Title
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Journal
Investigative Ophthalmology & Visual Science, 56(1)

ISSN
0146-0404

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Publication Date
2015-01-29

DOI
10.1167/iovs.14-15220

Peer reviewed

Metanx and Early Stages of Diabetic Retinopathy

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Submitted: July 13, 2014
Accepted: December 21, 2014

Citation: Liu H, Tang J, Lee CA, Kern TS. Metanx and early stages of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2015;56:647–653. DOI:10.1167/iovs.14-15220

Purpose. L-Methylfolate, pyridoxal 5′-phosphate, and methylcobalamin, individually have been reported to have beneficial effects on diabetes-induced defects. The possibility that combining these therapeutic approaches might have additional benefit led us to investigate the effect of Metanx against development of early stages of diabetic retinopathy in a mouse model.

Methods. C57BL/6J mice were made diabetic with streptozotocin, and some were given Metanx (a combination food product) mixed in the food at a dose of 5 mg/kg of body weight. Mice were killed at 2 months and 10 months of study for assessment of retinal function, retinal vascular histopathology, accumulation of albumin in neural retina, and biochemical and physiological abnormalities in retina.

Results. Two months of diabetes significantly increased leukostasis within retinal vessels and superoxide generation by the retina. Diabetes also significantly increased expression of intercellular adhesion molecule-1 (ICAM-1) and phosphorylation of IκB. Daily consumption of Metanx significantly inhibited all of these abnormalities. Ten months of diabetes significantly increased the degeneration of retinal capillaries and impaired visual function (spatial frequency threshold (SFT) and a parameter of contrast sensitivity) compared to nondiabetic controls. Daily consumption of Metanx for 10 months inhibited impairment of SFT but had no significant beneficial effect on capillary degeneration, pericyte loss, or the estimate of contrast sensitivity.

Conclusions. Metanx inhibited a diabetes-induced defect in retinal spatial frequency threshold and inhibited measures of oxidative stress and inflammation. It had no significant effect on contrast sensitivity or retinal capillary degeneration. Nutritional management with Metanx may help inhibit diabetes-induced defects in visual function.

Keywords: diabetic retinopathy, inflammation, Metanx, oxidative stress

Retinopathy is a common complication of diabetes and is the principal cause of blindness in the adult population. Available evidence strongly suggests that the processes leading to diabetic retinopathy (DR) are initiated by hyperglycemia, and reduction in glycemia has been found to inhibit the development and progression of the retinopathy. Nevertheless, improved achievement of glycemic control has been difficult for many patients, so effective supplemental therapies still are needed to inhibit the retinopathy.

Many efforts to inhibit diabetic retinopathy and other complications of diabetes have focused on highly specific therapeutic approaches to inhibit vascular lesions in the retina. Clinically, anti-VEGF therapies have had dramatically beneficial effects on macular edema. Preclinically, genetic approaches to alter expression of single enzymes (such as the inducible isoform of nitric oxide synthase [iNOS], 5-lipoxygenase, PPARα, and superoxide dismutase[1–5]) or pharmacologic inhibitors to target specific proteins (such as RAGE, p38 MAPK, and NADPH oxidase[6–8]) have shown significant inhibition of retinal capillary degeneration or permeability.

An alternate approach is to identify treatment regimens that work via multiple or less specific mechanisms. Metanx is a prescription medical food product that contains L-methylfolate, pyridoxal 5′-phosphate, and methylcobalamin, which are the active forms of vitamins B_9, B_6, and B_{12}. The active metabolite of folate, L-methylfolate, is known to stabilize and enhance production of tetrahydrobiopterin, a cofactor of iNOS, which inhibits uncoupling and associated superoxide generation.[9,10] Methylcobalamin (a coenzyme form of vitamin B_{12}) reacts with superoxide and inhibits glutathione depletion and peroxynitrite generation.[11] Pyridoxal phosphate has been reported to inhibit formation of advanced glycation end products (AGE), act as a metal chelator, and show antioxidant properties in diabetes[12] and has been studied as a potential treatment for neuropathy in diabetes and other conditions.[13–19] Consistent with these separate effects, Metanx has been reported to have beneficial effects on endothelial dysfunction and on peripheral nerves in diabetic rodents and patients.[15,16,18,19] Thus, Metanx therapy has shown effects that might be beneficial for diabetic retinopathy. A pilot clinical trial in which Metanx was administered daily as an intervention for 6 months to 7 patients with early to intermediate diabetic retinopathy[20] reportedly had statistically significant but modest effects on diabetes-induced alterations of retinal threshold sensitivity or central retinal thickness. Whether administration of this therapy for a longer duration or administration from the onset of diabetes would have had more favorable effects is not clear. In the present study, we evaluated the effects of Metanx on the

development of diabetes-induced alterations in retinal physiology, inflammation, histopathology, and visual function in mice.

