Podocyte-Specific Overexpression of the Antioxidant Metallothionein Reduces Diabetic Nephropathy

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ABSTRACT
Podocytes are critical components of the selective filtration barrier of the glomerulus and are susceptible to oxidative damage. For investigation of the role of oxidative stress and podocyte damage in diabetic nephropathy, transgenic mice that overexpress the antioxidant protein metallothionein (MT) specifically in podocytes (Nmt mice) were produced. MT expression was increased six- and 18-fold in glomeruli of two independent lines of Nmt mice, and podocyte-specific overexpression was confirmed. Glomerular morphology and urinary albumin excretion were normal in Nmt mice. OVE26 transgenic mice, a previously reported model of diabetic nephropathy, were crossed with Nmt mice to determine whether an antioxidant transgene targeted to podocytes could reduce diabetic nephropathy. Double-transgenic OVE26Nmt mice developed diabetes similar to OVE26 mice, but MT overexpression reduced podocyte damage, indicated by more podocytes, less glomerular cell death, and higher density of podocyte foot processes. In addition, expansion of glomerular and mesangial volume were significantly less in OVE26Nmt mice compared with OVE26 mice. Four-month-old OVE26Nmt mice had a 70 to 90% reduction in 24-h albumin excretion, but this protection does not seem to be permanent. These results provide evidence for the role of oxidative damage to the podocyte in diabetic mice and show that protection of the podocyte can reduce or delay primary features of diabetic nephropathy.

Diabetic nephropathy (DN) is the leading cause of ESRD. Several interventions1–3 slow the progression, but they do not permanently prevent DN. Improved treatment is hampered by incomplete understanding the mechanism of the disease. Glomeruli contain podocytes, mesangial cells, and endothelial cells. All of them exhibit abnormalities in DN. In addition, high levels of protein entering the tubules lead to tubular damage and fibrosis.4,5 Unraveling the sequence of events leading to advanced DN requires cell-specific manipulations in the context of accurate models of human disease.

Several molecular mechanisms have been implicated in DN. Data from cell and animal studies indicate that excessive production of reactive oxygen species (ROS) activates pathways of hyperglycemic damage6,7; therefore, ROS could be an important mediator of DN. If this is correct, then protection from ROS would reduce the pathology of DN. Craven et al.8 and DeRubertis et al.9 used a transgenic approach to test this hypothesis. They demonstrated that overexpression of the antioxidant superoxide dismutase 1 throughout the body reduced DN in streptozotocin-induced and db/db diabetic

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mice. These studies strengthen the hypothesis that ROS are significant in causing early DN; however, they were not designed to reveal the sites of antioxidant action. Because superoxide dismutase 1 was overexpressed in almost all cells, the protective effect could have been exerted on any cell in the glomerulus and in tubules or cells outside the kidney that mediate inflammation.

Podocytes are considered an important cell type in the development of DN. Reduction in glomerular podocyte number has been observed in patients with both early and late DN\(^{10,11}\) and in animal models.\(^{12}\) Although podocyte depletion is an early feature of human DN, it is not clear whether podocytes are primary targets of hyperglycemia or are lost secondary to damage in surrounding glomerular cells. Podocytes are known to be susceptible to oxidative damage,\(^{13,14}\) and diabetes increases oxidative stress. We hypothesized that hyperglycemia-induced ROS produce direct damage to podocytes and that antioxidant protection of only podocytes will be sufficient to reduce their damage and decrease DN. For testing of our hypothesis, a potent antioxidant protein, metallothionein (MT), was overexpressed specifically in podocytes of OVE26 diabetic mice. OVE26 mice are a model of severe early-onset diabetes.\(^{15}\) It develops characteristics of DN, including albuminuria similar to advanced human DN, fibrosis, and enlarged glomeruli.\(^{16}\) In this study, we showed that a cell-specific antioxidant reduces damage to the podocyte and lessens DN.

