Photobiomodulation Inhibits Long-term Structural and Functional Lesions of Diabetic Retinopathy

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Previous studies demonstrated that brief (3 to 4 min) daily application of light at 670 nm to diabetic rodents inhibited molecular and pathophysiologic processes implicated in the pathogenesis of diabetic retinopathy (DR) and reversed diabetic macular edema in small numbers of patients studied. Whether or not this therapy would inhibit the neural and vascular lesions that characterize the early stages of the retinopathy was unknown. We administered photobiomodulation (PBM) therapy daily for 8 months to streptozotocin-diabetic mice and assessed effects of PBM on visual function, retinal capillary permeability, and capillary degeneration using published methods. Vitamin D receptor and *Cyp24a1* transcripts were quantified by quantitative real-time PCR, and the abundance of c-Kit$^+$ stem cells in blood and retina were assessed. Long-term daily administration of PBM significantly inhibited the diabetes-induced leakage and degeneration of retinal capillaries and also significantly inhibited the diabetes-induced reduction in visual function. PBM also inhibited diabetes-induced reductions in retinal *Cyp24a1* mRNA levels and numbers of circulating stem cells (CD45$^-$/c-Kit$^+$), but these effects may not account for the beneficial effects of PBM on the retinopathy. PBM significantly inhibits the functional and histopathologic features of early DR, and these effects likely are mediated via multiple mechanisms.

The pathogenesis of diabetic retinopathy (DR) remains unclear, but hyperglycemia is regarded as the initiating event, and subsequent local oxidative stress and inflammation have been implicated in the development of early stages of the retinopathy (1–3). The focus of interest has been primarily on retinal vascular changes. Nevertheless, abnormalities of the neural retina also are known to develop (4,5), and at least some neuronal cells (photoreceptor cells) likely contribute to the pathogenesis of the observed vascular changes (6–8).

Therapeutic use of light (photobiomodulation [PBM]) in the visible far-red to near-infrared region of the spectrum (590–850 nm) (9,10) is being found to have beneficial effects in a variety of diseases, including wound healing, hypoxic injury, cerebral degeneration, Alzheimer disease, postoperative cognitive dysfunction, and retinal degeneration (10–20). Recent evidence from our group (21,22) also indicates that PBM inhibits the development of molecular abnormalities that contribute to the development of early lesions of DR in animals and resolve early (noncenter-involved) macular edema in two case series of patients with diabetes (23,24).

The mechanism by which PBM mediates observed beneficial effects remains under investigation. A number of investigations have focused on stimulation of cytochrome c oxidase, an enzyme that enhances the energy production by mitochondria in the respiratory electron transport chain (25–28), whereas other studies have shown the beneficial effects of PBM correlate with release of stem cells or progenitor cells, including enhancing proliferation of bone marrow mesenchymal stem cells and adipose-derived stem cells in vitro (29–31). Tuby et al. (32) reported low-level laser therapy recruited mesenchymal stem cells from the circulation to infarcted heart area and attenuated the scarring process. Other evidence (21,33–36) raises the possibility of an indirect effect, because beneficial effects could be observed even if light was blocked from shining directly on a particular area of the body by a shield. Thus, favorable effects of PBM may be mediated by both local and systemic effects.

In this study, our goal is to assess the long-term effects of PBM therapy on *1*) the morphologic vascular lesions that characterize early DR, *2*) the function of the neural retina

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via testing of visual psychophysics, and *3*) two possible systemic mechanisms by which PBM might mediate its beneficial effects on the retina (release of stem cells and activation of signaling from the vitamin D receptor [VDR]). Vitamin D and signaling via the VDR have strong anti-inflammatory and antioxidant actions (37,38).

RESEARCH DESIGN AND METHODS

Animals

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in ventilated microisolator cages under 12:12-h light/dark cycle. All animal procedures in this study adhered to the National Institute of Health *Guide for the Care and Use of Laboratory Animals*, the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and authorization of the Institutional Animal Care and Use Committee at Case Western Reserve University. Intraperitoneal injection of streptozotocin (STZ) in citrate buffer was used to induce diabetes in mice at the age of 2 to 3 months (60 mg/kg of body weight for 5 consecutive days) (39). Subcutaneous injection of insulin (0–0.2 units) was applied as needed to prevent weight loss while still allowing hyperglycemia. The onset of diabetes was defined as three consecutive measures of nonfasting blood glucose >250 mg/dL (~14 mmol/L). Both blood glucose and HbA_{1c} were monitored as described previously (21,40,41).

PBM

Cohorts of diabetic and nondiabetic mice were treated with far-red light generated by an array of light-emitting diodes (SpectraLife; Quantum Devices, Inc., Barneveld, WI). This device was determined to deliver 670-nm light at a power of 25 mW/cm^2 (irradiance) at the 50% intensity setting at a distance of 2 to 3 cm between the device and the animal (measured with spectroradiometer, specbos 1211UV; Dataoptics, Inc., Ypsilanti, MI). The mice were confined to a small plastic box (90 × 135 mm) that blocked ambient light, but did not have a cover between the animals and the light source. The light provided illumination evenly across the entire back of the animals. Under ambient room light in the animal room (250 lux), animals were irradiated for 240 s each day (~5 J/cm^2 dose daily). PBM was started immediately after confirmation of diabetes and administered daily between 10:00 and 11:30 A.M. Conditions of light delivery were similar to those in our prior studies (21,22).

