Quantitative Proteomic Analysis of Sokotrasterol Sulfate-stimulated Primary Human Endothelial Cells*§

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The endothelium forms a continuous monolayer at the interface between blood and tissue and contributes significantly to the sensing and transducing of signals between blood and tissue. New blood vessel formation, or angiogenesis, is initiated by the activation of endothelial cells and is an important process required for various pathological and physiological situations. This study used cleavable isotope-coded affinity tag reagents combined with mass spectrometry to investigate the molecular basis of a recently discovered angiogenesis-promoting steroid, sokotrasterol sulfate. Changes in the relative abundances of over 1000 proteins within human endothelial cells treated with sokotrasterol sulfate and vehicle-treated cells were identified and quantitated using this technique. A method that examines the entire ensemble of quantitative measurements was developed to identify proteins that showed a statistically significant change in relative abundance resulting from treatment with sokotrasterol sulfate. A total of 93 proteins was significantly up-regulated, and 37 were down-regulated in response to sokotrasterol sulfate stimulation of endothelial cells. Among the up-regulated proteins, several were identified that are novel to endothelial cells and are likely involved in cell communication and morphogenesis. These findings are consistent with a role for sokotrasterol sulfate in endothelial sprouting.

Angiogenesis, the formation of new blood vessels from existing vessels, is a complex process requiring modulation of multiple endothelial cell functions (1, 2). Endothelial cells line the interior of blood vessels and are responsible for initiating the growth of new vessels (1, 2). The formation of capillary sprouts from the existing microvasculature occurs secondary to an inciting stimulus resulting in increased vascular permeability, accumulation of extravascular fibrin, and local proteolytic degradation of the basement membrane (1, 2). Endothelial cells overlying the disrupted region become activated, change shape, and extend elongated processes into the surrounding tissue (1, 2). Directed migration toward the angiogenic stimulus results in the formation of a column of endothelial cells (1, 2). Just proximal to the migrating tip of the column is a region of proliferating cells that cause the sprout to increase in length (1, 2). Proximal to the proliferative zone, the endothelial cells undergo a second shape change, adhere tightly to each other, and begin to form a lumen resulting in a capillary plexus (1, 2). Fusion of individual sprouts at their tips closes the loop and circulates blood into the vascularized area (1, 2). Throughout this process the expression of various proteins is tightly regulated (1, 2).

The development of new blood vessels is required in various pathological and physiological processes including embryogenesis, organogenesis, ovarian follicle development, and tumor growth and metastasis (1, 3). Ischemic coronary artery disease is a major cause of morbidity and the leading cause of mortality in the industrialized world (4). The major reason for cardiovascular disease is the occlusion of blood vessels due to atherosclerosis (4). Neovascularization, or new blood vessel growth, is also required to bypass the occlusion of arteries caused by atherosclerosis (4). The effects of neovascularization in ischemic disease may be both beneficial and detrimental to the patient (5, 6). Lack of adequate neovascularization when arteries narrow can result in clinical symptoms of ischemia, and strategies to promote angiogenesis may improve symptoms and tissue function (7). In contrast, neovascularization of an atherosclerotic plaque can result in growth of the plaque and further luminal stenosis, or plaque hemorrhage and rupture (8, 9). While the beneficial effects of using angiogenic drugs to promote neovascularization in ischemic disease remains controversial, several clinical trials are under way to evaluate the benefits (9).

In an attempt to identify small molecules with the ability to stimulate blood vessel growth for potential use in therapeutic angiogenesis, we recently screened a library of crude marine extracts and identified a sulfated steroid called sokotrasterol
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sulfate (10). Sokotrasterol sulfate stimulation causes sprouting of endothelial cells in vitro in a dose-dependent manner and induces the development of new blood vessels in an in vivo chick chorioallantoic membrane model of angiogenesis. To ensure the safe use of angiogenic therapies, however, a thorough understanding of the mechanisms of neovascularization is important. To understand how sokotrasterol sulfate affects the endothelial cell and potentially understand its mechanism of angiogenic action, differential protein analysis using cleavable isotope-coded affinity tag (cICAT)2 reagents combined with mass spectrometry (11) was used to quantitate protein expression differences in primary human endothelial cells stimulated with sokotrasterol sulfate. In addition, a statistical analysis was performed to determine what magnitude of change in relative abundance between proteins extracted from the treated and control human umbilical vein endothelial cells (HUVECs) was significant. Previous studies using ICAT have arbitrarily selected a specific cut-off as a measure of significance; however, the results presented in this study show that the level of significance is likely to be different for every data set analyzed in this manner. In particular, membrane proteins such as Tie-1 and the αv integrin that are known to be required in the developing vasculature were shown to be up-regulated at the borderline level, bolstering the requirement for choosing optimal cut-off levels for up- and down-regulated proteins. Several other proteins, such as Dysferlin and E2F4, demonstrated borderline but significant up-regulation by sokotrasterol sulfate implicating these proteins in the process of angiogenesis induced by sokotrasterol sulfate.

