Insulin-Like Growth Factor Binding Protein-3 Mediates Vascular Repair by Enhancing Nitric Oxide Generation

Jennifer L. Kielczewski,* Yagna P.R. Jarajapu,* Evan L. McFarland,* Jun Cai, Aqeela Afzal, Sergio Li Calzi, Kyung Hee Chang, Todd Lydic, Lynn C. Shaw, Julia Busik, Jeffrey Hughes, Arturo J. Cardounel, Kenneth Wilson, Timothy J. Lyons, Michael E. Boulton, Robert N. Mames, Tailoi Chan-Ling,† Maria B. Grant†

Rationale: Insulin-like growth factor binding protein (IGFBP)-3 modulates vascular development by regulating endothelial progenitor cell (EPC) behavior, specifically stimulating EPC cell migration. This study was undertaken to investigate the mechanism of IGFBP-3 effects on EPC function and how IGFBP-3 mediates cytoprotection following vascular injury.

Objective: To examine the mechanism of IGFBP-3–mediated repair following vascular injury.

Methods and Results: We used 2 complementary vascular injury models: laser occlusion of retinal vessels in adult green fluorescent protein (GFP) chimeric mice and oxygen-induced retinopathy in mouse pups. Intravitreal injection of IGFBP-3–expressing plasmid into lasered GFP chimeric mice stimulated homing of EPCs, whereas reversing ischemia induced increases in macrophage infiltration. IGFBP-3 also reduced the retinal ceramide/sphingomyelin ratio that was increased following laser injury. In the OIR model, IGFBP-3 prevented cell death of resident vascular endothelial cells and EPCs, while simultaneously increasing astrocytic ensheathment of vessels. For EPCs to orchestrate repair, these cells must migrate into ischemic tissue. This migratory ability is mediated, in part, by endogenous NO generation. Thus, we asked whether the migratory effects of IGFBP-3 were attributable to stimulation of NO generation. IGFBP-3 increased endothelial NO synthase expression in human EPCs leading to NO generation. IGFBP-3 exposure also led to the redistribution of vasodilator-stimulated phosphoprotein, an NO regulated protein critical for cell migration. IGFBP-3–mediated NO generation required high-density lipoprotein receptor activation and stimulation of phosphatidylinositol 3-kinase/Akt pathway.

Conclusion: These studies support consideration of IGFBP-3 as a novel agent to restore the function of injured vasculature and restore NO generation. (Circ Res. 2009;105:897-905.)

Key Words: hematopoietic stem cells ■ nitric oxide ■ insulin-like growth factor binding protein-3 ■ vascular repair

Ins ischemic tissues, by definition, require improved or greater oxygen delivery. Most pharmacological therapies of ischemic retinopathies try to inhibit pathological neovascularization but do not correct the underlying lack of blood supply. A more judicious approach would be to develop a pharmacological intervention to repair or create more normal vessels, thus converting ischemic tissue to nonischemic tissue. The insulin-like growth factor (IGF) system has been implicated in vascular physiology, including in the retina, because unregulated levels of IGF-1 can lead to inadequate vascularization, as well as pathological ocular neovascularization.1–6

The effects of IGF-1 are mediated by the IGF-1 receptor (IGF-1R) and modulated by complex interactions with six different IGF binding proteins (IGFBPs), which function not only as transporter proteins and storage pools for IGF-1 within tissues but also as signaling molecules.7,8 IGFBP-3 has IGF-1–independent effects and autocrine and paracrine actions affecting cell mobility and survival.9,10 Moreover, IGFBP-3 levels are increased by hypoxia.5

Recently, we showed IGFBP-3 could serve as a modulator of vascular development by potently regulating bone marrow–derived cell (BMDC) function, specifically, stimulating
endothelial precursors to differentiate into endothelial cells and promoting their migration. Studies using the oxygen-induced retinopathy (OIR) model showed that IGFBP-3 prevented oxygen-induced vasoobliteration and vascular regression in vivo (during phase 1 of the model) and subsequently mitigated preretinal neovascularization (phase 2 of the model). Lotqvist et al showed that IGFBP-3–deficient mice undergoing the OIR model have increased retinal vessel loss compared to wild type controls. They also showed that premature infants with retinopathy of prematurity had lower levels of IGFBP-3 than infants with no retinopathy of prematurity, thus concluding that increased serum IGFBP-3 improved clinical outcome. These studies collectively support the role of IGFBP-3 as a critical regulator of vascular development.

In this study, we examined possible mechanism(s) by which IGFBP-3 modulated endothelial progenitor cell (EPC) behavior and mediated cytoprotection following vascular injury. For these studies, we used both in vitro systems and complementary in vivo vascular injury models: laser occlusion of retinal vessels in adult green fluorescent protein (GFP) chimeric mice and OIR in mouse pups. These 2 models were selected to examine the effects of IGFBP-3 on 2 distinct types of vasculature. The adult laser model represented the effects of IGFBP-3 on a stable vascular bed and the hyperoxia induced retinal injury model represented the effects of IGFBP-3 on an immature, unstable vascular bed, which was still undergoing active endothelial cell proliferation and migration.

**Methods**

**Bone Marrow Transplantation Procedure**

The care and use of animals is stated in the Online Data Supplemental material. The generation of the C57BL/6J.gfp chimeric mouse was previously described.

**Laser Injury to Vessels to Induce Experimental Neovascularization**

Two groups of C57BL/6J.gfp chimeric mice: laser only (n=15) and laser with IGFBP-3 injection (n=18) were subjected to laser injury in their right eye as previously described. A third group of chimeric mice received only IGFBP-3 injection into their right eye (n=16). The IGFBP-3 plasmid, under control of a proliferating endothelial cell–specific promoter, was packaged into liposomes as previously described. Immediately following laser treatment to the right eye, a total of 2 μL of IGFBP-3 plasmid (2 μg/μL) packaged in liposomes was delivered intravitreally into the right eye of the appropriate groups.

