Ambient pressure upregulates nitric oxide synthase in a phosphorylated-extracellular regulated kinase–and protein kinase C–dependent manner

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Purpose: **Using endothelial cell/smooth muscle cell (SMC) cocultures, we have demonstrated that pressurized endothelial cell coculture inhibits SMC proliferation and promotes apoptosis, and that this effect is transferable through pressurized endothelial medium. We now hypothesized that endothelial nitric oxide synthase (eNOS) plays a significant role in mediating these pressure-induced effects.**

Methods: **Conditioned media from endothelial cells and SMCs exposed to ambient and increased pressure were transferred to recipient SMCs. We counted cells after 5 days of incubation with these media and evaluated eNOS and inducible NOS (iNOS) levels by Western blot.**

Results: **Conditioned media from pressurized endothelial cells significantly decreased recipient SMC counts. This effect was sustained when *N*-nitro-L-arginine-methyl ester (L-NAME) was added to recipient cells but abolished when L-NAME was added to donor cells. SMCs were then exposed to control and pressurized conditions in monoculture or in coculture with endothelial cells. Pressure and coculture caused similar increase in iNOS levels but had no additive effect in combination. Finally, endothelial cells were exposed to control and pressurized environments. Pressure caused a 24% ± 1.6% increase in eNOS protein (P = .04, n = 12). This effect was sustained when cells were treated with L-NAME (32% ± 1.6% increase, P = .02) but abolished when endothelial cells were treated with calphostin C or PD98059 to block protein kinase C (PKC) or extracellular regulated kinase (ERK). Pressure also increased endothelial phosphorylated ERK (p-ERK) by 1.8-fold to 2.6-fold compared with control conditions after exposure of 2, 4, and 6 hours (P = .02, n = 4). This increase was sustained after pretreatment with calphostin C.**

Conclusion: **Pressure modulates endothelial cell effects on SMC growth by increasing eNOS in an ERK-dependent and PKC-dependent manner. (J Vasc Surg 2006;44:1076-84.)**

Clinical Relevance: **Intimal hyperplasia is the main cause for restenosis that complicates 10% to 30% of all such vascular procedures and 30% to 40% of endovascular procedures. This article provides some novel information about smooth muscle cell/endothelial cell interaction, one of the main regulators of vascular remodeling and intimal hyperplasia. The role of endothelial cell/smooth muscle cell interaction cannot be studied well in vivo because these interactions cannot be distinguished from other factors that coexist in vivo, such as flow dynamics, matrix proteins, inflammatory factors, and interactions with other cells in the vascular wall and in the bloodstream. In this work, we use pressure as a triggering stimulus to alter in vitro endothelial behavior and identify important changes in endothelial regulation of smooth muscle cell biology. The pathways involved in this process and discussed in this article could ultimately be used to manipulate endothelial cell/smooth muscle cell interaction in clinical disease.**

Despite significant medical advances, intimal hyperplasia remains a major cause of failure of endovascular and vascular reconstructions. Although numerous factors affect intimal hyperplasia, endothelial (EC) and vascular smooth muscle cell (SMC) interaction may be one of the most significant. The in vivo study of EC/SMC cross-talk is complex because it is impossible to separate the pure effects of EC/SMC interaction from all the other dynamic, humoral, cellular, and inflammatory factors that coexist in vascular environments in vivo. In vitro studies that use coculture models could theoretically achieve such separation; however, in most such models, ECs paradoxically stimulate SMC migration[1-4] and increase SMC proliferation.[5-7] In other words, in conventional coculture, ECs exhibit an aggressive form different from what is usually observed in vivo. In vitro, cocultured ECs promote rather than control SMC growth.[1,2]

We recently created a pressurized coculture model to study EC interaction with SMC in a pressurized environment. Pressure is known to affect EC growth in a bimodal fashion. Typically, ECs at lower pressures (60 mm Hg, /20 mm Hg, 100 mm Hg,/60 mm Hg or ≤80 mm Hg) proliferate more rapidly than ECs in atmospheric pressure, whereas higher pressures (cyclic 140 mm Hg/100 mm Hg

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or static 100 to 160 mm Hg) inhibit EC growth.[8] We exposed EC/SMC cocultures to 130 to 135 mm Hg ambient pressure, choosing in this way to study the inhibitory growth effect of pressure in the context of coculture in an effort to delineate pathways that might prevent intimal hyperplasia.