EXPERIMENTAL PROCEDURES

Materials—Ammonium bicarbonate (NH₄HCO₃), ammonium formate (NH₂HCO₂), guanidine hydrochloride, dibasic sodium phosphate (Na₂HPO₄), sodium chloride (NaCl), ammonium hydroxide (NH₄OH), Tris, sodium fluoride (NaF), sodium orthovanadate (Na₃VO₄), Triton X-100, and phenylmethylsulfonyl fluoride, and hexylamine hydrochloride in 50 mM NH₄HCO₃, pH 8.3. Each sample was chemically reduced by adding 5 µl of 100 mM Tris(2-carboxyethyl)phosphine hydrochloride followed by boiling in a water bath for 10 min. The reduced samples were transferred to vials containing either cICAT-13C₀ or cICAT-13C₉ dissolved in 20 µl of CH₃CN and incubated at 37 °C for 2 h. The two samples were combined, buffer-exchanged into 50 mM NH₄HCO₃, pH 8.3, using a D-Salt Excellulose plastic desalting column (Pierce), and digested with trypsin (Promega, Madison, WI) overnight at 37 °C using an enzyme to protein ratio of 1:50 (w/w). The digestion was quenched by boiling the samples in a water bath for 10 min and adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM.

Affinity Purification and Cleaving of cICAT-labeled Peptides—A 1.5-ml bed volume UltraLink immobilized monomeric avidin column was slurry-packed in a glass Pasteur pipette and equilibrated with 2× PBS (0.2 M sodium phosphate, 0.3 M NaCl, pH 7.2). The column was blocked with 2 mM o-biotin in 2× PBS, pH 7.2, the biotin was stripped from the reversible binding sites of the column according to the manufacturer’s instructions, and the column was re-equilibrated with 2× PBS, pH 7.2. The cICAT-labeled peptides were boiled for 5 min, cooled to room temperature, loaded onto the avidin column, and incubated for 15 min at ambient temperature. After washing the column with 10 bed volumes of each of 2× PBS, pH 7.2; 1× PBS, pH 7.2; and 50 mM NH₄HCO₃, pH 8.3, 20% CH₃CN, the cICAT-labeled peptides were eluted using 30% CH₃CN, 0.4% formic acid and lyophilized to dryness. The biotin moiety was cleaved from the cICAT-labeled peptides by treatment with the cleaving reagents provided by the manufacturer for 2 h at 37 °C and lyophilized to dryness.

Strong Cation Exchange Fractionation of cICAT-labeled Peptides—The lyophilized HUVEC cICAT-labeled peptide samples were dissolved in 170 µl of 0.1% formic acid, 25% CH₃CN and injected onto a strong cation exchange liquid chromatography (SCXLC) column (1 mm × 150 mm, polysulfoethyl A, Polycl Inc., Columbia, MD). The following NH₄HCO₃/CH₃CN multistep gradient was used to elute the cICAT-labeled peptides from the column at a flow rate of 50 µl/min: 2% mobile phase B for 8 min followed by a linear increase to 10% B in 32 min, then a linear increase to 60% B in 45 min, a steep increase to 100% B in 1 min, and maintenance at 100% B for 10 min. Mobile phase A was 25% CH₃CN, and mobile phase B was 25% CH₃CN, 0.5 M NH₄HCO₃, pH 3.0. Fractions were collected every minute for 96 min. Each SCXLC fraction was lyophilized and reconstituted in 20 µl of 0.1% trifluoroacetic acid prior to microcapillary reversed-phase (μRP) LC-MS/MS analysis.

