Retinal Not Systemic Oxidative and Inflammatory Stress Correlated with VEGF Expression in Rodent Models of Insulin Resistance and Diabetes

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Purpose. To correlate changes between VEGF expression with systemic and retinal oxidative stress and inflammation in rodent models of obesity induced insulin resistance and diabetes.

Methods. Retinal VEGF mRNA and protein levels were assessed by RT-PCR and VEGF ELISA, respectively. Urinary 8-hydroxydeoxyguanosine (8-OHdG), blood levels of C-reactive protein (CRP), malondialdehyde (MDA), and CD11b/c positive cell ratio were used as systemic inflammatory markers. Retinal expression of Nox2, Nox4, and p47phox mRNA levels were measured as oxidative stress markers. TNF-α, inter-cellular adhesion molecule-1 (ICAM-1), IL1β, and activation of nuclear factor κB (NF-κB) were used as retinal inflammatory markers.

Results. Retinal VEGF mRNA and protein expression increased in Zucker diabetic fatty ($ZDF^{fa/fa}$) rats and streptozotosin (STZ) induced diabetic Sprague-Dawley rats, after two months of disease, but not in Zucker fatty (ZF) rats. Systemic markers of oxidative stress and inflammation were elevated in insulin resistant and diabetic rats. Some oxidative stress and inflammatory markers (TNF-α, IL-6, ICAM-1, and IL1-β) were upregulated in the retina of $ZDF^{fa/fa}$ and STZ diabetic rats after 4 months of disease. In contrast, activation of NF-κB in the retina was observed in high fat fed nondiabetic and diabetic cis-$NF\text{-}κB^{EGFP}$ mice, ZF, $ZDF^{fa/fa}$, and STZ-induced diabetic rats.

Conclusions. Only persistent hyperglycemia and diabetes increased retinal VEGF expression. Some markers of inflammation and oxidative stress were elevated in the retina and systemic circulation of obese and insulin resistant rodents with and without diabetes. Induction of VEGF and its associated retinal pathologies by diabetes requires chronic hyperglycemia and factors in addition to inflammation and oxidative stress. (*Invest Ophthalmol Vis Sci.* 2012;53:8424–8432) DOI: 10.1167/iovs.12-10207

Diabetic retinopathy (DR) is observed in a majority of diabetic patients and is one of the most frequent causes of blindness in the United States.[1,2] Chronic hyperglycemia is thought to be the primary cause of DR, as supported by the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study.[3,4] Sequential pathologic changes in DR include increased vascular permeability, pericyte apoptosis, acellular capillaries, and aberrant retinal new vessel growth, or neovascularization.[5] VEGF is one of the most important endogenous angiogenic or permeability inducing polypeptides that respond to hypoxia under normal physiologic conditions.[6,7] Elevated levels of VEGF in the vitreous have been shown to correlate with the development of diabetic macular edema and proliferative diabetic retinopathy (PDR).[8,9] Further, treatments with VEGF inhibitors by intravitreous injections have been shown to protect visual acuity in diabetic macular edema and to regress neovascularization in PDR.[10,11] These findings have established VEGF elevation in the ocular fluids and tissues as an important validated marker for severe DR.

Previous reports have indicated that the induction of oxidative stress and inflammation by abnormal metabolites induced by diabetes may play a significant role in increasing VEGF expression in the retina of diabetic patients or animals.[12–14] The addition of reactive oxygen species (ROS) and inflammatory cytokines to vascular cells have also been shown to induce VEGF expression.[15] However, it is also well established that obesity-induced insulin resistant states without hyperglycemia will increase oxidative stress and inflammation in many tissues and systemic circulation, yet vascular pathologies of DR are rarely observed in obesity/insulin resistant states without hyperglycemia.[16,17] Thus, it is unclear whether elevation of ROS and inflammation are observed in the retina with obesity/insulin resistance and whether their elevation alone in the retina will increase VEGF expression without hyperglycemia.

In this study, we correlated the changes in systemic and retinal inflammation and oxidative stress with VEGF expression

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in the retina of various rodent models of diabetes and insulin resistance. The results suggested that inflammation or oxidation can occur early in both obesity and diabetes, but chronic exposure to hyperglycemia is essential for elevation of VEGF expression in the retina.

Methods

Animals and Induction of Diabetes

Nuclear factor κB (NF-κB)-dependent enhanced green fluorescent protein (GFP) transgenic mice (cis-NF-κB^{EGFP}) were produced as described previously.[18] Fourteen-week-old male Zucker diabetic fatty rats (ZDF$^{fa/fa}$) were used as models of obesity associated with type 2 diabetes, and Zucker nondiabetic lean rats (ZDF$^{fa/+}$) were used as their age-matched controls (Charles River Laboratories, Wilmington, MA). Six-month-old male Zucker fatty rats (ZF) and their age-matched lean controls (ZL) were used as models of insulin resistance (Charles River Laboratories). Diabetes was induced in C57/B6 mice and Sprague-Dawley (SD) rats by injection of streptozotosin (STZ; 55 mg/kg body weight), in 0.05 M/L citrate buffer (pH 4.5) or citrate buffer for controls, (Sigma-Aldrich, St. Louis, MO). Blood glucose levels were determined 2 days after the injections by glucose analyzer (Yellow Spring Instruments, Yellow Springs, OH). Glycemic levels of greater than 16.7 mM/L were defined as having diabetes. Obesity and insulin resistance in 8-week-old cis-NF-κB^{EGFP} mice were induced by a high fat diet, 42% from fat, for an additional 8 weeks prior to sacrifice (Harlan Teklad, Indianapolis, IN). All procedures involving animals conformed to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research and were approved by the Animal Use and Care Committee of Joslin Diabetes Center.

