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Pharmacologic manipulation of sphingosine kinase in retinal endothelial cells: Implications for angiogenic ocular diseases

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Abstract

Purpose—The increased vascular permeability and pathogenic angiogenesis observed in diabetic retinopathy are induced, at least in part, by local inflammation and vascular endothelial growth factor (VEGF). Therefore, inhibition of signaling from VEGF and tumor necrosis factor α (TNF α) is a promising approach to the treatment of this disease, as well as ocular diseases with similar etiologies, including age-related macular degeneration. A growing body of evidence demonstrates that sphingosine kinase (SK) plays an important role in cellular proliferation and angiogenesis. Therefore, we have examined the effects of SK inhibitors on the responses of retinal endothelial cells (RECs) to VEGF and TNF α , and their therapeutic efficacy in a diabetic retinopathy model.

Methods—The expression and function of SK in bovine and human RECs were examined by immunoblotting. The involvement of SK in mediating responses to VEGF and TNF α was examined using pharmacological inhibitors of SK in cellular and *in vivo* assays, including a 3-month streptozotocin-induced diabetic retinopathy model in rats.

Results—We demonstrate that SK is present and active in human and bovine RECs, and that SK activity in these cells is stimulated by VEGF. Inhibitors of SK block VEGF-induced production of sphingosine 1-phosphate, and markedly attenuate VEGF-induced proliferation and migration of RECs. Additionally, SK inhibitors are shown to block TNF α -induced expression of adhesion proteins, suppress VEGF-induced vascular leakage in an *in vivo* mouse model, and reduce retinal vascular leakage in the rat diabetic retinopathy model.

Conclusion—Overall, these studies demonstrate that inhibitors of SK attenuate the effects of proliferative and inflammatory stimuli on RECs both *in vitro* and *in vivo*, and so could be significant therapeutics in the treatment of diabetic retinopathy.

Keywords

Sphingosine kinase; Diabetic retinopathy; Macular degeneration; Retinal endothelial cells; Angiogenesis; VEGF; TNF α

Introduction

Diabetic retinopathy remains a leading cause of vision impairment¹. Although the underlying cellular and molecular mechanisms of diabetic retinopathy remain obscure, elevation in the expression of growth factors contributes to pathogenic angiogenesis in this disease. In particular, vascular endothelial growth factor (VEGF) appears to be a prominent contributor to the new vessel formation in the diabetic retina^{2,3}, and VEGF has been shown to be elevated in patients with proliferative diabetic retinopathy⁴. In addition to diabetic retinopathy, several other debilitating ocular diseases, including age-related macular degeneration and choroidal neovascularization, are associated with excessive angiogenesis that is mediated by VEGF and other growth factors⁵⁻⁹. Therefore, better understanding of the mechanisms by which these angiogenic factors exert their pathological effects should be useful for the development of new approaches to treating a variety of angiogenic ocular diseases.

In the retina, VEGF is expressed in the pigmented epithelium, the neurosensory retina, the pericytes and the vascular smooth muscle layer^{10,11}. VEGF induces endothelial cell proliferation, favoring the formation of new vessels in the retina¹². Additionally, VEGF facilitates fluid extravasation in the interstitium, where exudates form in the retinal tissue^{10,11}. VEGF also promotes the fenestration of endothelial cells, a process that can give rise to intercellular channels through which fluids can leak¹³, and disrupts tight junctions between cells¹⁴. The pro-inflammatory cytokine TNF α has also been demonstrated to play a role in diabetic retinopathy since it alters the cytoskeleton of endothelial cells, resulting in leaky barrier function and endothelial cell activation¹⁵. These changes in retinal endothelial cells are central in the pathologies of diabetic retinopathy.

The sphingolipid metabolism pathway is emerging as a promising target for rational anti-angiogenesis therapy. One of the most attractive sites of intervention in this pathway is the conversion of sphingosine to sphingosine-1-phosphate (S1P) by the enzyme sphingosine kinase (SK)¹⁶. SK is the key enzyme responsible for the production of S1P synthesis in mammalian cells, which facilitates cell survival and proliferation¹⁷ and mediates critical processes involved in angiogenesis and inflammation, including responses to VEGF¹⁸ and TNF α ^{19,20}. Therefore, inhibition of S1P production is a potentially important point of therapeutic intervention for diabetic retinopathy.

In the current study, SK expression and function were evaluated in human and bovine retinal endothelial cells (RECs). The abilities of newly-identified SK inhibitors to reduce VEGF-induced proliferation and microvessel formation, and responses to TNF α in these cells were demonstrated. An *in vivo* model of VEGF-induced vascular permeabilization was used to demonstrate the *in vivo* activity of the SK inhibitors, and their therapeutic potential was confirmed using a 3-month diabetic retinopathy study in rats. These demonstrations of significant SK activity in RECs and attenuation of responses to VEGF and TNF α by pharmacologically active SK inhibitors, combined with data from two animal models of vascular permeability, identifies SK as a new target for specific therapies to combat the pathology of diabetic retinopathy.

