Specific Involvement of Src Family Kinase Activation in the Pathogenesis of Retinal Neovascularization

Xiang Q. Werdich¹ and John S. Penn¹,²

PURPOSE. Src family kinases (SFKs) are membrane-attached non-receptor protein tyrosine kinases that link a variety of extracellular cues to intracellular signal pathways. The purpose of this study was to characterize the roles of SFKs in vascular endothelial growth factor (VEGF)-mediated retinal angiogenesis.

METHODS. Primary rat retinal glial Müller cells and bovine and human retinal microvascular endothelial cells (RMECs) were used in the in vitro studies. A rat model of retinopathy of prematurity (ROP) was used in the in vivo studies.

RESULTS. In vitro, SFKs were essential for hypoxia-induced VEGF expression in Müller cells and for VEGF signaling in RMECs. However, neither process required significant further phosphorylation of the SFK activation loop Tyr416. In vivo, in a rat model of ROP, a pronounced increase of retinal SFK Tyr416 phosphorylation was observed that was specifically associated with pathologic angiogenesis. These retinas also expressed significantly higher levels of VEGF than did those in healthy controls. Immunohistochemical analysis indicated that Müller cells were the major source of the elevated level of phospho-SFK Tyr416. Intravitreous injection of a selective SFK inhibitor, PP2, significantly reduced retinal VEGF and retinopathy in the ROP model, indicating that SFKs acted as important regulators in abnormal retinal angiogenesis.

CONCLUSIONS. Together, these data suggest that SFK activation through a Tyr416-dependent mechanism may be an important factor in the pathogenesis of retinal neovascularization. (Invest Ophthalmol Vis Sci. 2006;47:5047–5056) DOI:10.1167/iovs.05-1543

Retinal neovascularization is the leading cause of severe vision loss and irreparable blindness in developed countries, affecting people of all ages.¹ It is the characteristic pathologic event of diverse retinal diseases such as retinopathy of prematurity (ROP) and diabetic retinopathy. Retinal ischemia and hypoxia have been identified as major driving forces behind these angiogenic conditions, and hypoxia-inducible vascular endothelial growth factor (VEGF, or VEGF-A) is a crucial stimulator and regulator of the pathogenesis.²–⁴ In the vertebrate retina, Müller cells are the most abundant glial cells and play active roles in the maintenance of retinal extracellular homeostasis and the metabolic support of neurons.⁵ They are the major VEGF-secreting cell type in the retina during ischemia-induced neovascularization,⁶–⁷ though other retinal cells may contribute to a lesser extent.⁸–¹⁰ VEGF has a profound impact on multiple functions in endothelial cells, such as proliferation, migration, survival, tube formation, and vascular permeability.¹¹,¹² These biologic effects are mediated through the high-affinity tyrosine kinase receptors VEGFR-1 (flt-1) and, more important, VEGFR-2 (KDR/flk-1).¹¹,¹² VEGF stimulation leads to receptor dimerization and autophosphorylation. These events subsequently initiate intracellular signal cascades that activate various signal molecules, such as mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), and Akt/protein kinase B, which, among other signal molecules, are involved in the regulation of cell proliferation, migration, and survival, respectively.¹¹,¹² In the eye, retinal microvascular endothelial cells (RMECs) undergo dysregulated proliferation and differentiation in proliferative retinopathies such as those mentioned.⁴

As membrane-attached non-receptor protein tyrosine kinases, Src family kinases (SFKs) link a variety of extracellular cues to intracellular signal pathways.¹³ Family members Src, Fyn, and Yes are often coexpressed (e.g., in vascular endothelial cells).¹³–¹⁵ Recent studies have revealed that SFKs are involved in VEGF induction by hypoxia¹⁶ and in VEGF signaling.¹⁷–¹⁹ However, their roles in these signal cascades are not well characterized, particularly in the context of retinal angiogenesis. We believe that the characterization of SFKs in retinal VEGF induction and transduction pathways, in vitro and in vivo, is of great importance for understanding their roles in physiological retinal angiogenesis and in the pathogenesis of retinal neovascularization.

