Retinal blood flow dysregulation precedes neural retinal dysfunction in type 2 diabetic mice

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Retinal blood flow dysregulation precedes neural retinal dysfunction in type 2 diabetic mice

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We investigated and compared the susceptibility of retinal blood flow regulation and neural function in mice developing type 2 diabetes. The longitudinal changes in retinal neuronal function and blood flow responses to a 10-min systemic hyperoxia and a 3-min flicker stimulation were evaluated every 2 weeks in diabetic db/db mice and nondiabetic controls (db/m) from age 8 to 20 weeks. The retinal blood flow and neural activity were assessed using laser speckle flowgraphy and electroretinography (ERG), respectively. The db/db mice had significantly higher blood glucose levels and body weight. The resting retinal blood flow was steady and comparable between two groups throughout the study. Hyperoxia elicited a consistent decrease, and flicker light an increase, in retinal blood flow in db/m mice independent of age. However, these flow responses were significantly diminished in db/db mice at 8 weeks old and then the mice became unresponsive to stimulations at 12 weeks. Subsequently, the ERG implicit time for oscillatory potential was significantly increased at 14 weeks of age while the a-wave and b-wave amplitudes and implicit times remained unchanged. The deficiencies of flow regulation and neurovascular coupling in the retina appear to precede neural dysfunction in the mouse with type 2 diabetes.

Recent clinical observations for diabetes revealed that prior to the development of vascular lesions and visible retinopathy, retinal thinning occurs[1,2]. These results support the concept that diabetes (type 1 and type 2) evokes early neurodegeneration in the retina, which appears to occur concurrently or before the structure and morphological changes in retinal vasculature[3,4]. Interestingly, abnormal retinal circulation was also found in patients with type 1[5,6] and type 2[7] diabetes before visible retinopathy developed. Thus, early detection and treatment of abnormal retinal circulation may be a valuable strategy to minimize the development of diabetic retinopathy. However, in contrast to vascular lesions, the information on the temporal change of retinal vasomotor activity and blood flow regulation during the progression of diabetes is not currently available, and the relative susceptibility of retinal circulation versus neuroretinal function during diabetic insult remains unknown.

Retinal blood flow is known to be intrinsically regulated to maintain proper retinal function[8,9]. Systemic hyperoxia (100% oxygen inhalation) produces a reduction of retinal blood flow, a response mediated by the released vasoconstrictor endothelin-1 (ET-1)[10] from glia cells[11], linking oxygen homeostasis to retinal blood flow regulation. In addition, Riva et al. reported that retinal blood flow is increased when a flickering light stimulates the neuronal retina[12]. This hyperemic response was thought to be mediated by the linkage of neural activity and metabolism to blood flow regulation, i.e., neurovascular coupling, in the retina[13]. Clinical studies reported that diabetes blunts retinal blood flow responses to systemic hyperoxia[14] and flicker light[15], corresponding to the extent of pathological neovascularization and increased stage of retinopathy. However, it is unclear whether the altered vascular structure leads to flow dysregulation in diabetes, or vice versa. Interestingly, in both type 1 and type 2 diabetes, a close association between impairment of the vascular response to flicker light and abnormal ERG was noted in patients without retinopathy[16]. Moreover, a subtle decrease in capillary density was found to associate with neural function alterations in patients with type 2 diabetes who did not have visible lesion in the retina[17]. However, the extent of the relationship between these abnormalities remains unknown. It is worth noting that the aforementioned clinical studies were cross-sectional with various ages of diabetes. Thus, there is a need to determine the exact relationship between the alterations of neuronal activity and vascular function during progression of type 2 diabetes.

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In the present study, using a longitudinal approach, we examined the time course of development of flow dysregulation versus neural dysfunction in type 2 diabetes in the same subject. This study examined changes in retinal blood flow in response to hyperoxia and flicker light with laser speckle flowgraphy (LSFG) and evaluated neural retina function with ERG using a genetic type 2 diabetes mouse model, db/db[18], between the ages 8 weeks and 20 weeks old. We tested the hypothesis that retinal blood flow dysregulation precedes the development of retinal neuronal dysfunction in type 2 diabetes.

Results

Longitudinal assessment of systemic and ocular parameters. Bodyweight was significantly higher in the db/db mice than the nondiabetic db/m mice during the experiments (two-way repeated measures ANOVA; Fig. 1A). The blood glucose levels were relatively constant in all mice throughout the study, with about three folds higher in resting blood glucose in db/db mice (two-way repeated measures ANOVA; Fig. 1B). The systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MABP) were not different between db/m and db/db mice (Fig. 1C,D; two-way repeated measures ANOVA). The intraocular pressure (IOP) and ocular perfusion pressure (OPP) were not different between two groups of mice (Fig. 1E,F; two-way repeated measures ANOVA). These systemic and ocular parameters were not affected by age (one-way repeated measures ANOVA).

Longitudinal assessment of resting retinal blood flow. Figure 2 presents the stability of resting retinal blood flow. Both db/m and db/db mice exhibited a steady resting retinal blood flow throughout the experimental period (8 to 20 weeks) with no difference between the groups (two-way repeated measures ANOVA).

Longitudinal assessment of retinal blood flow response to systemic hyperoxia. On experiment day-1, after the measurement of baseline retinal blood flow, a 10-min systemic hyperoxia was imposed. The retinal blood flow decreased significantly with systemic hyperoxia in db/m mice at 8 weeks of age (one-way repeated measures ANOVA; Fig. 3A). This flow response pattern was consistently observed along with the growth of the animal to 20 weeks (Fig. 3B–G). The retinal blood flow returned to resting levels within 10 min after cessation of systemic hyperoxia (Fig. 3A–G). In 8 weeks old db/db mice, a reduction in retinal blood flow in response to hyperoxia was also observed; however, the response was significantly blunted compared to that in db/m mice (two-way repeated measures ANOVA; Fig. 3A). The hyperoxia-induced flow reduction was absent in db/db mice at 10 weeks, 12 weeks, and 14 weeks old (Fig. 3B–D). As the db/db mice grew older, there was a tendency to increase retinal blood flow in response to hyperoxia (Fig. 3E–G). No difference was observed in resting blood flows between db/db and db/m mice 10 min after the hyperoxia cessation.

