F-actin Capping (CapZ) and Other Contractile Saphenous Vein Smooth Muscle Proteins Are Altered by Hemodynamic Stress

A PROTEOMIC APPROACH*

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Increased force generation and smooth muscle remodeling follow the implantation of saphenous vein as an arterial bypass graft. Previously, we characterized and mapped 129 proteins in human saphenous vein medial smooth muscle using two-dimensional (2-D) PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Here, we focus on actin filament remodeling in response to simulated arterial flow. Human saphenous vein was exposed to simulated venous or arterial flow for 90 min in vitro, and the contractile medial smooth muscle was dissected out and subjected to 2-D gel electrophoresis using a non-linear immobilized pH 3–10 gradient in the first dimension. Proteins were analyzed quantitatively using PDQuest 2-D software. The actin polymerization inhibitor cytochalasin B (1 μM) prevented increases in force generation after 90 min of simulated arterial flow. At this time point, there were several consistent changes in actin filament-associated protein expression (seven paired vein samples). The heat shock protein HSP27, identified as a three-spot charge train, showed a 1.6-fold increase in abundance (p = 0.01), but with reduced representation of the phosphorylated Ser10 and Ser15Ser82 isoforms (p = 0.018). The abundance of actin-capping protein α2 subunit CapZ had decreased 3-fold, p = 0.04. A 19-kDa proteolytic fragment of actin increased 2-fold, p = 0.04. For the four-spot charge train of gelsolin, there was reduced representation of the more acidic isoforms, p = 0.022. The abundance of other proteins associated with actin filaments, including coflin and drestrin, remained unchanged after arterial flow. Actin filament remodeling with differential expression and/or post-translational modification of proteins involved in capping the barbed end of actin filaments, HSP27 and CapZ, is an early response of contractile saphenous vein smooth muscle cells to hemodynamic stress. The observed changes would favor the generation of contractile stress fibers. Molecular & Cellular Proteomics 3: 115–124, 2004.

Human saphenous vein (HSV) \(^1\) remains a common conduit of choice for bypass grafting in humans because HSV is readily accessed, relatively plentiful, easily harvested (1), and provides adequate flow to the recipient artery. In the arterial bypass situation, the vessel experiences an abrupt change from the low-pressure, minimally pulsatile venous circulation to the high-pressure, pulsatile arterial circulation. The adaptation of HSV to the altered hemodynamic environment is a crucial process in graft maturation and patency.

In the artery wall, smooth muscle cells are organized circumferentially and spirally, allowing efficient conduction of the arterial pulse. In contrast, the smooth muscle cells of saphenous vein are orientated both longitudinally and circumferentially. Re-orientation of the medial smooth muscle cells of saphenous vein into co-ordinated circumferential and spiral units is a crucial adaptive response observed in vein grafts. The thin-walled vein dilates in response to arterial pressure. Restoration of a smaller graft lumen with increase in wall thickness, to reduce wall tension, is achieved by migration of smooth muscle cells into the intima, with alteration to a synthetic, proliferative phenotype. This healing response of intimal hyperplasia can be locally excessive, particularly at the anastomoses, and is an important underlying cause of vein graft failure (2, 3). There are very early changes in the contractile properties of HSV following exposure to arterial hemodynamic stress.

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1 The abbreviations used are: HSV, human saphenous vein; HSP, heat shock protein; 2-D, two-dimensional; DTT, dithiothreitol; IEF, isoelectric focussing; IPG, immobilized pH gradient; 1-D, one-dimensional; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; ESI, electrospray ionization; EC\(_{50}\), effective concentration of agonist giving 50% of maximal response; MAPK, mitogen-activated protein kinase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; IP, immunoprecipitation; BSA, bovine serum albumin.
modynamics. These changes include sensitization to vasoconstrictors and increased force generation, which involve Rho-kinase signaling (4). These changes in contractile properties are similar to the myogenic response, where changes in the polymerization of actin and the formation of contractile stress fibers in vascular smooth muscle cells have been proposed as downstream of Rho-kinase and other signaling pathways (5). Contractile stress fiber formation then increases force production by vascular smooth muscle cells (5). Details of other signaling pathways and actin remodeling involved in the adaptive responses of HSV to arterial hemodynamics are largely uncharacterized.

The structure of saphenous vein lends itself to straightforward dissection of the medial smooth muscle away from the adventitia. A consequence of using dissected intact tissue is contamination by other cell types, e.g., endothelial and red blood cells in the case of medial smooth muscle cells, but this proved not to be a significant problem for proteomic analysis (6). Although pure populations of smooth muscle cells may be isolated readily from HSV, these cells are not contractile and exhibit a phenotype very different from the smooth muscle embedded in the connective tissue of the medial layer of blood vessels. Therefore, we considered that to progress our understanding of the changes in protein expression and/or post-translational modifications associated with the increased force generation of HSV smooth muscle in response to arterial hemodynamics, the proteomic analysis of media dissected from intact vein had considerable advantages.