Materials and Methods

Reagents

Unless otherwise noted, chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). [3-³H]Sphingosine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [6-³H]thymidine was from Amersham Biosciences (Piscataway, NJ). VEGF was purchased from R&D Systems (Minneapolis, MN) and human TNF α was from Sigma. Rabbit polyclonal antibodies were produced by Biosynthesis Inc. (Lewisville, TX)

against the peptide (TLMLTERRNHARELVRSEE) which was determined by BLAST searches to be unique to SK, yet conserved among the human and mouse enzymes. The SK inhibitors SKI-I (5-naphthalen-2-yl-2H-pyrazole-3-carboxylic acid (2-hydroxy-naphthalen-1-ylmethylene)-hydrazide, CAS 306301-68-8) and SKI-II (4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol, CAS 312636-16-1) were purchased from ChemBridge Corporation (San Diego, CA). SKI-V (2-(3,4-dihydroxy-benzylidene)-benzofuran-3-one) was synthesized as previously described²¹. Compound ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)-amide] was synthesized by conducting a Friedel-Craft condensation of 3-bromoadamantane-1-carboxylic acid with chlorobenzene, followed by 1,1'-carbonyldiimidazole-mediated amide formation with 4-aminomethylpyridine²². The identity and purity of the final compounds were confirmed by NMR and mass spectroscopy.

Cell Culture

Primary cultures of bovine RECs were isolated as previously described²³. Human RECs were purchased from Cell Technologies (catalog number ACBRI181, Kirkland, WA) and cultured under identical conditions as those described for bovine RECs. Briefly, the cells were maintained in growth medium consisting of Minimum Essential Medium with D-valine supplemented with 20% fetal calf serum (Gibco, Rockville, MD), 50 µg/mL of endothelial cell growth supplement (Vec Technologies, Renssler, NY), 16 U/mL heparin (Fisher Scientific, Pittsburg, PA), 0.01 mL/mL MEM vitamins and glutamine (Sigma, St. Louis, MO), and 0.02 mL/mL antibiotic/antimycotic (Gibco, Rockville, MD). The cells were plated on a 25 cm² tissue culture flask precoated with fibronectin (Sigma, St. Louis, MO) at 2 µg/cm² and were grown in a humidified incubator at 37°C. The medium was removed and fresh medium was added 24 hr following the plating. For experiments on VEGF and TNFα signaling, the culture medium was replaced with fresh MCDB 131 medium (Sigma, M8537) that lacked fetal calf serum, termed serum-starvation in the text. Human RECs were used as the primary model, with bovine RECs being used as a confirmation on multiple experiments, and the human and bovine cells behaved similarly in all experiments.

Western Blotting

Protein concentrations were determined using the fluorescamine assay²⁴ with bovine serum albumin as the standard. Samples were normalized for equal amounts of protein per lane (100 µg), separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. For SK analyses, membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 and probed with the anti-SK rabbit polyclonal antibody at a 1:50 dilution, washed and incubated with anti-mouse antibodies conjugated to horseradish peroxidase (1 hr in 3% nonfat milk). The blots were then washed 4 times for 5 min at room temperature, developed with SuperSignal development reagents (Pierce Biotechnology, Inc., Rockford, IL) and exposed to Kodak XAR film. As described in the Results section, the following antibodies, along with their appropriate horseradish peroxidase-conjugated secondary antibodies, were also used: Erk1/2 (Catalog number 9102, Cell Signaling Technology, Beverly, MA), phospho-Erk1/2 (Catalog number 9101, Cell Signaling Technology, Beverly, MA), E-selectin (Catalog number 59555, Sigma, St. Louis, MO), VCAM-1 (Catalog number CBL206, Chemicon International, Temecula, CA), Cox-2 (Catalog number SC-1745, Santa Cruz Biotechnology, Santa Cruz, CA) and NFκB (Catalog number 3031, Cell Signaling Technology, Beverly, MA).

Cellular S1P formation assay

Cells were grown to confluency in 24-well tissue culture plates and serum-starved for 24 hr as described above. Cells were then treated with 1% DMSO (as the drug vehicle), 20 µM dimethylsphingosine, or the indicated concentration of SKI-I, -II or -V for 4 hr. The cells were

then incubated with [^3H]sphingosine for 15 min, and the formation of [^3H]S1P was measured as previously described²¹. In some assays, the cells were serum-starved for 24 hr and then treated with VEGF (50 ng/mL) alone or in the presence of an SK inhibitor for an additional 12 hr. [^3H]Sphingosine was then added for 15 min, and its conversion to [^3H]S1P was measured as indicated above.

Cytotoxicity assays

The toxicity of the lead SK inhibitor toward confluent RECs was assessed to estimate the effects of the compound on microvessel integrity in a mature animal. Cells were grown to confluence in 96-well tissue culture plates and serum-starved for 24 hr as described above. Cells were then treated with varying concentrations of SKI-II for 12 hr (to parallel the SK activity assays described above) or with varying concentrations of SKI-II and 50 ng/mL of VEGF for 14 hr (to parallel the proliferation assays described below). Cell survival was determined using the sulforhodamine assay²⁵.