Longitudinal assessment of retinal blood flow response to flicker stimulation. On experiment day-2, the temporal change of retinal blood flow in response to a 3-min flicker light stimulation was assessed. In 8-week-old db/m mice, the flicker light caused a slow and steady increase in retinal blood flow by 30% above baseline (one-way repeated measures ANOVA; Fig. 4A). When the mice grew older, from around 10 weeks, the flow was increased promptly and then stabilized at about 30% above baseline after 60 s of light stimulation (Figs. 4B–G). The blood flow returned to baseline within 3 min after flicker stimulation cessation (Figs. 4A–G). In db/db mice, the flow response to flicker light was blunted at younger ages, i.e., 8-week (Fig. 4A) and 10-week old (Fig. 4B). By 12 weeks of age, the mice became unresponsive to stimulation (Fig. 4C two-way repeated-measures ANOVA). There was a tendency of reversing the light-stimulated flow response in db/db mice beyond 12 weeks old (Figs. 4D–G). At 3 min after cessation of the flicker light stimulation, no difference was observed in resting blood flows between db/db and db/m mice.

Longitudinal assessment of ERG parameters. On experiment day-3, the ERG was assessed. There were no significant changes in amplitude and implicit time of a-wave and b-wave ERG in both db/m and db/db mice with age (Fig. 5A–D; one-way and two-way repeated-measures ANOVA). While no significant differences were found between db/m and db/db mice in the implicit time of OP3 (Fig. 5G) and the ΣOP amplitude (Fig. 5H), the implicit times of OP1 were significantly increased in db/db mice at 14 weeks old (Fig. 5E; two-way repeated-measures ANOVA). From 14 to 20 weeks, post hoc comparison with Holm-Sidak test showed that the implicit time of OP2 was significantly prolonged in db/db mice compared with db/m mice (Fig. 5F).

Discussion

Our present findings provide the first longitudinal data on the deterioration of retinal blood flow regulation before the development of neural deficiency during type 2 diabetes progression in mice. We found that the resting retinal blood flow was not altered in db/db mice from 8 to 20 weeks of age. However, the retinal blood flow responses to systemic hyperoxia and flicker light stimulation were compromised in the early stage of diabetes before the presence of ERG abnormality.

A reduction of resting retinal blood flow has been reported, without a noticeable change in retinal arterial and venous diameters, in rats after one week of streptozotocin-induced diabetes[19,20]. However, no changes in resting retinal blood flow and vascular diameters were found in Akita type-1 diabetic mice from 5 to 13 weeks of age[21]. It is unclear whether differences in species, age, and/or the type 1 diabetes model contributed to the inconsistent results. Using magnetic resonance imaging technology, retinal blood flow was also found unaltered in Akita mice at 10 weeks old, but a significant reduction of resting flow associated with visual deficiency was noted at older ages (i.e., 30 weeks of age)[22]. Prolonged diabetes appears to contribute to reducing resting retinal blood

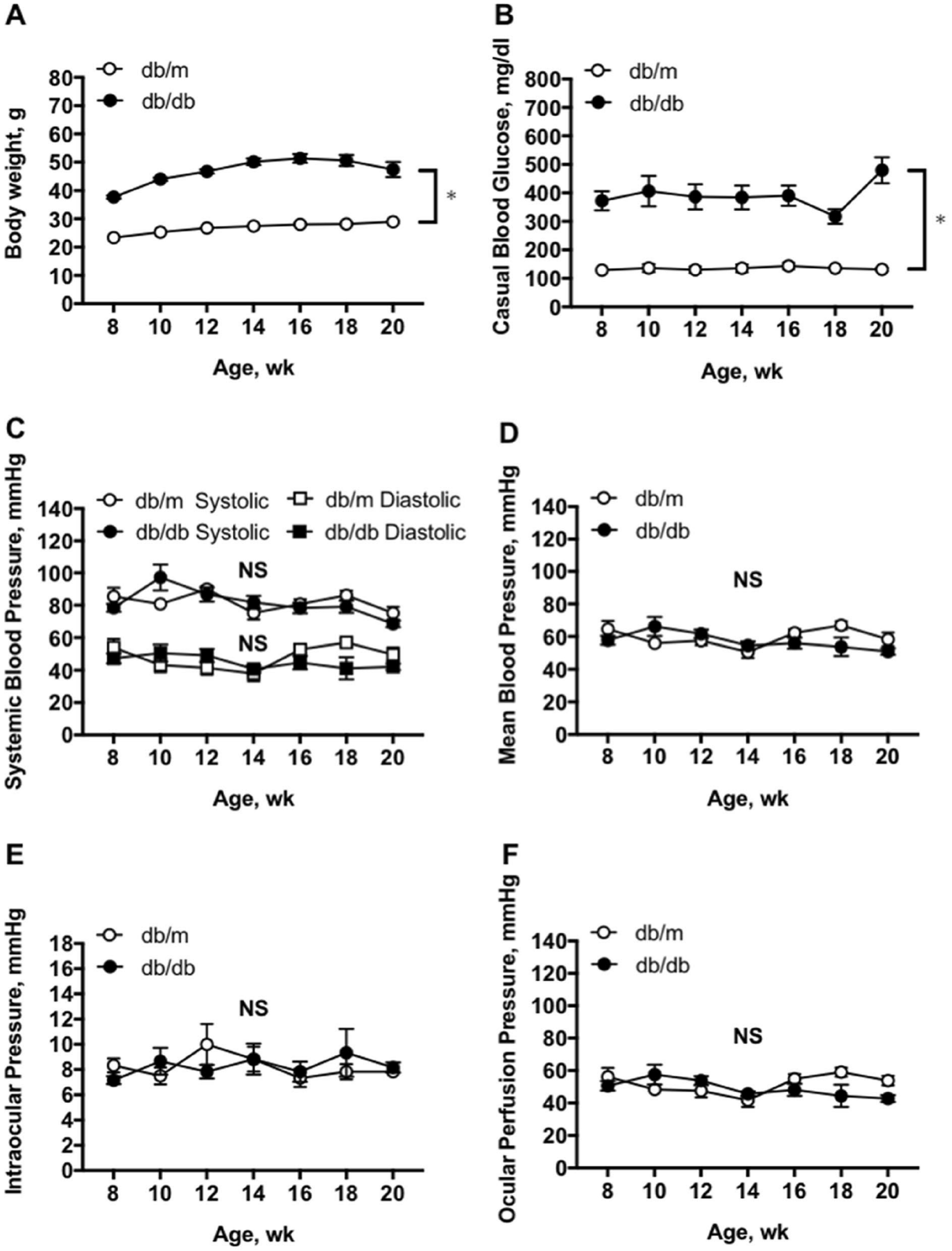

Figure 1. Average systemic and ocular parameters in db/db and db/m mice from 8 to 20 weeks of age. Significant increases were observed in body weight (**A**) and blood glucose (**B**) in db/db mice (n = 6) compared with nondiabetic db/m mice (n = 6) by two-way repeated measures ANOVA. In contrast, no significant differences were observed in systemic blood pressure (**C**), mean arterial blood pressure (**D**), intraocular pressure (**E**), and ocular perfusion pressure (**F**) between two animal groups during follow-up period. Data are expressed as the mean ± SEM; au = arbitrary unit; *P < 0.05 between groups; NS = not significant between groups.

