Suppressors of Cytokine-Signaling Proteins Induce Insulin Resistance in the Retina and Promote Survival of Retinal Cells

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OBJECTIVE—Suppressors of cytokine signaling (SOCS) are implicated in the etiology of diabetes, obesity, and metabolic syndrome. Here, we show that some SOCS members are induced, while others are constitutively expressed, in retina and examine whether persistent elevation of SOCS levels in retina by chronic inflammation or cellular stress predisposes to developing insulin resistance in retina, a condition implicated in diabetic retinopathy.

RESEARCH DESIGN AND METHODS—SOCS-mediated insulin resistance and neuroprotection in retina were investigated in *1*) an experimental uveitis model, *2*) SOCS1 transgenic rats, *3*) insulin-deficient diabetic rats, *4*) retinal cells depleted of SOCS6 or overexpressing SOCS1/SOCS3, and *5*) oxidative stress and light-induced retinal degeneration models.

RESULTS—We show that constitutive expression of SOCS6 protein in retinal neurons may improve glucose metabolism, while elevated SOCS1/SOCS3 expression during uveitis induces insulin resistance in neuroretina. SOCS-mediated insulin resistance, as indicated by its inhibition of basally active phosphoinositide 3-kinase/AKT signaling in retina, is validated in retina-specific SOCS1 transgenic rats and retinal cells overexpressing SOCS1/SOCS3. We further show that the SOCS3 level is elevated in retina by oxidative stress, metabolic stress of insulin-deficient diabetes, or light-induced retinal damage and protects ganglion cells from apoptosis, suggesting that upregulation of SOCS3 may be a common physiologic response of neuroretinal cells to cellular stress.

CONCLUSIONS—Our data suggest two-sided roles of SOCS proteins in retina. Whereas SOCS proteins may improve glucose metabolism, mitigate deleterious effects of inflammation, and promote neuroprotection, persistent SOCS3 expression caused by chronic inflammation or cellular stress can induce insulin resistance and inhibit neurotrophic factors, such as ciliary neurotrophic factor, leukemia inhibitory factor, and insulin, that are essential for retinal cell survival. ***Diabetes*** **57:1651–1658, 2008**

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EAU, experimental autoimmune uveitis; FOXO, forkhead transcription factor; GFP, green fluorescent protein; HIF, hypoxia-inducible factor; IFN, interferon; IL, interleukin; IRBP, interphotoreceptor retinoid binding protein; IRS, insulin receptor substrate; NIH, National Institutes of Health; PI3K, phosphatidylinositol 3-kinase; pSTAT, phosphorylated STAT; qRT-PCR, quantitative RT-PCR; RPA, ribonuclease protection assay; RGC, retinal ganglion cell; RPE, retinal pigment epithelium; siRNA, small interfering RNA; SOCS, suppressors of cytokine signaling; STAT, signal transducer and activator of transcription; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

Insulin resistance and chronic inflammation are implicated in the pathogenesis of diabetic retinopathy, a glucose-mediated microvascular disease that derives, in part, from the inability of retina to adapt to metabolic stress (1–3). Clinical evidence for a role of insulin resistance as a risk factor for diabetic retinopathy comes from several clinical trials, including the Diabetic Control and Complications Trial and the EURODIAB Prospective Complications Study (4,5). In insulin-deficient diabetic rats, progressive diminution of insulin receptor activity and a marked decrease in insulin receptor substrate (IRS) in retina precede appearance of early features of diabetic retinopathy and neuronal cell death (6). On the other hand, early features of diabetic retinopathy, such as vascular permeability, edema, and increase in inflammatory proteins, are hallmarks of ocular inflammation (1). Although a direct connection between chronic inflammation and insulin resistance in the etiology of diabetic retinopathy has yet to be established, studies on obesity or metabolic syndrome provide a paradigm for understanding the roles of these disparate processes in diabetic retinopathy (7,8). In hepatocytes or adipocytes, activation of insulin receptors recruits IRS proteins to the receptor complex, leading to activation of phosphatidylinositol 3-kinase (PI3K) and AKT signaling pathways, increased glucose metabolism, and cell growth (7). However, several reports (9–11) have shown that induction of suppressors of cytokine-signaling (SOCS) proteins by inflammatory cytokines desensitizes insulin signaling by targeting IRS1/IRS2 for degradation, leading to development of obesity, hepatic steatosis, or metabolic syndrome. It is therefore of note that IRS2 is essential for insulin signaling in retina (12), and SOCS proteins are elevated in retina during intraocular inflammatory disease (uveitis) (13), suggesting that SOCS-mediated degradation of IRS2 in retina may inhibit signaling downstream of the insulin receptor, providing a mechanistic link between inflammation, insulin resistance, and diabetic retinopathy.

SOCS proteins regulate intensity and duration of cytokine/growth factor signals and integrate multiple extracellular signals that may converge on target cells. The eight-member SOCS family of proteins attenuates cytokine signals through interactions with cytokine/growth factor receptors and signaling proteins, leading to proteosomal degradation of the receptor complex (14–16). SOCS pro-

teins are rapidly induced in many cell types in response to cytokines (interferon [IFN]-γ, interleukin [IL]-1β, and IL-6) or growth factors (ciliary neurotrophic factor, leukemia inhibitory factor, fibroblast growth factor, and insulin), and their effects are transient due to their short half-life (17–19). However, constitutive SOCS expression occurs in some tissues due to unabated stimulation by chronic inflammation or cellular stress, leading to silencing of critical cellular pathways and predisposition to organ-specific disease development.