We previously have described the changes in force generation when intact vein is exposed to arterial hemodynamics in vitro (4) and characterized and mapped protein expression in HSV medial smooth muscle using two-dimensional (2-D) PAGE and a combination of peptide mass fingerprinting by matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (MS) and partial amino acid sequencing by nanospray tandem MS (6). Changes in the proteome underlie many cardiovascular diseases. By understanding these changes, proteomics has the potential to reveal new therapeutic targets (7). Here, we provide evidence for early actin filament remodeling, detailing quantitative changes in smooth muscle protein expression, and post-translational modifications following exposure of HSV to arterial hemodynamics.

**EXPERIMENTAL PROCEDURES**

**HSV Samples**—Saphenous vein was harvested from patients undergoing aortocoronary or infrainguinal bypass, or high ligation of saphenous vein for correction of varicose veins, with consent and the approval of the Riverside Research Ethics Committee. The vein was transported to the laboratory in ice-cold Krebs solution. Diseased vein, which did not respond to phenylephrine (10 μM) with a contraction of >1 g, was discarded. Samples from current smokers and patients with diabetes were excluded. Paired segments of vein were exposed to simulated venous or arterial flow in vitro for 90 min as described previously (4). Medial dissection was performed and validated by immunostaining for smooth muscle cell actin, smooth muscle cell myosin, and CD31 as described previously (6).

**Preparation of Tissue Samples for 2-D PAGE**—Sample preparation was performed according to Weekes et al. (8) and Heinke et al. (9). Briefly, frozen dissected smooth muscle (70–110 mg) was pulverised under liquid N2 into a fine powder using a pestle and mortar. The resulting powder was collected, homogenized in lysis buffer (9.5 M urea, 2% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonic acid (CHAPS), 0.8% (w/v) Phospho-ate, pH 3–10, and 1% (w/v) dithiothreithol (DTT), 150 μM Mini Complete Protease Inhibitor Mix- ture (Roche Molecular Biochemicals, Indianapolis, IN), 10 μM Phos- phatase Inhibitor Mixture 1 and 2 (Sigma, Poole UK), in 1.5 ml Eppendorf tubes (for analytical gels, 150 μl of lysis buffer was added to 10 mg wet weight of tissue), sonicated at 4 °C for 5 min and centrifuged at 15,000 rpm at 20 °C for 1 h. The supernatant was harvested and, using a modification of the method described by Bradford (10), the protein concentration was determined (8). Solubi- lized protein samples were divided into 50-μl aliquots and stored at −70 °C.

**2-D PAGE**—2-D PAGE was performed according to Weekes et al. (8) and Heinke et al. (9). Isoelectric focusing (IEF) was performed using 180 mm, immobilized, non-linear pH gradient strips (IPG strips) of pH 3–10 (Amersham Biosciences, Piscataway, NJ). For quantitative analysis, analytical gels were run. These required a protein load of 100 μg per IPG DryStrip using an in-gel rehydration method, as described by Rabilloud et al. (11) and Sanchez et al. (12). Samples were diluted in rehydration solution (8 M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, and 0.2% (w/v) Phospho-ate, pH 3–10) and rehydrated overnight in a reswelling tray (Amersham Biosciences). Strips were focused at 0.05 mA/IPG strip for 60 kVh at 20 °C (13). Once IEF was completed the strips were equilibrated in 6 M urea containing 30% (v/v) glycerol, 2% (w/v) SDS, and 0.01% (w/v) bromphenol blue, with the addition of 1% (w/v) DTT for 15 min, followed by the same buffer without DTT, but with the addition of 4.8% (w/v) iodoacetamide for 15 min (14). SDS- PAGE was performed using 12% T, 2.6% C separating polyacryl- amide gels without a stacking gel using the Hoefer DALT system (15). The second dimension was carried out overnight at 20 mA/gel at 8 °C and was terminated when the bromphenol dye front had migrated off the lower end of the gels. After electrophoresis, analytical 2-D gels were fixed for a minimum of 1 h in a methanol:acetic acid:water solution (4:1:5 v/v/v). Analytical 2-D protein profiles (100 μg protein loadings) were visualized by silver staining using the OWL Silver Staining Kit (Insight Biotechnology Ltd., London, UK), while prepara- tive 2-D gels (400 μg protein loadings) were stained using the Plu- sine silver staining kit (Amersham Biosciences) with slight modific- ations to ensure compatibility with subsequent MS analysis (16).