Cell proliferation assay

Cells were grown to confluence in 24-well tissue culture plates and serum-starved overnight as described above. Cells were then treated with 50 ng/mL of VEGF and varying concentrations of SKI-II for 12 hr. At that time, 16 μCi of [^3H]thymidine was added to each well, and the cultures were incubated for an additional 2 hr. The media was then removed by aspiration, the cells were washed twice with cold PBS, and 0.8 mL of ice-cold 10% trichloroacetic acid was added to each well. After 10 minutes, the trichloroacetic acid was removed by aspiration and replaced with 0.4 mL of 40 $\mu\text{g/mL}$ Type I DNA (Sigma, St. Louis, MO) in 0.2 M NaOH. The samples were incubated at 37°C for 30 minutes, scraped into scintillation vials, and the amount of ^3H in the recovered genomic DNA was quantified by scintillation counting.

Microvessel formation assay

The basement membrane-like substrate, Matrigel (Becton-Dickinson, Franklin Lakes, NJ), was used to induce vessel-like tube formation from human RECs as in previous studies with other cell types²⁶. Briefly, 300 μL of Matrigel was pipetted into 24 well plates and allowed to gel at 37°C for 30 minutes. Human RECs were briefly trypsinized and plated onto the layer of Matrigel at an approximate density of 25,000 cells/cm². VEGF (50 ng/mL in PBS) with either DMSO or 20 μM SKI-II was added to the media immediately. After 18 hr at 37°C, images were digitally captured with a Retiga Ex camera with bright-field microscopy using a Nikon Eclipse TE300 microscope. The wells were plated in triplicate in each experiment with the experiment performed twice.

Vascular permeability assay

The effects of VEGF on vascular leakage *in vivo* were determined as described by Miles and Miles²⁷. These studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the IACUC at the Penn State College of Medicine. Groups of female athymic nude mice (approximately 20 g) were given intraperitoneal injections of DMSO alone, SKI-II (100 mg/kg of body weight) or ABC294640 (75 mg/kg) in a volume of 50 μL . In some experiments, ABC294640 was administered by oral gavage at a dose of 100 mg/kg. After 30 min, 100 μL of 0.5% Evan's blue dye in PBS was administered by tail vein injection. One half hr later, mice received the first of 3 sequential (every 30 minutes) intradermal injections of VEGF (400 ng in 20 μL of PBS per injection) on the left hind flank. As a control, similar injections of PBS were administered on the right hind flank. Thirty minutes after the last injection, leakage of the dye from the vasculature into the skin was assessed by measuring the length and width of the spots of blue-colored skin using calipers.

Diabetic retinopathy model in rats

Male Sprague-Dawley rats weighing 150-175 g were used. Diabetes was produced by intraperitoneal injection of streptozotocin (65 mg/kg in citrate buffer) after overnight fasting. Sham-injected non-diabetic animals were also carried as controls. Blood glucose was measured three days post-injection and animals with blood glucose over 250 mg/dL were used as diabetic rats for the study. Blood glucose levels and body weights were monitored weekly throughout the study. On Day 45, retinal vascular permeability was measured in a group of control and diabetic rats as previously described^{28,29}. Briefly, animals were weighed, anesthetized with ketamine/xylazine (80/0.8 mg/kg) and injected with fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA; Sigma catalog number A-9771) into the femoral vein. Following 30 min of FITC-BSA circulation, the rats were sacrificed by decapitation. Trunk blood was collected to measure the FITC-BSA concentration, and eyes were quickly enucleated. Each eye was placed in 4% paraformaldehyde for 1 hr and frozen in OCT embedding medium in a bath of isopentane and dry ice. The paraffin-embedded eyes were sectioned on a microtome making 10 µm sections. Sections were dewaxed and viewed with an Olympus OM-2 fluorescence microscope fitted with a Sony CLD video camera. Fluorescence intensities of digital images were measured using Leica Confocal Software (Version 2.61, build 1538, LCS Lite, 2004). The average retinal intensity for each eye was then normalized to non-injected controls analyzed in the same manner and to the plasma fluorescence of the animal. Through serial sectioning of the eye, this technique enables quantification of varied vascular permeability in the retina^{28,29}.

The remaining control animals were maintained for an additional 6 weeks, i.e. until Day 87, as were the remaining diabetic rats that were divided into untreated, low-dose ABC294640 (25 mg/kg) or high-dose ABC294640 (75 mg/kg) treatment groups. ABC294640 was administered by intraperitoneal injection (dissolved in 0.375% Tween-80) 5 days per week from Day 45 to Day 87. On Day 87, all remaining animals were tested for retinal vascular permeability as described above. Sections were also stained for SK immunoreactivity using the rabbit polyclonal antibodies described above, and counterstained for nuclei using Hoescht stain.

Results

Expression and activity of SK in RECs

The expression of SK in bovine and human RECs was analyzed by immunoblotting of whole cell lysates using polyclonal antibodies that cross-react with SK from multiple species. As indicated in Figure 1A, bovine RECs contained high levels of SK protein, exceeding that of endothelial cells from rat brain cortex and JC murine mammary carcinoma cells (ATCC number CRL-2116), which we have previously characterized³⁰. Similarly, several preparations of human RECs consistently expressed high amounts of SK (Figure 1B), demonstrating that endothelial cells from multiple species express this enzyme.