Microcapillary Reversed-phase LC-MS/MS of cICAT-labeled Peptides—Ten-centimeter-long μRPLC-electrospray ionization (ESI) columns were coupled on line with an ion trap mass spectrometer (LCQ Deca XP, ThermoElectron, San Jose, CA) to analyze the cICAT-labeled peptides extracted from the control and sokotrasterol sulfate-treated HUVECs. To construct the 10-cm-long μRPLC-ESI columns, 75-µm-inner diameter fused silica microcapillaries (Polymicro Tech-
Peptide Identification and Quantitation—The raw MS/MS data acquired on the ion trap mass spectrometer were searched using SEQUEST against the Homo sapiens proteome data base (28,816 entries) downloaded from the European Bioinformatics Institute (EBI) (www.ebi.ac.uk/proteome/index.html). The Archaea-derived data base (12,038 entries) utilized in the false-positive bioinformatic analysis was constructed using genomic sequence information from the following organisms: Aeropyrum pernix, Archaeoglobus fulgidus, Pyrobaculum aerophilum, Sulfolobus tokodaii, and Thermoplasma volcanium. Dynamic modifications for cysteinyl (Cys) residues were set by mass addictions of the cleaved cICAT labels (227.13 Da for the light label and 236.16 Da for the heavy label) in a single search. SEQUEST criteria were set as Xcorr \geq 1.9 for [M + H]^+ ions, \geq 2.2 for [M + 2H]^2+ ions, and \geq 2.9 for [M + 3H]^3+ ions and as \Delta C_n \geq 0.08 for the identification of fully tryptic peptides within the cICAT-labeled samples. The identified peptides were quantified using XPRESS (ThermoElectron), which calculates the relative abundances \(13C_9/13C_0\) in this data set) of peptides based on the area of their extracted ion chromatograms.

RESULTS

Various steroid compounds have been shown to have either pro- or antiangiogenic activity, but the mechanism by which these steroids function remains to be elucidated (14). There is some suggestion that estradiol functions by promoting the synthesis and release of other angiogenic factors to promote neovascularization (15, 16). The identification of a novel function for the sulfated steroid sokotrasterol sulfate prompted us to attempt to understand its mechanism of action. There is no information currently on the effect of sokotrasterol sulfate on the molecular profile of any cell type. Since the sprouting of new vessels is initiated at the level of the endothelial cell, primary human endothelial cells were treated with sokotrasterol sulfate or vehicle (Me2SO) to quantitate differential protein expression between these two conditions.

Crude protein extracts harvested from HUVECs treated with Me2SO as a control or with sokotrasterol sulfate (5 \(\mu\)g/ml) for 24 h were prepared and separately labeled (Fig. 1) with the isotopomerically distinct versions of the cICAT reagents (cICAT-\(^{13}C_9\) and cICAT-\(^{13}C_0\)). Both versions of the cICAT reagent contain a thiol-reactive group and a biotin moiety separated by a linker region in which the heavy version contains nine \(^{13}\)C atoms in place of nine \(^{12}\)C atoms in the light version. After cICAT labeling, the protein extracts were combined and digested with trypsin, and the cICAT-modified peptides were recovered using immobilized avidin chromatography. The cICAT-labeled tryptic peptides were resolved into 96 fractions by SCXLC that were subsequently analyzed by data-dependent \(\mu\)RPLC-MS/MS.

Central to comprehensive proteomic measurements is the ability to identify large numbers of peptides, hence proteins. It is generally accepted that no single chromatographic or electrophoretic procedure is capable of resolving the complex mixture of peptides that results from a global proteolytic digest of a proteome. Therefore, combining two or more orthogonal separation procedures dramatically improves the overall...
Fig. 2. Separation of cICAT-labeled peptides by SCXLC. a, fluorescence chromatogram of cICAT-labeled peptides separated by SCXLC. b, histogram illustrating the number of peptides (hatched bars) and proteins (solid bars) identified in the μRPLC-MS/MS analysis of the SCXLC fractions.
peak capacity of the measurement and results in larger numbers of peptides being identified from complex proteome digests (17). Fractionation is a critical aspect of successful global proteome investigations that utilize MS because while a mass spectrometer can perform mass measurements on several co-eluting peptides, conventional MS/MS cycle times for most instruments typically enable only a limited number of these peptides to be selected for collision-induced dissociation. Therefore, limiting the number of co-eluting peptides helps to increase the percentage of species that may be identified. Illustrated in Fig. 2a is the SCXLC chromatogram of the cICAT-labeled peptide digest from HUVECs. Fig. 2b depicts a histogram of the peptides and proteins identified in each μRPLC-MS/MS analysis of the 96 SCXLC fractions. There is good correlation between the number of peptides/proteins identified and the apparent fluorescence intensities of each of the SCXLC cICAT fractions. In total, 2162 unique cICAT-labeled peptides, corresponding to 1019 proteins, from HUVECs treated with vehicle or sokotrasterol sulfate were quantitated in this study (Supplemental Table I). Although the SEQUEST analysis resulted in 2162 uniquely identified peptides, some portion of these peptides may identify more than one protein due to sequence homology among orthologous proteins etc. Hence we have conducted a more detailed search of the peptides and proteins identified to account for these homology effects and find that 1602 peptides (74.1%) are found to uniquely identify a single protein (i.e. protein clusters) due to peptide sequence redundancy (i.e. 100% homology in more than one protein in the data base).