Methods

Experimental Animals

Male C57Bl/6J mice were randomly assigned to become diabetic or remain as nondiabetic controls. Diabetes was induced by five sequential daily intraperitoneal injections of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 60 mg/kg of body weight (BW). After hyperglycemia was verified at least three times during the second week after streptozotocin, diabetic mice randomly were assigned to remain as untreated diabetic controls or to be administered Metanx. Animals were divided equally among 4 groups (nondiabetic control, nondiabetic-fed Metanx, diabetic control, and diabetic-fed Metanx). All animals were fed Teklad 7004 diet (Harlan Laboratories, Indianapolis, IN, USA) ad libitum with or without Metanx. Metanx was provided by Pamlab, Inc. (Carson City, NV, USA) and was mixed into the food at a dose of 5 mg/kg of BW. Insulin was given as needed to prevent weight loss without preventing hyperglycemia and glucosuria (0–0.2 units of neutral protamine Hagedorn (NPH) insulin subcutaneously, 0–3 times per week). Glycohemoglobin (GHb) was measured using total glycated hemoglobin assay (Bio-Rad; Hercules, CA, USA) every 2 to 3 months and just before animals were killed. Food consumption and body weight were measured weekly. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research, as well as to institutional guidelines. Animals were studied for durations of 10 months or 2 months of diabetes in order to investigate effects of the therapy on retinal histopathology or molecular and physiologic changes, respectively. All experimental groups for the long-and short-term studies had $n = 20$ and $n = 12$ mice, respectively, at the start of the experiment. We observed no adverse effects of the therapy on body weight gain of the diabetic mice.

Lucigenin Assay of Superoxide

Superoxide was measured chemically using lucigenin (*bis-N*-methylacridinium nitrate) as we published previously.[4,7,21–23]

Leukostasis

At 2 months of diabetes, blood was removed from the vasculature of anesthetized animals by complete perfusion with PBS via a heart catheter. Animals were then perfused with fluorescein-coupled concanavalin A lectin (20 μg/mL in PBS; Vector Laboratories, Burlingame, CA, USA), as we and others have described previously.[22,24–26] Flat-mounted retinas were imaged by fluorescence microscopy, and the number of leukocytes adhering to the vascular wall were counted.

Accumulation of Albumin in Neural Retina

The amount of leakage of albumin into neural retina was used to estimate vascular permeability. At 2 months of diabetes, eyes were cryosectioned (10-μm slices), fixed in methanol for 10 minutes, and washed four times in PBS. Each section was incubated in sheep anti-mouse serum albumin (catalog no. AB8940, 1:2000 dilution; Abcam, Cambridge, MA, USA) for 2 hours. After being washed, sections were incubated in FITC-labeled secondary antibody for 90 minutes (catalog no. AB 6743, 1:1000 dilution; Abcam). Using fluorescence microscopy, we determined the average amount of fluorescence in each of four retinal layers (inner plexiform layer, inner nuclear layer, outer plexiform layer, and outer nuclear layer). Measurements were made in three replicate areas in each retinal layer, both at midretina and by optic nerve. The amount of fluorescence in each site was the average of 10 replicate measurements measured by software (NIS Elements BR version 3.00; Nikon, Tokyo, Japan).

Expression of Inflammatory Proteins

Retinas were isolated, sonicated, and centrifuged in the presence of protease inhibitors, and the supernatant was used for Western blot analysis. Samples (50 μg) were fractionated by SDS-PAGE and electroblotted to nitrocellulose membrane, and membranes were blocked in Tris-buffered saline containing 0.02% Tween 20 and 5% nonfat milk. Antibodies for ICAM-1 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), inducible isoform of nitric oxide synthase (iNOS, 1:1000 dilution; Santa Cruz Biotechnology), heme oxygenase 1 (HO-1, 1:200 dilution; Santa Cruz Biotechnology), IκBa and p-IκBa (1:500 and 1:200 dilution, respectively; Santa Cruz Biotechnology), eNOS (1:500 dilution; BD-Transduction Laboratories, Franklin Lakes, NJ, USA), and MCP-1 (1:1000 dilution; Abcam) were applied for 1 hour, followed by secondary antibody for 1 hour. After samples were washed, results were visualized by enhanced chemiluminescence. All data for protein expression from Western blot analysis were expressed as a protein of interest-to-actin ratio in the same sample.