In studies on experimental autoimmune uveitis (EAU), a model of human uveitis, we showed that SOCS1 and SOCS3 are induced in retina during uveitis (13,20). Therefore, in this study, we tested the hypothesis that SOCS proteins induced during uveitis can induce insulin resistance and inhibit prosurvival insulin signaling pathways in retina. In addition to intraocular inflammation, we found that factors that induce cellular stress (hypoxia, metabolic stress of diabetes) or photoreceptor damage (high intensity) induce SOCS3 expression in retina, confer partial protection to neuronal cells against apoptosis, and also desensitize insulin signaling in retina cells. We show for the first time that the SOCS6 protein is constitutively expressed in retinal neurons and may function to improve glucose metabolism in neuroretina. Together, these results suggest two-sided roles of SOCS proteins in retina. Benefits of SOCS-mediated protective adaptive responses to inflammation, light damage, hypoxia, or metabolic stress come at a cost, as persistent SOCS1/SOCS3 expression induced by uveitis or chronic stress can induce insulin resistance and trigger diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Animals and induction of diabetes. C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME). For induction of diabetes, overnight-fasted Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were given a single intravenous injection of streptozotocin (STZ) (55–65 mg/kg; Sigma) in freshly dissolved 10 mmol sodium citrate (pH 4.5). Diabetes was confirmed by blood glucose >250 mg/dl, and tissues were harvested 6 and 8 weeks after induction. Transgenic rat with targeted expression of SOCS1 using an opsin promoter element was generated and characterized as described (21). Mice/rats were housed in accordance with National Institutes of Health (NIH) guidelines on animal care.

Rat retina, organ culture, culture of human cell lines, and hypoxia studies. Rat (postnatal day 2 pups) organ cultures were prepared as described (22). Retina organs were stimulated with 100 units/ml IFN-γ (Pepro Tech, Rocky Hill, NJ) and/or 10 nmol/l insulin in Dulbecco's modified Eagle's medium (DMEM) at 37°C, 5% CO_2. Human Müller cell line (MI0-M1) (provided by G. Astrid Limb, London, U.K.) was cultured as described (23). Human retinal pigment epithelium cell line (ARPE-19) was cultured as described (24). Before stimulation, cells were placed in serum-free medium for 12 h and then stimulated with 100 units/ml IFN-γ (Pepro Tech), 10 ng/ml IL-4, or 10 nmol/l insulin (Roche). For hypoxia experiments, cells were placed in a hypoxic incubator (37°C, 1% O_2, 5% CO_2) for various amounts of time (0, 2, 12, or 24 h) as described (25). All experiments were done using cells within the first five passages, and under our experimental condition the cells were under hypoxic conditions after 2 h, as established by induction of hypoxia-inducible factor (HIF)-1α expression (26). Observation of cells by light microscopy after 24 h of hypoxia demonstrated that they were viable and healthy

EAU induction. We induced EAU in C57BL/6 mice by active immunization with 150 μg bovine interphotoreceptor retinoid binding protein (IRBP) and 300 μg human IRBP peptide (1–20) in complete Freund's adjuvant containing the *Mycobacterium tuberculosis* strain H37RA (2.5 mg/ml) (13). Eyes for histology were harvested 0, 14, and 21 days postimmunization, fixed in 10% buffered formalin. Sections were stained with hematoxylin and eosin.

Light treatment. Mice were dark-adapted overnight and then exposed to white light (5,000 lux) for 6–24 h. Each animal was housed in a separate well-ventilated transparent plastic cage so that one animal could not hide behind another. Temperature was kept at 25.0°C ± 1.0°C during light exposure.

Confocal microscopy. Sections blocked in 5% normal goat serum were incubated with rabbit polyclonal SOCS6 antibody (Zymed, San Francisco, CA). SOCS6-expressing cells were detected with Alexa-488 conjugated goat anti-rabbit secondary antibody containing DAPI (1 μg/ml) (Invitrogen, Carlsbad, CA) on a Leica-SP2 laser-scanning confocal microscope (Leica Microsystems, Exton, PA) as described (20).

RT-PCR and quantitative RT-PCR analysis. RNA samples were DNA free. cDNA was generated as described previously (17); each gene-specific primer pair used for RT-PCR analysis spans at least an intron. Quantitative RT-PCR (qRT-PCR) analysis was performed, as previously described (20), using primers and probes from Applied Biosystems. mRNA expression levels were normalized to levels of *β-actin* and *GAPDH* housekeeping genes. RT-PCR primers can be found in the online appendix (available at http://dx.doi.org/10.2337/db07-1761).

Western blot analyses. Preparation of whole-cell lysates from retina, organ cultures, or cell lines and Western blotting were as described (13). Blots were probed with antibodies specific to SOCS1, SOCS3, and SOCS6 (Zymed Laboratories, Inc., South San Francisco, CA); β-actin; p85; SOCS5 and SOCS7; forkhead transcription factor (FOXO) 1; signal transducer and activator of transcription (STAT) 3; vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, CA); phosphorylated STAT1 (pSTAT1) and pSTAT3; AKT and phosphorylated AKT (pAKT); and IRS-1 and IRS-2 (Cell Signaling, Beverly, MA). Signals were detected using an electrochemiluminescence system (Amersham, Arlington Heights, IL). Some blots were analyzed with the NIH Image Quant program. Each band was normalized to corresponding β-actin band and expressed in arbitrary relative protein expression units.

Ribonuclease protection assay. A ribonuclease protection assay (RPA) was performed with RNA (10 μg), [α-^{32}P]-UTP radiolabeled RNA probes, and a human SOCS RPA kit (BD Biosciences, San Diego, CA), as recommended by the manufacturer.

Small interfering RNA–mediated silencing of SOCS6 expression. Cells were cultured for 2–3 days to 60–70% confluency and electroporated with small interfering RNA (siRNA) oligonucleotides (Dharmacon, Lafayette, CO) by Amaxa Nucleofector Technology (Amaxa, Cologne, Germany), according to manufacturer's instructions. Cells were replated in six-well plates and maintained in medium for 48 h before stimulation with agonists.

Cytotoxic assay for detection of apoptosis in mock- and SOCS3-transfected retinal cells. Apoptosis was induced in ganglion cells (1×10^6 cells/ml) transfected with empty-vector or SOCS3 cDNA with a Nucleofector kit (Amaxa) by culturing for 1 and 3 h in medium containing 1 μmol/l staurosporin (Sigma). Cell death was assessed by fluorescence-activated cell sorter analysis of annexin-V – or 7-amino-actimycin D–labeled cells, according to the manufacturer (BD Biosciences).

