadoret *et al.* 1998; Weller *et al.* 1999; Davidson *et al.* 2000; Cesaro *et al.* 2001). PKC-α was linked to ERK in bile-acid-treated AA/C1 adenoma cells (McMillan *et al.* 2003), but not in HCT-116 cells (Qiao *et al.* 2000). PKC action appeared independent of ERK in our cells.

In conclusion, our data demonstrate that adherent SW620 cells proliferate more rapidly in response to a modest increase in pressure. In particular, the mitogenic effects of pressure require PKC, are independent of Src and are not blocked by phalloidin, precisely the reverse of the effects of pressure on adhesion. Thus, the effects of pressure on colon cancer cell proliferation are mediated by pathways different from the mitogenic effects of strain or the adhesion-promoting effects of pressure on colon cancer cells. However, both greater adhesion and enhanced proliferation could facilitate colon cancer implantation and growth. Tumour progression in intact organisms is a highly complex process, but these results suggest that forces such as pressure and strain, acting through different pathways, may promote colon cancer progression.

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