The enzymatic activity of SK in bovine RECs, and its sensitivity to a series of compounds that we have previously shown to inhibit human SK²¹ was next assessed. In these assays, SK activity was determined following a 5 hr pre-incubation with the test compound and a 15 minute reaction with [³H]sphingosine. The conversion of [³H]sphingosine to [³H]S1P was determined following a chloroform: methanol extraction as previously described²¹. Figure 2 demonstrates the excellent reproducibility of this assay, and the inhibition of SK by the positive control, dimethylsphingosine (DMS). Each of inhibitors of SK (SKI-I, SKI-II and SKI-V) was also active against the endogenous SK activity in the bovine RECs. Similar results were obtained with human RECs (data not shown).

Since SKI-II was the most efficacious inhibitor of SK in the RECs, this compound was used to characterize the biological effects of inhibiting SK in these cells. It was first necessary to determine the toxic effects of this compound toward cultures of RECs under conditions identical to subsequent signal studies. Therefore, human RECs were grown to confluence and then incubated in serum-free MCDB 131 medium for 24 hr. These conditions were chosen to mimic the state of endothelial cells in mature retinal microvasculature, and provide cultures that are sensitive to VEGF-induced proliferation (described below). As demonstrated in Figure 3, concentrations of SKI-II up to approximately 16 μ M had only minimal toxicity (< 10% cell kill) to the RECs after the exposures of either 12 or 14 hr, while concentrations up to at least 65 μ M resulted in cell kills of less than 25%. The low toxicity of SKI-II was confirmed by studies with the Molecular Probes Live/Dead cell assay, which uses calcein-AM conversion to fluorescent calcein by intracellular esterases to quantify live cells, and ethidium homodimer binding to nucleic acid in cells with a compromised plasma membrane to quantify dead cells (data not shown). For comparison, the toxicity of 72-hr exposure of RECs to SKI-II was assessed to evaluate the effects of prolonged suppression of SK activity in these cells. Treatment of the cells for 72 hr caused dose-dependent decreases in the survival of the RECs, with IC_{50} s of approximately 15 μ M for both human and bovine RECs (data not shown). These data demonstrate that transient suppression of SK activity does not induce cytotoxicity in the RECs, while protracted suppression of SK activity does. Therefore, all of the following signaling experiments were conducted with exposures to SKI-II of 14 hr or less to ensure the viability of the RECs.

Suppression of VEGF- and TNF α -signaling in RECs by SKI-II

We next tested the effects of VEGF on SK activity in bovine RECs using the same cell-based assay as above, except that serum was removed from the cultures 24 hr before the addition of the SK inhibitor and VEGF. This was done to reduce the background level of SK activity responding to growth factors in the serum. As shown in Figure 4, growth arrest by serum-starvation reduced the basal SK activity, i.e. conversion of [3 H]sphingosine to [3 H]S1P was lower than in controls shown in Figure 2. Treatment of the cells with VEGF stimulated SK activity, and this response was dose-dependently inhibited by SKI-II such that the response to VEGF was completely inhibited by concentrations of SKI-II of 1.3 μ M or higher. It is important to note that this concentration of SKI-II is significantly lower than the concentration required to induce cytotoxicity, even after prolonged exposure.

We next evaluated the effects of VEGF on the proliferation of human RECs by measuring the effects of the growth factor on the incorporation of [3 H]thymidine into the DNA of serum-starved cells. As shown in Figure 5A, VEGF significantly increased the incorporation of [3 H]thymidine into DNA. The effect of VEGF was dose-dependently inhibited by SKI-II, such that concentrations of 1.3 μ M or higher blocked the mitogenic response to VEGF. Therefore, the inhibition of VEGF-induced SK activity and proliferation are well-correlated in the RECs. The role of S1P in the antiproliferative effect of the SK inhibitor was examined by providing exogenous S1P to the cultures during the stimulation with VEGF. As indicated in Figure 5B, addition of 100 nM S1P in the presence of VEGF did not further stimulate cell proliferation, indicating that they utilize the same mechanism for promoting DNA synthesis. Importantly, the antiproliferative effect of SKI-II was abrogated by the presence of S1P indicating that this lipid is down-stream from the point blocked by the compound. Therefore, it is highly likely that the effects of SKI-II are in fact due to suppression of SK-mediated production of S1P in the RECs.

Western analyses were conducted to evaluate the effects of SKI-II on signaling proteins known to be regulated in endothelial cells by VEGF or TNF α . In these experiments, human RECs were serum-starved for 24 hr and then exposed to VEGF (50 ng/mL) or TNF α (100 ng/mL)

for either 15 min or 6 hr. Cell lysates from cells treated with VEGF for 15 min were then analyzed for levels of phosphorylated ERK1/2 as a measure of signaling through the Ras-mediated proliferation pathway. Samples from cells treated with TNF α were analyzed for activation of NF κ B, i.e. phosphoNF κ B, and the down-stream proteins, cyclooxygenase-2 (Cox-2), E-selectin and VCAM-1. As demonstrated in Figure 6, serum-starved human RECs maintained a small level of residual pERK1/2 that was eliminated by treatment of the cells with SKI-II. More importantly, VEGF promoted the rapid phosphorylation of Erk1/2, and this response was completely abrogated by treatment of the cells with SKI-II. Six hr of exposure to VEGF had no effect on the expression of phosphorylated NF κ B, Cox-2, VCAM-1 or E-selectin (data not shown).

