Reversibility of Structural and Functional Damage in a Model of Advanced Diabetic Nephropathy

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ABSTRACT

The reversibility of diabetic nephropathy remains controversial. Here, we tested whether replacing leptin could reverse the advanced diabetic nephropathy modeled by the leptin-deficient BTBR ob/ob mouse. Leptin replacement, but not inhibition of the renin-angiotensin-aldosterone system (RAAS), resulted in near-complete reversal of both structural (mesangial matrix expansion, mesangiolysis, basement membrane thickening, podocyte loss) and functional (proteinuria, accumulation of reactive oxygen species) measures of advanced diabetic nephropathy. Immunohistochemical labeling with the podocyte markers Wilms tumor 1 and p57 identified parietal epithelial cells as a possible source of regenerating podocytes. Thus, the leptin-deficient BTBR ob/ob mouse provides a model of advanced but reversible diabetic nephropathy for further study. These results also suggest that restoration of lost podocytes is possible but is not induced by RAAS inhibition, possibly explaining the limited efficacy of RAAS inhibitors in promoting repair of diabetic nephropathy.


Diabetic nephropathy (DN) is now the major cause of CKD and ESRD throughout the world and is the largest single cause of ESRD in the United States, accounting for nearly half of the patients entering dialysis each year.1–5 The mainstays of current therapy for DN are control of hyperglycemia and BP and inhibition of the renin-angiotensin-aldosterone system (RAAS).6,7 These therapies can be effective in slowing progression but have not been effective in reversing established complications, such as DN. The recently reported Renin–Angiotensin System Study, a prospective 5-year clinical trial in which early and sustained therapy with inhibitors of the RAAS in diabetic patients did not prevent development of DN, was particularly disappointing in this regard.8 Two of the major obstacles to progress in the treatment of DN are the lack of relevant animal models in which reversal of advanced DN can be tested and uncertainty about whether podocytes, a cell type that has long thought to be nonreplicating and nonrenewable and to be lost during development of DN, can be replaced and hence permit reconstitution of a normal glomerulus.9

In this study, we show that both of these obstacles can be overcome. We have recently characterized a new murine model of type 2 DN, the BTBR ob/ob leptin-deficient mouse, which better mirrors human DN than do most previous murine models.10,11 We have extended our previous characterization of this model by administering leptin to mice with advanced DN and demonstrating, uniquely among both experimental models and human DN, that DN can be reversed with pharmacologic therapy.

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We have previously demonstrated that podocyte loss occurs early in the BTBR ob/ob model and that this loss persists as DN progresses. We now show that the “nonproliferating” podocyte population can be restored and that this is linked to regression of DN. We then used the model to study mechanisms underlying the limited ability of RAAS inhibition to reverse the structural injury of DN and demonstrate that the inability of the two classes of RAAS inhibitors in wide clinical use to reverse DN may result from their inability to restore podocyte number/density.

RESULTS

Leptin Replacement, but Not RAAS Inhibition or Treatment with Hydralazine, Rapidly Reverses Diabetes, Obesity, and Manifestations of DN in BTBR ob/ob Mice

In these experiments, treatment began at 18 weeks of age, when DN was well established, and continued for 6 weeks. BTBR ob/ob mice have significantly elevated blood glucose levels and body weight compared with BTBR wild-type (WT) littermates. Leptin replacement results in rapid return to normoglycemia that is sustained and a significant decrease in body weight. Enalapril, losartan, or hydralazine treatment had no effect on body weight or blood glucose level (Table 1 and Supplementary Figure 1).

BTBR ob/ob mice develop progressive albuminuria, detected as early as 8 weeks of age, progressively increasing through 18 weeks of age. After 6 weeks of leptin replacement or enalapril treatment, beginning at week 18, albuminuria was reduced, with reduction most marked in mice receiving leptin replacement. Losartan treatment also resulted in reduced albuminuria, but the values were not statistically significant. Albuminuria did not decrease with hydralazine treatment (Table 1).

Urine albumin-to-creatinine ratio also decreased significantly in leptin-, enalapril-, and losartan-treated mice compared with untreated BTBR ob/ob control mice. Hydralazine treatment did not significantly reduce albumin-to-creatinine ratio (Table 1).

Serum BUN was significantly decreased in the leptin replacement group compared with the 24-week control BTBR ob/ob mice (Table 1). Enalapril, losartan, and hydralazine treatment did not result in significant changes in BUN levels (Table 1). Serum creatinine levels were significantly improved in BTBR ob/ob mice with leptin replacement. Creatinine levels improved in all other treatment modalities compared with 24-week-old control BTBR ob/ob (Table 1), but this did not reach statistical significance.

Insulin levels in BTBR ob/ob mice were significantly elevated at 18 weeks compared with those in BTBR WT mice. After 6 weeks of leptin treatment, insulin levels dropped to nearly normal levels (Supplemental Figure 2).

Serum leptin levels did not significantly differ between BTBR WT mice and leptin-treated BTBR ob/ob mice (mean ± SEM, 5078.67 ± 1125.55 versus 3583.07 ± 966.81 pg/ml), confirming that leptin administration achieved physiologic levels. Untreated control BTBR ob/ob mice had no detectable leptin.

Diabetes-induced renal hypertrophy was evidenced by gradually increasing kidney weight from 18-week-old to 24-week-old BTBR ob/ob mice. All treatments decreased kidney weight, but these changes were statistically significant only in the leptin replacement group (Table 1).