Generation of retinal pigment spithelium or retinal ganglion cells overexpressing SOCS1 or SOCS3. The rat retinal ganglion cell (RGC) line, RGC-5, was generated and characterized as described (27). Construction of SOCS1 or SOCS3 cDNA expression vectors has been described (18,28). RGC-5 and ARPE-19 cells (60–70% confluence) were electroporated by Amaxa Nucleofector Technology.

Statistical analysis. Experiments were performed at least three times. Figures show data from representative experiments or from combined experiments as indicated. The Student's *t* test was performed on the data as indicated. *P* values ≤0.05 are considered statistically significant.

RESULTS

Insulin signaling pathways of the retina are inhibited in EAU. SOCS1 and SOCS3 are implicated in the etiology of insulin resistance, obesity, and metabolic syndrome (10,29). Here, we have examined whether upregulation of SOCS1/SOCS3 expression in retina during intraocular inflammation (uveitis) predisposes to developing insulin resistance in retina. EAU was induced in C57BL/6 mice by immunization with IRBP, and disease was established by fundoscopy and histology (13,20). Initial clinical signs of EAU appear 7–12 days after immunization, and full-blown clinical disease characterized by IFN-γ secretion develops by postimmunization day 14 (Fig. 1*A*). Substantial reduction in inflammation is observed by postimmunization day 21 (Fig. 1*A*), with complete resolution of EAU occurring day 28 postimmunization (13). In line with published reports (13,20), SOCS1 and SOCS3 mRNA expression is induced in retina, with the highest levels detected at peak of the disease (data not shown). Western blot analysis

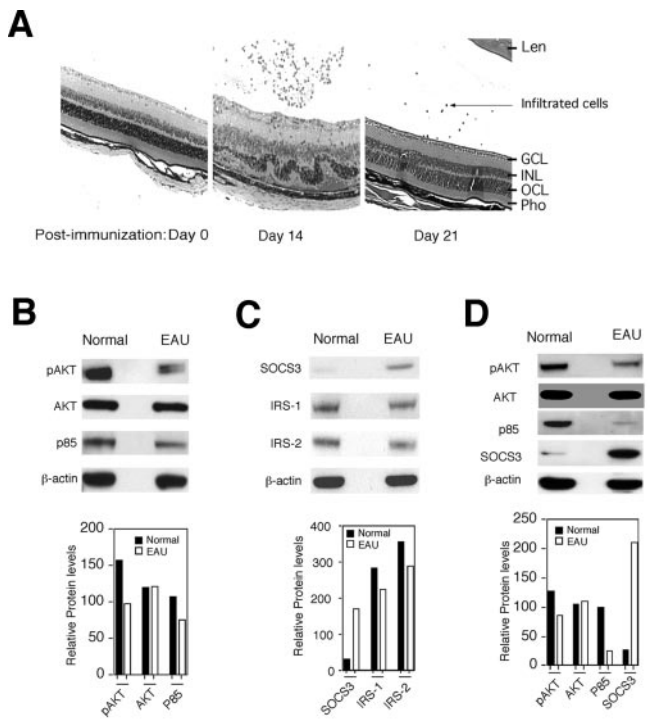

FIG. 1. Induction of SOCS1 and SOCS3 during EAU correlates with inhibition of insulin signaling in the retina. *A*: Six-week-old mouse was immunized with IRBP in complete Freund's adjuvant and 14 days later eyes were enucleated. Four-micron-thick sections were cut through the retina and stained with hematoxylin and eosin. *B*: Western blot analysis of whole-cell protein extract prepared from the retina (*B* and *C*) or spleen (*D*) of normal mouse or mouse with EAU. *B*, *C*, and *D* (*bottom panels*): Intensities of the Western blot bands were analyzed in a densitometer to quantify the relative protein expression levels.

shows that normal mouse retina has high basally active insulin receptor signaling, as indicated by constitutive expression of pAKT, IRS proteins, and p85 regulatory subunit of PI3K in retina (Fig. 1*B*). These analyses also show that an increase in SOCS3 protein during EAU correlates with decreased PI3K/AKT signaling in EAU retina, as indicated by diminution in levels of pAKT, p85, and IRS proteins (Fig. 1*B* and *C*). Increase in SOCS3 expression and decrease in PI3K/AKT signaling are also observed in EAU mouse spleen (Fig. 1*D*). Of note, the slight decrease in IRS2 in EAU retina is in concert with reports showing that IRS2 is required for survival/insulin signaling in photoreceptor cells (12) and sensitive to SOCS3-mediated degradation (11). These results establish a correlation between upregulation of SOCS1 and SOCS3 in retina during uveitis and diminished biological responses to insulin in retina.

SOCS genes are differentially regulated in the retina by proinflammatory cytokines. SOCS1 and SOCS3 expression in retina during uveitis is a negative-feedback response that mitigates retinal pathology by limiting intensity and duration of activities of proinflammatory cytokines produced by uveitogenic T-cells (13). However, inhibition of basal insulin/AKT signaling pathways in retina during EAU suggests that SOCS1/SOCS3 may also be induced in retinal cells by inflammatory cytokines. To establish that retinal cells express SOCS genes in response to proinflammatory cytokines, we stimulated ARPE-19 and MI0-M1 retinal cells with IFN-γ or IL-4. qRT-PCR (Fig. 2*A*) and RPA (Fig. 2*B*) unambiguously show that exposure of resident retinal cells to proinflammatory cytokines upregulates SOCS1/SOCS3 expression. Of particular interest is constitutive expression of SOCS5, SOCS6, and SOCS7 in human retinal cells (Fig. 2*B*) as well as mouse and rat retina (Fig. 2*C* and *D*). These results suggest that SOCS genes are differentially regulated in retina and may have distinct functions in the physiology of neuroretina. Because SOCS6 has been shown to improve glucose metabolism (30), we examined the spatial localization of SOCS6 protein in retina. We show that SOCS6 is localized to ganglion cell and photoreceptor layers of mouse retina (Fig. 2*E*) and retinal pigment epithelium (RPE). The anatomic localization of SOCS6 protein to regions of high metabolic demands in the retina is consistent with its role in supporting glucose metabolic pathways.

