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Abstract—The potential to promote neovascularization in ischemic tissues using exogenous agents has become an exciting area of therapeutics. In an attempt to identify novel small molecules with angiogenesis promoting activity, we screened a library of natural products and identified a sulfated steroid, sokotrasterol sulfate, that induces angiogenesis in vitro and in vivo. We show that sokotrasterol sulfate promotes endothelial sprouting in vitro, new blood vessel formation on the chick chorioallantoic membrane, and accelerates angiogenesis and reperfusion in a mouse hindlimb ischemia model. We demonstrate that sulfation of the steroid is critical for promoting angiogenesis, as the desulfated steroid exhibited no endothelial sprouting activity. We thus developed a chemically synthesized sokotrasterol sulfate analog, 2β,3α,6α-cholestanetrisulfate, that demonstrated equivalent activity in the hindlimb ischemia model and resulted in the generation of stable vessels that persisted following cessation of therapy. The function of sokotrasterol sulfate was dependent on cyclooxygenase-2 activity and vascular endothelial growth factor induction, as inhibition of either cyclooxygenase-2 or vascular endothelial growth factor blocked angiogenesis. Surface expression of $\alpha_v\beta_3$ integrin was also necessary for function, as neutralization of $\alpha_v\beta_3$ integrin, but not β_1 integrin, binding abrogated endothelial sprouting and antiapoptotic activity in response to sokotrasterol sulfate. Our findings indicate that sokotrasterol sulfate and its analogs can promote angiogenesis in vitro and in vivo and could potentially be used for promoting neovascularization to relieve the sequelae of vasoocclusive diseases. **(*Circ Res*. 2006;99:257-265.)**

Key Words: angiogenesis ■ ischemia ■ endothelium

The development of new blood vessels from an existing network of vessels, a process referred to as angiogenesis, contributes to various pathologies such as tumor progression and chronic inflammatory processes.[1] On the other hand, neovascularization in response to ischemia is a desirable response to alleviate the sequelae of tissue hypoxia.[2–5] Therapeutic angiogenesis is a promising strategy in the treatment of occlusive vascular disease, such as myocardial and limb ischemia. However, the outcome of early-phase clinical trials has been less than encouraging, possibly because of factors such as choice and formulation of the growth factor, duration of exposure, route of administration, and selection of patients.[6,7]

To date, clinical studies for promoting neovessel development in ischemic tissues have focused exclusively on the use of angiogenic growth factors, which are delivered using 2 main strategies.[6,7] One strategy has been to deliver recombinant proteins directly to the ischemic tissue through intramuscular injection or intraarterially. The alternate strategy has been to use gene therapy, either by direct transfer of expression vectors or through a cell-based approach. An optimum delivery strategy has not been devised, and the approach of using large proteins as therapeutic agents, although not without precedent, carries with it several disadvantages. The use of a small molecule for therapeutic angiogenesis would obviate many of the difficulties associated with the use of proteins or gene therapy.

Natural products or synthetic compounds based on natural product pharmacophores make up between 50% and 60% of all drugs currently used in human medicine and investigational new drugs under preclinical and clinical trial evaluation.[8] Marine sponges are 1 of the richest sources of new natural product structures.[9] There are currently more than a dozen compounds based on natural products isolated from sponges undergoing clinical trials or advanced preclinical evaluation.[9,10] The rich chemical diversity found in sponge-

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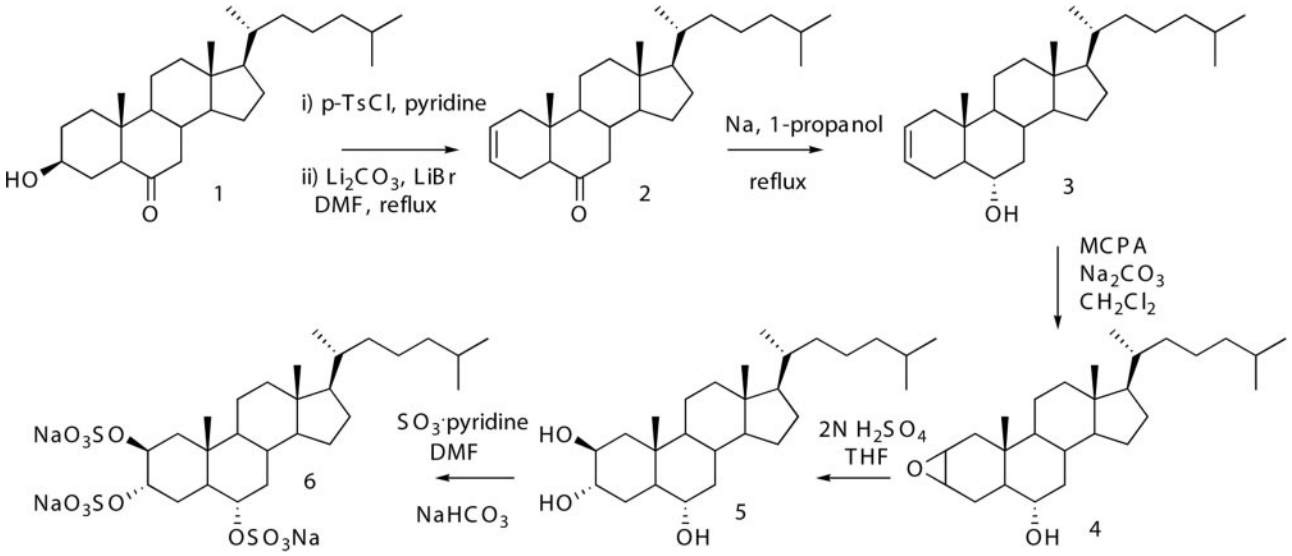

Figure 1. Schema depicting the steps required for synthesis of 2β,3α,6α-cholestanetrisulfate.

derived natural products, along with their wide spectrum of well-documented biological activities, prompted us to screen a library of marine sponge extracts for compounds that had proangiogenic activity. Further characterization of 1 of the extracts that promoted endothelial sprouting revealed a sulfated steroid, sokotrasterol sulfate, that was capable of inducing angiogenesis in the chick chorioallantoic membrane (CAM), as well as reperfusion in a mouse hindlimb ischemia model. Our studies demonstrate that the sulfate groups of this steroid are required for angiogenic activity. The action of sokotrasterol sulfate is dependent on cycloxygenase-2 (COX-2) activation, vascular endothelial growth factor (VEGF) induction, and the $\alpha_v\beta_3$ integrin. Finally, a synthetic analog of sokotrasterol sulfate promoted more rapid reperfusion, with longer-term stability of the vasculature, in a murine hindlimb ischemia model.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were harvested and cultured as previously described.[11]

Endothelial Sprouting Assays

Endothelial sprouting was assessed by a modification of the method of Nehls and Drenckhahn, as previously described.[12,13] For the initial screen, 60 marine extracts were examined, under conditions where the absence of angiogenic factors resulted in no sprouts being formed. Extracts were scored as either positive or negative with respect to sprouting activity.