**OIR Model Studies**

We used the OIR model developed by Smith et al. Mice were intravitreally injected with 0.5 μL (2 μg/μL) into one eye with either the IGFBP-3 plasmid or the cloning vector on postnatal day (P) 11. Some mice were euthanized on removal from hyperoxia at P12, whereas others were euthanized at P17. At both time points, eyes from mice injected with the plasmid expressing IGFBP-3 (n=6) were compared to eyes from mice injected with empty cloning vector (n=6) or the noninjected eye of the same animal (n=6). Mice were euthanized, and eyes were enucleated and fixed for analysis as previously reported. Details of immunohistochemistry procedures are given in the expanded Methods section in the Online Data Supplement, available at http://circres.ahajournals.org.

**Lipid Analysis by Nanoelectrospray Ionization/Tandem Mass Spectrometry**

Lipid species were detected using lipid class–specific precursor ion and neutral loss tandem mass spectrometry (MS/MS) scans as previously described and fatty acid constituents of individual lipid species were verified by product ion mode MS/MS in negative ion mode.

Refer to the supplemental methods section for details of the following studies: determination of NO production by DAF-FM fluorescence imaging, endothelial NO synthase (eNOS) activity, and in vitro functional studies in rat mesenteric and cerebral arteries.

**Results**

**IGFBP-3 Promotes Retinal Vascular Repair by Increasing BMDC Homing Following Mechanical Disruption by Laser in GFP Chimeric Mice**

In GFP chimeric mice receiving intravitreal injection of IGFBP-3 plasmid, GFP+ cells robustly participated in vascular remodeling (Figure 1A through 1C), with GFP+ cells differentiating into endothelial cells (Online Figure I, A through I). Greater GFP+ cell incorporation was clearly evident in IGFBP-3 plasmid–injected eyes than in noninjected control eyes (Figure 1A through 1C compared with Figure 1D through 1F). When the vasculature of GFP chimeric mice was injured by laser, significant numbers of circulating GFP+ cells took on the appearance of activated macrophages (Online Figure II). In the animals receiving both laser and IGFBP-3 plasmid (Online Figure II, D through F), the distribution of GFP+ cells exhibited a similar vascular pattern, as seen with IGFBP-3 alone (Online Figure II, A through C), but the number of activated macrophages was greatly reduced compared to laser alone treated mice. We also confirmed by mRNA expression that intravascular injection of the IGFBP-3–expressing plasmid resulted in high IGFBP-3 mRNA levels in laser-treated mice for up to 1 week post laser injury (Online Figure III).
IGFBP-3 Reduces Inflammation Following Laser Occlusion by Reducing the Retinal Ceramide/Sphingomyelin Ratio

Sphingolipids are lipid components of the membrane known to be highly expressed in the retina. The most abundant sphingolipid in the membrane, sphingomyelin, can be converted to ceramide under certain conditions, such as inflammation, and the ceramide/sphingomyelin ratio can be used as an indicator of the inflammatory/proapoptotic state. Laser treatment induced a 3.34-fold increase in ceramide/sphingomyelin ratio, consistent with proinflammatory and proapoptotic effects. IGFBP-3 returned ceramide/sphingomyelin ratio to control (untreated) levels (Figure 2). We found no difference in ceramide/sphingomyelin levels in the IGFBP-3 only–injected animals (Online Figure IV, B) compared to controls (Online Figure IV, A). We analyzed another member of the sphingolipid family, sphingosine-1-phosphate (S1P). The levels of retinal S1P were low in all groups, and there was no difference between the groups, suggesting that any elevation of S1P following injury had returned to baseline levels by three weeks (data not shown).

IGFBP-3 Reversed Lipid Profile Changes Induced by Laser Occlusion

Because lipid peroxidation and lipid metabolism changes are highly associated with a number of retinal pathologies, we next addressed the effect of laser treatment on the structural component of retinal membranes, specifically membrane phospholipids. Laser treatment induced a significant increase in total retinal incorporation of docosahexaenoic acid (DHA) (22:6n3) into glycerophosphatidylcholine and glycerophosphatidylinositol. All glycerophosphatidylcholine and glycerophosphatidylinositol changes were completely reversed by IGFBP-3 treatment (Online Figures V and VI, respectively).

IGFBP-3 Reduces Endothelial Cell Death Following Hyperoxia-Induced Injury

We next used the OIR model, which exhibits extensive endothelial cell loss by apoptosis, to evaluate whether IGFBP-3 could mediate cytoprotection following injury by reducing cell death. We injected IGFBP-3 plasmid into the vitreous of mouse pups (P1) and then placed pups in hyperoxia on P7 for 5 days following the standard OIR model. The retinas of mice injected with IGFBP-3 had significantly reduced endothelial cell death (Figure 3D through 3F) in both the hyperoxic (mid-peripheral, \( P < 0.05 \); peripheral, \( P < 0.05 \)) and hypoxic phases (phase 2) of the OIR model compared to controls (Figure 3A through 3C) (mid-peripheral, \( P < 0.05 \); peripheral, \( P < 0.05 \); 1-way ANOVA). This reduction in endothelial cell death was only evident in the mid-peripheral and peripheral retina as the oxygen-induced vascular regression associated with the OIR model results in very little vasculature in the central retina. IGFBP-3 did not reduce pericyte or astrocyte cell death (data not shown).

IGFBP-3 Increases Astrocytic Ensheathment of Neovasculature Following Hyperoxia-Induced Injury

We next asked whether IGFBP-3 could stabilize vessels during hyperoxia by increasing astrocytic ensheathment of retinal blood vessels. Compared with control plasmid–in-
IGFBP-3 generates NO via the scavenger receptor class B, type 1

IGFBP-3 has vascular-stabilizing effects by preventing endothelial cell death. A through C. Shown in green are TUNEL+ cells; blue indicates GS lectin+ endothelial cells; white arrows point to TUNEL+/GS lectin- endothelial cells. In comparison to controls (A through C), IGFBP-3–injected eyes (D through F) had significantly reduced endothelial cell death during the hyperoxic phase of the OIR model.