RT-PCR Analysis for Retinal RNA Expressions

Total RNA were isolated and purified from the retina by RNAeasy micro column with DNase treatment (Qiagen, Inc., Valencia, CA) and cDNA was synthesized using high capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA) or SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). The number of copies of transcript of VEGF (Forward: AGC CAG AAA ATC ACT GTG AGC C; Reverse: TTT AAC TCA AGC TGC CTC GCC; Probe: FAM-TGT TCC TGC AAA AAC ACA GAC TCG CGT TG-TAMRA), Nox2 (Forward: CAG TGC GTG TTG CTC AAC CAG AAT; Reverse: CAA TGG TGT GAA TGG CCG TGT GAA; Probe: FAM-TCG AAG ACA ACT GGA CAG GAA CCT TAC T-TAMRA), tumor necrosis factor-α (TNF-α) (Forward: TCT GTG CCT CAG CCT CTT CTC ATT; Reverse: AAC TGA TGA GAG GGA GCC CAT TTG; Probe: FAM-ATC GGT CCC AAC AAG GAG GAG AAG TT-TAMRA), ICAM-1 (Forward: ACA GCA GAC CAC TGT GCT TTG AGA; Reverse: ACT CGC TCT GGG AAC GAA TAC ACA; Probe: FAM-AGT CCT CGG CTT CTG CCA CCA TCA-TAMRA), and rat IL1β (Forward: TGCAGGC TTCGAGATGAAC; Reverse: GGGATTTTGTCGTTGCTTGTCR) in the retina were measured using Applied Biosystems (ABI) Taqman real-time PCR (Applied Biosystems). The p47phox (Forward: GTG AAG CCA TCG AGG TCA TTC; Reverse: CCC GCG GCT TCT AAT CTG T), Nox4 (Forward: GAA CCT CAA CTG CAG CCT GAT C; Reverse: CTT TTG TCC AAC AAT CTT CTT GTT CTC), IL-6 (Forward: CTT CAA TCC AGT TGC CTT CTT G; Reverse: AAT TAA GCC TCC GAC TTG TGA AG), gapdh (Forward: ATG TTC CAG TAT GAC TCC ACT CAC G; Reverse: GAA GAC ACC AGT AGA CTC CAC GAC A) mRNA expressions in the retina were evaluated by SYBR Green procedure (Applied Biosystems). Amplification and detection were performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems).

Urinary 8-OHdG, Plasma MDA, and Plasma CRP

Urinary 8-Hydroxydeoxyguanosine (8-OHdG) excretion was measured by new 8-OHdG Check ELISA Kit (Nikken Seil Co., Ltd., Shizuoka, Japan). Plasma malondialdehyde (MDA) and plasma C-reactive protein (CRP) concentrations were measured by Lipid Peroxidation Assay Kit (Oxi International, Inc., Foster City, CA) and by CRP, Rat Quantitative Kit (Helica, Fullerton, CA), respectively.

Monocyte Activation in Peripheral Blood

Peripheral blood was drawn from the abdominal large vein by heparinized syringe. Fresh drawn blood was treated by BD Pharm Lyse lysing solution (BD Biosciences, San Diego, CA) for 15 minutes to exclude red blood cells. Isolated leukocytes were used to analyze the monocyte activation by fluorescence-activated cell sorting (FACS) for the two color flow cytometry analysis, mouse PE-conjugated anti-rat CD11b/c monoclonal antibody, PE-conjugated mouse Immunoglobulin (IgG) 2a, κ isotype control (BD Biosciences) were used. Flow cytometry was performed on a BeckmanCoulter XL-MCL (BeckmanCoulter, Fullerton, CA). The percentage of population was calculated by the positive cell number in the total mononuclear cell number. Data were analyzed by Expo32 (BeckmanCoulter).

Immunoblot Analysis, VEGF ELISA, and NF-κB p65 DNA Binding Assay

Nuclear-specific proteins were isolated using the compartmental protein extraction kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Samples were dissolved in 0.5% NP-40, used after optimization studies. Proteins were separated by SDS-PAGE. Blots were subsequently incubated with anti–NF-κB (p65) (Santa Cruz Biotechnologies, Santa Cruz, CA) and anti-PCNA (Cell Signaling, Beverly, MA). Labeled protein bands were identified with enhanced chemiluminescent system (Amersham Biosciences, Piscataway, NJ). VEGF protein was measured by VEGF ELISA kit (R&D Systems, Minneapolis, MN).

Retina was homogenized and extracted protein was used in VEGF ELISA assay. The data were normalized to protein level. NF-κB p65 activation (DNA binding activity) was assessed using NF-κB p65 Transcription Factor Kit as per manufacturer's instructions (Pierce Biotechnology, Rockford, IL).

Data Analysis

The data are expressed as mean ± SD. Comparison among more than two groups was performed by one-way ANOVA followed by the analysis with paired or unpaired *t* test to evaluate statistical significance between the two groups. All analyses were performed using StatView (SAS Institute, Cary, CA). Statistical significance was defined as P less than 0.05.