SOCS1 and SOCS3 induce insulin resistance in retinal cells. To further examine whether cytokine-induced overexpression of SOCS proteins in retina causes insulin resistance, we exposed postnatal day 2 rat retinal organ cultures to IFN-γ and/or insulin for 12 h. As shown (Fig. 3*A*), a relatively high level of SOCS1 is induced by IFN-γ, while only a modest level of SOCS3 expression is induced by insulin or IFN-γ (Fig. 3*A*). However, marked elevation of SOCS3 by IFN-γ occurs in retinal cells concurrently stimulated by insulin (Fig. 3*A*). We further show that enhanced expression of SOCS1/SOCS3 proteins in retina derives, in part, from synergistic activation of STAT1 by insulin and IFN-γ (Fig. 3*C*). In concert with high metabolic requirements of retina (6,31), insulin signaling is constitutively active in retina, as indicated by detection of pAKT in freshly isolated primary retinal cells (Fig. 3*B*). However, pAKT activity of freshly isolated retinal cells is inhibited by IFN-γ, even in our insulin/IFN-γ coculture experiment, where cells are continuously stimulated by exogenous insulin (Fig. 3*B*, *lanes 3* and *4*). Potential mechanism(s) by which IFN-γ reduces AKT phosphorylation in both basal and insulin-stimulated states may be similar to those described in liver and adipose cells, where induction of SOCS1/SOCS3 by IL-6 or tumor necrosis factor-α desensitizes insulin signaling by targeting IRS1/IRS2 for degradation (9–11). Thus, diminution of constitutive pAKT in retinal cells (Fig. 3*A*) following IFN-γ–induced upregulation of SOCS1 and SOCS3 expression (Fig. 3*A*) suggests that IFN-γ secretion during uveitis may diminish biological responses of retinal cells to insulin in retina. We have also generated rat ganglion (Fig. 3*D*) or ARPE-19 (Fig. 3*E*) cells overexpressing SOCS1 or SOCS3, and either SOCS member suppresses AKT/PI3K activation by insulin, underscoring potential roles of these SOCS proteins in inducing insulin resistance in retinal cells. To directly examine effects of constitutive expression of SOCS1 in retina, we generated transgenic rats with targeted overexpression of SOCS1 in retina (21). Similar to our in vitro data, elevation of SOCS1 level in retina induces insulin resistance in transgenic rat retina as indicated by low basal pAKT activity in freshly isolated retinal cells and diminished ability of insulin to activate AKT pathways in transgenic retina (Fig. 3*F*).

SOCS3 is upregulated in retina by light damage, oxidative stress, and insulin-deficient diabetes. Desensitization of retinal cells to insulin signals by SOCS proteins led us to examine other cellular pathways that may cause elevation of SOCS proteins in retina. We therefore examined whether SOCS proteins are induced in retina in response to three distinct types of stress implicated in the development of retinal degenerative diseases: high-intensity light, oxidative stress, and metabolic stress