Diabetes-Induced Retinal Histopathology

Assessment of diabetes-induced retinal capillary disease has been previously described (39). Briefly, at 8 months of diabetes, mouse eyes were fixed in 10% buffered formalin (Sigma-Aldrich, St. Louis, MO) for at least 1 week, and retinas were isolated, washed overnight in water, digested in Tris buffer containing 40 units/mL elastase overnight, and mechanically brushed to dislodge retinal neurons from the vasculature. When totally cleaned of neural cells, the isolated vasculature was laid out on a glass microscope slide and stained with hematoxylin and periodic acid Schiff reagent. Degenerate (acellular) capillaries were quantitated in six to seven field areas corresponding to the midretina (original magnification ×200) in a masked manner. Acellular capillaries (reported per square millimeter of retinal area) were identified as capillary-sized vessel tubes having no nuclei along their length, as previously reported (42–44).

Leakage of Albumin Into Neural Retina

Accumulation of the blood protein, albumin, in the neural retina has been viewed as a marker of increased vascular permeability (41,45). At 8 months of diabetes, sterile fluorescein isothiocyanate (FITC)–BSA (50 μg/μL; Sigma-Aldrich) in PBS (NaCl, 0.138 mol/L; KCl, 0.0027 mol/L; pH 7.4) was injected into the tail vein of mice at 100 μg/g body weight. After 20 min, mice were euthanized, and their eyes were enucleated, fixed in 4% paraformaldehyde for 5 h, followed by incubation in increasing concentrations of sucrose (up to 20%; at least 2 h each), and then frozen in Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, CA). At least three noncontiguous cryosections of retina were cut from each eye, and average image pixel density in the neural retina was measured by computer-assisted fluorescence microscopy. Visible blood vessels were excluded from areas where fluorescence (pixel density) was measured. Leakage of albumin was estimated from measurements of fluorescence in the inner and outer plexiform layers and the inner nuclear layer of the neural retina. Average values of relative fluorescence units were normalized by expressing tissue fluorescence for each animal as a ratio to the relative fluorescence units of blood plasma from each animal. Vascular permeability in diabetes was expressed as the ratio of FITC-dextran concentration in neural retina of diabetic animals relative to that in nondiabetic animals.

Optokinetic Assessment of Photopic Visual Function

The spatial frequency threshold (an estimate of visual acuity) and contrast sensitivity threshold were measured with the Virtual Optokinetic system (OptoMotry; Cerebral-Mechanics, Inc., Medicine Hat, Alberta, Canada), as reported previously (40,46,47). The maximum spatial frequency capable of driving head tracking was determined as the spatial frequency threshold. The contrast sensitivity at 8 months of diabetes was measured at six spatial frequencies (0.031, 0.064, 0.092, 0.103, 0.192, and 0.272 cycles/degree) to detect functional defects in spatially sensitive retinal cells or in higher visual pathways. This was determined as the inverse of Michelson contrast without correction for luminance of the monitors. Each measurement was repeated several times to evaluate the reproducibility of responses. The experimenter was masked as to the identity of the experimental animals and groups.

Assessment of $CD45^-$/c-Kit^+ Cells

Blood was collected between 9:00 and 10:00 A.M. from anesthetized animals via heart puncture with syringes containing EDTA, immediately transferred to 7.2-mg EDTA vacutainers

(BD Biosciences, San Jose, CA), and stored on wet ice for up to 10 min. Erythrocytes were lysed by the addition of red blood cell lysis buffer (Invitrogen, Carlsbad, CA), and remaining cells were washed and analyzed with flow cytometry (BD Accuri C6 cytometer; BD Biosciences). Cells were stained with FITC–anti-mouse c-Kit and phycoerythrin anti-mouse CD45 antibodies (BD Pharmingen, San Jose, CA). Identically labeled isotype control antibodies (BD Pharmingen) served as negative controls. Circulating stem cells were gated as $CD45^{-/low}$/c-Kit^{+} cells (48) and expressed as a percentage of all $CD45^{+}$ cells. c-Kit^{+} cells also were identified in retinal whole mounts and counted manually. The antibody used was the same as that described above, and the immunohistochemical method used was described previously (44).

Quantitative Real-time PCR

Total RNA was isolated from mouse skin and retina with TRIzol reagent (Ambion, Carlsbad, CA) and RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. A total of 350 ng total RNA was reverse transcribed into cDNA using Iscript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed using FastStart Universal SYBR Green Master Mix (Roche, Mannheim, Germany) on an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) in triplicate. PCR conditions were 400 nmol primer for 10 min at 95°C, 45 cycles of 15 s at 95°C, and at 57°C for 30 s. The fold-change expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ fold-change formula normalized to β-actin and nondiabetic untreated controls. The following primer sets were used in this study: VDR (sense: 5′-CTTCCTAAGAGACTTCCCGAGAGA-3′ and antisense: 5′-GGCATTTATTTACAGCGGTACTTGT-3′); Cyp24a1 (sense: 5′-CATCGACCACCGCCTAGAGA-3′ and antisense: 5′-ACAGCAGCGTACAGTTCTTTC-3′); and β-actin (sense: 5′-GATTCCATACCCAAGAAGGAAGGCTG-3′ and antisense: 5′-AGCTGAGAGGGAAATCGTGCGT-3′).

Statistical Analysis

Statistical analysis was performed using ANOVA followed by Fisher test. A P value ≤ 0.05 was considered statistically significant. Comparisons of contrast sensitivity data between groups were analyzed by ANOVA with the repeated-measures test. Mann–Whitney test ($P \leq 0.05$) was used to compare the flow cytometry results between groups.