RESULTS

Nmt transgenic mice were developed to determine whether increased podocyte MT reduced DN. Mice were produced with a transgene designated Nmt, containing the human MTII gene regulated by the mouse nephrin promoter. Two transgenic lines, Nmt3 and Nmt7, were characterized (Figure 1). PCR of kidney RNA for human MT showed expression of the transgene in both lines but was highest in line Nmt7. Western blots of glomerular protein indicated that relative to FVB, MT was 18- and six-fold higher in Nmt7 and Nmt3 glomeruli, respectively. Immunohistochemistry showed MT staining clearly coincided with nuclear staining of the podocyte marker WT1. MT staining in nontransgenic glomeruli was almost undetectable.

OVE26 diabetic mice\(^{16}\) were crossed to Nmt mice to test the effect of the transgene on DN. Possible effects of podocyte MT overexpression on diabetes were assessed in OVE26Nmt3 mice (Table 1). The transgene did not reduce blood glucose in fed adult OVE26 mice. To confirm that chronic glycemia was unaffected, we also measured glycosylated hemoglobin. This was elevated to the same extent in OVE26 and OVE26Nmt3 mice, 9.8 and 10% A\(_{1c}\), respectively, which is more than two-fold higher than in nondiabetic mice; therefore, MT-induced changes in DN were not due to less severe diabetes.

MT effects on albuminuria were measured because severe and progressive albuminuria is the most striking component of DN in OVE26 mice. Albuminuria was measured at 2 and 4 mo of age (Figure 2). Between these ages, albuminuria in OVE26 mice increased more than 10-fold, but albuminuria in FVB mice was unchanged (note the different scales used for 2 and 4 mo). The Nmt3 transgene produced a significant reduction in the mild albuminuria seen in this diabetic model at 2 mo of age (Figure 2A). More important, the transgene slowed the progression to severe albuminuria that develops in 4-mo-old diabetic mice (Figure 2B). MT overexpression also reduced albuminuria in Nmt7 mice, although, surprisingly, it was not as effective as that seen in Nmt3 mice. At 2 mo of age, the reduction in albuminuria was NS (Figure 2C); however, at 4 mo of age, there was a four-fold, significant reduction in albuminuria (Figure 2D).
MT effects on diabetic podocytes and glomeruli were assessed in OVE26Nmt mice. In FVB and Nmt mice (Figure 3), podocytes exhibited typical ultrastructural features, but in OVE26 podocytes, significant changes were evident. The narrow foot processes of control podocytes were replaced by club-like extensions that often covered large areas of glomerular basement membrane (GBM; Figure 3B). Foot process density was 33% lower in OVE26 compared with FVB glomeruli ($P < 0.01$). Diabetic Nmt mice (OVE26Nmt7) were substantially protected from these changes. Foot process effacement and large club-like foot processes were less frequently seen in OVE26Nmt7 glomeruli. In OVE26Nmt7 mice, foot process density was significantly higher than in OVE26 mice ($P < 0.05$) and was within 17% of FVB density.

Western blots of glomerular protein (Figure 4) showed that diabetes reduced the level of podocyte slit diaphragm proteins. Nephrin levels in OVE26 samples were reduced to 11% of control, and nephrin was not restored in OVE26Nmt7 samples. Podocalyxin was less affected by diabetes than nephrin. In OVE26 glomeruli, podocalyxin declined to 59% of control. MT overexpression partially but significantly reversed the decline in podocalyxin, to 81% of control.