The ability to identify a protein depends directly on the extent to which the peptide identified possesses a unique sequence amid all proteins in the genome-derived data base. The rate of false-positive peptide (hence protein) identifications in “bottom-up” proteomic approaches is multifactorial with strong dependence on almost every aspect of the experimental execution. Such factors governing false-positive identifications include the quality and specificity of the proteolytic digest, the calibration and operation of the mass spectrometer, the bioinformatic algorithm used to search the MS-derived data, the interpretation of the bioinformatic output, and the accuracy and completeness of the genome-derived proteomic data base. It is thus necessary that proteomic data sets derived from the aforementioned experimental approach be subjected to false-positive analyses to determine the confidence of the reported peptide (protein) identifications. A false-positive analysis of the peptide (protein) identifications was conducted by searching the data from the μRPLC-MS/MS analyses of the SCXLC cICAT peptide fractions against an Archaea-derived proteomic data base utilizing identical bioinformatic filters for peptide identification (see “Experimental Procedures”). This false-positive analysis resulted in the identification of 22 unique peptides from the Archaea-derived proteomic data base as compared with 2162 unique peptides from the human proteomic data base. We therefore estimate a false-positive rate of 1% (percentage of false-positives = (22/2162) × 100) for the present cICAT peptide data set that reflects the high stringency of the SEQUEST scoring and filtering parameters used in this analysis.

The relative abundances of the cICAT-labeled peptides were quantitated based on the area of their extracted ion chromatograms reconstructed from the molecular ion MS scans of the 13C9/13C0 variants of each peptide. There can be no standard for what changes in relative abundance as measured by cICAT analyses represent statistically significant changes in peptide abundance ratios since the determination of cICAT ratios depends directly on multiple experimental factors. These include factors such as the extent to which the initial mixing of samples was equal, the relative efficiency of recovery of each of the cICAT peptide pairs from the sample preparation, the inherent resolution and accuracy of the MS instrumentation utilized, and the number of molecular ion scans across each cICAT peptide pair μRPLC peak. We sought to determine the threshold above and below which the measured cICAT peptide abundance ratios were statistically significant within this data set. The normalized percentage of the number of cICAT-labeled peptides identified within binned abundance ratios (e.g. 1.0–1.09, 1.1–1.19, etc.) was plotted (Fig. 3). Theoretically the distribution of the ICAT ratios should be a normal distribution defined as Equation 1,

\[
P = \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{(x - \mu)^2}{2\sigma^2}}
\]

where \(P\) is the normalized peptide percentage at a given cICAT peptide pair ratio \(x\), \(\mu\) is defined as the experimentally measured mean value of the total population of cICAT peptide pair ratios, and \(\sigma\) is standard deviation. The \(z\) score is defined as \(z = (x - \mu)/\sigma\). Using a non-linear least squares regression
### Proteins with increased expression levels in response to sokotrasterol sulphate