SFKs are 52- to 62kDa proteins consisting of six distinct functional domains: a Src homology 4 (SH4) domain, a unique domain, SH3 and SH2 protein-binding domains, a catalytic domain, and a negative regulatory carboxyl terminal tail.¹³ SFKs have two primary regulatory phosphorylation sites, Tyr527 in the negative regulatory tail and Tyr416 in the activation loop of the catalytic domain. Inactive SFKs are present in a restrained form through the intramolecular interaction of the SH2 domain with phospho-Tyr527 (pY527) and adjacent residues, which is critical for suppressing the kinase activity.¹³ When the intramolecular interactions are disrupted, dissociated pY527 may allow dephosphorylation. Tyr416 then undergoes autophosphorylation, which permits and stabilizes the active conformation and promotes the intrinsic kinase activity.¹⁵,²⁰ Autophosphorylation of Src at Tyr416 was shown to be directly correlated with its catalytic activity.²¹ In vivo, wild-type SFKs are strictly regulated and mainly present in the restrained state.²⁰ By coupling to signal molecules and phosphorylating substrates, SFKs participate in various signaling pathways.¹³ A recent study indicates that the regulation of SFK activity through a Tyr416-dependent mechanism may not always be required for SFK signaling. Cary et al.²² show that Tyr416-dependent regulation of Src activity is not important and may not occur in integrin-mediated events but that Src kinase activity is clearly required.

In the current study, we investigated the roles of SFKs in VEGF-mediated retinal angiogenic events, both in vitro in ret-
inal cell cultures and in vivo in a rat model of ROP. In vitro, we did not observe a significant increase of SFK Tyr
phosphorylation (also referred to as phospho-SFK Tyr416 or SFK pY416) in Müller cells exposed to hypoxia or in RMECs stimulated by VEGF. In vivo, SFKs were highly phosphorylated at the activation loop Tyr416 in retinas developing pathologic angiogenesis but not in those undergoing physiological intraretinal vascularization. SFK activation through a Tyr416-dependent mechanism may be an important factor in the pathogenesis of retinal neovascularization.

**Materials and Methods**

**Materials**

Recombinant human VEGF was purchased from R&D Systems (Minneapolis, MN). PP2, a selective SFK inhibitor, and the negative control, PP3, were purchased from Calbiochem (San Diego, CA). PP2 binds to the adenosine triphosphate (ATP) pocket of SFKs and is noncompetitive against ATP for the inhibition of SFKs. We obtained antibodies recognizing SFKs in general or individual Src, Fyn, Yes, or VEGFR-2 from Upstate Biotechnology (Waltham, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-SFK Tyr416 (SFK pY416) and anti-phospho-Erk1/2 from Cell Signaling (Beverly, MA), anti-phospho-FAK Tyr861 (FAK pY861) from Biosource (Camarillo, CA), and anti-vimentin and anti-gal fibrillary acidic protein (GFAP) from DAKO (Carpinteria, CA). Unless otherwise specified, all other reagents were purchased from Sigma, St. Louis, MO.

**Cell Culture**

Retinal Müller cells were isolated from postnatal day 8 Long-Evans rat pups, and cell identity was confirmed as previously described. Cells were routinely cultured with Dulbecco modified eagle medium (DMEM; Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1X antibiotic-antimycotic solution in a standard culture atmosphere of 5% CO2/95% air (20.9% oxygen; designated as normoxia).

Bovine RMECs (VEC Technologies, Rensselaer, NY) were routinely cultured in tissue flasks coated with 100 µg/ml fibronectin and 100 µg/ml hyaluronan acid and in MCDB-131 complete medium (VEC Technologies) containing 10% FBS. Human RMECs (Cell Systems, Kirkland, WA) were routinely cultured in tissue flasks coated with 0.1% gelatin and supplemented with endothelial growth medium (EGM; Cambrex, East Rutherford, NJ) supplemented with 10% FBS. Both cell types were cultured in standard cell culture atmosphere of 5% CO2/95% air (20.9% oxygen; designated as normoxia).

**Rat Model of ROP**

For the in vivo study, we used a well-established rat model of ROP. Litters of Sprague-Dawley rat pups were exposed to alternating periods of hyperoxia (50% oxygen) and hypoxia (10% oxygen) every 24 hours for 14 days (the 50%/10% oxygen treatment) and then were moved to room air. In other experiments, animals were exposed to a less extreme varying oxygen regimen with oxygen concentrations ranging from 20.9% to 2.5% were generated with the use of a laboratory CO2 incubator with O2 control (Isotemp; Kendro Laboratory, Asheville, NC). Culture medium and cell lysates were collected for VEGF and protein quantification, respectively. The anoxic condition was identified as the optimal hypoxic condition for induction of VEGF expression in rat Müller cell culture during an incubation period of 24 hours.