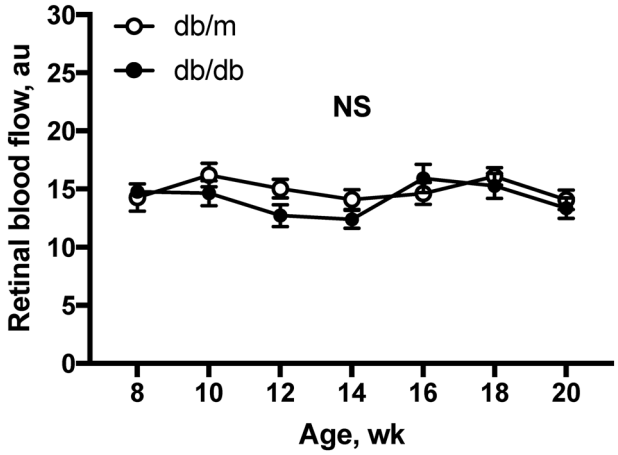

Figure 2. Retinal blood flow in db/db and db/m mice from 8 to 20 weeks old. Retinal blood flow (a.u) remained stable in both groups throughout, by one-way repeated measures ANOVA (P = 0.37 for db/m mice and P = 0.47 for db/db mice). No differences in resting retinal blood flows were observed (two-way repeated measures ANOVA). *NS* not significant between groups and within group.

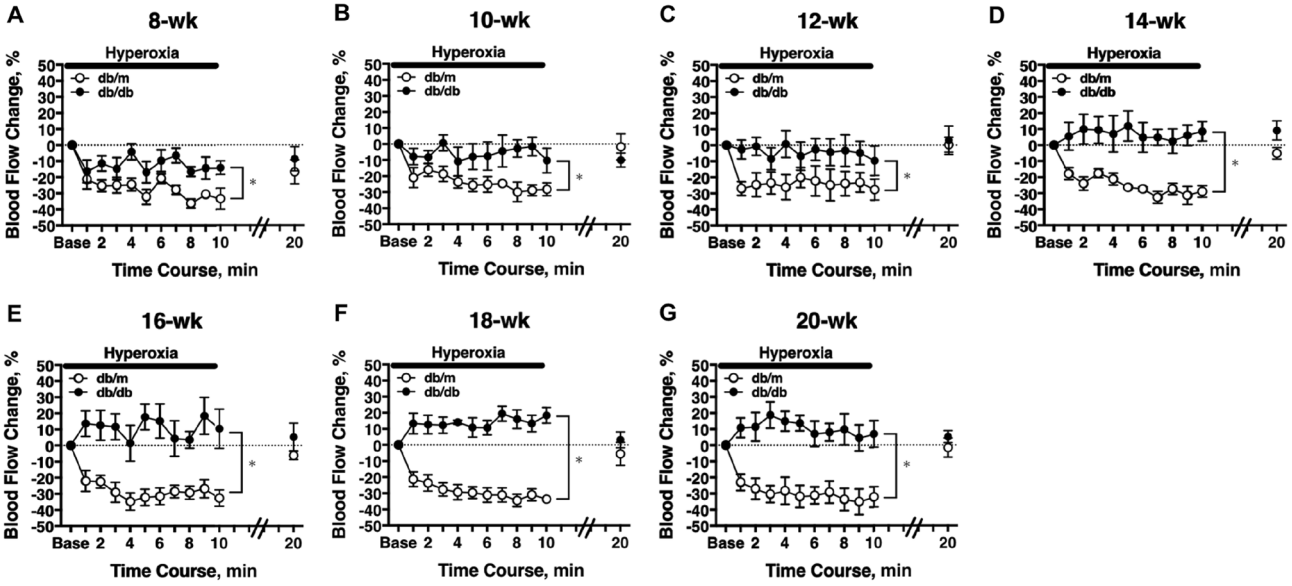

Figure 3. Retinal blood flow response to systemic hyperoxia. Longitudinal assessment of retinal blood flow in db/db and db/m mice from 8 to 20 weeks old. Retinal blood flow was significantly reduced from baseline during hyperoxia in db/m control mice (n = 6) from 8 weeks (**A**) to 20 weeks (**G**) of age. Reduction of retinal blood flow from baseline was observed in 8-week old db/db mice (A). No significant changes in retinal blood flow were noted in db/db mice (n = 6) at 10 weeks (**B**) and older (**C**–**G**) (one-way repeated measures ANOVA). The hyperoxia-induced flow response was significantly blunted in db/db mice for each age studied (two-way repeated-measures ANOVA). Following termination of hyperoxia, return of retinal blood flow to baseline levels occurred within 10 min, and no difference was observed between groups (two-way repeated measures ANOVA). *P < 0.05 between groups; Solid bar = period of hyperoxia.

flow. However, the temporal relationship between the flow alteration and the observed neural deficiency remains unclear as only two time points (i.e., 10 and 30 weeks) were examined in the above study. We measured the global change of retinal blood flow in type 2 diabetes every two weeks in the same subjects. Our results showed a steady resting retinal blood flow from 8 to 20 weeks of age with no differences between db/db mice and their age-matched controls. Although our current study did not extend the flow assessment beyond 20-week-olds, our findings indicate that type 2 diabetes, up to early adulthood, has little impact on the resting retinal blood flow.

The neurovascular coupling mechanism that optimally regulates retinal blood flow to match oxygen demand and metabolic activity of the retinal tissue is well-established[8,23]. The retinas respond to systemic hyperoxia with reduction of retinal perfusion[10,11,24,25] through the release of a potent vasoconstrictor ET-1[10,11] from neural glia cells[11] and the subsequent activation of ET-1 type A receptors (ET_AR) in retinal blood vessels[10,26,27].

