

Arterial pO₂ stimulates intimal hyperplasia and serum stimulates inward eutrophic remodeling in porcine saphenous veins cultured *ex vivo*

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Abstract *Ex vivo* culture of arteries and veins is an established tool for investigating mechanically induced remodeling. Porcine saphenous veins (PSV) cultured *ex vivo* with a venous mechanical environment, serum-supplemented cell-culture medium and standard cell-culture conditions (5% CO₂ and 95% balance air ~140 mmHg pO₂) develop intimal hyperplasia (IH), increased cellular proliferation, decreased compliance and exhibit inward eutrophic remodeling thereby suggesting that nonmechanical factors stimulate some changes observed *ex vivo*. Herein we explore

the contribution of exposure to greater than venous pO₂ and serum to these changes in cultured veins. Removing serum from culture medium did not inhibit development of IH, but did reduce cellular proliferation and inward eutrophic remodeling. In contrast, veins perfused using reduced pO₂ (75 mmHg) showed reduced IH. Among the statically cultured vessels, veins cultured at arterial pO₂ (95 mmHg) and above showed IH as well as increase in proliferation and vessel weight compared to fresh veins; veins cultured at venous pO₂ did not. Taken together, these data suggest that exposure of SV to arterial pO₂ stimulates IH and cellular proliferation independent of changes in the mechanical environment, which might provide insight into the etiology of IH in SV used as arterial grafts.

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1 Introduction

Under the appropriate culture conditions, conduit blood vessels cultured *ex vivo* remain viable and vasoactive for days (Porter et al. 1996b; Clerin et al. 2003; Gusic et al. 2005a,b). A primary benefit of these *ex-vivo* models is that they afford much better control and monitoring of the mechanical and chemical environments than possible with vessels *in vivo* while allowing the study of whole-vessel behavior not captured in cell culture. Capitalizing on these strengths of the *ex-vivo* culture systems, investigators have used them to study the independent contribution of mechanical factors (e.g., flow, pressure, and pulsatility) (Clerin et al. 2003; Han et al. 2003; Davis et al. 2005; Gusic et al. 2005a,b; Han et al. 2006; Gleasonm et al. 2007; Wayman et al. 2008; Yao et al. 2009) on different aspects of vascular remodeling and to understand

how multiple mechanical factors (e.g., pressure and axial loading) interactively regulate vascular remodeling (Nichol et al. 2005, 2009; Lawrence and Gooch 2009a,b). Many aspects of mechanically induced remodeling of blood vessels originally observed *in vivo* have also been observed in vessels cultured *ex vivo* including pressure-induced hypertrophy (Gusic et al. 2005a,b), axial loading-induced growth/remodeling (Clerin et al. 2003; Nichol et al. 2005, 2009; Gleason et al. 2007; Lawrence and Gooch 2009a,b), and flow-induced changes in luminal area (Nichol et al. 2005, 2009).

While much of the observed remodeling can be attributed to mechanically induced changes, some changes in the cultured vessel's dimension, composition, activity and mechanical properties appear to occur independently of changes in the mechanical environment. For example, vessels cultured *ex vivo* with mechanical environments similar to their native *in vivo* environments exhibit hypertrophy (Clerin et al. 2003; Gusic et al. 2005a,b), lengthen (Nichol et al. 2005, 2009; Gleason et al. 2007; Lawrence and Gooch 2009a,b), eutrophically remodel (Gusic et al. 2005a,b), develop intimal hyperplasia (Gusic et al. 2005a,b), as well as alter their extracellular matrix content (Gusic et al. 2005a,b), matrix metalloproteinase activity (Nichol et al. 2009) and biomechanical properties (Clerin et al. 2003; Gusic et al. 2005a,b; Lawrence and Gooch 2009a,b). There are at least three general explanations for these changes. First, it is possible that the *ex-vivo* culture environment fails to adequately replicate important aspects of the native mechanical environment and these differences stimulate the observed changes. Second, processes associated with the harvest of the vessel such as surgical trauma including disruption of the vasa vasorum or denervation might account for the observed remodeling. Here we conducted experiments to explore a third possibility—i.e., non-mechanical aspects of the *ex-vivo* culture system might account for some of the observed changes in cultured vessels. Saphenous veins (SV) are attractive vessels to use for these studies because (1) they are well studied in various *ex-vivo* culture systems (Porter et al. 1996a,b, 1998a,b, 1999, 2001, 2002; Gusic et al. 2005a,b), (2) they exhibit pronounced changes in culture which do not appear to be due to the mechanical environment (Porter et al. 1996b, 1999), and (3) their remodeling is an important component of both the success and failure of vein grafts used in coronary artery bypass grafting and peripheral revascularization (Bourassa 1991; Motwani and Topol 1998).

SV grafts rapidly develop intimal hyperplasia (IH) after a few weeks of grafting into the arterial circulation after coronary artery bypass grafting (CABG) (Motwani and Topol 1998). Although this initial IH does not significantly impede blood flow, it does provide the foundation for subsequent atherosclerosis and thrombosis (Motwani and Topol 1998). Thus inhibition of this initial and rapid IH is an attractive target

for improving vein graft performance. It is often suggested that this IH may be a pathological response to abrupt change in the hemodynamic environment as the vein goes from the relatively mild venous mechanical environment to the arterial environment with its increased circumferential tension, cyclic stretching, and high flow-induced stresses caused by the higher blood pressure and flow velocities (Dobrin and Endean 1989; Motwani and Topol 1998). To investigate the stimulus for IH further, a number of studies by others and our laboratory have employed porcine SV (PSV). We have previously reported that perfused PSV develop significant IH during *ex-vivo* culture using 5% CO₂/95% balance air (~140 mmHg pO₂ or normal cell culture atmospheres) and serum-containing medium (Gusic et al. 2005a,b). Collectively data from our prior work (Gusic et al. 2005a,b) and others (Porter et al. 1996a,b, 2001) suggest that the development of this observed IH in cultured PSV does not require exposure to an environment mimicking *in vivo* arterial mechanical conditions. In contrast, arterial levels of mechanical forces reduced the observed IH (Porter et al. 1996b; Gusic et al. 2005a,b). In addition, PSV cultured *ex vivo* under venous mechanical conditions exhibit inward eutrophic remodeling and reduced compliance (Porter et al. 1996b; Gusic et al. 2005a,b).

Based on these observations from SV cultured *ex vivo*, we speculated that non-mechanical factor(s) modulate the development of IH in PSV cultured *ex vivo*. Serum contains potent mitogens for cultured vascular SMC, such as platelet-derived growth factor, which is suspected to be involved in atherosclerosis (George et al. 1996). In addition to such biochemical factors, aspects of the chemical environment typically used for *ex-vivo* culture might stimulate IH. Cultured PSV are typically exposed to a dramatic increase in oxygen from venous levels (pO₂ = 40 mmHg) to that of 5% CO₂ balanced with air (pO₂ = 140 mmHg). In contrast to PSV, porcine arteries cultured *ex vivo*, which experience a smaller increase in pO₂, do not develop IH in culture (Clerin et al. 2002; Gusic et al. 2005a,b). Therefore, in this study, we sought to explore the role serum factors and elevated oxygen levels play in the changes in observed in PSV cultured *ex vivo*. This knowledge will aid in the design better *ex-vivo* culture systems and the interpretation of data from *ex-vivo* studies. While we are focusing on features of the *ex-vivo* environment that might induce remodeling in PSV, it should be noted that human SV (HSV) used as coronary artery bypass grafts *in vivo* are likely exposed to elevated platelet-derived growth factor (PDGF, due to platelet adhesion and thrombus formation) (George et al. 1996; Wang et al. 2005) and are exposed to elevated pO₂ due to exposure to arterial blood with a pO₂ of 95 mmHg. Thus results from these *ex-vivo* studies with PSV may provide insight into factors stimulating IH in HSV grafted into the arterial circulation (Schachner et al. 2006).

2 Materials and methods

2.1 Vessel harvest and preparation

All animal work was reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Pennsylvania and at the Ohio State University. PSV and femoral arteries were harvested aseptically from pigs (*Sus scrofa*). After harvesting, all vessels were washed in HEPES buffer (Invitrogen, Carlsbad, CA) and transported back to the laboratory in a gas-impermeable container with ~100 cc of Dulbecco's modified Eagle's medium pre-equilibrated with the desired gas mixture, representing either high or low pO₂ levels and immediately set up for culture. The time between vessel harvests to culture setup was typically less than 1 h.

