The Role of the NFAT Signaling Pathway in Retinal Neovascularization

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Purpose. The purpose of the present study was to investigate the role of nuclear factor of activated T cells (NFAT), a transcription factor downstream of VEGF, in angiogenic cell behaviors of human retinal microvascular endothelial cells (HRMEC), and to assess the efficacy of NFAT signaling inhibitors in a rat model of oxygen-induced retinopathy (OIR).

Methods. Human retinal microvascular endothelial cells were treated with VEGF in the presence or absence of the NFAT inhibitor of NFAT-calcineurin association-6 (INCA-6), and NFAT translocation was evaluated using immunocytochemistry (ICC). Human retinal microvascular endothelial cells were treated with increasing doses of INCA-6, and cell proliferation and tube formation were assessed. Rats subjected to OIR were administered increasing doses of INCA-6 or the CN inhibitor FK-506, and the retinal neovascular area was measured.

Results. Nuclear factor of activated T-cells c1 was translocated to the nucleus of HRMEC treated with VEGF, and INCA-6 treatment blocked translocation. Inhibitor of NFAT-calcineurin association-6inhibited HRMEC proliferation and tube formation in a dose-dependent manner. Both INCA-6 and FK-506 treatment significantly reduced pathologic neovascularization in OIR.

Conclusions. This investigation has demonstrated that in HRMEC, NFATc1 is activated downstream of VEGF signaling and NFAT signaling plays a key role in angiogenic cell behaviors. In addition, NFAT inhibition is shown to be highly efficacious in an OIR model. These findings indicate that the NFAT signaling pathway may serve as a suitable therapeutic target for the treatment of neovascular eye disease.

Keywords: NFAT, retinal microvascular endothelial cells, oxygen-induced retinopathy, transcription factors, neovascularization

Angiogenesis is the formation of new capillaries from existing blood vessels.[1] Under normal physiologic conditions, angiogenesis is tightly controlled and plays important roles in reproduction, development, growth, and healing.[2,3] However, persistent angiogenesis is also involved in numerous pathologic processes such as tumor growth and retinopathies.[3,4] In the pathologic context, cells respond to angiogenic stimuli and eventually establish microvascular networks that either lack patency or display hyperpermeability.[5] This pathologic angiogenesis is referred to as neovascularization (NV), a common feature of retinopathy of prematurity (ROP), diabetic retinopathy (DR), and AMD, the leading causes of blindness in infants, working age individuals, and the elderly, respectively.[6,7] Retinal NV represents a common outcome from multifaceted and disparate processes, and typically occurs at a relatively late stage in the pathology of these disorders resulting in irreversible vision loss, and as such it remains the primary target for current therapeutic intervention.

In the retina, NV typically arises due to ischemia-induced hypoxia that results in the upregulated production of proangiogenic growth factors. Of the growth factors associated with retinal angiogenesis, VEGF, is believed to be the critical regulator, and has been under intense investigation in the ophthalmic field since its identification.[8] Vascular endothelial growth factor is a 46 kDa homodimeric glycoprotein associated with angiogenic endothelial cell behaviors such as survival, proliferation, migration, and vasopermeability.[9]

One signaling pathway known to act downstream of VEGF is regulated by the nuclear factor of activated T-cell (NFAT) family of transcription factors.[10–12] The NFAT family is a set of five proteins grouped for their similarity to the Rel Homology Domain (RHD)/nuclear factor kappa B (NFκB) family of transcription factors.[13] Within this family, NFATc refers to the four isoforms (NFATc1, NFATc2, NFATc3, and NFATc4) regulated by intracellular Ca^{2+} through the serine phosphatase calcineurin (CN).[14] Calcineurin regulation of NFATc is controlled through its binding to a conserved Ca^{2+}/CN-dependent translocation (CAT) regulatory domain, consisting of a 300 amino acid region located at the N-terminus of the DNA binding domain, and encoded by a single exon in all four proteins. When static, the NFAT regulatory domain is heavily phosphorylated, but when CN is activated it dephosphorylates the domain revealing a nuclear localization sequence, and NFAT translocates to the nucleus where it begins to accumulate,