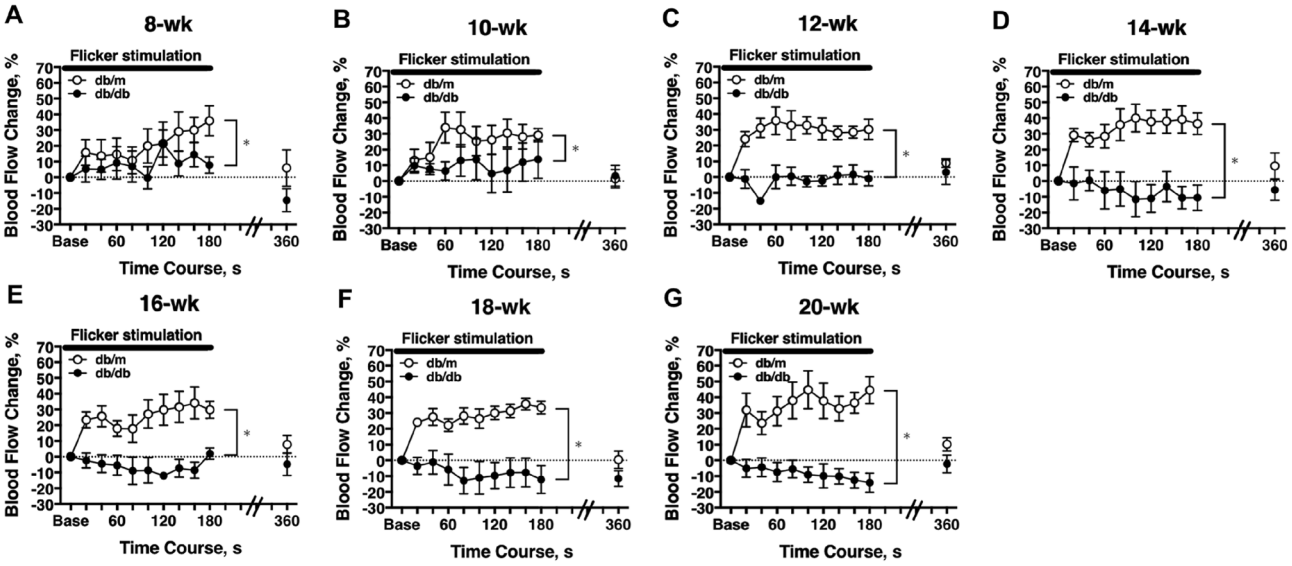

Figure 4. Retinal blood flow response to flicker stimulation. Retinal blood flow was longitudinally assessed in db/db (n = 6) and db/m (n = 6) mice from 8 to 20 weeks of age. During the period of flicker light stimulation, retinal blood flow increased significantly in db/m mice from 8 weeks (**A**) to 20 weeks (**G**) of age and in db/db mice at 8-week (**A**) and 10-week (**B**) old. When the db/db mice grew older (**C**–**G**), retinal blood flow changes in response to flicker light were not significant (one-way repeated measures ANOVA). The flicker-induced flow response was significantly blunted in db/db mice for each age studied (two-way repeated measures ANOVA). The retinal blood flow in db/m mice returned to baseline at three minutes after termination of light stimulation, and there was no difference between groups (two-way repeated -measures ANOVA). *$P < 0.05$ between groups; Solid bar = period of flicker stimulation.

Administration of ET-1 significantly reduces retinal blood flow in healthy humans without affecting retinal arterial and venous diameters, suggesting the main action of ET-1 in the retinal microcirculation[28]. ET-1 does not contribute to the maintenance of retinal vascular tone or diameter in healthy human subjects during rest[28]. However, the ET-1 levels in vitreous fluid[29–31], retinal tissue[32,33], and plasma[34–36] are elevated in subjects with diabetes. Interestingly, a sevenfold increase in vascular ET-1 mRNA was reported in type 1 diabetic mice[37]. Administration of ET_AR blockers in animals with early diabetes prevents a decrease in the retinal blood flow, suggesting the contribution of upregulated ET-1 system to early vascular complication in diabetic retinas[38,39].

In the present study, we did not observe a decrease in resting retinal blood flow; however, the blood flow response to hyperoxia was significantly blunted in diabetic mice. Initially, the extent of flow reduction was reduced around 8 weeks old (early stage of diabetes). Following this, the retinal circulation became unresponsive to stimulation (10–12 weeks) and tended to reverse the flow response (i.e., increase in flow) at the later stages of diabetes (14–20 weeks) (Fig. 3). Because ET-1 has been shown to be responsible for the reduction of retinal blood flow during hyperoxia[10,40], the observed impairment of this flow response in the present study might be explained by the diminished vascular responsiveness to ET-1, possibly due to ET_AR desensitization[34] in vascular smooth muscle cells[41–46] and/or pericytes[47] which are pre-exposed to the elevated level of ET-1 in diabetes. In line with this speculation, a reduced mesenteric[35] and retinal[48] vascular responsiveness to ET-1 has been reported in diabetic rats. Moreover, the hyperglycemic insult might also contribute to the reduction of flow response to hyperoxia by impairing ET-1-mediated biochemical signaling in retinal pericytes[49]. Collectively, these findings suggest that the diabetic insult may gradually compromise the coupling between glial cells and the vasoconstriction to ET-1 at downstream terminal microvessels, where the pericyte is abundant and dominant for blood flow regulation[50]. Our findings are in line with observations in other studies of reduced retinal blood flow response to hyperoxia in patients with diabetes, with or without retinopathy[24,51,52]. Further mechanistic studies are warranted to investigate how hyperglycemia and diabetes exert negative impacts on the retinal microvascular function related to ET-1 overproduction.

Riva et al. were the first to show that the increase of optic nerve head blood flow evoked by a flickering light is associated with the reduction of retinal pO_2, possibly due to increased metabolic activity of ganglion cells[12]. The increased neuronal activity can consequently increase retinal blood flow by triggering release of vasodilators such as nitric oxide (NO) and/or arachidonic acid metabolites[53,54]. Clinical studies found that the vasodilation elicited by flicker light is reduced in patients with diabetes, correlating with an advanced stage of retinopathy[15,55]. In fact, some patients with diabetes (both type 1 and type 2) showed reduced flicker-induced flow response before the clinical appearance of retinopathy[15], possibly reflecting neural dysfunction in the inner retina[16]. However, the development of flow dysregulation in response to flicker light, in relation to neural dysfunction, during the progression of diabetes is not known. There also has been no report concerning the impact of type 2 diabetes on retinal blood flow regulation in db/db mice, an animal model widely used for retinal disease research. We found that the hyperemia induced by flicker light was gradually diminished with age in db/db mice (Fig. 4). Interestingly, the compromised flow response was already present in the 8-week-old mice, the youngest age studied,