2.2 Ex-vivo perfusion

The ex-vivo perfusion system has been previously described (Clerin et al. 2003; Gusic et al. 2005a,b; Nichol et al. 2005, 2009; Lawrence and Gooch 2009a,b) and is similar to those used by others (Porter et al. 1996b; Han et al. 2003, 2006; Davis et al. 2005; Gleason et al. 2007; Wayman et al. 2008; Yao et al. 2009). PSV isolated from 25–35 kg pigs were cultured in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, CA), 100 µg/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and 25 mM HEPES (all from Invitrogen Corp., Rockville, MD). A subset of vessels was perfused with serum-free medium, which contained 2% of bovine serum albumin (Sigma, MO).

Culture medium was typically equilibrated with a gas mixture consisting of 5% CO₂ / 95% air resulting in a pO₂ of ~140 mmHg, which is somewhat higher than ~95 mmHg found in the arterial circulation and much higher than the ~40 mmHg found in the venous circulation. Perfusion conditions consisted of steady, venous levels of flow (10 mL/min) and a pressure (25 mmHg) that was slightly greater than typical in vivo venous pressures. To test the response of PSV to lower oxygen environments, PSV were perfused with medium bubbled with a 10% CO₂/90% N₂ gas mixture. This resulted in a pO₂ of ~75 mmHg representing an intermediate between typical arterial and venous concentrations. The oxygen tension achieved was roughly half of that in typical, 5% CO₂/ air studies (~140 mmHg); but greater than in-vivo venous conditions (~40 mmHg). PSV perfused ex vivo with serum-free culture medium or with serum-containing culture medium at a reduced pO₂ were cultured at the same time as other PSV used to study the effects of mechanical factors on IH (Gusic et al. 2005a,b). Thus, select data from these published studies provide appropriate control

groups to compare the results presented here. All perfusion experiments were run for 7 days, which we have previously shown to be long enough for changes in PSV dimensions and mechanical properties to occur (Gusic et al. 2005a,b). At least five vessels were subjected to each experimental condition.

2.3 Histological analysis

At the completion of experiments, vein length and mass were measured, and sections of veins were fixed at zero pressure in either 70% ethanol or formalin overnight, dehydrated, embedded in paraffin, cut into 5 µm thick sections, and mounted on glass slides. Elastin staining (Accustain, Sigma, MO) was used according to manufacturer's instructions, and the intimal and medial areas of vein cross-sections, which were delineated by the external and internal elastic lamina, were measured using Scion Image (Scion, MD). Proliferating cells were stained with monoclonal mouse PC10 antibody recognizing proliferating cell nuclear antigen/HRP (PCNA, 1:1, DAKO). To account for the cell type which accounted for the IH, both SMC and EC were counterstained with markers identifying each cell type. SMC were identified with an antibody against the SMC lineage marker myosin heavy chain (SM-MHC), and EC were identified by antibodies against CD31. In addition the in situ Cell Death Detection, POD kit (TUNEL, Roche, IN) was used as directed. TUNEL and PCNA stained sections were counterstained with DAPI (Vector Labs, CA).

2.4 Wet weight, dry weight and collagen assay

Vessel segments (~2–3 cm long) were weighed to provide wet weight, lyophilized, and weighed to provide dry weight and stored at –20°C until processing. Frozen vessel segments were digested with papain (25 mg/ml papain, 10 mM cysteine in buffered solution, both from Sigma, MO) at 60°C for 16 h. Collagen content was determined using a modified version of the protocol of Woessner previously adapted for this tissue (Woessner 1961; Gusic et al. 2005a,b).

2.5 Vein Mechanics

At the completion of the one-week ex-vivo perfusion culture, transverse sections of the vein were taken for histology and the remaining vein segment was subjected to mechanical testing. As described previously (Gusic et al. 2005a,b), pressure and outer-diameter measurements of veins were recorded using a modified protocol of Cox et al. (1978). The medium in the vessel chamber was replaced with phosphate-buffered saline (PBS). Calcium-free Hanks' balanced salt solution (HBSS) from a second

reservoir was diverted to the vein by means of three-way stopcocks and a second peristaltic pump. A pressure head of approximately 80 mmHg was generated and the vein was allowed to incubate for 1 h at this transmural pressure. At the end of this period, the vein was preconditioned by subjecting it to 10 continuous cycles of inflation and deflation at a rate of 2 mmHg/s between 10 and 100 mmHg, and then three inflation responses were recorded at 1 mmHg/s between 10 and 100 mmHg. The vein was then pressurized again to 80 mmHg and norepinephrine (NE) (10^{-4} M, Sigma, MO) was added to the vessel bath and the vein was allowed to constrict for 2 min, after which the pressure was lowered to 10 mmHg and maintained at this level until no changes in diameter were observed. The vessel bath was drained, rinsed, and replaced with calcium-free PBS, supplemented with 2 mM ethylene glycol-bis(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma, MO). The vein was again pressurized to 80 mmHg and was allowed to incubate at this pressure for 30 min. At the end of this period, the vein was preconditioned and then three inflation responses were recorded at 1 mmHg/s between 10 and 100 mmHg. At the end of the testing, veins were removed from the vessel chamber, blotted lightly with gauze, measured and weighed.

A pseudo-elastic model for an incompressible, homogeneous, cylindrical, isotropic vein wall, with a Poisson's ratio of 0.5, was assumed to look at the mean behavior of the vein wall in order to compare changes that may occur with remodeling. The inner radius, R_i , of the vein was calculated using the measured outer radius, the vein mass, length and an assumed density of 1.06 g/cm^3 . Wall thickness, h , was calculated as $(R_o - R_i)$. Luminal area was calculated at a pressure of 10 mmHg under calcium-free conditions. The compliance was calculated as the change in luminal area induced by a transmural pressure change (ΔP) of 20 mmHg, and the distensibility was calculated as $\text{Distensibility} = \frac{2\Delta R_i}{(R_i \Delta P)}$.

Laplace's law for a thin-walled vessel was employed to estimate the circumferential stress $\sigma = PR_i/h$ in the vein wall, as veins typically display relatively thin walls. Measurements of thickness to radius ratios for fresh saphenous veins averaged ~ 0.1 , while measurements from cultured veins ranged from 0.1 to 0.2.

Circumferential strain (ε) was calculated as the change in internal radius from that measured at 10 mmHg or

$$\varepsilon = (R_i - R_{i(10 \text{ mmHg})})/R_{i(10 \text{ mmHg})}$$

2.6 Ex-vivo static culture

While the perfused system is optimal to study mechanical factors (Clerin et al. 2002; Gusic et al. 2005a,b; Nichol et al. 2005; Lawrence and Gooch 2009a,b) it is a complex sys-

tem and cumbersome to operate. To conduct extensive studies on the role of pO_2 in a timely manner, we adopted a much simpler non-perfused SV organ culture system widely used by others (Soyombo et al. 1990; Porter et al. 1996b, 1998a,b, 1999, 2001, 2002; Jeremy et al. 1997; Corpataux et al. 2005; Mekontso-Dessap et al. 2006). Porcine vessels from pigs 60–65 kg weight were cultured inside sterile 100-mm plastic Petri dishes (Nalgene Nunc, Fisher Scientific), pre-coated with ~ 7 mm of Sylgard 184 resin (Dow Corning Corporation, USA). Intact PSV selected for culture, were cleaned from adherent tissues, opened longitudinally and pinned with 26-gauge 5/8"-stainless-steel needles to the Sylgard with the luminal surface facing up. PSV were cultured in culture medium as described earlier pre-equilibrated with the desired gas mixture. PSV were cultured at 37°C in Petri dishes, placed on an orbital shaker at 30 RPM and housed inside O_2 -, CO_2 -, N_2 -and humidity-controlled incubator (NUAIRE 4950). Veins were exposed to a pO_2 of 140 and 75 mmHg, to match the perfused conditions as well as arterial pO_2 (95 mmHg) and venous pO_2 (40 mmHg). Porcine femoral arteries were included as control vessels for culture at arterial pO_2 (95 mmHg) since arteries experience the same pO_2 level in vivo. Spent medium was replaced every 2 days with fresh medium pre-equilibrated with the desired pO_2 . Samples of spent culture medium equilibrated with the desired pO_2 were sampled every 2 days in a blood-gas analyzer (Rapid lab, Siemens Healthcare, USA) for determination of pCO_2 , pO_2 and pH. After 14 days of static culture, PSV were fixed at zero pressure overnight in 70% ethanol or formalin and processed as required for histological and other analyses.