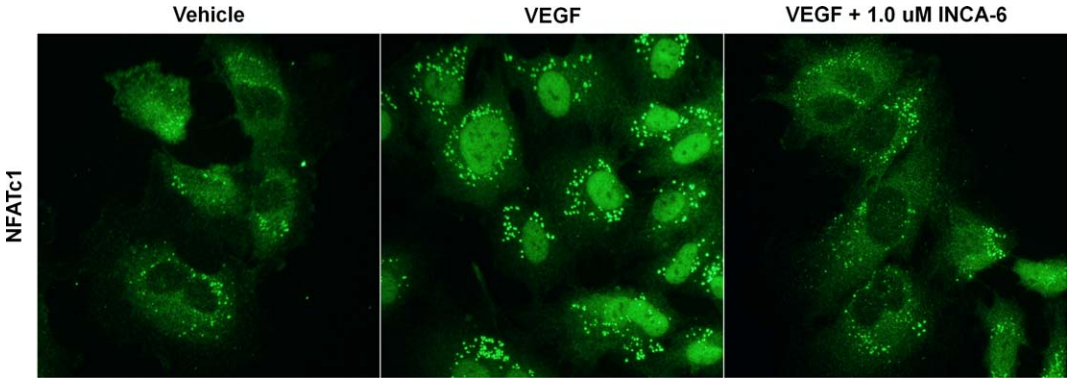

FIGURE 1. Vascular endothelial growth factor stimulates NFATc1 nuclear translocation in HRMEC. Vascular endothelial growth factor treatment (25 ng/mL) resulted in nuclear translocation of NFATc1 after 30 minutes, and this was effectively inhibited by treatment with INCA-6 (1.0 μM).

demonstrating an increased affinity for its target DNA sites.[15,16] Efficient dephosphorylation of NFAT has been shown to require a direct docking interaction with CN, and the protein is quickly rephosphorylated by NFAT kinases in the nucleus when CN is blocked, emphasizing its dependence and sensitivity to CN activity and Ca^{2+} dynamics.[17,18] Due to the tight association between CN and NFAT activity, the compound inhibitor of NFAT-calcineurin association-6 (INCA-6) is commonly used in experimental conditions to inhibit NFAT signaling.[19-21] Inhibitor of NFAT-calcineurin association-6 is a small organic molecule (9,10-dihydro-9,10[1′,2′]-benzenoanthracene-1,4-dione) that competitively binds to the discrete NFAT binding site of CN, blocking NFAT dephosphorylation without altering other CN phosphatase activity.[22,23]

Various groups have determined a direct role for NFAT transcriptional activity in angiogenic gene expression, angiogenic cell behaviors, and the angiogenic component of tumor growth.[12,24–28] However, it remains to be determined whether NFAT plays a similar role in ocular NV. In this study, immunocytochemistry was used to verify that VEGF stimulates NFAT nuclear translocation in primary human retinal microvascular endothelial cells (HRMEC). Secondly, the effect of NFAT antagonism on VEGF-induced endothelial cell proliferation and tube formation in HRMEC was investigated. Lastly, to investigate the therapeutic potential of NFAT antagonism for the treatment of ocular NV, a rat model of oxygen-induced retinopathy (OIR) was used to assess the efficacy of NFAT inhibition. These studies will help define the role of NFAT signaling in mediating pathologic ocular angiogenesis.

METHODS

HRMEC Culture

Primary HRMEC (Cell Systems, Kirkland, WA) were cultured in flasks coated with attachment factor (Cell Signaling, Danvers, MA). Growth medium consisted of endothelial basal medium (EBM; Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), and endothelial cell growth supplements (EGM SingleQuots; Lonza). All cultures were incubated at 37°C, in 5% CO_2, and 95% relative humidity. Passages 5 to 8 were used for these experiments.

Immunocytochemistry

Human retinal microvascular endothelial cells were cultured on multi-well glass slides to near confluence, before being serum starved (serum-free EBM) for 12 hours. Cells were then treated with 25 ng/mL VEGF (Millipore, Billerica, MA) in the presence or absence of the inhibitor INCA-6 (R&D Systems, Minneapolis, MN) at 1.0 μM. Thirty minutes after treatment, cells were fixed with 4% paraformaldehyde and 1% Triton X-100 in PBS. Wells were then incubated with primary antibody (NFATc1: sc-13033; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After incubation, wells were washed and incubated with secondary antibody. Samples were viewed and imaged with a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss Microscopy LLC, Thornwood, NY).