Table 1. Representative laboratory data for BTBR ob/ob control and treatment mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>24-Week-Old BTBR ob/ob Mice</th>
<th>18-Week-Old BTBR ob/ob Mice</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Hydralazine</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Losartan</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Enalapril</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum BUN (mg/dl)</td>
<td>13.83 ± 1.23</td>
<td>34.07 ± 4.97</td>
<td>64.07 ± 11.17</td>
<td>55.50 ± 7.59</td>
<td>53.46 ± 8.72</td>
<td>42.08 ± 7.58</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>0.17 ± 0.02</td>
<td>0.32 ± 0.05</td>
<td>0.29 ± 0.08</td>
<td>0.23 ± 0.07</td>
<td>0.20 ± 0.04</td>
<td>0.18 ± 0.05</td>
</tr>
</tbody>
</table>
| Body weight (g)   | 31.99 ± 0.70                 | 65.57 ± 0.77                | 60.24 ± 1.28                                        | 68.23 ± 2.84                                      | 65.96 ± 1.87                                      | 64.20 ± 1.52                                  | 50.90 ± 2.12
| Kidney weight (g) | 0.25 ± 0.01                  | 0.31 ± 0.02                 | 0.50 ± 0.08                                        | 0.37 ± 0.02                                      | 0.38 ± 0.01                                      | 0.38 ± 0.01                                  | 0.28 ± 0.01
| Kidney/body weight (%) | 0.78 ± 0.02       | 0.49 ± 0.02                 | 0.73 ± 0.18                                        | 0.54 ± 0.02                                      | 0.57 ± 0.02                                      | 0.59 ± 0.03                                  | 0.56 ± 0.04
| Blood glucose (mg/dl) | 161.0 ± 25.1            | 485.8 ± 27.3                | 503.4 ± 77.9                                       | 462.0 ± 31.1                                      | 463.3 ± 43.4                                      | 491.9 ± 32.7                                  | 139.7 ± 9.7
| Albuminuria/24 hr, μg | 84.6 ± 15.8              | 680.1 ± 61.4                | 740.7 ± 167.1                                      | 773.7 ± 222.6                                    | 632.6 ± 100.6                                    | 400.2 ± 74.2                                  | 175.0 ± 74.8
| Urine albumin-to-creatinine ratio (μg/mg) | 24.1 ± 5.2               | 236.1 ± 29.6                | 386.2 ± 73.3                                       | 303.9 ± 73.7                                     | 239.9 ± 33.6                                     | 199.2 ± 49.2                                  | 69.7 ± 34.1

Controls include 18- and 24-week-old BTBR ob/ob mice and BTBR WT mice. Treatments include BTBR ob/ob plus leptin replacement, enalapril, losartan, and hydralazine. Unless otherwise noted, data are expressed as mean ± SEM.

A P < 0.01 versus 24-week-old BTBR ob/ob mice.
B P < 0.01 versus 24-week-old BTBR WT mice.
C P ≤ 0.001 versus 24-week-old BTBR ob/ob mice.
D P < 0.05 versus 24-week-old BTBR WT mice.
E P < 0.001 versus 24-week-old BTBR ob/ob mice.
Mesangial matrix expansion was quantitated by measurement of matrix stained for collagen IV and by silver methenamine. Leptin replacement significantly reduced the mesangial matrix accumulation seen in BTBR ob/ob mice to levels equivalent to those of nondiabetic BTBR WT mice. Enalapril, losartan, and hydralazine did not significantly decrease the mesangial matrix expansion, although treatment with enalapril and losartan prevented further increase in mesangial matrix beyond that already present in 18-week-old BTBR ob/ob mice (Table 2 and Figures 1 and 2).

Mesangiolysis results in both expansion of mesangial regions, with loss of normally compact silver-staining mesangial matrix demonstrable by light microscopy, and corresponding areas of lucency demonstrable by electron microscopy (Figure 3). Mesangiolysis disrupts sites where capillary loops are anchored to the mesangium, leading to marked dilatation/ballooning of the untethered capillary loops. Both mesangiolysis and the consequent capillary wall dilatation can be detected in silver methenamine–stained sections of BTBR ob/ob mice (Figure 2A). Leptin replacement significantly reduced the extent of mesangiolysis compared with 24-week-old BTBR ob/ob control mice, unlike treatment with enalapril, losartan, and hydralazine (Table 2 and Figure 2B).

Leptin replacement reduced the thickening of glomerular capillary basement membranes in BTBR ob/ob mice compared with untreated BTBR ob/ob mice at 24 weeks (169.15 ± 1.78 versus 181.36 ± 3.76 nm), but this reduction did not reach statistical significance in the limited number of mice used for this analysis (n = 3 each) (Figure 3, B and C).

The number of Mac-2 expressing monocyte/macrophages infiltrating glomeruli was reduced in all treatment groups except the hydralazine group (Table 2).

BTBR ob/ob mice develop mild tubulointerstitial fibrosis quantifiable with picrosirius red staining. Leptin, enalapril, losartan, and hydralazine treatment significantly reduced interstitial fibrosis in these mice compared with 24-week-old control BTBR ob/ob mice (Table 2).

### Leptin Replacement, but Not RAAS Inhibition, Results in Restoration of Podocyte Density and Podocyte Number

Progression of DN is characterized by significant loss of podocyte density, quantified as Wilms tumor 1 (WT-1)–expressing cells divided by mean glomerular volume. Using the method of Sanden et al., which involves thick and thin paraffin sections immunostained with WT-1, we previously demonstrated that BTBR ob/ob mice with DN show marked reduction in the total number and density of podocytes per glomerulus compared with BTBR WT controls at 20 weeks of age. In the current study, podocyte density was restored in leptin-treated mice compared with BTBR ob/ob control mice and was similar to that in WT mice of similar age (Figure 4). In contrast, treatment with enalapril, losartan, or hydralazine did not significantly alter podocyte density compared with 24-week-old BTBR ob/ob mice (Figure 4, A and B). Use of this method of podocyte counting showed no significant differences in total podocyte numbers. Glomerular volume was significantly reduced in leptin-treated BTBR ob/ob mice compared with 24-week-old control BTBR ob/ob mice (Figure 4B), contributing to the observed changes in podocyte density. Treatment with enalapril, losartan, and hydralazine did not significantly change glomerular volume. Residual podocytes in 24-week-old BTBR ob/ob mice show partial effacement of foot processes overlying capillary basement membranes, which are largely, but not completely, restored to normal in leptin-treated mice compared with BTBR WT mice (Figure 3C).