RESULTS

Animals

Diabetes was successfully induced in mice by injection with STZ based on HbA_{1c} and blood glucose levels. There were significant differences between STZ-treated mice and nondiabetic control mice in nonfasting blood glucose levels, HbA_{1c}, and body weight (Table 1). PBM application to diabetic animals had no significant effect on body weight, nonfasting blood glucose, or HbA_{1c} compared with the untreated diabetic control group.

Table 1—Clinical data of nondiabetic (N) and diabetic mice (D) with or without PBM

	N	D	N+PBM	D+PBM
Body weight (g)	45 ± 6	29 ± 2*	42 ± 5	27 ± 3*
Nonfasting blood glucose (mg/dL)	150 ± 22	473 ± 64*	157 ± 22	455 ± 66*
HbA_{1c}				
%	3.4 ± 0.1	12.0 ± 0.3*	3.3 ± 0.1	11.8 ± 0.5*
mmol/mol	14 ± 1.1	108 ± 2.2	13 ± 1.1	105 ± 5.5*

Data are mean ± SD. n = 13–16 animals in each group. *$P < 0.001$ compared with N.

Effects of PBM on Structural and Functional Lesions of DR

Although long-term (8 months) diabetes did not result in any change in retinal thickness or loss of photoreceptors, it did result in a significant ($P < 0.0001$) increase in the number of degenerate (acellular) capillaries in the retina of untreated animals (Fig. 1). Diabetes of 8 months also resulted in a significant ($P < 0.01$) increase in the amount of albumin extravasated into the nonvascular retina (i.e., in the neural retina between vessels) in each of the three layers of the retinal vascular plexus (in the inner and outer plexiform layers and at the border of the inner plexiform and inner nuclear layers) (Fig. 2). Daily illumination of the animals with 670-nm light for 4 min/day significantly inhibited the diabetes-induced degeneration of retinal capillaries and albumin accumulation in the inner nuclear and outer plexiform layers of the retina.

Visual Function

To determine whether the diabetes-induced reduction in visual function is influenced by PBM, an optokinetic system was used to measure spatial frequency threshold and contrast sensitivity at six different spatial frequencies (Fig. 3) in mice diabetic for 8 months. As reported previously (47), diabetic mice displayed significantly lower spatial frequency threshold and contrast sensitivity than normal diabetic mice ($P < 0.001$). Both measures of visual function were largely preserved in diabetic mice treated daily with PBM for the entire duration of diabetes, with the effect being statistically different from that in the diabetic control group ($P < 0.001$). There was no marked difference in visual function between the untreated nondiabetic group and nondiabetic mice receiving PBM. These results indicate that PBM can play an important role in preserving normal neural function in diabetes.

Because our previous study of PBM in DR (22) provided no support for the prevailing hypothesis that beneficial effects of PBM were mediated via stimulation of mitochondrial cytochrome oxidase activity, we conducted pilot studies to determine if other possible effects of the light therapy (increasing numbers of circulating stem cells and stimulation of vitamin D signaling) were associated with the

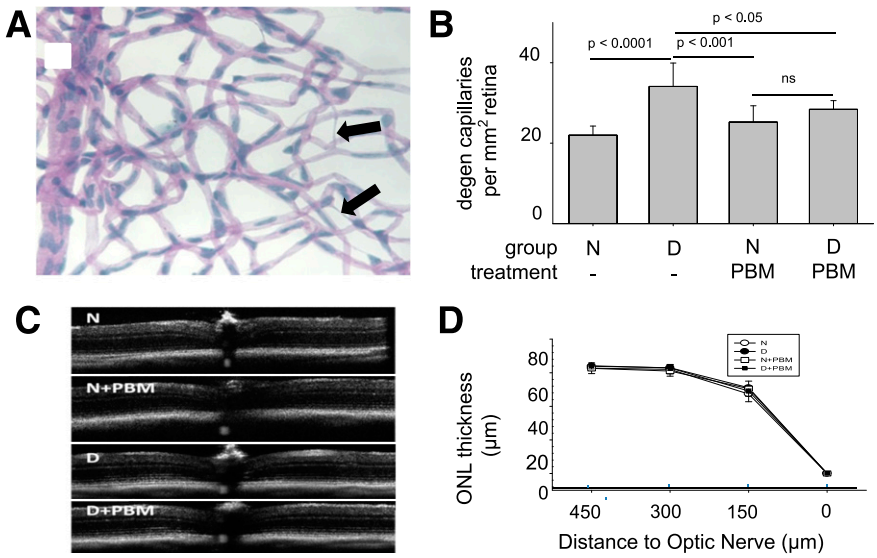

Figure 1—Diabetes (D) of 8 months' duration significantly increased the number of degenerate (acellular) capillaries in the retinal vasculature compared with that in nondiabetic (N) mice, and daily application of PBM at 670 nm for 4 min/day to diabetic mice for the 8 months significantly inhibited the retinal capillary degeneration. *A*: Representative micrograph of the isolated retinal microvessels from a diabetic mouse indicating degenerate capillaries (arrows). *B*: Summary of numbers of degenerate (degen) retinal capillaries in the various groups. Optical coherence microscopy of mouse retinas (*C*) and graphical summary of thickness of the outer nuclear layer (ONL; *D*) in the experimental groups at 8 months of diabetes (10 months of age). n = 7/group. Data expressed as mean ± SD.

observed beneficial effects on functional and structural end points of the retinopathy.