HRMEC Proliferation

Human retinal microvascular endothelial cells were seeded at 3×10^3 cells/well in a 96-well plate containing standard growth medium for 8 hours to allow them to settle and attach. Cells were then serum-starved for 12 hours before being treated with either 10% serum or VEGF (25 ng/mL in medium containing 0.5% serum) in the presence or absence of the inhibitor INCA-6 (0.5, 1.0, or 2.5 μM). After 24 hours of treatment, cells were labeled with BrdU labeling solution for an additional 12 hours, and BrdU incorporation was quantified using a colorimetric BrdU ELISA (Roche, Indianapolis, IN), according to the manufacturer's instructions. Absorbance values were normalized to the 0.5% serum control for each experiment.

HRMEC Tube Formation

Twenty-four–well tissue culture plates were coated with 350 μL of growth factor-reduced Matrigel (Becton Dickenson, Franklin Lakes, NJ). Human retinal microvascular endothelial cells were seeded at 2.5×10^4 cells/well and treated with 10% serum in the presence or absence of INCA-6 (1.0 or 2.5 μM). Tubes were imaged 12 hours after treatment, using a Nikon Eclipse Ti microscope (Nikon, Melville, NY) and Nikon DS-Fi1 camera at 2× magnification. ImageJ software (National Institutes of Health, Bethesda, MD) was used to determine the mean tube length per unit area, and these values were normalized to a 0.5% serum control for each experiment.

Oxygen-Induced Retinopathy

All animal procedures used in this study were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Within 8 hours after birth, litters of Sprague-Dawley