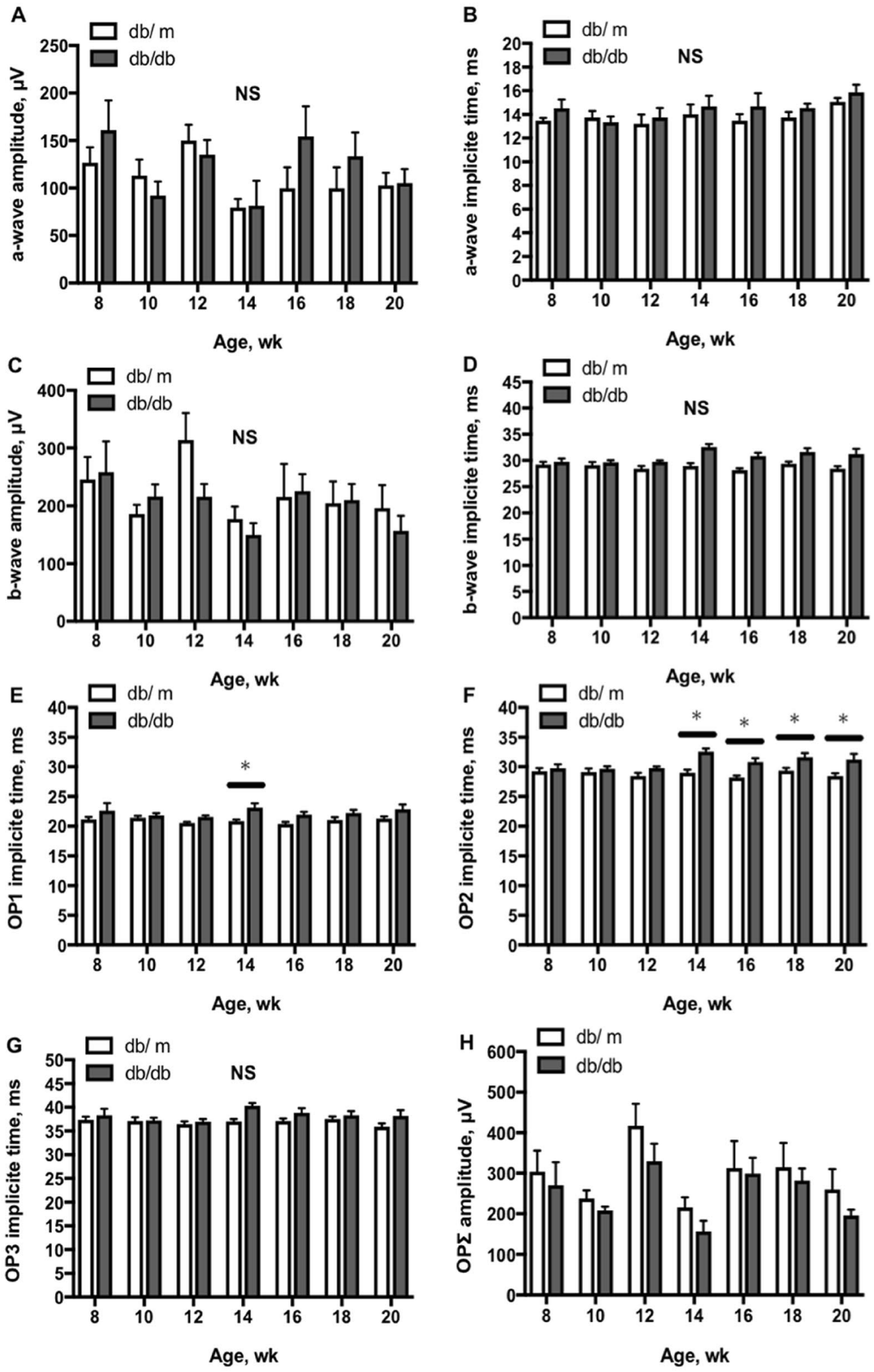

Figure 5. Longitudinal assessment of ERG in db/db and db/m mice from 8 to 20 weeks of age. There were no significant differences in implicit time and amplitude of a-wave (**A**,**B**) and b-wave (**C**,**D**) between db/m (n = 6) and db/db (n = 6) mice. There were no significant changes in the implicit times of OP3 (**G**) waves and the total amplitudes of OP-waves (ΣOP) (H) between db/m and db/db mice (two-way repeated -measures ANOVA). The implicit times of OP1 (**E**) were significantly increased in db/db mice at 14 weeks old and the implicit times of OP2 waves (**F**) were significantly increased in db/db mice from 14 to 20 weeks old (two-way repeated -measures ANOVA). *P < 0.05, between groups.

where blood glucose level was already significantly elevated (Fig. 1). Because the onset of hyperglycemia in db/db mice has been reported to be at 4 weeks of age[18], it is likely that the deterioration of flow regulation might have begun at early diabetes around 4–6 weeks old. This speculation is supported by finding vasomotor dysregulation of both retinal arterioles[56,57] and venules[29] after induction of type 1 diabetes for only 2 weeks. Although the NO and arachidonic acid metabolites can participate in flicker-induced vasodilation[53] and increased retinal blood flow[54], the mechanisms underlying neurovascular uncoupling and flow dysregulation in the diabetic retina are incompletely understood. Interestingly, a recent study in diabetic pigs demonstrated the impairment of endothelium-dependent NO-mediated dilation of retinal arterioles by upregulated vascular arginase[57], an enzyme that competes with NO synthase for their common substrate L-arginine, and thus consequently compromises NO production and vasodilation[58]. Because the vasodilation mediated by arachidonic acid[59] appears to be intact in retinal arterioles isolated from diabetic animals[57], the observed retinal blood flow dysregulation in response to flicker light is likely due, in part, to NO deficiency. Nonetheless, contribution from other factors cannot be excluded because a progressive reduction of neuroretinal thickness and the loss of ganglion cells become apparent in db/db mice at 16- and 24-week of age[18]. These structural changes may worsen neurovascular uncoupling and completely exhaust retinal blood flow regulation in advanced stages of diabetes (i.e., age 10–12 weeks and older) as shown in the present study (Figs. 4 and 5).

It has been reported that OPs are the most sensitive ERG parameters, reflecting the changes in microvascular function[9,60]. The OPs are likely to originate from the inner retinal neuron activity, which is known to be vulnerable to ischemia[60]. The OP electroactivity could also be derived from retinal glial cells, amacrine, and interplexiform cells, which are sensitive to hyperglycemic insults[61]. Our results agree with previous clinical studies showing that the prolonged OP implicit times are present earlier than the decrease of amplitudes in diabetic patients[60]. We further showed that the onset of OP abnormality (at 14-week old; Fig. 5) occurred at the time after the complete exhaustion of retinal blood flow regulation (at 10- to 12-week old; Figs. 3 and 4), suggesting a possible link of flow dysregulation to the development of neural dysfunction in the inner retina during diabetic insult. On the other hand, we found that there were no significant alterations in both a- and b-wave amplitudes and implicit times in db/db mice (Fig. 5). These results are inconsistent with a previous report by Bogdanov et al. on the appearance of reduced b-wave amplitudes and prolonged implicit times in db/db mice at 16 and 24 weeks of age[18]. Although the reason for this discrepancy is unclear, we noticed that the variability of b-wave amplitudes was greater than the variability of its implicit times across different ages and the coefficient of variation of b-wave amplitudes in our study was higher than that of Bogdanov's study at 16 and 24 wks. The difference in the number of animals studied, n = 15 in Bodganvo's study[18] vs. n = 6 in our study, might have had an impact on the power of the analysis. Nevertheless, our data support the idea that retinal blood flow dysregulation precedes the development of neural dysfunction during the progression of type 2 diabetes. Our findings also indicate that the retina exhibits an intrinsic ability to maintain a steady resting blood flow but fails to react to metabolic disturbances by adjusting blood flow accordingly during diabetic insult.