Results

Physiologic Characteristics of Experimental Groups

Body weights of ZF rats were greater than ZL rats by 2.1 ± 0.6-fold. Plasma insulin, cholesterol, free fatty acids, and triglyceride levels of ZF rats were elevated by 1.7 ± 0.2-fold, 5.3 ± 1.4-fold, 3.0 ± 0.7-fold, and 9.1 ± 3.8-fold, respectively, compared with ZL rats ($P < 0.05$, Table 1). In the ZDF$^{fa/fa}$ rats, a type 2 diabetic model, blood levels of glucose, cholesterol, and triglycerides were increased by 4.5 ± 1.5-fold, 1.8 ± 0.2-fold, and 7.2 ± 2.2-fold, respectively, compared with ZDF$^{fa/+}$ nondiabetic rats ($P < 0.05$, Table 1). After induction of diabetes in Sprague-Dawley rats for 2 months, body weights of diabetic Sprague-Dawley rats were significantly less than the nondiabetic Sprague-Dawley rats by 20 ± 5% and insulin was decreased by 93 ± 10% in diabetic Sprague-Dawley rats compared with nondiabetic Sprague-Dawley rats ($P < 0.05$, Table 1). The final body weights of Sprague-Dawley diabetic rats were still greater than their initial weights indicating that

Table 1. General Characteristics of the Rat Experimental Groups

	ZL	ZF	ZDF$^{fa/+}$ (Lean)	ZDF$^{fa/fa}$ (Diabetic)	Cont. (2M)	STZ (2M)	Cont. (4M)	STZ (4M)
Number	7	7	6	6	5	5	7	7
Body weight, g	400 ± 20	830 ± 130*	385 ± 6	406 ± 5‡	283 ± 6	226 ± 13‖	475 ± 29	355 ± 20#
Blood glucose, g	90 ± 10	99 ± 13	89 ± 11	402 ± 16§	151 ± 5	559 ± 57¶	128 ± 24	473 ± 82**
Insulin, ng/mL	5.2 ± 2.6	36 ± 3†	4.0 ± 0.9	2.4 ± 0.9	4.3 ± 1.6	0.3 ± 0.1¶	ND	ND
FFA, mEq/L	0.4 ± 0.2	1.3 ± 0.1†	ND	ND	ND	ND	ND	ND
T-Cho, mg/dL	57 ± 7	474 ± 50†	44 ± 14	75 ± 37‡	21 ± 12	38 ± 27	ND	ND
TG, mg/dL	131 ± 10	1198 ± 80†	38 ± 2	275 ± 21§	0.9 ± 0.7	2.1 ± 2.3	ND	ND

Cont., nondiabetic Sprague-Dawley rats; ND, not done; FFA, free fatty acids; T-Cho, total cholesterol; TG, triglycerides. The data are expressed as the mean ± SD.

* $P < 0.05$.

†$P < 0.001$ versus ZL rats.

‡ $P < 0.05$.

§$P < 0.001$ versus ZDFfa/+ lean rats.

‖ $P < 0.05$.

¶$P < 0.001$ versus nondiabetic Sprague-Dawley rats (2M).

# $P < 0.05$.

**$P < 0.001$ versus nondiabetic Sprague-Dawley rats (4M).

the difference in body weights were not due to starvation. Blood glucose levels were significantly elevated in diabetic Sprague-Dawley rats at 559 ± 57 mg/dL and 473 ± 82 mg/dL at 2 and 4 months, respectively.

VEGF Expression in the Retina

The expression of VEGF-β mRNA levels were elevated in the retina of diabetic ZDF$^{fa/fa}$, which have had diabetes for 8 weeks compared with nondiabetic ZDF$^{fa/-}$ rats by 1.6 ± 0.2-fold ($P < 0.05$, Fig. 1). Retinal VEGF protein levels were 34.3 ± 12.4 pg/mg protein in diabetic ZDF$^{fa/fa}$ versus 16.2 ± 2.2 pg/mg in nondiabetic control ZDF$^{fa/-}$ rats ($P < 0.05$, Fig. 1B). In contrast, no differences in VEGF-β mRNA or protein expression were detected between ZL and ZF rats. In Sprague-Dawley rats, the induction of diabetes did not significantly increase VEGF-β mRNA expression until after 2 months of diabetes by 1.3 ± 0.3-fold ($P < 0.05$, Fig. 1), which persisted at 4 months of diabetes by 1.4 ± 0.2-fold ($P < 0.05$, Fig. 1). No significant increases in retinal VEGF-β mRNA expression were observed after 1 month of diabetes in Sprague-Dawley rats. Consistent with the mRNA levels, retinal VEGF protein significantly increased at 4 months (diabetic 26.2 ± 3.5 pg/mg versus nondiabetic rats 21.4 ± 4.2 pg/mg ($P < 0.05$, Fig. 1B).

Systemic Oxidative Stress and Inflammation in the Experimental Groups

Systemic markers of oxidative stress and inflammation were assessed in fatty and diabetic rats and compared with their

