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## Functional Deficits Precede Structural Lesions in Mice With High-Fat Diet–Induced Diabetic Retinopathy

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Obesity predisposes to human type 2 diabetes, the most common cause of diabetic retinopathy. To determine if high-fat diet-induced diabetes in mice can model retinal disease, we weaned mice to chow or a high-fat diet and tested the hypothesis that diet-induced metabolic disease promotes retinopathy. Compared with controls, mice fed a diet providing 42% of energy as fat developed obesity-related glucose intolerance by 6 months. There was no evidence of microvascular disease until 12 months, when trypsin digests and dye leakage assays showed high fat-fed mice had greater atrophic capillaries, pericyte ghosts, and permeability than controls. However, electroretinographic dysfunction began at 6 months in high fat-fed mice, manifested by increased latencies and reduced amplitudes of oscillatory potentials compared with controls. These electroretinographic abnormalities were correlated with glucose intolerance. Unexpectedly, retinas from high fat-fed mice manifested striking induction of stress kinase and neural inflammasome activation at 3 months, before the development of systemic glucose intolerance, electroretinographic defects, or microvascular disease. These results suggest that retinal disease in the diabetic milieu may progress through inflammatory and neuroretinal stages long before the development of vascular lesions representing the classic hallmark of diabetic retinopathy, establishing a model for assessing novel interventions to treat eye disease.

Despite new approaches, diabetic retinopathy (DR) continues to ravage vision (1). Vascular endothelial growth factor (VEGF) plays a definitive role in the disease, and its antagonism with neutralizing molecules represents an authentic advance in diabetes care (2,3). Yet, in practice and in clinical trials, anti-VEGF agents are not always efficacious, require frequent administration, and may have adverse effects (4). VEGF antagonism in mice may disrupt long-term visual function (5). Other therapies are destructive or are associated with serious adverse effects (6). The growing rate of diabetic visual complications represents an unmet need. The development of novel treatments is limited by a surprising lack of relevant models for retinopathy in obesity-related type 2 diabetes and an incomplete understanding of the sequential evolution of the disease.

Rodent models of DR can be simplified into those that are spontaneous due to genetic background (including the db/db and Ins2<sup>Akita</sup> mice and the ZDF and GK rats) (7,8) and those that require induction, such as models using the  $\beta$ -cell toxin streptozotocin (STZ) (9). STZ can be used in disease-free animals and acts quickly. A disadvantage is that this drug is a potent alkylating agent, with off-target effects that include immunosuppression (10) and toxicity to the kidney (11), liver (12), and brain (13–15). Sometimes considered to model human type 1 diabetes, mice given STZ fail to reproduce key features of the disease. Notably, humans with type 1 diabetes die without insulin; STZ-treated mice do not require insulin for survival.

Compared with the STZ-administered mouse, the Ins2<sup>Akita</sup> mouse is more reflective of the physiology of type 1 diabetes. However, this model is the subject of variable reports regarding eye phenotypes, ranging from mild changes (16) to advanced abnormalities that include neovascular features (7,17). The widely used rodent type 2

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the work is not altered.

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diabetes model, db/db, is caused by a leptin-receptor mutation. However, leptin signaling affects the retina (18), suggesting that these animals are not an ideal model for obesity-related DR.

A high-fat diet (HFD) promotes obesity, insulin resistance, and diabetes in rodents (19). Curiously, although some studies have used HFDs to study eye disease, we are unaware of any linking HFD-induced diabetes to retinal microvascular disease. We studied retinal structure and function over time to determine if diabetes induced by feeding without administration of a potential neurotoxin could model retinal disease in mice. Our findings show that HFD feeding produces retinopathy characterized by sequential inflammation, neurologic dysfunction, and then vascular dysfunction.

#### **RESEARCH DESIGN AND METHODS**

#### Animals

Protocols followed the Association for Research in Vision and Ophthalmology Statement for the Use of Animals and were approved by Washington University in St. Louis. C57BL/6J mice were free of RD1 or RD8 mutations. Animals were weaned onto Purina 4043 chow (13% kcal from fat, 62% kcal from carbohydrate, 25% kcal from protein) or Harlan Teklad TD 88137 HFD (42% kcal from fat, 43% kcal from carbohydrate, and 15% kcal from protein).