**Quantification of VEGF Concentration**

VEGF concentrations (in picograms per milliliter) in Müller cell culture media or in retinal homogenates were determined using a colorimetric VEGF ELISA assay kit (R&D Systems) according to the manufacturer’s instructions. The VEGF concentrations (in picograms per milliliter) were normalized to the total protein concentrations (in milligrams per milliliter) of Müller cell lysates or retinal homogenates.

**Immunoprecipitation**

Cell lysates were centrifuged at 12,000g for 15 minutes at 4°C. Supernatants were precleared with protein A and G agarose (Pierce). Cell extracts, 1 mg per sample, were then incubated with the primary antibody for 2 hours at 4°C. Immunocomplexes were captured by protein A and G agarose (Pierce). Cell extracts, 1 mg per sample, were then incubated with the primary antibody for 2 hours at 4°C. Immunocomplexes were captured by protein A and G agarose (Pierce). Cell extracts, 1 mg per sample, were then incubated with the primary antibody for 2 hours at 4°C. Immunocomplexes were captured by protein A and G agarose (Pierce). Cell extracts, 1 mg per sample, were then incubated with the primary antibody for 2 hours at 4°C. Immunocomplexes were captured by protein A and G agarose (Pierce). Cell extracts, 1 mg per sample, were then incubated with the primary antibody for 2 hours at 4°C. Immunocomplexes were captured by protein A and G agarose (Pierce). Cell extracts, 1 mg per sample, were then incubated with the primary antibody for 2 hours at 4°C. Immunocomplexes were captured by protein A and G agarose (Pierce). Cell extracts, 1 mg per sample, were then incubated with the primary antibody for 2 hours at 4°C. Immunocomplexes were captured by protein A and G agarose (Pierce).

**In Vitro Kinase Assay**

The in vitro activity of SFKs was determined with a tyrosine kinase activity assay kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. Cells and retinas were lysed with cold lysis buffer II (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, pH 7.4) containing protease and phosphatase inhibitors (1 mM EDTA, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM Na3VO4, 1 mM NaN3). Protein concentrations were determined with a bicinechonic acid protein assay (BCA; Pierce, Rockford, IL).
ml), and 10 μL ATP/MgCl₂ solution (5 mM ATP, 50 mM MgCl₂) at 30°C for 60 minutes. The reaction was terminated with the addition of 10 μL of 120 mM EDTA. A reaction mix of 50 μL was transferred to streptavidin-coated 96-well plates. The fraction of phosphorylated substrate was visualized using a phosphotyrosine monoclonal antibody conjugated to HRP and an ensuing chromogenic substrate reaction.

**Interference of SFK Expression with siRNAs in Human RMECs**

Src siRNA, corresponding to the human Src cDNA sequence AA-GCACTTGGCCAGCTATGA, and Fyn siRNA, corresponding to the human Fyn cDNA sequence AAGATGGTGACCGGAGCTA, were chemically synthesized with symmetric 3’ dTdT overhangs (Qiagen; Valencia, CA). Validated human Yes siRNA, β-actin siRNA, and nonsense control siRNA were purchased (Ambion, Austin, TX).

siRNAs were transfected into human RMECs with the use of a human lung microvascular endothelial cell kit (Nucleofector; Amaxa, Gaithersburg, MD) and a Nucleofector device (Amaxa). Cells were treated with VEGF 24 hours after transfection.

The use of these siRNAs in human RMECs was described in detail elsewhere. Briefly, transfection of Src siRNA (300 nM) alone induced 66% specific downregulation of Src expression, 65% downregulation of Fyn expression by Fyn siRNA (300 nM), and 60% downregulation of Yes expression by Yes siRNA (300 nM) 24 hours after transfection. Simultaneous delivery of Src, Fyn, and Yes siRNAs significantly reduced protein expression of Src, Fyn, and Yes by 40% to 50%, individually, 24 hours after transfection. Nonsense control siRNA was used at 300 nM or 900 nM. Transfection of siRNAs at concentrations up to 1 μM showed no toxicity in human RMECs.

**Immunohistochemistry and Isolectin Histochemistry**

Deparaffinized 5-μm-thick transverse sections of retinal tissue were antigen retrieved with target retrieval solution (Dako). After blocking in blocking buffer (SuperBlock; Pierce), the sections were incubated with the primary antibody at 4°C overnight and then with fluorescein isothiocyanate (FITC)-conjugated or rhodamine red-X (RRX)-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at room temperature for 2 hours. Retinal sections incubated in blocking buffer (SuperBlock; Pierce) without the primary antibody served as negative controls. Retinal blood vessels were visualized with FITC-conjugated Griffonia (Bandeiraea) simplicifolia isoleciton B4 according to previously described procedures. Slides were observed and images were taken with a confocal microscope (LSM510; Carl Zeiss Micromaging, Thornwood, NY).