Spatial Frequency Threshold and Contrast Sensitivity

Spatial frequency threshold was determined as described originally by Prusky et al.[27] The spatial frequency started at 0.1 cycle/degree (c/d) with 100% contrast, and was systematically increased (0.1, 0.2, 0.3, and 0.4 c/d) until the animal no longer responded, then the interval between the two last stimuli (last stimulus seen and stimulus not seen) was reduced by half until the animal could again detect the stimulus, at which point the staircase reversed. The response threshold was crossed three times. Reported spatial frequency threshold was determined with a precision of ±0.001 c/d. One staircase was done for each direction of rotation of the optokinetic stimulus, with the resulting two thresholds averaged. An abbreviated assay for contrast sensitivity (measured at the single point (0.064 c/d) was measured to detect functional defects in spatially sensitive retinal cells or in higher visual pathways. It was determined as the inverse of Michelson contrast, without correction for luminance of the monitors and was plotted on a log–log graph. Animals were not anesthetized and were initially placed in the device without recording the response to allow animal training. Each measurement was repeated several times to evaluate the reproducibility of responses. Spatial frequency threshold and abbreviated measure of contrast sensitivity were measured in wild-type diabetic animals and in diabetic animals treated with Metanx (and age-matched nondiabetic controls) at 10 months of diabetes. Our previous studies[28] demonstrated reductions in spatial frequency threshold and contrast sensitivity in diabetic mice.

Diabetes-Induced Retinal Histopathology

After 10 months of diabetes, eyes were fixed in formalin, and one retina from each animal was isolated, washed in running water overnight, and digested for 2 hours in Difco crude trypsin solution (catalog no. 15090-046; Invitrogen Corp., Burlington, ON, Canada) as we have reported previously.[7,29,30] When totally cleaned of neural cells, the isolated vasculature was laid out on a glass microscope slide, dried overnight,

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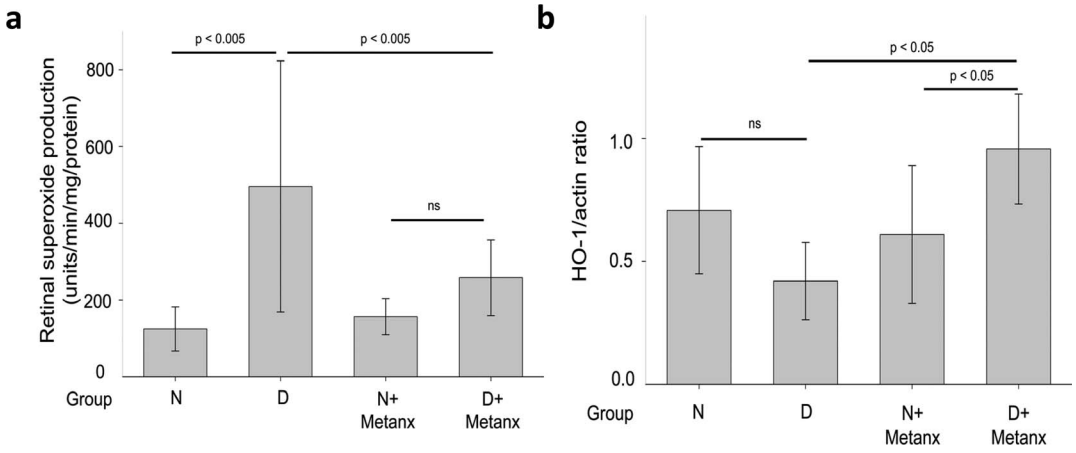

Figure 1. Diabetes (D) of 2 months' duration causes oxidative stress in retinas of mice compared to nondiabetic (N) controls, and Metanx (L-methylfolate, pyridoxal 5′-phosphate, and methylcobalamin) mixed into the food at a dose of 5 mg/kg BW significantly inhibited the diabetes-induced increase in retinal generation of superoxide (**a**) and caused upregulation of the protective enzyme heme oxygenase 1 (HO-1) in the retina (**b**). Superoxide was assayed by the lucigenin method, and HO-1 expression was assessed by Western blot analysis. Data are means ± SD; $n = 5$ to 10 in all groups.

stained with hematoxylin and periodic acid-Schiff stain, dehydrated, and coverslipped. Degenerated (acellular) capillaries were quantitated in 6- to 7-field areas corresponding to the midretina (Magnification: ×200) in a masked manner. Acellular capillaries were identified as capillary-sized vessel tubes having no nuclei anywhere along their length, and were reported as per square millimeter of retinal area. Pericyte ghosts were estimated from the prevalence of protruding "bumps" in the capillary basement membranes from which pericytes had disappeared. At least 1000 capillary cells (endothelial cells and pericytes) in 5 field areas in the midretina (Magnification: ×400) in a masked manner were examined. Ghosts on any already acellular vessel were excluded.

Statistical Analysis

Data are means ± SD. Statistical analysis was performed using ANOVA, followed by Fisher's test. A P value of <0.05 was considered statistically significant.