### Table 2. Morphometric data of BTBR ob/ob mice in each treatment group and control BTBR ob/ob and BTBR WT mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>24-Week-Old BTBR ob/ob WT Mice</th>
<th>18-Week-Old BTBR ob/ob Mice</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Hydralazine</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Losartan</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Enalapril</th>
<th>24-Week-Old BTBR ob/ob Mice Treated with Leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTA occupied by silver-stained matrix (%)</td>
<td>5.96 ± 0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.12 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.04 ± 0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.22 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.26 ± 0.55&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.70 ± 0.37&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>GTA occupied by collagen type IV-stained matrix (%)</td>
<td>11.33 ± 1.80&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.43 ± 0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.51 ± 1.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.54 ± 0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.92 ± 0.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.83 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glomeruli with mesangiolysis (%)</td>
<td>3.2 ± 1.36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.71 ± 7.23</td>
<td>36.33 ± 13.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>29.71 ± 12.05</td>
<td>28.76 ± 7.12</td>
<td>27.67 ± 3.56</td>
</tr>
<tr>
<td>GTA occupied by α-SMA–expressing cell (%)</td>
<td>0.19 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94 ± 0.38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.51 ± 0.14</td>
<td>0.20 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.31 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mac-2 positive cells/GTA</td>
<td>0.62 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.82 ± 0.40</td>
<td>3.33 ± 0.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.63 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.60 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ki67-positive cells/GTA</td>
<td>0.40 ± 0.03</td>
<td>0.81 ± 0.22</td>
<td>1.79 ± 0.61</td>
<td>1.28 ± 0.39</td>
<td>1.04 ± 0.09</td>
<td>1.31 ± 0.60</td>
</tr>
<tr>
<td>Picrosirius red–stained in interstitial areas (%)</td>
<td>0.09 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.19</td>
<td>0.97 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.33 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
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Data are expressed as mean ± SEM. GTA, glomerular tuft area.

<sup>a</sup>P < 0.001 versus 24-week-old BTBR ob/ob mice.

<sup>b</sup>P < 0.001 versus 24-week-old BTBR WT mice.

<sup>c</sup>P < 0.05 versus 24-week-old BTBR ob/ob mice.

<sup>d</sup>P < 0.05 versus 24-week-old BTBR WT mice.

<sup>e</sup>P < 0.01 versus 24-week-old BTBR ob/ob mice.
In recognition of controversies in the literature over methods for enumerating podocytes, along with recent data demonstrating that WT-1 may not be specific for podocytes because other kidney cells (including parietal epithelial cells [PECs]) may express this marker, immunohistochemical staining was performed on serial histologic sections to mark p57-positive cells. Linear tracing methods have previously shown p57 to be both a sensitive and a specific marker of podocytes in murine glomeruli. Taniguchi et al. demonstrated the utility of p57 as a podocyte-specific marker by using nephrin-Cre green fluorescent protein (GFP) mice that express GFP under the podocyte-specific nephrin promoter. In both control and diseased mice treated with an antipodocyte antibody, the number of podocytes immunohistochemically stained positive for p57, WT-1, and GFP were similar. With use of established stereologic methods (fractionator/dissector), total podocyte numbers (p57-positive glomerular cells) were counted in BTBR WT, BTBR ob/ob, and BTBR ob/ob with leptin treatment. Total podocyte numbers were reduced in 24-week-old BTBR ob/ob mice compared with BTBR WT mice (50.4±2.8 versus 61.1±2.7 p57-positive podocytes per glomerulus; P=0.03), and leptin treatment restored those numbers to WT mouse levels (60.2±2.4 p57-positive podocytes per glomerulus; P=0.82 versus BTBR WT and P=0.03 versus BTBR ob/ob) (Figure 5). These findings parallel our measurements showing restoration of podocyte density.

As one further method of validation, one BTBR ob/ob and one BTBR WT mouse from the p57-stained groups were used to obtain serial 1-micron-thick plastic embedded sections (“thick” sections as used for electron microscopy), stained with toluidine blue, and podocyte profiles were counted using published stereologic methods (Supplemental Figure 3). The numbers obtained from this counting did not significantly differ from the numbers obtained using the p57-immunostained serial sections. However, as illustrated in Supplemental Figure 3, this method has limitations in its accuracy when substantial mesangial expansion is present. Such expansion obscures the anatomic identification of podocytes based solely on their location. For this reason, this approach was not used in additional mice.

Figure 1. Leptin replacement results in reduction of collagen IV positive mesangial matrix. (A) Representative glomeruli showing mesangial matrix accumulation in BTBR ob/ob mice with leptin replacement was significantly decreased compared with 24-week-old BTBR ob/ob control mice. BTBR ob/ob mice treated with hydralazine, losartan, or enalapril showed no difference in mesangial matrix collagen IV immunostaining. Original magnification, ×400. (B) Morphometric analysis of glomerular tuft area (GTA) occupied by type IV collagen immunostaining of mesangial matrix. +++ P<0.001 versus 18- and 24-week-old BTBR ob/ob mice, *** P<0.001 versus 24-week-old BTBR WT mice.

Leptin Replacement Increases WT-1 Expression and Proliferation of PECs

WT-1– and Ki67-positive PECs were quantitated in all groups (Figure 6). Leptin treatment of BTBR ob/ob mice resulted in a significant increase of WT-1 expression in PECs lining the Bowman capsule (Figure 6B). Double-label immunohistochemistry
showed individual cells expressing both the podocyte marker WT-1 and the PEC marker claudin-1 (Figure 6). There was a trend toward increased numbers of Ki67-positive, proliferating PECs in the leptin-treated mice compared with 18- and 24-week-old BTBR ob/ob mice (Ki67-positive PECs/glomerulus: leptin-treated BTBR ob/ob, 0.118; 18-week-old BTBR ob/ob, 0.04083; 24-week-old BTBR ob/ob, 0.0718; hydralazine-treated BTBR ob/ob, 0.09314; enalapril-treated BTBR ob/ob, 0.09163; losartan-treated BTBR ob/ob, 0.088; 24-week-old BTBR WT, 0.02375; P=0.10), although this trend did not reach statistical significance. Proliferation of podocytes (WT-1–expressing cells) located within the glomerular tuft and not on the Bowman capsule was not present. p57-positive PECs were seen in only the leptin-treated mice but were much less prevalent than WT-1–positive PECs (Figure 5).