**Mass Spectrometry**—In gel trypsinolysis was performed using an Investigator Progest (Genomic Solutions, Huntington, UK) robotic digestion system, as previously described (17). The resulting mixtures of peptides were characterized by MALDI MS, using a TooFSpec 2E instrument (Micromass, Manchester, UK) from α-cyano-4-hydroxy- cinnamic acid matrix and by tandem nano electrospray ionization (ESI) MS on a Micromass Q-Tof spectrometer (18) interfaced to a Micromass CapLC chromatograph. Mass spectra were searched against Swiss-Prot/TrEMBL using Protein Lynx Global Server as de- scribed (18). Amino acid sequences were deduced semi-manually from the product ion spectra, with the assistance of the FindTag program in BioLynx (Micromass).

**Image Analysis**—Computer-assisted image analysis was performed as described previously (6). Briefly silver-stained gels were scanned at 100-μm resolution using a Molecular Dynamics Personal SI Laser Densitometer (Sunnyvale, CA). Protein spots were analyzed quantitatively using the PDQuest 2-D software version 6.2.0 (Bio-Rad, Hercules, CA). Background and vertical streaks were removed from each gel image, spots digitized by Gaussian fit, giving rise to a synthetic image. Protein abundance was quantified by measuring the
normalized optical density (optical density of a protein spot of interest taken as a fraction of the total optical density of valid spots on the gel) of each protein spot.

**Separation of Proteins Using One-dimensional (1-D) SDS-PAGE**—12% (w/v) acrylamide gels were poured into a Mini-PROTEAN II cell system (Bio-Rad, Herts, United Kingdom) and allowed to polymerize for 45 min. Isobutyl alcohol (Sigma, St. Louis, MO) was placed along the top of the resolving gel to aid polymerization. The layer of alcohol was removed, and a 4% stacking gel was poured and allowed to polymerize for 45 min at room temperature. Smooth muscle tissue lysates and CapZ eluates were denatured and reduced by being boiled in a 1:1 ratio with Laemmli loading buffer (246 mM Tris Base-HCl (pH 6.8), 10% w/v SDS, 2.5% v/v glycerol, 8% v/v β-mercaptoethanol, 0.1% w/v bromphenol blue) for 5 min. Lysates were then subjected to electrophoresis at 20 mA (200 V) per gel until protein ran to the end of the stacking gel. The current was then increased to 25 mA (200 V) per gel. Equal protein loading between lanes was confirmed by determining protein concentration of lysates using a modification of the method described by Bradford (10), as for 2-D PAGE (8). Electrophoresis was terminated when the dye front had migrated off the lower end of the gel.

**CapZ Immunoprecipitation**—Equal amounts of protein were taken from paired (venous and arterial flow) smooth muscle lysates (ranging from 150 to 350 μg). Urea is not compatible with immunoprecipitation, so lysates were diluted 20-fold with 20 mM Tris-HCl, pH 7.0, containing 1% Triton X-100, 300 mM NaCl, 2 mM EGTA, 5 mM DTT (immunoprecipitation buffer (IP buffer)). Protease and phosphatase inhibitors were also included. Lysates were precooled of endogenous immunoglobulins by rotation with 50 μl each of protein A and protein G Sepharose (Amersham Biosciences) for 10 min at 4°C. The Sepharose was then pelleted by centrifugation for 5 min at 3000 rpm, and the resultant supernatant was transferred to a fresh tube. A monoclonal anti-CapZ antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) was then added at a final dilution of 1:100, followed by 200 μl protein A Sepharose. Samples were then rotated for 4 h at 4°C. After this time, the protein A Sepharose complex was washed once by rotation with 8 ml ice-cold IP buffer, transferred to a fresh tube, and the Sepharose complex was pelleted as above. IP buffer was removed and CapZ protein eluted from the Sepharose by vigorous agitation at room temperature with 100 μl of O’Farrell lysis buffer (8.5 μl urea, 2%, w/v) CHAPS, 0.8% w/v) Pharmalyte, pH 3–10, and 1% (w/v) DTT, 150 μg/ml Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals). Eluates were then snap frozen in liquid N2 until further processing.