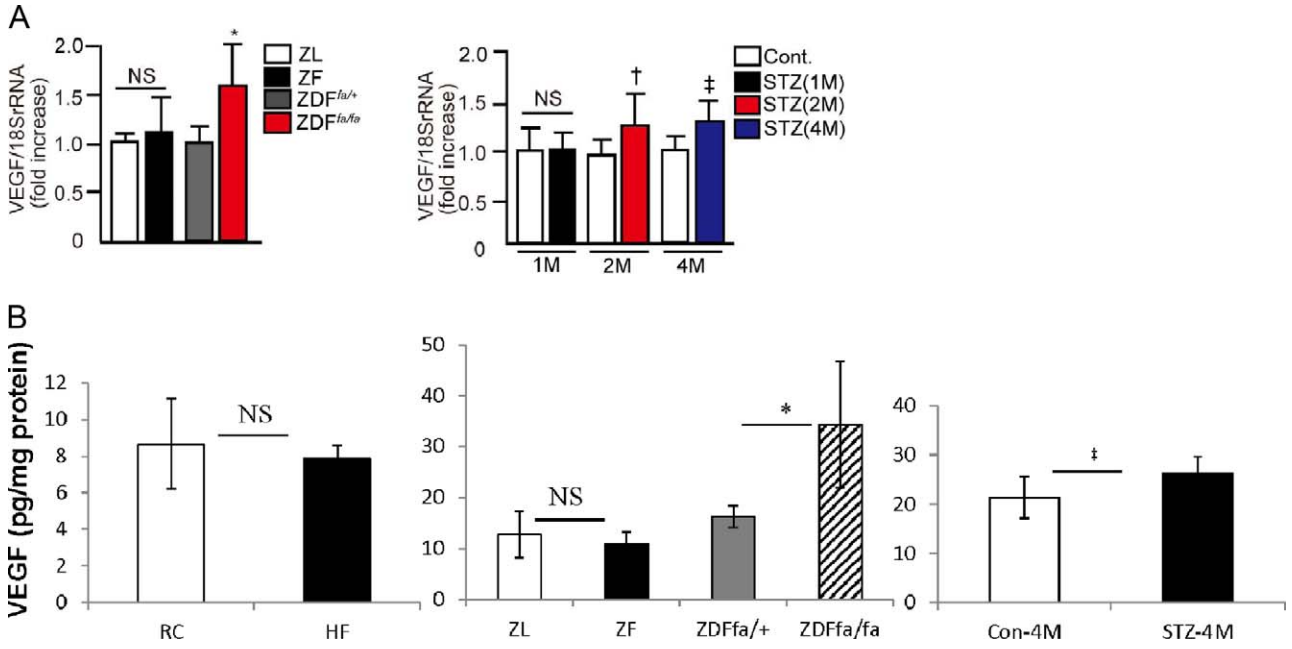

Figure 1. Expression of VEGF mRNA and protein in the retina. VEGF mRNA (**A**) and protein expression (**B**) in the retina of ZL, ZF, ZDF$^{fa/+}$ lean, ZDF$^{fa/fa}$, nondiabetic Sprague-Dawley and STZ-induced diabetic Sprague-Dawley rats after several months of disease duration. *$P < 0.05$ versus ZDF$^{fa/+}$ lean rats. †$P < 0.05$ versus nondiabetic Sprague-Dawley rats (2M). ‡$P < 0.05$ versus nondiabetic Sprague-Dawley rats (4M). One of three independent experiments is shown. These data are expressed as mean ± SD. NS, not significant; STZ, STZ-induced diabetic Sprague-Dawley rats; M, months.

Table 2. Systemic Oxidative Stress and Inflammatory Markers

	ZL	ZF	$ZDF^{fa/+}$ (lean)	$ZDF^{fa/fa}$ (diabetic)	Cont. (2M)	STZ (2M)
Number	7	7	6	6	5	5
MDA, μM	0.7 ± 0.1	1.1 ± 0.3*	1.2 ± 0.5	2.5 ± 0.6†	0.9 ± 0.2	1.6 ± 0.5‡
CRP, mg/mL	303 ± 45	385 ± 83*	334 ± 55	392 ± 55†	252 ± 57	290 ± 70
CD11b/c positive population, %	15.3 ± 1.5	39.7 ± 8.6*	15.6 ± 1.7	29.5 ± 3.8†	13.1 ± 3.5	23.8 ± 6.6‡
Urinary 8-OHdG, ng/d	144 ± 64	381 ± 107*	325 ± 113	2603 ± 1130†	267 ± 119	1188 ± 334‡

The data are expressed as the mean ± SD.

* $P < 0.05$ versus ZL rats.

† $P < 0.05$ versus $ZDF^{fa/+}$ lean rats.

‡ $P < 0.05$ versus nondiabetic Sprague-Dawley rats (2M).

respective controls. Oxidative stress markers as measured by plasma MDA levels were significantly increased in ZF, $ZDF^{fa/fa}$, and diabetic Sprague-Dawley rats with 2 months of diabetes by 1.6 ± 0.3-fold, 2.1 ± 0.8-fold, and 1.7 ± 0.4-fold, respectively, when compared with ZL, $ZDF^{fa/+}$ lean and nondiabetic Sprague-Dawley rat controls ($P < 0.05$, Table 1). Similar to MDA, urinary oxidative stress markers 8-OHdG excretion was also increased in ZF, $ZDF^{fa/fa}$, and diabetic Sprague-Dawley rats with 2 months of diabetes by 2.6 ± 1.2-fold, 8.0 ± 2.1-fold, and 4.4 ± 1.3-fold, respectively, when compared with ZL, $ZDF^{fa/+}$ lean and nondiabetic Sprague-Dawley rats ($P < 0.05$, Table 2).

Several systemic inflammatory markers were assessed including circulating monocyte activation using CD11b/c labeling and plasma CRP levels. Circulating monocytes positive for CD11b/c, as measured by FACS, were increased in ZF, $ZDF^{fa/fa}$, and diabetic Sprague-Dawley rats by 1.9 ± 0.4-fold, 2.6 ± 0.2-fold, and 1.8 ± 0.5-fold, respectively, when compared with ZL, $ZDF^{fa/+}$ lean and nondiabetic Sprague-Dawley rats with 2 months of diabetes ($P < 0.05$, Table 1). Similarly, plasma levels of CRP were also increased in ZF and $ZDF^{fa/fa}$ rats by 1.3 ± 0.5-fold and 1.2 ± 0.1-fold, respectively, when compared with ZL and $ZDF^{fa/+}$ lean rats. In contrast, plasma CRP levels were not significantly increased in Sprague-Dawley rats with 2 months of diabetes compared with Sprague-Dawley nondiabetic rats ($P < 0.05$, Table 1).

