Blocking Mammalian Target of Rapamycin (mTOR) Attenuates HIF-1α Pathways Engaged-Vascular Endothelial Growth Factor (VEGF) in Diabetic Retinopathy

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Key Words
HIF-1α • VEGF • Diabetic retinopathy • mTOR

Abstract
Background/Aims: Prior studies demonstrate that hypoxia inducible factor subtype 1α (HIF-1α) in retinal tissues is involved in development of diabetic retinopathy (DR). In this report, we particularly examined the role played by mammalian target of rapamycin (mTOR) in regulating expression of HIF-1α and its downstream pathway, namely vascular endothelial growth factor (VEGF). Methods: Streptozotocin (STZ) was systemically injected to induce hyperglycemia in rats. ELISA and Western Blot analysis were employed to determine the levels of HIF-1α and VEGF as well as expression of mTOR pathways in retinal tissues of control rats and STZ rats. Results: Our results show that HIF-1α and VEGF as well as VEGF receptor subtype 2 (VEGFR-2) were increased in STZ rats. Also, the protein expression of p-mTOR, mTOR-mediated phosphorylation of 4E–binding protein 4 (4E-BP1), p70 ribosomal S6 protein kinase 1 (S6K1) pathways were amplified in diabetic retina compared with controls. Blocking mTOR by using rapamycin significantly attenuated activities of HIF-1α and VEGF signaling pathways. Conclusion: Our data for the first time revealed specific signaling pathways engaged in the development of DR, including the activation of mTOR and HIF-1α -VEGF mechanism. Targeting one or more of these signaling molecules may present new opportunities for treatment and management of DR often observed in clinics.

Introduction
Diabetic retinopathy (DR) is a microvascular complication associated with chronic exposure to hyperglycemia and is a major cause of blindness worldwide [1]. Although clinical assessment and retinal autopsy of diabetic patients provide information on the features and progression of DR [2, 3], the underlying pathophysiological mechanism responsible for the disease cannot be clarified. In order to have a better understanding of the development of DR at the molecular and cellular levels, a variety of animal models have widely been used...
[4]. They include pharmacological induction of hyperglycemia and spontaneous diabetic rodents. Nevertheless, it is important to determine substrates involved in pathophysiological mechanism of DR.

Hypoxia inducible factor-1 (HIF-1) as an important endogenous signaling protein contributes to physiologic changes of homeostasis under conditions of oxygen deprivation [5]. Accumulated subunit HIF-1α modulates the expression of several target genes in tissues under hypoxic conditions [6-8]. One of the important protein molecules responsible for the neovascularization is vascular endothelial growth factor (VEGF), which is resulted from increased HIF-1α formation due to ischemic hypoxia; and both HIF-1α and VEGF have been found in the retina of diabetic animals and humans to be involved in the pathophysiology of DR [9-11]. Importantly, blocking or silencing HIF-1α-regulated VEGF pathway has been reported to have potential benefits to DR [10].

Mammalian target of rapamycin (mTOR) is a serine threonine protein kinase. Activation of mTOR, in particular, mTOR complex 1 (mTORC1) that is more sensitive to rapamycin, leads to promotion of the phosphorylation of downstream effectors, such as p70 ribosomal S6 protein kinase (p70S6K) and this further governs mRNA translation [12]. The mTORC1 is well known for its critical roles in the regulation of protein synthesis and growth and further the compelling evidence supports the widespread dysregulation of mTOR in development of DR [13].

Therefore, in light of the key role of mTOR in regulating ischemic hypoxia in DR, we first examined responsiveness of HIF-1α, VEGF and VEGF receptor subtype 2 (VEGFR-2) in the retinal tissues of non-diabetic rats and diabetic rats induced by injection of streptozotocin (STZ). We further examined the protein expression of mTOR and its downstream pathways in diabetic retina. Then, we determined the role played by mTOR in regulating HIF-1α-VEGF in DR. We examined the effects of intraocular injection of mTOR inhibitor, rapamycin, on the levels of HIF-1α, VEGF and VEGFR-2 in diabetic retina. Moreover, we examined the effects of rapamycin on protein expression of Caspase-3 indicating cell apoptosis. Our hypothesis was that inhibiting mTOR blunts the upregulation of HIF-1α-VEGF signaling pathway as well as Caspase-3 evoked by hyperglycemia in STZ rats.