**Anti-phosphorylation/CapZ Immunoblotting**—Proteins from 1-D SDS-PAGE gels were transferred onto polyvinylidene difluoride Immobilon-P transfer membrane (Millipore, Bedford, MA), using the Mini-PROTEAN II system (Bio-Rad), according to manufacturer’s instructions. The membrane was then removed, washed briefly in TBS-Tween (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (w/v) Tween-20 (Sigma)] containing 0.1% (w/v) bovine serum albumin (BSA) and then incubated in blocking buffer (TBS-Tween plus 5% BSA) with gentle shaking for 6 h at room temperature. The membrane was then incubated overnight at 4°C with a monoclonal anti-phosphoserine or anti-phosphothreonine antibody (Sigma) (diluted 1:2000 in blocking buffer) with gentle shaking. Membrane was then washed for 4 h at room temperature in TBS-Tween plus 0.1% (w/v) BSA, changing the wash solution every 15 min. Membrane was then incubated at room temperature for 2 h with horseradish peroxidase-labeled rabbit antiamouse IgG diluted 1:2000 in TBS-Tween plus 0.1% (w/v) BSA. Membrane was washed four times for 5, 10, 10, and 5 min with TBS-Tween plus 0.1% (w/v) BSA. Proteins were detected using enhanced chemiluminescence (Amersham Biosciences) exposing membrane to Hyperfilm ECL x-ray film (Amersham Biosciences).

**Organ Chamber Studies**—Vein rings were mounted in organ chambers as previously described (4). Briefly, vein rings 5 mm in length were mounted in a 10-ml organ chamber suspended between two 0.2-mm stainless steel wire stirrups, the upper one being attached to an isometric force transducer. Vein rings were stretched at 0.5-g intervals until the optimum-length tension relationship was obtained, usually 1.5–2 g. The changes in tension generated by the rings were recorded in both electronic format (MacLab Software; AD Instruments, Oxford, UK) and a hard copy (Student Oscillograph, Harvard Apparatus, Kent, UK). Responses to KCl (data not shown) and phenylephrine were determined as described previously (19).

**Statistical Analysis**—Pair-wise comparisons of protein abundance were performed using Wilcoxon signed rank test to allow for the possibility of non-parametric distribution of protein abundance. Regression analysis was used to analyze the relative abundance of phosphorylated isoforms of heat shock protein HSP27 and acidic gelsolin.

**RESULTS**

**The Effect of Cytochalasin B on Force Generation in Vein Rings**—Freshly isolated vein rings generated >2 g tension in response to KCl or phenylephrine in the presence of low concentrations of cytochalasin B (0.3–1 μM), but at higher concentrations vein rings generated progressively lower tensions. The inclusion of cytochalasin B (1 μM) in vein perfusate had no effect on peak tension after 90 min of venous flow conditions, but reduced peak tension after 90 min of arterial flow conditions, from 6.7 ± 1.4 to 3.8 ± 1.1 g (n = 4, p < 0.05; Table I). The apparent increase in EC50 for phenylephrine after arterial flow in the presence of cytochalasin B was not significant. These results indicated that actin (re) polymerization was necessary to support the increased force generation after 90 min of simulated arterial flow.

**Gel Reproducibility**—The mean integrated optical density of protein spots detected on seven analytical gels loaded with samples from seven separate smooth muscle dissections was 87,117 ± 5,491.8 (individual values 61,848, 87,742, 115,915, 87,996, 85,924, 85,143, and 85,251, respectively), indicative of good inter-gel reproducibility (individual gel images not shown).

**Changes in Protein Expression in HSV Smooth Muscle After Arterial Flow**—A screening scan of seven pairs (venous and arterial flow) of images identified three consistent changes after arterial flow: i) alteration in the abundance and charge of gelsolin, ii) a decreased abundance of CapZ, and iii) an increased abundance of a 19-kDa actin fragment. These very clear changes dictated the further detailed investigations and analyses of HSP27 and other proteins associated with muscle contraction, identified on the published smooth muscle cell protein map (6). The proteins investigated are identified in Fig. 1 on a preparative gel obtained from HSV smooth muscle after 90 min of simulated arterial flow, and the changes before and after simulated arterial flow are detailed in Fig. 2 and Table II.
vivo (20), and spots 12, 13, and 14 represent HSP27 in three different phosphorylation states, spot 14 being the most acidic (and most phosphorylated). The pI of spot 12 was estimated as 6.02, in excellent agreement with the theoretical value of 5.98 (calculated with the Expasy compute pI/M program). The observed pIs of the more acidic spots 13 and 14 were 5.62 and 5.45, respectively, which would be consistent with the presence of one (calculated pI \( /H11011 \) 5.8) and two (calculated pI \( /H11015 \) 5.6) phosphate groups.