Circulating CD45$^-$/c-Kit$^+$ Cells

To investigate whether stem cells are associated with the beneficial effects of PBM, blood was collected from mice diabetic for 3 months (5 months of age; the duration of diabetes when blood was collected for this initial study was arbitrary), and numbers of CD45$^-$/c-Kit$^+$ cells in peripheral blood were determined by flow cytometry. CD45$^-$/c-Kit$^+$ cells were found to be decreased in circulating blood from untreated diabetic mice compared with nondiabetic controls (Fig. 4 and Supplementary Fig. 1). Diabetic mice that had received PBM soon after the onset of diabetes, in contrast, had a moderate increase of CD45$^-$/c-Kit$^+$ cells ($P < 0.05$) compared with untreated diabetic mice. Efforts to detect and quantitate c-Kit$^+$ cells in the retina in diabetes showed that the c-Kit$^+$ cells were very rare in all experimental

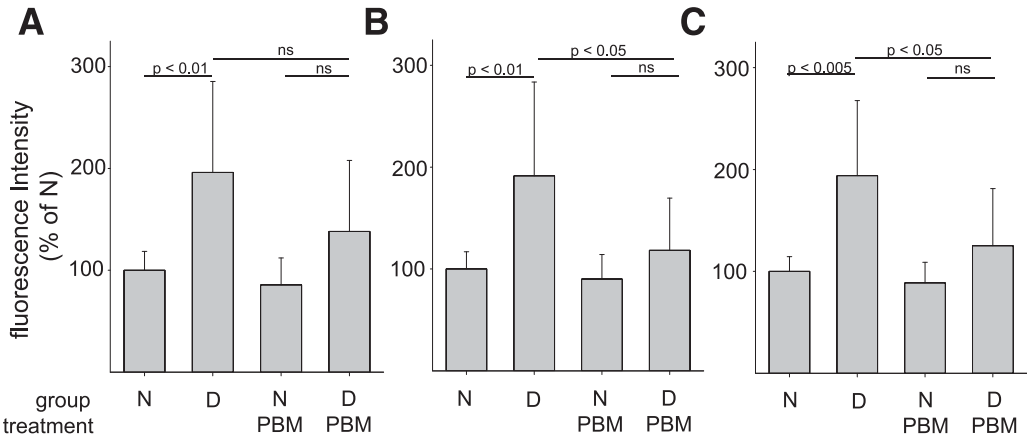

Figure 2—Diabetes (D) of 8 months' duration significantly increased accumulation of FITC-BSA in the inner plexiform (*A*), inner nuclear (*B*), and outer plexiform (*C*) layers of the mouse retina compared with that in nondiabetic (N) mice. PBM from the onset of diabetes significantly inhibited the extravascular fluorescence in the inner nuclear and outer plexiform layers of the retina. FITC-albumin was injected intravenously, allowed to circulate for 20 min, and then average fluorescence was measured from cross-sections from a large area of each layer, taking care to avoid obvious microvessels. n = 6 to 7. Data expressed as mean ± SD.

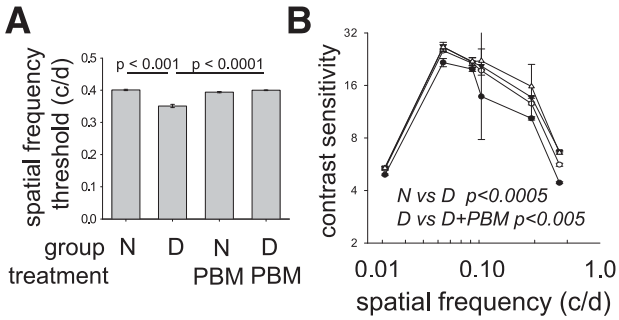

Figure 3—PBM significantly inhibited the diabetes-induced reductions in spatial frequency threshold (*A*) and contrast sensitivity (*B*) at 8 months' duration of diabetes. Contrast sensitivity was measured at six different frequencies from 0.032 to 0.272 cycles/degree (c/d). Diabetic (D) mice had significantly lower visual function than did nondiabetic (N) mice, whereas diabetic mice receiving PBM therapy 4 min/day had significantly enhanced spatial frequency threshold and contrast sensitivity compared with diabetic controls. No significant difference between N and N+PBM was detected. PBM was initiated immediately after onset of diabetes and applied daily for >30 weeks when visual function was tested. Data expressed as mean ± SEM. n = 4–6 in all groups. △, N; ●, D; ▼, N+PBM; ○, D+PBM.

groups (zero to one immunopositive cells per retina), and no obvious effects of diabetes or PBM were observed. Thus, although the PBM-induced increase in circulating $CD45^-$/c-Kit^+ in diabetes was associated with the observed beneficial effects of PBM on the retinopathy, the lack of significant accumulation of these cells in the retina in any of the groups offers no support to the postulate that c-Kit^+ cells played an important role in the inhibition of the retinopathy by PBM.

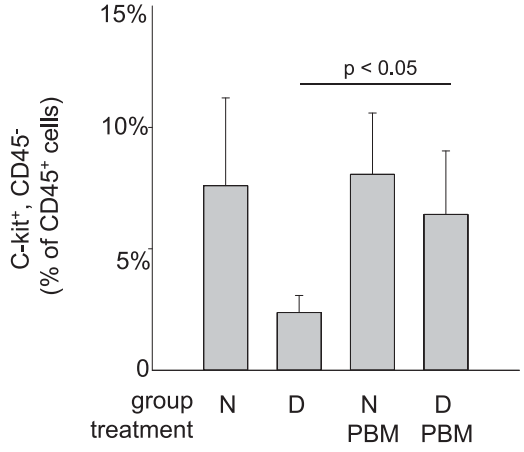

Figure 4—Effect of PBM exposure on circulating stem cells in mice at 14 to 15 weeks of diabetes (5 to 6 months of age). Representative flow histogram of stem cells in peripheral blood are summarized graphically for nondiabetic (N), diabetic (D), and nondiabetic and diabetic mice treated with PBM. Circulating stem cells were gated as $CD45^-$/c-Kit^+ cells following immunolabeling. Representative flow data are provided in the Supplementary Fig. 1. PBM significantly increased the number of circulating $CD45^-$/c-Kit^+ cells in diabetic mice compared with that in untreated diabetic controls. Results are reported as mean ± SEM. n = 5 animals in each column.