It should be noted that VEGF regulates vascular development, angiogenesis and lymphangiogenesis by binding to a number of VEGFRs [14, 15]. There are three main subtypes of VEGFR, numbered 1, 2 and 3. The function of VEGFR-1 is less defined, although it is required for the recruitment of hematopoietic stem cells and the migration of monocytes and macrophages [14, 15]. VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF and it is critical for vascular endothelial cell development and regulates vascular endothelial function [14]. VEGFR-3 regulates lymphatic endothelial cell function and mediates lymphangiogenesis in response to VEGF [14, 15]. Considerable evidence shows VEGFR-2 specific intracellular signal cascades leading to proliferation, migration, survival and increased permeability, each of which contributes to the angiogenic response [14]. Accordingly, we examined VEGFR-2 in this report.

Materials and Methods

Animals and diabetes

All experimental procedures were in accordance with the guidelines of the International Association for the Study of Pain and were approved by the Animal Research Committee of our medical institution. Male Sprague-Dawley rats weighing 150-200 g were used in this study. STZ was freshly dissolved in 0.9% sterile saline and diabetes was induced by a single injection of STZ (60 mg/kg i.p., Sigma Co.) as described previously [16]. Diabetes was confirmed by measurements of blood glucose concentrations in samples obtained from the tail vein four weeks after injection of STZ. It should be noted that rats whose blood glucose concentration was > 350 mg/dl were included in the study. Age- and body weight-matched rats with saline injection were used as controls. In a subset of experiments, rapamycin (Sigma Co; 1 µg once every two days) was given by intraocular injection. Note that intraocular injection of saline was served as
control. At different time courses after injection of STZ, the retinal tissues were removed for the following measurements.

**ELISA**

The levels of HIF-1α were determined using an ELISA assay kit (Abcam Co., Cambridge, MA, USA) according to the provided description and modification.

**Preparation of extracts from tissue.** All the retinal tissues from individual rats were sampled for the analysis. Tissue lysates were typically prepared by homogenization of tissue that was first minced and thoroughly rinsed in PBS to remove blood. Wet tissue was homogenized in 500 µL of chilled cell extraction buffer and incubated on ice for 20 minutes. Total protein was then extracted by homogenizing (18,000 x g for 20 minutes at 4°C) retinal sample in ice-cold radioimmunoprecipitation assay buffer with protease inhibitor cocktail kit. Then, the pellets were discarded and the supernatants were transferred into clean tubes for measurements of protein concentrations using a bicinchoninic acid assay reagent kit. Results were expressed as pg/mg protein.

**Plate preparation.** Polystyrene 96-well microtitre immunoplates were coated with affinity-purified polyclonal rabbit anti-HIF-1α antibody. Parallel wells were coated with purified rabbit IgG for evaluation of nonspecific signal. After overnight incubation at room temperature and 2 hours of incubation with the coating buffer containing 50 mM carbonate buffer (pH 9.5) in 2% BSA, plate were washed with 50 mM Tris-HCl. After extensive washing, the diluted samples and the HIF-1α standard solutions were distributed in each plate and left at room temperature overnight. The plates were then washed and incubated with anti-HIF-1α galactosidase per well. Then, the plates were washed and incubated with substrate solution. After an incubation of 2 hours at 37°C, the optical density was measured using an ELISA reader. Likewise, this method was also employed to examine the levels of VEGF according to the provided description and modification (Abcam Co.).

**Western Blot Analysis**

The protein expression of VEGFR-2, Caspase-3, mTOR, S6K1, 4E-BP1 (their respective phosphorylated forms, namely p-mTOR, p-S6K1, p-4E-BP1) was determined by using a standard Western Blot analysis. In brief, the retinal tissues from individual rats were sampled. Total protein was then extracted by homogenizing sample in ice-cold radioimmunoprecipitation assay buffer with protease inhibitor cocktail kit. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit. After being denatured by heating at 95°C in an SDS sample buffer, the supernatant samples was loaded onto 4-20% Mini-Protean TGX Precast gels and then electrically transferred to a polyvinylidene fluoride membrane. Membranes were incubated with rabbit anti-VEGFR-2 antibody, rabbit anti-p-mTOR/p-S6K1/p-4E-BP1 antibodies; rabbit anti-mTOR/S6K1/4E-BP1 antibodies; and rabbit anti- Caspase-3 (1:500, obtained from Abcam Co.) After being fully washed, the membrane was incubated with horseradish peroxidase-linked anti-rabbit secondary antibody (1:250) and visualized for immunoreactivity. The membrane was also processed to detect β-actin for equal loading. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. The film was then scanned and the optical densities of protein bands were analyzed using the Scion image software, and values for densities of immunoreactive bands/β-actin band densities from the same lane were determined. Each of the values was then normalized to a control sample.