ESI MS confirmed the lack of phosphorylation of spot 12, because signals were observed at \( m/z \) 538.25 (2+) and \( m/z \) 481.19 (2+), corresponding to the peptides GPS\(_{15}\)WDPR and QLS\(_{82}\)SGVSEIR, which include serines 15 and 82, the known phosphorylation sites of HSP27 (21, 22). The sequences of these peptides were verified by tandem MS sequencing of their respective precursor ions. An additional peptide containing Ser\(_{15}\) (GPS\(_{15}\)WDPRFDWYHFSR; \( m/z \) 634.92), attributable to incomplete cleavage at Arg\(_{20}\), was sequenced. Phosphorylation is readily detected by MS because phosphopeptides undergo characteristic fragmentation, including facile neutral loss of phosphoric acid (\( H_3PO_4; \) mass 98 Da). No such phosphate-specific fragmentation processes were observed in the tandem mass spectra of any of the other peptides detected, suggesting the absence of additional, previously undescribed phosphorylations. The presence of a signal at \( m/z \) 578.24 (absent from spot 12) in the

![Identification of proteins from 2-D gels.](image)

**TABLE I**

| Flow conditions                | Cytochalasin B (1 \( \mu \)M) | EC\(_{50}\) phenylephrine before perfusion | EC\(_{50}\) phenylephrine after 90 min perfusion | Max tension induced by phenylephrine before perfusion | Max tension induced by phenylephrine after 90 min perfusion |
|-------------------------------|-------------------------------|------------------------------------------|-----------------------------------------------|------------------------------------------------------|----------------------------------------------------------|
| Venous                        | No                            | ND                                       | ND                                            | 2.7 ± 0.5                                            | 2.9 ± 0.4                                                |
| Significant decrease in EC\(_{50}\) \( p < 0.05 \). |
| Mean pressure, 20 mmHg         | No                            | ND                                       | ND                                            | 2.6 ± 0.6                                            | 2.2 ± 0.3                                                |
| Significant increase in peak tension, \( p = 0.02 \). |
| Nonpulsatile flow rate, 10–20 ml/min | Yes                          | 10 ± 2                                   | 9 ± 2                                         | 3.0 ± 0.6                                            | 6.7 ± 1.4                                                |
| Arterial                      | No                            | 11 ± 3                                   | 0.9 ± 0.2\(^a\)                              | 2.6 ± 0.5                                            | 3.8 ± 1.1                                                |
| Significant increase in peak tension, \( p < 0.01 \). |
| Mean pressure, 100 mmHg        | Yes                           | 9 ± 2                                    | 3 ± 3                                         | 2.6 ± 0.5                                            | 6.7 ± 1.4                                                |
| Pulsatile (90 cpm) flow rate, 200–225 ml/min | Yes                          | 10 ± 3                                   | 9 ± 2                                         | 3.0 ± 0.6                                            | 6.7 ± 1.4                                                |

FIG. 1. Identification of proteins from 2-D gels. A 2-D preparative gel image of HSV smooth muscle lysate, after exposure to 90 min arterial flow, showing 18 identified protein spots involved in actin filament dynamics.
digested by the enzyme trypsin. The resulting digest of spot 13 was consistent with the addition of a phosphate group to QLS82SVGEIR; tandem MS sequencing confirmed the sequence of the peptide, and localized the modification to the Ser82. This peptide was also detected in the digest of spot 14 and was accompanied by a triply charged ion at \( m/z \) 661.58 (absent from spots 12 and 13), corresponding to phosphorylation of GPS15WDPFRDWYPHSR. Tandem MS confirmed that the phosphorylation site was Ser15.

**TABLE II**

| Spot | Protein identity                          | After 90 min venous flow conditions spot intensity | After 90 min arterial flow conditions spot intensity |
|------|------------------------------------------|--------------------------------------------------|----------------------------------------------------|
| 1    | Gelsolin (actin depolymerisation factor) | 524.2 ± 160.4                                    | 453.4 ± 121.9                                      |
| 2    | Gelsolin (actin depolymerisation factor) | 1793.5 ± 441.4                                   | 1376.0 ± 209.5                                    |
| 3    | Gelsolin (actin depolymerisation factor) | 3853.6 ± 257.6                                   | 3374.4 ± 634.7*                                   |
| 4    | Gelsolin (actin depolymerisation factor) | 2530.0 ± 245.4                                   | 2393.4 ± 260.4                                    |
| 5    | Integrin-linked protein kinase 1          | 121.4 ± 29.5                                     | 151.1 ± 41.3                                      |
| 6    | Elongation factor 1-α1                   | 310.1 ± 134.4                                    | 725.6 ± 394.1                                     |
| 7    | α-Actinin-2-associated LIM protein        | 96.3 ± 18                                        | 70.5 ± 48.9                                       |
| 8    | Cofilin, muscle isoform (Cofilin-2)       | 707.8 ± 187.1                                    | 697.9 ± 208.4                                     |
| 9    | Cofilin, nonmuscle isoform (Cofilin 1)    | 1254.3 ± 158                                     | 1273.9 ± 257.5                                    |
| 10   | Destrin (actin depolymerising factor)     | 4556.4 ± 792.3                                   | 5220.3 ± 482.7                                    |
| 11   | Actin fragment (C-terminal)               | 177 ± 72.3                                       | 406.7 ± 58.6*                                     |
| 12   | HSP27                                     | 2225.0 ± 394.5                                   | 2997.5 ± 339.5*                                  |
| 13   | HSP27                                     | 792.5 ± 200.9                                    | 1678.9 ± 512.5                                    |
| 14   | HSP27                                     | 475.7 ± 177.4                                    | 755.8 ± 264.2                                     |
| 15   | Rho GDP-dissociation inhibitor 1 (Rho GDI 1) | 306.7 ± 85.7                                    | 358.6 ± 123.4                                    |
| 16   | F-actin capping protein α-1 subunit (CapZ) | _b                                          | _                                                  |
| 17   | F-actin capping protein α-2 subunit (CapZ) | 1123.2 ± 494.1                                   | 351.2 ± 95.6*                                     |
| 18   | Creatine kinase B chain                   | 1249.9 ± 391.1                                   | 1423.5 ± 441.7                                    |