VDR Signaling

Production of vitamin D by sunlight and subsequent signaling via the VDR is well recognized to have important biologic effects (49,50). To explore the possibility that PBM might act systemically by activating the VDR signaling, we measured expression of VDR and a gene regulated by VDR signaling (*Cyp24a1*) in both skin and retina. As shown in Fig. 5*A* and *B*, VDR mRNA levels did not exhibit any significant change due to diabetes or PBM in either skin or retina. In contrast, *Cyp24a1* mRNA levels in retina were significantly decreased in diabetic controls compared with nondiabetic controls, and exposure of diabetic animals to PBM inhibited the diabetes-induced loss of *Cyp24a1* (Fig. 5*D*). Thus, preservation of retinal levels of *Cyp24a1* by PBM at this duration was at least associated with the observed beneficial effects of PBM on structural and functional lesions of DR.

DISCUSSION

The current clinical approach to inhibit development of retinopathy and other complications of diabetes uses exogenous insulin or oral medications to maintain blood glucose concentrations as close to normal as possible. Such good glycemic control is difficult to achieve and maintain for many patients with diabetes, so there has been continued interest in identification of alternate ways to inhibit the development of diabetic complications.

Increased permeability of the retinal vasculature, progressive degeneration of the retinal capillaries, and altered function of the neural retina, as evidenced by altered visual function, are major abnormalities that characterize the early stages of DR. All three abnormalities develop together in diabetes, but it is unclear whether or not the vascular and neural abnormalities of diabetes share a similar pathogenesis (47). The current study shows that PBM therapy for only 4 min/day significantly inhibited all three of these clinically important lesions of DR. PBM exerts these beneficial effects noninvasively, is inexpensive, and is easy to administer. The 8-month duration of daily PBM therapy in the current study is among the longest durations in which PBM has been evaluated, and it is important that no adverse effects of the therapy were observed.

Mechanisms by which PBM affect cellular biologic processes are still under investigation. A number of studies have demonstrated a beneficial effect of PBM on activity of cytochrome oxidase (51,52), a critical enzyme in the mitochondrial electron transport chain. In retinas of diabetic animals, however, we previously reported that PBM inhibited the diabetes-induced increases in retinal oxidative stress and inflammation by mechanisms that seemed not to involve cytochrome oxidase (22). Thus, we looked for other changes mediated by PBM that might give a clue pertaining how the light therapy exerts the observed beneficial effects.

PBM has been shown by our group (21) to have beneficial effects on the retina in diabetes even if there is not direct exposure of the light with eye, and others have made

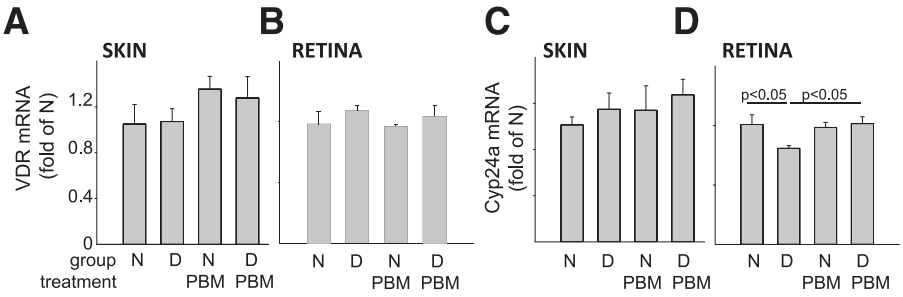

Figure 5—Abundance of VDR in mouse skin (*A*) and retina (*B*) and *Cyp24a1* in skin (*C*) and retina (*D*) as determined by quantitative real-time PCR and normalized to β-actin. No significant changes were observed in VDR mRNA levels in the skin and retina among groups, but diabetes significantly reduced mRNA for *Cyp24a1* in retina, and PBM inhibited that reduction. PBM was initiated immediately after onset of diabetes and continued for 14 to 15 weeks. D, diabetic mice; N, nondiabetic mice. Data are presented as mean ± SEM. n = 6 to 7 animals/group.

similar observations in other tissues (33–36,53,54). We reasoned that if observed beneficial effects of PBM were not directly mediated on the eye, then skin would be a logical place where the light energy would be absorbed. Thus, we investigated the possibility that the PBM might exert beneficial effects in vivo via VDR signaling in skin. Our initial studies focused on effects of diabetes and PBM on expression of the VDR and *Cyp24a1*, which encodes the hydroxylase that converts 1,25-dihydroxyvitamin D3 into inactive 24-hydroxylated products. The wavelength, which most strongly stimulates generation of vitamin D (~300 nm [55]), is much shorter than the wavelength used by us in this study, suggesting that direct signaling through the VDR as a result of PBM therapy is unlikely. Nevertheless, we did find evidence that expression of a gene regulated by vitamin D and the VDR (*Cyp24a1*) was subnormal in diabetes in the retina but preserved by PBM. Neither the inhibitory effects of diabetes nor the beneficial effects of PBM on retinal *Cyp24a1* were shared by skin.