Our laboratory has previously developed an EC/SMC coculture model by plating SMCs in six-well plates and placing inserts on top of these wells. The inserts have endothelial cells growing on semipermeable membranes that pass nutrients but do not allow cell contact. In this way, ECs and SMCs share the same media and can have humoral interaction but cannot physically touch each other. Using this model, we have previously reported that cocultured SMCs in ambient environment grew more rapidly than monocultured SMCs. When cocultured SMCs were placed in a high-pressure environment, the growth-promoting effect caused by ECs was reversed.[9]

Pressurized cocultured SMC exhibited decreased cell counts by Coulter counter compared with cell counts observed in pressurized monocultured SMC, ambient pressure monocultured SMC, or pressurized SMC in coculture with ECs. This decrease in cell counts was accompanied with decreased c-myc levels in pressurized cocultured SMCs, suggestive of decreased proliferative activity.[10] The same pressurized cocultured SMCs also exhibited increased apoptotic activity assessed by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining fluorescence microscopy as well as acridine-orange staining.[10,11]

A similar growth pattern was reproduced in recipient SMCs in ambient pressure when conditioned media from control ECs, pressurized ECs, and control and pressurized SMC donor cultures were transferred to the recipient SMCs.[10] In such studies, media from control ECs caused the highest increase in growth, and media from pressurized ECs cause the most prominent growth inhibition in the recipient SMCs.[10] These previous data suggests that pressure converts the endothelial behavior towards the cocultured SMC from an aggressive one seen in conventional in vitro environment to a regulatory, vessel-friendly one seen in our pressurized model.

In the present article, we report our test of the hypothesis that this transferable factor is some molecule related to nitric oxide (NO), postulating that the dramatic effects seen in pressurized coculture are more due to endothelial changes by pressure than due to myocytic humoral changes by pressurized coculture. We assessed indirectly the effects of pressure and EC coculture on NO, a significant effector known to be influenced by mechanical forces.[12,13] We focused on endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) regulation and evaluated NOS levels in EC cultures and SMC monocultures and cocultures (with EC) in different pressure environments. We also evaluated the effect of NOS activity inhibition by *N*-nitro-L-arginine-methyl ester (L-NAME) on the effects of conditioned medium from pressurized ECs and SMCs that is then transferred to separate SMC monocultures.

Finally, we hypothesized that if eNOS was changed by pressure, this effect might be mediated by extracellular regulated kinase (ERK) or protein kinase C (PKC) activation, since the literature suggests that vascular and other cells respond to mechanical stimuli such shear stress and strain through mitogen-activated protein kinase (MAPK) or PKC activation.[14-16] We performed additional studies to test this further hypothesis.

MATERIALS AND METHODS

Pressure apparatus. The chamber used for pressurized cultures has been described elsewhere.[10] This is a custom-made chamber made of stainless steel that fits into a cell culture incubator. A carefully regulated gas mixture creates a humidified culture environment with 5% carbon dioxide at 37°C. The pressure levels in the pressure chamber can be easily adjusted according to the experimental design and were set here at 130 to 135 mm Hg. The literature suggests that pressure affects ECs in a bimodal fashion. Typically, ECs at lower pressures (60 mm Hg/20 mm Hg, 100 mm Hg/60 mm Hg or mean ≤80 mm Hg) proliferate more rapidly than ECs in atmospheric pressure, whereas higher pressures (cyclic 140 mm Hg/100 mm Hg or static 100 to 160 mm Hg) inhibit EC growth.[8] The inhibitory effect of high pressure at 130 to 135 mm Hg has been validated in our model in previous experiments.[10] We therefore now used the same pressure regimen to test the hypothesis underlying the present investigation. Pressure was constantly measured by an analog pressure monitor. Chamber conditions were monitored in both pressure environments with regular media testing for pH and pCO_2.

Cell cultures. Rat aortic endothelial and smooth muscle cells were harvested and grown (8 to 15 passages) from primary culture in Dulbecco Modified Eagle's Medium (DMEM) supplemented with penicillin, streptomycin, and 10% or 5% calf serum.

EC/SMC cocultures. The coculture model has been described previously.[9,10] In this project, we used the coculture model only for the experiments designed to measure iNOS levels. In brief, endothelial cultures were established by plating ECs on a 15-μm-thick polycarbonate membrane with 3.0-μm pores (Corning Costar Brand Transwell Plate inserts, Corning, NY) at a subconfluent density of 150,000 cells/well. SMCs were plated separately at density of 50,000-cells/well on six-well plates (Corning). After overnight incubation, the inserts with attached ECs were transferred to six-well plates to form EC/SMC cocultures.

SMC cell baseline density was assessed by cell counting with a Coulter counter before coculture formation at day 0. One milliliter of complete medium was placed in each insert and 2 mL in each coculture well, and 3 mL of complete medium was placed in each well of independent SMC culture. The SMCs were not in direct contact with the ECs, but they shared the same media and nutritional factors.

Studies with conditioned media. Smooth muscle and endothelial cells were seeded in T-75 flasks, treated with fresh media containing 10% fetal bovine serum, and

allowed to achieve 70% confluence. The culture medium was then changed to 8 mL of new medium containing 5% fetal bovine serum, and flasks were then maintained at control ambient pressure or in pressurized (130 to 135 mm Hg) chambers. We chose to expose donor cells to pressure for 5 days, because in previous work,[9,10] we achieved maximal difference in cell counts or TUNEL-positive cell counts in pressurized EC/SMC cocultures after exposing the cells to pressure for 5 days.