Sponge Material

Specimens of *Topsentia ophirhaphidites* were collected by hand using self-contained underwater breathing apparatus (SCUBA) equipment at a depth of 15 to 20 m in Prince Rupert Bay, southwest of Glánvillia, near Portsmouth, Commonwealth of Dominica in June 1997. Freshly collected sponge was frozen on site and transported frozen to Vancouver. The sponge was identified by Prof Rob van Soest, University of Amsterdam, and a voucher sample has been deposited at the Zoological Museum of Amsterdam (POR ZMA 17182).

Isolation

The thawed sponge sample (120 g) was cut into small pieces and immersed in and subsequently extracted repeatedly with methanol (MeOH) (3×250 mL). The combined MeOH extracts were concentrated in vacuo to yield 4.0 g of a pale green amorphous solid. The extract was then partitioned between EtOAc (3×75 mL) and H_2O (125 mL). The aqueous extract was evaporated to dryness, and the resulting 3.1 g of pale yellow solid was chromatographed 3 times on Sephadex LH-20 using first 1:1 MeOH/H_2O as eluent, then MeOH as eluent, and finally 20:5:1 EtOAc/MeOH/H_2O as eluent. Pure sokotrasterol sulfate (81.3 mg) was obtained as the active component and was identified by standard spectroscopic analysis.[14]

Preparation of Sokotrasterol

Sokotrasterol sulfate (40.1 mg) in 1:1 MeOH/H_2O (4 mL) was treated with 1 mL of 2 N HCl. The solution was stirred for 16 hours at 70°C. On cooling, the reaction mixture was extracted with CH_2Cl_2 (3×2 mL). The combined CH_2Cl_2 extracts were concentrated in vacuo to yield 29.8 mg of a mixture of sokotrasterol and sokotrasterol sulfate. This material was fractionated with silica gel flash chromatography using a 2-g Sep-Pak (Waters), with a step gradient from 95:5 CH_2Cl_2/MeOH to MeOH. A fraction containing pure sokotrasterol (8.0 mg) was eluted with 9:1 CH_2Cl_2/MeOH.

Synthesis of 2β,3α,6α-Cholestanetrisulfate

2β,3α,6α-Cholestanetrisulfate (cholestanetrisulfate) was synthesized as outlined in Figure 1. Details of the synthesis are presented in the online data supplement, available at http://circres.ahajournals.org.

Chick CAM Assay

The chick CAM assay was performed as previously described.[15,16] On day 8, 20 mL of sokotrasterol sulfate (20 to 60 mg/mL) was loaded onto 2-mm^3 gelatin sponges (Gelfoam, Pharmacia Upjohn), which were then placed on the surface of the developing CAM. Sponges containing vehicle alone (20 μL of PBS containing DMSO) were used as negative controls. Eggs were resealed and returned to the incubator. On day 10, digital images of the CAMs were captured and analyzed for quantitation of angiogenesis.

Mouse Hindlimb Ischemia Model

All protocols were approved by The University of British Columbia Committee on Animal Care, Vancouver, British Columbia. C57Bl/6 male and female mice weighing between 25 to 35 g were provided by Charles River Laboratories, Quebec, Canada. Unilateral hindlimb ischemia was created in animals that were 10 to 12 weeks of age as

previously described.[17] All animals were anesthetized using inhalational isoflurane (Bimeda-MTC). After dissection of the neurovascular bundle of the right hindlimb, the proximal and distal portions of the femoral artery were ligated, and the ligated portion of the femoral artery was excised and removed. Sokotrasterol sulfate or cholestanetrisulfate was administered at 100 μg per dose, 3 times per week into the overlying muscle. The left hindlimb was sham operated in all animals.

Laser Doppler Perfusion Imaging

Hindlimb blood flow was monitored by laser Doppler perfusion imaging (LDPI) performed using the Periscan PIM II Laser Doppler Perfusion Imager High Resolution model (PIM II) (Perimed, Stockholm, Sweden). The velocity/perfusion images were subdivided into 6 different intervals set to have a user-defined color scale (dark blue representing lowest perfusion and red representing greatest perfusion) ranging from 0 to 7.0 V, and the perfusion index values were expressed in volts. Scans were performed on anesthetized mice as described for ligation above. For all LDPI measurements, mice were kept on a heating pad maintained at 37°C for 10 minutes, as well as during scanning, to minimize variations during perfusion scans. A perfusion ratio was calculated by dividing the mean perfusion value of the ischemic hindlimb by the mean perfusion value of an identical region in the nonischemic hindlimb from the same scan.

Migration Assay

The ability of HUVECs to migrate toward fibroblast growth factor (FGF)-2, sokotrasterol sulfate, and sokotrasterol was measured with the use of a Transwell filter assay as previously described.[12,15]

Flow Cytometry

Flow cytometry analysis of sokotrasterol or DMSO-treated cells was performed as previously described.[15] LM609 primary antibody was obtained from Chemicon, and mouse IgG2a isotype control was obtained from Sigma.

Survival Assays

HUVECs were seeded on 96-well plates at a density of 25 000 cells per well in MCDB131 medium containing 1% calf serum, with either 5 μg/mL sokotrasterol sulfate or DMSO. Medium was replaced daily. At days 0, 2, 4, 7, and 10, viable cell numbers were estimated by neutral red uptake as previously described.[18]

Immunofluorescent Staining

Immunofluorescent staining of HUVECs with anti–5-bromodeoxyuridine (anti-BrdU) (Molecular Probes) or anti–activated caspase-3 (BD Pharmingen) was performed using previously described methods.[11]

Reverse-Transcription Polymerase Chain Reaction

RT-PCR was performed in semiquantitative fashion, as previously described.[12] Details of the primers used and cycling conditions are given in the online data supplement.