**Assessment of Retinal Neovascularization**

Retinal vasculature was stained using a histochemical method for detecting adenosine diphosphatase (ADPase) activity. Stained retinas were whole-mounted onto slides.

The severity of retinal pathologic neovascular development was semiquantified by the clock-hour method. Three masked investigators independently evaluated the number of clock hours containing abnormal blood vessel growth. Their assessments were averaged for each retina. Data were expressed in values varying from 0 (no abnormality) to 12 (involvement of entire retinal circumference). Retinas of each retina. Data were expressed in values varying from 0 (no abnormality) to 12 (involvement of entire retinal circumference). Retinas of each retina. Data were expressed in values varying from 0 (no abnormality) to 12 (involvement of entire retinal circumference). Retinas of each retina. Data were expressed in values varying from 0 (no abnormality) to 12 (involvement of entire retinal circumference). Retinas of each retina. Data were expressed in values varying from 0 (no abnormality) to 12 (involvement of entire retinal circumference). Retinas of each retina. 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**Statistical Analysis**

Data were analyzed with commercial software (StatView: Abacus Concepts, Berkeley, CA). Analysis of variance and t tests were used to analyze parametric data such as VEGF concentration, kinase activity, and normalized densitometric values. Nonparametric data for retinal neovascularization obtained by the clock-hour method were analyzed with the Mann–Whitney U test. For each test, P < 0.05 was considered significant.
Therefore, SFKs are required for VEGF production under normoxic and hypoxic conditions. However, the latter condition is more sensitive to SFK inhibition. Blocking SFK activity may not result in complete inhibition of VEGF expression.\textsuperscript{34} In addition, hypoxia-mediated VEGF induction occurred without a significant increase in SFK Tyr416 phosphorylation.

**VEGF Stimulation Does Not Significantly Increase SFK Tyr416 Phosphorylation in RMECs**

In bovine and human RMECs cultured in growth medium (data not shown) or serum-free medium (Fig. 2), we observed a constant level of SFK pY416. These wild-type SFKs could actually phosphorylate substrates as measured in vitro using a kinase activity assay (Fig. 2B). VEGF stimulation did not significantly increase SFK pY416 in any cell type or in any time course or dose–response studies. In addition, the catalytic activity of SFKs was not changed significantly (Figs. 2A–B). Furthermore, pY416 of individual Src, Fyn, or Yes did not change (Fig. 2C). Tyr861 of FAK is a major phosphorylation target of SFKs.\textsuperscript{37} Consistently, FAK pY861 showed no increase on VEGF stimulation (Fig. 2B). Nevertheless, VEGF did significantly activate the MAPK signal molecules Erk1 (p\textsuperscript{44MAPK}) and Erk2 (p\textsuperscript{42MAPK}), as demonstrated in Figs. 2B, 2D, and 3A. We considered the possibility that the culture conditions were not optimal for SFK quiescence and that the preexisting background signal of SFK pY416 might have masked a significant response of SFKs to VEGF stimulation. To address this issue, we conducted experiments under modified culture and stimulation conditions. These conditions included a broad range of VEGF stimulation times (30 seconds to 24 hours), different phosphatase inhibitors, and different starvation or growth surface coatings. The results of these experiments were consistent: VEGF stimulation did not significantly enhance phosphorylation of SFKs at the activation loop Tyr416, nor was the resting state of SFK pY416 (Fig. 2D) or the kinase activity (data not shown) ever completely quiescent.

Nevertheless, pretreatment with PP2 significantly blocked VEGF-induced phosphorylation of Erk1/2 in bovine and human RMECs in a dose-dependent manner (Fig. 3A). PP2 showed no influence on the basal level of phospho-Erk1/2 when VEGF was not added. The negative control PP3 exerted no impact on Erk1/2 activation by VEGF (Fig. 3A).

**FIGURE 2.** In vitro, VEGF signaling in RMECs does not increase SFK Tyr416 phosphorylation. Cells were serum starved overnight. Unless specifically noted, cells were stimulated by 25 ng/ml VEGF for 10 minutes. Each experiment was independently repeated at least three times. (A) Time course and dose–response studies in bovine RMECs using Western blot analysis. (B) Time course study using Western blot analysis and in vitro catalytic activity of SFKs in human RMECs. SFK catalytic activity was not significantly increased in response to VEGF stimulation ($P > 0.05$). (C) Time course study of individual SFK members Sqc and Fyn in bovine RMECs. The protein expression level of Yes was lower than that of Sqc and Fyn (data not shown). IP, immunoprecipitation; IB, immunoblotting. (D) Representative Western blot analysis of experiments under modified culture conditions. Cells were cultured on plates coated with various concentrations of fibronectin (FN).