The number of podocytes per glomerulus was reduced by approximately 44% in OVE26 mice (Figure 5) compared with FVB control. In OVE26Nmt3 glomeruli, podocyte counts were increased by 39% over OVE26 and significantly restored to within approximately 25% of control. The podocyte counts are subject to error because the maximal midplanar area of the glomerulus was not determined. In fact, we found that select-

<table>
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<th>OVE26Nmt</th>
<th>FVB</th>
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Figure 2. MT overexpression reduces albuminuria in diabetic mice. (A and B) Line Nmt3 results at 2 and 4 mo of age, respectively. (C and D) Line Nmt7 results at 2 and 4 mo of age, respectively. The asterisks indicate that OVE26Nmt is less than OVE26 ($**P < 0.05$; $*P < 0.01$) by two way ANOVA. In all panels, OVE26 groups were higher than non-OVE26 groups ($P < 0.01$). Note that the scales are more than 10-fold higher at 4 mo than at 2 mo; $n = ±8$ per group.
ing just the largest 25% of glomerular cross-sections for analysis produced an approximately 20% higher calculated number of podocytes per glomerulus than when the analysis was done on all glomerular cross-sections (data not shown). This was true for all experimental groups. Also, more precise protocols such as the thick and thin section\textsuperscript{17} method or the disector/fractionator methods\textsuperscript{18} were not used. For determination of whether the apparent reduction in the number of podocytes was related to increased cell death, sections were subject to terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining. Diabetes increased TUNEL-positive glomerular cells by approximately 20-fold over FVB, and this induction of cell death was significantly decreased by MT overexpression (Figure 6). The proteinase K treatment required for TUNEL staining prevented double staining with podocyte markers; however, the peripheral localization and cellular morphology were similar to cells stained with podocyte markers.

Two of the most striking features of the OVE26 glomerulus are expansion of the glomerulus and an increase in mesangial matrix.\textsuperscript{16} Consistent with our previous observations, OVE26 glomerular volume was increased to 200% of control and matrix volume was increased to 270% of control (Figure 7). Overexpression of MT in podocytes of OVE26Nmt3 glomeruli partially but significantly reduced the diabetes-induced increase in both glomerular and matrix volume ($P < 0.03$).

**DISCUSSION**

The goals of this study were to determine whether podocytes are a direct target of diabetes and to determine whether protection of just the podocyte reduced DN. These questions were addressed by targeting the antioxidant protein MT specifically to podocytes. Our results demonstrate that podocytes are a direct target of oxidative stress and that a major part of DN pathology originates in the podocyte.

Previous work demonstrated that oxidant stress contributes to DN\textsuperscript{8,19–21} and that systemic antioxidants\textsuperscript{8,9,22–24} are beneficial; however, those studies were not designed to show which renal cells were critical to the response. To address this, we limited MT transgene expression to podocytes with the nephrin promoter.\textsuperscript{25} MT was selected for overexpression because it is such a potent antioxidant: On a molar basis, it is 50 times more potent than glutathione,\textsuperscript{26,27} and MT is unique in providing protection against hydroxyl radical, peroxynitrite, superoxide, and hydrogen peroxide.\textsuperscript{28,29} Also, we previously showed that MT provides antioxidant protection from diabetes in transgenic heart\textsuperscript{30} and transgenic pancreatic $\beta$ cells.\textsuperscript{31}

Because only podocytes overexpressed MT, we are certain that reduced DN was due to direct protection of the podocyte. One limitation of this approach is that only approximately 20% of glomerular cells are podocytes. This makes it very difficult to measure changes in glomerular oxidative damage in such a small fraction of protected cells.