Statistical Analysis

A Student *t* test was used to determine statistical significance between 2 groups. To determine statistical significance with multiple groups, a 1-way ANOVA with a Tukey test for multiple comparisons was used. Statistical significance was taken at $P \leq 0.05$.

Results

Identification of Sokotrasterol Sulfate As an Agent That Promotes Endothelial Sprouting

To determine whether we could identify molecules that promote vascularization from a repository of marine extracts, we used a previously described endothelial sprouting assay.[12,13,15] Of 60 extracts tested, 3 showed endothelial sprouting activity. One extract that showed endothelial sprouting activity was selected for further study. A previously identified sulfated steroid, sokotrasterol sulfate, was found in extracts from the Dominican marine sponge *T ophirhaphidites* (Figure 2A).[14] Sokotrasterol sulfate showed dose-dependent endothelial sprouting activity, which peaked at a concentration of 5 mg/mL (Figure 2B and 2C).

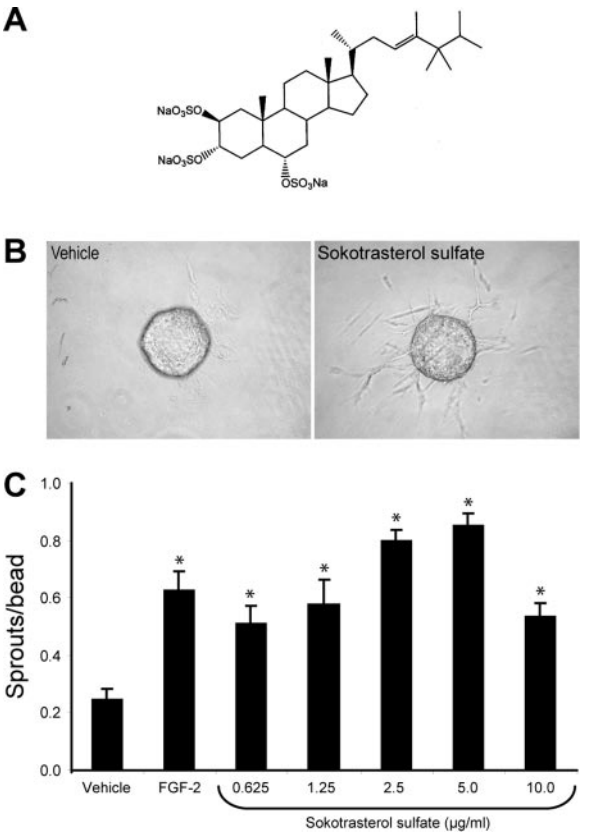

Figure 2. Sokotrasterol sulfate promotes endothelial sprouting in vitro. A, Chemical structure of sokotrasterol sulfate. B, Example of the microcarrier bead endothelial sprouting assay demonstrating sprout formation by sokotrasterol sulfate. C, Endothelial sprout formation was quantitated after 3 days of incubation by counting the number of tube-like structures per microcarrier bead (sprouts per bead). Data are the means±SD from 7 independent experiments, each done in triplicate. *$P<0.01$, comparison with vehicle control.

Sokotrasterol Sulfate Promotes Neovascularization In Vivo

To determine whether sokotrasterol sulfate was also able to promote neovascularization in vivo, we used a chick CAM assay. Gelatin sponges were embedded with sokotrasterol sulfate at the concentrations indicated and placed on the chick CAM. The angiogenic index was calculated by quantitating the number of vessels entering the sponge and dividing by the length of the perimeter of the sponge. As seen in Figure 3A and 3B, sokotrasterol sulfate promoted angiogenesis on the chick CAM at similar levels to FGF-2 (15 ng/mL).

To evaluate the potential of sokotrasterol sulfate to induce neovessels in ischemic tissues, we used a murine hindlimb ischemia model where the femoral artery was ligated in 1 hindlimb and a sham operation was conducted on the con-