Results

Glycemia was elevated in all diabetic groups to a similar amount. Average glycated hemoglobin over the entire duration of the 10-month experiment was 3.3 ± 0.2%, 3.4 ± 0.2%, 12.0 ± 0.9%, and 12.2 ± 0.7% for the nondiabetic groups treated without and with Metanx and diabetic groups treated without and with Metanx, respectively. Final BW in these same groups were 46 ± 5, 49 ± 7, 27 ± 3, and 26 ± 2 g, respectively. Data from the 2-month experiment were similar (but not shown). Administration of Metanx had no adverse effect on the health of animals. During the long-term experiments, some animals treated with Metanx (three nondiabetics and five diabetics) died; cause of death was not determined.

Diabetes-induced increases in oxidative stress and local inflammation have been strongly implicated in the pathogenesis of early stages of diabetic retinopathy. Thus, we measured the effect of Metanx on parameters of these abnormalities in retinas of diabetic mice at 2 months after induction of diabetes.

Generation of superoxide by the retina in diabetes is a well-accepted indicator of oxidative stress in retinal tissue. In this experiment, there was a clear increase in this retinal superoxide generation by diabetes, and Metanx strongly inhibited that increase (Fig. 1a). HO-1, a prosurvival enzyme induced under several conditions of oxidative stress[31–34] tended to be decreased in retina by diabetes (although not significantly) but was significantly induced by Metanx (Fig. 1b).

Because increased expression of adhesion molecules on endothelial cells plays a prominent role in inflammation via adhesion of white blood cells to the vessel wall (leukostasis), we measured the effects of diabetes and therapy on leukostasis and expression of ICAM-1 in retina. Figure 2 shows that diabetes of 2 months' duration resulted in a significant increase in leukostasis ($P < 0.001$) and significantly increased expression of retinal ICAM-1. Consistent with the increase in ICAM-1 expression, diabetes of 2 months' duration significantly increased the phosphorylated IκB-to-total IκB ratio in retinas. ICAM-1 expression is regulated by NFκB, and IκB is an important regulator of NFκB activation. Metanx significantly inhibited all of these diabetes-induced abnormalities. Diabetes did not significantly alter expression of several other inflammatory proteins in the retina (iNOS, eNOS, MCP-1) at the duration of diabetes evaluated in this experiment, so we could not assess the effect of Metanx on those parameters (not shown).

We also assessed the effects of the therapy on long-term complications in retinal neural function and vascular structure.

Visual Function

Diabetes of 10 months' duration significantly inhibited spatial frequency threshold, a psychophysical measure that assesses function of both retinal and central visual pathways ($P < 0.01$) (Fig. 3a), and Metanx significantly inhibited this defect in visual function ($P < 0.01$). An abbreviated assay for contrast sensitivity (measured at a single point [0.064 c/d]) also was measured, and diabetes likewise induced a significant reduction also in this parameter ($P < 0.05$), but Metanx had no demonstrable effect on this abnormality (Fig. 3b).

Vascular Structure and Function

As expected, diabetes caused the expected degeneration of retinal capillaries and capillary pericytes (both, $P < 0.01$) (Figs. 4a, 4b). Metanx did not significantly inhibit either of these characteristic histopathologic signs of diabetic retinopathy. Accumulation of plasma protein in the neural retina tended to be increased in the group of mice that were diabetic for 10