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IGFBP-3 Generates NO via the Scavenger Receptor Class B, Type 1

IGFBP-3 has both promitotic and proapoptotic effects. Such diverse biological effects can be mediated by activation of scavenger receptors.20–24 We asked whether IGFBP-3 may be initiating its vascular protective effect by binding to the scavenger receptor class B, type 1 (SR-B1), which is the high-density lipoprotein (HDL) receptor.25,26 Activation of this receptor results in NO generation.27 In human umbilical vein endothelial cells (HUVECs), IGFBP-3 at a physiological concentration (100 ng/mL) increased NO release as seen by an increase in DAF-FM fluorescence (Figure 5). HDL at the physiological concentration of 1 mg/mL similarly increased NO generation, as did the combination of IGFBP-3 and HDL (P<0.05 for all treatments). In HUVECs, pretreatment with SR-B1 blocking antibody (SR-B1-Ab, 1:100) prevented NO release by IGFBP-3 (P<0.02) and, as expected, decreased the NO production in response to HDL (P<0.0001), as well as to the combination of both agents (P<0.01). Similar results were observed in human lung microvascular endothelial cells (HMVEC-L), whereas in the RGC-5 cells that do not express SR-B1, IGFBP-3–mediated NO release was not altered with coadministration of HDL and was not affected by the addition of SR-B1-Ab.

IGFBP-3 increased NO production in human CD34+ cells (Figure 6), and in contrast to the response in HUVECs and HMVEC-L cells, the response to IGFBP-3 was greater than the response to HDL. When both agents were added simultaneously to CD34+ cells, the response was intermediate, less than with IGFBP-3, but greater than with HDL (Figure 6). We next confirmed the effect of IGFBP-3 on NO generation was attributable to increasing phosphorylation of eNOS at Ser1177 (Online Figure VII, A and B) in human CD34+ cells.

Our studies thus far show that IGFBP-3 stimulates NO release independent of HDL in 2 distinct endothelial cell types and in endothelial progenitors and that this effect is mediated by the SR-B1 receptor. To examine the signaling pathways by which SR-B1 activation by IGFBP-3 induces NO generation, we used selective pharmacological inhibitors to block phosphatidylinositol 3-kinase (PI3K) or Akt. IGFBP-3–mediated NO generation that was sensitive to blockade of SR-B1 or eNOS (by N-nitro-L-arginine methyl ester [L-NAME]) and was significantly decreased by either pretreatment with the PI3K inhibitor LY294002 (P<0.0001) or the Akt inhibitor triciribine (P<0.01) (Figure 7A and 7B). We further confirmed these observations using a biochemical assay of eNOS activity involving determination of the conversion of l-arginine to l-citrulline in HUVECs. IGFBP-3 (100 ng/mL) stimulated eNOS activity was significantly reduced by pretreatment with SR-B1-Ab or LY294002 or triciribine (P<0.05) (Figure 7C). We next examined the effect of IGFBP-3 on phosphorylation of eNOS at Ser1177 in HUVECs. IGFBP-3 increased eNOS phosphorylation, which was blocked by pretreatment with SR-B1-Ab or triciribine significantly (Online Figure VIII). These results support that IGFBP-3 induces eNOS activation and NO generation, which is dependent on the SR-B1–PI3K–Akt signaling pathway.

IGFBP-3 has also been shown to increase the activity of the sphingosine kinase (SphK)-1, the enzyme responsible for the generation of the potent angiogenic factor sphingosine-1-phosphate (S1P).7 Addition of the SphK inhibitor N,N-dimethylsphingosine resulted in a reduction in NO generation in response to IGFBP-3, as well as HDL, supporting that S1P generation was contributing to the effects of both HDL and IGFBP-3 on NO generation. CD34+ cells, HUVECs, and HMVEC-L cells express S1P receptors, primarily S1P receptor 1, and express both SphK1 and SphK2 (Online Figure IX, A and B).

IGFBP-3 Causes Vasodilatation via SR-B1–Dependent NO Release

One of the primary functions of NO in the vasculature is to stimulate vasodilatation and numerous vascular protective agents stimulate NO generation. To determine whether IGFBP-3 mediates its protective effects by promoting NO-mediated vasodilatation, we performed studies using intact arterial segments from 2 different vascular beds and assessed whether the NO released by IGFBP-3 produced a functional response in vitro. Rat mesenteric arterial segments (diameter, 276±11 μm; n=12) preconstricted with phenylephrine were completely relaxed to carbachol, indicating the presence of functional endothelium. HDL (1 mg/mL) produced marked relaxation in the phenylephrine precontracted arteries. IGFBP-3 (100 ng/mL) produced a relaxant effect, however, weaker than HDL (Figure 8A and 8B). Phenylephrine precontraction was decreased by HDL and IGFBP-3 to 58±8%
(n=4; P<0.0001) and 81±7% (n=4; P<0.001) of the control, respectively. After IGFBP-3 treatment, addition of HDL resulted in an additional relaxation causing a total decrease in precontraction to 51±7% (n=3) (Figure 8D). Conversely, addition of IGFBP-3 after HDL treatment did not cause any further relaxation (data not shown). Furthermore, in a separate set of experiments, we observed IGFBP-3 did not produce any relaxation in arteries pretreated with L-NAME, a commonly used inhibitor of eNOS. (Figure 8C and 8D).

In another set of experiments, we pressurized rat posterior cerebral arteries (passive diameter, 175±5 μm; n=15) in vitro at 70 mm Hg and evaluated the effect of IGFBP-3 on pressure induced constriction (decrease in intraluminal diameter) by applying it intraluminally. Pressurized arteries showed stable constriction in response to pressure and this constriction was significantly decreased by intraluminal IGFBP-3 (100 ng/mL). In the presence of intraluminal SR-B1-Ab or L-NAME, dilation by IGFBP-3 (decrease in constriction) was not observed (Figure 8E), suggesting that
IGFBP-3 can cause arterial dilatation via SR-B1 dependent endothelial NO release in the arterial wall.

**IGFBP-3–Mediated NO Generation Stimulates Vasodilator-Stimulated Phosphoprotein Redistribution**

Previously, we demonstrated that a critical function of NO is to promote EPC migration by regulating the distribution of the cytoskeletal protein vasodilator-stimulated phosphoprotein (VASP). VASP plays a pivotal role in promoting actin filament elongation at the leading edge of the cell by forming an active molecular motor complex that propels motility. Migration of BMDCs into areas of ischemia is paramount to their ability to initiate and orchestrate repair. IGFBP-3–stimulated migration was mediated by NO generation and blocked by the addition of the NO scavenger 2-(4-carboxyphenol)-4,4,5,5-tetramethylimidazoline-1-oxide (data not shown). To examine this further, we asked whether treatment with IGFBP-3 resulted in redistribution of VASP. Compared to control conditions (Online Figure X, A), IGFBP-3 treatment resulted in redistribution to the lamellipodia VASP fluorescence to the periphery (Online Figure X, C), which was entirely eliminated by pretreatment with an NO scavenger (Online Figure X, E).