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In bovine and human RMECs cultured in growth medium (data not shown) or serum-free medium (Fig. 2), we observed a constant level of SFK pY416. These wild-type SFKs could actually phosphorylate substrates as measured in vitro using a kinase activity assay (Fig. 2B). VEGF stimulation did not significantly increase SFK pY416 in any cell type or in any time course or dose–response studies. In addition, the catalytic activity of SFKs was not changed significantly (Figs. 2A–B). Furthermore, pY416 of individual Src, Fyn, or Yes did not change (Fig. 2C). Tyr861 of FAK is a major phosphorylation target of SFKs. Consistently, FAK pY861 showed no increase on VEGF stimulation (Fig. 2B). Nevertheless, VEGF did significantly activate the MAPK signal molecules Erk1 (p\textsuperscript{44MAPK}) and Erk2 (p\textsuperscript{42MAPK}), as demonstrated in Figs. 2B, 2D, and 3A. We considered the possibility that the culture conditions were not optimal for SFK quiescence and that the preexisting background signal of SFK pY416 might have masked a significant response of SFKs to VEGF stimulation. To address this issue, we conducted experiments under modified culture and stimulation conditions. These conditions included a broad range of VEGF stimulation times (30 seconds to 24 hours), different phosphatase inhibitors, and different starvation or growth surface coatings. The results of these experiments were consistent: VEGF stimulation did not significantly enhance phosphorylation of SFKs at the activation loop Tyr416, nor was the resting state of SFK pY416 (Fig. 2D) or the kinase activity (data not shown) ever completely quiescent.

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To exclude the possibility that the reduced Erk1/2 activation during PP2 exposure was a result of a nonspecific inhibition of proteins other than SFKs, we used the gene-specific RNA interference (RNAi) technique and efficiently knocked down Src, Fyn, and Yes individually or simultaneously in human RMECs.\textsuperscript{30} VEGF-induced phosphorylation of Erk1/2 was significantly reduced in cells deficient in individual Src, Fyn, or Yes and in cells deficient in all three SFKs ($P < 0.05$) (Figs. 3B–C). Simultaneous delivery of multiple siRNAs reduced gene-silencing effects of individual siRNA sequences.\textsuperscript{38,39} The reduced knockdown of the individual SFK members in Src, Fyn, and Yes triple knockdown cells might have contributed to the absence of an additive inhibition effect on Erk1/2 activation. The remaining activities of SFKs that were not completely abolished by RNAi might have contributed, at least in part, to the observed Erk1/2 activation in these cells. Therefore, gene-specific downregulation of SFKs by RNAi in human RMECs confirmed that SFKs were indeed required for VEGF signaling through the Ras-MEK-Erk pathway and that Src, Fyn, and Yes all contributed.

In addition, with the use of immunoprecipitation analysis, we observed that VEGF induced rapid and substantial recruitment of SFKs to VEGFR2 in bovine and human RMECs. Two minutes after stimulation in human RMECs, the amount of VEGFR2-associated SFKs was significantly increased 1.8-fold.
Thus, SFKs were important molecular components of VEGF signal cascades, which occurred without an increase of SFK Tyr416 phosphorylation.

**Increased Retinal SFK Tyr416 Phosphorylation Correlates with Pathologic Retinal Angiogenesis**

Previously, our laboratory demonstrated in newborn rat pups that a higher amplitude of fluctuating inspired oxygen can induce greater incidence and severity of retinopathy. The 50%/10% oxygen treatment produced an average retinal avascular area of 29.4% of the total retinal area after oxygen exposure and 100% incidence of retinopathy on postexposure day 6 (Fig. 5A). An oxygen regimen cycling between 40% and 15% oxygen constituted the maximum oxygen variation possible without producing retinopathy (Fig. 5A), despite a retinal avascular area of 14.3% after oxygen exposure.

In the retinas of 50%/10% oxygen-treated pups, VEGF expression was significantly increased at all tested postexposure ages compared with room air controls (Fig. 5B). Immediately after exposure, oxygen-treated animals showed the highest retinal VEGF level—a 6.6-fold increase compared to their room air controls. VEGF levels decreased during the first day after exposure and then gradually increased before decreasing again, as measured on day 6 after exposure. In animals treated with the less severe regimen of 40%/15% oxygen, there was a 2.7-fold increase in the retinal VEGF level compared with the room air control immediately after exposure. This difference, however, became insignificant after 2 days in room air (Fig. 5B).