2.7 Analysis of 4-Hydroxynonenal (4-HNE) by immunostaining and Western Blotting

In addition to the other histological analysis performed as described earlier, PSV cultured statically were also analyzed for the lipid peroxidation marker, 4-Hydroxynonenal (4-HNE). 4-HNE immunostaining was performed on paraffin-tissue sections to semi-quantitatively compare the extent of lipid peroxidation in vessel sections using polyclonal antibodies recognizing 4-HNE adducts (Bethyl Labs, Montgomery, TX). Previously frozen tissue from freshly isolated and cultured HSV was homogenized and lysed for Western blot analysis using 4-HNE polyclonal antibodies to detect and quantify levels of 4-HNE produced in experimental samples and in controls (Axxora, San Diego, CA).

2.8 Data analysis

All data are reported as means \pm SD. Differences from fresh and cultured veins in both perfused and statically cultured

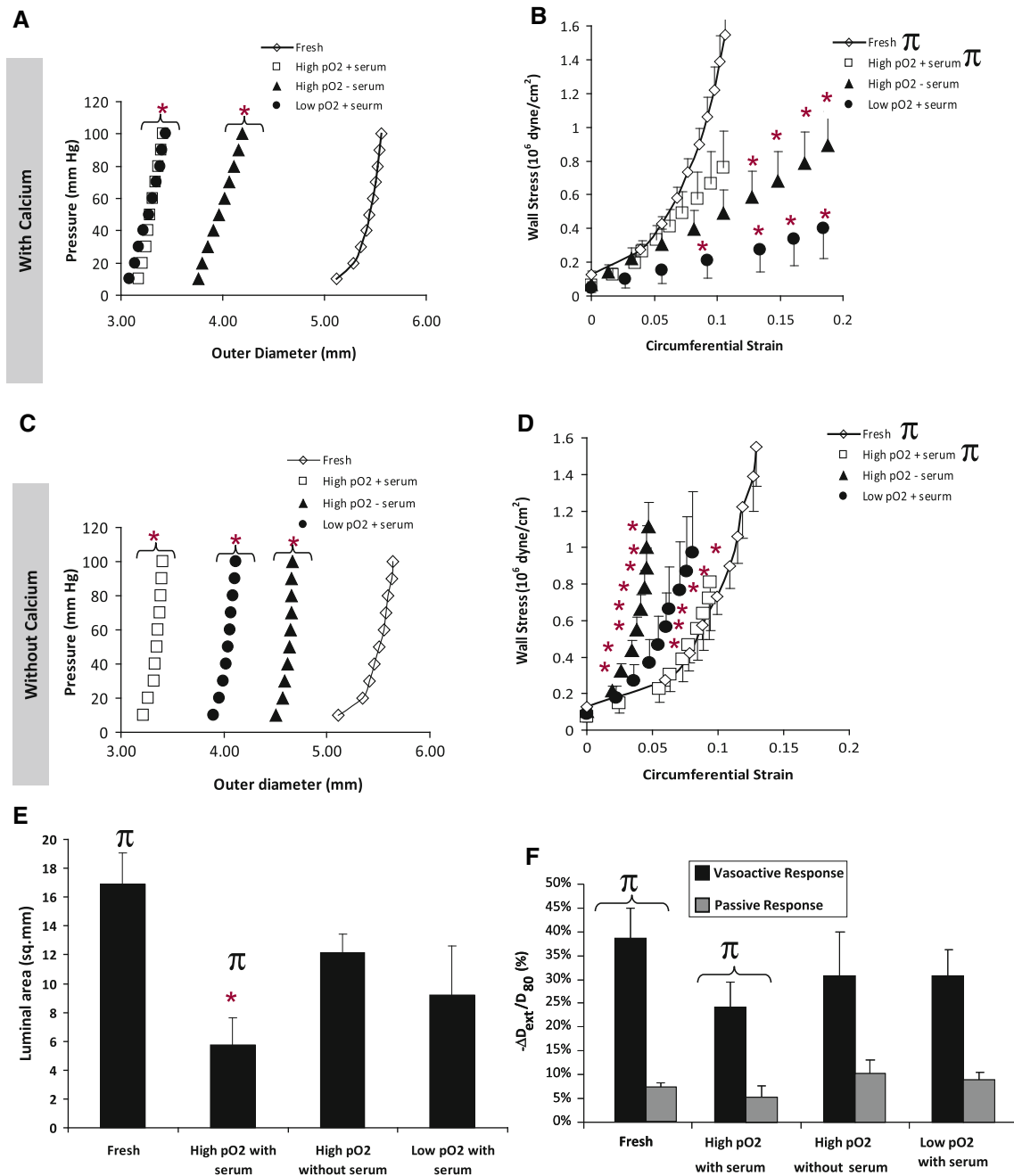


Fig. 1 Freshly isolated and cultured PSV after culture under native venous mechanical conditions in a perfused system ex-vivo **a, c** Pressure-diameter relationships of fresh and perfused veins mechanically tested with **(a)** and without **(c)** calcium. SD of vessel diameter across all groups was $13 \pm 5\%$. **b, d** Wall stress versus strain mechanically tested with **(b)** and without **(d)** calcium. Error bars represent SD. **e** Luminal area calculated from pressure-diameter curves above in the presence of calcium at a pressure of 10 mmHg. Error bars represent SD. π denotes values published Gusic et al. (2005a,b) and have

been reported for comparison. **f** Vasoactivity and normal distension for fresh and perfused veins. Black bars represent the measured change in external diameter from a pressure of 80–10 mmHg after stimulation with norepinephrine (vasoactive response). Grey bars represent the measured change in external diameter at the same pressure change as a passive response. *denotes data statistically different from fresh vessels ($p < 0.05$) where indicated. $n = 7$ for fresh, and $n = 5$ for PSV cultured at high pO₂ with or without no serum, and PSV cultured at low pO₂

vessels were tested using the Dunnett’s Test used as a post hoc analysis. Specifically in the statically cultured vessels group differences among groups were tested using Dunnett’s with

a one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. For each experiment or condition $n \geq 6$ PSV were used except as noted otherwise.

Table 1 Intimal, medial and total wall areas, cell proliferation and cell death index for freshly isolated and cultured PSV in a perfused flow system *ex vivo*

	Intimal area (mm ²)	Medial area (mm ²)	Total wall area (mm ²)	Cell proliferation index (%)	Cell death index (%) TUNEL
Freshly isolated SV <i>n</i> = 7	0.06 ± 0.01 ^π	1.08 ± 0.05 ^π	1.16 ± 0.05 ^π	Intima: 3.5 ± 1.8 ^π Media: 1.1 ± 0.1 ^π	Intima: 0 ^π Media: 0 ^π
Cultured with high pO ₂ (140 mmHg) <i>n</i> = 5	0.16 ± 0.02 ^{*π}	1.27 ± 0.04 ^π	1.48 ± 0.03 ^π	Intima: 25.2 ± 16.3 ^{*π} Media: 6.9 ± 1.6 ^{*π}	Intima: 4.2 ± 2.9 ^π Media: 1.8 ± 0.8 ^π
Cultured with no serum at high pO ₂ (140 mmHg) <i>n</i> = 5	0.14 ± 0.05	1.40 ± 0.21 [*]	1.59 ± 0.21 [*]	Intima: 3.1 ± 1.7 Media: 1.0 ± 0.5 [#]	Intima: 3.1 ± 2.3 Media: 0.5 ± 0.2
Cultured with low pO ₂ (75 mmHg) <i>n</i> = 5	0.08 ± 0.03	1.17 ± 0.02	1.29 ± 0.02	Intima: 8.4 ± 3.5 Media: 0.9 ± 0.9 [#]	Intima: 14.3 ± 3.7 [*] Media: 6.4 ± 4.3

All data are shown as mean ± SD. *indicates $p < 0.05$ relative to fresh veins, and # indicates $p < 0.05$ relative to veins cultured at 140 mmHg with fresh data excluded in the analysis. ^π denotes values that that been previously published [Gusic et al. (2005a,b)] and have been reported here only for comparison

3 Results

3.1 Perfused vessels

3.1.1 Pressure-diameter curves and biomechanics

Pressure versus outer diameter relationships revealed that all groups of cultured PSV had smaller outer diameters than freshly isolated PSV (Fig. 1a). The decrease in outer diameter was not solely due to vasoconstriction, since when tested with calcium-free medium, which should relax the veins, cultured PSV still exhibited smaller outer diameters than freshly isolated PSV (Fig. 1c). Comparing pressure-diameter curves in the presence and absence of calcium revealed that freshly isolated PSV as well as PSV cultured under high pO₂ with serum had little basal tone (Fig. 1a, c). In contrast, veins cultured under low pO₂ with serum or high pO₂ without serum exhibited greater basal tone (Fig. 1a, c).