**Discussion**

In this study, we identified cellular and signaling mechanisms responsible for the vascular protective effects of IGFBP-3. We examined the effects of IGFBP-3 on 2 distinct types of vasculature, a stable vascular bed (adult chimeric mice undergoing laser occlusion) and an immature, unstable vascular bed undergoing active endothelial cell proliferation and migration (the hyperoxia induced retinal injury model).

In the adult model, we show IGFBP-3 enhances repair by recruiting BMDCs to sites of laser occlusion within the ischemic retina. Even in the absence of retinal injury, over-expression of IGFBP-3 by the resident retinal endothelium promoted extravasation of BMDCs from the circulation into the perivascular region and their incorporation into areas of vascular remodeling. The reparative effects of IGFBP-3 are not limited to promoting BMDC homing, because IGFBP-3...
IGFBP-3 promotes cell migration.11 In this study, we showed that following IGFBP-3 treatment redistribution of VASP occurred to focal adhesions and pseudopodia, which we quantified (Online Figure X). The rapid increase in VASP (within 15 minutes) suggests that VASP redistribution occurred, rather than a change in VASP protein expression.

Our studies support that SR-B1 mediates IGFBP-3–induced NO generation and that this occurs independent of HDL. Activation of eNOS by phosphorylation at Ser1177 by IGFBP-3 is dependent on activation of SR-B1 and the downstream signaling pathway involving PI3K and Akt activity. We further show that SR-B1–dependent release of NO by IGFBP-3 modulates vascular reactivity in intact artery preparations from rat mesenteric and cerebral vascular beds resulting in decreased tone or dilation, and this effect is potentially vascular protective. Based on these studies, we postulate that SR-B1 activation by IGFBP-3 in the retinal vasculature generates NO, which is critical for vasodilatation, facilitating increased blood flow thereby infiltration of BMDCs to the sites of ischemia. NO released by IGFBP-3 in circulating EPCs and resident endothelial cells may modulate the function of BMDCs in an autocrine, as well as a paracrine manner hence, contributing to vascular repair.

In agreement with Granata et al in HUVECs,7 our studies in CD34+ cells support the notion that IGFBP-3–mediated NO generation is also dependent on activation of SphK1, because we observed that NO generation is blocked by SphK inhibition. S1P-mediated NO generation occurs by activation of endothelial differentiation gene receptors, also known as the S1P receptors. S1P, much like IGFBP-3, increases NO generation, thus promoting migration of cells.30 Moreover, S1P, like IGFBP-3, has direct vascular protective effects. In the blood, S1P is associated with lipoproteins including low-density lipoprotein, very-low-density lipoprotein, and HDL, with the majority of S1P being bound to HDL.31,32 Our studies support that in EPCs, both IGFBP-3 and HDL activate SphK1, because inhibiting SphK1 with N,N-dimethylsphingosine resulted in loss of NO generation in response to either agents (Figure 6).

Although we observed no increase in S1P in vivo in response to increase in IGFBP-3, we performed our measurements 3 weeks following retinal injury, and we suspect that any acute rise in S1P would have returned to baseline levels by this point. Despite our inability to detect S1P changes in any acute rise in S1P would have returned to baseline levels following response to increase in IGFBP-3, we performed our measurements 3 weeks following retinal injury, and we suspect that any acute rise in S1P would have returned to baseline levels by this point. Despite our inability to detect S1P changes in vivo, we observed a significant increase in S1P shortly after injury, which persisted at 3 weeks. The laser-treated eyes had a higher ceramide/sphingomyelin ratio, consistent with an inflammatory, proapoptotic state. However, this increase in ceramide/sphingomyelin ratio was completely normalized by IGFBP-3. This suggests IGFBP-3 mediates vascular protection through inhibition of inflammation, allowing IGFBP-3 to promote the recruitment of the reparative BMDC population, EPCs, rather than an inflammatory population. As observed in our studies using the adult laser injury model, IGFBP-3 reduces the number of inflammatory cells, specifically macrophages. We also found a striking increase in DHA-containing phospholipids in laser-treated eyes, indicating oxidative damage to the retina. These changes were completely normalized by increasing expression of IGFBP-3. In addition, there was an increase in DHA-containing phospholipids in laser-treated eyes, and IGFBP-3 treatment returned the phospholipid
profile to normal levels, further supporting the role of IGFBP-3 in the repair of retinal vasculature.

Although this study has identified a new receptor system that mediates the effects of IGFBP-3 with respect to NO generation, IGFBP-3 has been shown to bind to several other receptors including the transforming growth factor-β receptor V, transferrin receptor, and low-density lipoprotein receptor–related protein (LRP). However when IGFBP-3 binds to LRP-1, it is degraded. Hence, the diverse physiological effects of IGFBP-3 are likely to be mediated by distinct receptor systems.

In summary, our data support the hypothesis that IGFBP-3 mediates functional revascularization in the retina by promoting the homing of beneficial EPCs, while reducing the number of detrimental inflammatory cells, such as macrophages. Once EPCs are routed to areas of damage, IGFBP-3 enhances their incorporation and differentiation into endothelial cells, which facilitates vessel formation. IGFBP-3 serves to protect the resident endothelium and EPC from cell death and increases astrocyte ensheathment to enhance vessel barrier properties and autoregulatory capacity. Our studies suggest that these beneficial effects may be mediated by increased NO generation which occurs via IGFBP-3 binding of SR-B1 and subsequent activation of the P13K–Akt pathway. Our studies also support a second mechanism by which IGFBP-3 stimulates NO generation by activation of Sphk-1. We postulate that IGFBP-3 may modulate interactions between the scavenger receptor system and the S1P receptor system and may serve to regulate both physiological and pathological angiogenesis and vascular remodeling. Our findings support that IGFBP-3 in the circulation and in tissues may represent an endogenous vascular protective protein and following vascular injury increased levels of IGFBP-3 could promote proper revascularization.