These are the first results to demonstrate that protection of only the podocyte reduces albuminuria. At 4 mo of age, the MT transgene decreased albuminuria by 10-fold in OVE26Nmt3 mice and three-fold in OVE26Nmt7 mice; however, MT definitely did not eliminate albuminuria, which remained several-fold elevated, compared with FVB mice. Preliminary results at 7 mo of age obtained from one OVE26Nmt3 mouse and four OVE26Nmt7 mice revealed that three of them had 24-h albumin levels exceeding 5000 $\mu$g/d. These studies indicate that MT can delay albuminuria but cannot prevent it.
Unexpected, the low-expressing transgenic line Nmt3 was more effective than the high-expressing line Nmt7. The reduced benefit in the high-expressing line suggests that too much MT can be detrimental. In support of this proposition, we assayed four mice homozygous for the Nmt7 transgene. Three of four mice developed elevated albuminuria (>250 μg/24 h), and one had overt albuminuria (2100 μg/24 h). Also, cross-breeding a single allele of the Nmt7 transgene onto mice carrying a new podocyte-specific green fluorescence protein transgene (unpublished data) increased albuminuria four-fold \((P < 0.05)\) over the green fluorescence protein transgene alone. All of these results suggest that MT, at a sufficient dosage, becomes detrimental to podocytes. Detrimental effects of MT are not unique to podocytes, because we previously observed negative consequences of excess MT in pancreatic \(β\) cells.\(^{32}\) An alternative or additional explanation for the lower protective efficacy in the Nmt7 line might be the site of integration of the transgene. Transgenes insert into the genome at random sites.\(^{33}\) It is possible that the insertion site of Nmt7 may have disrupted a mouse gene involved in normal glomerular function, thereby reducing its protective potency. The albuminuria we observed in homozygous Nmt7 mice is consistent with insertional mutagenesis into a gene relevant to albuminuria. Until the site of insertion of Nmt7 is determined, it will not be possible to distinguish the relative impact of insertional mutagenesis from the impact of excessive MT expression.

Damage to diabetic OVE26 podocytes is similar to what has been described in patients with diabetes\(^{34,35}\) and other models\(^{1,2}\). Podocyte death increased, numbers declined, foot processes density dropped, and podocyte-specific proteins decreased. Most of the podocyte parameters assayed in this study were at least partially benefited by MT. Podocyte foot process density was significantly higher in OVE26Nmt glomeruli compared with OVE26 glomeruli. This coincided with 50% lower TUNEL. These results demonstrated that direct antioxidant protection reduced podocyte death and foot process damage. They also indicate that MT helped preserve the number of podocytes. A limitation of this study is that the most recent and precise methods for enumerating podocytes\(^{17,18}\) were not used; therefore, these podocyte counts are subject to error.

MT benefit to slit diaphragm proteins was less impressive. OVE26 glomeruli had 10% of normal nephrin content and 40% of normal podocalyxin. MT overexpression was unable to improve nephrin content but brought podocalyxin values to within 20% of control. Apparently, the threshold for restoring podocalyxin and nephrin expression is markedly different.

All glomerular structures and cells are affected by diabetes. The volumes of the glomerulus and the mesangial matrix increase in DN. MT overexpression significantly reduced both of these pathologies. Because MT overexpression is limited to podocytes, it follows that protection of the rest of the glomerulus is secondary to MT actions in the podocyte. H\(_2\)O\(_2\) from podocytes has been shown to reach the GBM and likely damage it.\(^{36}\) Because MT scavenges ROS, overexpression of MT should

![Figure 4](image-url). Altered expression of slit diaphragm proteins in isolated glomeruli. (A) Nephrin, podocalyxin, and GAPDH protein in glomeruli; FVB (F), OVE26 (O), and OVE26Nmt7 (ON). (B and C) Expression of nephrin (B) and podocalyxin (C), normalized to GAPDH expression. OVE26 and OVE26Nmt7 are always less than FVB (*) and podocalyxin OVE26Nmt7 is greater than OVE26 (**) \((P < 0.05)\) by Kruskal Wallis ANOVA.
reduce release of free radicals generated in podocytes. Also, injured podocytes release factors that promote growth of mesangial cells. Because MT reduces podocyte injury, this should lessen release of growth factors. Wharram et al. demonstrated that a reduction in podocyte number induced mesangial expansion and glomerular sclerosis. Because there is less podocyte loss in OVE26Nmt glomeruli, this reduced mesangial expansion and glomerular sclerosis.

In summary, these are the first results to show that podocyte-targeted treatment provides protection from structural and functional aspects of DN. This confirms the importance of oxidative stress to glomerular damage and shows the central role of podocyte injury to the pathology of DN.