No media changes were performed during this period. Pressurized and ambient donor ECs and SMCs in T-75 flasks were treated separately with 2.5 to 5 mM of L-NAME, which inhibits eNOS activity and NO formation. In choosing to study the effects of 2.5 to 5 mM L-NAME, we were guided by other authors' reports of similar experiments with L-NAME using similar coculture models.[17] We tested 2.5 mM and 5 mM concentrations. In each case, we observed similar responses and did not observe obvious toxic effects in either endothelial or smooth muscle cell cultures. There were no floating cells, and a lactate dehydrogenase activity assay showed that lactate dehydrogenase activity was not increased.

In addition, cells treated with L-NAME exhibited similar if not higher cell counts than control cells. Conditioned medium at the end of the 5-day period was diluted 8:1 with fresh medium (5% fetal bovine serum) and transferred to six-well plates containing recipient SMCs plated overnight at 10,000 cells/well. Conditioned medium was changed every other day. Recipient SMCs were treated for 5 days. At the end of this period, recipient SMCs were trypsinized, and cell number was assessed by counting cells using a Coulter counter.

Western blot analysis for iNOS and eNOS. In previous work, decreases in cell counts of the cocultured SMCs occurred on day 3 and were maximal on day 5 of exposure to pressure. We observed a similar pattern when we evaluated SMC apoptosis by TUNEL.[9,18] We believe that pressure-induced changes in intracellular and intercellular signals, including changes in eNOS/iNOS, must occur earlier than the maximal effects on cell number or apoptosis, or both, if they are to mediate these effects. We therefore measured eNOS and iNOS levels at day 3.

We first evaluated iNOS regulation by pressure, EC coculture, or the combination of pressure and coculture by exposing SMCs to four conditions: (1) monocultures in a control environment (SMC/0), monocultures at 130 to 135 mm Hg pressure (SMC/0-P), EC coculture in control environment (EC/SMC), and pressurized EC coculture at 130 to 135 mm Hg (SMC/EC-P).

SMC proteins were isolated by scraping SMC at 4°C using 30 μL of a commercially available preparation of protease inhibitors that contains AESBF-[4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride]-serine proteases, aprotinin, bestatin hydrochloride, E-64-[n(trans-epoxysuccinyl)-L-leucine 4 guanidinobutylamide], and pepstatin (100 μL/3 wells, Sigma/Aldrich # P8340, St. Louis, Mo) mixed with 3 mL of the lysis buffer.

Cells were homogenized for 1 minute, and then incubated at 4°C. Protein content was quantified by spectrophotometry. Samples (with equal protein content) were resolved on 10% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Mass). Membranes were incubated overnight at 4°C with 5% milk in phosphate-buffered saline (PBS) to block nonspecific binding sites, rinsed in 0.1% Triton X-100 in PBS (PBST), and incubated for 2 hours with primary antibody against rat iNOS (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) diluted at 1:200 to 1:300. Blots were then rinsed for 10 minutes three times with PBST and incubated in secondary antibody (1:1000 in 5% milk) for 1 hour. After washing the membranes again three times in PBST for 10 minutes, we visualized antibody binding using the enhanced chemiluminescence method (Pierce, Rockford, Ill). For quantification, films were digitally scanned using a ScanJet 6300C scanner (Hewlett Packard, Palo Alto, Calif).

Membranes were also stripped and reprobed for actin. Actin band density then served as a protein loading control. Image scans were analyzed with the UN-SCAN-IT gel Automated Digitizing System 5.1 (Silk Scientific, Inc, Orem, Utah). Protein band density was normalized according to actin band density derived from the analysis of the same endothelial or myocytic protein. All exposures used for analysis were within the linear range of the system.

In separate studies, we evaluated eNOS regulation by pressure in endothelial cells. For these studies, we compared ECs monocultured under control or 130 to 135 mm Hg pressure for 3 days. EC cultures were treated with: (1) dimethylsulfoxide (DMSO) (control), (2) 100 nM of light-activated calphostin C, a specific PKC inhibitor, (3) 20 μM of PD98059 an inhibitor of MEK (mitogen activated ERK-activating kinase) activity that prevents subsequent ERK activation, or (4) 2.5 mM L-NAME to inhibit NO. Endothelial protein was harvested on day 3 using the same protocol used for myocytic protein and then analyzed for eNOS after a 2-hour incubation in primary antibody against rat eNOS (1:200 dilution; Santa Cruz Biotechnology), followed by a 1-hour incubation in secondary antibody (1:1000 dilution in 5% milk).