With the use of Western blot analysis, we found that phosphorylation of the SFK activation loop Tyr416 was significantly increased in the retinas of 50%/10% oxygen-treated pups compared with room air controls on various postexposure days (Fig. 5C, first panel). Immediately after exposure, SFK pY416 was observed at the highest level and later gradually decreased in a pattern consistent with the retinal VEGF profile. SFK protein levels showed no significant difference between oxygen-treated retinas and room air controls (Fig. 5C, second panel). Tyr861 of FAK (Fig. 5C, third panel) was highly phosphorylated.

**Figure 4.** In vitro, VEGF signaling in RMECs induces recruitment of SFKs to VEGFR-2. Serum-starved human RMECs were treated with 25 ng/mL VEGF. The experiment was independently repeated five times. (A) Representative Western blot analysis. IP, immunoprecipitation; IB, immunoblotting. (B) Quantitative analysis of Western blots. *Significantly different from samples without VEGF treatment (P < 0.02).
phorylated in these oxygen-treated retinas, with a profile similar to that of SFK pY416. Retinal phospho-Erk1/2 showed no significant difference between oxygen-treated samples and room air controls. Unlike in the 50%/10% retinas, we did not observe any increase of SFK pY416 or FAK pY861 in the 40%/15% oxygen treatment group compared with room air controls (Fig. 5D).

Thus, significantly increased phosphorylation of the SFK activation loop Tyr416 was specifically observed in the retinas of the 50%/10% oxygen treatment group that would either soon develop retinopathy or already showed the presence of retinopathy. It was not observed in the retinas of the 40%/15% oxygen treatment group that would not develop retinopathy.

Retinal Immunohistochemical Staining Suggests that Müller Cells Are a Primary Source of Elevated Retinal SFK pY416

To further investigate the cellular source of the elevated retinal SFK pY416 seen in the 50%/10% oxygen treatment group on Western blot analysis, we performed immunohistochemical analysis of retinal transverse sections using confocal microscopy. In the retinas of the 50%/10% oxygen treatment group harvested immediately after oxygen exposure, many cell somas located around the midline of the inner nuclear layer (INL) were positively stained for SFK pY416. The age-matched room air control exhibited no staining in this region (Fig. 6). This location corresponded to the leading edge of vascular growth at this stage of development and was precisely where neovascularization occurred in the following days. The immunoreactive staining pattern displayed cells with an irregular shape containing processes projecting from the main trunk. The cells were not positively stained for isoelectin B4, a marker for blood vessels. The location and morphology strongly suggest that these cells are Müller cells. The increased SFK pY416 in the INL tended to subside during the postexposure period (data not shown), which was consistent with the Western blot profile of SFK pY416.

We also observed that the normal intraretinal vascular beds, including superficial and deep networks, were positively stained for SFK pY416. The staining pattern was consistent among tissues harvested from 50%/10% oxygen-treated rats and from room air controls on various postexposure days (Fig. 7 and data not shown). On day 6 after exposure, abnormal blood vessel growth was seen in the retinas of the 50%/10% oxygen treatment group but not in room air controls. The pathologic vessels penetrated the inner limiting membrane, grew into the vitreous, and formed preretinal neovascular tufts. These tufts were also positively stained by the SFK pY416 antibody (Figs. 7G, 7J, 7M). Double labeling of SFK pY416 with isoelectin B4 demonstrated colocalization (Figs. 7G–I), which indicated that these SFK pY416-positive cells were hyperplastic endothelial cells. During the development of the retinal vascular network, astrocytes function as a guide for endothelial cell migration and are closely associated with blood vessels, but they failed to accompany the proliferating endothelial cells into preretinal neovascular tufts (Figs. 7J–L). Double labeling of SFK pY416 with vimentin, an intermediate filament protein normally ex-
pressed in Müller cells, showed no significant colocalization (Figs. 7M–O).