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Disclosures
None.
References


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Supplement Material

Supplemental Methods

Animals

All animals were treated in accordance with the principles in The Guiding Principles in the Care and Use of Animals (NIH) and all protocols were approved by the University of Florida IACUC. C57BL/6J.gfp transgenic mice homozygous for GFP were used in the laser induced neovascularization model and pregnant C57L/6J mice (E14) were utilized in the OIR model. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the institutional animal care facilities at the University of Florida.

Immunohistochemistry for laser induced experimental neovascularization

Immunohistochemistry was performed on the neural retinas as previously described. 1 Retinas from GFP+ chimeric animals were stained with: Griffonia Simplicifolia isolectin B4 (Sigma, St. Louis, MO) for detection of endothelial cells 2, 3 and activated microglia/macrophages, 2, 4 NG2 (Chemicon, Temecula, CA) for detection of pericytes and smooth muscle cells and S-100 and GFAP (Sigma, St. Louis, MO) for detection of astrocytes.

Immunohistochemistry for OIR model

Cell death (TUNEL analysis) of astrocytes (S-100 and GFAP) 5, endothelial cells (GS isolectin B4), pericytes (NG2) 6 and microglia (GS isolectin B4) were determined using quadruple marker immunohistochemistry on retinal wholemounts and cryosections in the central, mid-peripheral and peripheral regions of the retina.

Evaluation of endothelial, pericyte and astrocyte cell death in the OIR model

Retinas were examined after exposure to a high oxygen environment (P12) and after 5 days return to room air (P17). Cell death (TUNEL analysis) of endothelial cells (GS isolectin B4) 7 was determined on retinal wholemounts. OIR animals were stained with an in situ cell death detection kit (F. Hoffman-La Roche Basel, Switzerland) based on the TUNEL method to label nuclei undergoing DNA fragmentation, followed by GS isolectin B4 for vascular endothelial cells (Sigma), NG2 for pericytes (Chemicon) or S100 for astrocytes (Sigma).

Statistical analysis of endothelial, pericyte and astrocyte cell death

Representative fields of view from the central, mid-peripheral and peripheral retinas were counted using a 20x objective as the field of view for analysis as modified from Hughes and Chan-Ling. 6 In each field of view TUNEL+/GS Lectin+ vascular endothelial cells, TUNEL+/NG2+ pericytes, and TUNEL+/S-100+ for astrocytes were counted. The data is collected as mean ± standard deviation (SD) where N=6 per experimental group, and the statistical significance of differences among mean values was determined by one-way ANOVA and the Tukey HSD multiple comparison post-hoc test. ANOVA statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, IL). A p-value of less than 0.05 was considered statistically significant.
Confocal microscopy
Retinas of laser injured animals were examined using a Leica-argon krypton laser mounted on a Leica DMRBE epifluorescence photomicroscope (Leica, Wetzlar, Germany) using OpenLab Imaging software. Retinas of OIR animals were examined using a Zeiss LSM 510 META confocal microscope. Images were processed with Adobe Photoshop software.

Quantification of astrocyte ensheathment
The frequency of S-100 ensheathment of retinal vessels was determined using a modified method previously described for determining the desmin ensheathment ratio. Representative fields of view from the mid-peripheral retina were counted using a 20x objective as the field of view for analysis. Each confocal image was overlaid with a 10 X 10 equally spaced grid using Adobe Photoshop V5.0. The grid was superimposed onto each image. The occurrence of S-100 labeling relative to lectin labeling at the 100 intersection points yielded the percentage of astrocyte ensheathment. The data was collected as mean ± standard deviation (SD) where n=6 per experimental group and the statistical significance of differences among mean values was determined by t-Test: two-sample assuming unequal variances. A p-value of less than 0.05 was considered statistically significant.

Cell culture
Mobilized normal human CD34+ cells (Lonza, Walkersville, MD), Human Microvascular Endothelial Lung Cells (HMVEC-L) (Lonza, Walkersville, MD) and Human Umbilical Vein Endothelial Cells (HUVEC) (ATCC, Manasas, VA) were maintained as previously described.

Western Blotting
Human CD34+ cells from 3 different donors were thawed according to suppliers suggested instructions (Lonza) and maintained in culture for 5 five days with StemSpan™ media and cocktail (Stem Cell Technologies, Vancouver, BC, Canada). Cell growth, protein isolation and analysis of immunoblots was performed on an Odyssey LI-COR infrared imaging scanner (LI-COR) was carried out as previously described.

RT-PCR Analysis
Total mRNA of the various four cells types were isolated using the Total RNA Mini Kit (Bio-Rad, Hercules, CA). The mRNA was transcribed using an iScript cDNA Synthesis Kit (BioRad, Hercules, CA), and real-time PCR was performed using ABI Master Mix (ABI Biosystems, Foster City, CA). Primers for S1PR’s 1-5, α-actin, SR-B1, Sphk1, and Sphk2 were purchased from ABI systems. All samples were normalized to α-actin or TATABP (ABI Biosystems, Foster City, CA). Real-time PCR was performed on an ABI 7500 Fast PCR machine for 60 cycles. All reactions were performed in triplicate.

Lipid analysis by nESI-MS/MS
Prior to analysis, lipid extracts were appropriately diluted in isopropanol/methanol/chloroform (4:2:1 v/v/v) containing 20 mM ammonium hydroxide. Internal standards added to the lipid extracts were GPCho (23:0/23:0) (Avanti Polar Lipids, Alabaster, AL) at 300 nM, and Ceramide/Sphingomyelin Internal Standard Mixture I (Avanti Polar Lipids, Alabaster, AL) at 12.5 nM. All lipid samples were analyzed using a Thermo model TSQ Quantum Ultra triple quadruple mass spectrometer (San Jose, CA), and lipidomic data was corrected for $^{13}$C isotope effects and quantitated against the internal standards as previously described $^{11,12}$.