When tested in the presence of calcium, the stress-strain curves for all cultured vessels were similar to or rightward shifted (Fig. 1b) from freshly isolated veins. Since different groups of vessels had different amounts of basal tone and since the amount of basal tone affects the reference diameter for strain calculations, stress-strain curves were calculated from pressure-diameter curves generated in the absence of calcium (Fig. 1d). Removing calcium from the medium used for biomechanics testing had the greatest impact on the stress-strain curves for the groups of veins that had the greatest basal tone (i.e., no serum group or low pO₂ group). Luminal and wall areas were calculated from the outer diameter measured at a pressure of 10 mmHg (calcium free) as well as the wet weight and length of the PSV. The luminal areas for all groups of cultured PSV were smaller than freshly isolated PSV. PSV cultured with serum and high pO₂ lost more than 10 mm²

or two thirds of their luminal area (Fig. 1e). This dramatic decrease in luminal areas was associated with only a modest increase (less than 0.5 mm²) in wall area (Table 1) suggesting that the decrease in luminal area was due to inward eutrophic remodeling. All groups of PSV contracted in response to norepinephrine with no statistically significant differences between the groups (Fig. 1f).

Cultured PSV were less compliant than freshly isolated veins, especially at pressures less than 30 mmHg (Fig. 2a, c). In contrast, distensibility was more similar among all groups of veins, especially under calcium-free conditions (Fig. 2b, d). The fact that *ex-vivo* culture had greater effects on compliance than distensibility suggests that effects on compliance were largely due to changes in the veins' dimensions—especially the decrease in inner diameter—and not changes in their material properties.

3.1.2 Histological analysis and collagen content

All perfused veins remained intact and patent (Fig. 3). Veins cultured using 140 mmHg pO₂ displayed an intimal area significantly greater than fresh veins (Fig. 3a vs. b and Fig. 4a). Exclusion of serum, by substituting albumin for serum, had a very modest affect on reducing this increase in intimal area (Fig. 3c vs. b and Fig. 4a). However, culture at low pO₂ (75 mmHg) inhibited this increase in intimal area (Fig. 3d vs. b and Fig. 4a). To account for the variation in the initial sizes of the veins and for the possibility of growth of the entire vein, the intimal area was normalized to the medial area. Veins cultured at 140 mmHg pO₂ displayed an intimal/medial ratio that was significantly greater than that of fresh veins (Fig. 4b). Removal of serum had only a modest effect (not significant) on intimal/medial ratio, but reducing pO₂ (from 140 to 75 mmHg) significantly reduced the intimal/medial ratio (Fig. 4b). It should be noted that

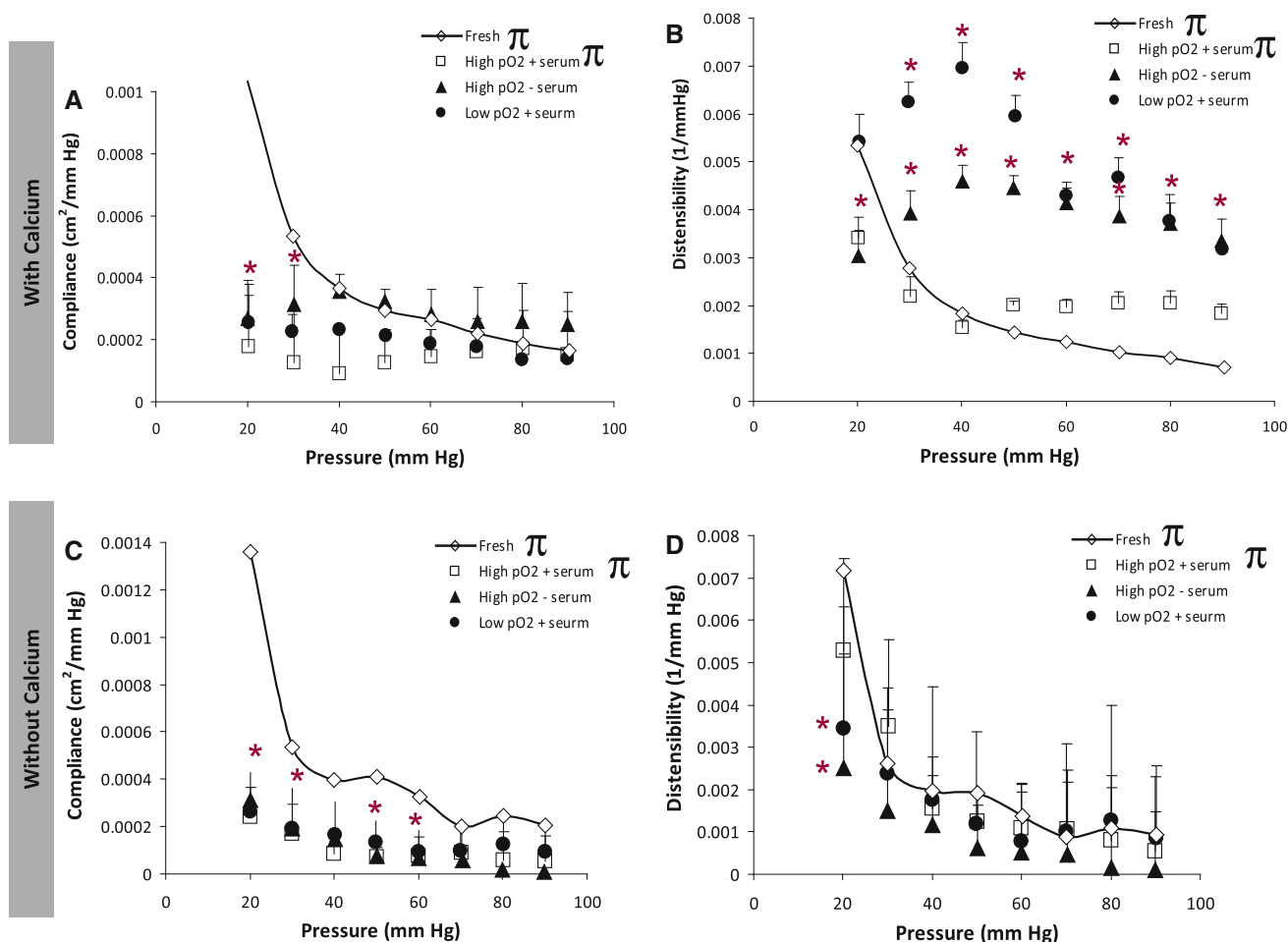


Fig. 2 Freshly isolated and cultured PSV after culture under native venous mechanical conditions in a perfused system ex-vivo **a-d** Compliance and distensibility for fresh veins (solid line) and veins perfused under different conditions tested either with and without calcium. π denotes values published [Gusic et al. (2005a,b)] and have been

reported for comparison. *denotes data statistically different from fresh vessels ($p < 0.05$). Error bars represent SD in all graphs. $n = 7$ for fresh, and $n = 5$ for PSV cultured at high pO₂ with or without no serum, and PSV cultured at low pO₂

Porcine SV cultured in a perfused flow system ex vivo using native venous mechanical conditions

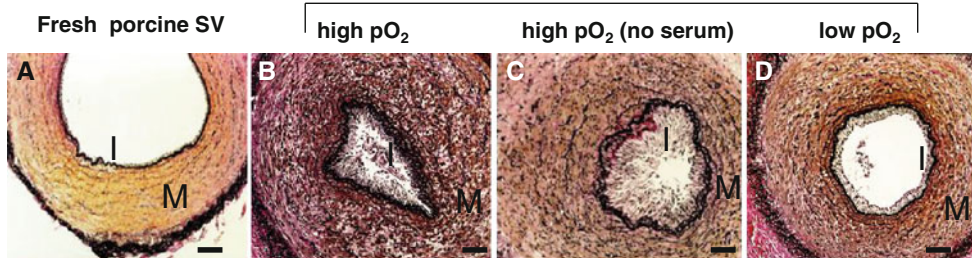


Fig. 3 Freshly isolated and cultured porcine saphenous veins after culture under native venous mechanical conditions in a perfused system ex vivo, stained using Elastin stain (Accustain). (I) stands for intima and

(M) stands for media. Scale bars depict 100 μ m. $n = 7$ for fresh, and $n = 5$ for PSV cultured at high pO₂ with or without no serum, and PSV cultured at low pO₂

tissues shrink during processing for histology so the intima and medial areas underestimate the areas in the vessels before processing. Relative to freshly isolated veins, cell proliferation was elevated 8-fold in the intima, and 7-fold in the

media in veins cultured at 140 mmHg pO₂ (Table 1). Culture at 75 mmHg pO₂ did stimulate cell proliferation 2-fold in the intima in the vessel intima relative to fresh veins which was not statistically significant (Table 1). Culture at high pO₂,