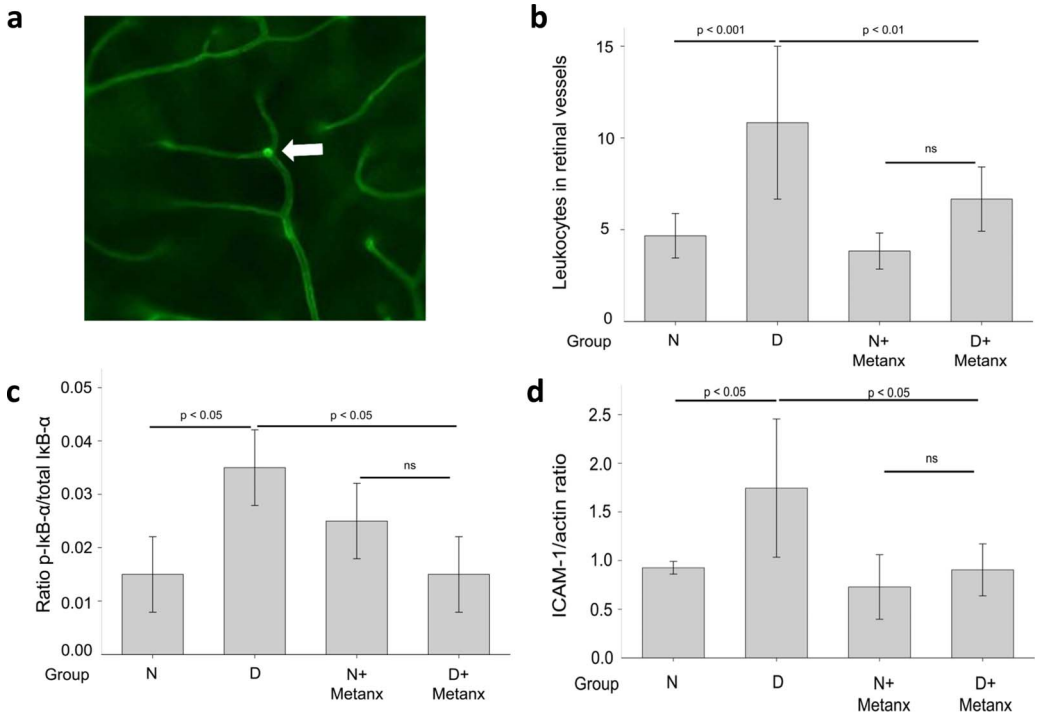

Figure 2. Diabetes of 2 months' duration caused a proinflammatory state in the retina, and Metanx inhibited those abnormalities. An example of leukostasis (leukocyte adherent to the vascular wall [*white arrow*]) in a retinal capillary of a diabetic mouse is shown (**a**), and the number of adherent leukocytes per retina was quantitated (**b**). Leukostasis and the vascular wall are visualized by infusion of concanavalin A-FITC. Leukostasis (**b**), IκB activation (a regulator of the proinflammatory transcription factor NFκB) (**c**), and expression of ICAM-1 (adhesion molecule that leukocytes adhere to in leukostasis) (**d**) were all significantly increased above normal levels in the retina by 2 months after diabetes onset, and Metanx significantly inhibited all of these abnormalities. NFκB activation was estimated from the increased phosphorylated IκB alpha (p-IκB-α)-to-total IκB-α ratio. The brightness of the photomicrograph of leukostasis was enhanced to aid viewing, but no other changes were made to the photo. Data are means ± SD; $n = 3$ to 6 in all groups.

months, but the increase was not statistically significant (not shown), so we are unable to conclude anything about effects of Metanx on this parameter.

Discussion

Preclinical evidence shows that targeting of specific enzymes can have considerable beneficial effect in a variety of diseases, including diabetes. Nevertheless, extensive testing and resources are needed to demonstrate the selectivity and safety of such therapies. Complications of diabetes develop slowly, so we tested the possibility that a combination of therapies, each potentially having less specific and powerful effects, might inhibit development of the complications such as retinopathy.

Metanx is a medical food that has been prescribed for the clinical dietary management of endothelial dysfunction. It contains high concentrations folate (3 mg), vitamin B12 (2 mg), and vitamin B6 (35 mg). The rationale for this preparation in diabetes is that tissue function might be impaired by nutrient deficiencies, especially of water soluble nutrients that are lost during polyuria. Vitamin B_{12} deficiencies are common in

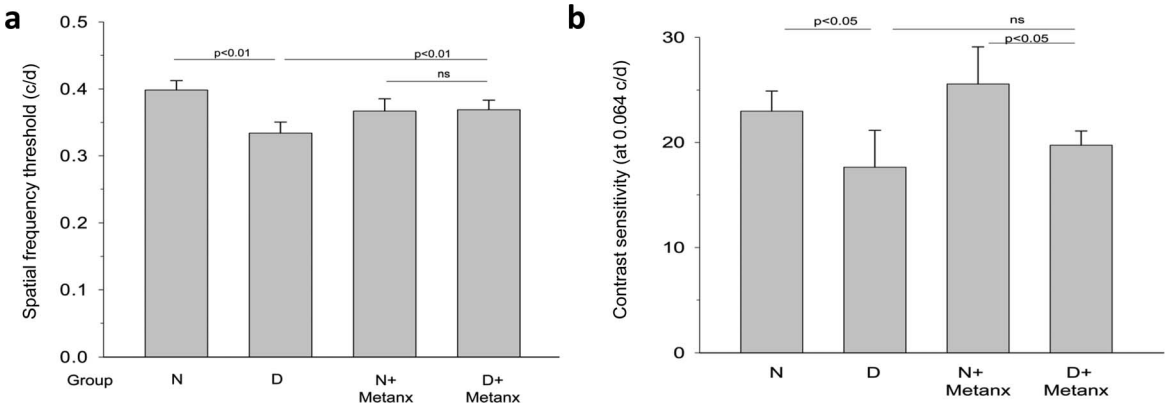

Figure 3. Diabetes impairs visual function in diabetic mice, and Metanx has beneficial effects. At 10 months, diabetes significantly impaired (**a**) the spatial frequency threshold (a marker of visual acuity) and (**b**) an abbreviated assay for contrast sensitivity (measured at 0.064 c/d) in mice. Daily consumption of Metanx significantly inhibited the defect in spatial frequency threshold but had no apparent effect on the defect in contrast sensitivity, measured at 0.064 c/d. Data are means ± SD; $n = 4$ to 5 in all groups.