#### **Antibodies and PCR Primers**

For Western blotting, we used monoclonal rat IgG against mouse NACHT, LRR, and PYD domain-containing protein (NALP3, encoded by the Nlrp3 gene and also referred to as pyrin domain-containing protein 3 [NLRP3]) (cat. no. MAB7578, R&D Systems, Minneapolis, MN), polyclonal goat IgG against mouse interleukin (IL)-1B (cat. no. AF-401-NA, R&D Systems), polyclonal rabbit IgG against mouse caspase-1 p10 (M20) subunit (cat. no. sc-514, Santa Cruz Biotechnology, Dallas, TX), polyclonal rabbit IgG against phosphorylated Thr183/Thr185 c-Jun N-terminal kinase (JNK) (cat. no. 9251, Cell Signaling Technology, Beverly, MA), and polyclonal rabbit IgG against JNK/stress activated protein kinase (cat. no. 9252, Cell Signaling Technology). For immunostaining, anti-NALP3 was used at 1:100, anti-adaptor protein apoptosis-associated specklike protein containing CARD (ASC) (cat. no. AL177, Adipogen Corporation, San Diego, CA) was used at 1:200, and anti-Iba1 (cat. no. 019-19741, Wako, Richmond, VA) was used at 1:400. PCR primers were as follows:

- *Vegf-a*: sense 5'-AATGCTTTCTCCGCTCTGAA-3', antisense 5'-GCTTCCTACAGCACAGCAGA-3'
- *Icam-1*: sense 5'-AACAGTTCACCTGCACGGAC-3', antisense 5'-GTCACCGTTGTGATCCCTG-3'
- *Gfap*: sense 5'-TTTCTCGGATCTGGAGGTTG-3', antisense 5'-AGATCGCCACCTACAGGAAA-3'
- *Gapdh*: sense 5'-TGCACCCCAACTGCTTAGC-3', antisense 5'-GGCATGGACTGTGGTCATGAG-3'

- $\beta$ -actin: sense 5'-TCCATCATGAAGTGTGACGT-3', antisense 5'-GAGCAATGATCTTGATCTTCAT-3'
- *Rpl32*: sense 5′-GGCTTTTCGGTTCTTAGAGGA-3′, antisense 5′-TTCCTGGTCCACAATGTCAA-3′

Gene expression data were normalized to the mean of Gapdh,  $\beta$ -actin, and ribosomal protein L32.

#### **Metabolic Parameters**

Body composition was assessed by MRI (3-in-1 instrument; EchoMRI, Houston, TX). Glucose and insulin tolerance tests were performed after a 6-h fast in animals housed with hardwood bedding and treated intraperitoneally with 1 g/kg dextrose or 0.75 units/kg regular human insulin. Glucose was measured 10, 30, 60, and 120 min later.

#### **Retinal Morphologic Assay**

Eyes from male and female mice were fixed in 4% paraformaldehyde at 4°C for 48 h, embedded in paraffin, sectioned into 4- $\mu$ m slices, and stained with hematoxylin and eosin. For trypsin digests, isolated retinas were washed overnight with distilled water at 37°C and then incubated with 1% porcine trypsin in 1 mol/L Tris-EDTA buffer (pH 7.5) for 3 h. Neuroglial elements were washed away by dripping tap water at 25°C. Remaining vascular skeletons were air dried, incubated with 10% formalin at 25°C for 10 min, stained with periodic acid Schiff base and hematoxylin, and then mounted.

#### **Vascular Permeability Assays**

Under anesthesia, Evans Blue (EB) dye (25 mg/kg of 1% solution) was injected into the left iliac vein with a 32-gauge needle and allowed to circulate for 180 min. Mice were killed, and whole blood was removed from the left ventricle with a 29-gauge needle. Plasma was isolated at 10,000g for 10 min with removal of 10  $\mu$ L of the supernatant, which was diluted 1:100 in formamide. From enucleated globes, 3 µL of aqueous fluid was removed from the anterior chamber with a 10-µL capacity Hamilton syringe and diluted into 97 µL of formamide. Samples were incubated in formamide at room temperature overnight. EB absorbance was determined by subtracting absorbance at 740 nm from that at 620 nm. The results were generated by comparison with a standard curve, normalized to the plasma concentration, and expressed as ratios between aqueous solution and plasma. Retinas were then isolated from remaining fixed globes, flat mounted, and imaged with a Leica DMI4000B inverted fluorescence microscope. Fluorescein angiography was performed by administering sodium fluorescein (5 mg/kg of 10% w/v; cat. no. 17478-253-10, Akorn, Lake Forest, IL) to anesthetized 6- or 12-month-old animals. Fundus photographs were obtained 2 min later with a Micron III camera (Phoenix, Pleasanton, CA).