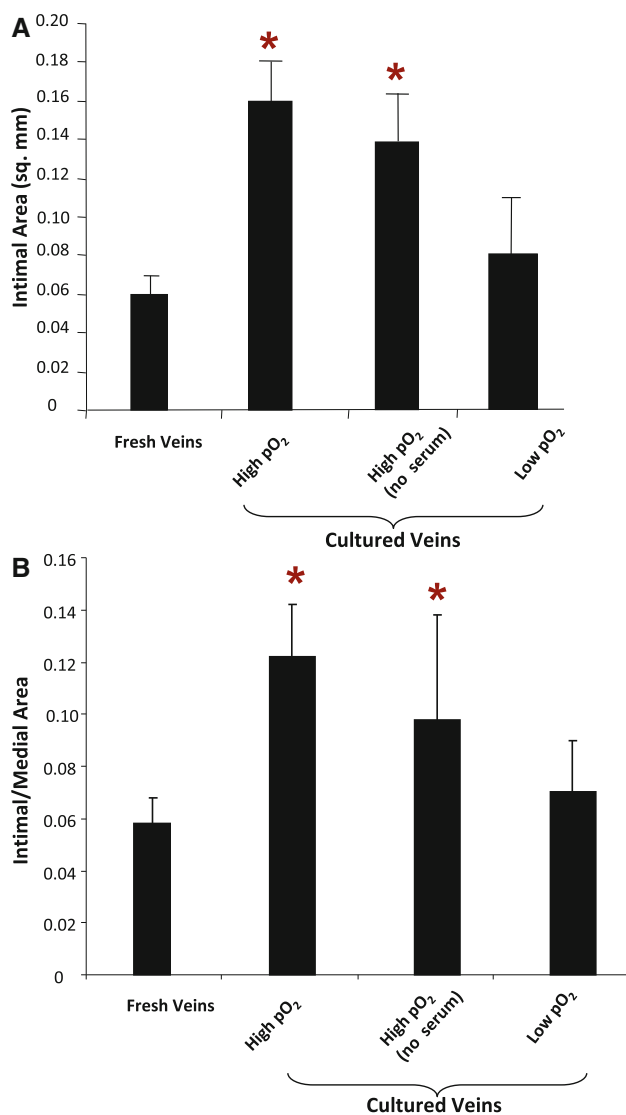


Fig. 4 Quantification of intimal and medial areas for fresh and cultured veins which were perfused under venous mechanical conditions at variable pO₂, as indicated. **a** Quantification of intimal area (square. mm). **b** Ratio of intimal to medial area. *denotes statistically significant ($p < 0.05$) compared to other unmarked groups. $n = 7$ for fresh, and $n = 5$ for PSV cultured at high pO₂ with or without no serum, and PSV cultured at low pO₂

140 mmHg without serum did not stimulate cell proliferation in the intima or media compared to fresh vessels (Table 1). When counter stained with antibodies for smooth muscle myosin heavy chain (MHC), 80 ± 15 % of the proliferating cells in the neointimal region, and 65 ± 10 % in the media stained positively (Supplementary Fig. 1). None of these proliferating cells reacted with the antibody for endothelial cell marker (CD31) (images not shown). Cultured veins displayed low and similar numbers of TUNEL stained cells (Table 1) except for veins cultured at 75 mmHg pO₂ which show significantly larger number of TUNEL positive cells in both the intima and the medial layers. While perfused veins did not

significantly increase their mass relative to freshly isolated veins, all perfused veins displayed significant increases in their collagen content (Table 2). All the perfused vessels showed inward eutrophic remodeling which lead to overall reduction in lumen area. To accommodate for this decrease in lumen area, the wall thickness was increased. When analyzed, all cultured vessels showed decrease in circumferential length and increase in wall thickness. From the fresh vessel, a part of which was cut and perfused for culture ex vivo; the circumferential length decreased from 300 ± 20 μm to 200 ± 10 μm increasing the wall thickness from 100 ± 5 μm to 150 ± 20 μm when measured in at least eight vessels. This however did not affect the resultant trends in total wall areas in cultured vessels calculated from histological analysis as shown earlier (Table 1), or the wet weights of the vessels normalized to their respective axial lengths (Table 2).

3.2 Non-perfused vessels

3.2.1 Chemical culture conditions

By placing the cultured veins in a pO₂-controlled incubator, the pO₂ of the culture medium could be varied from venous (~40 mmHg) to near atmospheric levels (140 mmHg) (Table 3). These changes in culture medium pO₂ were achieved without changing pCO₂ or pH (Table 3).

3.2.2 Intimal and medial dimensions

PSV (Fig. 5 a–e) and femoral arteries (Fig. 5f, g) cultured in the non-perfused culture system for 2 weeks maintained viability as evidenced by the maintenance of normal cellular structure in the vessel wall and low rates of TUNEL staining (Table 4). As sometimes seen in perfused vessels exhibiting IH, in static veins IH was sometimes associated with disruption of the IEL and invasion of the underlying medial cells (Fig. 5d, e). On the contrary porcine femoral arteries cultured at arterial pO₂ did not show any evidence of IH (Fig. 5g) when compared to their fresh vessel controls (Fig. 5f). PSV cultured at pO₂ levels from 75–140 mmHg exhibit dose-dependant increase in intimal and medial areas (Fig. 6). PSV cultured at venous pO₂ of 40 mmHg did not exhibit increased intimal or medial areas (Fig. 6). The increases in medial area in statically cultured PSV are greater than the change in medial area observed with ex vivo perfused vessels at the corresponding pO₂.

3.2.3 Cell indices and dry weight

Proliferation was elevated in veins cultured at 75–140 mmHg pO₂ compared to freshly harvested PSV (Fig. 7). Cell

Table 2 Wall thickness, wet weights and collagen contents for freshly isolated and cultured veins in a perfused flow system ex vivo

	Wall thickness (mm)	Wet weight per length (mg/mm)	% Collagen per wet weight
Freshly isolated SV <i>n</i> = 7	2.5 ± 0.5	4.8 ± 0.4 ^π	7.7 ± 0.3 ^π
Cultured with high pO ₂ (140 mmHg) <i>n</i> = 5	3.0 ± 1.0	5.1 ± 0.2 ^π	10.3 ± 1.3 ^{π*}
Cultured with no serum at high pO ₂ (140 mmHg) <i>n</i> = 5	3.5 ± 1.0	5.3 ± 0.7	10.2 ± 1.1 [*]
Cultured with low pO ₂ (75 mmHg) <i>n</i> = 5	4.0 ± 1.0 [*]	4.7 ± 0.2	12.3 ± 0.3 [*]

All data are shown as mean ± SD

* indicates *p* < 0.05 relative to fresh veins. ^π denotes values that that been previously published [Gusic et al. (2005a,b)] and have been reported here only for comparison

Table 3 Maintenance of pH, pO₂ and pCO₂ in a static ex-vivo culture system

Set points	pH	pO ₂ (mmHg)	pCO ₂ (mmHg)
Standard cell culture atmosphere (140 mmHg)	7.34	150 ± 8	36.6 ± 0.8
Arterial (95 mmHg)	7.35	104 ± 5	35.8 ± 0.6
Venous (40 mmHg)	7.36	42 ± 4	34.6 ± 0.8