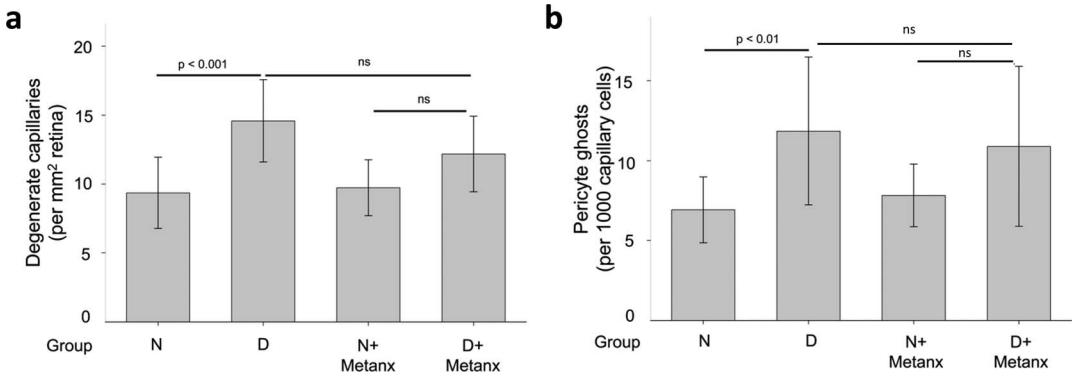

Figure 4. Administration of Metanx for 10 months did not significantly inhibit the diabetes-induced increase in (**a**) degenerated (acellular) capillaries or (**b**) pericyte loss (as indicated by "ghosts," where pericytes used to be). Both of these lesions are characteristic of diabetic retinopathy in animals and patients. Data are means ± SD; $n = 8$ in all groups.

patients with diabetes, especially those using metformin, since metformin inhibits the absorption of cobalamin (the precursor to vitamin B_{12}).[17]

Previous studies demonstrated that Metanx corrected nitric oxide synthase uncoupling and oxidative stress in vascular endothelium and peripheral nerve,[18] and it also inhibited diabetes-induced defects in sensory (but not sciatic motor) nerve conduction velocity, and thermal and mechanical hypoalgesia. Metanx also increased intraepidermal nerve fiber density in the diabetic animals. Metanx has been shown to have benefit also for diabetic peripheral neuropathy in patients.[15,16] A multicenter, randomized, double-blind, placebo-controlled trial of Metanx involving 214 patients with type 2 diabetes and neuropathy was conducted for 24 weeks of treatment.[19] Patients receiving the therapy consistently reported symptomatic relief and improved quality-of-life scores, although the therapy did not show a significant effect on vibration perception threshold during this duration.

Based on these findings in peripheral nerve, we speculated that this nutrient-based therapy might have beneficial effects also in diabetic retinopathy. Diabetes is known to cause oxidative and nitrosative stress in the retina, and these abnormalities have been implicated in development of early vascular lesions of diabetic retinopathy in animals.[35] The present study demonstrated that the combination of L-methylfolate, pyridoxal 5′-phosphate, and methylcobalamin had significant beneficial effects on several diabetes-induced molecular and functional abnormalities of diabetic retinopathy. Specifically, Metanx significantly inhibited the diabetes-induced increase in generation of superoxide and induction of molecular abnormalities that are characteristic of inflammation in the retina (leukostasis, ICAM-1 expression, and p-IκB-to-IκB ratio). The effects of Metanx on these abnormalities were measured at 2 months of diabetes but not in the long-term studies (the tiny retinas were reserved for histopathology analyses in the long-term studies), so we cannot say whether effects of the therapy became weaker or stronger with time, or if superoxide generation is causally related to the diabetes-induced defects in visual function. After 10 months of Metanx therapy, diabetes-induced alterations in the function of retinal neurons were significantly less in the Metanx-treated group (as shown by the inhibition of the impairment of SFT in diabetes), but despite randomization into experimental groups, it is possible that the animals selected to get Metanx always had lower SFT.