| Protein name | Swiss-Prot accession no. | -Fold increase in protein levels |
|--------------|-------------------------|---------------------------------|
| 14-3-3 protein σ/δ | P29312 | 1.74 (5) |
| 180-kDa transmembrane PLA2 receptor | Q13018 | 3.03 |
| 2-Oxoglutarate dehydrogenase E1 component, mitochondrial precursor | Q00218 | 1.75 |
| 60 S acidic ribosomal protein P0 | P05388 | 1.78 (5) |
| 60 S ribosomal protein L10a | P53025 | 3.80 (4) |
| Acidic leucine-rich nuclear phosphoprotein 32 family member A | P39687 | 3.33 |
| Actin filament-associated protein | Q9HBV1 | 2.78 |
| Adapter-related protein complex 2 α1 subunit | O95782 | 1.77 (7) |
| Adenine phosphoribosyltransferase | P07741 | 1.79 |
| ADP-ribosylation factor 6 | P26438 | 7.69 |
| Alcohol dehydrogenase (NADP+) | P14550 | 1.65 (5) |
| Annexin A5 | P08758 | 3.33 |
| Aspartate aminotransferase, cytoplasmic | P17174 | 3.57 |
| ATP-dependent RNA helicase A | Q08211 | 1.96 (2) |
| β-Arrestin 1 | P49407 | 3.57 |
| Bifunctional aminoacyl-tRNA synthetase | P07814 | 2.24 (7) |
| Calcium-transporting ATPase type 2C, member 1 | P98194 | 2.44 |
| CD166 antigen precursor | Q13740 | 2.70 |
| CDC16 | Q96AE6 | 2.38 |
| Chloride intracellular channel protein 1 | Q95596 | 2.51 (13) |
| Coflin, non-muscle isoform | Q95664 | 1.90 (16) |
| Contactin-associated protein 1 precursor | Q95675 | 2.44 |
| CTL2 gene | Q8IWA5 | 2.04 |
| Cytosolic acetoacetyl-coenzyme A thiolase | Q16146 | 2.13 |
| d-3-Phosphoglycerate dehydrogenase | O43175 | 1.82 |
| Dihydropteridine reductase | P09417 | 3.03 |
| Dihydroxyacetone phosphate acyltransferase | O15228 | 2.50 |
| DNA mismatch repair protein Msh2 | P43246 | 4.35 |
| DNA-dependent protein kinase catalytic subunit | P78527 | 1.73 (19) |
| Down syndrome critical region protein 2 | Q9S456 | 1.67 (2) |
| Dysterlin | O75923 | 1.75 (2) |
| Eukaryotic translation initiation factor 3 subunit 9 | P55884 | 2.14 (4) |
| Exocyst complex component Sec10 | Q90471 | 2.78 |
| FLJ00365 protein | Q8NF37 | 7.10 (2) |
| Follistatin-related protein 1 precursor | Q12841 | 1.77 (3) |
| Glutamate dehydrogenase 1, mitochondrial precursor | P00367 | 1.94 (4) |
| Glutathione peroxidase 1 | P07203 | 1.87 (3) |
| Glycerolaldehyde-3-phosphate dehydrogenase, muscle | P00345 | 3.19 (17) |
| Group X secretory phospholipase A2 precursor | O15496 | 100.0 (2) |
| Heat shock-related 70-kDa protein 2 | P54652 | 1.75 |
| Hedgehog-interacting protein (hypothetical protein) | Q96QV1 | 1.75 |
| Homeobox protein HB9 | P50219 | 12.50 |
| Human full-length cDNA clone CS0DC022YA24 of neuroblastoma (fragment) | Q86U01 | 1.72 |
| Hypothetical protein | Q95328 | 1.82 (2) |
| Hypothetical protein (fragment) | Q8N3G2 | 1.72 |
| Hypothetical protein (putative α-mannosyltransferase) | Q9BV10 | 2.56 |
| Hypothetical protein CGI-99 | Q9Y224 | 1.89 |
| Hypothetical protein FLJ12647 | Q9H9N0 | 1.92 |
| Hypothetical protein FLJ12980 | Q9H974 | 3.33 |
| Hypothetical protein FLJ20397 | Q86Y56 | 2.16 (4) |
| Hypothetical protein FLJ32019 | Q96MR1 | 2.08 |
| Hypothetical protein KIAA0095 | Q14705 | 3.45 |
| Hypothetical protein KIAA0765 | O94863 | 9.09 |
| Hypothetical protein KIAA1440 (fragment) | Q92PA8 | 1.72 (2) |
| Integrin, precursor | P06756 | 1.65 (3) |
| Isoctirate dehydrogenase (NADP), cytoplasmic | P07874 | 1.92 |
| Laminin β-1 chain precursor | P07942 | 2.28 (5) |
| Laminin γ-1 chain precursor | P11047 | 2.27 |
| Leucine-rich glioma-inactivated protein 1 precursor | O95907 | 16.25 (3) |
method, $\mu$ and $\sigma$ can be obtained from the data series of the normalized peptide percentage and the experimentally determined quantitation ratios of the cICAT-labeled peptide pairs. The cICAT-labeled peptide abundance ratio above which an increase or decrease in abundance should be considered as statistically significant is defined as $\frac{z}{\sigma}$ (i.e. $2\sigma$ above or below the measured mean ratio). Using these criteria, we determined that the threshold above which a measured cICAT peptide pair abundance ratio reflects a statistically significant increase in abundance should be considered to be $>1.645$, and the threshold below which a measured cICAT peptide pair abundance ratio reflects a statistically significant decrease in abundance should be considered to be $<0.487$ for these specific data. Using these criteria 93 proteins were significantly induced, and 37 proteins were significantly repressed in HUVECs responding to 24 h of treatment with sokotrasterol sulfate (Tables I and II, respectively).

To validate this approach, two proteins were chosen, Dysferlin and the transcription factor E2F4, that lie at the threshold of what would be considered significant up-regulation, a 1.75-fold increase (Table I and Fig. 4). Fig. 4a illustrates the tandem mass spectra of the observed light and heavy ICAT tryptic peptide $[M+2H]^2$ isotopomeric ions of Dysferlin. Fig. 4b depicts the reconstructed ion chromatograms of the pair of Dysferlin cICAT-labeled peptide molecular ion isotopomers. Similarly Fig. 4c shows the tandem mass spectra of the $[M+2H]^2$ ion of the heavy labeled peptide of E2F4, while the reconstructed ion chromatogram of the cICAT-labeled peptide molecular ions of E2F4 is presented in Fig. 4d. We confirmed by immunoblotting that both Dysferlin and E2F4 are up-regulated by sokotrasterol sulfate at 8, 24, and 48 h, and densitometric scanning normalized to a protein that did not change in expression, $\alpha$-tubulin, demonstrated a similar degree of induction (within the limits of biological and experimental variability) at 24 h as compared with the ratio determined by the extracted ion chromatograms reconstructed from the MS scans (Fig. 5).