**Mesangial Expansion and Podocyte Loss Are Associated with Accumulations of Reactive Oxygen Species**

Accumulation of superoxide was demonstrated in glomeruli of BTBR ob/ob mice by staining tissue sections with dihydroethidium (DHE) (Figure 7). DHE produces 2-hydroxyethidium when oxidized by O$_2^-$, which appears as red fluorescent nuclear staining. After 8 weeks with a control saline pump, BTBR ob/ob mice at 20 weeks and at 28 weeks showed markedly increased DHE staining compared with BTBR WT mice (Figure 7). Treatment with leptin reversed the glomerular accumulation of superoxide (Figure 7). Double staining with DHE and WT-1 demonstrated that many of the cells positive for superoxide are podocytes (Figure 7). DHE staining of the hydralazine, enalapril, and losartan groups showed very elevated accumulation of reactive oxygen species only in the enalapril-treated mice; levels in the hydralazine and losartan treatment groups did not significantly differ from those in the WT mice (Supplemental Figure 4).

Lipid-related oxidative stress was also increased in glomeruli of BTBR ob/ob mice with DN, demonstrated by increased immunostaining for 4-hydroxynonenal (4-HNE) (Supplemental Figure 5). Similar to the results with DHE, glomeruli of BTBR
**BTBR ob/ob Mice Do Not Develop Hypertension**

As previously reported, BTBR ob/ob mice are hypotensive relative to BTBR WT and heterozygous BTBR ob/+ mice, probably as a direct consequence of leptin deficiency. The systolic pressures of BTBR ob/ob mice at 24 weeks were significantly lower than those of BTBR WT mice: 89.8±4.32/66.5±3.31 versus 120±8.08/83.7±5.95 mmHg (P<0.05). Mice treated for 6 weeks with enalapril, losartan, and hydralazine had BP similar to those of untreated BTBR ob/ob mice (enalapril, 84.72±9.38/53.63±9.7 mmHg; losartan, 80.26±5.01/55.2±8.14 mmHg; hydralazine, 81.25±2.75/50±4.18 mmHg; P=NS).

**BTBR ob/ob Mice Express ObRa within Glomeruli**

RNA isolated from whole cortex and from isolated glomeruli was used to perform RT-PCR using primers to both the short form of the leptin receptor, ObRa, and the long form, ObRb. Expression of ObRa was detectable in BTBR ob/ob and BTBR WT mice in both cortex and isolated glomeruli, whereas there was no detectable expression of ObRb (Figure 8). We further showed by immunohistochemistry that in leptin-treated BTBR ob/ob mice, phosphorylated-Stat 3 (pStat3) is upregulated in intrinsic renal cells in both glomeruli and tubular epithelium (Figure 8), an indication that there is possible leptin-mediated signaling through the leptin receptor ObRa. Double immunolabeling of tissue sections with the PEC marker...
claudin-1 or the podocyte marker nephrin and pStat3 showed de novo expression of pStat3 in both of these cell types in leptintreated mice but not untreated diabetic mice (Supplemental Figure 6).

**DISCUSSION**

The first major outcome of this study is establishment of a model of reversible DN in the BTBR ob/ob mouse. The BTBR mouse strain with the ob/ob leptin deficiency mutation is a model of type 2 diabetes and obesity. This strain develops glomerular injury similar to human DN; lesions are detectable as early as 8–10 weeks of age and progress to morphologically advanced lesions by 18 weeks, with corresponding renal dysfunction evidenced by albuminuria. Nephropathy in this model is characterized by podocyte loss, diffuse and focally nodular mesangial sclerosis, mesangiolysis, glomerular capillary microaneurysm formation, glomerular and tubular basement membrane thickening, absence of immune deposits, and mild to moderate interstitial fibrosis.

The treatment options for human DN remain severely limited. Currently, even the best treatment regimens, using inhibitors of the RAAS system, delay the onset of ESRD in patients with DN but do not reverse or even stabilize the disease process. However, a major paradigm shift in our understanding of DN came from the demonstration in humans of reversal of DN in eight diabetic recipients of pancreatic (but not renal) transplants, 10 years after normalization of the glycemic milieu. There was dramatic improvement in the glomerular features of nodular glomerulosclerosis, mesangial thickening, basement membrane thickening, and interstitial fibrosis, all characteristic of advanced DN previously thought to be irreversible. In the 13 years since the publication of that landmark study, no other therapeutic interventions have been identified that result in similarly dramatic reversal of morphologically advanced DN, either in experimental models or humans. Lesser degrees of mesangial expansion have regressed in streptozotocin-induced DN in rats by pancreas transplantation, but the investigators could not repeat these findings in a subsequent study. Here, we demonstrate that the BTBR ob/ob mouse is a robust animal model of DN that recapitulates the features of advanced human DN and its reversibility when underlying metabolic abnormalities are corrected.

The restoration of leptin in BTBR ob/ob mice resulted in rapid reversal of the diabetic state and obesity. After leptin
replacement, mice became normoglycemic, with a return to insulin levels found in control BTBR WT mice, indicating resolution of much of their underlying insulin resistance. The improvement in metabolic abnormalities was accompanied by rapid and nearly complete reversal of both functional and structural abnormalities of DN, including proteinuria, renal hypertrophy, mesangial matrix accumulation, mesangiolysis, and interstitial fibrosis.