n = 6 for all groups shown

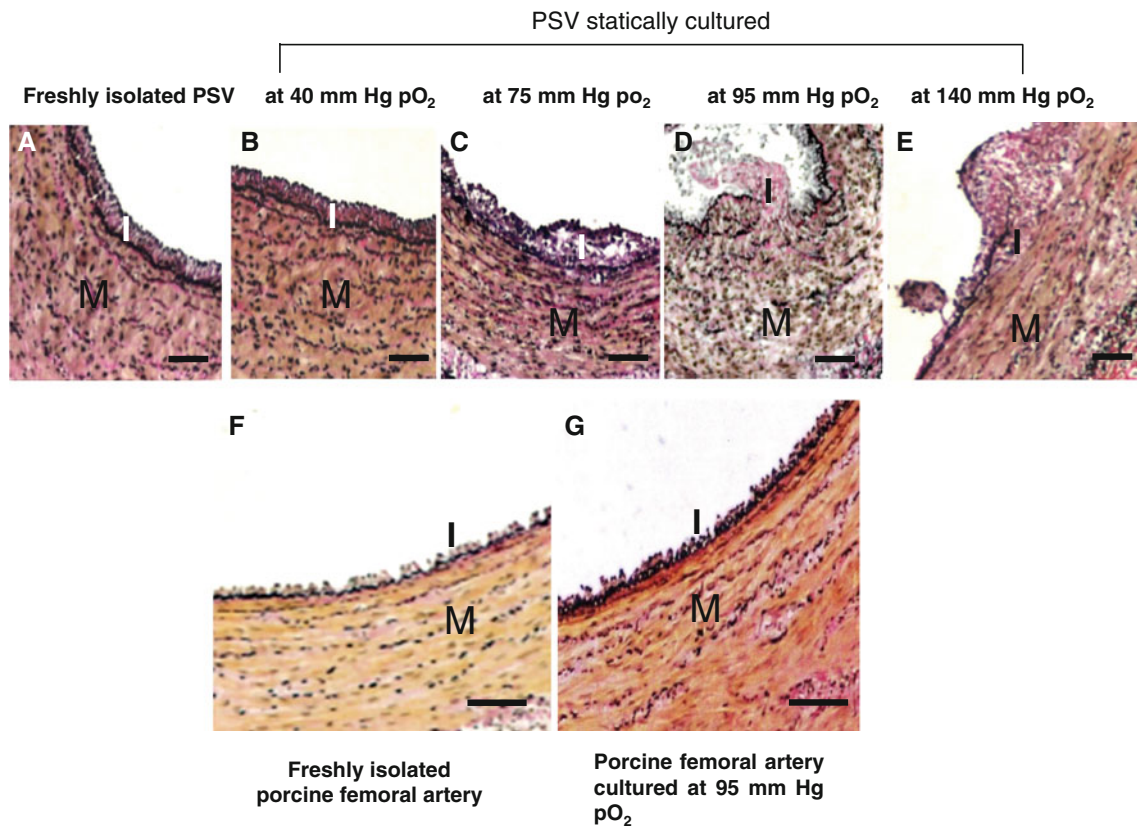


Fig. 5 Freshly isolated and statically cultured PSV (a–e) and femoral arteries (f, g) stained using Elastin stain (Accustain). (i) stands for intima and (m) stands for media. The veins and arteries were statically cultured at variable pO₂ in a static ex-vivo culture system. Scale bars denote 100 μm

proliferation was elevated in the intima and media by 4-fold and 8-fold, 14-fold and 18-fold, and 22-fold and 24-fold respectively in PSV cultured at 75, 95 and 140 mmHg pO₂ respectively. All PSV cultured with increased pO₂

showed low and similar percentage of TUNEL positive nuclei (Table 4). Quantification of DAPI-stained nuclei revealed that culture at higher pO₂ increased cell density by 2-fold in both the intima and media relative to freshly isolated vessels

Table 4 Cell death index (TUNEL) and DAPI (cell counts) for freshly isolated and cultured veins in a static culture system ex-vivo

	Cell death index (%)	DAPI (No./mm ²)
Freshly isolated SV	0	Intima: 50 ± 10 Media: 60 ± 14
Cultured at 40 mmHg pO ₂	2.2 ± 0.4	Intima: 40 ± 17 Media: 60 ± 15
Cultured at 75 mmHg pO ₂	2.2 ± 0.2	Intima: 60 ± 17 Media: 70 ± 15
Cultured at 95 mmHg pO ₂	2.4 ± 0.1	Intima: 80 ± 10* Media: 90 ± 10*
Cultured at 140 mmHg pO ₂	2.8 ± 0.3	Intima: 95 ± 15* Media: 140 ± 12*

All data are shown as mean ± SEM

* indicates $p < 0.05$ relative to fresh veins. $n = 6$ for all groups shown

(Table 4). In contrast, the numbers of DAPI-stained nuclei in the intima and in the media of vessels cultured at venous pO₂ were comparable to freshly isolated vessels (Table 4). The observed increases in intimal and medial area for PSV cultured at 75–140 mmHg pO₂ were associated with an increase in wet and dry weight of the tissue (Fig. 8).

3.2.4 4-HNE adduct formation

PSV cultured at 140 mmHg pO₂ showed a greater intensity of 4-HNE immunostaining and western blot band intensity when compared to fresh PSV or PSV cultured at 40 mmHg pO₂ (Fig. 9). The protein adduct intensity of 4-HNE was undetectable in fresh PSV (Fig. 9).

4 Discussion

Saphenous veins cultured ex vivo with mechanical conditions intended to mimic their native environment rapidly develop IH, alter their biomechanical properties, and exhibit inward eutrophic remodeling (Gusic et al. 2005a,b). It is difficult to understand these changes in the cultured vessels as resulting solely from mechanically induced remodeling, which led us to consider other aspects of the ex-vivo culture environment that might stimulate these changes.

4.1 Role of elevated pO₂

The oxygen tension typically used in tissue culture is significantly greater than venous pO₂. We hypothesized that exposure to this elevated pO₂ stimulated some of the changes observed in cultured veins. Consistent with this hypothesis, relative to PSV culture with 140 mmHg, PSV cultured under native venous mechanical conditions for 7 days with lower oxygen tensions exhibited reduced IH, reduced proliferation in the intima and media, and less disruption of the IEL. PSV cultured statically for 2 weeks at venous pO₂ were indistinguishable from freshly isolated PSV with respect to these parameters. The dramatic impact that exposure to elevated pO₂ had on IH in PSV was not expected since it is widely believed that aspects of the mechanical environment are the

primary stimuli for IH in SV used as grafts in the arterial circulation (Dobrin PB and Endean 1989; Motwani and Topol 1998; Grabellus et al. 2007). There are other studies, however, that also suggest that exposure to an arterial mechanical environment is not a primary cause of IH in SV. Porter et al. reported that relative to static conditions, HSV cultured ex vivo with a combination of venous levels of pressure and shear stress developed less IH; arterial levels of pressure and shear stress further reduced IH (Porter et al. 1996b). Similarly, using perfused PSV, we reported similar observations to Porter et al. We further explored the role of specific flow-induced shear stress, which is greater in the arterial circulation than the venous circulation. Across five sets of mechanical conditions, there was a monotonic decrease in IH with increasing flow-induced shear stress (Gusic et al. 2005a,b). These ex vivo observations of elevated average shear stresses inhibiting IH in PSV are consistent with the clinical observation that HSV used for CABG have better patency when installed in areas with good distal outflow (Motwani and Topol 1998) as well as the notion that steady and pulsatile shear stress is athero-protective for arteries (Davies 1995). Relative to freshly isolated PSV, cellular proliferation was elevated in the media of PSV cultured under native venous mechanical conditions with serum and at 140 mmHg pO₂. In PSV perfused for 7 days under venous mechanical conditions, the increase in proliferation was accompanied by only an 18% increase in wall area, which was reduced to an 8% increase by culturing with a pO₂ of 75 mmHg (Table 1). The increased cell death observed in PSV due to culture at low levels of pO₂ (75 mmHg), may have potentially offset the elevated cellular proliferation and resulted in reduced IH. However, in statically cultured veins, decreasing pO₂ inhibited IH without increasing cell death. In PSV cultured statically for 2 weeks at 140 mmHg pO₂, the increase in medial area was much greater ~150% (Fig. 6b). It is possible that the greater increase in medial area with the statically perfused veins is partially due to the longer culture duration. We chose to statically culture veins for 2 weeks instead of the 1 week we used for the perfused veins, to facilitate comparison of our results to a number of reports by others with vessels cultured statically for 2 weeks (Soyombo et al. 1990; Masood et al. 1997; Mekontso-Dessau et al. 2006).