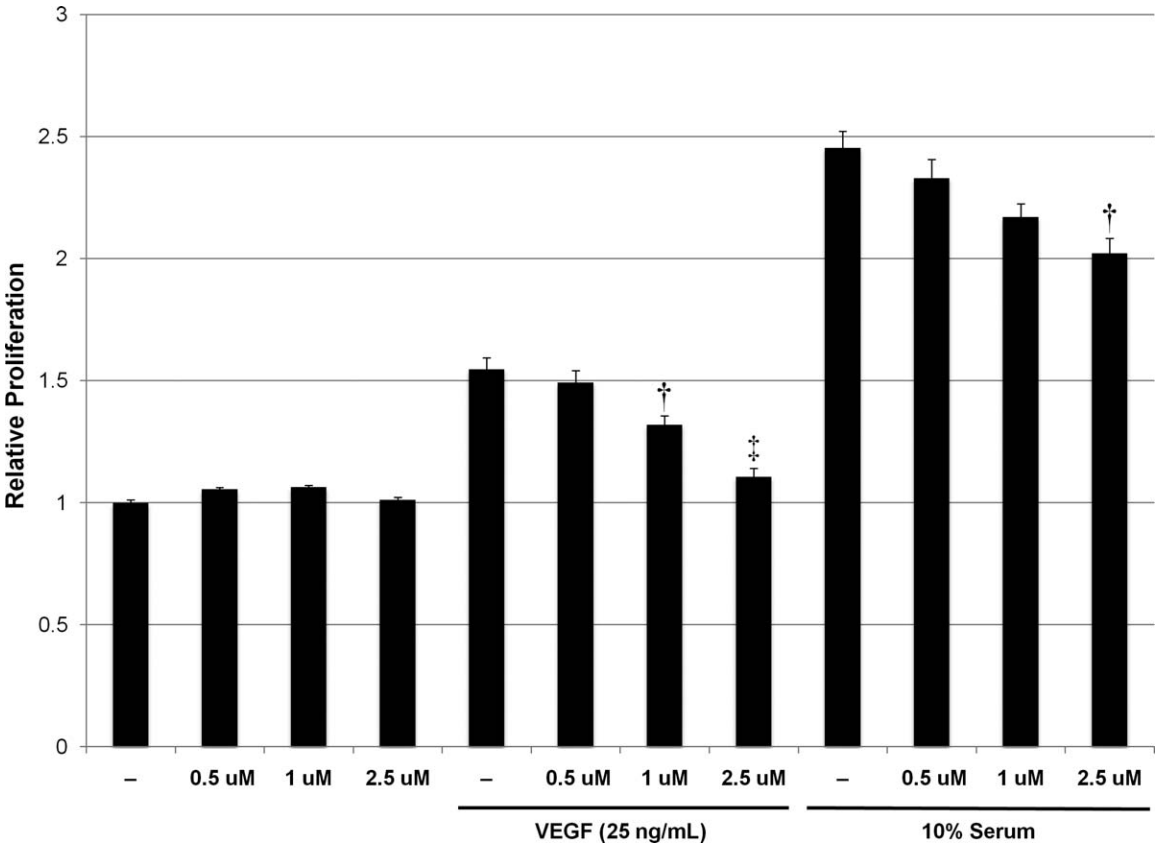

FIGURE 2. The effect of NFAT inhibitor INCA-6 on HRMEC proliferation. Human retinal microvascular endothelial cell proliferation was stimulated with either VEGF (25 ng/mL on a 0.5% serum background) or 10% serum. Inhibitor of NFAT-calcineurin association-6 treatment significantly decreased both VEGF and serum-induced HRMEC proliferation, but did not affect baseline proliferation. Each *bar* represents the mean ± SEM ($n = 21$). ‡$P < 0.001$. †$P < 0.01$.

rat pups and their mothers (Charles Rivers Laboratories, Wilmington, MA) were transferred to oxygen exposure chambers in which they were subjected to alternating 24 hour periods of 50% and 10% oxygen for 14 days.[29] On postnatal day 14 (14[0]), the oxygen-exposed rats were removed to room air.

Intravitreal Injections

Rats were anesthetized by isoflurane (Butler Animal Health Supply, Dublin, OH) inhalation and a drop of 0.5% proparacaine (Allergan, Hormigueros, Puerto Rico) was topically applied to the cornea before intravitreal injection. The globe was penetrated approximately 0.5-mm posterior to the ora ciliaris, using a 30-gauge needle with a 19° bevel and 10-μL syringe (Hamilton Co., Reno, NV). The needle was advanced to the posterior vitreous at a steep angle to avoid contact with the lens. The injection bolus (5 μL) was delivered near the trunk of the hyaloid artery proximal to the posterior pole of the retina.[30–32] After injection, a topical antibiotic suspension (Vigamox; Alcon Laboratories, Fort Worth, TX) was applied. Noninjected eyes were also treated with topical proparacaine and antibiotic to control for the potential of these agents to influence retinal vessel growth. Subsets of oxygen-exposed rats were administered vehicle (0.1% Dimethyl sulfoxide [DMSO] in PBS), INCA-6 (2.5, 5.0, or 25.0 μM), or FK-506 (2.5, 5.0, or 25.0 μM; R&D Systems) by intravitreal injection on days 14(0) and 14(3). Noninjected animals were used as controls. Age-matched room air pups, also received two injections of either vehicle, INCA-6 (25.0 μM), or FK-506 (25.0 μM) on days 14(0) and 14(3).

Quantification of Retinal Neovascularization

On day 14(6), all rats were killed and their retinas dissected. After dissection, the retinal vasculature was stained for adenosine diphosphatase (ADPase) activity, according to well-established procedures.[29,30] Images of ADPase-stained retinas were digitized, and preretinal vessel tufts were measured by computer-assisted image analysis. Data are reported in square millimeters. The data shown is normalized to NV values from vehicle-treated eyes of each experimental group.

Statistical Analyses

Data were analyzed with commercial software (JMP; SAS Institute, Cary, NC) using ANOVA and Dunnet's post hoc analyses with probability less than or equal to 0.05 considered statistically significant.

RESULTS

VEGF Induction of NFATc1 Nuclear Translocation in HRMEC

To determine whether VEGF induced NFAT translocation occurs in HRMEC, cells were cultured on chamber slides and

treated with 25 ng/mL of VEGF for 30 minutes before being fixed and stained for NFAT isoforms c1-4. While all four calcineurin-dependent NFAT isoforms displayed immunoreactivity in HRMEC (data not shown), only NFATc1 exhibited clear translocation to the nucleus in response to VEGF (Fig. 1). Vascular endothelial growth factor stimulation was effectively blocked using the NFAT inhibitor INCA-6 at 1.0 μM.