**Determination of NO production by DAF-FM fluorescence imaging**

NO production was quantified in HUVEC’s, HMVEC-L’s, rat retinal ganglion cells (RGC-5’s) and human cord blood-derived CD34+ cells using NO sensitive cell permeant fluorescent dye DAF-FM. HUVEC’s, HMVEC-L and RGC-5’s were plated on coverslip-bottomed dishes (MatTek, Ashland, MA). CD34+ cells in suspension were loaded with DAF-FM diacetate (Invitrogen) (10 µM for HUVEC’s, HMVEC-L’s and RGC-5’s; 20 µM for CD34+ cells) for 30-45 minutes in Dulbecco’s phosphate buffered saline with Ca$^{2+}$ and Mg$^{2+}$ (Mediatech, Inc., Manassas, VA) supplemented with glucose (1 mg/ml) and L-arginine (1 mM). DAF-FM-loaded cells in dishes were placed on the stage of Axiovert inverted microscope with 20X fluor objective (Zeiss) for fluorescence imaging. In the case of CD34+ cells, approximately 75 µl of cell suspension was placed in the coverslip-bottomed dish to perform imaging. Fluorescent images were obtained using a computer-controlled monochromator excitation light source (TILL Polychrome II, TILL-Photonics, Martinsried, Germany) and a cooled CCD camera with exposure control. Images were analyzed and fluorescence was measured in arbitrary units using Till Vision.

To evaluate the effect of IGFBP-3 and/or HDL on NO production, cells were treated with these agents 30 minutes after cells were loaded with DAF-FM and further incubated for 30 minutes. Some dishes were incubated with a scavenger receptor blocking antibody (SRB1-Ab) for 30 minutes before loading cells with DAF-FM. Changes in DAF fluorescence with different treatments were expressed as percent change with respect to cells that were used as either time or vehicle control i.e. cells that received no treatments, but loaded with DAF-FM. Results were evaluated for statistical significance by one-way ANOVA.

**Evaluation of eNOS activity**

Activation of eNOS by IGFBP-3 was evaluated by measuring L-citrulline synthesis in HUVEC’s using radioactive L-arginine as substrate. Briefly, cell suspension was incubated with L-[^14]C]arginine at 37°C with constant agitation in the presence or absence of 500 µM L-NAME, a nitric oxide synthase inhibitor. Following incubation, cells were lysed by sonication for 10 seconds and sample suspension was run through 1-mL columns of Dowex AG50WX-8 (Na+ form). Radioactivity corresponding to[^14]C]citrulline within the eluate was quantified by liquid scintillation counting. Enzyme activity was expressed as L-NAME inhibitable radioactivity/mg of cell protein. In order to evaluate the effects of different blockers on IGFBP-3-stimulated eNOS activity, cell suspension was incubated with blocker for 30 minutes before the addition of IGFBP-3.

**Functional studies in rat small mesenteric arteries**
Vasodilatory effect of IGFBP-3 was evaluated in wire-mounted or pressurized artery preparations. Third order mesenteric arterial segments were isolated from male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) aged 12 – 14 weeks and mounted in a four channel small vessel wire myograph (Danish Myotech, Aarhus, Denmark) for isometric tension measurements in PSS at 37°C as previously described. Tension (mN) developed in the arteries was continuously recorded by the software program Chart (ADInstruments, Colorado Springs, CO). The resting tension–internal circumference (IC) relation was determined and the resting tension was set to a normalized IC of L0.9 as described by. To evaluate the relaxation responses to IGFBP-3 and HDL, arteries were preconstricted with submaximal concentration of phenylephrine (3 μM).

**Functional studies in rat cerebral arteries**

In another set of experiments rat posterior cerebral arteries were studied to evaluate the effect of IGFBP-3 on pressure induced constriction. Rats were anesthetized with pentobarbital sodium (160 mg/kg ip) and killed by decapitation. The brain was removed and placed in an ice cold oxygenated physiological saline solution (PSS). Posterior cerebral arteries were isolated and mounted in an arteriograph, as described elsewhere, using a pressure servo-null system (Living Systems, Burlington, VT). Diameter was measured with a calibrated video-caliper system. The arteries were slowly pressurized to 70 mmHg under no flow conditions and warmed to 37°C during superfusion (3 ml/min) with PCSF bubbled with 21% O2-5% CO2-74% N2 (pH 7.3–7.4 in the bath). All experiments were done in endothelium intact arteries. After ~30 minutes of equilibration, the arteries showed stable constriction. Pressure induced constriction was calculated as follows:

\[
\text{Pressure-induced constriction} \times 100 = \frac{(D_p - D_a)}{D_p} \\
\]

where Da is diameter of the arterial segment with active tone and Dp is passive diameter in the presence of calcium free PSS at a given intraluminal pressure.

To evaluate the effect of IGFBP-3, arteries were mounted in the arteriograph with both the glass cannulae filled with the solution of IGFBP-3 or of the vehicle and the pressure induced constriction was evaluated with IGFBP-3 present in the arterial lumen. To evaluate the effect of a blocker, arterial lumen was first exposed to the blocker for 30 minutes and then IGFBP-3 along with the blocker was applied.

**VASP Immunocytochemistry**

HMVEC-Ls were cultured on fibronectin-coated coverslips (BD Biosciences) and processed as described in supplemental methods. Cells were then left untreated (Control); treated for 15 minutes with 100ng/ml IGFBP-3 (Upstate Biotechnology, Lake Placid, NY) (IGFBP-3); or with IGFBP-3 preceded by 1 hour pretreatment with 100μM L-NAME, a non-selective inhibitor of nitric oxide synthase (L-NAME / IGFBP-3). At the end of the treatment, medium was removed, and cells were fixed for 10 minutes at room temperature in 4% paraformaldehyde (PFA) in PBS, supplemented with calcium and magnesium ions, and adjusted to pH 7.4. Cells were then washed in PBS and permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature. After three additional washes in PBS, cells were treated with 10% normal goat serum with 1% BSA in PBS for 1 hour at room temperature to block nonspecific antigens.
Cells were then incubated with 5 µg/ml mouse anti-VASP antibody (BD Biosciences) in blocking solution overnight at 4°C and then with FITC-labeled goat anti-mouse IgG1 (Abcam, Cambridge, MA) at 1:200 dilution in blocking solution for 1 hour at room temperature. Samples were then mounted with Vectashield ® mounting medium containing 4’,6-diamino-2-phenylindole (DAPI) for nuclear DNA staining (Vector Laboratories). Samples were examined by fluorescence microscopy (Zeiss Axioplan 2), using a Zeiss Plan-neofluar 40X (NA=1.30) oil objective. Micrographs were captured using a QImaging® Retiga-EXi Fast 1394 12 bit cooled CCD digital camera (QImaging, Surrey, BC, Canada) and processed using Openlab imaging software (Improvision, Waltham, MA) for Macintosh.