Exposure of the human RECs to TNF α for 6 hr did not affect levels of pNF κ B in these cells, and caused a moderate increase in the expression of Cox-2 (Figure 7A). Interestingly, expression of pNF κ B and Cox-2 was enhanced by treatment of the cells with SKI-II, and this may reflect ability of ceramide, elevated by inhibition of SK, to activate NF κ B³¹⁻³⁴ and induce the expression of Cox-2^{35,36}. TNF α caused marked up-regulation of the expression of both E-selectin and VCAM-1 (Figure 7A), and these responses were completely blocked in cells co-treated with SKI-II. Interestingly, treatment of the cells with an SK inhibitor blocked TNF α -induced prostaglandin E₂ production, indicating that the increased expression of Cox-2 does not result in increased amounts of inflammatory prostaglandins (data not shown). In contrast with the results with VEGF, addition of exogenous S1P did not restore the ability of TNF α to induce the expression of the adherence proteins (Figure 7B). These results indicate that VEGF-mediated responses can be mimicked by extracellular S1P interacting with cell surface, G protein-coupled receptors, whereas, signaling through TNF α is dependent on the intracellular generation of S1P. In either case, reduction of S1P by inhibition of SK appears to be an effective means for interfering with proliferative processes induced by VEGF and inflammatory processes induced by TNF α .

Suppression of VEGF-induced REC migration and *in vivo* vascular leakage by SKI-II

Since VEGF-mediated angiogenesis and vascular leakage are critical processes in the pathology of diabetic retinopathy, we next evaluated the effects of SKI-II on the cellular and *in vivo* effects of VEGF on RECs. In the first series of experiments, the effects of SKI-II on the VEGF-induced formation of microvessels by human RECs were evaluated. As demonstrated in Figure 8, plating of RECs on Matrigel-coated dishes allowed the cells to migrate to form flat cellular networks. Addition of VEGF to the cultures induced the formation of vessel-like tubes that were more elongated and three-dimensional than the networks in control cultures. Addition of SKI-II caused a marked reduction of the formation of networks and tubes in control and VEGF-treated cultures. Isolated cells were commonly visible in the SKI-II-treated cultures, whereas single cells were rarely seen in the VEGF-alone treated cultures. Thus, inhibition of SK effectively blocks REC migration and thereby prevents VEGF from promoting the assembly of these cells into microvessels.

In the second series of experiments, the Miles assay was used as an acute model of VEGF-induced vascular permeability for testing the effects of SKI-II in an *in vivo* model (Figure 9). In this model, Evan's Blue dye is administered intravenously and rapidly binds to albumin. Administration of an intradermal bolus of VEGF results in leakage of the protein-bound dye into the skin indicating a local increase in vascular permeability (Figure 9B). When SKI-II was administered by intraperitoneal injection one hr before the VEGF treatment, vascular leakage (determined three hr later) was markedly reduced (Figure 9A). The extent of vascular leakage was quantified by measuring the blue area, and Figure 9C demonstrates that SKI-II inhibited the *in vivo* response to VEGF by more than 80%.

ABC294640 is an aryladamantane recently synthesized as part of our medicinal chemistry efforts to optimize SK inhibitors for the treatment of cancer and inflammatory diseases²². This compound has excellent anti-SK activity *in vitro* and in intact cells, along with excellent pharmacologic properties, including high oral bioavailability. We have demonstrated that ABC294640 has therapeutic activity in *in vivo* models of inflammatory bowel disease³⁷ and rheumatoid arthritis (French and Smith, unpublished data). Therefore, we have also evaluated this compound in the VEGF-induced vascular leakage model. As indicated in Figure 9D, either intraperitoneal or oral administration of ABC294640 suppressed the ability of VEGF to promote dye leakage into mouse skin. Therefore, structurally diverse SK inhibitors have a common ability to suppress *in vivo* vascular leakage in response to VEGF.

Reduction of vascular leakage by ABC 294640 in a rat model of diabetic retinopathy

The data thus far show that inhibition of SK substantially reduces VEGF-induced endothelial cell migration *in vitro* and vascular permeabilization *in vivo*. A rat model of diabetes was used to translate these results into a specific model of retinopathy. Hyperglycemic rats were left untreated for 45 days to allow the progression of retinopathy. At that time, control and diabetic rats were evaluated for retinal vascular permeability by measuring the leakage of FITC-labeled BSA into the retina using quantitative image analyses. As indicated in Figure 10, the diabetic animals had substantial increases in the leakage of the labeled BSA into the inner plexiform and outer nuclear layers of the retina. The signal in the photoreceptor layer is auto-fluorescence unrelated to the FITC-BSA. Quantification of the images indicated that there is an approximately 4-fold increase in the amount of FITC-BSA leakage in the retinas from diabetic rats (Figure 10C). Therefore, substantial vascular damage was present before the initiation of treatment with the SK inhibitor.

During the progression of the experiment, the diabetic rats experienced marked toxicity from the uncontrolled hyperglycemia resulting in the need to euthanize some animals in all of the treatment groups. It should be noted that although the health status of the diabetic rats was severely compromised, animals receiving ABC294640 (at either the 25 or 75 mg/kg dose) did not demonstrate increased toxicity compared with the untreated diabetic rats. All of the rats were sacrificed on Day 87 and retinopathy was measured as the leakage of FITC-BSA into the retina. As indicated in Figure 11, retinal vascular permeability in the diabetic rats was significantly elevated compared with the control rats. Diabetic animals that had been treated with the SK inhibitor ABC294640, at either dose, had substantially reduced levels of FITC-BSA leakage than did the untreated diabetic rats. This effect of the compound was manifested in both the inner plexiform layer and the outer nuclear layer of the retina.