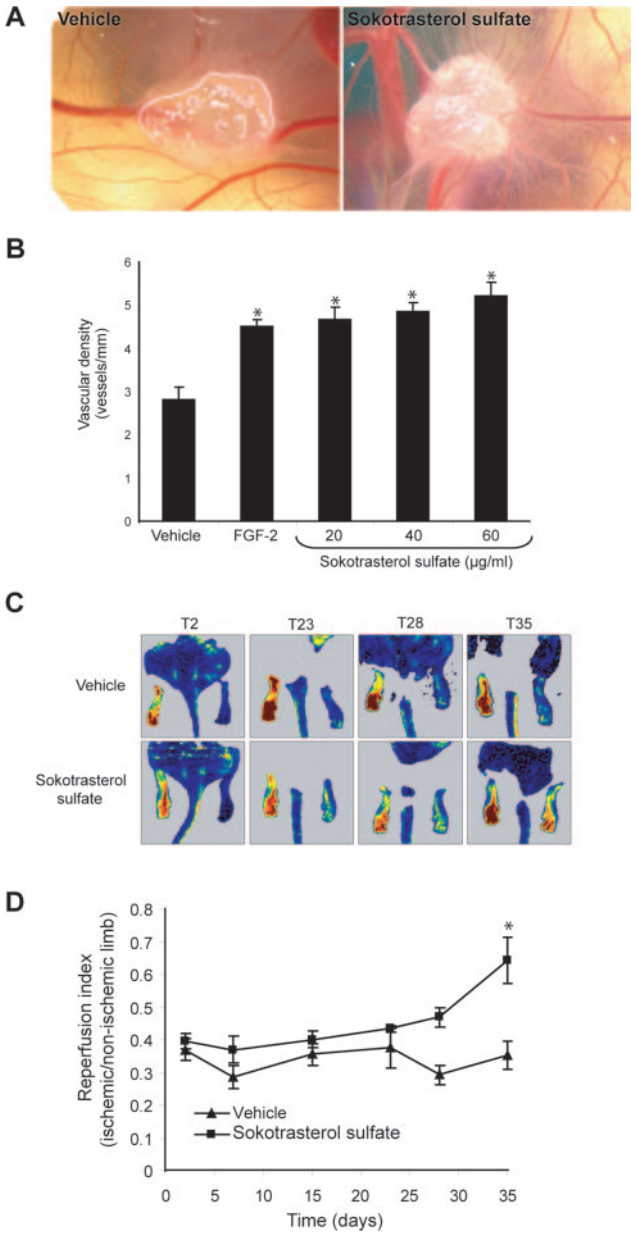

Figure 3. Sokotrasterol sulfate promotes angiogenesis in vivo. A, Sokotrasterol sulfate was loaded onto gelatin sponges, and the sponges were placed on the CAMs of day-8 chick embryos. As controls, sponges containing vehicle or FGF-2 (15 ng/mL) were placed on CAMs. B, Quantitation of angiogenesis was performed on day 10 by counting the number of vessels that entered the sponge and dividing by the perimeter of the sponge (vessels per millimeter). Data shown are means±SE (n=7). *$P<0.01$, comparison with vehicle control. C, Vascular reperfusion in response to sokotrasterol sulfate (100 μg per dose, 3 times per week) or vehicle (DMSO) was measured by LDPI following femoral ligation. Examples of imaged limbs are shown at different time points. D, Reperfusion following hindlimb ischemia was quantitated at various times and plotted as the reperfusion index. The area analyzed over the ischemic limb was superimposed on the control (sham-operated) limb, and the mean values for each limb were calculated by the LDPI software. The reperfusion index for each time point and animal was taken as the values for the ischemic limb expressed as a ratio of the values for the nonischemic limb. Results shown are the mean±SE (n=6 for each group). *$P<0.01$, comparison with vehicle control.

tralateral limb. Sokotrasterol sulfate (100 μg per dose) was injected 3 times per week into the overlying muscle of the ischemic hindlimb. As demonstrated in Figure 3C and 3D, sokotrasterol sulfate was able to accelerate the reperfusion of ischemic limbs following femoral ligation as measured by LDPI. We verified that the vascular density was increased in the sokotrasterol sulfate-treated compared with the vehicle-treated animals by quantitating the microvessel/myofiber ratio (4.89±0.11 versus 3.98±0.094, $P<0.0001$) by immunostaining for CD31.

The Sulfate Groups Are Required for the Function of Sokotrasterol Sulfate

To determine whether the sulfate groups on sokotrasterol sulfate were required for the endothelial sprouting activity, the compound was desulfated to generate sokotrasterol, and mono- (3β-cholestanesulfate) and disulfated (2β-3α-cholestanedisulfate) cholestane derivatives were synthesized. Interestingly complete desulfation of sokotrasterol sulfate inhibited the ability of sokotrasterol to promote endothelial sprouting, whereas even partial sulfation of cholestane was able to promote sprouting activity, indicating that the sulfate groups are required for the sprouting function of sokotrasterol sulfate (Figure 4A).

In order for capillaries to sprout, endothelial cells need to migrate toward a stimulus.[19] To examine whether enhanced migration could explain the sokotrasterol sulfate-induced sprouting, we performed chemotaxis assays using Transwell filters. Our findings demonstrate that sokotrasterol sulfate promotes endothelial migration, but migration is inhibited by desulfation of sokotrasterol (Figure 4B). These findings suggest that the promigratory activity contributes to endothelial sprouting and that the sulfate groups are essential for the function of sokotrasterol sulfate.

Because endothelial proliferation is also required for extension of the capillary stalk, we tested whether sokotrasterol sulfate could promote endothelial proliferation. HUVECs were stimulated with vehicle, sokotrasterol sulfate (5 μg/mL), or VEGF (30 ng/mL), and cell numbers were estimated 24 hours later by neutral red incorporation. Sokotrasterol sulfate showed similar proliferative capacity as that induced by VEGF (Figure 4C), suggesting that this compound is also able of promoting endothelial proliferation in the angiogenic process.

A Synthetic Analog of Sokotrasterol Sulfate Is Capable of Promoting Hindlimb Revascularization

We devised a simple strategy to synthesize cholestanetrisulfate, a trisulfated analog of sokotrasterol sulfate, to determine whether the synthetic compound would mimic the effects of sokotrasterol sulfate in the murine hindlimb ischemia model. As demonstrated in Figure 5A and 5B, cholestanetrisulfate (100 μg per dose, 3 times per week into the overlying muscle) showed significant reperfusion activity within 3 weeks of administration. Following an initial compensatory vasodilation[6] reflected by the increased early reperfusion common to the cholestanetrisulfate and vehicle-treated hindlimbs (Figure 5B, day 2 to 7), there was increased neovessel-driven reper-