**Intravitreous Injection of PP2 Significantly Reduces Retinopathy in a Rat Model of ROP**

The importance of SFKs in retinal neovascularization is evidenced by our in vitro and in vivo findings. In vitro experiments identified 10 μM PP2 as the optimal concentration for inhibiting SFK-mediated VEGF induction by hypoxia in Müller cells and VEGF signaling in RMECs. When 50%/10% oxygen-treated rats were removed to room air, intravitreous injection of 10 μM PP2 significantly inhibited retinal SFK catalytic activity \((P = 0.001)\) and VEGF levels \((P = 0.01)\) on day 3 after exposure, in contrast to the vehicle control injection (Fig. 8). On day 6 after exposure, retinopathy was markedly reduced \((P = 0.006)\) by 33%. The severity of retinopathy semiquantified by the clock-hour method showed a median of 4 with a range of 2 to 7.5 clock hours for the PP2-injected eyes and a median of 6 with a range of 2 to 10 clock hours for the vehicle-injected eyes. The size of the retinal area involving pathologic neovascularization was also markedly reduced, as shown in Figure 9. No significant difference in retinopathy was observed between vehicle and PP2-injected eyes. Thus, blocking the catalytic activity of SFKs is effective in the inhibition of retinal neovascularization.

**DISCUSSION**

Our findings clearly demonstrate that SFKs are involved in VEGF-mediated retinal neovascularization. In vitro, SFKs were required for VEGF expression under hypoxia in Müller cells and VEGF signaling in RMECs. In vivo, in a rat model of ROP, the activation loop Tyr416 of SFKs was highly phosphorylated and paralleled the increased retinal VEGF. Immunohistochemical analysis revealed that the elevated SFK pY416 originated mainly from the somas of Müller cells, which have been previously identified as the predominant source of VEGF expression during retinal neovascularization.6,7 A significant inhibition of retinopathy by intravitreous injection of a selective SFK inhibitor, PP2, in the 50%/10% oxygen-treated rats confirmed that SFKs were important regulators of abnormal retinal blood vessel growth.

It is generally believed that, on stimulation, the catalytic activity of SFKs is increased by the phosphorylation of Tyr416. This would allow them to phosphorylate substrates and to participate in various signaling events.13 However, recent evidence shows that the regulation of SFK activity through a Tyr416-dependent mechanism is not always required for SFK signaling. Cary et al.22 did not detect increased Src catalytic activity or Tyr416 phosphorylation in Src-mediated integrin signaling. They showed that a Src Y416F mutant was able to fully rescue integrin-mediated FAK phosphorylation, cell spreading, and migration in src−/−fyn−/−yes−/− fibroblasts. Although it has been shown that Src is activated by Tyr416 phosphorylation in hypoxia-induced VEGF expression in U87 glioma cells and kidney 293 cells,16 we found that SFK-mediated VEGF expression under hypoxia in Müller cells and VEGF signaling in RMECs could occur without an increase of Tyr416 phosphorylation.

Our in vitro data showed that VEGF expression under hypoxia in Müller cells required SFK activity, which, however, was not correlated with an increase in Tyr416 phosphorylation. In bovine and human RMECs, VEGF stimulation could activate signal molecules Erk1/2 without increasing SFK Tyr416 phosphorylation. These findings were consistent with our in vivo data from rats treated with the 40%/15% oxygen regimen. During the postexposure period, there was a continuous process of hypoxia-induced retinal VEGF expression and intraretinal vascularization. However, we did not observe any increase of SFK pY416 in these retinas compared with age-matched room air controls, in which the retinal vasculature had fully developed. In addition, even though the retinas of the 50%/10% oxygen treatment group showed a 6.6-fold increase in VEGF level compared with that of the room air controls, SFK pY416 was positively detected in intraretinal vessels and preretinal vascular tufts in the retinas of oxygen-untreated and -treated animals. We did not observe any obvious difference in staining pattern. Thus, SFK-mediated VEGF expression under hypoxia in Müller cells and VEGF signaling in RMECs were not correlated with an increase in Tyr416 phosphorylation in vitro or in vivo.

Wild-type SFKs may exist in four possible forms, depending on the phosphorylation states of Tyr527 and Tyr416.20 It has been shown that Src, when unphosphorylated at Tyr416, retains a basal kinase activity and can readily phosphorylate substrates.24 In addition, the phospho-Tyr416 form of Src, even when in the intramolecular SH2-pY527 complex, retains approximately 20% of its catalytic activity and can phosphorylate...
substrates. In quiescent Müller cell and RMEC cultures, we consistently detected SFK pY416, indicating that at least a fraction of cellular SFKs were phosphorylated at Tyr416. In fact, these wild-type SFKs could phosphorylate substrates in vitro. We believe that both the SFK basal kinase activity and the pY416-related activity might have contributed to this catalytic activity in vitro. We propose that, in some circumstances, wild-type SFKs retain a baseline kinase activity that is physiologically relevant. This baseline kinase activity is sufficient for signal transduction through SFKs. Upregulation of SFK activity by Tyr416 phosphorylation is not important or may not occur. As evidence, using an in vitro kinase assay, Takahashi and Shibuya reported that SFKs were not activated by VEGF in sinusoidal endothelial cells. Conversely, Le Boeuf et al. demonstrated that Src was significantly phosphorylated at Tyr416 on VEGF stimulation in HUVECs. Tissue-specific characteristics of different types of endothelial cells might have contributed to the difference. Nevertheless, an undetected in-