Immunohistochemistry with the SK antibody described above was used to evaluate the expression of SK in the retinas of these animals (Figure 12). Fluorescence in the retinal pigment epithelium and the outer segment was non-specific since it was present in samples incubated in the absence of the SK antibody. Retinal sections from control rats had only low levels of specific staining for SK; whereas, SK expression was markedly elevated in the ganglion cell layer and in specific cell bodies and projections at the interface of the inner nuclear layer and the inner plexiform layer. Elevated SK expression was also observed in both the low-dose and the high-dose ABC294640-treated animals. Therefore, the long-term hyperglycemic state appears to be associated with elevation of retinal SK levels that are not normalized by treatment with the SK inhibitor. This expression data indicate that ABC294640 very effectively suppresses SK activity in the diabetic retina, thereby preventing the increased vascular permeability normally present in retinopathy.

Discussion

Understanding the mechanisms by which VEGF and other growth factors and cytokines regulate REC structure and function are critically important in identifying new targets for the treatment and/or prevention of angiogenic ocular diseases, such as diabetic retinopathy and age-related macular degeneration. Since several studies have shown that S1P is a critical second messenger that exerts proliferative and angiogenic actions in other models, and SK is the cellular enzyme responsible for S1P biosynthesis, we have focused on SK as a new target for treating these diseases. A PubMed search currently indicates that nearly 700 papers have explored the effects of VEGF on retinal endothelial cells; however, only 1 previous paper has considered the role of SK in mediating angiogenic responses in these cells³⁸. In this case, adenoviral-mediated delivery of SK did not directly promote neovascularization; however, no confirmation of SK expression or elevation of S1P levels was provided. Similarly, the role of SK in VEGF signaling has been studied in other types of endothelial cells³⁹⁻⁴³; however, it is clear that endothelial cells isolated from different organs, as well as from macrovasculature or microvasculature of the same organ, can have different functional properties⁴⁴⁻⁴⁷. Therefore, we have examined the expression of SK and evaluated its role in mediating responses of two types of RECs to VEGF and TNF α using newly-identified SK inhibitors.

Our data demonstrate that SK is present and functioning in human and bovine RECs, and that VEGF-induced SK activity can be inhibited by SKI-II, resulting in prevention of VEGF-induced signaling through the Erk pathway and cell proliferation. We have also demonstrated that the SK inhibitor blocks TNF α -mediated induction of adhesion molecules, and markedly reduces VEGF-induced vascular leakiness in an *in vivo* model. Most importantly, we provide the first *in vivo* demonstration that SK expression is increased in the retinas of diabetic animals, and that inhibition of SK strongly suppresses retinal vascular leakage in diabetic rats. Overall, these results indicate that SK plays a key role in REC angiogenesis and vascular permeability, which are hallmark pathologies of several ocular diseases.

The mechanistic pathway between occupancy of VEGF receptors and increased REC permeability remains to be fully elucidated. Others have demonstrated that VEGF-induced activation of SK can be mediated by protein kinase C¹⁸, and it has recently been demonstrated that VEGF-induced permeability across a bovine REC monolayer can be blocked by a protein kinase C inhibitor, although protein kinase C activation alone does not induce permeability (Antonetti, unpublished data). VEGF has also been shown to induce the expression of S1P receptors on endothelial cells, associated with enhanced responses to S1P, and the potentiation of its angiogenic actions⁴¹. These effects are also attenuated by the addition of a protein kinase C inhibitor⁴¹. Therefore, protein kinase C and SK each represent a new target for the development of drugs to block undesired angiogenesis induced by VEGF. However, the ability of the SK inhibitor to block the induction of the adhesion molecules VCAM-1 and E-selectin on RECs exposed to TNF α indicates that this target has the advantage of also reducing signals for the recruitment of inflammatory cells at the sites of vascular damage in ocular diseases.

Several studies have recently demonstrated that treatment of endothelial cells with S1P promotes barrier integrity⁴⁸⁻⁵⁰, and this may seem contrary to the known ability of VEGF to increase vascular leakiness^{13,14,51}. This may simply reflect the sum of the pleiotropic actions of VEGF, including the phosphorylation of tight junctional proteins such as occludin¹⁴, or may result from compartmentalization of S1P. In the later case, it is clear that the signaling mechanisms for S1P are complex, with distinct pathways for intracellular and extracellular S1P. Intracellular S1P is produced by the action of SK in response to stimuli such as growth factors and inflammatory cytokines, as described above. The intracellular receptor for S1P has not been determined, although the ability of S1P to signal through the Ras pathway may be due to its ability to inhibit the actions of the GTPase-Activating Protein that turns off Ras