The current study has some limitations. First, the study was performed under anesthesia with isoflurane but the impact of this anesthetic on retinal blood flow regulation is unclear. Because isoflurane, like other general anesthetics, can suppress central nervous activity and cardiovascular system, its broad impact on circulation is expected under high concentrations. Unfortunately, to the best of our knowledge, there was no study to examine the influence of isoflurane on retinal blood flow regulation. However, a previous study reported that there is no substantial difference in mouse ERG parameters between isoflurane and ketamine anesthesia[62]. In the present study, it is worth noting that all data were derived and compared under experimental conditions with the same level of anesthesia, which did not alter systemic or ocular parameters across different ages (Figs. 1 and 2). Therefore, we believe that isoflurane anesthesia might only have had a little effect, if any at all, in our study. Second, it is recognized that db/db mice are one of the most widely used animal models for type 2 diabetes research by developing hyperglycemia, hyperphagia, obesity, hyperinsulinemia, insulin resistance, and hyperlipidemia. However, it should be cautious to extend and translate our current findings to human application because there are some dissimilarities in phenotypes and pathophysiology between db/db mice and human patients with advanced diabetes[63].

In conclusions, we found that the retina fails to regulate blood flow in response to systemic hyperoxia and flicker stimulations at the early stage of type 2 diabetes in db/db mice without apparent changes in resting retinal perfusion. Although the resting blood flow is maintained, retinal blood flow dysregulation in response to metabolic disturbances is manifested before a noticeable change in neuroretinal function. Although the underlying mechanisms responsible for retinal neurovascular uncoupling in diabetes remain largely unexplored, early detection and treatment of this flow dysregulation might help to preserve retinal tissue from developing irreversible retinopathy.

Materials and methods

Animal preparation. The Nihon University Ethical Committee approved the animal experiments, which were carried out according to the tenets of the Association of Research in Vision and Ophthalmology. We also confirmed that this study was performed in accordance with ARRIVE guidelines (https://arriveguidelines.org).

The 7-week-old male C57BL/KsJ-db/db mice (BKS.Cg-Dock7^{m}+/+Lepr^{db}/J; n = 6) and db/m (congenic non-diabetic littermates, n = 6) control mice were acquired from Charles River Laboratories JAPAN, Inc. (Yokohama, Japan) one week before the study. We used only male db/db mice because diabetes is more severe in male than in female db/db mice[63]. Blood glucose levels were measured from the tail vein (glucose assay kit; Abbott Laboratories, Abbott Park, IL). Mice were housed in a temperature-controlled room with a 12-h dark and light cycle with free access to food and water. Throughout the experiment, the mice were anesthetized with continuous inhaled 2% isoflurane (Pfizer, Tokyo, Japan) at a flow rate of 1.5 L/min. The pupils were dilated with 0.5% tropicamide

(Santen Pharmaceutical Co., Osaka, Japan). Rectal temperature was measured and a heated blanket was used to maintain temperature between 37 °C and 38 °C.

Systemic blood pressure and intraocular pressure measurements. Systemic blood pressure (BP) and intraocular pressure (IOP) were measured at 30 min following anesthesia induction. An automatic sphygmomanometer (THC-31, Softron, Tokyo, Japan) was used to measure systolic BP (SBP) and diastolic BP (DBP) at the tail. The IOP was measured by a handheld tonometer (TonolabTV02, ME Technical, Tokyo, Japan). The mean arterial BP (MABP) was derived from the standard formula: MABP = DBP + (SBP-DBP)/3. Ocular perfusion pressure (OPP) was calculated using the formula OPP = MABP − IOP[64], due to the animals' prone position during the experiments.

Retinal blood flow measurement. Retinal blood flow was measured with the LSFG-Micro system (Softcare Co., Ltd., Fukutsu, Japan), which is designed for small animals[64]. The LSFG-Micro system is equipped with a standard charge-coupled device camera (700 × 480 pixels) and a diode laser (830-nm wavelength) attached to a stereomicroscope (SZ61TR, Olympus Corporation, Tokyo, Japan). The underlying principle of the LSFG-Micro is the same as that of LSFG, which has been used in humans[65] and animals[64] for quantitative estimation of ocular (optic nerve head, choroid, and retina) blood flow. In brief, the mean blur rate (MBR) represents a relative index of blood velocity and is generated from the blurring of the speckle pattern formed by the backscattered light of the coherent laser by moving blood cells. The MBRs acquired from the vascular area around and at the optical nerve head (ONH) (Fig. 1B) reflect the entire retinal circulation and are used as an index of retinal blood flow[66].

In the present study, the MBRs were obtained as follows: The mice were positioned on a stand with the right eye facing downward. A cover glass was gently placed on the left cornea with a drop of viscoelastic material. The margin of the ONH was indicated by manually placing a rubber o-ring (1.37-mm diameter) over the ONH fundus image. The MBRs were acquired within the o-ring area continuously at 30 frames/second. The vessels' average MBR was analyzed with an LSFG analyzer software (version 3.2.19.0, Softcare Co., Ltd., Fukutsu, Japan).

Induction of systemic hyperoxia. Systemic hyperoxia was induced by inhalation of 100% oxygen over 10-min, as described in our previous studies[11,64]. The baseline value was determined as the mean of three consecutive flow measurements, obtained at 1-min intervals for 3 min before the initiation of hyperoxia. Retinal blood flow measurements were made every minute for 20 min during hyperoxia (10-min stimulation) and after the termination of hyperoxia (10-min recovery)[64].

Flicker light stimulation. We used a 12 Hz-flicker stimulation as this frequency triggers a maximal response of retinal blood flow in mice[64]. The ambient light was reduced to 1 lx or less before induction of flicker stimulation. The mice were dark-adapted for 2 h, with a light intensity for flicker stimulation of 30 lx for the rod-dominant mouse retina, as reported previously[64]. The retinal blood flow was measured with 20 s intervals throughout both the 3-min flicker stimulation and the 3-min recovery. The baseline value was calculated using the mean of three consecutive flow measurements obtained in 1 min (20-s intervals) before initiation of flicker light stimulation.