Analysis of cellular VASP levels in HMVEC-Ls

Cells were processed as described in supplemental methods. The analysis was then performed on TIFF images of the HMVEC-Ls that were mounted with VectaShield (Vector Laboratories) containing DAPI for nuclear staining (blue) and immuno-stained with an antibody to VASP (BD Biosciences, green) (Online Figure 10 A,C,&E) and analysis of the images was performed using Image J (W. Rasband, NIH, http://rsb.info.nih.gov/ij/) and are detailed in the supplemental methods. For each cell, three measurements were made by drawing a line from the outer edge of the nuclear membrane to the outer edge of the plasma membrane. A fluorescence profile was determined by plotting the profile of each line. This yielded a set of distance coordinates (X-values) and fluorescence values (Y-values) for each line. This was done for a set of five cells for each condition and resulted in 15 sets of X-Y values for each condition. The sets of distance values (X’s) were normalized by setting the distance from the nucleus to the outer plasma membrane of each set to one and then adjusting the remaining distance values relative to this value so that all X-values are within the range of zero and one. The sets of fluorescent values (Y’s) were normalized by taking the largest value of each set and setting it to 100 and then adjusting the remaining Y-values relative to this value so that all Y-values are within the range of zero and 100. Each set of 15-X-Y coordinates were then graphed using Excel (Microsoft) and a graph with fifteen plots for each condition was obtained (Online Figure 10 B, D &F). The area under each curve was determined for each condition at the relative distance from the nucleus of 0.2 – 0.25, 0.5 – 0.55 and 0.95 – 1.0 using Scion Image (based on NIH Image for MacIntosh by W. Rasband at NIH and modified for Windows by Scion Corporation, www.scioncorp.com). These areas were averaged and the standard error of the mean was determined. The results were graphed in Excel (Online Figure 10H).
References


Online Figure Legends

Online Figure 1: Injection of IGFBP-3 leads to greater stem cell homing and incorporation into damaged vasculature in an adult mouse ocular neovascularization model. The green stain is GFP+ cells and the blue represents GS isolecitin B4 for detection of endothelial cells and activated microglia/macrophages. (A-C) represents laser only retina with 2 laser burn sites indicated by pigment changes and tissue damage (dashed circles) and surrounding vascular remodeling. (D-F) illustrates IGFBP-3 injected plus laser retina showing large incorporation of GFP+ cells, at and around the site of laser injury, participating in wound healing response. GFP+ cells incorporate into lectin positive vessels and also differentiation into GS Lectin labeled endothelial cells (white arrows). (G-I) illustrates IGFBP-3 injected eyes showing significant GFP+ BMDC incorporation into GS Lectin labeled vessels (white arrows).

Online Figure 2: A-F: Representative confocal images of mouse retina flatmounts demonstrating IGFBP-3 stimulates GFP+ BMDC incorporation into vessels and differentiation into macrophages in the retinal vasculature. The red stain is rhodamine agglutinin and the green represents GFP+ cells following staining with an anti-GFP antibody (conjugated to Alexa 488). (A-C) GFP+ cells are associated with retinal vessels in IGFBP-3 injected eyes. Inset shows, at high magnification, GFP+ BMDC incorporation into the retinal vasculature and in the perivascular area. (D-F) GFP+ macrophages were present in the parenchyma of the laser plus IGFBP-3 treated eyes, while GFP+ BMDC also incorporated into the retinal vasculature. (G-I) GFP alone (G), GS-lectin (H), co-visualization showed that all non-vascular associated GFP+ cells were GS isolecitin B4+, indicative of their macrophage character (I). (J-M) representative confocal images of transverse retinal cryosections. The green stain is GFP+ BMDC. (J) GFP chimeric control mouse retina showing no evident GFP+ cells within the retina. (K) represents IGFBP-3 only retina showing marked incorporation of GFP+ cells within 3 predominant layers of the retina. (L) represents laser only. (M) depicts laser plus IGFBP-3 injected retinas, showing GFP+ cells associated with the superficial and deep vascular plexii. GFP+ cells were also found in the photoreceptor layer, retinal pigment epithelial layer and choroid. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium.

Online Figure 3: Time course of IGFBP-3 expression in mouse retina undergoing laser injury followed by injection with IGFBP-3 expressing plasmid. RT-PCR analysis of mouse retina evaluating IGFBP-3 mRNA expression over a 3 week time course. IGFBP-3 mRNA remains highly expressed in the treated right eye (OD) of mice subjected to laser injury followed by intravitreal injection of IGFBP-3 up to 1 week compared to the contralateral control eye (OS). By 3 weeks, IGFBP-3 expression levels in the treated eye (OD) returned to normal control levels. (*P<0.05).

Online Figure 4: Representative ceramide and SM MS/MS spectra from control, IGFBP-3, laser or laser and IGFBP-3 treated mice. (A) control retinal ceramide (top) and sphingomyelin (bottom) molecular species detected by precursor ion scanning of m/z 264 and m/z 184, respectively, after alkaline hydrolysis of glycerophospholipids. I.S.,
internal standard. **(B)** IGFBP-3 expressing retina ceramide (top) and sphingomyelin (bottom) molecular species detected by precursor ion scanning of m/z 264 and m/z 184, respectively, after alkaline hydrolysis of glycerophospholipids. I.S., internal standard. **(C)** Laser injured retina ceramide (top) and sphingomyelin (bottom) molecular species detected by precursor ion scanning of m/z 264 and m/z 184, respectively, after alkaline hydrolysis of glycerophospholipids. I.S., internal standard. **(D)** Retina from laser and IGFBP-3 treated mice. Ceramide (top) and sphingomyelin (bottom) molecular species detected by precursor ion scanning of m/z 264 and m/z 184, respectively, after alkaline hydrolysis of glycerophospholipids. I.S., internal standard.