activity⁵². In contrast, it is well-established that extracellular S1P mediates its effects by activating one or more G protein-coupled receptors⁵³⁻⁵⁵, most likely S1PR1 and S1PR3 on endothelial cells⁴⁸. These S1P receptors promote calcium mobilization within the cells, and are uncoupled from their effector G proteins by treatment of the cells with Pertussis toxin. The extracellular S1P may be either exported from within the cell or synthesized by extracellular SK⁵⁶, but is unlikely to have access to intracellular targets because of its charge and polarity. Therefore, studies that involve the addition of S1P, or the analog FTY-720P, cause calcium-mediated changes in the cytoskeleton that affect the integrity of tight junctions⁴⁴. This is a distinctly different process than the activation of proliferative and NFκB-mediated pathways that are stimulated by intracellular S1P. The degree to which either pathway dominates will be dependent on levels of expression of the cell-surface S1P receptor subtypes and the intracellular S1P target(s), as well as their respective down-stream effectors. This is difficult to establish in a cell culture model; however, data presented here demonstrate that the SK inhibitor is able to block VEGF-induced vascular leakage *in vivo*, indicating that depletion of intracellular S1P should be therapeutically useful.

Although it has been previously demonstrated that S1P is a critical messenger in angiogenesis, the use of specific inhibitors of SK as anti-angiogenic compounds for ocular diseases has not been previously addressed. This is largely due to the heretofore lack of potent, specific SK inhibitors that are pharmacologically active. We have recently identified well-characterized and synthetically amenable non-lipid specific inhibitors of SK that are effective at low micromolar concentrations²¹. Although the SK inhibitors used in the current studies were originally identified as potential antitumor agents²¹, we hypothesize that they will also be useful for the treatment and/or prevention of other angiogenic and/or inflammatory diseases³⁷. In the present case, SK inhibitors have wide potential utility for blocking the proliferative effects of S1P in response to VEGF as well as deleterious TNFα-mediated cellular processes. As demonstrated in the currently reported diabetic retinopathy model, these inhibitions are expected to reduce or prevent both the clinically observed neovascularization and vascular leakage that are the hallmark pathologies in diabetic retinopathy and age-related macular degeneration. The present report is the first proof-of-principle demonstration that pharmacological inhibition of SK attenuates angiogenic and inflammatory responses of RECs, resulting in therapeutic activity in an accepted model of diabetic retinopathy. Continued development of these compounds and other SK inhibitors will allow assessment of their efficacies in human retinopathy.

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Abbreviations

Cox-2	cyclooxygenase-2
DMS	N,N-dimethylsphingosine
DMSO	dimethylsulfoxide
PBS	phosphate-buffered saline
REC	retinal endothelial cell
S1P	sphingosine 1-phosphate

SK	sphingosine kinase
TNFα	transforming growth factor- α
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

Figure 1. Expression of SK in RECs

Panel A. Cell lysates were prepared from JC murine mammary adenocarcinoma cells (Lane 1), bovine RECs (Lane 2) or rat brain cortical endothelial cells (Lane 3) and analyzed by western blotting using antibodies against SK as described in the Materials and Methods section. Immunoreactive bands representing SK are shown. **Panel B.** Cell lysates were prepared from four independent preparations of human RECs (lanes 1-4) and evaluated for expression of SK as indicated above. In each case, equal amounts of protein were loaded as confirmed by reprobing with an anti-actin antibody.

Figure 2. Inhibition of cellular SK activity in RECs

Bovine RECs were incubated with 20 μ M DMS or 25 μ g/mL SKI- I, SKI-II or SKI-V (approximately 80 μ M for each compound) for 4 hr before the addition of 0.4 μ Ci of [³H] sphingosine. After 15 min, cells were lysed and extracted with chloroform: methanol. The total amounts of [³H]sphingosine in the organic phase and [³H]S1P in the aqueous phase were then determined as described in the Materials and Method section. Values represent the mean $\pm$ SD of duplicate samples in a typical experiment.

Figure 3. Toxicity of SKI-II toward HRECs

Human RECs were serum-starved for 24 hr and then left untreated for 12 hr (■) or incubated with 50 ng/mL VEGF (▲) for 14 hr in the presence of the indicated concentration of SKI-II. These protocols were chosen to exactly match conditions in SK activity and proliferation experiments described below. After incubation, the percentages of cells that survived the treatment were determined as described in the Materials and Method section. Values represent the mean $\pm$ SD cell survival in triplicate samples in a typical experiment.

Figure 4. Effects of SKI-II on VEGF-stimulated SK activity in RECs

Bovine RECs were serum-starved for 24 hr and then left untreated or incubated with VEGF (50 ng/mL) in the presence of DMS (D) or the indicated concentration (in μ M) of SKI-II for 12 hr. [³H]Sphingosine was then added to the cells and its conversion to [³H]S1P was determined as described in the Materials and Method section. Values represent the mean $\pm$ SD of duplicate samples in a typical experiment.

Figure 5. Effects of SKI-II on VEGF-stimulated proliferation of RECs

Panel A. Human RECs were serum-starved for 24 hr and then left untreated or incubated with VEGF (50 ng/mL) in the presence of the indicated concentration (in μM) of SKI-II for 12 hr. [^3H]Thymidine was then added and the cultures were incubated an additional 2 hr. **Panel B.** Human RECs were serum-starved and incubated with the indicated concentration of SKI-II as above. The cells were then treated with VEGF (solid bars) or VEGF plus 100 nM S1P (cross-hatched bars) for 12 hr, followed by labeling with [^3H]thymidine for 2 hr. The amount of [^3H]thymidine incorporated into DNA was determined as described in the Materials and Method section. Values represent the mean $\pm$ SD of duplicate samples in a typical experiment.