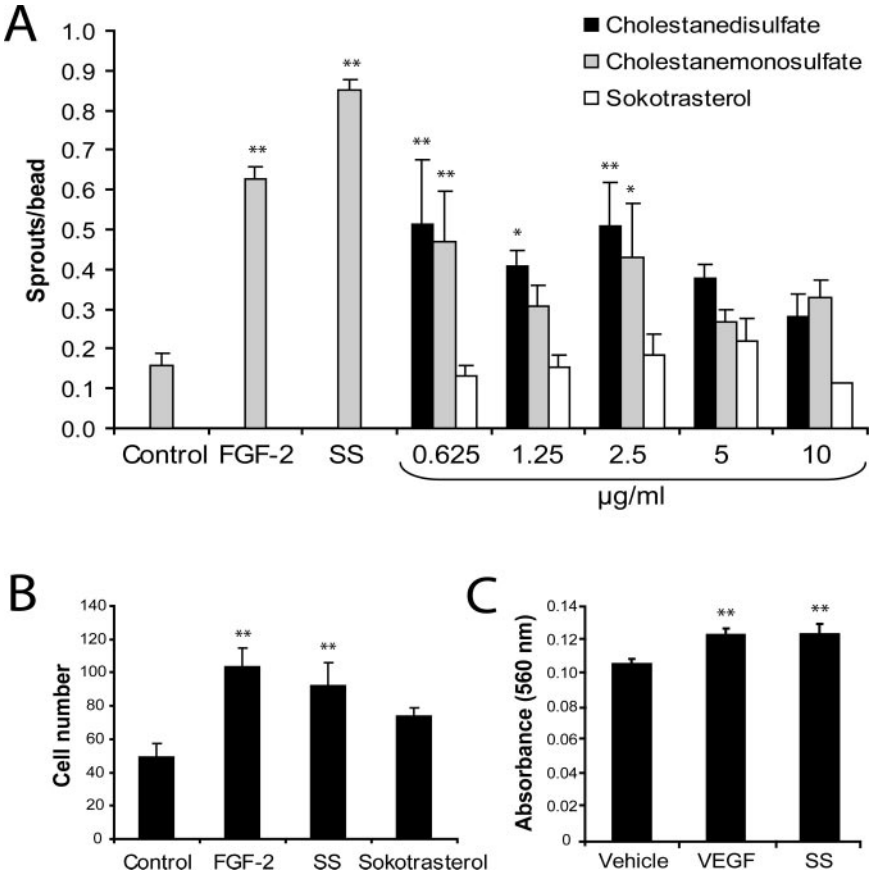

Figure 4. The sulfate moieties are required for the function of sokotrasterol sulfate. A, Endothelial sprout formation was quantitated after 3 days of incubation with cholestane mono- and disulfate or sokotrasterol (lacking all sulfate moieties) by counting the number of sprouts per microcarrier bead. Data are the means±SD from 3 independent experiments, each done in triplicate. B, Migration of HUVECs toward vehicle, FGF-2 (1 ng/mL), sokotrasterol sulfate (2.5 µg/mL), or sokotrasterol (2.5 µg/mL) was assayed using a modified Boyden chamber assay. Following 16 hours of incubation, cells that had migrated and adhered to the underside of the filter were stained and counted. Data shown are the mean±SD of 3 experiments, each done in duplicate. C, HUVECs proliferation was estimate by neutral red incorporation 24 hours after stimulation with vehicle (DMSO), VEGF (30 ng/mL), or sokotrasterol sulfate (5 µg/mL). Data shown are the mean±SE of 3 experiments, each done in sextuplicate. *$P<0.05$, **$P<0.01$, comparison with vehicle control.

fusion seen in the cholestanetrisulfate-treated animals (Figure 5B, day 12 to 26).

To determine whether the neovessels that were regenerated were stable, after 26 days (11 doses)—when there was a significant difference in reperfusion of the cholestanetrisulfate-treated ischemic limb—administration of cholestanetrisulfate was discontinued, but hindlimb perfusion continued to be monitored. Although the increase in reperfusion diminished following cessation of cholestanetrisulfate treatment, the established perfusion remained stable with a continued slight increase until day 35 (Figure 5). At day 35, the microvessel/myofiber ratio was increased in the cholestanetrisulfate-treated animals compared with the vehicle-treated mice (5.05±0.11 versus 4.15±0.099, $P<0.0001$). Thus an easily synthesized analog of sokotrasterol sulfate is able to accelerate revascularization and generate stable vessels following hindlimb ischemia.

$\alpha_v\beta_3$ Integrin Is Required for the Sprouting Activity of Sokotrasterol Sulfate

To identify proteins regulated by sokotrasterol sulfate, we recently conducted a differential protein analysis.[20] Comparison between HUVECs treated with sokotrasterol sulfate for 24 hours and vehicle-treated HUVECs demonstrated a significant increase in the expression of α_v integrin. To determine whether expression of $\alpha_v\beta_3$ protein was upregulated at the cell surface by sokotrasterol sulfate, HUVECs were stimulated with sokotrasterol sulfate for 24 hours and flow cytometry was performed. Sokotrasterol sulfate promoted surface expression of $\alpha_v\beta_3$ integrin as seen in Figure 6A.

We next determined whether surface expression of $\alpha_v\beta_3$ integrin played a functional role in sokotrasterol sulfate-mediated endothelial sprouting. Using the endothelial sprouting model, we investigated the effect of the function-blocking $\alpha_v\beta_3$ integrin antibody LM609 on sokotrasterol sulfate-induced sprouting. Microcarrier beads were coated with either LM609 or an isotype control antibody, and the matrix was supplemented with the relevant antibody at the initiation of the sprouting assay. As shown in Figure 6B, inhibition of $\alpha_v\beta_3$ function with LM609 abrogated sokotrasterol sulfate-induced sprouting.

$\alpha_v\beta_3$ integrin antagonists have been reported to block angiogenesis by inducing apoptosis of migrating endothelial cells.[21–23] We thus tested whether sokotrasterol sulfate promoted endothelial survival in a matrix-dependent manner.[24–26] HUVECs were plated on either fibrinogen ($\alpha_v\beta_3$ integrin counter receptor) or collagen I (β_1 integrin counter receptor) and then subjected to serum starvation. Whereas sokotrasterol sulfate was able to protect endothelial cells adherent to fibrinogen, this survival activity was not observed when cells were plated on collagen I (Figure 6C). To confirm that sokotrasterol sulfate protects endothelial cells from apoptosis in an integrin-specific manner, we quantitated the proportion of caspase 3–positive HUVECs 48 hours following serum depletion. As seen in Figure 6D, sokotrasterol sulfate inhibited caspase 3 activation when cells were plated on fibrinogen, but not when they were plated on collagen I. The requirement for the engagement of $\alpha_v\beta_3$ integrin for the cytoprotective effect of sokotrasterol sulfate was confirmed