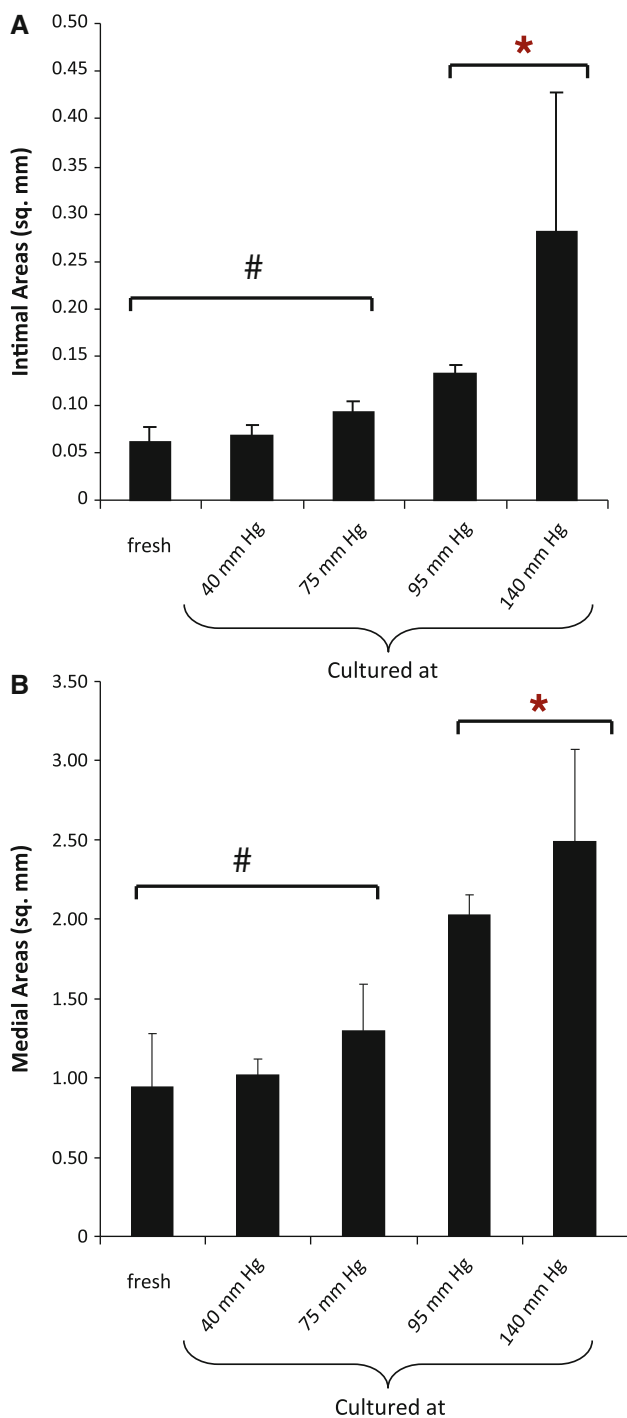


Fig. 6 Quantification of (a) intimal and (b) medial areas from PSV either freshly isolated or cultured in a static system ex-vivo. Cultured veins were maintained at variable pO₂ (140, 95, 75 and 40mmHg) as shown above. *n* = 8 vessels for all area measurements. *p* < 0.05 denotes statistically significant. Groups marked with an *are statistically different from groups marked with an #. There were no intragroup differences in both groups

In addition, the larger increase in wall area was likely partially due to increased water content. This was because the increase in dry weight of the tissue was a more modest 30%.

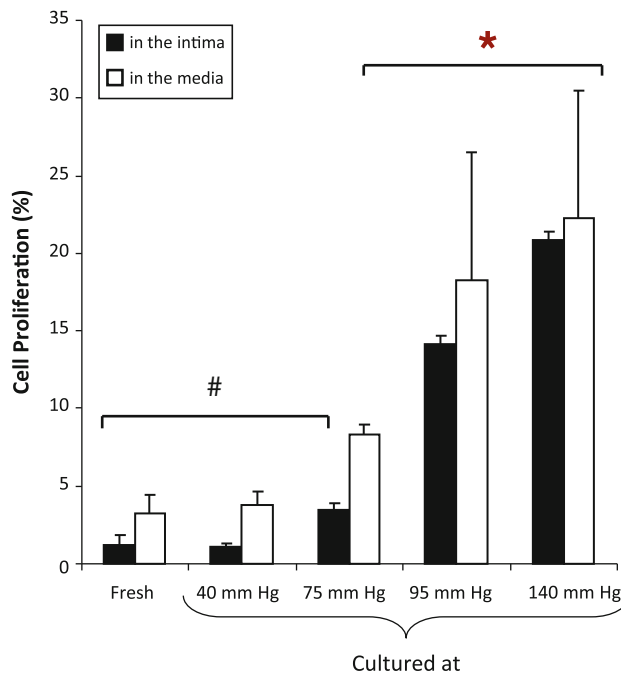


Fig. 7 Quantification of cell proliferation or mitotic index from porcine saphenous veins either freshly isolated or cultured in a static system ex-vivo. Cultured veins were maintained at variable pO₂ (140, 95, 75 and 40mmHg) as shown above. *n* = 8 vessels for all measurements. *p* < 0.05 denotes statistically significant. Groups marked with an # are statistically different from groups marked with an *. There were no intragroup differences in groups marked with an #. In groups marked with an * cell proliferation in the media in PSV cultured at 95 and 140 mmHg pO₂ both were statistically different from cell proliferation in the media of vessels cultured at 75 mmHg pO₂. groups. Cell proliferation in the intima when compared in between PSV cultured at 95 and 140 mmHg pO₂ were not statistically different

In addition to affecting cell proliferation, intimal and in some cases medial area, veins cultured at reduced pO₂ had noticeably different stress-strain curves both in the presence and absence of calcium relative to freshly isolated veins or those cultured in the presence of high pO₂ and serum. Relative to veins cultured with higher pO₂ and serum, those cultured with lower pO₂ and serum exhibited greater basal tone as evidenced by the shift in the pressure-diameter and stress-strain curves for these vessels upon the removal of calcium. Even in the presence of higher pO₂, veins cultured in the absence of serum exhibited basal tone indicating a non-linear interaction between pO₂ and serum.

Arteries are typically cultured ex vivo with a pO₂ of 140mmHg, which is greater than the 95 mmHg of arterial blood. While this increase in pO₂ for cultured arteries is not as great as that for cultured veins, it is possible the exposure to elevated pO₂ might have some effects on cultured arteries. While we do not see IH in pig carotid arteries perfused ex vivo for 1 week at 140 mmHg (Clerin et al. 2002, 2003; Lawrence et al. 2003; Lawrence and Gooch 2009a,b; Nichol et al. 2005,

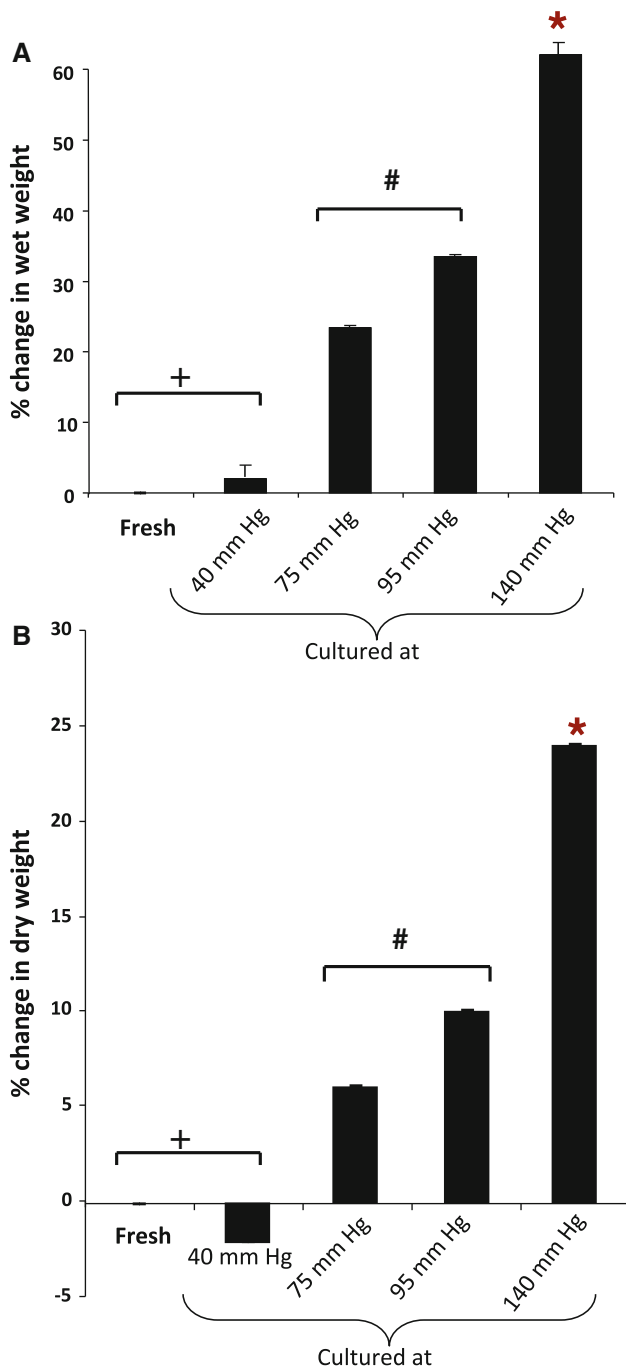


Fig. 8 Quantification of change in wet weight (a) and dry weight (b) of PSV statically cultured at variable pO_2 compared to freshly isolated veins ex vivo. $n = 8$ vessels for all measurements. $p < 0.05$ denotes statistically significant. Groups marked with an * are statistically different from groups marked with an # or a +. Groups marked with an # are statistically different from group marked with a +. No intragroup differences were observed in # or + groups

2009), other groups have reported that human arteries including the internal mammary artery and radial artery cultured statically for 2 weeks with 140 mmHg develop IH but that the amount of IH was less than 15% of the IH seen in HSV

cultured at the same pO_2 (Holt et al. 1992, 1993; Mekontso-Dessap et al. 2006). Thus it is recommended that the pO_2 used for culturing arteries be controlled at arterial levels or, at least, that the potential consequences of culturing arteries at pO_2 different than their in vivo environment should be considered.