Some studies have suggested that stem cells can be mobilized by PBM or low-level laser therapy and thus might participate in beneficial effects of the light therapy (29,32,34,56). Consistent with that, it has been reported that light therapy increased the proliferation of c-Kit$^+$ cells in the infarcted heart (32) and other tissues. In our hands, PBM did not increase the number of circulating stem cells in nondiabetic animals, potentially because the animals in our study were considerably older than those in most studies when the blood was collected for analysis. To determine if availability of hematopoietic stem cells in response to PBM is associated with the observed protective effects of PBM in diabetic mice, c-Kit$^+$ cells in the circulation were assessed by flow cytometry, and the number of c-Kit$^+$ cells in the retina was quantitated in retinal whole mounts. Diabetes significantly reduced the number of c-Kit$^+$ cells in the circulation, whereas diabetic mice treated with PBM had a higher number of these cells in the circulation compared with control diabetic mice. It is not clear whether the observed reduction in circulating CD45$^-$/c-Kit$^+$ cells in diabetes was a result of a true reduction in circulating numbers of this cell population that was enhanced by PBM or was instead a phase shift in the diurnal release of those cells from bone marrow that was corrected by the PBM. However, essentially no c-Kit$^+$ cells were observed within the neural retina or retinal vasculature in any groups. The lack of accumulation of these cells within the neural retina is not altogether surprising, because we previously reported that diabetes did not increase the accumulation of any leukocyte cell types in the neural retina (44), and stem cells likely lose their stem markers within the environment of a tissue. Nevertheless, the lack of accumulation of these cells within the retina does not provide support for a possible contribution of stem cells in the observed beneficial effect of PBM on the retinopathy.

Previously, we reported that PBM therapy from the onset of disease or as an intervention therapy inhibited the oxidative stress and inflammation that develop in the retina in diabetes, as well as upregulating survival pathways (21,22). Others have reported similar findings in other conditions (52), and these effects might be mediated by effects on signaling molecules and activation of transcription factors, including nuclear factor-κB. These effects of PBM to inhibit the diabetes-induced increase in oxidative stress and inflammation-associated proteins likely play an important role in the observed inhibition of at least the dysfunction and degeneration of the retinal vasculature in DR, because genetic or pharmacologic inhibition of oxidative stress or inflammation previously was shown to inhibit development of these vascular lesions (1,43,44,57–64). Thus, our present working hypothesis is that the observed beneficial effects of PBM against the development of retinopathy in diabetic animals likely are secondary to PBM-induced changes in transcription or activity of critical enzymes having antioxidant and anti-inflammatory effects.

In summary, PBM for only 4 min/day significantly inhibited the development of several clinically accepted and important lesions of DR. In particular, daily administration of PBM for 8 months significantly inhibited the diabetes-induced increase in vascular permeability and capillary degeneration that is characteristic of DR. Moreover, PBM protected against the reduction in visual function that is caused by diabetes. These findings have important implications for PBM as a potential adjunct therapy to

inhibit DR and possibly other degenerative processes. It seems likely that PBM is having beneficial effects on the retina via multiple mechanisms.