Western blot analysis for phosphorylated (activated) ERK. Endothelial cells were exposed to ambient and high pressure (130 to 135 mm Hg) for 2, 4, and 6 hours before analysis for activated phosphorylated-ERK (p-ERK) and ERK protein. Protein was harvested using the previously described protocol. Cellular protein was then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and probed with antibodies specific for the phosphorylated forms of ERK1/2 (pERK1/2, dilution 1:1000; Cell Signaling Technology, Danvers, Mass) to determine the activation status of ERK by pressure. As a control, the total amount of ERK1/2 was determined by Western blot analysis using an anti-ERK1 antibody that also cross-reacts with ERK2 (1:1000; Cell Signaling Technology). Goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase was used

as a secondary antibody, and the membrane was developed by a chemiluminescent detection method as explained above. The band density ratio of activated content of ERK1/2 to total ERK from each condition was also normalized to this first experimental condition (2 hours of exposure to no pressure).

In separate experiments, ECs were treated with 100 nM of DMSO (control) or light-activated calphostin C (to achieve PKC inhibition) and exposed to ambient pressure and a high-pressure environment for 4 hours. Western blot analysis of the endothelial protein assessed the effect of calphostin C on ERK1/2 activation in these two different pressure conditions.

Statistical analysis. Each experiment involving cell counts was done in independent triplicate wells. The cell count/well reflects the arithmetic average from three to four counts by Coulter counter. Each experiment was repeated three times for nine separate and independent results, which were considered as n = 9 and pooled for statistical analysis. Western blot analysis for each separate experiment was repeated between 4 and 12 times, depending upon the particular study. Data are expressed as ± SEM. Western blot for iNOS was repeated three times; however, the observed difference reached statistical significance at $P \leq .01$.

For the studies involving conditioned media and cell counts, two comparisons were performed separately in the control and L-NAME group. The first comparison was between the SMCs treated with media from control SMCs, and SMCs treated with media from pressurized SMCs. The second comparison was between SMCs treated with media from control ECs, and SMC treated with media from pressurized ECs. This set of studies was analyzed by *t* test with Bonferroni correction for multiple tests. According to the Bonferroni correction for two comparisons, values of $P < .018$ were considered statistically significant. Statistical analysis of the Western blots was performed using *t* test with Mann-Whitney modification or analysis of variance (ANOVA) as applicable. $P \leq .05$ was considered statistically significant.

RESULTS

Nitric oxide inhibition attenuates growth inhibitory properties of pressurized ECs. We first performed control studies with SMCs treated with conditioned media from atmospheric (nonpressurized) SMCs and ECs and media from pressurized ECs and SMCs. Media from pressurized ECs decreased the cell count of SMCs by 33.5% compared with the effects on SMC cell counts of media from control ECs. This difference was statistically significant ($P < .01$, n = 9) (Fig 1, *a*). The results from these control groups are consistent with previous published observations.[9,10]

This pressure-induced, endothelially derived, growth-inhibiting effect was abolished when donor cells were pretreated with L-NAME ($P = .8$, n = 9) (Fig 1, *b*). The growth inhibiting effect persisted when L-NAME was added to recipient cells (Fig 1, *c*). Pressure caused no changes in the trophic properties of the conditioned media that were originally derived from SMC. L-NAME did not alter this effect.

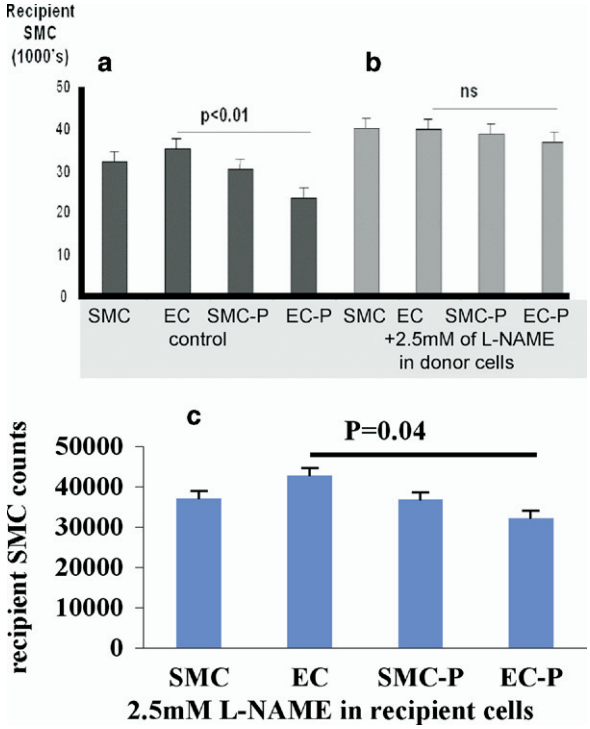

Fig 1. Conditioned media from pressurized endothelial cells *(EC-P)* decreased the cell counts of recipient smooth muscle cell *(SMC)* cultures compared with conditioned media from ambient endothelial cells *(EC)* ($P \leq .001$). Media from atmospheric *(SMC)* and pressurized *(SMC-P)* had similar effect **(a)**. This reversed trophic effect of conditioned medium from EC-P was abolished when the donor cells were pretreated with 2.5 to 5 mM of *N*-nitro-L-arginine-methyl ester *(L-NAME)* a nitric oxide inhibitor **(b)**. The addition of L-NAME to pressurized endothelial media after its removal from the recipient cells had no effect on the properties of the pressurized media **(c)**. Data are expressed as ± SEM.