#### Electroretinography

A UTAS BigShot System (LKC Technologies, Inc., Gaithersburg, MD) was used. Mice (≥5 for each group) were dark adapted

overnight. Under red light illumination, animals were anesthetized with ketamine (80 mg/kg total body mass) and xylazine (15 mg/kg lean body mass). Pupils were dilated with 1% atropine sulfate. Body temperature was maintained at  $37^{\circ}$ C with a heating pad. Contact lens electrodes were placed bilaterally with appropriate reference and ground electrodes.

The stimulus consisted of a full-field white light flash (10 µs) in darkness or in the presence of dim  $(30.0 \text{ candela } [cd]/m^2)$  background illumination after 10 min adaptation time. The response was recorded over 231 ms plus 25 ms of pretrial baseline. Between 5 and 10 repeated trials were averaged for each luminance, with 10 repeats used for the dimmest flashes, and 5 for the brightest. Raw data were processed using MATLAB software (MathWorks, Natick, MA). The amplitude of the a-wave was measured from the average pretrial baseline to the most negative point of the average trace, and the b-wave amplitude was measured from that point to the highest positive point, after subtracting oscillatory potentials (OPs). The eye with the larger recorded b-wave amplitude was used for each mouse. OPs were isolated using a digital Butterworth 25 Hz high-pass filter and quantified using root-mean-square analysis of filtered waves, normalized to the maximal b-wave amplitude. The log luminance of the stimulus  $(\log [cd \cdot s/m^2])$  was calculated based on the manufacturer's calibrations.

#### **Statistical Analysis**

Distributions of electroretinography (ERG) responses across diet groups at different light intensities are described by means  $\pm$  SEM. Differences were compared using two-way ANOVA, followed by a Bonferroni post hoc test for multiple comparisons.

#### RESULTS

#### HFD Feeding Produces a Type 2 Diabetes Phenotype

HFD feeding accelerates weight gain and adiposity (19). Significant gains in total weight in mice fed the HFD compared with controls were measured at 6 months. This difference progressed through 12 months of age (Fig. 1A). Nearly all of the weight difference between mice on chow and the HFD was due to increased adiposity (Fig. 1B and C). Animals fed the HFD developed abnormal glucose metabolism by 6 months of age that persisted through 12 months of age, as assessed by glucose tolerance tests (Fig. 1D-G) and insulin tolerance tests (Fig. 1H).

Animals fed the HFD developed hyperinsulinemia that peaked at 6 months of age and was less marked by 12 months (Fig. 1*I*). Consistent with other models of HFD-induced obesity, this pattern likely reflects initial  $\beta$ -cell hypersecretion, followed by  $\beta$ -cell dysfunction and loss mirroring the progression of human type 2 diabetes.

#### Gross Retinal Morphology Is Preserved After Prolonged HFD-Induced Diabetes

STZ induces thinning of the inner retina as soon as 2 months after diabetes induction (20). To determine if HFD-induced diabetes promotes inner retinal cell loss, we compared cross-sectional retinal thickness between chow-fed and HFD-fed mice at 12 months of age. As shown in Fig. 2A and B, there were no apparent changes in total retinal thickness or in the thickness of any individual retinal laminae. In the juxtapapillary retina, maximal total thickness was 235.6  $\pm$  66.3  $\mu$ m (mean  $\pm$  SD) in the chow group and 229.9  $\pm$  70.9  $\mu$ m in the HFD group (P > 0.99). Maximal juxtapapillary inner nuclear layer thickness was 34.8  $\pm$  10.4  $\mu$ m in the chow group and 30.5  $\pm$  13.2  $\mu$ m in the HFD group (*P* > 0.99). These retinal layers were quantified at various distances from the optic nerve in dorsal (negative distances) and ventral (positive distances) directions, as shown in Fig. 2C and D. There were no differences in any of the major retinal laminae maximal thicknesses, including the retinal ganglion cell layer (12.0  $\pm$  5.1  $\mu$ m chow vs. 11.5  $\pm$  3.7  $\mu$ m HFD, *P* > 0.99), the inner plexiform layer (54.6  $\pm$  21.2  $\mu$ m chow vs. 48.2  $\pm$  9.6  $\mu$ m HFD, P > 0.99), the outer plexiform layer (15.8  $\pm$  6.0  $\mu$ m chow vs. 16.0  $\pm$  5.5 µm HFD, *P* > 0.99), or the outer nuclear layer (59.3  $\pm$  17.2  $\mu m$  chow vs. 55.4  $\pm$  21.2  $\mu m$  HFD, P >0.99). These results differ from those obtained with animals given STZ but are consistent with human data showing no thinning of the inner nuclear retina in early diabetes (21,22). Therefore, the HFD-fed mouse may model human DR in its early stages, when interventions are likely to provide benefit, with greater fidelity than in the mouse given STZ.