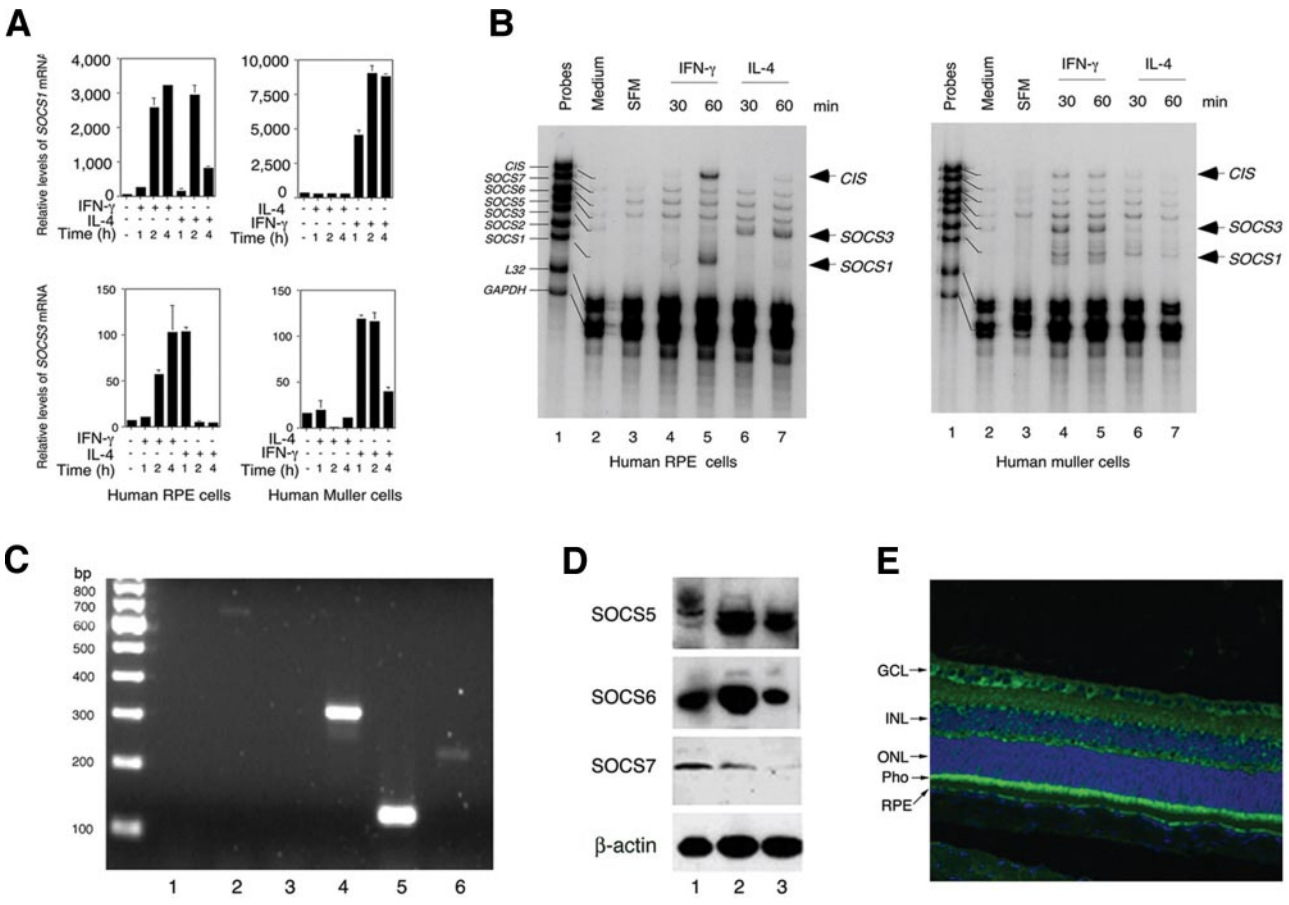

FIG. 2. Expression of SOCS family genes is differentially regulated in the retina. *A*: Induction of *SOCS* expression in human retinal cell lines by proinflammatory cytokines was detected by qRT-PCR (*A*) or RPA (*B*). *C*: RT-PCR analysis of *SOCS* expression in mouse retina: SOCS1 (*lane 1*), SOCS2 (*lane 2*), SOCS3 (*lane 3*), SOCS5 (*lane 4*), SOCS6 (*lane 5*), and SOCS7 (*lane 6*). *D*: Detection of SOCS5, SOCS6, and SOCS7 proteins in the retina and retinal cell lines by Western blot analysis: rat retina (*lane 1*), human ARPE-19 cell line (*lane 2*), and human Müller cell line (*lane 3*). *E*: Localization of SOCS6 protein expression in the mouse retina by immunohistochemical analysis: GLC, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; Pho, photoreceptors.

induced by insulin-deficient diabetes. For light-induced retina damage, mice were exposed to high-intensity light for varying amounts of time, ranging from 24 h (Fig. 4*A*) to 6.5 h (Fig. 4*B*). RT-PCR (Fig. 4*A*) and Western blot (Fig. 4*B*) analysis of mouse retina revealed that exposure of retinal cells to high-intensity light that induces apoptotic death of retina cells in vivo (32) elicits a rapid activation of STAT3, upregulation of VEGF, and SOCS3 expression. In three separate experiments, activation of STAT3/VEGF expression precedes peak expression of SOCS3 (Fig. 4*B*). Thus, consistent with published reports (33,34), light-induced VEGF may activate STAT3 and induce SOCS3 expression. It may well be that light-induced changes in SOCS3/STAT3 plays a role in protecting photoreceptor cells from light-induced apoptosis. Similarly, culturing retinal cells under hypoxic condition induces VEGF and HIF-1α expression, with HIF-1α expression observed as early as 2 h, while high-level VEGF expression is detected at 12 h (Fig. 4*C*). On the other hand, genetic studies (35,36) show that STAT3 is required for VEGF and HIF-1α expression, suggesting that VEGF may be regulated by STAT3 and under negative-feedback regulation by SOCS3 in retina. We also investigated whether insulin deficiency in retina is associated with elevation of SOCS proteins in retina. Diabetes was induced by injection of STZ, and by week 8 postinjection rats developed large focal retinal degeneration, retinal folds, and thinning of retinal layers (Fig. 4*D*). Analyses of STZ-induced diabetic (glucose level >400 mg/dl) or nondiabetic rat retinae reveal that SOCS3 mRNA (Fig. 4*E*) and protein (Fig. 4*F*) are substantially elevated in all STZ-induced diabetic rat retina examined. Interestingly, increase in SOCS3 in diabetic retina is accompanied by decrease in pAKT. Remarkably, retinal cells selectively induce SOCS3 but not other SOCS family members (Fig. 4*B*) in response to light damage, oxidative stress, or diabetes-induced retinal damage, suggesting that SOCS3 may play a role in protecting the neuroretina from cellular stress. In fact, we provide direct evidence that SOCS3 confers protection to retinal cells from apoptosis. Survival of retinal ganglion cells transfected with empty-vector or SOCS3 cDNA was assessed following treatment with the apoptosis-inducing chemical staurosporin. Apoptotic and necrotic cells were assessed by annexin-V and 7-amino-actimycin D staining assays, respectively, and enhanced survival of SOCS3 transfected cells (45.8 vs. 30.8%) and reduced number of SOCS3 transfectants undergoing apoptosis (39.7 vs. 59.9%) after 3 h treatment indicate that SOCS3 protects retinal cells from apoptosis (Fig. 4*G*).

SOCS6 activates the prosurvival AKT pathway and inhibits the proapoptotic FOXO1 protein. In the retina, we observe a dichotomous pattern of SOCS expression: whereas SOCS1, SOCS2, SOCS3, and cytokine-induced SH2 protein (CIS) are barely detectable, SOCS5,