The Effect of NFAT Inhibition on VEGF-Induced HRMEC Proliferation

In order to investigate the contribution of NFAT signaling to VEGF-induced HRMEC proliferation, HRMEC were treated with VEGF and increasing concentrations (0.5, 1.0, 2.5 μM) of the NFAT inhibitor INCA-6 (Fig. 2). Inhibitor of NFAT-calcineurin association-6 significantly inhibited VEGF-induced proliferation at 1.0 ($P < 0.003$) and 2.5 μM ($P < 0.001$) concentrations. Proliferation was also measured under serum-stimulated conditions, where INCA-6 inhibition was also observed at the 2.5 μM ($P < 0.003$) concentration. Notably INCA-6 treatment did not alter baseline proliferation at any of the tested concentrations in the absence of VEGF or serum stimulation.

The Effect of NFAT Inhibition on HRMEC Tube Formation

To investigate the contribution of NFAT signaling in HRMEC tube formation, HRMEC in growth medium with 10% serum were treated with increasing concentrations of the NFAT inhibitor INCA-6 (Fig. 3). Inhibitor of NFAT-calcineurin association-6 significantly inhibited tube formation at 1.0 ($P < 0.001$) and 2.5 μM ($P < 0.0001$) concentrations.

The Effect of NFAT Inhibition on OIR Severity

Figures 2 and 3 demonstrate that NFAT inhibition influences the HRMEC response to VEGF and serum stimulation in in vitro models of angiogenesis. Accordingly, the efficacy of the NFAT inhibitor INCA-6 was tested in the rat model of OIR. Nuclear factor of activated T-cells inhibition by INCA-6 decreased the severity of OIR in a dose dependent manner (Fig. 4). Significant inhibition was seen at 5.0 and 25.0 μM concentrations ($P < 0.03$). The CN inhibitor FK-506 was also tested in the OIR model, and a dose-dependent effect of efficacy was again observed, with significant inhibition seen at 5.0 ($P < 0.05$) and 25.0 μM concentrations ($P < 0.02$). To determine whether treatment with these inhibitors had an effect on normal vascular development, total vascular area of treated retinas was also assessed and no significant changes were observed in any of the treatment groups (Supplementary Fig. S1). Additionally, potential toxicity of INCA-6 and FK-506 treatment was assessed by identical treatment of room air animals with 25.0 μM concentrations of both inhibitors, and no effect was seen on the rate or architecture of early physiologic retinal vascular development (Supplementary Fig. S2).

Discussion

The NFAT family of transcription factors is composed of four unique isoforms, regulated through interaction with the intracellular calcium signaling phosphatase, CN. These isoforms are differentially expressed depending on tissue and cell type, and they mediate diverse physiologic and pathophysiologic processes. Nuclear factor of activated T-cells signaling is known to play key roles in the inflammatory response,[33–35] cardiac development,[26,28,36] angiogenesis,[35,37] cancer metastasis,[24,38,39] and bone homeostasis.[40] Here we demonstrate that NFAT signaling plays a critical role both in in vitro experiments that model retinal angiogenic cell behavior and in an in vivo model of retinal angiogenesis. To our knowledge, this is the first study to examine and demonstrate a role for NFAT signaling in retinal angiogenesis.

Nuclear factor of activated T-cells activation occurs as a result of CN binding and dephosphorylation of a nuclear localization sequence leading to nuclear translocation of the activated isoform. This process is triggered by cellular calcium influx, and, thus, can be stimulated by treatment with ionophores, such as ionomycin. Vascular endothelial growth factor is another activator of the CN/NFAT signaling pathway through VEGF receptor activation of phospholipase C that results in increased intracellular calcium,[41,42] and has been shown to stimulate NFAT translocation in various cellular contexts. Notably, VEGF stimulates NFATc1 translocation in human pulmonary valve endothelial cells,[11] and NFATc2 translocation in human umbilical vein and intestinal microvascular endothelial cells.[10,43] Given the prominent role of VEGF in ocular NV we tested its effect on HRMEC, noting that while all four CN-dependent isoforms are present in HRMEC, only NFATc1 was translocated to the nucleus as a result of VEGF treatment (Fig. 1). This VEGF-induced NFATc1 translocation was abolished by the NFAT-specific inhibitor INCA-6, which prevents CN binding and subsequent dephosphorylation. Furthermore, we demonstrated that INCA-6 treatment inhibits both VEGF- and serum-induced cell proliferation, as well as serum-induced tube formation (Figs. 2, 3) at concentrations consistent with those reported in the literature.[19–21]