In previous articles, we observed that pressure decreases the growth of EC-cocultured SMCs. This growth effect was also reproduced in separate SMC cultures treated with conditioned media from pressurized ECs.[10] Taking these previous observations together with this new result suggests that the growth-inhibiting transferable factor is blocked by eNOS inhibition in donor ECs and not affected by blocking iNOS in the recipient cultures.

Pressure induces eNOS in a PKC-dependent and ERK1/2-dependent manner. Because NO seemed a likely candidate to contribute to the trophic effect of pressurized ECs on SMC growth, we sought to determine whether pressure induces eNOS and whether PKC or ERK activation could contribute to this effect. We did not directly measure NO but concentrated on investigating eNOS regulation by pressure. Fig 2, *A* demonstrates a typical Western

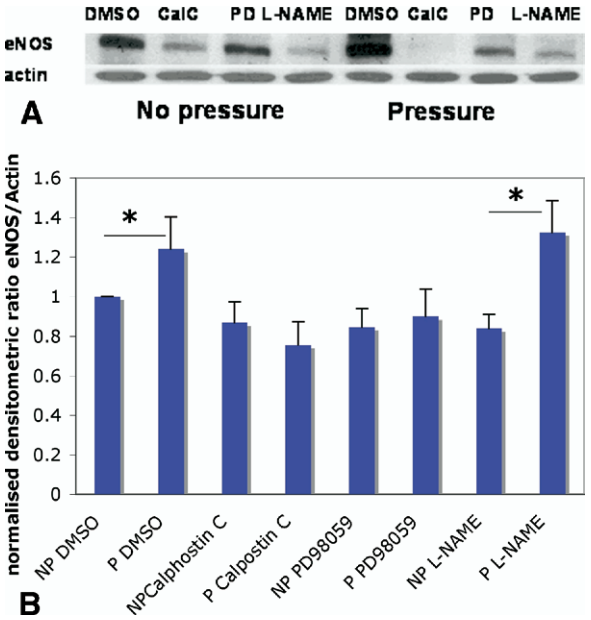

Fig 2. Endothelial cell (EC) protein exposed to ambient *(NP)* and 135 mm Hg pressure *(P)* was analyzed for endothelial nitric oxide synthase *(eNOS)*. Endothelial cells were treated with dimethylsulfoxide *(DMSO)* (vehicle control), calphostin C *(CalC)* to block protein kinase C, PD98059 *(PD)* to block extracellular signal-regulated kinase, or *N*-nitro-L-arginine-methyl ester *(L-NAME)* to inhibit nitric oxide. **A,** Shows typical western blot for eNOS from EC protein from ambient and high-pressure conditions. **B,** Pressure increased eNOS in the control and L-NAME group (**P* = .04 and ***P* = .02, n =12). Calphostin C and PD98509 blocked this effect, suggesting that pressurized EC exhibit increased levels of eNOS via a PKC and ERK dependent pathways. Data are expressed as ± SEM.

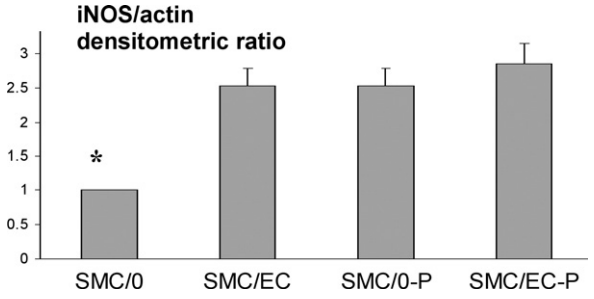

Fig 3. Smooth muscle cell (SMC) protein was analyzed for inducible nitric oxide synthase *(iNOS)*. Endothelial cell *(EC)* coculture and pressure increased to a similar degree iNOS levels. However, the combination of the two conditions in the form of pressurized coculture had no additive effect on iNOS (**P* < .01 bars 2, 3, and 4 vs first bar, n = 3 experiments). *SMC/0,* SMCs grown in control conditions; *SMC/EC,* SMCs in coculture with ECs; SMC/0P control SMCs exposed to pressure; *SMC/EC-P,* SMCs in pressure coculture with ECs. Data are expressed as ± SEM.

blot for eNOS from ECs in atmospheric and pressurized conditions treated with DMSO (control), calphostin C (PKC inhibitor), PD98059 (MEK inhibitor that prevents ERK activation), or L-NAME. Fig 2, *B* summarizes the densitometric analysis of 12 similar Western blots. Exposure to a high-pressure environment increased the levels of endothelial eNOS by 24% ± 1.6% (*P* = .04, n = 12). This effect did not change when cells were treated with L-NAME, (32% ± 1.6% increase, *P* = .02); however, the effect of pressure on eNOS was abolished when ECs were treated with calphostin C or PD98059.This observation suggested that pressure-induced upregulation of eNOS requires ERK and PKC activity.