**Statistical analysis**

All data were analyzed using a two-way repeated-measures analysis of variance. Values were presented as means ± standard error of mean (SEM). For all analyses, differences were considered significant at \( P < 0.05 \). All statistical analyses were performed by using SPSS for Windows version 13.0 (SPSS, USA).

**Results**

**Measurements of Blood Glucose**

We examined blood glucose in control rats and rats 0-28 days after injection of STZ. Figure 1 shows that animals developed hyperglycemia 21 days after STZ (\( P < 0.05 \) vs. control rats) as compared with saline control rats.
Figure 1. Showing effects of STZ injection on blood glucose. Hyperglycemia was induced in rats 21 and 28 days after STZ injection. *P < 0.05, indicated STZ-rats vs. control rats. Note that blood glucose was not examined > 28 days after STZ injection. The number of animals is indicated in each bar.

Levels of HIF-1α and VEGF

Figure 2 shows that the levels of HIF-1α and VEGF were significantly increased in the retinal tissues of STZ rats (P < 0.05 vs. control rats, n = 6-12 in each group) as compared with saline control group (n = 8). The increases were observed 21 and 28 days after injection of hyperglycemia as compared with control animals. *P < 0.05, indicated rats injected with STZ vs. control rats (n = 8).

Expression of mTOR Pathway

Figure 3 demonstrates the protein expression of p-mTOR, p-S6K1 and p-4E-BP1 as well as mTOR, S6K1 and 4E-BP1 in control rats and STZ rats 28 days after its injection. STZ induced- hyperglycemia significantly increased the protein levels of p-mTOR and mTOR-mediated p-S6K1 and p-4E-BP1 in the retinal tissues as compared with control rats (P < 0.05, n = 8-10 in each group; STZ rats vs. control rats, n = 6-10 in each group). Also, the ratio of p-mTOR, p-S6K1 and p-4E-BP1 levels vs. total protein of mTOR, S6K1 and 4E-BP1 levels was significantly increased in STZ rats.

Effects of Blocking mTOR on Expression of VEGFR-2 and Caspase-3

We also examined the effects of blocking mTOR on expression of VEGFR-2 and Caspase-3. Figure 4 demonstrates that the protein expression of VEGFR-2 and Caspase-3 was significantly increased in STZ rats with intraocular injection of saline (n = 6-10 in each group) as compared with control rats (n = 6-8 in each group). When rapamycin was injected in STZ
rats, the amplified activities of VEGFR-2 and Caspase-3 evoked by STZ were significantly attenuated (n = 8-10 in each group). In addition, Fig. 4 shows that mTOR signaling pathway was inhibited by rapamycin because as an indicator of mTOR expression, p-S6K1, the ratio of p-S6K1 and S6K1, were significantly decreased after application of rapamycin.
Discussion

In the present study, we first examined the levels of HIF-1α and VEGF in control rats and STZ rats, demonstrating that STZ increased HIF-1α and VEGF. Also, expression of p-mTOR and its downstream pathways, namely p-S6K1 and p-4E-BP1 was upregulated in STZ rats. In order to determine the role played by mTOR in regulating HIF-1α-VEGFR2 signal pathways, we then examined the effects of intraocular injection rapamycin on the levels of VEGFR-2 in retinal tissues of STZ (Fig. 4). In addition, we examined the effects of rapamycin on protein expression of Caspase-3. Our data further showed that inhibiting mTOR can blunt the upregulation of VEGFR2 as well as Caspase-3 pathways evoked by STZ.

There is solid evidence for an early decrease in oxygen tension in DR, suggesting that the retina is physiologically exposed to hypoxia and becomes more hypoxic even early in the evolution of diabetes (i.e. 3 weeks after experimental diabetes induction) [17]. Also, retinal flow is decreased in patients with diabetes before any retinal changes are observed [18], and it becomes more profound in patients with severe DR. The profound hypoxic environment present in DR further induces HIF-1α in both humans and in animal models of diabetes [19]. Thus, a rat model of STZ injection-induced DR was widely employed to investigate the mechanisms responsible for the disease and potential drugs used to treat DR in the previous studies [4]. Consistent with the previous notion, in the current study, using the same rat model our data showed that HIF-1α was significantly increased in the retinal tissues of rats 21-28 days after injection of STZ with being companied with STZ induced-hyperglycemia.