**Online Figure 5: Correction of laser induced changes in total retinal GPCho by IGFBP-3.** Laser treatment induced a significant increase in total retinal incorporation of DHA (22:6n3) into glycerophosphatidylcholine (GPCho). Upon laser treatment, 16:0/22:6 and 18:0/22:6 GPCho were significantly higher at the expense of 16:0/16:0 and 16:0/18:0. Moreover, laser treatment induced substantial increases in 22:6 containing lysolipids consistent with oxidative damage of the tissue. IGFBP-3 treatment completely reversed these changes in phospholipid profile and lysolipid content and returned them to normal control level. Control (A), laser treated (B), or laser treated with IGFBP-3 (C) retinal lipid extracts were analyzed for GPCho molecular species by precursor ion scanning of m/z 184 mass spectrometry. Peak areas were quantitated against an internal standard, 23:0/23:0 GPCho (I.S.), after correction for 13C isotope effects. Fatty acid constituents were confirmed in GPCho [M+Cl]- species by –ve precursor ion scanning of m/z corresponding to deprotonated fatty acyl species. Representative spectra of at least 3 independent experiments are shown.

**Online Figure 6: Correction of laser induced changes in total retinal GPIns species by IGFBP-3.** Upon laser treatment, GPIns 16:0/18:0 and GPIns 18:1/18:1 each comprised approximately 15 mol% of all GPIns species present (C), while these same species were completely absent from untreated control retinas (A). Furthermore, GPIns 18:0/22:6 species increased two-fold in laser treated retinas (C), from ~10 mol% in untreated retinas (A) to ~20 mol% in laser treated retinas (C). A dramatic reduction of GPIns 16:0/20:4, from ~30 mol% to ~5 mol%, was also observed in laser treated retinas. All GPIns changes were completely reversed by IGFBP-3 treatment. Control (A), laser treated with IGFBP-3 (B), or laser treated (C) retinal lipid extracts were analyzed for GPIns molecular species by precursor ion scan of m/z 241.1 mass spectrometry. Fatty acid constituents were confirmed in GPIns [M+Cl]- species by –ve precursor ion scanning of m/z corresponding to deprotonated fatty acyl species. Representative spectra of 3 independent experiments are shown.

**Online Figure 7: IGFBP-3 increases phosphorylation of eNOS at Ser<sup>1177</sup> in human CD34<sup>+</sup> cells and HUVECs.** (A) Western Blot analysis depicting eNOS phosphorylation in human CD34<sup>+</sup> cells treated with 100 ng/ml of IGFBP-3 at 10, 30 and 60 minutes. Protein expression levels were normalized to coflin. (B) Quantification of western blot in panel A. There is a time dependent increase in eNOS phosphorylation at Ser<sup>1177</sup> in human CD34<sup>+</sup> cells (P<0.05 and P<0.01 at 30 and 60 min, respectively).
**Online Figure 8:** Western Blot analysis depicting eNOS phosphorylation at Ser\(^{1177}\) in HUVECs treated with 100 ng/ml of IGFBP-3 for 45 minutes. Protein expression levels were normalized to pan-cadherin. Phosphorylation was significantly increased by IGFBP-3 compared to untreated cells (P<0.05). In the presence of blockers SRB1-Ab or triciribin, the phosphorylation was significantly decreased (P<0.05).

**Online Figure 9:** S1P receptors (S1PRs), Sphingosine kinase (SphK) 1 and 2 and SR-B1 expression in human CD34\(^+\) cells and mature endothelial cells. S1PR1 and SphK1, as well as SR-B1 are highly expressed in CD34\(^+\) cells and mature endothelial cells. **(A)** RT-PCR showing expression of S1PR’s 1-5 in normal human CD34\(^+\) cells, human microvascular lung endothelial cells (HMVEC-L), and human umbilical vein endothelial cells (HUVEC’s) **(B)** RT-PCR depicting expression of SphK 1 and 2 in normal untreated human CD34\(^+\) cells, human macrovascular lung endothelial cells (HMVEC-L), and human umbilical vein endothelial cells (HUVEC’s).

**Online 10:** IGFBP-3-mediated VASP redistribution in human microvascular endothelial cells from the lung (HMVEC-L). HMVEC-L, cultured on fibronectin-coated coverslips, were treated with IGFBP-3 and VASP biodistribution was detected by immunofluorescence **(A, C, E, G)** and quantified as described in the methods section. Immunostaining was quantified by Image J as the intensity of green fluorescence. For each cell, three series of fluorescence measurements were made at selected distances from the nucleus of 0.2 – 0.25, 0.5 – 0.55 and 0.95 – 1.0 and the fluorescence profile determined. The area under each curve was determined for each condition, which showed that IGFBP-3 treatment increased the redistribution of VASP fluorescence to the periphery. This effect was blocked by pretreatment with L-NAME. **(A)** Untreated control cells showing uniform VASP localization (green) along the actin filaments throughout the cytoplasm. **(C)** IGFBP-3 induced VASP redistribution to lamellipodia at the leading edge of microvascular endothelial cells. **(E)** Pretreatment with an inhibitor of nitric oxide synthase (L-NAME) abolishes the effect of IGFBP-3 on VASP redistribution. **(G)** Control in which the VASP primary antibody was omitted. **(B, D, F)** Quantification of VASP biodistribution in A, C, and E, respectively. **(H)** Area under the curve calculated from the three areas of interest in B, D, and F (region shaded blue). Representative results from three independent experiments are shown. Green: VASP; Blue: DAPI (nuclei). (Scale bars = 25 \(\mu\)m).
Online Figure 3
Online Figure 4
Online Figure 5
Online Figure 7

A

Phospho-eNOS (ser1177)
Cofilin

B

![Graph showing phospho-eNOS/Coilin protein ratio over time.](image)

- 0 min
- 10 min
- 30 min
- 60 min

*Significant change from 0 min.
Online Figure 8
Online Figure 9

A

Receptor/BA mRNA relative to SIP1 (%)

HUVEC  CD34+  HMVEC-L

SIPR1  SIPR2  SIPR3  SIPR4  SIPR5

B

Receptor/BA mRNA relative to SPHK1 (%)

HUVEC  CD34+  HMVEC-L

SPHK1  SPHK2