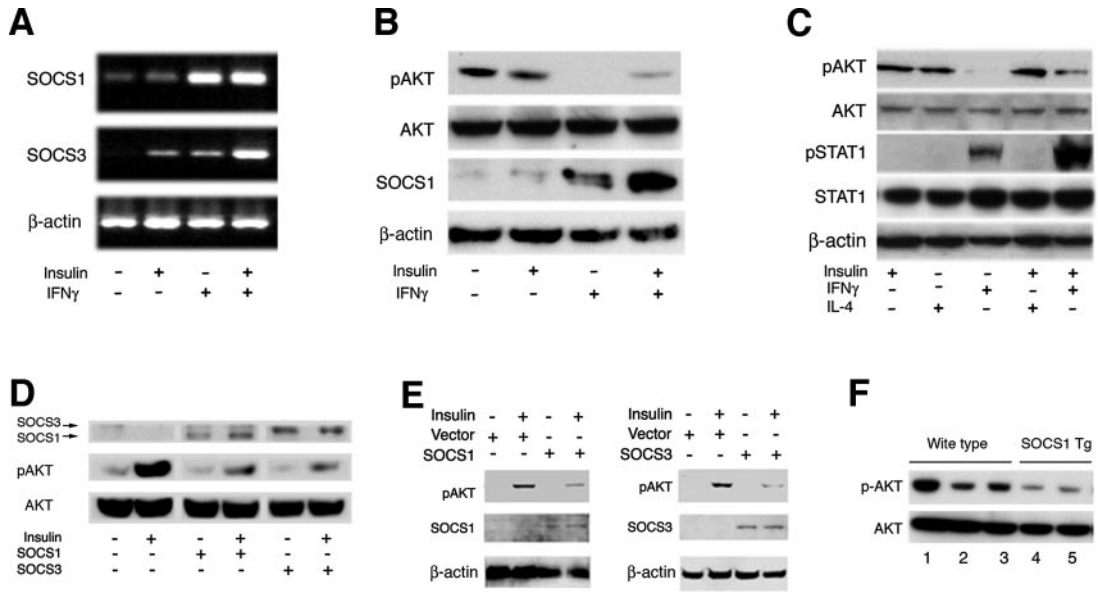

FIG. 3. SOCS1 or SOCS3 induces insulin resistance in retinal cells. *A*: RT-PCR analysis showing synergistic induction of SOCS3 expression in rat retina organ culture by IFN-γ and insulin. Western blot analysis of extracts from rat organ cultures stimulated with insulin and/or IFN-γ (*B*) or insulin, IL-4, and/or IFN-γ (*C*). Western blot analysis showing inhibition of insulin-induced pAKT signaling in rat ganglion (*D*) or human RPE cells overexpressing SOCS1 or SOCS3 (*E*). *F*: Western blot analysis showing inhibition of insulin-induced activation of AKT signaling in a transgenic rat strain with targeted overexpression of SOCS1 in the retina. Retina organs were cultured for 12 h in normal serum in the absence (*lanes 2* and *4*) or presence of 10 nmol/l insulin (*lanes 3* and *5*). *Lane 1*, freshly isolated retina from wild-type rat.

SOCS6, and SOCS7 are constitutively expressed (Fig. 2*C* and *D*). The spatial localization of SOCS6 in ganglion and photoreceptor cell layers is of particular interest as these regions of retina are indispensable components of the visual apparatus and major sites of retinal degenerative changes in diabetic retinopathy. Small interfering RNA (siRNA)-mediated silencing of SOCS6 expression was therefore used to investigate functional relevance of SOCS6 in ocular cells. Optimal condition for efficient siRNA transfection into ARPE-19 cells was established with green fluorescent protein (GFP)-siRNA oligonucleotides (Fig. 5*A*), and 1.5 μg SOCS6-siRNA was found to induce an 85% reduction of endogenous SOCS6 RNA expression without appreciable effect on cell viability (Fig.

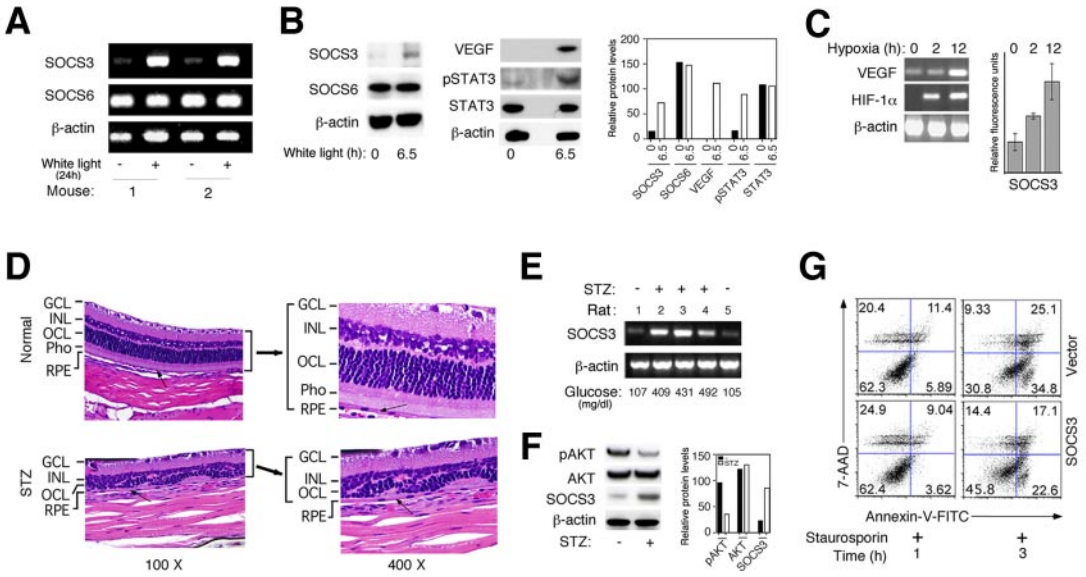

FIG. 4. Retinal cells induce expression of SOCS3 in response to light damage, hypoxia, or insulin-deficient diabetes. *A*: RT-PCR analysis of mouse retina following 24 h exposure to high-intensity light (5,000 lux). *B*: Western blot analysis of retina following 6.5 h exposure of mice to high-intensity light (5,000 lux). *Right panel*: Intensities of the Western blot bands were analyzed in a densitometer to quantify the relative protein expression levels. These results are from three independent experiments. *C*: Mouse retina explants were propagated for varying amounts of time under hypoxia condition. Induction of VEGF and HIF-1α expression was detected by RT-PCR, while SOCS3 expression was by qRT-PCR analysis. These results are from three independent experiments. *D*: Histology of rat retina showing development of retinal degeneration in 8-week STZ-induced diabetic rats but not in non–STZ-induced control rat. RT-PCR (note: each lane represents an individual rat) (*E*) and Western blot (*F*) analysis of *SOCS3* expression in retina of normal or insulin-deficient diabetic rats. *Right panel*: Intensities of the Western blot bands were analyzed in a densitometer to quantify the relative protein expression levels. *G*: Annexin V–fluorescein isothiocyanate histograms showing percentage of apoptotic cells following treatment with staurosporin. Necrotic cells were detected by fluorescence-activated cell sorter analysis of 7-amino-actimycin D labeled.