Characterization of Inflammatory Markers in the Retina

Markers of inflammation in the retina with the induction of diabetes or insulin resistant states were characterized. Expression of TNF-α mRNA levels were not increased in the retina of ZF rats when compared with ZL rats after 2 months on the fatty diet. In contrast, TNF-α mRNA levels were elevated in the retinas of $ZDF^{fa/fa}$ rats compared with nondiabetic $ZDF^{fa/-}$ rats. For Sprague-Dawley rats, the induction of diabetes by STZ for 1 and 2 months did not increase TNF-α mRNA levels in the retina, but, 4 months of diabetes did increase its levels by 3.0 ± 1.4-fold ($P < 0.05$, Fig. 2A). Similarly, IL-6 mRNA expression was not increased in the retina of ZF rats after 1 month of diabetes, but it did elevate significantly in the retina of $ZDF^{fa/fa}$ rats, after 2 and 4 months of diabetes and in Sprague-Dawley rats by 2.8 ± 0.3-fold and 9.0 ± 1.5-fold, respectively ($P < 0.05$, Fig. 2B). ICAM-1 mRNA expression was not increased in the retina of ZF rats, or after 1 month and 2 months of diabetes in Sprague-Dawley rats. However, ICAM-1 mRNA expression was increased in the retina of diabetic $ZDF^{fa/fa}$ rats and Sprague-Dawley rats after 4 months of diabetes by 2.8 ± 1.3-fold and 5.3 ± 1.8-fold, respectively ($P < 0.05$, Fig. 2C). Auto-inflammatory marker IL1β mRNA increased significantly after 4 months of diabetes in Sprague-Dawley rats ($P < 0.05$, Fig. 2D). Retinal IL1β mRNA levels did not differ between ZL and ZF rats. In contrast, levels of IL1β mRNA were increased by 1.6 ± 0.3-fold in diabetic $ZDF^{fa/fa}$ rats versus controls ($P < 0.05$, Fig. 2D).

Evaluation of Oxidative Stress Markers in the Retina

Expression of mRNA levels of NOX2, NOX4, and p47phox, assessed in the retina were not changed in insulin resistant ZF rats compared with their ZL controls. In contrast, the mRNA levels of NOX2, NOX4, and p47phox were elevated significantly by 2.5 ± 0.4-fold, 1.3 ± 0.1-fold, and 2.2 ± 0.7-fold in $ZDF^{fa/fa}$ compared with nondiabetic $ZDF^{fa/wt}$ rats. In diabetic Sprague-Dawley rats, mRNA levels of NOX2 and NOX4 were not significantly elevated until 4 months of diabetes. For p47phox mRNA levels, significant elevations of 1.5 ± 0.6-fold and 3.1 ± 1.2-fold were noted in the retina of diabetic Sprague-Dawley rats after 2 and 4 months of diabetes, respectively, but not after 1 month of disease.

Involvement of NF-κB Activation in the Retina

Previous reports have indicated that both increase in inflammatory cytokines and oxidants are associated with NF-κB activation, especially with diabetes or insulin resistance.[12,19] In addition, high glucose levels can activate NF-κB in retinal vascular cells.[20] Thus, we evaluated whether the expression of p65 subunit of NF-κB, a marker of its activation, was increased in the retina of various diabetic and insulin resistant rodent models. Immunoblot study showed increases in p65 subunit of NF-κB in the retina of ZF rats, $ZDF^{fa/fa}$ rats, and diabetic Sprague-Dawley rats of 4 months duration by 3.6 ± 0.3-fold, 4.6 ± 0.4-fold, and 6.4 ± 0.2-fold, respectively, when compared with ZL, $ZDF^{fa/+}$ lean, and nondiabetic Sprague-Dawley rats ($P < 0.05$, Fig. 4A). We also evaluated the effect of insulin resistance and diabetes in cis-$NF\text{-}\kappa B^{EGFP}$ mice, which will increase GFP expression in tissues that activated NF-κB.[21] After 2 months of high fat feeding to induce obesity without diabetes or 2 months of STZ-induced diabetes, GFP positive areas in the retina of cis-$NF\text{-}\kappa B^{EGFP}$ mice increased by 5.8 ± 2.8-fold and 5.6 ± 2.0-fold, respectively ($P < 0.05$, Fig. 4B). The findings of NF-κB activation were further confirmed by the measurement of NF-κB DNA binding activity, which were significantly increased in high fat feeding mice, ZF and $ZDF^{fa/fa}$ compared with their respective control groups ($P < 0.05$, see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10207/-/DCSupplemental). These results suggest that both diabetes and insulin resistance can activate NF-κB in the retina.