* AU, arbitrary units.

b Unable to be quantified.

**Fig. 2.** Quantification of proteins from 2-D gels. Significant differential expression of four out of a total of 18 protein spots that were quantified from seven paired analytical 2-D gels, pH 3–10 NL (\( n = 7 \) paired samples, pre-versus post-arterial flow), is highlighted, the expression pre- and post-arterial flow being shown in the left and right side, respectively, in each detail panel. Protein abundance was quantified using the PDQuest 2-D software version 6.2.0 (Bio-Rad) by measuring the normalized optical density of each protein spot. Protein spot identities and optical density values are presented in Table II.
fore, spot 12 contains unphosphorylated HSP27, spot 13 the Ser82 phosphorylated isoform, and spot 14 the biphosphorylated Ser15 Ser82 isoform. The relative abundance of these isoforms was $\frac{12}{H_{11022}} = \frac{13}{H_{11022}} = \frac{14}{H_{11022}}$. There was a significant increase in the relative abundance of the less phosphorylated isoforms (spots 12 and 13) after arterial flow, regression analysis, $p = 0.018$. There was an absolute increase in the abundance (1.3-fold) of spot 12 after arterial flow, $p = 0.04$ (Fig. 2), but the apparent doubling in abundance of spot 13 was only of borderline significance, $p = 0.06$ (Table II). There also was a significant 1.6-fold increase shown in the total amount of HSP27 (i.e. addition of spots 12, 13, and 14), $p = 0.01$ (Table II).

CapZ (F-actin Capping Protein)—The change of greatest magnitude was the 3-fold decrease in abundance of CapZ $\alpha_1$ subunit after 90 min arterial flow, $p = 0.04$ (Fig. 2 and Table II). This change was confirmed by Western blotting (Fig. 3). Examination of the sequence data of CapZ (Swiss-Prot/TrEMBL Protein Knowledgebase) identified several potential serine ($n = 16$) or threonine ($n = 8$) phosphorylation sites in the $\alpha_2$ subunit. Western blotting indicated that the CapZ $\alpha_2$ subunit in spot 17 was both threonine and serine phosphorylated (data not shown). The 2-D gel image (Fig. 1) reveals several proteins in the vicinity of spot 17 that have similar molecular masses. None of these spots were identified as CapZ $\alpha_2$ subunit. Therefore, the 3-fold decrease in CapZ $\alpha_2$ subunit (spot 17) after arterial flow conditions reflects an absolute decrease in abundance rather than post-translational modification(s) leading to a change in pI. Although spot 16 was identified as CapZ $\alpha_1$ subunit on preparative gels, the staining on analytical gels was insufficient to permit quantification.

Other Proteins Associated with Contractile Functions—Nine other proteins associated with smooth muscle contractile mechanisms, identified on the HSV map (6), were chosen and quantified from seven paired gels of HSV medial smooth muscle after venous and arterial flow (Fig. 1 and Table II). The most obvious visually detected change, after arterial flow, was an increase in spot 11. Spot 11 (and the corresponding feature from a duplicate gel) were identified by MALDI peptide mass fingerprinting as $\alpha$-actin. The migration on the gel suggested an apparent mass of about 19 kDa, and the eight peptides, matching to within 40 ppm of their calculated values, clustered between residues 241 and 375 of the $\alpha$-actin sequence. This indicates that spot 11 represents an N-terminally truncated fragment of actin. Image analysis confirmed a 2-fold increase in abundance of spot 11, $p = 0.03$ (Fig. 2 and Table II). The gelsolin charge train consisted of four quantifiable spots (Fig. 4), of which spot 3 was the most abundant and the third most acidic: the abundance of this spot decreased after arterial flow conditions, $p = 0.022$ (regression analysis). The relative abundance of other proteins, including cofilin and destrin, was unchanged (Table II).