ERG recording. Prior to the ERG, the mice were dark-adapted for a minimum of 12 h and then transferred to a room with dim red light. The full-field ERGs were recorded with PuREC (Mayo, Inazawa, Japan) under systemic anesthesia with isoflurane. The ground electrode was attached at the tail, the reference electrode in the mouth, and bilateral corneal electrodes were placed on the corneal surface. The 3.0 cd s/m^2 flash was used to achieve a maximal response of both the cones and rods as previously reported[64]. The amplitude of the a-wave was measured from the baseline of the a-wave to the most negative trough, and the amplitude of the b-wave was measured from the trough of the a-wave to the positive peak of the b-wave[64]. The implicit times of the a- and b-wave were measured from the onset of the stimulus to the trough of the a-wave and from the trough of the a-wave to the peak of the b-wave, respectively. Oscillatory potentials (OPs) are small high-frequency oscillation wavelets superimposed on the b-wave's ascending limb. The OP wavelets were labeled as OP1 to OP3 consecutively, starting at the first detected positive peak. The amplitudes (peak positive amplitude – peak negative amplitude of previous peak) and implicit times of OPs were measured[67]. The OP amplitudes were calculated by adding the first 3 positive wavelets and presented as ΣOP amplitude[9,18,68].

Experimental protocols. We performed the following protocols in each animal for longitudinal assessments of retinal blood flow regulation and neural function on three consecutive days, every 2 weeks from 8 to 20 weeks of age. The systemic hyperoxia response of retinal blood flow carried out on day 1, and the following day, the response to flicker light stimulation was conducted. The ERG recording was made on day 3. We verified that the systemic BP, IOP, and OPP were not altered by hyperoxia or flicker light stimulation in mice in a previous study[64]. An independent masked observer (AK) performed all data calculations and analyses.

Statistical analysis. Data are expressed as mean ± standard error of the mean, and n value represents the number of animals studied. Retinal blood flow changes were calculated as percentage changes from the baseline. Normality of data distribution was verified by the Kolmogorov–Smirnov test. The significance of experimental intervention across different time points within and between groups were analyzed by one-way or two-way repeated-measures analysis of variance (ANOVA). This was followed by the Dunnett's test or Holm-Sidak test, where appropriate. A P-value < 0.05 was considered statistically significant.

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T.N. wrote the main manuscript text and J.H. and M.W. prepared all figures. A.K. performed the statistical analysis. H.Y., S.Y., and L.K. reviewed the manuscript.

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論文和文要約

糖尿病網膜症は糖尿病細小血管症の一つであり、現在でも成人の失明原因の主因となっている。長期経過で病期が進行する糖尿病網膜症では早期からの血糖コントロールが重要となるが、眼科的な介入は網膜症が進行して虚血がある程度進行してからの網膜光凝固術や硝子体手術、黄斑浮腫に対する薬物の硝子体注射などの侵襲的治療のみである。また、進行した増殖糖尿病網膜症に対してこれらを施行しても、すでに廃絶した視機能を回復させることは現状困難である。私は糖尿病網膜症の予防および低侵襲的な早期治療介入により糖尿病患者が良好な視機能を保ち天寿を全うできることを理想とし、その実現の一歩として糖尿病網膜症の早期診断および病態解明を目指した。

糖尿病網膜症は細小血管障害に基づくと考えられてきたが、最近では網膜症発症前から神経細胞で構成される網膜の菲薄化を認めることが報告されている[1,2]。さらに 1 型および 2 型糖尿病患者において網膜症発症前から網膜血流障害を認めることも報告されている[5,6,7]。網膜循環障害の早期発見、治療は糖尿病網膜症の発症予防に重要と考えられるが、糖尿病の進行過程における網膜循環と網膜神経機能の相互関係については不明のままである。網膜血流は、適切な網膜機能を維持するために本質的に調節されていることが知られている[8,9]。網膜血流調節には神経細胞やグリアが密接に関与しており、この現象は神経血管連関（neurovascular coupling）として認知されている[13]。糖尿病ではこれらが障害されていると考えられているが、詳細は不明である。私は 2 型糖尿病で網膜血流調節不全が網膜神経機能障害に先行するという仮説を立て、網膜神経、網膜グリア、網膜血流の中で、特にこれまで評価困難であった網膜グリア機能をフリッカー刺激と高酸素負荷の 2 つの負荷試験を用いて非侵襲的に評価し、網膜電図（ERG）を用いた神経機能評価と併せて経時的に検討することとした。

1 実験系の樹立

疾患における検討を行う前に、まずは実験系の樹立を目指した。実験動物には、モデル作成が容易であり、さらに週齢を揃えることで罹病期間と病態の関連の解明に利点があるマウスを用いた。マウスは眼球サイズが非常に小さいため、これまで網膜血流測定の報告がなされていなかったが、私は血流測定機器として眼科において臨床で汎用されている Laser Speckle Flowgraphy（LSFG）を小動物用に改良した LSFG-micro を用いることで、マウス網膜血流の定量的測定を実現したことを報告した。この先行研究では、生後 8–20 週齢の正常マウス 8 頭で安静時網膜血流およびフリッカー刺激による網膜血流増加反応、高酸素負荷による網膜血流減少反応、さらに ERG による網膜神経機能が測定期間中に変化せず長期的に安定していたことが確認できた。LSFG-micro を用いてマウス網膜血流の長期的な評価を可能にしたこの報告は世界で初めてであり、疾患動物や治療効果判定などの次なる検討の基盤となるものとなった。

2 2 型糖尿病モデルマウスへの応用

本研究では、この独自の評価法を糖尿病モデルマウスに応用した。糖尿病群としてレプチン受容体遺伝子変異による過食で肥満を誘発させた 2 型糖尿病モデル db/db マウスを 6 頭用いた。対照群にはヘテロタイプの db/m を 6 頭用いた。8–20 週齢までの偶数週に、安静時網膜血流測定、フリッカー刺激および高酸素負荷下での網膜血流測定、ERG、さらに体重、随時血糖、血圧、眼圧、眼灌流圧を測定した。実験は 3 日に渡って行い、第 1 日目に行った高酸素負荷試験では、100％酸素を吸入させ 10 分間の吸入中および終了後 10 分間、1 分毎に網膜血流を連続測定した。第 2 日目に行ったフリッカー刺激負荷試験では、フリッカー白色光を 12Hz に設定し 3 分間の刺激中および終了後 3 分間、20 秒毎に網膜血流を連続測定した。そして第 3 日目に ERG を施行した。両負荷試験では安静時網膜血流からの変化率を毎時算出しグラフ化した。群間比較の統計解析には Two-way Repeated Measured ANOVA を用いた。

体重および随時血糖は実験期間中の全ての週齢において糖尿病群で有意に高値であった。血圧、眼圧、眼灌流圧には両群間で有意差はなかった。刺激負荷前の安静時網膜血流は両群いずれも実験期間を通じて安定しており、両群間での有意差は認めなかった。 高酸素負荷試験では 8 週齢の対照群において網膜血流は有意に減少し、負荷終了後に徐々にベースラインまで戻った。この血流反応は 20 週齢まで持続した。一方で 8 週齢の糖尿病群では網膜血流減少が観察されたものの対照群の反応と比較して有意に減弱しており、さらに 10 週齢、12 週齢、14 週齢では血流減少はみられず、それ以降の週齢では逆に血流が増加する傾向にあった。フリッカー刺激負荷試験では 8 週齢の対照群において網膜血流は有意に増加し、