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Author Contributions. Y.C., Y.D., H.L., and A.V. performed molecular and physiologic analyses and analyzed data. Y.C., A.V., and T.S.K. wrote the manuscript. J.T. evaluated histopathology. T.S.K. designed the experiments and analyzed data. T.S.K. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Tang J, Kern TS. Inflammation in diabetic retinopathy. Prog Retin Eye Res 2011;30:343–358
2. Kern TS, Tang J, Berkowitz BA. Validation of structural and functional lesions of diabetic retinopathy in mice. Mol Vis 2010;16:2121–2131
3. Zheng L, Kern T. Role of nitric oxide, superoxide, peroxynitrite and PARP in diabetic retinopathy. Front Biosci 2009;14:3974–3987
4. Kern TS, Barber AJ. Retinal ganglion cells in diabetes. J Physiol 2008;586: 4401–4408
5. Jackson GR, Scott IU, Quillen DA, Walter LE, Gardner TW. Inner retinal visual dysfunction is a sensitive marker of non-proliferative diabetic retinopathy. Br J Ophthalmol 2012;96:699–703
6. Liu H, Tang J, Du Y, et al. Photoreceptor cells influence retinal vascular degeneration in mouse models of retinal degeneration and diabetes. Invest Ophthalmol Vis Sci 2016;57:4272–4281
7. Kern TS, Berkowitz BA. Photoreceptors in diabetic retinopathy. J Diabetes Investig 2015;6:371–380
8. Tonade D, Liu H, Palczewski K, Kern TS. Photoreceptor cells produce inflammatory products that contribute to retinal vascular permeability in a mouse model of diabetes. Diabetologia 2017;60:2111–2120
9. Chung H, Dai T, Sharma SK, Huang YY, Carroll JD, Hamblin MR. The nuts and bolts of low-level laser (light) therapy. Ann Biomed Eng 2012;40:516–533
10. Desmet KD, Paz DA, Corry JJ, et al. Clinical and experimental applications of NIR-LED photobiomodulation. Photomed Laser Surg 2006;24:121–128
11. Eells JT, Wong-Riley MT, VerHoeve J, et al. Mitochondrial signal transduction in accelerated wound and retinal healing by near-infrared light therapy. Mitochondrion 2004;4:559–567
12. Liang HL, Whelan HT, Eells JT, et al. Photobiomodulation partially rescues visual cortical neurons from cyanide-induced apoptosis. Neuroscience 2006;139:639–649
13. Soriano F, Campana V, Moya M, et al. Photobiomodulation of pain and inflammation in microcrystalline arthropathies: experimental and clinical results. Photomed Laser Surg 2006;24:140–150
14. Oliveira PC, Meireles GC, dos Santos NR, et al. The use of light photobiomodulation on the treatment of second-degree burns: a histological study of a rodent model. Photomed Laser Surg 2008;26:289–299
15. Hashmi JT, Huang YY, Osmani BZ, Sharma SK, Naeser MA, Hamblin MR. Role of low-level laser therapy in neurorehabilitation. PM R 2010;2(Suppl. 2):S292–S305
16. Peplow PV, Chung TY, Baxter GD. Laser photobiomodulation of wound healing: a review of experimental studies in mouse and rat animal models. Photomed Laser Surg 2010;28:291–325
17. Albarracin R, Eells J, Valter K. Photobiomodulation protects the retina from light-induced photoreceptor degeneration. Invest Ophthalmol Vis Sci 2011;52:3582–3592
18. Muili KA, Gopalakrishnan S, Meyer SL, Eells JT, Lyons JA. Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by photobiomodulation induced by 670 nm light. PLoS One 2012;7:e30655
19. Muili KA, Gopalakrishnan S, Eells JT, Lyons JA. Photobiomodulation induced by 670 nm light ameliorates MOG35-55 induced EAE in female C57BL/6 mice: a role for remediation of nitrosative stress. PLoS One 2013;8:e67358
20. Eells JT, Gopalakrishnan S, Valter K. Near-infrared photobiomodulation in retinal injury and disease. Adv Exp Med Biol 2016;854:437–441
21. Saliba A, Du Y, Liu H, et al. Photobiomodulation mitigates diabetes-induced retinopathy by direct and indirect mechanisms: evidence from intervention studies in pigmented mice. PLoS One 2015;10:e0139003
22. Tang J, Du Y, Lee CA, Talahalli R, Eells JT, Kern TS. Low-intensity far-red light inhibits early lesions that contribute to diabetic retinopathy: in vivo and in vitro. Invest Ophthalmol Vis Sci 2013;54:3681–3690
23. Tang J, Herda AA, Kern TS. Photobiomodulation in the treatment of patients with non-center-involving diabetic macular oedema. Br J Ophthalmol 2014;98: 1013–1015
24. Eells JT, Gopalakrishnan S, Connor TB. 670 nm photomodulation as a therapy for diabetic macular edema: a pilot study (ARVO abstract). Invest Ophthalmol Vis Sci 2017;58:932
25. Wong-Riley MT, Liang HL, Eells JT, et al. Photobiomodulation directly benefits primary neurons functionally inactivated by toxins: role of cytochrome c oxidase. J Biol Chem 2005;280:4761–4771
26. Karu TI, Pyatibrat LV, Kolyakov SF, Afanasyeva NI. Absorption measurements of a cell monolayer relevant to phototherapy: reduction of cytochrome c oxidase under near IR radiation. J Photochem Photobiol B 2005;81:98–106
27. Zhang R, Mio Y, Pratt PF, et al. Near infrared light protects cardiomyocytes from hypoxia and reoxygenation injury by a nitric oxide dependent mechanism. J Mol Cell Cardiol 2009;46:4–14
28. Poyton RO, Ball KA. Therapeutic photobiomodulation: nitric oxide and a novel function of mitochondrial cytochrome c oxidase. Discov Med 2011;11:154–159
29. Tuby H, Maltz L, Oron U. Low-level laser irradiation (LLLI) promotes proliferation of mesenchymal and cardiac stem cells in culture. Lasers Surg Med 2007;39:373–378
30. Mvula B, Mathope T, Moore T, Abrahamse H. The effect of low level laser irradiation on adult human adipose derived stem cells. Lasers Med Sci 2008;23: 277–282
31. Li WT, Leu YC, Wu JL. Red-light light-emitting diode irradiation increases the proliferation and osteogenic differentiation of rat bone marrow mesenchymal stem cells. Photomed Laser Surg 2010;28(Suppl. 1):S157–S165
32. Tuby H, Maltz L, Oron U. Induction of autologous mesenchymal stem cells in the bone marrow by low-level laser therapy has profound beneficial effects on the infarcted rat heart. Lasers Surg Med 2011;43:401–409
33. Schindl A, Heinze G, Schindl M, Pernerstorfer-Schön H, Schindl L. Systemic effects of low-intensity laser irradiation on skin microcirculation in patients with diabetic microangiopathy. Microvasc Res 2002;64:240–246
34. Stone J, Johnstone D, Mitrofanis J. The helmet experiment in Parkinson's disease: an observation of the mechanism of neuroprotection by near infra-red light. In Proceedings of the 9th World Association for Laser Therapy Conference, Gold Coast, Surfers Paradise, Australia, 2012, p. 17–20
35. Johnstone DM, el Massri N, Moro C, et al. Indirect application of near infrared light induces neuroprotection in a mouse model of parkinsonism - an abscopal neuroprotective effect. Neuroscience 2014;274:93–101
36. Johnstone DM, Mitrofanis J, Stone J. Targeting the body to protect the brain: inducing neuroprotection with remotely-applied near infrared light. Neural Regen Res 2015;10:349–351
37. Goncalves de Carvalho CM, Ribeiro SM. Aging, low-grade systemic inflammation and vitamin D: a mini-review. Eur J Clin Nutr 2017;71:434–440
38. Mehmood ZH, Papandreou D. An updated mini review of vitamin D and obesity: adipogenesis and inflammation state. Open Access Maced J Med Sci 2016;4:526–532