Figure 6. Effects of SKI-II on VEGF-induced Erk phosphorylation in RECs

Human RECs were serum-starved for 24 hr and then left untreated (Lanes 1 and 3) or incubated with 10 μ M SKI-II (Lanes 2 and 4) for 12 hr. VEGF (50 ng/mL) was then added to samples shown in Lanes 3 and 4 for 15 minutes before cell lysates were prepared and evaluated for the expression of phospho-Erk1/2 and total Erk1/2 protein as described in the Materials and Methods section.

Figure 7. Effects of SKI-II on TNF α -induced Cox-2 and adhesion molecule expression in RECs
Panel A. Human RECs were serum-starved for 24 hr and then left untreated (Lanes 1 and 2) or incubated with 20 μ M SKI-II (Lane 3) for 12 hr. TNF α (100 ng/mL) was then added to samples shown in Lanes 2 and 3 for 6 hr before cell lysates were prepared. **Panel B.** Human RECs were serum-starved for 24 hr and then left untreated (Lanes 1 and 2) or incubated with 20 μ M SKI-II (Lanes 3 and 4) for 12 hr. TNF α (100 ng/mL) was then added to samples shown in Lanes 2 and 3, while TNF α plus 100 nM S1P was added to the sample shown in Lane 4 for 6 hr before cell lysates were prepared. Samples were evaluated for the expression of E-selectin, VCAM-1, Cox-2, phospho-NF κ B or actin as described in the Materials and Methods section.

Figure 8. Effects of VEGF and SKI-II on vessel-like tube formation by RECs

Human RECs were plated into Matrigel-coated wells and treated with DMSO (as the solvent control), VEGF (50 ng/mL) or VEGF plus 20 μ M SKI-II. After 18 hr at 37 °C, the cells were digitally imaged as described in the Materials and Methods section. Representative fields are shown.

Figure 9. Effects of SK inhibitors on VEGF-induced vascular leakage

Nude mice were treated with either DMSO (as the solvent control) or an SK inhibitor. After 30 min, Evan's Blue dye was injected intravenously and the animals received subsequent subcutaneous injections of either PBS or 400 ng of VEGF as described in the Materials and Methods section. **Panel A.** A representative mouse treated with 100 mg/kg of SKI-II with the left hind flank showing subcutaneous injection sites of VEGF is shown. The right flank was injected with PBS to serve as a control. **Panel B.** A representative control animal showing the typical, marked VEGF-induced leakage on the left hind flank and injection site mark on the right, PBS control injection side is shown. **Panel C.** The areas of vascular leakage from animals in part A and B were quantified ($n = 3$ and 5 for control and SKI-II-treated animals, respectively). **Panel D.** Nude mice were injected intraperitoneally with carrier (Control, open bar) or 75 mg/kg ABC294640 (hatched bar) or given 100 mg/kg ABC294640 by oral gavage (solid bar), followed by administration of Evan's Blue dye and VEGF as indicated above. Values represent the mean $\pm$ SD areas of vascular leakage. * $p < 0.01$.

Figure 10. Increased retinal vascular permeability in diabetic rats

Sprague-Dawley rats were injected with buffer (Control) or streptozotocin (Diabetic) and left untreated for 45 days. At that time, animals were injected intravenously with FITC-BSA, and after 30 min the animals were sacrificed. Each retina was harvested, sectioned and imaged by fluorescence microscopy as described in the Materials and Methods section. **Panel A** indicates a representative section from a Control rat, while **Panel B** is an image from a typical Diabetic rat. The inner plexiform (IP), outer nuclear and auto-fluorescing photoreceptor (PE) layers are labeled. In sections of retina from Control rats, fluorescence is limited to the lumens of blood vessels, whereas sections from Diabetic rats showed more even distribution throughout the retina, indicative of vascular leakage. **Panel C.** The relative light intensity of FITC-BSA in the inner plexiform and outer nuclear layers from Control (open bars) and Diabetic (shaded bars) rats were quantified. Values represent the mean $\pm$ sd for 4 rats.

Figure 11. Effects of ABC 294640 on retinal vascular permeability in diabetic rats

From Day 45 through Day 87, Control (open bars) and Diabetic rats were treated with solvent (shaded bars) or ABC294640 at 25 mg/kg (horizontal-hatched bars) or 75 mg/kg (cross-hatched bars). On Day 87, retinal leakage in each animal was measured as indicated in the Materials and Methods section. Values represent the mean $\pm$ sd for 3-5 rats per group.

Figure 12. Sphingosine kinase expression in retinas of Control and Diabetic rats

Retinal sections were prepared on Day 87 from Control (Panel A) or Diabetic rats from the solvent (Panel B) or ABC294640 at 25 mg/kg (Panel C) or 75 mg/kg (Panel D) treatment groups. The sections were stained for SK using rabbit polyclonal antibody and secondary Cy5 linked anti-rabbit antibody (Red) and nuclei were stained using Hoescht (Blue). Images are representative of 3-5 rats per group.