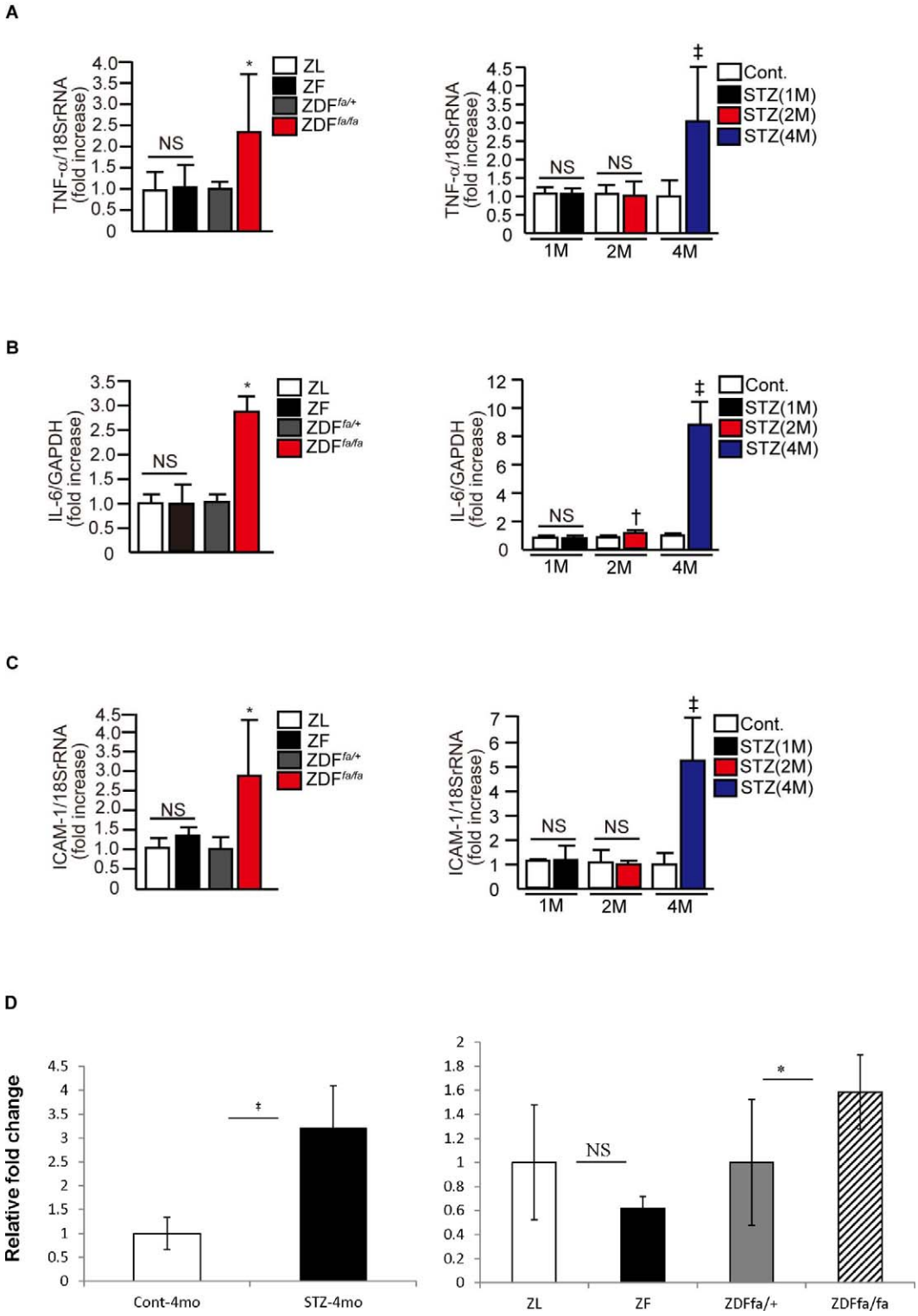

Figure 2. Evaluation of retinal inflammation. Effect of diabetes and insulin resistance on the levels of various inflammatory markers in the retina. TNF-α mRNA (**A**), IL-6 (**B**), ICAM-1 (**C**), and IL1β (**D**) expressions in the retina of ZL, ZF, ZDF$^{fa/+}$ lean, ZDF$^{fa/fa}$, nondiabetic Sprague-Dawley, and STZ-induced diabetic Sprague-Dawley rats. $^{*}P < 0.05$ versus ZDF$^{fa/+}$ lean rats. $^{\dagger}P < 0.05$ versus nondiabetic Sprague-Dawley rats (2M). $^{\ddagger}P < 0.05$ versus nondiabetic Sprague-Dawley rats (4M). Results of three independent experiments are shown. These data are expressed as mean ± SD.

Discussion

This study correlated, for the first time, changes in systemic and retinal indices of oxidative stress and inflammation associated with either diet induced insulin resistance or diabetes on retinal VEGF expression. Past studies have focused mainly on the activation of these pathways by diabetes alone and their correlation to increasing retinal VEGF expression, which is a pathogenic marker for diabetic macular edema and proliferative retinopathy.[2,8,9,22–25] The elevated expression of