4.2 Role of serum

Relative to veins cultured under native venous mechanical conditions at 140 mmHg pO_2 , veins cultured without serum at this pO_2 set point did not display reduced levels of IH but did show reduced levels of proliferation throughout the vessel wall. This was likely due to the removal of potent growth factors found in serum. Levels of cell death were relatively low in veins cultured in 140 mmHg pO_2 without serum and the veins maintained vasoactivity, suggesting that smooth muscle cells were not adversely affected by culture without serum. Inward eutrophic remodeling in veins cultured at 140 mmHg pO_2 without serum was the least among all perfused veins which suggests a role for serum in the observed eutrophic remodeling.

4.3 Changes not due to elevated pO_2 or serum

Removal of serum reduced the amount of inward remodeling but did not block it completely in perfused veins. Thus all perfused veins exhibited some degree of inward eutrophic remodeling. Similarly, all perfused veins had a greater collagen content and exhibited less compliance than freshly isolated veins. Though the absolute levels are low, all groups of perfused veins exhibited increased cell death relative to freshly isolated veins. All of these changes appear to be induced by some aspect of the ex-vivo perfusion culture that is not associated with change in either pO_2 or serum. The causes of these pO_2 - and serum-independent changes are not clear. Possible causes include disruption of the vasa vasorum, denervation, mechanical factors associated with vessel harvest, and exposure to non-serum factors in the culture medium. Surgical removal of the saphenous vein results in a loss of continuity of the adventitial vasa vasorum (Dashwood et al. 2004), and disruption of the sympathetic innervation (Loesch and Dashwood 2009), both of which have been suggested to mediate vein remodeling in vivo. Harvesting veins and pressure testing them for leaks briefly exposes veins to an altered mechanical environment, which may result in subsequent remodeling during culture. For example, both axial and circumferential stretch has been reported to up-regulate molecular mediator of vascular remodeling including matrix metalloproteinase (MMP), MMP-2 and MMP-9 (Chesler et al. 1999; Nichol et al. 2009). Axial stretch of HSV ex vivo for less than 10 s results in elevated MMP levels that peaks 3 days later (Meng et al. 1999). Thus a mechanical stimulus present

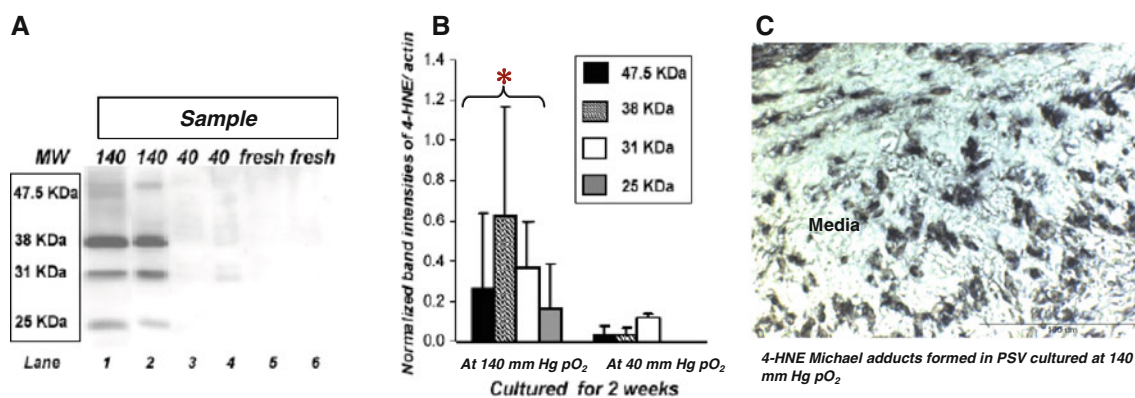


Fig. 9 Quantification of 4-HNE adduct intensity. Expression of 4-HNE normalized with actin in PSV either freshly isolated or cultured at variable pO₂. **a** Western blot bands for 4-HNE protein adducts varying from 25–48 KDa. Samples marked with ‘140’ indicate porcine SV cultured at 140 mmHg, and ‘40’ indicate those cultured at 40 mmHg. Freshly isolated PSV are indicated by ‘fresh’. **b** Expression of 4-HNE band intensities normalized to β-actin, *n* = 8 for all groups. Normalized

4-HNE adduct band intensities of PSV cultured at 140 mmHg pO₂ are marked with *and are significantly different from the other group cultured at 40 mmHg pO₂. In the freshly isolated PSV no bands for 4-HNE were detected, and so no quantification for this group is included on the graph shown. **c** Immunostained 4-HNE Michael adducts formed in PSV cultured at 140 mmHg pO₂

during vessel harvest or preparation might manifest itself as remodeling days later. Finally, it is possible that biochemical factors other than those in serum might stimulate the observed changes. It is possible that the observed changes in the cultured vessels that do not initially appear to be due to changes in the mechanical environment are actually due to our inability to adequately reproduce the in vivo mechanical environment in the ex-vivo perfusion system. For example, though we attempted to reproduce/control many aspects of mechanical environment (e.g., flow, pressure, axial stretch ratio) to match venous conditions in vivo, it is possible that relatively small differences between the in vivo and ex vivo values of these parameters might be responsible for some forms of the remodeling observed. It is possible that additional mechanical factors that are not present or well controlled in our ex-vivo system might stimulate remodeling. Two examples of such factors that have been suggested to stimulate vascular remodeling include flexure, (i.e., bending of blood vessels often due to tissue motion) (Vorp et al. 1999) as well as tethering or compressive forces of the tissue acting on the adventitia (Jackson et al. 2002).

4.4 Role of pO₂-induced IH and potential relevance to vein graft failure following CABG

The observation that veins cultured ex vivo remodel independent of changes in the pO₂, presence of serum, or well-defined changes in the mechanical environment suggests that caution should be taken when interpreting data from such systems. Despite this limitation, ex-vivo culture of vessels remain an attractive tool to study vascular remodeling, especially as it allows for isolation and control of variables that would be very difficult to accomplish in vivo. pO₂ and the

mechanical environment are examples of variables that are difficult to decouple in vivo. HSV used for CABG or peripheral revascularization are exposed to both arterial pO₂ and higher pressures and flows associated with the arterial circulation. By controlling pO₂ independent of the mechanical environments, exposure of veins to arterial pO₂ was shown to stimulate IH and increase SMC proliferation across a variety of mechanical environments. Though the molecular mechanisms relating an increase in pO₂ and IH are not known, increased oxidative stress due to the former could be one possible mechanism. While pO₂ is not a widely recognized contributor to IH, the role of oxidative stress has often been discussed in the context of a number of vascular diseases including atherosclerosis and restenosis (Griendling et al. 2000; Lassegue et al. 2001; Jeremy et al. 2002; Sorescu et al. 2001, 2002a; Sorescu and Griendling 2002). Due to oxidative stress, superoxide production is elevated in porcine and human vein grafts, relative to arterial grafts (West et al. 2001). Therefore, we cultured porcine femoral arteries alongside PSV at the same level of pO₂ (arterial pO₂ ~95 mmHg), based upon the hypothesis that an arterial pO₂ environment would not impose oxidative stress on the artery but only on the PSV which was removed from a low venous pO₂ and placed within an arterial pO₂ environment. Oxidative stress can lead to many biochemical perturbations, including protein or DNA damage (Jaimes et al. 2001) or lipid peroxidation (Usatyuk et al. 2006). Lipid peroxidation products such as 4-HNE can in turn lead to activation of downstream signaling pathways such as ERK, JNK, and p38 MAPK (Usatyuk et al. 2006), which are also involved in cellular proliferation and migration (Nelson et al. 1998). In our study, the enhanced intensity of the 4-HNE adducts formed in porcine SV cultured at 140 mmHg pO₂ compared to the other groups

implicate that oxygen sensitive pathways such as MAPK might be involved in this pO₂-induced intimal hyperplasia evident in porcine SV cultured at higher than venous pO₂. Further these 4-HNE adducts were mostly located in the neointimal and medial region of the cultured PSV. Since this region is mostly made of SMC, it can be inferred that this pO₂-induced IH involves redox-dependant 4-HNE pathways which regulate smooth muscle cell proliferation (Kakishita and Hattori 2001). Our findings therefore not only suggest a significant role for elevated pO₂ in the pathological remodeling of veins ex vivo, but also in the development of IH in HSV grafted into the arterial circulation in vivo.

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