Role of blood pressure and the renin-angiotensin system in development of diabetic nephropathy (DN) in eNOS$^{-/-}$ db/db mice

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Zhang MZ, Wang S, Yang S, Yang H, Fan X, Takahashi T, Harris RC. Role of blood pressure and the renin-angiotensin system in development of diabetic nephropathy (DN) in eNOS$^{-/-}$ db/db mice. Am J Physiol Renal Physiol 302: F433–F438, 2012. First published November 23, 2011; doi:10.1152/ajprenal.00292.2011.—Randomized clinical trials have clearly shown that inhibition of the renin-angiotensin system (RAS) will slow the rate of progression of diabetic nephropathy, but controversy remains about whether the observed beneficial effects result from more than control of blood pressure. Deletion of eNOS in a model of type II diabetes, db/db mice (eNOS$^{-/-}$ db/db), induces an accelerated nephropathy and provides an excellent model of human diabetic nephropathy. As is frequently seen in type II diabetes, blood pressure is moderately elevated in eNOS$^{-/-}$ db/db mice. To determine the role of elevated blood pressure per se vs. additional deleterious effects of the RAS in mediation of disease progression, 8-wk-old eNOS$^{-/-}$ db/db mice were randomly divided into three groups: vehicle, treatment with the angiotensin-converting enzyme inhibitor (ACEI) captopril, or treatment with “triple therapy” (hydralazine, reserpine, hydrochlorothiazide), and the animals were euthanized after treatment for 12 wk. Blood pressure was reduced to comparable levels with ACE inhibition or triple therapy. Although both treatment regimens decreased development of diabetic nephropathy, ACE inhibition led to more profound reductions in albuminuria, glomerulosclerosis, markers of tubulointerstitial injury, macropage infiltration, and markers of inflammation. Therefore, this animal model suggests that while there is an important role for blood pressure control, RAS blockade provides additional benefits in slowing the progression of diabetic nephropathy.

METHODS

Animals

Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Vanderbilt University. Type 2 diabetes eNOS$^{-/-}$ db/db mice on the C57BLKS/J (BKS) background were generated as described previously (23). Genotyping was performed by PCR. At 8 wk of age, after urine collection and blood pressure measurement, the animals were divided into three groups (n = 5/group): vehicle, captopril, and triple therapy. Blood pressure was comparable among the groups before the initiation of the experiment. Captopril was given in the drinking water (0.75 mg/ml). Triple therapy was achieved by giving drinking water containing 80 µg/ml hydralazine, 30 µg/ml hydrochlorothiazide, 5 µg/ml reserpine. After 12 wk of treatment (20 wk of age), the animals were euthanized after urine collection and blood pressure measurement.

Measurements of Blood Pressure, Blood Glucose and Creatinine, and Albuminuria

Systolic blood pressure (SBP) was measured in conscious, trained mice at room temperature using a tail-cuff monitor (BP-2000 BP Analysis system, Visitech Systems). In addition, in a subset of mice, blood pressures were monitored by carotid catheterization. In brief, mice were anesthetized with 80 µg/g ketamine (Fort Dodge Laboratories) and 8 µg/g inactin (BYK) by intraperitoneal (ip) administration. Mice were placed on a temperature-controlled pad. PE-10 tubing was inserted into the right carotid artery, tunneled under the skin, exteriorized, secured at the back of the neck, filled with heparinized saline, and sealed. The catheterized mice were housed individually, and blood pressure measurements were made 24 and 48 h after surgery with a Blood Pressure Analyzer (Micro-Med, Louisville, KY) (12). Blood pressure was recorded for 3 h continuously at the same time of the day, and data recorded were averaged. Data are presented as mean arterial pressure (MAP). Blood glucose was determined using a OneTouch glucometer and test strips (LifeScan, Milpitas, CA). Serum creatinine was measured by a previously described HPLC method (20). Spot urine was collected from individually caged mice using polycarbonate metabolic cages. Urinary albumin and creatinine excretion was determined using Albuwell-M kits (Exocell, Philadelphia, PA).