#### Time-Dependent Retinal Vascular Damage Is Associated With HFD-Induced Diabetes

Vascular stigmata of human nonproliferative DR that are visible by ophthalmoscopy, such as microaneurysms, dot-blot hemorrhages, venous beading, and intraretinal microvascular anomalies, are absent in most rodent retinopathy models. Histopathologic features, such as capillary basement membrane thickening, loss of pericytes, and atrophy of small capillaries, are present in diabetic animals (23). To determine whether animals fed an HFD develop vascular lesions typical of rodent diabetes models, we analyzed trypsin-digested skeletons of microvessels. Digested retinas from chow- or HFD-fed animals were flat mounted and stained as shown in Fig. 3A. A masked observer scored the number of atrophic capillaries or pericyte ghosts per high-power field (Fig. 3B). There were no differences in the mean number of atrophic capillaries or pericyte ghosts between HFD-fed animals and chow-fed controls at 6 months of age (Fig. 3C and D). At 12 months of age, however, HFD animals showed more atrophic capillaries (P < 0.001) and pericyte ghosts (P < 0.01) compared with controls.



**Figure 1**—HFD-fed mice develop obesity and glucose intolerance. Male C57BL/6J mice were fed standard chow or an HFD providing 42% of energy from fat. *A*: HFD-fed mice develop significant increases in body mass compared with chow-fed controls beginning at 6 months of age. *B*: Whole-body adiposity was measured using MRI, showing that mice fed the HFD have significantly more fat mass compared with controls beginning at 6 months. *C*: Measurements from MRI demonstrate mild increases in lean mass in HFD-fed animals at 6 months but not at 12 months of age. Traces from intraperitoneal GTTs performed after 6 h of fasting at 3 (*D*), 6 (*E*), and 12 (*F*) months of age are shown. *G*: Calculated AUC for GTTs at each indicated age. *H*: Mean  $\pm$  SEM were calculated for AUCs of intraperitoneal insulin tolerance tests performed on animals fed each diet at the indicated ages. *I*: Insulin levels from fasting serum samples were measured using an ELISA-based method at 3, 6, and 12 months of age. Values represent mean  $\pm$  SEM from at least five independent experiments in each group. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\**P* < 0.0001. Mos, months.

#### Permeability of Retinal Vessels Is Increased by HFD Feeding

The EB dye normally stays within the plasma compartment for hours. In experimental diabetes, abnormal EB leakage is readily detected in the neural retina and vitreous (24). To determine whether HFD-fed animals develop disruptions in the blood-retina barrier (BRB) characteristic of DR, we used a modified EB dye leakage assay. At 3 h after dye circulation, we determined BRB disruption by the presence of blue color within the anterior chamber of enucleated globes and in flatmounted retinas. As shown in Fig. 4A, the anterior chambers of 12-month-old animals fed the HFD were bluer than those of age-matched chow-fed controls after an identical period of dye circulation. Retinal flat mounts from HFD-fed or chow-fed animals demonstrated distinctive dye patterns using fluorescence

microscopy. In chow-fed animals, EB was mostly detected in the microvascular space with discrete patterns of fluorescence (Fig. 4B and D). In older animals fed the HFD, we detected a more diffuse, "ground-glass" background fluorescence indicative of EB leakage (Fig. 4C and E). HFD-fed animals had more EB leakage into the aqueous compartment than controls at 12 months (P < 0.05) but not 6 months (Fig. 4F). An independent technique, fluorescein angiography, showed patterns consistent with increased leakage in HFD-fed mice compared with chow-fed controls in 12-month-old animals (Supplementary Fig. 1), suggesting vascular leakage in vivo with HFD feeding. Dye leakage at 12 months in HFD-fed animals coincided with elevations in typical markers of retinal endothelial damage and gliosis, consistent with other models of DR (Fig. 4*G*–*I*).