Our search for mediators of DN reversal has led us to the podocyte. We focused on podocyte alterations and their repair for the following reasons, as recently reviewed:21 (1) Podocyte numbers are reduced in humans with type 1 and type 2 diabetes who have DN, and this reduction in podocytes may precede and even predict the onset of clinically detectable proteinuria;22 (2) podocyte loss or podocyte dysfunction can be sufficient to induce mesangiolysis in both humans and mice,23–27 and repeated mesangiolysis with exuberant repair is central to the development of nodular mesangial sclerosis in DN;28,29 and (3) podocytes are terminally differentiated cells with extremely little, if any, capacity to proliferate,30 which has been taken to mean that the pool of podocytes in mature glomeruli is fixed; hence, depletion of podocytes in DN, whether by apoptosis or detachment, is postulated to lead to irreversible glomerulosclerosis.

Accordingly, the second major finding of our study is that the obstacle of terminal differentiation of podocytes can be overcome and podocyte number and density can be restored, concomitant with reversal of proteinuria and other structural alterations of DN. These data challenge concepts, widely accepted until recently, that considered the pool of mature podocytes to be fixed and nonregenerating, along with the consequent implication that glomerular injuries characterized by podocyte loss are irreversible. The current study did not unequivocally identify the source of the regenerated podocytes; however, we obtained some evidence suggesting that glomerular PECs lining the Bowman capsule function as a progenitor cell niche for podocytes, and under proper circumstances can assume a podocyte phenotype and mobilize to populate the glomerular tuft at sites exclusive to podocytes.31–33 The de novo expression of WT-1 and p57, the expression of which has been widely accepted as specific for podocytes within the glomerulus, is present in numerous PECs of mice (as determined by their location lining the Bowman capsule and by the double immunolabeling studies identifying transitional cells expressing both the PEC phenotypic marker claudin-1 and the podocyte marker WT-1) with regression of DN, but is only rarely identified in control BTBR WT mice and BTBR ob/ob mice with advanced DN (Figure 4). This finding suggests that transdifferentiation of PECs

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Figure 5. p57-positive podocytes were counted in serial sections using the fractionator/dissector method. One hundred serial sections were cut and stained with an antibody specific for p57, a second specific marker of podocytes. p57-positive podocytes seen in both sections of the dissector pair were not counted, ensuring that each podocyte was counted only once. Use of this method confirmed that BTBR ob/ob mice had significantly fewer total podocytes per glomerulus and that podocyte number was restored with 6 weeks of leptin treatment. p57-positive PECs were seen only in leptin treated mice (arrow). Original magnification, ×400.
into podocytes may be a key element of the regression of DN and indicates that PECs may be a potential reservoir for repopulation of glomerular podocytes that were previously lost during the evolution of DN. We show that these transitional cells are actively proliferating (expressing Ki67), indicating their capacity to serve as a reservoir of cells that can be mobilized to restore lost podocytes. The leading alternative possibility—that regeneration comes from local proliferation of established podocytes—is excluded by extensive data that conclusively establish the limited proliferative capacity of this cell type (reviewed by Jefferson et al.9), and by the data in our study demonstrating the absence of significant proliferation of podocytes.

A theoretical caveat for these findings is the use of immunohistochemically detected markers of differentiated podocytes to enumerate their presence or loss. This approach could be confounded by phenotypic modulation of injured podocytes resulting in loss of such markers, although evidence that this occurs in vivo is lacking, and such methods remain the standard approach for podocyte quantification in experimental models.12 Because of concurrent changes in glomerular volume with leptin administration, we could not clearly distinguish whether the changes in podocyte number detected by immunolabeling were due to changes in absolute podocyte number or a podocyte density using our WT-1 counting method using thick and thin sections. Direct counting of podocytes by a fractionator-dissector approach allowed us to demonstrate an absolute decrease in podocyte number in BTBR ob/ob mice compared and BTBR WT mice and an increase in podocyte number in BTBR ob/ob mice treated with leptin. The studies of patients receiving pancreatic transplants did not address the issue of podocyte number; hence, we are unable to compare these findings in mice with the situation in reversible human DN. To our knowledge, this is the first demonstration that the podocyte population in DN may be dynamic and that podocyte number and density regenerate during regression of DN.

Our studies build on the landmark contributions of the Moeller and Romagnani groups, which have shown the potential for podocytes to regenerate from PEC progenitor populations. Our immunohistochemical studies showing expression of markers usually limited to podocytes within glomeruli by cells located along the Bowman capsule are consistent with such regeneration. The advances made in the current studies include the demonstration that podocyte can be restored during a disease state and that this restoration is a feature of injury reversal. Furthermore, we demonstrate that lost podocyte populations can be fully restored in conjunction with restoration of normal function (as measured by proteinuria). Additional evidence supporting such a scenario is that superoxide accumulation in podocytes and accumulation of other oxidized species in glomeruli, such as 4-HNE, both thought to be early and key pathogenic events in the evolution of DN and specifically thought to mediate early podocyte loss,34–38 also are reversed concurrently with podocyte regeneration. That reduction of podocyte superoxide is not sufficient for restoration of normal podocyte number or density is evidenced by the reduction in superoxide accumulation demonstrated in cohorts of hydralazine- and losartan-treated BTBR ob/ob mice, in which podocytes were not restored.