VEGF is an important signaling protein playing a role in regulating the neovascularization and as a downstream product of HIF-1α, HIF-1α contributes to VEGF formation due to ischemic hypoxia [20]. VEGF and its receptors VEGFRs play an important role in ocular pathologic angiogenesis, and it has been reported that inhibiting this system is a promising therapeutic strategy for these potentially blinding diseases [21, 22]. In addition, compounds that modulate signaling pathways upstream and downstream of VEGF represent promising anti-angiogenic strategies for vasoproliferative retinal diseases. Rapamycin can oppose the VEGF-induced signaling pathways and also exhibits antiangiogenic effects [23, 24]. The anti-angiogenic effects of rapamycin have been demonstrated in animal models [25, 26]. Amplified HIF-1α and VEGF have been found in the retina of diabetic animals and humans to be involved in the pathophysiology process of DR [9-11]. Nevertheless, how mTOR pathway contributes to pathophysiological process of DR via HIF-1α and VEGF are not fully understood. Data of our current study show that inhibiting of mTOR by rapamycin decreases upregulated VEGFR-2, suggesting that enhancement of mTOR activity in the retina leads to upregulation of VEGFR-2 signaling pathway.

Caspases, a family of thiol proteases, play an important regulating the apoptotic cascade, and high glucose activates retinal caspases [27]. The processing of pro-caspase-3 to its active form is considered a key processing in the death-signaling cascade. Caspase-3 is a predominant target involved in the reactive oxygen species-mediated high glucose induced apoptosis in human endothelial cells [28]. Thus, in the current study, we also determined the levels of Caspase-3 in retinal tissues as an indicator of cellular apoptosis and we found that STZ increased Caspase-3 in the diabetic retina 28 days after induction of hyperglycemia. Moreover, inhibiting retinal mTOR decreased the amplified expression of Caspase-3 evoked by STZ injection.

Prior studies in humans and animal models have demonstrated that cell loss is generally involved in initial diseased tissues in development of the DR [29]. It is indicated that apoptosis is engaged in the pathological process of diabetes-induced tissue loss in DR, and a greater number of cells that are stained with terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) are found in the retina [30, 31]. Also, sequential activation of Caspase-3 plays an important role in the execution-phase of cell apoptosis linked to cell death in DR model. Nevertheless, as an important factor in mediating cell apoptosis the role played by HIF-1α in regulating Caspase-3 expression in engagement of pathophysiological process of DR is lacking. Data of our present study provide the evidence that blocking mTOR...
pathways decreases enhancements of Caspase-3 observed in diabetic retina. Inhibition of the enzymatic activity of Caspase-3 likely provides a mechanism to attenuate the cellular apoptosis. Overall, we suggest that enhancement of HIF-1α, VEGF and VEGFR-2 in the retina contributes to the tissue damage via mTOR pathway and blocking mTOR attenuates VEGF system thereby blunting cellular injury during development of DR.

**Conclusion**

We have provided evidence that STZ-induced diabetes amplifies the levels of HIF-1α, VEGF, VEGFR-2 as well as mTOR and its downstream pathways in the diabetic retina. In the process of STZ-induced DR, mTOR plays an important role in upregulating HIF-1α and VEGF pathway, and this leads to augmented Caspase-3 expression, which is involved in consequent retinal damages in animals. Thus, blocking mTOR has beneficial effects on development of DR. The results may offer promising clues for the developments of new therapeutic strategies to target specific mTOR and HIF-1α-VEGF pathways for managing intractable symptoms observed in patients with DR.

**Disclosure Statement**

None.

**References**

1. Cheung N, Mitchell P, Wong TY: Diabetic retinopathy. Lancet 2010;376:124-136.
2. Li YJ, Jiang Q, Cao GF, Yao J, Yan B: Repertoires of autophagy in the pathogenesis of ocular diseases. Cell Physiol Biochem 2015;35:1663-1676.
3. Qing S, Yuan S, Yun C, Hui H, Mao P, Wen F, Ding Y, Liu Q: Serum miRNA biomarkers serve as a fingerprint for proliferative diabetic retinopathy. Cell Physiol Biochem 2014;34:1733-1740.
4. Lai AK, Lo AC: Animal models of diabetic retinopathy: summary and comparison. J Diabetes Res 2013;2013:106594.
5. Wang GL, Jiang BH, Rue EA, Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA 1995;92:5510-5514.
6. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC: Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 2004;10:858-864.
7. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL: Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998;12:149-162.
8. Kelly BD, Hackett SE, Hirota K, Oshima Y, Cai Z, Berg-Dixon S, Rowan A, Yan Z, Campochiaro PA, Semenza GL: Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1 alpha. Circ Res 2003;93:1074-1081.
9. Catrina SB: Impaired hypoxia-inducible factor (HIF) regulation by hyperglycemia. J Mol Med (Berl) 2014;92:1025-1034.
10. Ling S, Birnbaum Y, Nanhwan MK, Thomas B, Bajaj M, Ye Y: MicroRNA-dependent cross-talk between VEGF and HIF1alpha in the diabetic retina. Cell Signal 2013;25:2840-2847.
11. Wang X, Wang G, Wang Y: Intravitreal vascular endothelial growth factor and hypoxia-inducible factor 1a in patients with proliferative diabetic retinopathy. Am J Ophthalmol 2009;148:883-889.
12. Hay N, Sonenberg N: Upstream and downstream of mTOR. Genes Dev 2004;18:1926-1945.
13 Yagasaki R, Nakahara T, Ushikubo H, Mori A, Sakamoto K, Ishii K: Anti-angiogenic effects of mammalian target of rapamycin inhibitors in a mouse model of oxygen-induced retinopathy. Biol Pharm Bull 2014;37:1838-1842.