**Figure 2**—Animals fed the HFD or chow have no differences in gross retinal thickness. Total and laminar retinal thicknesses were measured using hematoxylin and eosin–stained paraffin-embedded retinal sections obtained from 12-month-old animals. Representative examples of stained retinas from whole-globe hemisections obtained at the optic nerve from aged chow-fed (*A*) or HFD-fed (*B*) animals. Measurements of total retinal thickness (photoreceptor outer segments – the internal limiting membrane distance) (*C*) or of the inner nuclear layer (*D*) in 12-month-old animals fed the HFD or chow. All thicknesses were measured at the indicated distances from the optic nerve head. Distances marked as negative (–) and positive (+) were taken toward the dorsal and ventral borders of the retina, respectively. Scale bars = 25  $\mu$ m. Values represent mean distances measurements in  $\mu$ m  $\pm$  SEM, from 5 animals (10 eyes) in each group. No significant differences were observed between groups for any thickness comparison by ANOVA.

#### **HFD Feeding Promotes Retinal Neural Dysfunction**

Although the hallmark of DR is vascular disease, antecedent or coincident neural deficits also occur in humans (25–28). Whether neural effects are involved in the etiology of DR in rodents is less clear. To determine whether neurologic dysfunction occurs early in HFD-induced DR, we performed full-field, stimulus-evoked scotopic and photopic ERGs in HFD-fed mice and chow-fed controls.

After dark adaption overnight, scotopic ERG was performed with full-field white stimulus given across increasing intensities. Thereafter, a 10-min period of light adaptation was performed against a dim white background of  $30\text{-}cd/m^2$  luminance. Photopic ERG was recorded at increasing stimulus intensities. Raw ERG data were then analyzed for a-wave (scotopic reading only) and b-wave (in both scotopic and photopic conditions) amplitudes and implicit times. The raw waves were processed through a digital high-pass filter to isolate early OPs, as shown in Fig. 5A.

As expected, maximal ERG responses to white light stimuli for both a-waves (Fig. 5B, D, and F) and b-waves

(Fig. 5*C*, *E*, and *G*) declined with age. At no age did we observe a difference in amplitudes or implicit times in scotopic a-waves or b-waves between HFD and control groups. There were no differences in photopic b-wave characteristics between different groups at any age (data not shown). Amplitudes of summed OPs did not differ between dietary groups at 3 or 6 months of age (Fig. 6*A* and *B*). However, at 12 months of age, average OP amplitudes were significantly reduced in HFD-fed animals compared with chow-fed controls at all stimulus intensities (P < 0.05) except for the dimmest ( $-2.4 \log [cd \cdot s/m^2]$ ) and brightest (0.89 log  $[cd \cdot s/m^2]$ ). Examples of the preferential decline in OP amplitudes in HFD-fed animals compared with controls, with relative preservation of a-wave and b-wave amplitudes, are shown in Fig. 6*G*.

HFD feeding promotes insulin resistance (Fig. 1), but individual mice show variable glucose tolerance responses. To determine whether mice with greater glucose intolerance (i.e., higher area under the curve [AUC] values during a glucose tolerance test [GTT]) also have a greater reduction in OP amplitudes, we correlated OP amplitudes with



**Figure 3**—At an advanced age, HFD-fed mice develop typical lesions of DR. Retinal vasculature networks were analyzed in 6- and 12month-old animals by trypsin digest of isolated and fixed retinas, followed by periodic acid Schiff/hematoxylin staining. *A*: Representative example, under low-power magnification, of a trypsin-digested and stained retina. Grading of vascular lesions was performed under highpower magnification in the midperipheral retina at standardized areas across all samples, as indicated by the black boxes. *B*: Shown are examples of healthy-appearing pericytes (top three panels, arrowheads) or typical examples of vascular pathologies, including pericyte ghosts (middle three panels, arrows) and atrophic capillaries (bottom panels). Scale bar = 25  $\mu$ m. HFD-fed animals develop significantly more atrophic capillaries (*C*) and pericyte ghosts (*D*) at 12 months, but not 6 months, of age, compared with chow-fed controls. Shown are quantifications of vascular lesions, as assessed by a masked grader. Values represent mean  $\pm$  SEM from 10 independent experiments in each group. \*\**P* < 0.01, \*\*\**P* < 0.001 by ANOVA.

AUC of the GTT for individual mice at different ages. At 3 months (Fig. 6*D*), no correlation was found between glucose tolerance and OP amplitudes in either dietary group. However, at 6 months (Fig. 6*E*), the AUC of the GTT was correlated with lower OP amplitudes in the HFD-group (Pearson  $R^2 = 0.60$ , P = 0.04). In contrast, no such correlation was found for chow-fed animals of the same age ( $R^2 = 0.02$ , P = 0.81). At 12 months (Fig. 6*F*), the correlation between glucose tolerance and lower OP amplitudes was also present in HFD animals (Pearson  $R^2 = 0.51$ , P = 0.009) but not in chow-fed controls ( $R^2 = 0.12$ , P = 0.5).