刺激終了後に徐々にベースラインまで戻った。この血流反応は 20 週齢まで持続した。一方で 8 週齢、10 週齢の糖尿病群では網膜血流増加が観察されたものの対照群の反応と比較して有意に減弱しており、12 週齢では血流増加はみられず、それ以降では逆に血流が減少する傾向にあった。ERG 波形解析では、a 波および b 波の振幅と潜時は両群間で変化しなかった。律動様小波(OP)は OP3 の潜時と OP の振幅の総和には両群間に有意差はみられなかったが、OP1の潜時に関しては 14 週齢、OP2 の潜時に関しては 14 週齢以降の糖尿病群で対照群と比較して有意な延長を認めた。

本研究結果は、db/db マウスの糖尿病進行過程において、ERG で捉えられる網膜神経障害発症に先行して、網膜血流負荷試験に対する網膜血流調節障害を認めたことを示唆する。

本研究では db/db マウスで安静時網膜血流は観察期間中に変化しなかった。既報では、STZ で糖尿病を誘発した 1 週間後のラットで安静時網膜血流減少の報告[19,20]がある一方、Akita1 型糖尿病マウスでは 5–13 週齢の間で変化がないという報告[21]や、10 週齢では変化がなく 30 週齢で減少するという報告[22]などもあり様々である。これらの違いが動物の種や週齢の違いによるものかどうかは不明であるが、本研究で採用したマウスの週齢（8–20 週齢）は、ヒトではおよそ幼少期から若年成人の時期に相当すると考えられ、本研究結果はヒトで成人早期に相当すると考えられる 20 週齢の db/db マウスではまだ安静時網膜血流に影響を与えなかったことを示していると推測される。

本研究では db/db マウスで高酸素負荷に対する網膜血流反応が 8 週齢の段階から既に障害されていた。高酸素負荷では、グリアからの強力な血管収縮剤 ET-1 の放出と ET-1 タイプ A 受容体（ETAR）の活性化を介して、網膜血流の減少を伴うことが知られている[10,11,24,25,26,27]。糖尿病ラットでは網膜動脈において ET-1 に対する血流反応の減弱が報告されている[35,48]。また糖尿病患者において高酸素吸入に対する網膜血流反応が、網膜症の有無に関わらず減弱したことも報告されている[24,51,52]。本研究結果における詳細なメカニズムは不明であるが、これらの基礎研究や臨床研究と一致した結果が得られたと言える。

フリッカー刺激による血流増加反応には神経細胞やグリアからの神経型一酸化合成(Nitric oxide:NO)酵素(nNOS)を介した NO やアラキドン酸代謝物の関与が知られているが[53,54]、糖尿病網膜症における網膜血流調節不全の根底にある詳細なメカニズムは不明なままである。本研究では 8 週齢の時点の db/db マウスで既に高血糖を認め、同時にフリッカー誘発網膜血流反応も障害されており、糖尿病初期から網膜血流調節不全が始まっていたことが考えられる。このことは 1 型糖尿病誘発後わずか 2 週間で網膜の血流調整不全を認めた過去の報告からも裏付けられる[56,57,29]。さらに既報では 16 週齢と 24 週齢の db/db マウスで神経網膜の形態変化が報告されており[18]、本研究で 12 週齢以降にフリッカー誘発網膜血流反応がさらに悪化したことから、糖尿病進行期で網膜血流調節がさらに障害された可能性が考えられる。

本研究では ERG において db/db マウスで 14 週齢から OP の潜時が延長した。ERG は眼科の実臨床で古くから汎用されている機器である。被験者を十分に暗順応させた後に暗所で眼前に光を照射することで得られる波形は、網膜内の神経細胞の光受容体が光を感受することで生じる電気信号を捉えたものである。最初の陰性波である a 波は網膜外層の桿体・錐体視細胞の過分極を、次に現れる陽性波の b 波は中間層の双極細胞などの脱分極を反映する。b 波の立ち上がりに関与する律動波である OP は、アマクリン細胞などを含む網膜内層の機能を反映していると認知されている。このように ERG 成分は網膜内でその起源が異なるため、各 ERG 成分を評価することで網膜各層の機能を判定することが可能である。OP の波形に関与するアマクリン細胞は高血糖に感受性があり、糖尿病患者で潜時が延長することが知られている[60,61]。本研究結果はこれらと合致する。また既報では db/db マウスで b 波の振幅低下と潜時延長を認めた報告がされているが[18]、本研究では a 波、b 波の振幅と潜時に変化はみられなかった。本研究の検討では既報と比べサンプル数が少なかったため、統計差が検出されにくかった可能性がある。しかしながら、本研究の結果では OP の潜時延長が 14 週齢から出現しており、網膜神経機能障害が網膜血流調節不全に遅れて出現したことが言える。

本研究にはいくつかの限界がある。ERG における OP の潜時延長が網膜血流反応障害と直結して引き起こされたか、或いは独立してやや遅れて生じたかについては現時点では明らかでない。また本研究では全ての測定にイソフルラン吸入麻酔を用いている。イソフルランは他の全身麻酔薬と同様に、中枢神経系や心血管系の活動を抑制するため、高濃度下では循環への幅広い影響が予想されるが、イソフルランが網膜血流調節に与える影響は不明である。しかしながらマウスの ERG 波形に関しては、イソフルラン麻酔下とケタミン麻酔下で差が生じなかったことは既に報告されている[62]。また本研究

では全ての測定結果が、同濃度の麻酔条件下で群間比較されており、全身血圧や眼局所パラメーターを変化させなかった。このことから本研究では、イソフルラン麻酔の影響はたとえあったとしても僅かではないかと予想している。過食により糖尿病を誘発する db /db マウスは、2 型糖尿病研究に汎用されている動物モデルの 1 つであるが、db / db マウスと進行した糖尿病患者との間には表現型や病態生理にいくつかの相違があるため、本研究結果を拡張してヒトへ応用することには注意が必要である[63]。また、高血糖による糖毒性がもたらす影響においては不明であるが、その解明には血糖降下薬にて血糖を改善させた群との比較など、今後さらなる研究が必要と考えている。

本研究で私は、2 型糖尿病マウスの糖尿病初期段階において網膜血流調節不全が網膜神経機能障害に先行するということを発見した。糖尿病における網膜血流調節不全の早期発見は網膜症の発症予防に役立つ可能性がある。