To further characterize proteins differentially regulated by sokotrasterol sulfate, proteins were divided according to the

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**TABLE I—continued**

| Protein name$^a$ | Swiss-Prot accession no. | -Fold increase in protein levels$^b$ |
|------------------|--------------------------|-------------------------------------|
| Mitochondrial 60 S ribosomal protein L3 | P09001 | 1.72 (2) |
| Multisynthetase complex auxiliary component p43 | Q12904 | 1.89 (3) |
| Myosin heavy chain, non-muscle type A | P35579 | 2.10 (14) |
| Myosin light chain alkali, non-muscle isoform | P16475 | 1.75 (2) |
| $N$-Acetylglycosamine-6-sulfatase precursor | P15586 | 6.25 |
| NUP196 nucleoporin | Q8WYB0 | 2.27 |
| P2X purinoceptor 4 | Q99571 | 1.82 |
| PP5242 | Q8WY64 | 100.00 |
| Probable serine protease HTRA4 precursor | P83105 | 5.00 |
| Protein transport protein SEC61 $\beta$ subunit | P38391 | 1.75 |
| Protein-tyrosine phosphatase $\beta$ precursor | P23467 | 1.69 |
| RanBP7/importin 7 | Q95373 | 1.68 (3) |
| Ran GTPase-activating protein 1 | P46060 | 1.72 (3) |
| Ras-related protein Rab-2A | P20936 | 1.96 |
| SAM domain and HD domain-containing protein 1 | P08886 | 2.08 |
| Similar to ubiquitin-protein isopeptide ligase (E3) | Q9Y323 | 1.89 (2) |
| Small nuclear ribonucleoprotein Sm D3 | Q8TC15 | 7.14 |
| S phase kinase-associated protein 1A | P43351 | 2.00 |
| Splicing factor U2AF 35-kDa subunit | P34991 | 2.44 |
| SUPT5H protein | Q01081 | 1.81 (4) |
| SWI/SNF complex 155-kDa subunit | Q00267 | 1.81 (4) |
| Symplekin | Q92922 | 3.03 |
| Thioredoxin domain-containing protein 5 precursor | Q8NBS9 | 2.38 |
| Transcription factor E2F4 | O16254 | 1.75 |
| Transducin $\beta$-like 2 protein | Q9Y4P3 | 1.67 |
| Transcriptional coactivator tubedown-100 | Q8NEV2 | 1.96 |
| Translation initiation factor eIF-2B $\delta$ subunit | Q9U10 | 1.67 |
| Triose-phosphate isomerase | P00938 | 5.28 (23) |
| Tumor differentially expressed 1 protein-like | Q9NRX5 | 25.00 |
| Tyrosine-protein kinase receptor Tie-1 precursor | P35590 | 1.72 (2) |
| Uracil 5$'$-monophosphate synthase | P11172 | 1.78 (2) |
| Vascular ATP synthase subunit B, kidney isoform | P15313 | 1.92 |
| Vezatrin | Q0HBM0 | 1.69 |

$^a$ PLA2, phospholipase A2; eIF, eukaryotic initiation factor; HD, histidine aspartate; SAM, sterile alpha motif.

$^b$ Number of peptides identified is shown in parentheses.
gene ontology classes of molecular function, cellular compartment, and biological process. Sokotrasterol sulfate-induced proteins in HUVECs are classified in Fig. 6, and down-regulated proteins are classified in Fig. 7. As one would expect in this particular investigation, proteins involved in responding to external stimuli and in cell communication, including cell adhesion molecules, were more likely to be overexpressed than underexpressed in response to sokotrasterol sulfate. Interestingly at least one protein associated with signaling cell death, tumor necrosis factor receptor superfamily member 1B, was down-regulated by sokotrasterol sulfate. The distribution of proteins within cellular compartments was diverse and similar between proteins that were overexpressed and those that were underexpressed except that twice as many membrane proteins were found to be up-regulated, consistent with the gene ontology classification according to biological process. The up-regulated membrane proteins include molecules such as Dysferlin, which to date have not been associated with angiogenesis, as well as other proteins, such as the receptor tyrosine kinase Tie-1 and the αv integrin subunit, which are both known to be required for vascular development but have not previously been shown to be up-regulated by any steroid molecule (2).