Notably, serum includes a number of proproliferative factors in addition to VEGF, such as epidermal growth factor (EGF), FGF, and insulin-like growth factor 1 (IGF-1). Under the conditions used in our HRMEC proliferation experiments, serum stimulation induced a greater proliferative response than VEGF stimulation. Moreover, our optimal INCA-6 treatment completely inhibited VEGF-induced HRMEC proliferation, but it was less effective in inhibiting serum-induced proliferation. One explanation for these findings is that the inhibition of serum-stimulated proliferation by INCA-6 was largely or wholly due to inhibition of the contribution of VEGF. Together, these findings indicate that NFAT is not simply a global inhibitor of cell proliferation and further suggest that INCA-6 was not cytotoxic at the concentrations used. We conclude that NFAT signaling mediates angiogenic cell behaviors in a cell type known to contribute to pathologic ocular NV and in response to a stimulus known to contribute to ocular NV.

As an initial proof of principal that targeting NFAT signaling has therapeutic potential, we tested INCA-6 in a rat model of OIR and showed that the inhibitor significantly reduced the severity of NV (Fig. 4). Given this finding, we also tested the efficacy of the CN inhibitor FK-506. This compound binds the immunophilin FKBP12 to form a complex that binds CN and prevents its activation and phosphatase activity.[44–46] Thus, FK-506 inhibits the NFAT signaling pathway slightly upstream from INCA-6, and our finding that it caused a similar reduction in NV further confirms the role of NFAT in this important pathologic process. Moreover, FK-506 (also known as Tacrolimus and marketed as Prograf and Advagraf) is approved for clinical use, both as an immunosuppressant to prevent organ rejection in organ and tissue transplant patients,[47,48] and as a therapy for patients with severe refractory uveitis.[49,50] This feature could facilitate future translation to clinical use targeting retinal angiogenesis, but currently serves to highlight the efficacy and utility of targeting this pathway therapeutically. The 70% inhibition of NV exhibited by FK-506/Tacrolimus is impressive in comparison to other pharmacologic inhibitors evaluated in over two decades of testing in our lab.

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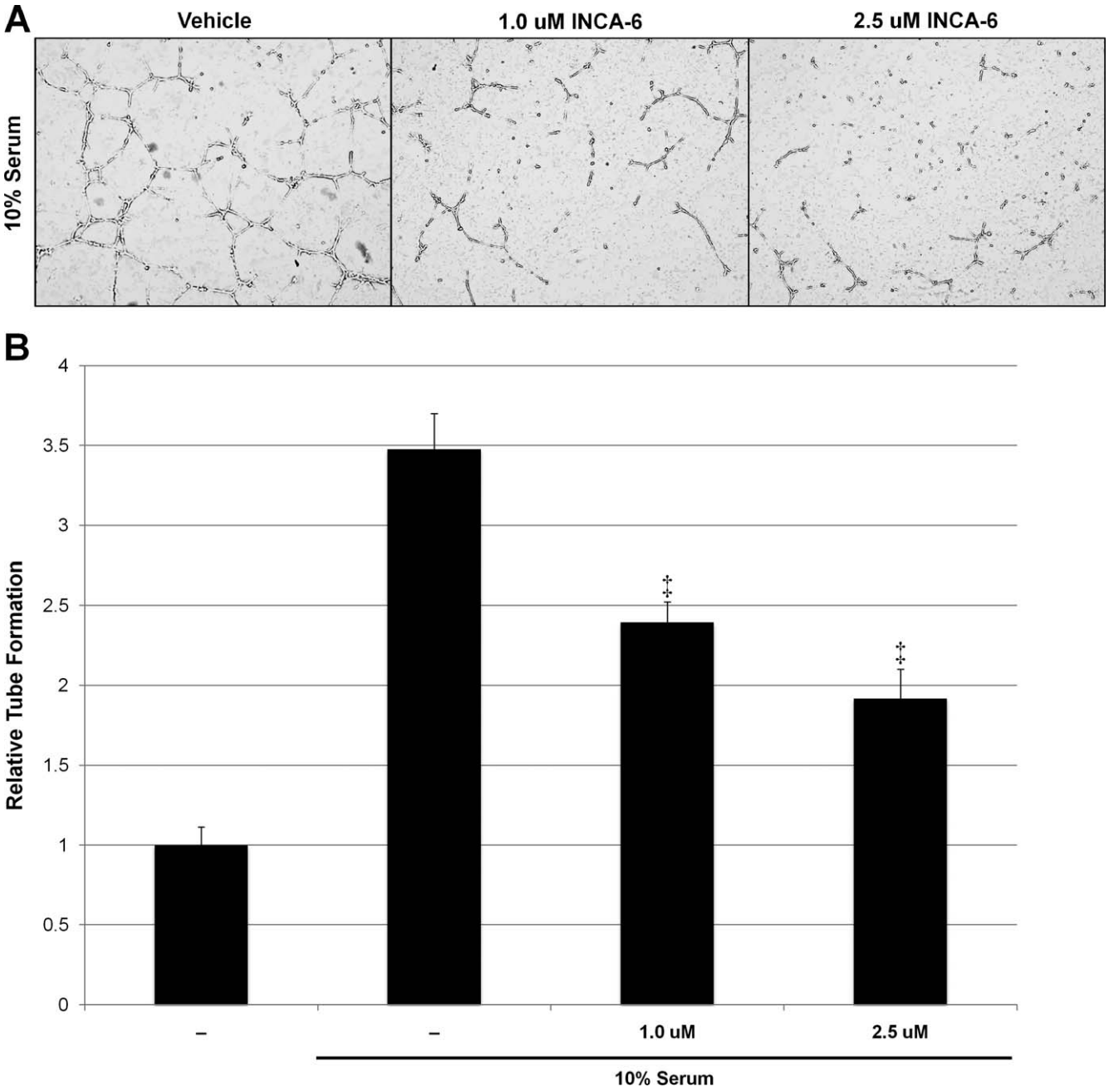