Pressure and coculture and their combination promote myocytic NOS similarly. We sought in parallel to assess how SMC iNOS is regulated by pressure or EC coculture, or both. We measured iNOS protein levels via Western blots of SMC protein derived from SMCs grown in control conditions (SMC/0), in coculture with ECs in control conditions (SMC/EC), from SMCs exposed to pressure (SMC/0-P), and from SMCs in pressurized coculture with ECs (SMC/EC-P). Application of pressure or the presence of endothelial cells similarly increased 2.5-fold (*P* < .05) myocytic iNOS compared with baseline iNOS in monocultured SMC at ambient pressure (SMC/0). However, combining both coculture and increased pressure (SMC/EC-P) had no additive effect on the protein increase (Fig 3).

We have previously[9,10,17] demonstrated that cocultured SMCs in pressurized EC/SMC cocultures exhibit dramatically decreased proliferative and enhanced apoptotic activity compared with monocultured control SMCs and also compared with EC-cocultured SMCs at ambient pressure and monocultured pressurized SMCs. Because we now demonstrate that iNOS levels do not change among cocultured, pressurized, and pressurized cocultured SMC, it seems likely that the increased SMC apoptosis and decelerated SMC proliferation in pressurized SMC coculture that we had previously described in comparison with all three other SMC groups may be linked to upregulated eNOS levels in pressurized ECs and cannot be explained by myocytic iNOS regulation.

Pressure induces early phosphorylation of ERK1/2 in a PKC-independent manner. To further investigate the pressure regulation of endothelial ERK, we analyzed endothelial protein harvested from endothelial cells exposed to atmospheric pressure or to 135 mm Hg for 2, 4, and 6 hours. ERK activation in response to various stimuli has been described either downstream of PKC activation or independent of the PKC pathway.[19-24] To explore these two possibilities, we treated ECs with DMSO (control) or light-activated calphostin C before exposure to pressure and assay for ERK activation.

A typical Western blot analysis for p-ERK1 and p-ERK2 is shown in Fig 4, *A*. Total ERK levels were constant throughout all conditions consistently in all four experiments. However, we observed increased activation of ERK1 and ERK2 (p-ERK 42 and 44) in re-

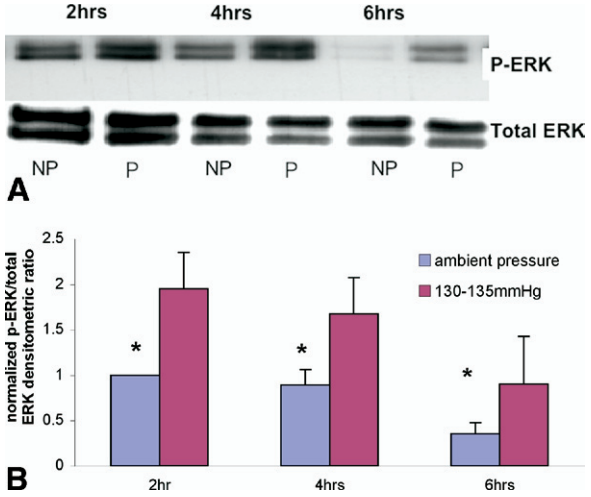

Fig 4. A, Typical Western blot analysis and summary of four such Western blots of endothelial protein exposed for 2, 4, and 6 hours to control pressure *(NP)* and high pressure at 130 to 135 mm Hg *(P)*. **B,** Pressure in each time-point increased activated phosphorylated extracellular signal-regulated kinase *(p-ERK)* 1/2 content over total ERK1/2 compared with control conditions (*$P < .02$, analysis of variance, n = 4). Data are expressed as ± SEM.