Prompted by this correlation, we determined whether OP kinetics may be affected in the HFD-induced diabetes model by measuring the latency time for each of the first four OPs recorded on scotopic ERG at 3, 6, and 12 months (Fig. 7). Beginning at 6 months of age, a significant delay in implicit time for the first two OPs (OP1 and OP2) elicited by a  $-0.60 \log (\text{cd} \cdot \text{s/m}^2)$  white light flash was observed in HFD-fed animals (27.1  $\pm$  2.2 ms) compared with controls (24.3  $\pm$  1.8) at OP1 (P < 0.05). This

delay persisted through 12 months, with an average OP1 implicit time of 25.6  $\pm$  2.0 ms in HFD-fed animals compared with 24.2  $\pm$  1.4 ms in chow-fed controls (P < 0.05) and an average OP2 implicit time of 32.0  $\pm$  2.6 ms in HFD-fed animals compared with 30.5  $\pm$  1.9 ms in chow-fed controls (P < 0.05). These data are consistent with the notion that diet-induced insulin resistance promotes early neurologic deficits in the retina. By ERG analysis, these deficits are manifested as reductions in amplitude and delays in OP responses to white light stimuli under scotopic conditions. Thus, electrophysiologic abnormalities detected at 6 months precede the development of vascular abnormalities at 12 months (Figs. 3 and 4) in a diabetes model induced by a clinically relevant diet without genetic manipulations or administration of toxins.

To investigate potential mechanisms responsible for these ERG defects, we examined retinal expression of factors known to be altered in metabolic disease. NLRP3 is an important regulator of cellular responses to obesityrelates diabetes (29). NLRP3 inflammasomes, classically



**Figure 4**—HFD feeding promotes retinal vascular permeability. EB dye (25 mg/kg) was injected intravenously into anesthetized animals and allowed to circulate for 180 min. *A*: Enucleated globes are shown from chow-fed (left) or HFD-fed (right) animals. Leakage of the EB dye into the anterior chamber is visible in the eye from a 12-month-old animal fed the HFD compared with the relatively clear aqueous fluid in the control animal. After dye circulation, retinal flat mounts were imaged by fluorescence microscopy. Dye leakage from capillaries into the neural retina is represented by a less defined pattern of fluorescence within the vascular space and a more diffuse "ground-glass" appearance to the background fluorescence. Shown are illustrative examples from chow-fed animals (*B* and *D*) and HFD-fed animals (*C* and *E*) at 12 months of age. *F*: Quantification of dye fluorescence from aqueous paracentesis samples demonstrates significantly greater leakage in HFD-fed animals compared with controls at 12 months of age but not at 6 months of age. Dye leakage at 12 months coincides with elevated expression of VEGF-A (*G*), intercellular adhesion molecule-1 (ICAM-1) (*H*), and glial fibrillary acidic protein (GFAP) (*l*), as assessed by RT-PCR from retinal lysates. Values represent mean  $\pm$  SEM. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 by ANOVA.

thought to direct immune responses against infectious agents, also respond to sterile damage-associated molecular patterns. These include oxidative stressors derived from excess fatty acids and glucose in diabetes (30,31). The HFD, compared with chow, robustly increased NLRP3 protein in the retina at 3 months, before the development of neural or vascular defects, an effect that persisted at 6 and 12 months (Fig. 8A and B). The cleaved, active forms of the NLRP3-dependent effectors IL-1 $\beta$  (mature IL-1 $\beta$ , 17 kDa) and caspase-1 (10 kDa) were increased in retinas



**Figure 5**—HFD feeding does not alter major retinal responses to light stimuli. ERG responses were recorded from animals in scotopic conditions after overnight dark adaptation. *A*: A high-pass filter was applied to the raw ERG to isolate the OPs for separate analysis from the a-wave and b-wave measurements. ERG a-wave amplitudes recorded at 3, 6, and 12 months of age are plotted against stimulus luminance (*B*, *D*, and *F*), with corresponding b-wave amplitudes (*C*, *E*, and *G*). Values represent the mean  $\pm$  SEM from at least five animals at each age. Decline in amplitudes of responses are seen with increasing age. At all tested ages, no significant differences were observed between animals on different diets with regard to either major ERG component (ANOVA).