FIGURE 3. The effect of NFAT inhibitor INCA-6 on HRMEC tube formation. Human retinal microvascular endothelial cell tube formation was stimulated with 10% serum. Inhibitor of NFAT-calcineurin association-6 treatment significantly decreased tube formation. (**A**) Contrast enhanced representative images from treated wells. (**B**) Quantification of tube measurements. Each *bar* represents the mean ± SEM ($n = 9$). ‡$P < 0.001$.

Interestingly, the efficacy observed using either inhibitor in our in vivo model was greater than what might be predicted based on inhibition of HRMEC angiogenic behaviors in our in vitro experiments. One explanation might be that NFAT inhibition affects another, unrelated aspect of NV. Indeed, preliminary studies in our lab (Bretz CA, et al. *IOVS* 2011;52:ARVO E-Abstract 3592), and in the context of corneal NV,[51] suggest a potential role for NFAT signaling in the regulation of retinal VEGF expression. To test whether our CN/NFAT inhibitors achieved significant efficacy in part by inhibiting VEGF expression, we measured in vivo retinal VEGF protein levels after INCA-6 treatment (Supplementary Fig. S3) as well as the effect of INCA-6 on hypoxia-induced Müller cell VEGF production in vitro (Supplementary Fig. S4) and found no effect. This suggests that the efficacy observed in vivo is largely due to inhibition of VEGF-stimulated NFAT signaling in HRMEC, though there could be additional, as yet unidentified, mechanisms that also contribute. While the downstream targets of NFAT signaling are beyond the scope of this study, there are a number of potential targets that we are currently investigating. For example, cyclooxegenase-2 (COX-2), hypoxia inducible factor alpha (HIF1a), VEGFR1, tissue factor, matrix metalloproteinase (MMP) 2, and MMP-9 are all known products of NFAT gene transcription, and all have defined roles in retinal angiogenesis.[10,52–55]