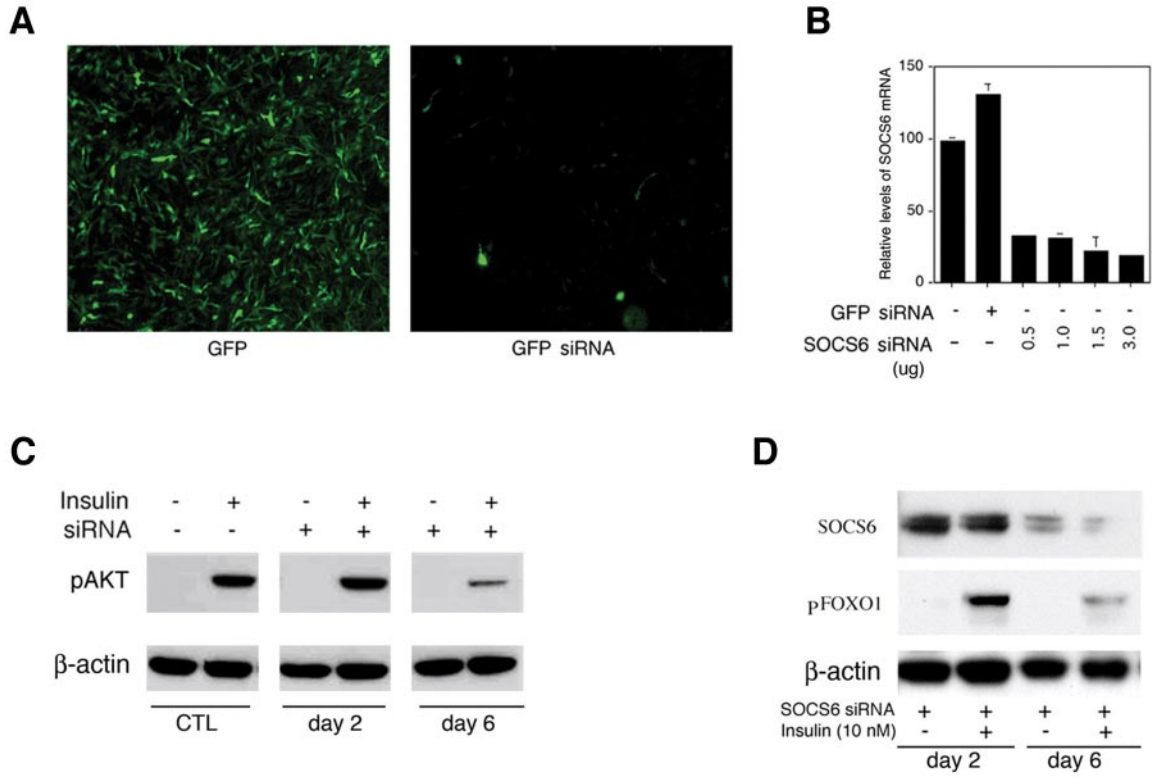

FIG. 5. siRNA-mediated silencing of SOCS6 expression inhibits insulin-induced activation of AKT signaling in human retinal cells. *A*: Verification of siRNA transfection efficiency in human RPE cells (ARPE-19) transfected with GFP plasmid and GFP-specific siRNA. *B*: qRT-PCR analysis of SOCS6 mRNA expression in ARPE-19 days after transfection with GFP-specific (GFP siRNA) or SOCS6-specific (SOCS6 siRNA) siRNA. Western blot analyses showing: inhibition of insulin-induced pAKT signaling (*C*) or inhibition of FOXO1 inactivation in day 6 SOCS6-siRNA–transfected ARPE-19 (*D*).

5*B*). We show that maximum depletion of the endogenous SOCS6 protein that occurs consistently in day 6–transfected cells coincides temporarily with inhibition of insulin-induced activation of AKT (Fig. 5*C*). Dependence of pAKT signaling on SOCS6 is of functional relevance because this pathway, downstream of activated insulin/IRS2, is essential for retinal cell survival (12). A well-characterized prosurvival pathway induced by insulin/IRS2 derives from obligatory inactivation of FOXO1 by AKT kinases, resulting in inhibition of proapoptotic and growth inhibitory functions of this forkhead family transcription factor (37). We therefore investigated whether SOCS6 is involved in regulating this pathway. Silencing of SOCS6 results in blockage of insulin/PI3K/AKT-mediated inactivation of FOXO1 (Fig. 5*D*), suggesting a potential role of SOCS6 in promoting insulin-induced survival of retinal cells.

DISCUSSION

In a previous study (38,39), we showed that transgenic rats with targeted expression of IFN-γ in the retina induced constitutively high levels of SOCS1 and SOCS3 in the retina and by 3 months of age the rats developed severe retinal degeneration. In this study, we have tested the hypothesis that chronic intraocular inflammation or other sources of cellular stress that induce persistent SOCS expression in retina might be risk factors for the development of retinal diseases. Particular emphasis is on the potential role of persistent stimulation of SOCS expression in retina by inflammatory cytokines, oxidative stress, light damage, or metabolic stress in the development of insulin resistance, an important complication of diabetes and contributory factor of the potentially blinding disease diabetic retinopathy.