Figure 6. Expression of WT-1 by PECs in BTBR ob/ob mice with leptin replacement. (A) Double immunostaining with WT-1 (gray/black), which stains nuclei of podocytes, and claudin-1 (red/brown), which stains cytoplasm and cell junctions of PECs, is shown in low- and high-power views of representative glomeruli. BTBR ob/ob mice with leptin replacement demonstrate de novo expression of WT-1 by PECs lining the Bowman capsule. Arrows point to cells that express both WT-1, a podocyte marker, and the PEC marker claudin-1. Original magnifications, ×400 and ×1000. (B) Graphic representation of quantitation of WT-1–positive PECs in study groups. **P<0.01 versus BTBR WT mice, ++ P<0.01 versus 18- and 24-week-old BTBR ob/ob mice treated with hydralazine, losartan, and enalapril; δδ P<0.01 versus 18- and 24-week-old BTBR ob/ob mice treated with hydralazine, losartan, and enalapril.
Therapies to antagonize the renin-angiotensin system, via angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers, are a cornerstone in the management of DN but have limited efficacy.7 Having a model that is permissive for reversal of DN allowed us to probe mechanisms underlying the limitations of RAAS for treatment of DN. RAAS inhibition with an angiotensin-converting enzyme inhibitor (enalapril) or an angiotensin-receptor blocker (losartan) for 6 weeks beginning at week 18 in BTBR\textit{ob/ob} mice produces a functional benefit but does not reverse the structural injuries of DN, analogous to the experience in human DN. Proteinuria is reduced substantially, but neither the process of mesangiolysis nor mesangial volumes are restored to baseline as they are with leptin administration. Unlike the case with leptin administration, RAAS inhibition did not result in restoration of podocytes. This inability to promote podocyte regeneration may identify a critical limitation of RAAS inhibition and offers one mechanism by which this widely used therapeutic approach fails to alter the structural lesions of DN. An important constraint on such a conclusion is that it is based on a treatment regimen of 6 weeks’ duration. Although such a duration is sufficient to reverse DN with leptin administration, treatment with RAAS inhibitors may require longer duration to effect such change, although the experience that long-term administration of RAAS inhibitors in diabetic patients does not reverse or prevent structural injury indicates otherwise.36

Mesangiolysis is a key injury in the development and progression of nodular mesangial sclerosis, the most characteristic lesion of advanced human DN.28,29,39,40 BTBR\textit{ob/ob} mice develop mesangiolysis, and leptin administration results in significant reduction of this process. Studies of mutant mice by Matsusaka\textit{et al.}, in which podocyte death is inducible, have shown that such injury is sufficient to result in mesangiolysis;27 complementary studies of progressive podocyte dysfunction manifesting as diminished production of the trophic growth factor vascular endothelial growth factor also show that this dysfunction leads to the development of mesangiolysis.25 The time points chosen for our morphologic studies did not allow us to evaluate whether podocyte restoration precedes the reduction in mesangiolysis. Nonetheless, we speculate that because podocyte loss or dysfunction can be a proximate cause of the mesangiolysis, as has been shown in humans and rodents,23,24,27 the loss of podocytes early in the progression of DN is a contributing proximate cause of the mesangiolysis of DN, which, in turn, contributes to the mesangial matrix accumulation characteristic of DN. It is then the restoration of

Figure 7. Superoxides are upregulated in BTBR\textit{ob/ob} mice. (A) Representative glomeruli stained with DHE show little to no superoxide in BTBR WT mice. Superoxide (red stain) is markedly increased in glomeruli of BTBR\textit{ob/ob} mice at 28 weeks. Leptin replacement for 8 weeks abolished the glomerular superoxide. (B) Glomerulus from 24 week BTBR\textit{ob/ob} mouse stained with DHE (red) and WT-1 (green). Merged pictures show WT-1–positive podocytes that are also positive for superoxide (arrows). Original magnification, ×400.
Figure 8. ObRa, the short form of the leptin receptor, is expressed in glomeruli of BTBR WT and BTBR ob/ob mice. RT-PCR was performed using RNA from whole cortex and from isolated glomeruli using primers specific for ObRa and ObRb, the long form of the leptin receptor. Levels of ObRa, but not ObRb, were detectable in glomeruli of BTBR WT and BTBR ob/ob mice (upper panel). Immunohistochemistry performed using an antibody specific for phosphorylated-Stat3 shows upregulated expression in BTBR ob/ob mice treated with leptin within intrinsic renal cells, within glomeruli and tubular epithelium (bottom panel), indicating signaling either directly through leptin or through some other intermediate mediator. Original magnification, ×400.

functional podocytes that abrogates this injury process and allows reversal of DN during a reparative phase.

Finally, our studies address, to a limited degree, the key question of whether it is the restoration of a normal metabolic milieu or a direct effect of leptin that enables the reversal of diabetic nephropathy in the BTBR model. The identification of leptin receptors within isolated glomeruli from BTBR mice establishes the possibility of a direct leptin effect. The demonstration of pStat3, a key downstream molecule in the leptin signaling pathway, provides stronger evidence that leptin signaling in the kidney contributes, to an unknown degree, to reversal of nephropathy, although it is recognized that the pStat3 could be the result of other signaling pathways. Studies to distinguish these two possibilities are underway.

In aggregate, these studies support a new paradigm that podocytes in DN can regenerate, and that concurrent reversal of DN is an attainable goal. They support, but do not prove, a central role for podocyte loss in mediating at least some structural manifestations of DN. They demonstrate for the first time that a lack of effect on podocyte restoration may underlie the limited efficacy of RAAS inhibitors in promoting repair of DN and that this may help us understand limitations of these therapeutics when used to treat human DN. These studies identify PECs as a possible progenitor cell population for restoration of podocytes in DN and suggest that therapeutics to reduce podocyte loss or promote mobilization of PECs to restore podocyte density and/or number may reverse or possibly prevent DN.

**CONCISE METHODS**

**Animals**

The experimental protocol was reviewed and approved by the Animal Care Committee of the University of Washington.

BTBR ob/ob mice are a well studied model of type 2 diabetes, and the nephropathy that develops in these mice has been characterized previously. Mice were maintained in specific pathogen-free facility with a temperature-controlled room, regulated with 12-hour light/dark cycle and free access to water and food (standard chow diet: Picolab Rodent Diet 20, Brentwood, MO) containing 0.2% glucose, 0.33% Na, 20% protein, 4.5% fat, and 11.9% calories from fat.