**Figure 6**—Aged HFD-fed mice have decreased OP amplitudes compared with controls. Visual function in HFD- or chow-fed animals was assessed at 3, 6, and 12 months of age by ERG under scotopic, dark-adapted conditions. OPs were extracted from the raw ERG trace using a high-pass filter. The root mean square (RMS) of all OP peaks and troughs were normalized to the amplitude of the maximal b wave. RMS of OP amplitudes are plotted against stimulus luminance at 3 (*A*), 6 (*B*), and 12 (*C*) months of age. At 12 months of age, mice fed the HFD demonstrate significant declines in OP amplitudes relative to controls. Values represent the mean  $\pm$  SEM from at least five animals at each age. \**P* < 0.05 by ANOVA. *D*–*F*: Reductions in OP amplitudes correlate with glucose intolerance beginning at 6 months of age. Correlation plots of normalized OP amplitude with the AUC for the intraperitoneal GTT are shown for animals at 3 (*D*), 6 (*E*), and 12 (*F*) months of age. Each data point represents a unique animal. Pearson correlation analyses were performed for animals fed each diet at each tested age, with *R*<sup>2</sup> and *P* values shown in the lower right corner of each graph. *G*: Representative ERG tracings from mice fed each of the two indicated diets, with the HFD-fed mice displaying reductions in OP amplitudes compared with chow-fed controls.

from HFD-fed but not chow-fed animals at all time points (Fig. 8A, *C*, and *D*). Phosphorylated JNK, an important stress signal in diabetes, was increased in HFD compared with control retinas (Fig. 8*E*).

Inflammasome activity contributes to retinal disease (30,32,33), with retinal induction of NLRP3 thought to occur in endothelium or macrophages. Imaging of NLRP3 and its adaptor protein ASC unexpectedly demonstrated colocalization of these inflammatory mediators in the inner nuclear and ganglion cell layers (Fig. 8F). These signals predominantly occur within cell bodies of ganglion cells and inner nuclear cells (Fig. 8F) rather than in vascular tissues. Stress signaling in this context likely reflects a chronic inflammatory change affecting multiple cell types. Consistent with this scenario, increased Iba1, reflecting microglial activation, was detected in HFD-fed mice compared with controls at 12 months (Supplementary Fig. 2). This signal was found in the deeper portions of the inner



**Figure 7**—Delays in OP implicit times are seen in HFD animals before the development of vascular lesions. Implicit times of the first four OPs (OP1–4) were measured from ERGs generated by a 0.25 cd  $\cdot$  s/m<sup>2</sup> white light stimulus. Recordings were performed at each indicated age. At 6 months of age, HFD-fed mice show significant delays in the implicit times of the first two OP peaks compared with chow-fed mice. This difference persists through 12 months of age. Shown are mean values ± SEM for each of the four measured peaks, with individual values for each animal tested superimposed (at least five animals were used in each diet group at each age). \**P* < 0.05 by ANOVA.

plexiform layer, distinct from ganglion cell bodies. These results suggest that HFD feeding induces a chronic inflammatory program that includes stress signaling in the inner retina, at least in part due to NLRP3 activity and JNK kinase activation, before the development of ERG and vascular defects. The temporal sequence of inflammatory activation, systemic glucose intolerance, ERG deficits, and vascular abnormalities in our mouse model suggests that HFD-initiated retinal stress signaling contributes to diabetes-related eye disease.

#### DISCUSSION

DR affects blood vessels. In humans and mice, the earliest visible signs of the disease are vascular. Yet, neurologic activity in the retina is metabolically demanding (34), raising the possibility that retinal neural function might be deranged in the setting of diabetes. If so, early neural insults could be related to vascular damage in the retina. In the current work, we provide evidence that retinal function is compromised in diet-induced diabetes in mice before the development of detectable defects in the retinal vasculature.

Using a clinically relevant diet, we show that the development of obesity, glucose intolerance, and insulin resistance in mice is associated with microvascular atrophy and pericyte damage by 1 year of age. Using ERG, we show that a functional neurologic deficit occurs by 6 months, before vascular injury. This functional abnormality persists to 12 months and is correlated with glucose intolerance, consistent with a temporal relationship linking metabolism, neural dysfunction, and the eventual development of microvascular disease. We also provide evidence that aberrant stress signaling induced by the HFD precedes ERG and retinal microvascular defects.