We show here that induction of SOCS1 and SOCS3 expression during uveitis inhibits signaling downstream of insulin receptor in the retina (Fig. 1*B* and *C*), and the reduction in constitutive insulin signaling derives in part from a decrease in levels of pAKT, p85 (regulatory subunit of PI3K), and IRS proteins in the retina. We further show that insulin synergizes with IFN-γ to enhance SOCS1/SOCS3 expression in retinal cells, suggesting that copious amounts of IFN-γ secreted in retina by inflammatory cells during uveitis (13) may synergize with insulin to elevate levels of SOCS1/SOCS3 proteins, resulting in desensitization of retinal cells to insulin signals. The role of SOCS in desensitizing retinal cells to insulin signals is further established by studies showing that forced overexpression of SOCS1 or SOCS3 protein in rat retinal ganglion cells, human RPE cells, or primary rat retinal cells inhibits insulin-induced PI3K/AKT signaling, an important indicator of the development of insulin resistance (Fig. 3). Insulin resistance in fresh retina organ cultures (Fig. 3*B* and *C*), in SOCS1- or SOCS3-transfected retinal cells (Fig. 3*D* and *E*), or during EAU (Fig. 1) is validated in transgenic rats with targeted overexpression of SOCS1 in the retina (Fig. 3*F*). It is remarkable that all SOCS1 transgenic rats exhibit low basal insulin/AKT signaling activity and are unable to induce appreciable PI3K/AKT signaling even after stimulation by exogenous insulin. Although SOCS1 and SOCS3 have been implicated in pathogenesis of type 2 diabetes (40), obesity (40), hepatic steatosis (8), and metabolic syndrome (41), this is the first report showing

that elevated levels of SOCS proteins during uveitis diminish basally active insulin and AKT signaling in retina.

In addition to inflammation, high-intensity light, oxidative stress, and metabolic stress of insulin-deficient diabetes also induce SOCS3 protein in the retina but not SOCS1, SOCS6, or other SOCS members. As SOCS3 inhibits signals downstream of the insulin receptor in retinal cells (Fig. 3*D* and *E*), these results suggest that other physiologic states that elevate SOCS3 levels may contribute to development of insulin resistance in retina. Furthermore, in our rodent models of light-induced retinal degeneration or hypoxia, VEGF, a survival factor for retinal neurons (42,43), is rapidly upregulated in retina, in part through STAT3-dependent mechanisms (Fig. 4*B* and *C*), consistent with requirement of STAT3 for transcription of VEGF and HIF-1α mRNA (35,36,44). While VEGF may provide prosurvival functions in retina, accumulation of VEGF in the eye is implicated in the development of retinopathies, and anti-VEGF antibody is effective in treatment of diabetic retinal neovascularization (45). These diametrically polar effects of VEGF in the retina suggest that STAT3-induced VEGF expression is exquisitely regulated in the retina, as activated STAT3 is preferentially localized to neovascular retinal vessels in a mouse model of ischemia-induced retinal neovascularization (46). However, as STAT3 promotes proliferation and prevents apoptosis of some cell types (47), we cannot rule out the possibility that pathways activated in the retina by STAT3 may play a role in protecting photoreceptor cells from light-induced apoptosis. SOCS3 is a major inhibitor of STAT3, and we observed a temporal correlation between STAT3-induced VEGF expression and SOCS3 induction in light-treated retina (Fig. 4*B*) or ocular cells subjected to oxidative stress (Fig. 4*C*), suggesting that stress-induced activation of STAT3 and VEGF expression are under negative feedback regulation by SOCS3. Physiological relevance of SOCS3 as a stress response factor is further underscored by the high constitutive SOCS3 mRNA (Fig. 4*E*) and protein (Fig. 4*F*) expression in degenerating retina (Fig. 4*D*) of insulin-deficient STZ-induced diabetic rats and by a recent report showing that loss of SOCS3-mediated feedback regulation converts STAT3 function from antiapoptotic to proapoptotic (47).

In this study, we also investigated possible roles of other SOCS members in insulin signaling. We found that SOCS5, SOCS6, and SOCS7 proteins are constitutively expressed in the retina, suggesting that they may have housekeeping functions in the retina (Fig. 2*C–E*). Surprisingly, depletion of endogenous SOCS6 in ARPE-16 cells inhibits insulin signaling, suggesting that constitutive SOCS6 expression may serve to maintain high basal insulin/AKT signaling in retina and improve glucose metabolism to meet the high metabolic demands of the retina. This is in line with recent findings (35) indicating that SOCS6 improves glucose metabolism by overcoming the inhibitory effects of p85 monomers on PI3K-dependent signaling pathways. The anatomic localization of SOCS6 expression in the inner retina with sparse vascularity, low oxygen tension, and exclusive dependence on glycolysis or in photoreceptors that are engaged in the high energy–requiring process of phototransduction is consistent with its role in glucose metabolism. Besides potentiation of AKT signaling, SOCS6 may also promote survival of retinal cells. In insulin-sensitive cells, progrowth/prosurvival functions of insulin necessitate inhibition of proapoptotic/growth inhibitory functions of forkhead transcription factors. We show here that insulin-induced inactivation of FOXO1 is inhibited in SOCS6-silenced ARPE-19 cells (Fig. 5*D*), suggesting that SOCS6 may be involved in regulating FOXO1 in retina.

Retinal dystrophies, including diabetic retinopathy, cause severe vision impairment or blindness, and development of effective treatment will benefit from better understanding of cellular pathways that inhibit neuronal cell death or repair of damaged neurons. Molecular cues that influence the decision to allow death of irreversibly damaged photoreceptors or provide survival signals that rescue partially damaged cells are unknown, and signals from neurotrophic factors that mediate reparative processes in retina are suspected to play important roles. Retinal neurotrophic factors include ciliary neurotrophic factor, leukemia inhibitory factor, and VEGF, and they all mediate their effects by activating Janus kinase/STAT pathways and are under negative-feedback regulation by SOCS proteins. It is therefore remarkable that SOCS3 is induced in retina in response to infection, light damage, oxidative stress, or insulin-deficient diabetes. Data presented thus suggest two-sided roles of SOCS proteins in retina. Whereas transient induction of SOCS3 may be a protective adaptive response to inflammation, light damage, hypoxia, or metabolic stress, persistently high SOCS3 levels in retina is potentially pathogenic because it would inhibit survival signals that emanate from neurotrophic factors. Nonetheless, of the SOCS proteins, SOCS3 might prove to be useful in inhibiting inflammation, VEGF, and angiogenesis, while targeted delivery of SOCS6 to retinal cells may improve glucose metabolism in diabetic subjects and confer protection against metabolic stress.

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