**FIGURE 7.** Normal intraretinal blood vessels and pathologic preretinal vascular tufts were positively stained for SFK pY416. Retinas were harvested on postexposure day 0 (A–C, room air retina; D–F, retinas of the 50%/10% oxygen treatment group) and on postexposure day 6 (G–O, retinas of the 50/10% oxygen treatment group). Sections were double stained for SFK pY416 with rhodamine red X (red, A, D, G, J, M) and for isolecin B4 (B, E, H), GFAP (K), and vimentin (N) with FITC (green). Micrographs (C, F, I, L, O) demonstrate the overlay of the double labeling (costaining, yellow).

**FIGURE 8.** Intravitreous injection of PP2 significantly reduces retinal SFK catalytic activity and VEGF levels in the rat model of ROP. Intravitreous injection was administered on removal of animals to room air, PP2, 10 μM; vehicle, 0.1% DMSO. Retinas were harvested on postexposure day 3. (A) In vitro SFK catalytic activity (n = 8). (B) Retinal VEGF levels (n = 8).
theless, activated SFKs in Müller cells may also participate in physiological course of oxygen-induced retinal neovascularization. Nevertheless, activated SFKs and excessive VEGF production by Müller cells in the retinas of the 50%/10% oxygen treatment group, some evidence does support this hypothesis. First, SFK activation in these retinas was correlated with drastically increased retinal VEGF levels as measured after oxygen exposure. Second, SFKs were activated in the somas of Müller cells, in which increased VEGF expression has been localized in the ischemic retina. Third, the distribution of cells with increased levels of SFK pY416 approximated the peripheral avascular retinal region. More significantly, in U87 glioma cells, overexpression of Src resulted in increased VEGF transcription under hypoxia. Transfection with v-Src constitutively increased the VEGF mRNA level even in the absence of hypoxia. During the pathophysiological course of oxygen-induced retinal neovascularization, SFK activation may serve as a switch that turns on excessive VEGF expression and leads to proliferative retinopathy, which is devoid of physiological intraretinal vascularization. Nevertheless, activated SFKs in Müller cells may also participate in other undefined pathophysiological events. As an example, glial cells have been shown to influence blood–brain barrier properties. However, it remains possible that SFK activation in the retinas that develop retinopathy is a consequence, rather than a cause, of pathologic or physiological events.

In the rat model of ROP, intravitreous injection of PP2 significantly inhibited retinopathy. It is likely that PP2 exerted its impact through the VEGF expression pathway in Müller cells (Figs. 1, 8) and the downstream signaling pathway in RMECs (Fig. 3). For proof of principle, we injected PP2 only once on removal of the animals to room air. Notably, at this time, retinal SFK pY416 and catalytic activity and VEGF expression were already at the highest levels, which likely predisposed the retinas for neovascularization. In addition, the existing high level of retinal VEGF might partially explain the incomplete inhibition of VEGF by PP2 seen on postexposure day 3. Further characterization of SFK activity during the oxygen treatment will provide a better definition of the critical moment for optimal inhibition of aberrant SFK signaling and the resultant efficacy.

Significant increases of SFK expression and catalytic activity have been observed in a variety of tumors. This elevated SFK activity was correlated with the stage and metastatic potential of some neoplasias, thus identifying the activated SFKs as potential targets for intervention in tumorigenesis. Here, we report that significantly increased phosphorylation of the activation loop Tyr416 of SFKs was associated with the pathogenesis of retinopathy in a rat model of ROP, but not in physiological intraretinal vascularization (e.g., in the 40%/15% oxygen treatment group). The unknown mechanism of SFK activation and its roles in the pathophysiological course warrant further characterization. Ultimately, this may lead to the identification of important molecular and cellular differences between physiological retinal vascular development and pathologic retinal neovascularization. Detailed knowledge of SFK activation and subsequent abnormal cellular functions may facilitate the rational design of therapeutic strategies that selectively target pathologic events yet minimize the unfavorable impact on physiological cellular functions.

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