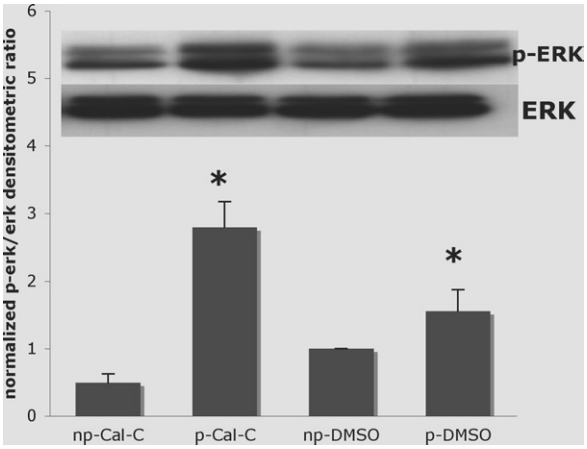

Fig 5. Summary of 4 similar Western blot analyses for phosphorylated extracellular signal-regulated kinase *(p-ERK)* from endothelial cells exposed to ambient *(np)* and 130 to 135 mm Hg pressure *(p)* for 4 hours. Cells were treated with 100 nm calphostin C *(Cal-C)* to block protein kinase C activation. Control cells were treated with dimethylsulfoxide. Each Western blot demonstrated a similar effect: endothelial cell treatment with calphostin C does not attenuate the increase in ERK activation by pressure. Indeed, there is a fivefold increase in p-ERK in pressurized endothelial cells treated with calphostin C compared with levels of ERK activation in endothelial cells treated with calphostin C at ambient pressure ($P < .05$ by analysis of variance). These findings suggest that pressure-induced ERK activation is independent of protein kinase C.

sponse to pressure at each time point of experimental exposure. Fig 4, *B* summarizes the densitometric assessment of activated ERK expressed as the ratio of p-ERK to total ERK normalized to the densitometric ratio obtained from the first condition (2 hours of ambient pressure). At each time point, 130 to 135 mm Hg of pressure caused a 1.5-fold to 2.3-fold increase in p-ERK (42 and 44) compared with ambient pressure (ANOVA $P = .02$; n = 4).

In separate experiments, PKC activation was inhibited by treating cells with calphostin C and exposing them to ambient and high pressure for 4 hours. PKC inhibition did not decrease the pressure-induced activation of ERK (p-ERK expression). We noticed a fivefold increase of p-ERK by high pressure in ECs treated with calphostin C ($P < .05$ ANOVA, n = 4) (Fig 5). This suggests that although pressure increases eNOS through activation of ERK and PKC, PKC activation does not affect ERK. Thus, in these particular conditions in ECs, ERK activation in response to increased pressure is not an event downstream of PKC activation.

DISCUSSION

In this study, we have been able to better elucidate the pressure-induced events that may modulate SMC proliferation and EC and SMC interaction in a pressurized coculture model. Specifically, we observed that pressure causes pressurized ECs to produce transferable mediators that, through conditioned media, decrease the cell count of recipient SMCs compared with conditioned media from ECs grown in ambient conditions. This paracrine effect seems likely to be NO-dependent, because the antiproliferative effect of pressurized conditioned media is abolished when donor ECs are treated with L-NAME. Furthermore, pressure increased eNOS levels in pressurized ECs, through the independent activation of ERK and PKC. ERK activation was not PKC-dependent.

Role of myocytic NOS in pressurized and cocultured SMC growth changes. Myocytic NO regulation does not seem to be a major growth effector in our coculture model, or at least is not likely to be linked to the pressure-induced changes observed in endothelially cocultured SMCs. Although pressure and endothelial coculture do cause iNOS induction in SMCs compared with baseline conditions, the intensity of this effect is similar in both conditions. Moreover, the combination of both conditions in the form of pressurized coculture does not cause any additional increase in iNOS levels (Fig 3). This similar iNOS induction in all three conditions compared with control SMC growing in monocultures in a control pressure (baseline) environment does not correlate with the observation that each one of these conditions influences SMC proliferation and apoptosis differently, leading to growth promotion in EC-cocultured SMCs, no significant growth change in pressurized SMCs, and maximal growth inhibition in pressurized cocultured SMCs.[9,10] Thus, iNOS changes are probably not responsible for the different growth patterns displayed by pressurized, cocultured, and pressurized-cocultured SMC.

NOS induction by pressure and endothelial cells. In vivo, NO is produced mainly in constitutive form by

eNOS and in inducible form (iNOS) by SMC, glial, and other cells. NO regulates vascular tone,[25] downregulates endothelin,[26] increases SMC apoptosis,[16,27,28] and decreases SMC proliferation.[27,29,30] Shear stress, cyclic strain, and repetitive deformation[31,32] consistently induce NO directly or increase NOS levels from vascular cells. However, the effects of pure pressure on NO induction outside the context of clinical hypertension are not well understood. Our data suggest that pressure induces not only eNOS but also iNOS synthesis in SMCs. Moreover, it is interesting that the sole presence of ECs causes induction of iNOS in cocultured SMCs. This effect could be mediated through endothelin activation, as iNOS stimulation by endothelin has been observed in other cell types such as chondrocytes and primary glial cells.[33-36]

The NO effect is transferable through endothelially conditioned medium. Conditioned media from pressurized ECs exerts growth-inhibitory effects on recipient SMCs compared to the effects of media from control ECs. This transferable growth-inhibitory effect is abolished when donor cells are treated with L-NAME. Thus, the transferable factor from pressurized ECs that causes SMCs not to grow is probably linked to eNOS activity. NO is probably the responsible initial pressure-induced mediator, although we have not measured NO levels in our system. However, NO is not transferable through the conditioned media because it is a very unstable molecule with a half-life of only 2 to 4 seconds before it converts to an inactive molecule. Thus, NO is not likely to be maintained in active form in conditioned media.