CONCISE METHODS

Development of Nmt Transgenic Mice and Breeding to OVE26 Mice

The podocyte-specific MT transgene, designated Nmt, was constructed by ligating 8300 bp of the mouse nephrin promoter to a 2400-bp fragment containing all exons of human MTII. Transgenic mice were generated in FVB embryos, as described previously. Male diabetic OVE26 mice were cross-bred with Nmt mice to produce double-transgenic OVE26Nmt mice. All mice were maintained on the inbred FVB background. Animal procedures adhered to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee.

Glucose, A1c, and Albumin Assays
Nonfasting blood glucose was measured using OneTouch Glucometer (Johnson & Johnson Company, Milpitas, CA). Glycated hemoglobin level was measured using an INVVIEW A1c monitor (Metrika, Sunnyvale, CA). For determination of urinary albumin excretion, individual mice were placed for 24 h in metabolic cages with access to food and 10% liquid diet (Glucerna; Abbot Laboratories, Abbott Park, IL). Urine albumin was measured by mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX).

Immunohistochemistry and TUNEL Staining

MT was localized on formalin-fixed, paraffin-embedded tissue with MT antibody (1:40, mouse, monoclonal; DAKO, Glostrup, Denmark). Podocytes were identified with WT1 antibody (1:50; rabbit
polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA). Apoptotic nuclei were detected by TUNEL assay following the manufacturer’s protocol (Chemicon, Temecula, CA).

Western Blot of Glomerular Proteins
Glomeruli were isolated after cardiac perfusion with Dynal magnetic beads (Dynabeads M450; Dynal Biotech ASA, Oslo, Norway).39 Either 2.5 or 5.0 μg of glomerular protein was separated on 4 to 12% gradient gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membrane. Antibodies used were nephrin (1:1000, guinea pig; Fitzgerald, Concord, MA), podocalyxin (1:3000; Alpha Diagnostic Int. Inc., San Antonio, TX), and MT (1:1000 mouse; DakoCytonation, Glostrup, Denmark).

Determination of Podocyte Number and Kidney Histopathology
The number of podocytes per glomerulus was estimated from podocyte density multiplied by the glomerular volume.40 In brief, digital images of random glomeruli were obtained at ×400 by an observer who was blind to the identity of the section. Podocyte density was determined from a count of the number of WT1-positive nuclei per glomerular section. The volume of this glomerular cross-section was obtained from the area of the cross-section multiplied by the thickness of the section. The cross-sectional area of the glomerular tuft (AG) was determined from outlines of the tuft using the program Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) and a micrometer image to confirm dimensions in micrometers. Glomerular volume (VG) was calculated from the cross-sectional area with the formula from Wiebel11: VG = β/k (AG)3/2; β = 1.38 is the shape coefficient for a sphere and k = 1.1 is the size distribution coefficient.17 Twenty consecutive glomerular cross-sections were photographed and counted per mouse by an observer who was blind to the sample identity. Four mice were analyzed for each genotype. The procedures for determining mesangial and glomerular volume have previously been described.16

Scoring of Apoptotic Nuclei In Situ
The total number of the TUNEL-positive cells from all of the glomerular profiles visible in a kidney section were summed; apoptosis was scored as the number of TUNEL-positive cells per 100 glomerular profiles.

Electron Microscopy and Morphometry
Kidneys were collected and prepared as described previously.16 Glomerular areas were selected randomly by technicians who were blind regarding animal type. Mean podocyte foot process density was calculated from transmission electron microscopy prints by a modification of the method of Mifsud et al.42 Foot processes were counted along segments of GBM. Counts were made on 20 electron micrographs from at least three glomeruli in each mouse. Micrographs were scanned, and the length of each GBM segment was measured using ImageJ software. An average of 2500 foot processes were counted for each group.

Statistical Analysis
Data are expressed as means ± SEM. Statistical comparisons were performed by t test for comparison between two groups and one- or two-way ANOVA for comparison among several groups using SigmaStat software (Systat, San Jose, CA). Significance was defined as P < 0.05.

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