### TABLE II

| Protein name | Swiss-Prot accession no. | -Fold increase in protein levels |
|--------------|--------------------------|---------------------------------|
| 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase δ1 | P51178 | 0.42 |
| 40 S ribosomal protein S4, Y isom form | P22090 | 0.17 |
| ADP-sugar pyrophosphatase YSA1H | Q8UKK9 | 0.31 |
| Amidophosphoribosyltransferase precursor | Q06203 | 0.41 |
| Branched-chain amino acid aminotransferase, cytosolic | P54687 | 0.45 |
| CGI-31 protein | Q9Y320 | 0.35 (2) |
| Chromatin-specific transcription elongation factor FACT 140-kDa subunit | Q9YSB9 | 0.48 (2) |
| Coronin-like protein p57 | P31146 | 0.22 |
| Cyclin G1 | P51959 | 0.03 |
| CYR61 protein precursor | Q00622 | 0.46 |
| DnaJ homolog subfamily A member 1 | P31689 | 0.28 (2) |
| Docking protein 1 | Q99704 | 0.39 |
| Dystroglycan precursor | Q14118 | 0.45 |
| Estrogen sulfotransferase | P49888 | 0.30 |
| Eukaryotic translation initiation factor 5 | P55010 | 0.04 (2) |
| Exosome complex exonuclease RRP45 | Q06265 | 0.27 |
| Hypothetical protein FLJ22673 | Q9HE20 | 0.43 |
| Hypothetical protein FLJ25701 (similar to RIKEN cDNA 0610011N22) | Q96BQ5 | 0.44 |
| Hypothetical protein KIAA0564 (fragment) | O60310 | 0.18 |
| MYB-binding protein 1A | Q9P0V5 | 0.43 |
| Myc-associated zinc finger protein | P56270 | 0.45 |
| NDRG1-binding protein | Q92597 | 0.39 |
| Nucleoporin-like protein RIP | P52594 | 0.29 |
| Probable ATP-dependent RNA helicase p47 | Q13838 | 0.36 |
| Proteinase-activated receptor 1 precursor | P25116 | 0.11 |
| Pumilio 2 | Q9HANZ | 0.34 |
| Putative polypeptide N-acetylgalactosaminyltransferase | Q8NFV9 | 0.34 |
| Ryanodine receptor 2 | Q92736 | 0.04 |
| SF21 protein | Q8NH2E | 0.48 (2) |
| SMC6 protein | Q86SB8 | 0.18 (2) |
| STE20/SPS1-related proline-alanine-rich protein kinase | Q8UEW8 | 0.45 |
| Structural maintenance of chromosome 1-like 1 protein | Q14683 | 0.39 |
| Structure-specific recognition protein 1 | Q8N945 | 0.32 |
| SWI/SNF chromatin remodeling complex subunit OSA2 | Q8NFDF | 0.33 |
| Thyroid receptor-interacting protein 6 | Q15654 | 0.43 |
| Tumor necrosis factor receptor superfamily member 1B precursor | P20333 | 0.05 |
| WAP four-disulfide core domain protein 12 precursor | Q8WWY7 | 0.10 |

* Number of peptides identified is shown in parentheses.

**DISCUSSION**

Ischemic coronary artery disease is a major cause of morbidity and the leading cause of mortality in the Western world (4). Current therapeutic options for patients with advanced ischemic heart disease include medical therapy or coronary revascularization by percutaneous coronary angioplasty or bypass surgery (4, 18). However, a significant number of these patients are not candidates for standard revascularization procedures or have incomplete revascularization with these procedures. As a result many of these patients have residual...
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(a) Dysferlin \(^{13}C_0\) vs. \(^{13}C_9\)

(b) Relative abundance

(c) Dysferlin \(^{13}C_9\) vs. E2F4 \(^{13}C_9\)

(d) Relative abundance

\(^{13}C_9\) vs. \(^{13}C_0\) = 1.75:1
symptoms of ischemia despite therapy (18). Furthermore the incidence of restenosis or reocclusion in patients who have had invasive revascularization procedures remains distressingly high (18). Hence the ability to promote neovascularization in a non-surgical manner is an important goal (19).

Various steroids have been shown to have either proangiogenic or antiangiogenic activity (14, 20–22). Thus, our discovery of sokotrasterol sulfate as a proangiogenic agent prompted us to elucidate its effects on the expression of proteins potentially involved in angiogenesis. Since the initiating events in angiogenesis are directed at the endothelial cell, we chose to study the effects of sokotrasterol sulfate on differential protein expression in primary endothelial cells.