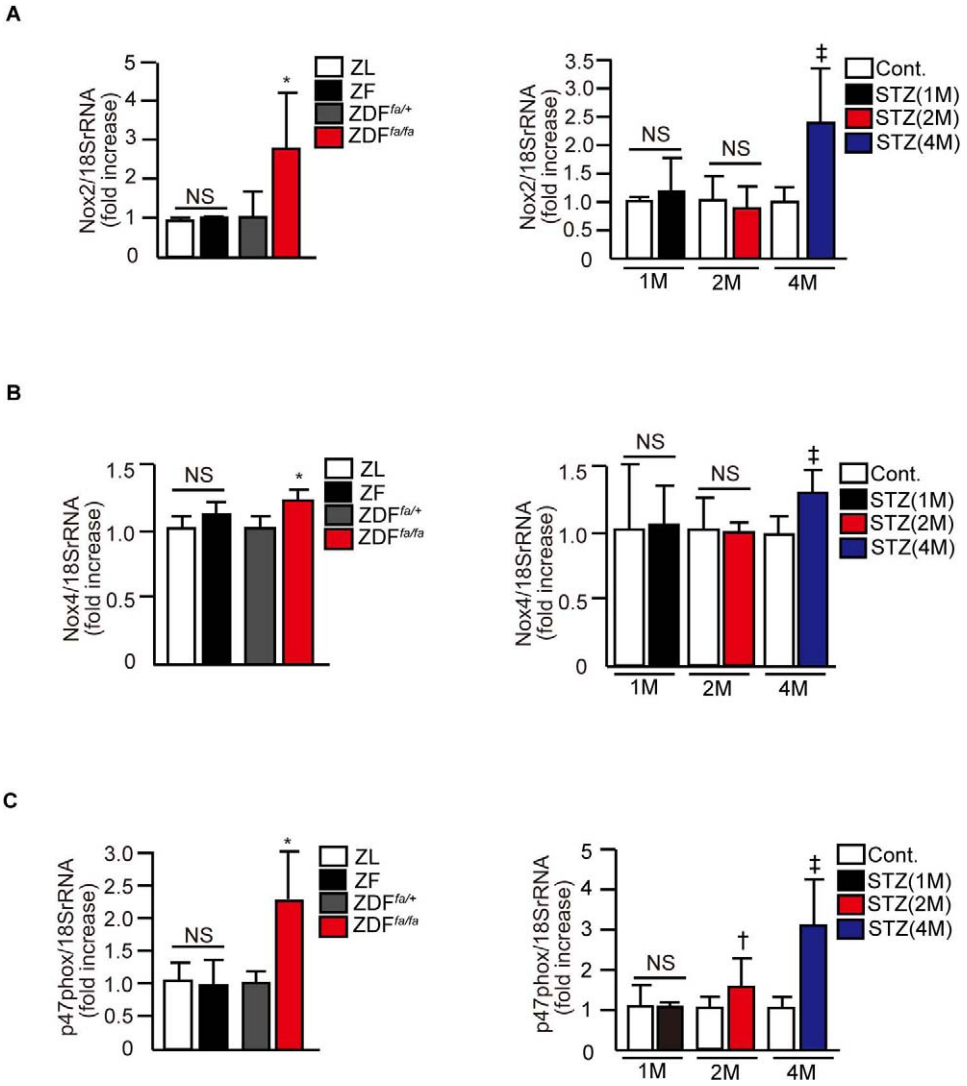

Figure 3. Evaluation of oxidative stress in the retina. Characterization of changes in mRNA levels of Nox2 (**A**), Nox4 (**B**), and p47phox (**C**) expressions in the retina of ZL, ZF, ZDF$^{fa/+}$ lean, ZDF$^{fa/fa}$, nondiabetic Sprague-Dawley, and STZ-induced diabetic Sprague-Dawley rats. $^{*}P < 0.05$ versus ZDF$^{fa/+}$ lean rats. $^{\dagger}P < 0.05$ versus nondiabetic Sprague-Dawley rats (2M). $^{\ddagger}P < 0.05$ versus nondiabetic Sprague-Dawley rats (4M). Results of three independent experiments are shown. These data are expressed as mean ± SD.

VEGF in the retina or vitreous is the only retinal molecular marker of DR, which has been validated by clinical trials using anti-VEGF therapy.[10,11] Multiple studies have associated changes in oxidative stress and inflammation with various retinal changes including elevated VEGF expression in DR, in animal models of diabetes, or in retinal vascular cells exposed to elevated glucose levels.[13,14,24–28] However, it has not been established that these changes are essential or additive for inducing elevated retinal VEGF expression by diabetes in vivo.

The results from this comparative analysis in rodent models of diabetes and obesity associated insulin resistance showed that VEGF expression in the retina was only induced by diabetes, but not by obesity or insulin resistant states alone where hyperglycemia is not present. Surprisingly, the increases in VEGF expression were not observed until after 2 months of diabetes and were not significant in obese rats (ZF) without diabetes even though systemic markers of oxidative stress and inflammation were clearly elevated. The finding of elevated systemic inflammation markers and oxidative stress in insulin resistant states and diabetes are not surprising since obesity, hyperlipidemia, and elevated free fatty acids, in addition to hyperglycemia, have all been reported to induce the expression of inflammatory cytokines, toll-like receptors, and oxidant formation in many tissues including the blood vessels.[16,29,30] We only studied ZF rats at 24 to 26 weeks, which may not be long enough to detect changes in retinal VEGF expression. However, it is likely that obesity and insulin resistance without diabetes will cause significant VEGF elevation since it is very rare that obese patients with metabolic syndrome, but without diabetes develop macular edema or proliferative diabetic retinopathy.

Elevation of retinal VEGF expression correlated only with increased levels of inflammatory cytokines and oxidases in the retina after they persisted for 2 to 4 months. This is clearly supported by the elevation of all the indicators of oxidative stress and inflammation in ZDF rats and STZ diabetic rats only after 4 months of disease duration. At 2 months of disease duration, some evidence of increased systemic oxidative stress and inflammation were present such as the elevation of p47phox in the retina, which correlated with increases in VEGF expression. In contrast to measurements of inflammatory cytokines and oxidative stress, diabetes was able to induce