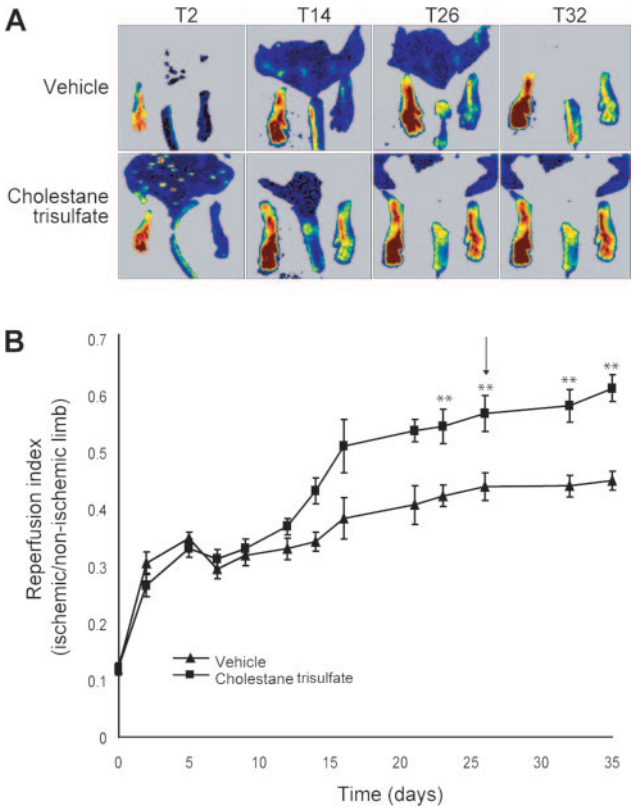

Figure 5. A synthetic sokotrasterol sulfate analog promotes revascularization in vivo. Vascular reperfusion in response to cholestanetrisulfate (100 μg per dose, 3 times per week) or vehicle (DMSO) was measured by LDPI following femoral ligation. A, Examples of imaged limbs are shown at different time points. B, Reperfusion following hindlimb ischemia was quantitated at various times and plotted as the reperfusion index. The area analyzed over the ischemic limb was superimposed on the control (sham-operated) limb, and the mean values for each limb were calculated by the LDPI software. The reperfusion index for each time point and animal was taken as the values for the ischemic limb expressed as a ratio of the values for the nonischemic limb. Results shown are the mean±SE (n=6 for each group). *$P<0.05$, **$P<0.01$, comparison with vehicle control.

by blocking the integrin with LM609, which abrogated the antiapoptotic effect of sokotrasterol sulfate on endothelial cells seeded on fibrinogen but not on collagen I (Figure 6E).

Activation of COX-2 and Induction of VEGF Is Required for Sokotrasterol Sulfate–Induced Angiogenesis

To determine whether other proangiogenic molecules were involved in the activity of sokotrasterol sulfate, we performed semiquantitative RT-PCR on HUVECs and primary human foreskin fibroblasts for angiopoietin-1, $VEGF_{121}$, $VEGF_{165}$, COX-1, and COX-2, followed by densitometric scanning of the bands obtained. Interestingly, COX-2, $VEGF_{121}$, and $VEGF_{165}$ were upregulated in response to either sokotrasterol sulfate or cholestanetrisulfate, whereas angiopoietin-1 and COX-1 were not affected (Figure 7A). Fibroblasts did not show any response to either compound (Figure 7B).

A functional link between $\alpha_v\beta_3$ integrin–mediated endothelial migration and COX-2 has been demonstrated.[27,28] The generation of prostaglandin (PG) E_2 by COX-2 is reported to be proangiogenic, and inhibition of COX-2 using nonsteroidal antiinflammatory drugs (NSAIDs) inhibits angiogenesis.[29,30] We first confirmed that cholestanetrisulfate/sokotrasterol sulfate could induce PGE_2 in HUVECs (vehicle control=265.5±64.7 pg/mL, cholestanetrisulfate=317.1±67.4 pg/mL following 24 hours of stimulation, n=8, $P=0.001$). To determine whether inhibition of COX-2 would block endothelial sprouting in response to sokotrasterol sulfate/cholestanetrisulfate, we used the selective COX-2 inhibitor SC-236 (5 mmol/L) in the microcarrier bead assay. Figure 8A demonstrates that inhibition of COX-2 abrogates sokotrasterol sulfate/cholestanetrisulfate-induced endothelial sprouting, thus implicating a role for COX-2 in sokotrasterol sulfate-induced angiogenesis. To verify that COX-2 is also required for sokotrasterol sulfate proangiogenic activity in vivo, chick CAM assays were performed. As shown in Figure 7B, the COX-2 inhibitor SC-236 (100 mmol/L) blocked sokotrasterol sulfate–mediated angiogenesis on the chick CAM.

Although $VEGF_{121}$ and $VEGF_{165}$ induction by sokotrasterol sulfate/cholestanetrisulfate did not achieve statistical significance, the fact that both isoforms were induced in HUVECs by both the natural and synthetic compound and because COX-2 is a known inducer of VEGF,[31] we attempted to determine whether VEGF inhibition (using a peptide inhibitor, $Flt_{2\text{-}11}$)[32] would block sokotrasterol sulfate/cholestanetrisulfate-induced angiogenesis. Figure 7C demonstrates that the VEGF inhibitor abrogates both sokotrasterol sulfate– and cholestanetrisulfate-induced angiogenesis on the chick CAM. Thus these compounds appear to act at least in part through the induction of COX-2 and VEGF.

Discussion

Natural product structures have high chemical diversity, biochemical specificity, and other molecular properties that make them favorable as lead structures for drug discovery.[33] The complexity and broad distribution of molecular properties have likely been selected through evolutionary pressures to interact with a wide variety of proteins and other biological targets for specific purposes.[33] In this article, we report the identification of a novel angiogenic steroid, sokotrasterol sulfate, that promotes neovascularization of ischemic muscle and demonstrate that a synthetic analog (cholestanetrisulfate) is just as efficacious in accelerating angiogenesis and reperfusion of ischemic hindlimbs in a murine model. To our knowledge, this is the first identification of a proangiogenic compound derived from a natural product.

Interestingly, various steroids have been demonstrated previously to have either angiogenesis-promoting or -inhibiting activity.[34,35] With sokotrasterol sulfate, the sulfate moieties appear to have a critical role in promoting endothelial sprouting, which suggests that the positive or negative effect of a steroid on angiogenesis is dependent on chemical modifications of the steroid ring. However, the mechanism of action of various steroids on angiogenesis remains to be elucidated. In the case of estradiol, which acts as a promoter of angiogenesis and may be effective in stimulating neovascularization in a hindlimb ischemia model, it has been suggested that the angiogenesis-promoting effect is indirect