39. Veenstra A, Liu H, Lee CA, Du Y, Tang J, Kern TS. Diabetic retinopathy: retina-specific methods for maintenance of diabetic rodents and evaluation of vascular histopathology and aolecular abnormalities. Curr Protoc Mouse Biol 2015;5:247–270
40. Liu H, Tang J, Lee CA, Kern TS. Metanx and early stages of diabetic retinopathy. Invest Ophthalmol Vis Sci 2015;56:647–653
41. Du Y, Cramer M, Lee CA, et al. Adrenergic and serotonin receptors affect retinal superoxide generation in diabetic mice: relationship to capillary degeneration and permeability. FASEB J 2015;29:2194–2204
42. Veenstra AA, Tang J, Kern TS. Antagonism of CD11b with neutrophil inhibitory factor (NIF) inhibits vascular lesions in diabetic retinopathy. PLoS One 2013;8:e78405
43. Talahalli R, Zarini S, Tang J, et al. Leukocytes regulate retinal capillary degeneration in the diabetic mouse via generation of leukotrienes. J Leukoc Biol 2013; 93:135–143
44. Li G, Veenstra AA, Talahalli RR, et al. Marrow-derived cells regulate the development of early diabetic retinopathy and tactile allodynia in mice. Diabetes 2012; 61:3294–3303
45. Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW; Penn State Retina Research Group. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Diabetes 1998;47:1953–1959
46. Prusky GT, Alam NM, Beekman S, Douglas RM. Rapid quantification of adult and developing mouse spatial vision using a virtual optomotor system. Invest Ophthalmol Vis Sci 2004;45:4611–4616
47. Lee CA, Li G, Patel MD, et al. Diabetes-induced impairment in visual function in mice: contributions of p38 MAPK, RAGE, leukocytes, and aldose reductase. Invest Ophthalmol Vis Sci 2013;93:135–143
48. Bertrand JY, Giroux S, Golub R, et al. Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. Proc Natl Acad Sci U S A 2005;102:134–139
49. Bikle DD. Extraskeletal actions of vitamin D. Ann N Y Acad Sci 2016;1376:29–52
50. Caprio M, Infante M, Calanchini M, Mammi C, Fabbri A. Vitamin D: not just the bone. Evidence for beneficial pleiotropic extraskeletal effects. Eat Weight Dis 2017; 22:27–41
51. Wong-Riley MT, Bai X, Buchmann E, Whelan HT. Light-emitting diode treatment reverses the effect of TTX on cytochrome oxidase in neurons. Neuroreport 2001;12: 3033–3037
52. de Freitas LF, Hamblin MR. Proposed mechanisms of photobiomodulation or low-level light therapy. IEEE J Sel Top Quantum Electron 2016;22:7000417.
53. Rochkind S, Rousso M, Nissan M, Villarreal M, Barr-Nea L, Rees DG. Systemic effects of low-power laser irradiation on the peripheral and central nervous system, cutaneous wounds, and burns. Lasers Surg Med 1989;9:174–182
54. Rodrigo SM, Cunha A, Pozza DH, et al. Analysis of the systemic effect of red and infrared laser therapy on wound repair. Photomed Laser Surg 2009;27:929–935
55. Olds WJ, Lucas RM, Kimlin MG. Action spectrum for vitamin D synthesis [article online], 2010. Available from https://www.niwa.co.nz/sites/niwa.co.nz/files/action_spectrum_for_vit_d_synthesis.pdf. Accessed 25 November 2017
56. Tuby H, Maltz L, Oron U. Implantation of low-level laser irradiated mesenchymal stem cells into the infarcted rat heart is associated with reduction in infarct size and enhanced angiogenesis. Photomed Laser Surg 2009;27:227–233
57. Du Y, Tang J, Li G, et al. Effects of p38 MAPK inhibition on early stages of diabetic retinopathy and sensory nerve function [published correction appears in Invest Ophthalmol Vis Sci 2011;52:6057]. Invest Ophthalmol Vis Sci 2010;51:2158–2164
58. Berkowitz BA, Gradianu M, Bissig D, Kern TS, Roberts R. Retinal ion regulation in a mouse model of diabetic retinopathy: natural history and the effect of Cu/Zn superoxide dismutase overexpression. Invest Ophthalmol Vis Sci 2009;50:2351–2358
59. Gubitosi-Klug RA, Talahalli R, Du Y, Nadler JL, Kern TS. 5-Lipoxygenase, but not 12/15-lipoxygenase, contributes to degeneration of retinal capillaries in a mouse model of diabetic retinopathy. Diabetes 2008;57:1387–1393
60. Zheng L, Howell SJ, Hatala DA, Huang K, Kern TS. Salicylate-based anti-inflammatory drugs inhibit the early lesion of diabetic retinopathy. Diabetes 2007;56: 337–345
61. Zheng L, Du Y, Miller C, et al. Critical role of inducible nitric oxide synthase in degeneration of retinal capillaries in mice with streptozotocin-induced diabetes. Diabetologia 2007;50:1987–1996
62. Kanwar M, Chan PS, Kern TS, Kowluru RA. Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. Invest Ophthalmol Vis Sci 2007;48:3805–3811
63. Joussen AM, Poulaki V, Le ML, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 2004;18:1450–1452
64. Kowluru RA, Tang J, Kern TS. Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. Diabetes 2001;50:1938–1942