Although Metanx had a statistically significant effect on SFT in our study, it had no similar effect on an abbreviated parameter of contrast sensitivity. This seems surprising, because visual acuity (which SFT approximates) is related to contrast sensitivity at maximal contrast. Diabetes impairs SFT, especially at high spatial frequencies,[28] so the effects of therapy on contrast sensitivity at the spatial frequency tested in these studies might not be reflective of results at higher spatial frequencies, or it seems possible that SFT is more sensitive to oxidative stress than is contrast sensitivity.

Metanx also has been reported to have some benefit for the retina in a small series of type 2 diabetic patients having mild to moderate nonproliferative diabetic retinopathy.[20] In that open-label, uncontrolled, prospective 6-month study, five of the seven patients that completed the trial showed statistically significant (but modest) improvements in mean threshold retinal sensitivity and mean central retinal thickness. Whether or not longer duration of treatment or a higher dose might yield better therapeutic results was not tested.

Very little of the retina is occupied by blood vessels, so the diabetes-induced defects in superoxide and inflammation observed in the present study likely resides within retinal neuroglia. Nevertheless, therapies which inhibited those retinal defects in previous studies have been found also to inhibit retinal vascular degeneration,[1–3,25,35–37] suggesting a link between oxidative stress, inflammation, and degeneration of retinal capillaries in diabetes. The present study thus yielded unexpected results, in that inhibition of the diabetes-induced increase in superoxide, leukostasis, activation of NFκB and expression of ICAM-1 by Metanx was not associated with protection of the retinal vasculature. These results seem at odds with previous findings that diabetics lacking ICAM-1 or over-expressing antioxidant enzymes were protected from the vascular degeneration,[1,25,38] suggesting that perhaps much more complete elimination of ICAM-1 (such as by genetic deletion) or superoxide is required to inhibit the vascular histopathology, whereas partial inhibition using Metanx was not sufficient to be vasoprotective. The present findings are not sufficient to conclude that oxidative stress or inflammation are not related to the capillary degeneration or impaired visual function in diabetes.

Previous research[28] provided evidence that therapies that significantly inhibited retinal capillary degeneration in diabetic rodents do not necessarily have a comparable effect on contrast sensitivity or SFT (except for animals deficient in aldose reductase, which resulted in both defects being inhibited). In the present study, the opposite is reported, in that a parameter of neural function (SFT) was inhibited by a therapy that had no effect on capillary degeneration. Findings from both studies are consistent with the possibility that the pathogenesis of the vascular lesions of diabetic retinopathy might differ in some respects from the pathogenesis of the visual function defects in diabetes.

Treatment regimens that work by multiple mechanisms have potential to be of benefit especially in slowly developing complications, such as in diabetes, because individual pathways might not need to be strongly inhibited. Nevertheless, this premise remains to be clearly demonstrated. The present studies demonstrated the concept that poly-therapy using a nutritional food might have relevance for the inhibition of aspects of diabetic retinopathy. Whether or not additional benefits will become evident at higher doses or at longer durations of study remain to be demonstrated, for the present effect was only partial. Good glycemic control is still the important therapeutic goal, but treatments such as with Metanx may provide additional functional protection for those unable to achieve or maintain such perfect glycemic control.

Acknowledgments

Supported by a research grant from Pamlab, Inc., PHS Grants EY00300 and EY022938, and a grant from the Medical Research Service of the Department of Veterans' Affairs.

Disclosure: **H. Liu**, None; **J. Tang**, None; **C.A. Lee**, None; **T.S. Kern**, Pamlab, Inc. (F)