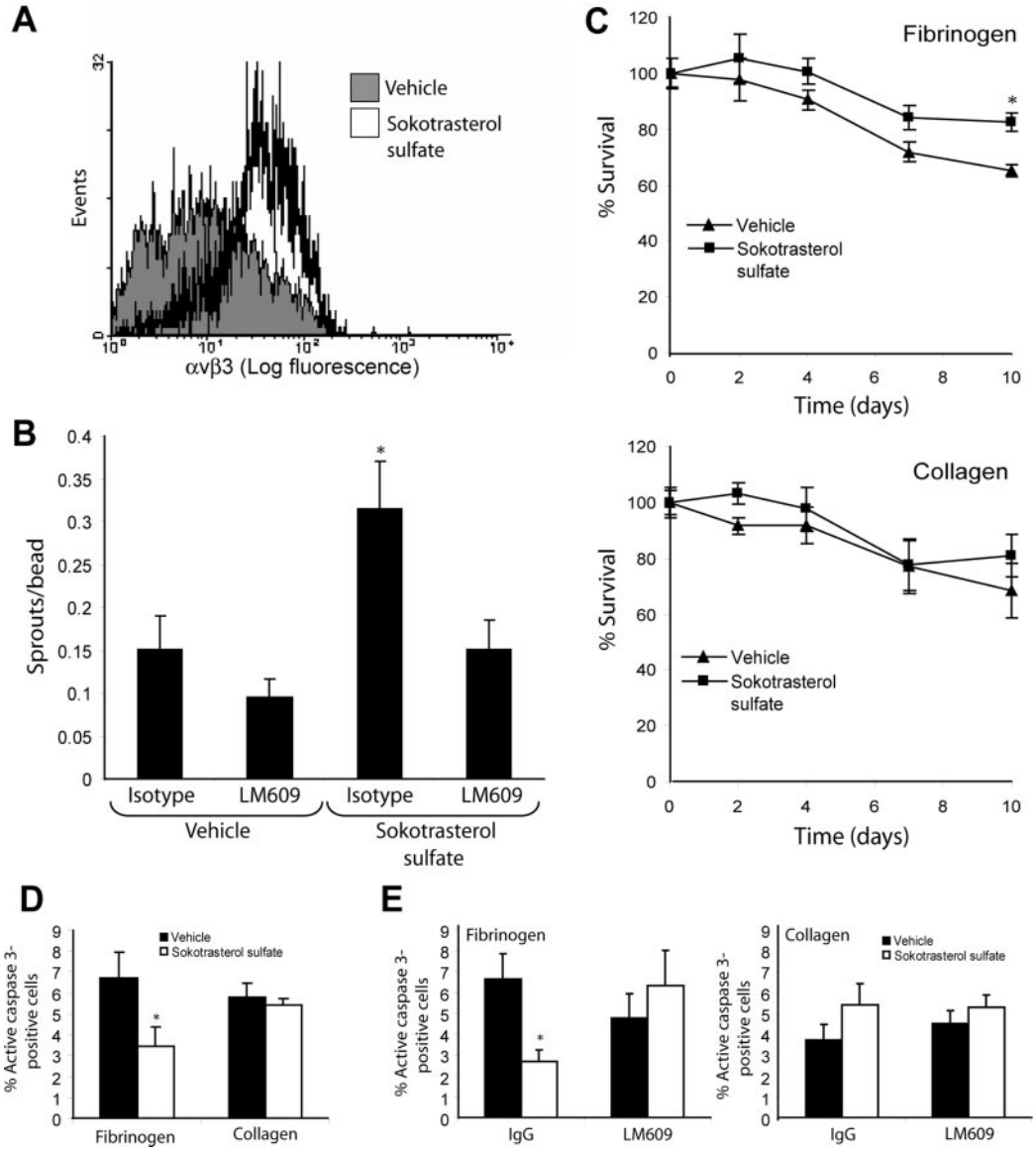

Figure 6. The activity of sokotrasterol sulfate is dependent on the $\alpha_v\beta_3$ integrin. A, Flow cytometric analysis of surface expression of $\alpha_v\beta_3$ integrin following stimulation with sokotrasterol sulfate (5 μg/mL) or vehicle. B, Microcarrier beads were coated with either an $\alpha_v\beta_3$ integrin–blocking antibody (LM609) or an isotype control antibody, then seeded with HUVECs. Sokotrasterol sulfate (5 μg/mL) or vehicle was added to the gel and the overlying medium in the presence of additional antibody (1 μg/mL). Endothelial sprout formation was quantitated after 3 days of incubation by counting the number of sprouts per microcarrier bead. Data are the means±SD from 4 independent experiments, each done in sextuplicate. C, The antiapoptotic activity of sokotrasterol sulfate on HUVECs was assessed by plating cells on an $\alpha_v\beta_3$ integrin (fibrinogen) or β_1 integrin (collagen I) substrate and serum starving cultures. Cell viability was measured by neutral red uptake and expressed as a proportion of cells plated at the initiation of the experiment. Results are the mean±SE of 3 independent experiments, each done in triplicate. D, HUVECs were cultured as in C, and the proportion of cells expressing activated caspase 3 as a proportion of the total number of cells was assayed. Caspase 3 activity was determined by immunostaining with an anti–active caspase 3 antibody. Results are the mean±SE of 3 independent experiments, each done in duplicate. E, The role of $\alpha_v\beta_3$ integrin on sokotrasterol sulfate–mediated HUVECs survival on fibrinogen or collagen was assayed as in D in the presence of an $\alpha_v\beta_3$ integrin–neutralizing antibody (LM609) or an isotype control antibody, and caspase 3 activation was quantitated. Results are the mean±SE of 3 independent experiments, each done in duplicate. *$P<0.05$, comparison with vehicle control.

through the release of VEGF.[35–37] Our findings demonstrate that VEGF is also involved in sokotrasterol sulfate/cholestanetrisulfate-mediated angiogenesis. We also demonstrate a requirement for COX-2 and the $\alpha_v\beta_3$ integrin in sokotrasterol sulfate/cholestanetrisulfate-initiated angiogenesis. The fact that fibroblasts did not respond to sokotrasterol sulfate/cholestanetrisulfate suggest that the effects of these compounds are selective.