**Experimental Design**

Six cohorts of mice were studied: (1) BTBR ob/ob mice that received subcutaneous implantation of osmotic mini-pumps (Alzet 2006, Durect Corp., Cupertino, CA) containing leptin at age 18 weeks (n=7); leptin was released from these pumps at a rate of 0.15 μL per hour at a dose of 250 PM per day; (2) BTBR ob/ob mice undergoing inhibition of the RAAS by administration of the angiotensin-converting enzyme inhibitor enalapril (Sigma-Aldrich, St. Louis, MO) at 125 mg/L in drinking water, a dose equal to 30 mg/kg body weight per day, previously established to be effective in mice44 (n=9); (3) BTBR ob/ob mice undergoing inhibition of the RAAS by the angiotensin II type 1 receptor blocker losartan (Merck, White House Station, NJ) at 100 mg/L in drinking water, equal to 25 mg/kg body weight per day (n=7); (4) a control group of BTBR ob/ob mice treated with the antihypertensive drug hydralazine (Sigma-Aldrich), 200 mg/L in drinking water, equal to 50 mg/kg body weight per day (n=7); (5) a control group of 24-week-old BTBR ob/ob mice that did not undergo therapeutic interventions (n=6); and (6) a control group of normoglycemic 24-week-old BTBR WT mice (n=5). All treatments were initiated at 18 weeks of age and continued for 6 weeks until 24 weeks of age.

In separate pilot studies, osmotic pumps (Alzet 2004, Durect Corp.) containing leptin or saline were implanted in BTBR ob/ob mice at 20 weeks and continued to 28 weeks of age, with pumps being replaced after 4 weeks (n=3). Kidneys from control 20-week-old BTBR WT and BTBR ob/ob mice and saline- or leptin-treated BTBR ob/ob mice at 28 weeks were used for reactive oxygen species studies.

**Blood Chemistry**

Blood samples were collected by retro-orbital bleeding at time of euthanasia. Blood glucose levels were monitored weekly using an ACCU-CHEK Ativa Kit (Roche Diagnostics, Indianapolis, IN) beginning at 18 weeks to establish baselines for each mouse. BUN was measured using a liquid urea nitrogen reagent set (Pointe Scientific, Inc., Canton, MI). Serum creatinine levels were measured by an HPLC-based method, as recommended by the Animal Models of Diabetic Complications Consortium at the Yale University Mouse Metabolic Phenotyping Center (MMPC) metabolic testing core. Serum insulin levels were measured...
using an ELISA-based assay by the University of Washington MMPC metabolic core. Leptin levels were measured in serum using a commercial ELISA kit, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

**Urine Measurements**
Timed (6-hour) urine collections were obtained beginning at the start of treatment and weekly thereafter. Urine samples were evaluated for proteinuria using the albumin-to-creatinine ratio, as previously described. Albuminuria was measured using mouse albumin ELISA quantitation (Bethyl Laboratories, Montgomery, TX) and creatinine using the Creatinine Companion kit (Exocell) according to the protocols of the manufacturer.

Body weight was measured weekly.

**BP Measurement**
BP was measured using the CODA 6 noninvasive tail-cuff system (Kent Scientific, Torrington, CT) on conscious mice, as previously described. BP was measured at the start of treatment at 18 weeks and before euthanasia at 24 weeks. The accuracy of BP measurements obtained using the CODA 6 have been validated in several studies that used comparisons to BP measurements done by invasive telemetry.

**Tissue Collection and Histologic and Electron Microscopic Studies**
Kidney tissue was obtained at euthanasia. Tissues were divided and portions fixed in 10% neutral buffer formalin, in methyl Carnoy solution or 1/2× Karnovsky solution, then processed and embedded in paraffin or Eponate resin using routine protocols. Formalin-fixed, paraffin-embedded kidney tissue was sectioned and stained with hematoxylin and eosin, periodic acid-Schiff, silver methenamine, and picrosirius red. Karnovsky-fixed tissue was used for electron microscopic study and measurements of basement membrane thickness. Portions of kidney were snap-frozen and stored at −80°C and used for immunofluorescence studies as previously described. In a subset of animals, magnetic beads (Dynabeads M-450 Tosylactivated beads, Life Technologies, Grand Island NY) were used to isolate glomeruli as published.

**Immunohistochemistry**
Four-micron sections of formalin or methyl Carnoy-fixed, paraffin-embedded tissue were immunostained as described previously. Antibodies used for immunohistochemistry were (1) goat anti-collagen IV (Southern Biotechnology, Birmingham, AL); (2) rat anti-Mac-2, a marker of monocytes/macrophages (Cedarlane, Hornby, Ontario, Canada); (3) mouse anti-α-smooth muscle actin (α-SMA), clone 1A4 (Sigma, St. Louis, MO); (4) rabbit anti-Ki67, a marker of proliferating cells (clone SP6, Neomarkers, Fremont, CA); (5) rabbit polyclonal antibody to WT-1 protein, a specific marker of podocyte nuclei in glomeruli (Santa Cruz Biotechnology, Santa Cruz, CA); (6) rabbit monoclonal antibody to WT-1 protein (Epitomics, Burlingame, CA); (7) rabbit polyclonal anti-p57 (Santa Cruz Biotechnology); (8) rabbit anti-HNE, a marker of reactive oxygen species production (Alpha Diagnostics, San Antonio, TX); and (9) phospho-STAT3 (Tyr705) XP rabbit mAb (Cell Signaling, Danvers, MA). Double immunohistochemistry was performed by first performing WT-1, nephrin, or claudin-1 immunohistochemistry and development with Vector SG (Vector Laboratories, Burlingame, CA), resulting in a black/gray reaction product. Slides were then blocked with 3% hydrogen peroxide and donkey antirabbit antibody (Jackson Immunoresearch, West Grove, PA), followed by immunohistochemistry for claudin-1 (Abcam, San Francisco, CA) or pSTAT3 and development with ImmPACT DAB substrate (Vector Laboratories) to give a brown reaction product. Secondary reagents used for immunohistochemistry were ImmPRESS antirabbit horseradish peroxidase (HRP) (Vector Laboratories), ImmPRESS goat HRP (Vector Laboratories), MM HRP-Polymer kit (Biocare, Concord, CA), and Polylink-1 HRP rat NM kit (Golden Bridge International, Mukilteo, WA).