Consistent with a role in endothelial sprouting and tube formation rather than in proliferation, many of the proteins identified to be up-regulated by sokotrasterol sulfate include molecules associated with cell communication and morphogenesis. For instance CD166/ALCAM, which has not previously been identified in endothelial cells, is reported to be important in directed cell migration, axonal guidance, and branching development (23–25). Our cICAT data showing up-regulation of ALCAM by sokotrasterol sulfate fits well with recent findings demonstrating coordinated events in neuronal and vascular development and shared molecules utilized in the integration of these processes (26, 27). Similarly homeobox proteins are implicated in both vascular and neuronal development (28). In particular the homeoprotein HB9 has been shown to be necessary for axonal pathfinding in a subset of neurons (29). Prior to this study there had been no previous documentation of HB9 being expressed in or involved in endothelial sprouting or angiogenesis. Thus our data showing sokotrasterol sulfate-induced expression of HB9 provides new information to be further investigated in angiogenic experiments.

Interestingly our findings have arisen in a setting where the endothelial cells were cultured in a two-dimensional tissue culture plate, whereas the morphogenic process of vascular sprouting is a three-dimensional process. Up-regulation of the above molecules by differential protein analysis suggests a powerful application for comparative proteomics in modeling in vivo events using in vitro models. Our data also indicate that some of the findings may be applicable more generally to angiogenesis secondary to other inciting stimuli, while other proteins may be more specific to sokotrasterol sulfate-induced angiogenesis.

The choice of what magnitude of change constitutes a biologically significant increase or decrease in protein expression as measured using ICAT reagents has generally been chosen arbitrarily. Abundance ratios varying from a 1.5- to 2-fold differential increase or decrease have been selected in previous studies to represent a statistically significant change in protein abundance (30, 31). However, such an arbitrary decision may exclude proteins that although not greatly increased in quantity may represent significant changes biologically. On the other hand under certain experimental conditions a 1.5-fold differential expression may not be stringent enough to exclude proteins that do not display a significant change in expression. In this study a statistical method was used to determine the cut-off levels for proteins differentially expressed in response to sokotrasterol sulfate, and we have verified that our cut-off level of ~1.7-fold increase in expression level is reproducible by immu-
noblotting. For instance, the up-regulation of E2F4 is consistent with a morphogenic effect of sokotrasterol sulfate. Although transcription factors of the E2F family are known to be necessary for the G1/S transition during the cell cycle, E2F4 has also been shown to be critical for nerve growth factor-induced differentiation and has been reported to be required for neurite outgrowth independent of cell growth in response to nerve growth factor (32). This finding reveals yet another potential connection between neuronal and vascular development. The second molecule validated, Dysferlin, is expressed on the membrane of skeletal muscle cells and is essential for maintaining the structural stability of the plasma membrane. This protein is mutated in limb-girdle muscular dystrophy and has not previously been shown to be expressed in endothelial cells (33). While the functional relevance of this membrane protein remains to be explored, it is tempting to speculate that Dysferlin may be required to maintain the integrity of the plasma membrane during the migratory phase of angiogenesis where the cell

Fig. 6. Distribution of proteins differentially up-regulated in endothelial cells by sokotrasterol sulfate. Shown is the classification of proteins according to the gene ontology categories of molecular function (a), cellular compartment (b), and biological process (c).
is actively modulating plasma membrane protein expression and function. Interestingly at least two other proteins that are up-regulated by sokotrasterol sulfate, Tie-1 and αv integrin, are functionally necessary for angiogenesis (2). Inhibitors against αvβ3 integrin are currently undergoing clinical trials as anti-angiogenic therapy in cancer; however, neither αvβ3 nor Tie-1 has previously been shown to be up-regulated by any steroid compound (34). These biologically relevant findings would have been missed had a cut-off value of 2-fold increase been chosen. This strategy of determining significantly over- or underexpression thus implicates other proteins at this borderline level that may be potentially important in vascular development.

Very few studies have attempted to undertake global proteomic analysis of endothelial cells (35–38). In addition to differential proteomic analysis that we performed, this is the most extensive global proteomic analysis of primary endothelial cells reported thus far. For instance, in a recent profiling of primary HUVEC cultures using two-dimensional gel electrophoresis, 53 proteins were identified (37). In a more directed approach targeted to identify constituents of
endothelial caveolae and rafts, 25 bona fide raft proteins were identified (36). In this study, we identified nine of these 25 raft proteins (36). We also identified multiple other membrane proteins not previously known to be expressed in endothelial cells, such as Dysferlin and ALCAM, thus providing further impetus for the cICAT approach as undertaken in this study.

Finally these findings will be important not only to investigators interested in promoting angiogenesis but also to those involved in inhibiting angiogenesis. Attempts to block angiogenesis to inhibit tumor growth and metastasis have recently proven efficacious in metastatic renal and colon cancer and have provided impetus for a rapid burgeoning of this area of research (39, 40). The identification of molecules that are differentially up-regulated during angiogenesis could provide potential targets toward which antiangiogenic therapeutic agents can be designed.

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