The relationship between early ERG dysfunction and retinopathy development is less clear in other mouse models. In the db/db mouse, which develops a disease similar to type 2 diabetes due to leptin receptor deficiency, massive reductions in b-wave amplitude have been recorded at  $\sim$ 6 months of age (35). These findings coincide with vascular defects, including morphological abnormalities and abnormal leakage. However, leptin signaling has important trophic and angiogenic effects in the retina that may account for the ERG deficits seen in these mice (18). In the STZ diabetes model, ERG and structural vascular abnormalities have both been identified (36–38), but STZ causes off-target neurologic effects (14,15). Moreover, STZ causes rapid onset of severe disease that would obscure a potential early neural contribution. Retinal inflammation and degeneration were recently demonstrated in diet-induced metabolic disease in rodents (39,40). A new rat model of type 2 diabetes manifests features of retinal inflammation and vascular disease coincident with severe metabolic derangements (41). These newer studies did not report ERG responses.

The current HFD-fed model is characterized by slowonset disease, mirroring the pathophysiology of human type 2 diabetes complications such as retinopathy. Even after 1 year of age, HFD mice do not lose inner retinal thickness, confirming that the disease in these animals is mild and similar to what is seen in early human retinopathy (21,22). Our findings that OP disruptions precede the appearance of vascular disease are consistent with data from humans, where electrophysiology is impaired before visible microvascular disease, with clinical impairment frequently involving these same waveforms (26,28,42).

Our ERG changes are small but exquisitely specific to OPs. Unlike the catastrophic ERG effects caused by mutations in visual cycle proteins, ERG deficits in diabetes models are expected to be modest. Rodent full-field ERG represents a summed response of the neural retina to light. However, as in human DR, experimentally induced DR is not expected to be uniform. In humans, multifocal



**Figure 8**—HFD promotes early inflammasome activation in the inner retina. *A*–*E*: Retinal lysates from HFD-fed or chow-fed animals at each indicated age were probed for NLRP3, IL-1 $\beta$ , immature caspase-1 (45 kD), active caspase-1 (10 kD), and phosphorylated (p)JNK and total JNK. NLRP3, IL-1 $\beta$ , and pJNK levels were normalized against total JNK, whereas caspase-1 p10 was quantitated relative to the corresponding levels of immature isoform (p45). Values represent mean  $\pm$  SEM from three independent experiments. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.001. Mo, months. *F*: Representative retinal sections from 3-month-old animals coimmunostained for the adaptor protein ASC and NLRP3. Staining for both molecules is strongest within the retinal ganglion cell and inner nuclear layers. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; RGCL, retinal ganglion cell layer. *G*: A high-power image from a HFD-fed animal shows specks (arrows) in retinal ganglion cell and inner nuclear layers (\*). Scale bar = 20  $\mu$ m.

ERG identifies preferentially affected regions that subsequently develop classic vascular lesions of retinopathy (43,44). Analogous multifocal recording capability is not available in rodents. Structural and functional assays of blood vessel integrity offer greater spatial resolution compared with visual function assays. Given the low resolution of full-field ERG in mice, it is striking that functional deficits correlated with glucose intolerance precede the detection of vascular defects. OP characteristics can lead to artifacts in b-wave recordings (45,46), but no b-wave changes between groups were detected.

Altered OPs likely reflect perturbations of the inner retina, specifically dysfunctional amacrine, bipolar, and retinal ganglion cells (47,48). Many studies during the past half century have reported decreased OP amplitude as the earliest sign of DR (26,28,42). Consistent with these observations, our findings suggest that inner retinal networks may be important early targets of disease. Alternatively, early ERG dysfunction in humans and mice could be caused by microvascular disease that cannot be detected with current technologies. Another possibility is that diet-induced diabetes perturbs neuroglial-vascular coupling mediated by arachidonic acid metabolites, nitric oxide, semaphorin 3a, and VEGF, all affected by diabetes (49–52).

Inflammation (NLRP3, cleaved IL-1 $\beta$ , cleaved caspase-1, phosphorylated JNK, and ASC recruitment) was induced in the inner retina by the HFD before the development

of OP abnormalities or detectable vascular disease. Obesity-related diabetes promotes inflammation, and our work demonstrated this effect in the retina. Possible priming mechanisms contributing to inflammasome activation include delivery of fatty acids (29,53), altered de novo lipogenesis (54), or disrupted redox chemistry (31). Our current findings are only associative. Additional studies will be required to define HFD-induced relationships between cellular effectors. But this work links inflammatory activation in a temporal sequence leading to ocular defects, providing rationale for manipulating the inflammasome to treat retinopathy.

In short, the HFD-fed mouse model could be useful for studying early neuronal, glial, or neurovascular dysfunction underlying retinal disease in type 2 diabetes. Identifying novel targets or biomarkers in this model could facilitate the development of diseasemodifying therapies for this devastating cause of blindness.

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