DISCUSSION

The implantation of saphenous vein into the arterial circulation, to bypass occlusive atherosclerotic arterial disease, exposes the tissue to an abrupt change in hemodynamic stress. Many of the endothelial cell responses to hemody-
namic stress have been described using cultured cells. However, the pathology of vein grafts is associated with smooth muscle cell migration and proliferation, but the contractile phenotype of this cell is lost in culture. The proteomic approach, using intact tissue, offers a unique approach to investigate the protein responses of venous smooth muscle to hemodynamic stress, complementing pharmacological investigations. We have extended our previous studies to show that the actin polymerization inhibitor cytochalasin B prevents the increase in agonist-induced tension of vein exposed to simulated arterial flow for 90 min. Moreover, we have identified changes in abundance and/or post-translational modification of at least two proteins associated with capping of the barbed ends of actin filaments: an almost 2-fold increase in the concentration of at least two proteins associated with capping of the barbed ends of actin filaments: an almost 2-fold increase in HSP27, accompanied by decreased phosphorylation, and a barbed ends of actin filaments: an almost 2-fold increase in cation of at least two proteins associated with capping of the simulated arterial flow for 90 min. Moreover, we have identified changes in abundance and/or post-translational modification of at least two proteins associated with capping of the barbed ends of actin filaments: an almost 2-fold increase in HSP27, accompanied by decreased phosphorylation, and a reduction in CapZ α2 subunit abundance. There also was a reduced representation of the more acidic isoforms of gelsolin. The importance of actin filament dynamics as an early response to arterial flow was further emphasized by the change in abundance of an N-terminally truncated fragment of α-actin.

When cells change shape, move, or generate stress fibers, they recruit unpolymerised cytoplasmic actin into new or pre-existing filaments. Monomer assembly and polymerization are the dominant processes at the fast-growing, barbed end, while actin filament disassembly is the principal process at the slow-growing pointed end. F-actin capping protein, also known as CapZ, is an αβ heterodimer, and association of the α and β subunits is required for barbed end actin filament capping activity to limit growth of the actin filament at this end (23, 24). Recent evidence indicates that CapZ regulates protein kinase C signaling and calcium sensitivity in cardiac muscle (25). Down-regulation of CapZ increased cardiac myofilament calcium sensitivity, inhibited protein kinase C-mediated control of myofilament activation, and decreased myofilament-associated protein kinase C-β (25). It also has been proposed that HSP27 caps the barbed end of actin filaments to stabilize the filament, with overexpression of HSP27 resulting in increased amounts of polymerized actin (26, 27). When HSP27 is phosphorylated, it may bind to tropomyosin to support actin-myosin interaction and facilitate smooth muscle contraction (28). In addition, there is evidence that the p38 mitogen-activated protein kinase (MAPK)/HSP27 pathway influences the force generation of vascular smooth muscle (29). Specifically, Yamboliev et al. infer that endothelin-1-induced contraction of canine pulmonary artery depends, in part, on HSP27 phosphorylation (29). Gelsolin is another capping protein, activated by calcium, to sever the noncovalent bonds between the subunits of actin filaments, to promote rapid filament shortening. Therefore, CapZ, HSP27, and gelsolin are all likely to play an important role in altering the contractile function and motility of vascular smooth muscle (Fig. 5).

Previously, we have shown that circumferential deformations, imposed by simulated arterial flow, enhance the contractility of venous smooth muscle (4). The enhanced force generation can be attenuated by low concentrations of cytochalasin B, a reagent that inhibits actin polymerization and attenuates the myogenic response. Our proteomic data provide evidence that simulated arterial flow increases the expression of HSP27 in venous smooth muscle, particularly the unphosphorylated and monophosphorylated (Ser62) isoforms. The increased concentration of unphosphorylated HSP27 (spot 12) could function to stabilize new contractile stress fibers, while increased amounts of the monophosphorylated (Ser62) isoform (spot 13) provide increased substrate concentration for the kinases that appear to be involved in generation of the biphosphorylated (Ser15Ser82) isoform (spot 14) required to bind to tropomyosin to promote actin-myosin interaction and contraction (28, 29). Hence, increases in spot 14 would only be observed in response to vasoconstrictors. This is in keeping with the corollary of our findings, that increased phosphorylation of HSP27 has been reported in the poorly contractile hearts of patients with dilated cardiomyopathy (20).

There was an absolute 3-fold decrease in CapZ (α2 subunit) in saphenous vein smooth muscle after 90 min of simulated arterial flow. In the absence of data concerning the half-life of CapZ, we speculate that this may arise through targeted degradation. This subunit was expressed at much higher levels than the homologous α1 subunit. Therefore, the α2 subunit would appear to be an important functional effector of barbed end capping in venous smooth muscle. The reduction in concentration of this subunit, following arterial hemodynamics, would facilitate polymerization at the fast growing end of actin filaments. The acute distension or stretching of venous smooth muscle results in calcium influx (30). This would activate a diversity of cellular signaling pathways as well as promoting the filament-severing activity of gelsolin. In the context of the recent findings of Pyle et al. (25), it would appear that CapZ has a critical role in both cell signaling and the regulation of actin in myofilament contractility (Fig. 5).