Tissues frozen in optimal cutting temperature were sectioned at 5 μm and stained with dDHE, a second marker of accumulation of reactive oxygen species, in Kreb HEPES buffer (20 mM HEPES, pH 7.4; 128 mM NaCl, 2.5 mM KCl, 2.7 mM CaCl2, 1 mM MgCl2, 16 mM glucose) for 30 minutes at 37°C. Glomerular DHE-positive staining was scored semiquantitatively on a scale of 0 (no staining) to 3 (robust glomerular cell staining). Sections used for double staining were first incubated with rabbit anti-WT-1 (Santa Cruz), followed by AlexaFluor 488-conjugated antirabbit (Invitrogen, Carlsbad, CA) and then incubated in DHE as detailed above.

Negative controls for immunohistochemistry included both substitution of the primary antibody with an isotype-matched irrelevant immunoglobulin or antisera from the same species and substitution with PBS.

**Quantitative Analysis of Glomerular and Interstitial Lesions**
To quantitate glomerular changes for each animal, 15 (0.1 mm2) sections were systematically digitized using a 40× microscope objective and examined. Glomeruli were consecutively encountered by moving the microscope stage with an S-shape path.

Glomerular mesangial expansion, matrix accumulation/regression, and mesangial cell activation were established using three complementary methods: (1) mesangial area occupied by silver methenamine-stained matrix, (2) mesangial area occupied by matrix immunostained to detect type IV collagen accumulation, and (3) immunostaining to detect mesangial cell expression of α-SMA. The cross-sectional glomerular tuft area (independent of the urinary space) was quantified, as was the total area positive for matrix, collagen IV, or α-SMA by computer image analysis (Image Pro Plus, Media Cybernetics, Bethesda, MD) for each glomerular cross-section as previously described.

Mesangiolysis was determined by assessing the silver methenamine-stained glomeruli and scoring at least 50 glomeruli per animal. Mesangiolysis was scored as present when lucency and dissolution of the normally compact silver-staining mesangial matrix was present, and/or there was dilatation/ballooning of adjacent capillary loops.

Picrosirius red-stained sections were used to evaluate concurrent interstitial fibrosis. Stained tissue sections were photographed under polarized light to achieve maximum brightness, and the percentage positive interstitial staining per area was quantified using Image Pro Plus image.

Ki67-positive proliferating cells, WT-1–positive cells, and Mac-2–expressing monocytes/macrophages within glomeruli were...
quantified by counting positive cells at a magnification of 40× in 50 consecutive glomeruli, as previously described. Additionally, Ki67- and WT-1–positive cells within the parietal cell layers lining the Bowman capsule were separately counted in a minimum of 50 glomerular cross-sections and expressed as average number of cells per parietal layer of the Bowman capsule.

Estimation of Glomerular Volume and Podocyte Density
The thick- and thin-section method of Sanden et al.54 was used to evaluate podocyte density using WT-1 antibody as a marker of podocyte nuclei. Briefly, two histologic slides of formalin-fixed kidney (one 3 μm thick and one 9 μm thick) were stained with WT-1 antibody to label glomerular podocytes. Images from 50 consecutive glomeruli from 3-μm and 9-μm sections were photographed with a digital camera under 40× magnification. Images were then imported into Image Pro Plus software for counting WT-1–expressing cells (exclusive of WT-1–expressing parietal cells as described above) and morphometric analysis.

The mean glomerular volume was derived with the Weibel formula55 using the glomerular cross-sectional area of the 50 consecutively measured glomerular profiles, as previously described.10 The value for glomerular volume per WT-1–positive nucleus was calculated and designated as GV/P. Calculation of the number of WT-1–positive nuclei per glomerulus and GV/P was adjusted to reflect the calibrated thickness difference of the microtome used to cut the tissue sections, as outlined by Sanden et al.54 This then allowed us to calculate the mean number of WT-1–expressing podocytes per glomerulus tuft, equal to the mean glomerular volume divided by GV/P.

To additionally validate podocyte counting, we collected 100 serial 3-micron paraffin sections, placing 4 sections per slide from BTBR ob/ob (n=4), BTBR WT (n=4), and BTBR ob/ob leptin-treated mice (n=4). Every other slide was stained with anti-p57 antibody to label podocytes, and the whole slide was scanned using a Bioimagene iScan Coreo (Ventana Medical Systems, Tucson, AZ). A minimum of 10 glomeruli per animal were counted, comparing section 1 to section 2, section 2 to section 1, section 3 to section 4, and section 4 to section 3 on each slide. If a positively stained podocyte was seen in one section but not the adjacent section, it was counted. Glomeruli were chosen so that the entire glomerulus was counted beginning when it first was apparent on the slide until it disappeared. Additionally, one BTBR ob/ob and one BTBR WT mouse from the above groups were processed and embedded for electron microscopy, and serial 1-micron-thick sections were cut and stained with toluidine blue for counting of podocyte profiles according to published fractionator/dissector methods.14,15

RNA Preparation and PCR
Isolation of RNA, reverse transcription, and PCR from total cortical and glomerular RNA were prepared as described previously.56,57 Primers used for PCR were ObRa-F 5’-ACA CTG TTA ATT TCA CAC CAG AG-3’, ObRa-R 5’-AGT CAT TCA AAC CAT TAG TTT AGG-3’, ObRb-F 5’-GTG TGA GCA TCT CTC GTG GAG-3’, ObRb-R 5’-ACC ACA CCA GAC CCT GTA AG-3’.

Statistical Analyses
All data are expressed as mean ± SEM. Statistical analysis of the data for multiple groups was performed by one-way ANOVA, the Tukey-Kramer multiple comparisons test, and two-tailed t test with Mann-Whitney test, using the GraphPad Prism 5.0 program for Windows (GraphPad, La Jolla, CA). Differences with P<0.05 were considered significant.

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DISCLOSURES
None.

REFERENCES


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