References

1. 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.
2. 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.
3. 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.
4. 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.
5. Hu Y, Chen Y, Ding L, et al. Pathogenic role of diabetes-induced PPAR-alpha down-regulation in microvascular dysfunction. *Proc Natl Acad Sci U S A*. 2013;110:15401–15406.
6. Li G, Tang J, Du Y, Lee CA, Kern TS. Beneficial effects of RAGE-Ig fusion protein on early diabetic retinopathy and tactile allodynia. *Mol Vis*. 2011;17:3156–3165.
7. Du Y, Tang J, Li G, et al. Effects of p38 MAPK inhibition on early stages of diabetic retinopathy and sensory nerve function. *Invest Ophthalmol Vis Sci*. 2010;51:2158–2164.
8. Al-Shabrawey M, Rojas M, Sanders T, et al. Role of NADPH oxidase in retinal vascular inflammation. *Invest Ophthalmol Vis Sci*. 2008;49:3239–3244.
9. Antoniades C, Shirodaria C, Warrick N, et al. 5-methyltetrahydrofolate rapidly improves endothelial function and decreases superoxide production in human vessels: effects on vascular tetrahydrobiopterin availability and endothelial nitric oxide synthase coupling. *Circulation*. 2006;114:1193–1201.
10. Miller AL. The methylation, neurotransmitter, and antioxidant connections between folate and depression. *Altern Med Rev*. 2008;13:216–226.
11. Moreira ES, Brasch NE, Yun J. Vitamin B12 protects against superoxide-induced cell injury in human aortic endothelial cells. *Free Radic Biol Med*. 2011;51:876–883.
12. Nakamura S, Li H, Adijiang A, Pischetsrieder M, Niwa T. Pyridoxal phosphate prevents progression of diabetic nephropathy. *Nephrol Dial Transplant*. 2007;22:2165–2174.
13. Levin ER, Hanscom TA, Fisher M, et al. The influence of pyridoxine in diabetic peripheral neuropathy. *Diabetes Care*. 1981;4:606–609.
14. Misumi J, Nagano M, Kaisaku J, Hitoshi T. Effects of vitamin B12 and B6 on 2,5-hexanedione-induced neuropathy. *Arch Toxicol*. 1985;56:204–206.
15. Walker MJ Jr, Morris LM, Cheng D. Improvement of cutaneous sensitivity in diabetic peripheral neuropathy with combination L-methylfolate, methylcobalamin, and pyridoxal 5′-phosphate. *Rev Neurol Dis*. 2010;7:132–139.
16. Jacobs AM, Cheng D. Management of diabetic small-fiber neuropathy with combination L-methylfolate, methylcobalamin, and pyridoxal 5′-phosphate. *Rev Neurol Dis*. 2011;8:39–47.
17. Vinik AI. A medicinal food provides food for thought in managing diabetic neuropathy. *Am J Med*. 2013;126:95–96.
18. Shevalye H, Watcho P, Stavniichuk R, Dyukova E, Lupachyk S, Obrosova IG. Metanx alleviates multiple manifestations of peripheral neuropathy and increases intraepidermal nerve fiber density in Zucker diabetic fatty rats. *Diabetes*. 2012;61:2126–2133.
19. Fonseca VA, Lavery LA, Thethi TK, et al. Metanx in type 2 diabetes with peripheral neuropathy: a randomized trial. *Am J Med*. 2013;126:141–149.
20. Smolek MK, Notaroberto NF, Jaramillo AG, Pradillo LR. Intervention with vitamins in patients with nonproliferative diabetic retinopathy: a pilot study. *Clin Ophthalmol*. 2013;7:1451–1458.
21. Kern TS, Du Y, Miller CM, Hatala DA, Levin LA. Overexpression of Bcl-2 in vascular endothelium inhibits the microvascular lesions of diabetic retinopathy. *Am J Pathol*. 2010;176:2550–2558.
22. Kern TS, Miller CM, Du Y, et al. Topical administration of nepafenac inhibits diabetes-induced retinal microvascular disease and underlying abnormalities of retinal metabolism and physiology. *Diabetes*. 2007;56:373–379.
23. Du Y, Miller CM, Kern TS. Hyperglycemia increases mitochondrial superoxide in retina and retinal cells. *Free Radic Biol Med*. 2003;35:1491–1499.
24. Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP. Leukocyte-mediated endothelial cell injury and death in the diabetic retina. *Am J Pathol*. 2001;158:147–152.
25. 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.
26. 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.
27. 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.
28. 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.
29. Engerman RL, Kern TS. Aldose reductase inhibition fails to prevent retinopathy in diabetic and galactosemic dogs. *Diabetes*. 1993;42:820–825.
30. 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.
31. Kutty RG, Kutty G, Wiggert B, Chader GE, Darrow RM, Organisciak DT. Induction of heme oxygenase 1 in the retina by intense visible light: suppression by the antioxidant dimethylurea. *Proc Natl Acad Sci U S A*. 1995;92:1177–1181.

32. Motterlini R, Foresti R, Intaglietta M, Winslow RM. NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am J Physiol*. 1996;270:H107–H114.
33. Mandal P, Park PH, McMullen MR, Pratt BT, Nagy LE. The anti-inflammatory effects of adiponectin are mediated via a heme oxygenase-1-dependent pathway in rat Kupffer cells. *Hepatology*. 2010;51:1420–1429.
34. Mandal P, Roychowdhury S, Park PH, Pratt BT, Roger T, Nagy LE. Adiponectin and heme oxygenase-1 suppress TLR4/MyD88-independent signaling in rat Kupffer cells and in mice after chronic ethanol exposure. *J Immunol*. 2010;185:4928–4937.
35. Zheng L, Kern T. Role of nitric oxide, superoxide, peroxynitrite and poly(ADP-ribose) polymerase in diabetic retinopathy. *Front Biosci*. 2009;14:3974–3987.
36. Kern TS, Tang J, Mizutani M, et al. Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. *Invest Ophthalmol Vis Sci*. 2000;41:3972–3978.
37. 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.
38. 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.

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