The transferable factor actually present in the media of pressurized ECs is likely to be some other factor regulated by endogenous NO. This factor is derived from ECs and not from SMCs, because media from pressurized myocytes causes no change in the growth pattern of recipient cells compared with myocytic media from control conditions. Furthermore, pretreatment with L-NAME does not affect the mitotic properties of the SMC-derived media. Further characterization of this endothelial transferable factor warrants future investigation.

Finally there is a significant body of emerging literature demonstrating the effects of peroxynitrites on vascular cells. These extremely unstable molecules are produced during conditions of increased oxidative stress. Peroxynitrites are known to cause accelerated SMC apoptosis and play a significant role in vascular remodeling.[37] Although other mechanical forces such as shear stress[38,39] are known to cause oxidative stress, the effects of pressure on oxidative stress is not yet known. It is quite likely though that peroxynitrites produced by pressurized ECs may play a role in our system. However because these molecules are very unstable, they cannot be maintained in the conditioned media and thus if they do cause changes in our system, these changes occur through the regulation of another more stable transferable intermediate factor.

Endothelial NOS dependence on ERK and PKC pathways. Our present results suggest that both PKC and ERK1/2 activation are likely to be involved in mediating the pressure effect on eNOS. Moreover, in contrast to some previous reports in response to other stimuli, pressure-induced endothelial ERK1/2 activation does not seem to require PKC activation.

PKC affects eNOS and NO activity in opposite ways in different settings. PKC activation may inhibit NO release.[40-42] However, PKC activation induced mechanically, hypoxically, or through platelet-derived growth factor may induce eNOS synthesis or NO release in endothelial and other cells,[43-45] in a manner similar to our observations.

Our results also suggest that pressure activates ERK1/2 that then induces eNOS. ERK phosphorylation usually occurs via a PKC-dependent pathway[21,46] and a PKC-independent pathway.[47] Mechanical stresses such as shear stress and cyclic strain have been reported to cause ERK activation via PKC-dependent pathways[43,48,49] that lead to cell proliferation. In our model, however, pressure-induced activation occurs through a PKC-independent pathway. This observation is consistent with other reports of pressure-induced ERK activation in pressurized rabbit aortic tissue ex vivo through a PKC-independent pathway.[50]

We studied pressure and coculture-induced NO-related effects on SMC indirectly by assessing regulation of eNOS and iNOS expression. We have not directly assayed NO release or NOS activation, nor have we evaluated other potentially important signals that also induce NOS activation in response to other stimuli and might be relevant here. For instance, the PI3-kinase-Akt pathway is sensitive to mechanostransduction,[51,52] activates NO release by phosphorylating eNOS,[53] and influences ET1-induced NOS via the p38 pathway.[54] This could also conceivably be involved in the effects described here, because we believe that ET1 may be responsible for the iNOS increases caused by EC coculture (Fig 3). Similarly PKA is also sensitive to mechanotransduction but causes eNOS activation and NO release in a PI3-AKT–independent pathway.[55] Finally other pathways induced by mechanotransduction (shear stress), such as Src that induces eNOS expression,[56] but not eNOS activation and NO release,[39] have also not been examined in our coculture model. Future studies will be necessary to determine whether these other signal proteins might also be important in our system.

CONCLUSION

Our results demonstrate that pressure increases eNOS levels in pressurized ECs though activation of separate PKC and ERK signals. These changes seem to affect the paracrine properties of EC, enhancing the ability of ECs to inhibit SMC growth. These observations emphasize the likely importance of extracellular pressure as an independent effector of vascular endothelial behavior and the differences in mechanotransduction of pressure effects on EC compared with the effects of shear stress and strain. In previous studies, we have seen that pressure causes unique effects on endothelial and smooth muscle cells, increasing apoptosis and decreasing EC and SMC proliferation[9,10,18] in a manner opposite to the predominantly trophic and anti-apoptotic effects of cyclic strain as extrapolated from vast

literature dedicated to cyclic strain.[51,57-59] In this study, we saw that ERK1/2 activation was induced through a different pathway. Thus, we believe that pressure as a separate hemodynamic entity merits more thorough investigation. Because EC/SMC interactions are important regulators of atherogenesis in vivo, PKC-dependent and ERK-dependent stimulation of endothelial NO release in response to extracellular pressure may play an important role in vascular remodeling and the formation of intimal hyperplasia.

AUTHOR CONTRIBUTIONS

Conception and design: AV
Analysis and interpretation: AV, MB
Data collection: AV, YJ, RR
Writing the article: AV
Critical revision of the article: MB
Final approval of the article: AV, MB
Statistical analysis: AV, RR
Obtained funding: AV, MB
Overall responsibility: AV

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