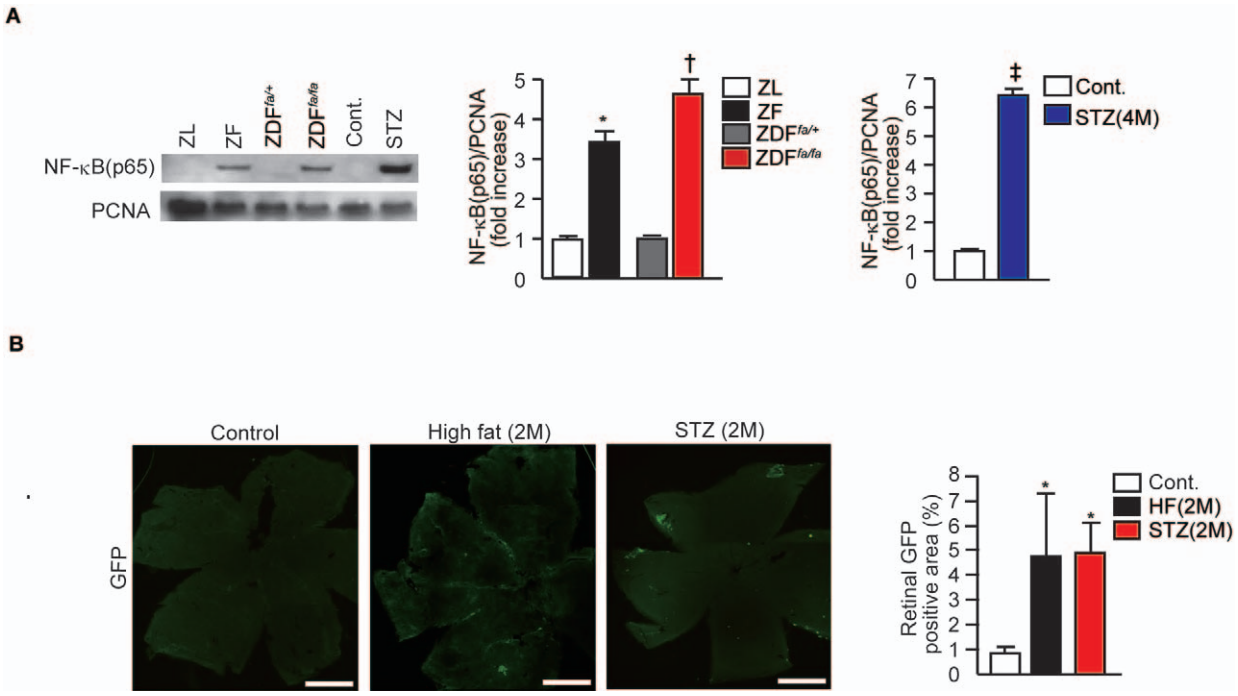

Figure 4. Activation of NF-κB in the retina of insulin resistant and diabetic rodents. (**A**) Representative immunoblots of NF-κB (p65) from retinal nuclear proteins of ZL, ZF, ZDF$^{fa/+}$, diabetes ZDF$^{fa/fa}$, control, and STZ diabetic Sprague-Dawley rats. Data from three experiments were quantitated by densitometry. $^{*}P < 0.05$ versus ZL rats. $^{\dagger}P < 0.05$ versus ZDF$^{fa/+}$ lean rats. $^{\ddagger}P < 0.05$ versus nondiabetic Sprague-Dawley rats (4M). Gel is from one of three independent experiments. These data are expressed as mean ± SD. (**B**) Activation of NF-κB in the retina of cis-NF-κB^{EGFP} transgenic mice. cis-NF-κB^{EGFP} mice were fed a high fat diet for two months or had diabetes induced by STZ for two months, EGFP fluorescence was assessed using digital fluorescence microscopy. Retina was digitally photographed, and the images were imported into Image-J software (National Institutes of Health) and analyzed morphometrically. $^{*}P < 0.05$ versus control mice (2M). One of three independent experiments is shown. The results from three independent experiments were quantitated and expressed as mean ± SD. HF, high fat feeding mice.

NF-κB activation in the retina as measured by both the binding and activation assays and NF-κB–GFP expression at 2 and 4 months of diabetes and in ZDF rats. However, our results clearly showed that insulin resistance and obesity without diabetes and hyperglycemia also activated NF-κB and possibly inflammation in the retina and shown in Figure 4 using ZF rats and high fat fed mice for 2 months. It is not surprising that insulin resistance or obesity could also induce inflammatory changes in the retina, since, it was previously reported that increases in leucocyte adhesion can be observed in both the retinal and systemic vessels in obese and insulin resistant animals similar to those observed in diabetes.[14,31,32] These increases in leucocyte adhesion are correlated with the elevation of ICAM expression or inflammation markers.[33,34] Thus, it is possible that increases in inflammation or oxidative stress alone are not sufficient to induce VEGF expression in vivo, without the presence of additional severe capillary endothelial dysfunction to induce hypoxia or decrease retinal perfusion induced by diabetes. These data also suggest that diabetes or hyperglycemia is activating other pathologic changes besides NF-κB, inflammation, or oxidative stress that will lead to capillary dysfunction and acellular capillaries. This conclusion is supported by clinical data, which clearly indicate that specific vascular lesions of diabetic retinopathy are rarely detected in people with obesity and severe insulin resistance without diabetes, although they clearly can manifest inflammation markers and oxidative stress.[17,18] In addition, there are multiple diseases, which cause inflammation of the vasculature, yet they don't manifest retinal vascular lesions similar to DR.[35] These findings strongly support the idea that hyperglycemia or diabetes is essential for the development of DR, and its progression requires hyperglycemia to activate mechanisms in addition to inflammation and oxidative stress. It is also possible that other metabolic changes induced specifically by diabetes could also play a role with hyperglycemia to induce changes in VEGF expression.

The presence of hyperglycemia in inducing retinal VEGF expression is clearly very important, which is likely due to its adverse effects on retinal pericytes and endothelial cells to cause pericyte apoptosis, acellular capillaries, and reduction in retinal perfusion.[5,20,35–38] However, unlike pericyte apoptosis, the formation of acellular capillaries may not be specific to DR since other inhibitors such as ischemic reperfusion may also induce acellular capillary formation. Previously, we have reported ZF rats, derived from a different rodent colony, which had mean plasma glucose levels that were significantly higher, by 50%, than its control ZL rats, had significant increases in VEGF expression.[39] In contrast, the mean plasma glucose levels in the present study were not different between ZL and ZF rats, resulting in the lack of VEGF levels being elevated. These data again supported the necessity of hyperglycemia to cause significant retinal vascular pathologies in order to induce VEGF expression in the retina. We have reported that one potential mechanism to induce pericyte dysfunction and apoptosis, independent of oxidative and inflammatory stress, is the activation of SHP-1 by hyperglycemia.[40] The activation of SHP-1, a tyrosine phosphatase, which can be induced by hyperglycemia and PKC-δ, inhibited the actions of platelet derived growth factor, a pericyte survival factor, causing pericyte apoptosis by mechanisms independent of inflammation, oxidative stress and NF-κB.[40]

In summary, both hyperglycemia and obesity related insulin resistance can elevate systemic and retinal oxidative stress and inflammation. However, hyperglycemia or diabetes are necessary to increase retinal VEGF expression, possibly by activating additional mechanisms that can induce significant capillary pathology, hypoperfusion, and hypoxia. Thus, future effective therapeutic interventions for diabetic retinopathy will require neutralizing hyperglycemia's multiple adverse effects in addition to anti-oxidative and anti-inflammatory agents.

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