performed by measurement of the length from optic disc to the edge of retinal vessels. Both SJW and hypericin treatment had no effect on the length of vessels under these conditions (Figure 3D).

St. John's Wort and hypericin reduce extracellular signal-regulated kinase phosphorylation in retinas: Because ERK plays important roles in the angiogenic responses, including retinal neovascularization [14,23], we used western blot analysis to assess the phosphorylation of ERK at Thr-202/Tyr-204 in retinas in each experimental group. SJW treatment markedly suppressed ERK phosphorylation in ischemic retina compared with vehicle (Figure 4). Similarly, ERK phosphorylation was diminished by treatment with hypericin (Figure 4). Both SJW and hypericin treatment had no effect on total ERK protein levels.

DISCUSSION

Our data provide in vivo evidence that SJW and its active ingredient, hypericin, inhibit the development of ischemic retinal neovascularization in a mouse model of ischemic retinopathy without affecting physiologic vascularization. Furthermore, the effects correlated with the ability of both SJW and hypericin to inhibit ERK phosphorylation under ischemic conditions. These data suggest that both SJW and hypericin could attenuate pathological retinal neovascularization through its ability to suppress ERK activation.

It has been shown that hypericin suppresses several angiogenic responses of bovine endothelial cells including endothelial growth, migration, and differentiation into vascular-like structure [24]. Hypericin also reduces proliferation and tube formation in choroidal endothelial cells [25]. Hypericin is reported to inhibit fibroblast growth factor

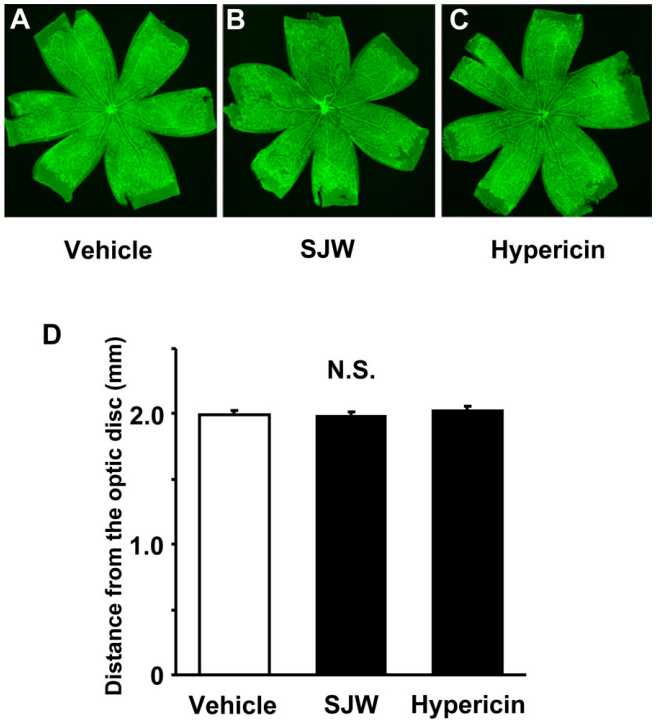

Figure 3. Effect of St. John's Wort and hypericin on physiologic vascularization in the retina. A-C: Shown are representative images of mouse retinas immunostained with isolectin B4 after treatment with 15 mg/kg/day St. John's Wort (SJW; n=18), 45 µg/kg/day hypericin (n=16), or vehicle (n=18) from P3 to P7. Magnification is X 5. D: Measurement of length of vasculature from the optic disc to the edge of the vascular front. Data are presented as mean ± SEM. N.S. means the data was not statistically significant.