The molecular events of actin filament assembly are difficult to examine in vivo. Gelsolin is a downstream effector of rac-mediated fibroblast motility (31). However, there is no information available to indicate whether gelsolin function depends on gelsolin phosphorylation. There were four spots in the gelsolin charge train that could be quantified on analytical gels (Fig. 4), and two further more acidic spots of low abundance were observed on preparative gels. The online prediction algorithm PhosphoBase indicates that human gelsolin contains the following potential phosphorylation consensus sequences: 13 serine, 10 threonine, and 8 tyrosine. It has been demonstrated in vitro that the proto-oncogene pp60-src phosphorylates gelsolin purified from human plasma at Tyr438 in the presence of phosphatidylinositol 4,5-bisphosphate or lysophosphatidic acid (32). A further four minor phosphorylation sites were also identified: Tyr59, Tyr382, Tyr576, and Tyr624 (32). Our data indicate that hemodynamic stress...
Figure 5. Integration of findings into a hypothesis of how specific changes in protein expression contribute to the phenotype of heightened contractility. a, Actin filament exposed to venous flow. b–e, Hypothesized actin filament dynamics after exposure to arterial flow. b, A decrease in expression of CapZ after 90 min arterial flow may initiate an increase in actin filament polymerization at the barbed end. It may also serve to facilitate calcium sensitivity in smooth muscle, a phenomenon previously demonstrated, in HSV smooth muscle, in our laboratory (4). In parallel, an increased concentration of monophosphorylated (Ser82) HSP27 may permit more rapid agonist-induced phosphorylation of HSP27 by providing increased substrate concentration for kinases involved in generating biphosphorylated (Ser15,Ser82) HSP27. c, Biphosphorylated HSP27 binds to tropomyosin to promote actin-myosin interaction and contraction. An increase in biphosphorylated HSP27 would ensure increased interaction with tropomyosin, thus potentially increasing the rate of venous smooth muscle contraction when exposed to arterial flow, as has been demonstrated previously (4). This, concomitant with activation of MAPK pathways, such as the p38 MAPK/HSP27, known to influence vascular smooth muscle contraction (28), may contribute to increased smooth muscle contraction after HSV has been exposed to arterial flow for even short time periods. d, Total gelsolin abundance remaining unchanged after arterial flow, with a decrease in expression of the more acidic isoforms, may maintain the rapid formation of short actin filaments by eliminating individual actin subunits, thus creating an increased amount of proteolytic actin fragments in the process. e, Synergy of the changes depicted in b–d would be likely to promote the observed increased force generation and sensitivity to vasoconstrictors of HSV smooth muscle after exposure to arterial hemodynamics.
man saphenous vein rather than the labor- and time-intensive technique, are the principal reason for not having conducted proteomic analyses at earlier time points, or in the presence of cytochalasin B or external stents. We have separated proteins in the first dimension and quantified differential protein expression using a pH 3–10 gradient. Separation and resolution could have been further maximized using IPG strips covering a variety of pH ranges. Generally wide pH 3–10 gradients are used to give an overview of the protein profile of a sample. Narrow range IPGs (e.g. pH 4–7, 6–9, 4.0–5.0, 4.5–5.5, 5.0–6.0, 5.5–6.7) have the capability of “pulling apart” this protein profile and increasing the resolution, and thereby increasing proteomic coverage, in particular regions (35). Previously, we characterized and mapped protein expression in HSV medial smooth muscle using 2-D PAGE and MS (6). We used a pH 3–10 gradient and found that only one protein spot, out of a total of 129 identified protein spots, produced two distinct spectra, i.e. was a “mixture” of two proteins. Therefore, it did not appear necessary to generate further 2-D gel images using narrower pH gradients in the present study.

It is difficult to examine the molecular responses to hemodynamic stress of contractile human vascular smooth muscle. We have demonstrated the feasibility of complementing pharmacology with proteomics to identify changes in abundance and post-translational modification of the proteins involved in actin filament remodeling and force generation in intact vein. The decrease in CapZ and the differential expression and phosphorylation of HSP27 may contribute synergistically to the altered contractile mechanism of HSV after arterial flow as hypothesized in Fig. 5.

The changes in protein expression found in the present study may have significant implications in the emerging concept that elements of the cytoskeleton not only shape the internal superstructure of the cell but also participate in intracellular signaling pathways.

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