There is increasing evidence that "therapeutic angiogenesis" should be targeting arteriogenesis, ie, the outward remodeling of collateral vessels, rather than angiogenesis per se, the development of microvessels from the existing microvasculature.[38] Others have argued that, in fact, all of the agents that have been labeled as being proangiogenic also perform an arteriogenic function, and thus the distinction between these 2 processes for therapeutic neovascularization is somewhat

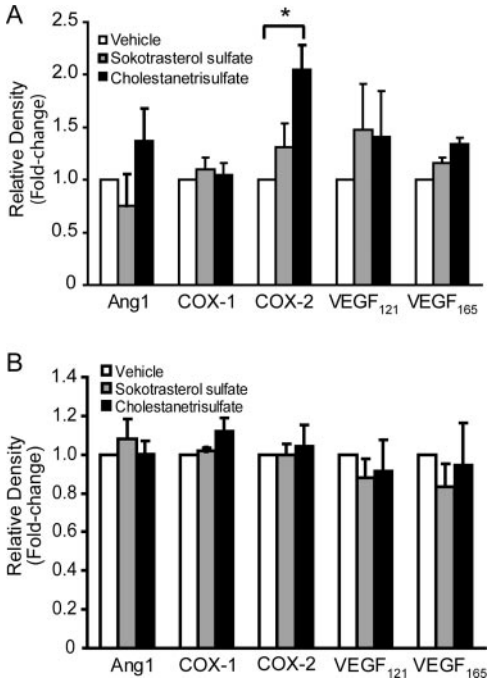

Figure 7. Sokotrasterol sulfate and cholestanetrisulfate induce expression of COX-2 and VEGF. HUVECs (A) or human foreskin fibroblasts (B) were stimulated with vehicle (DMSO), sokotrasterol sulfate (5 μg/mL), or cholestanetrisulfate (5 μg/mL) for 24 hours. Following RT-PCR and agarose gel electrophoresis, amplicons were quantified by densitometry. Results are the mean±SE of 3 independent experiments. *$P<0.01$.

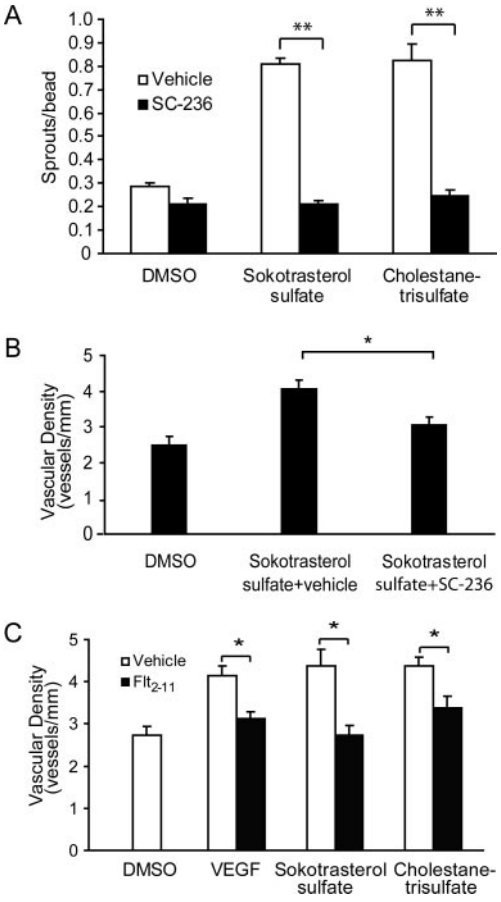

Figure 8. Sokotrasterol sulfate requires COX-2 and VEGF activity to induce angiogenesis. A, Endothelial sprout formation was quantitated after 3 days of incubation with sokotrasterol sulfate, cholestanetrisulfate, or solvent (DMSO) in the presence of a selective COX-2 inhibitor, SC-236 (5 mmol/L), or vehicle by counting the number of sprouts per microcarrier bead. Data are the means±SE from 3 independent experiments, each done in sextuplicate. B, Angiogenesis in the chick CAM was used to assay the requirement for COX-2 in sokotrasterol sulfate–triggered (40 μg/mL) angiogenesis. Sokotrasterol sulfate was loaded onto gelatin sponges in the presence of SC-236 (100 mmol/L) or vehicle, and the sponges were placed on the CAMs of day-8 embryos. As a negative control, sponges containing DMSO only were placed on CAMs. Quantitation of angiogenesis was performed on day 10. At least 9 CAMs per group from 3 independent experiments were tested. Data shown are means±SE. C, Angiogenesis in the chick CAM was used to assay the requirement for VEGF in sokotrasterol sulfate–triggered (40 μg/mL) and cholestanetrisulfate-triggered (40 μg/mL) angiogenesis. Gelatin sponges loaded with vehicle (DMSO) or the VEGF inhibitor (Flt_{2-11}, 500 μg/mL) were placed on the chick CAMs and vessel density enumerated. VEGF (30 ng/mL) was used as a control to demonstrate activity of the inhibitor against VEGF-driven angiogenesis. At least 8 CAMs per group from 3 independent experiments were tested. Data shown are means±SE. *$P<0.01$, **$P<0.001$.

artificial.[6] Indeed, it is likely that both processes will be required to take flow from enlarging collaterals to the microenvironment of the ischemic tissue.[38,39] Regardless of the process that is more important for effective therapeutic neovascularization, our data demonstrate that sokotrasterol sulfate and its synthetic analog, cholestanetrisulfate, are both efficacious in significantly improving perfusion to ischemic muscle. Furthermore, following multiple doses over ≈3 weeks, cholestanetrisulfate-treated limbs showed vasculature that was stable, in that perfusion to the ischemic limb was maintained well above that of the vehicle-treated limb for at least 10 days following the cessation of therapy. Furthermore, we did not observe any obvious toxic side effects with this therapy over the timeline of the experiment. There was no difference in animal weights, and, grossly, the organs appeared normal at the termination of the study.

The synthetic analog cholestanetrisulfate can be easily synthesized from commercially available 6-ketocholestanol in 6 chemical steps that all proceed in greater than 90% yield. Therefore, up to gram quantities of cholestanetrisulfate can be easily prepared for further biological evaluation. The ease of synthesis and favorable yield makes the use of sokotrasterol sulfate analogs in therapeutic angiogenesis a viable option for clinical use. Taken together, our findings indicate that sokotrasterol sulfate and its analogs could potentially add to the choice of therapeutics that are being clinically tested for promoting neovascularization to relieve the sequelae of vasoocclusive diseases.

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Disclosures

I.P., M.R., R.J.A., and A.K. have filed a patent for the use of sokotrasterol sulfate, cholestanetrisulfate, and derivatives in ischemia.

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