14 Holmes K, Roberts OL, Thomas AM, Cross MJ: Vascular endothelial growth factor receptor-2: Structure, function, intracellular signalling and therapeutic inhibition. Cell Signal 2007;19:2003-2012.

15 Stuttfeld E, Ballmer-Hofer K: Structure and function of VEGF receptors. IUBMB Life 2009;61:915-922.

16 Yang W, Yu X, Zhang Q, Lu Q, Wang J, Cui W, Zheng Y, Wang X, Luo D: Attenuation of streptozotocin-induced diabetic retinopathy with low molecular weight fucoidan via inhibition of vascular endothelial growth factor. Exp Eye Res 2013;115:96-105.

17 Edlund J, Hansell P, Fasching A, Liss P, Weis J, Glickson JD, Palm F: Reduced oxygenation in diabetic rat kidneys measured by T2 * weighted magnetic resonance micro-imaging. Adv Exp Med Biol 2009;645:199-204.

18 Bursell SE, Clermont AC, Kinsey BT, Simonson DC, Aiello LM, Wolpert HA: Retinal blood flow changes in patients with insulin-dependent diabetes mellitus and no diabetic retinopathy. Invest Ophthalmol Vis Sci 1996;37:886-897.

19 Kondo T, Kahn CR: Altered insulin signaling in retinal tissue in diabetic states. J Biol Chem 2004;279:1246-1251.

20 Kim Y-W, Byzova TV: Oxidative stress in angiogenesis and vascular disease. Blood 2014;123:625-631.

21 Finger RP, Guymer RH, Gillies MC, Keeffe JE: The Impact of Anti–Vascular Endothelial Growth Factor Treatment on Quality of Life in Neovascular Age-Related Macular Degeneration. Ophthalmol 2014:121:1246-1251.

22 Mintz-Hittner HA, Kennedy KA, Chuang AZ: Efficacy of intravitreal bevacizumab for stage 3+ retinopathy of prematurity. N Engl J Med 2011;364:603-615.

23 Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch KW, Geissler EK: Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002;8:128-135.

24 Hudson CC, Liu M, Chiang GG, Ottermes DM, Loomis DC, Kaper F, Giaccia AJ, Abraham RT: Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin. Mol Cell Biol 2002;22:7004-7014.

25 Dejneka NS, Kuroki AM, Fosnot J, Tang W, Tolentino MJ, Bennett J: Systemic rapamycin inhibits retinal and choroidal neovascularization in mice. Mol Vis 2004;10:964-972.

26 Shi W, Gao H, Xie L, Wang S: Sustained intraocular rapamycin delivery effectively prevents high-risk corneal allograft rejection and neovascularization in rabbits. Invest Ophthalmol Vis Sci 2006;47:3339-3344.

27 Mohr S, Xi X, Tang J, Kern TS: Caspase activation in retinas of diabetic and galactosemic mice and diabetic patients. Diabetes 2002;51:1172-1179.

28 Ho FM, Liu SH, Liau CS, Huang PJ, Lin-Shiau SY: High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3. Circulation 2000;101:2618-2624.

29 Kowluru RA, Koppolu P: Diabetes-induced activation of caspase-3 in retina: effect of antioxidant therapy. Free Radic Res 2002;36:993-999.

30 Barber AJ, Lieth E, Khin SA, Antontelli DA, Buchanan AG, Gardner TW: Neuronal apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 1998;102:783-791.

31 Kern TS, Tang J, Mizutani M, Kowluru RA, Nagaraj RH, Romeo G, Podesta F, Lorenzi M: Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. Invest Ophthalmol Vis Sci 2000;41:3972-3978.