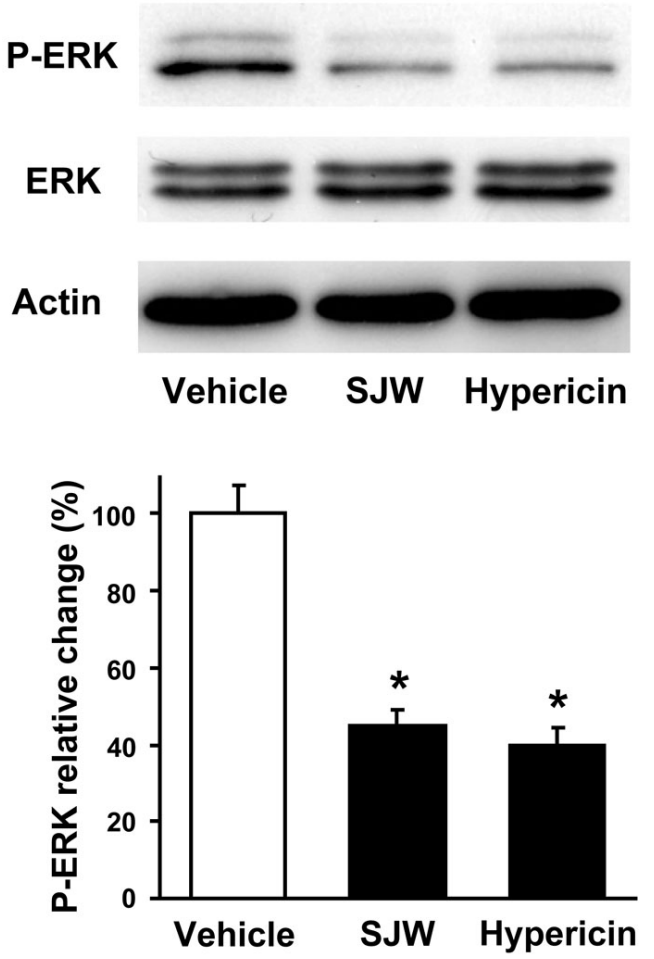

Figure 4. St. John's Wort and hypericin inhibit extracellular signal-regulated kinase activation in retina. Retinas were harvested from eyes of mice following gavage administration of vehicle, 15 mg/kg/day St. John's Wort (SJW) or 45 µg/kg/day hypericin from P12 through P17. Levels of phosphorylated ERK (P-ERK), total ERK, and actin were determined by western blot analysis. Representative blots from three experiments are shown. Phosphorylation levels of ERK were quantified by NIH image analysis. Immunoblots were normalized to total loaded protein. Data are presented as mean ± SEM. Asterisk (*) indicates $p<0.01$ versus vehicle treatment.

-2-induced angiogenesis in a rat corneal assay in vivo [26]. Consistent with the antiangiogenic effects of hypericin under nonischemic conditions, the present study indicates that ischemia-induced retinal neovascularization is effectively diminished after treatment with hypericin. Collectively, these data suggest that hypericin can act as a general repressor of angiogenesis under pathological conditions.

In the present study, a therapeutic dose of SJW significantly attenuated retinal neovascularization in vivo. Hypericin also inhibited retinal neovascularization in a dose-dependent manner. Hypericin at the lowest dose was equally or less effective at inhibiting retinal neovascularization than SJW. Because this dose of hypericin was approximately proportional to that present in the dose of SJW, hypericin may partly account for the antiangiogenic actions of SJW. SJW also includes another active constituent, hyperforin, which is reported to mimic the antidepressive effects of SJW [17]. It has been reported that hyperforin inhibits vessel growth in vivo determined by chorioallantoic membrane assay, and that it also suppresses angiogenic responses including proliferation and tube-like structure formation in cultured endothelial cells [27]. In addition, SJW contains several flavonoids, such as rutin and quercetin. These flavonoids may also inhibit angiogenesis in vivo and in vitro [28,29]. Thus, SJW has several active components showing antiangiogenic properties.

VEGF is critical for the determination of neovascularization in ischemic ocular diseases, including diabetic retinopathy [2,5,15], and inhibition of VEGF signaling results in reduction of retinal angiogenesis [6,14, 15,30,31]. VEGF stimulation leads to ERK activation in endothelial cells [7-9]. In this regard, hypericin has been shown to suppress ERK activation in both cancer and endothelial cell lines [18,26]. In line with these observations, our data show that hypericin suppressed ERK phosphorylation in a mouse model of OIR. Therefore, the inhibitory actions of hypericin on neovascularization may be mediated partly by its ability to suppress VEGF-stimulated signaling.

In summary, we show that both hypericin and SJW protect against pathological retinal neovascularization in mice with ischemic retinopathy. The beneficial effects of SJW and hypericin are associated with reduced ERK activation in retina. Thus, these agents could be potentially useful for treatment of ischemic ocular diseases.

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