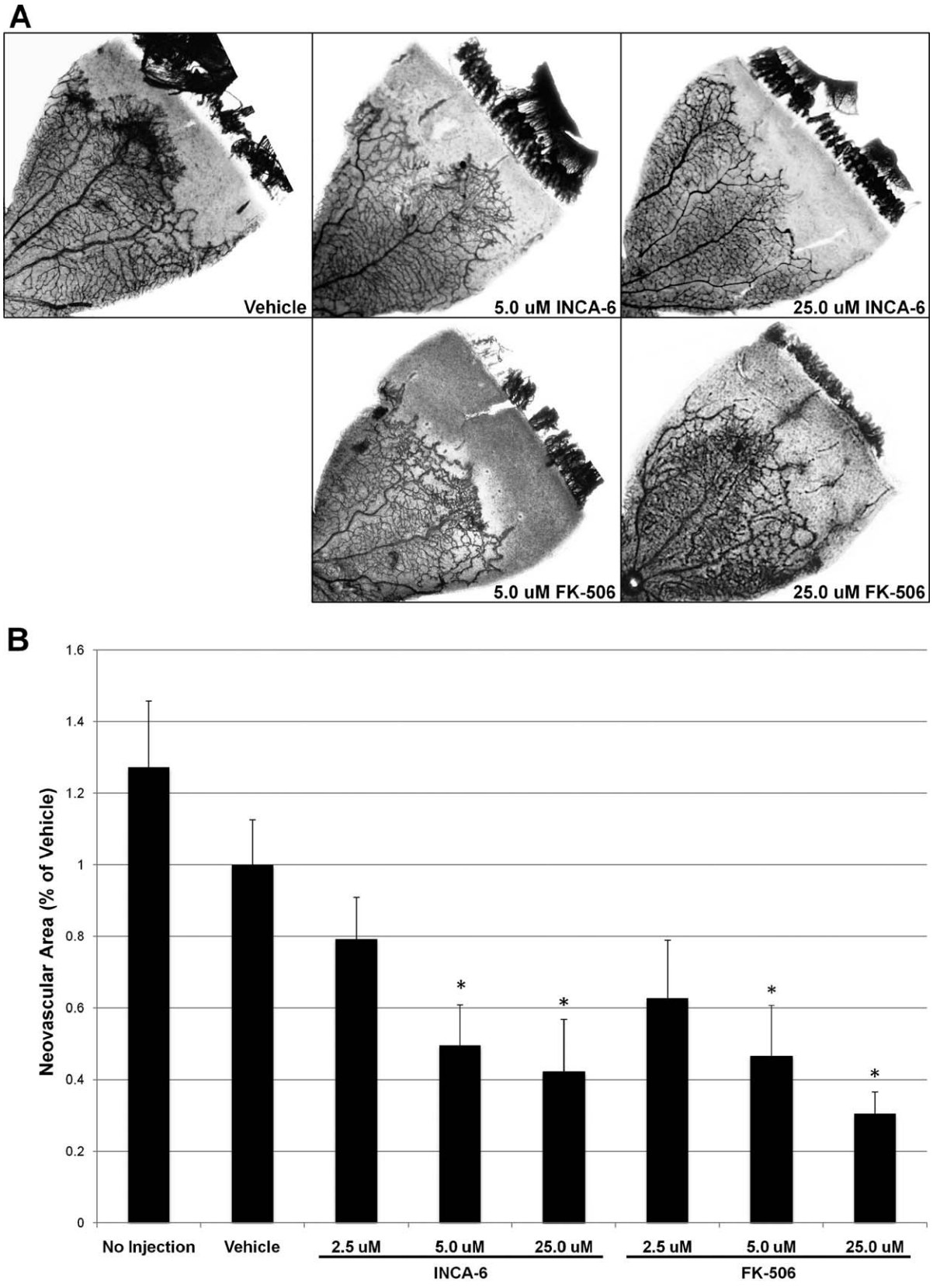

FIGURE 4. The effect of inhibitors INCA-6 and FK-506 on the severity of OIR in the rat. Inhibitor of NFAT-calcineurin association-6 and FK-506 significantly inhibited the severity of OIR in a dose-dependent manner. (**A**) Representative quadrants from treatment groups. (**B**) Quantification of neovascular area, relative to the vehicle-injected control. Each *bar* represents the mean ± SEM (for no injection, $n = 16$; for vehicle $n = 23$; for INCA-6 2.5 μM, $n = 12$; for INCA-6 5.0 μM, $n = 16$; for INCA-6 25.0 μM, $n = 11$; and for FK-506 2.5, 5.0, and 25.0 μM, $n = 7$). $^*P < 0.05$.

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We have identified the presence of a VEGF-activated NFAT isoform in HRMEC and showed a clear effect of NFAT inhibition in both in vitro angiogenic cell behaviors and an in vivo model of ocular NV. These findings suggest that NFAT signaling exerts a pro-angiogenic influence in HRMEC downstream of VEGF and indicate that NFAT inhibition may present a rational therapeutic target for ocular diseases in which angiogenesis plays an important pathologic role.

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