Antibodies

Monoclonal rat anti-mouse F4/80 (catalog no. MCA497R) was purchased from AbD Serotec; monoclonal anti-mouse Tim-1 (Kim-1) antibody (a marker of renal tubular injury, catalog no. MAB1817) was from R&D Systems; affinity-purified rabbit anti-human interleukin 4 receptor-α (IL4RA, or CD124, catalog no. NBP1-00884) was from Novus Biologicals; and rabbit anti-nitrotyrosine and 4-hydroxynonenal (4-HNE) antibodies were from Santa Cruz Biotechnology.
Immunohistochemistry and Western Blot Analysis

Under deep anesthesia with Nembutal (70 mg/kg ip; Abbot Laboratories), mice were exsanguinated with ~10 ml heparinized saline (0.9% NaCl, 2 U/ml heparin) through a transcardiac aortic cannula. One kidney was removed for Western blot analysis and quantitative (q) PCR. The other kidney was perfused with fixative containing 3.7% formaldehyde, 0.01 M sodium m-periodate, 0.04 M sodium phosphate, 1% acetic acid, and 0.1 M NaCl. The fixed kidneys were dehydrated through a graded series of ethanol, embedded in paraffin, sectioned at 4-μm thickness, and mounted on glass slides. The slides were deparaffinized, rehydrated, and stained with different antibodies, as previously described (5). Based on the distinctive density and color of immunostaining in video images, the number, size, and position of stained cells were quantified using the BIOQUANT True-Color Windows System (R & M Biometrics, Nashville, TN) as previously described (22). Western blot analysis was carried out as described previously (6).

Histological Analysis

Glomerular injury. Periodic acid-Schiff-stained slides were evaluated without knowledge of the identity of the various groups. A semiquantitative index was used to evaluate the degree of glomerular sclerosis. Each glomerulus on a single section was graded from 0 to 4, where 0 represents no lesion, and 1, 2, 3, and 4 represent sclerosis, involving ≤25, 25–50, 50–75, or >75% of the glomerular tuft area, respectively.

Interstitial fibrotic analysis. Picrosirius red-stained slides were evaluated without knowledge of the identity of the various groups. Interstitial fibrosis was quantified using the BIOQUANT True-Color Windows System. Interstitial fibrosis in vehicle-treated animals was used as 100%.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from kidneys using TRIzol reagents (Invitrogen) according to the manufacturer’s instructions. qPCR was performed using a TaqMan real-time PCR machine (7900HT, Applied Biosystems). The Master Mix and all gene probes were also purchased from Applied Biosystems. The probes used in the experiments included mouse S18 (Mm02601778), renin (Mm02342889), angiotensinogen (AGT; Mm00599662), ACE1 (Mm00802048), ACE2 (mm01159003), Mas (Mm0062713), ANG II type 1a (AT1a; Mm01166161), AT1b (Mm01701115), AT2 (Mm01341373), transforming growth factor (TGF)-β (Mm00441726), TNF-α (Mm99999068), IL-1α (Mm00439621), and inducible nitric oxide synthase (iNOS; Mm00440502).

Statistical Analyses

All values are presented as means ± SE. A Bonferroni t-test corrected for multiple comparisons was used for statistical analysis, and differences were considered significant at P < 0.05.

RESULTS

By 8 wk of age, SBP was significantly higher in eNOS<sup>_--</sup> db/db mice than wild-type db/db mice [145 ± 6 (n = 15) vs. 120 ± 3 mmHg, n = 5, P < 0.01] as was albuminuria (837 ± 79 vs. 46 ± 13 μg/mg albumin/creatinine, P < 0.01). To determine the role of the RAS vs. blood pressure elevations per se in the development of diabetic nephropathy in the eNOS<sup>_--</sup> db/db mice, we treated eNOS<sup>_--</sup> db/db mice with either the ACE inhibitor (ACEI) captopril (n = 5) or with a triple therapy regimen consisting of hydralazine, reserpine, and hydrochlorothiazide (n = 5) for 12 wk. Fasting blood sugars were comparable among the groups both at the initiation of the study and after 12 wk of drug administration (vehicle vs. captopril vs. triple therapy: 8 wk of age: 263 ± 34 vs. 279 ± 29 vs. 301 ± 19 mg/dl; 20 wk of age: 268 ± 33 vs. 338 ± 25 vs. 316 ± 32 mg/dl). At the initiation of the study, SBP was comparable among the groups (vehicle vs. captopril vs. triple therapy: 143 ± 4 vs. 144 ± 4 vs. 147 ± 3 mmHg, n = 5/group). At the end of 12 wk of treatment, SBP remained elevated in vehicle-treated eNOS<sup>_--</sup> db/db mice (146 ± 6 mmHg) but was comparably decreased in the captopril-treated (102 ± 5 mmHg) and triple therapy-treated mice (106 ± 4 mmHg) (Fig. 1A). In an additional subset of mice, after treatment for 12 wk (from 8 to 20 wk of age), blood pressures were measured by using carotid catheterization. As indicated in Fig. 1B, at 20 wk of age, MAP was reduced to similar levels by captopril and triple therapy (vehicle vs. captopril vs. triple therapy: 142 ± 2 vs. 103 ± 3 vs. 103 ± 2 mmHg, n = 4/group). We recognize

Fig. 1. Effects of captopril and triple therapy on blood pressure and albuminuria in endothelial nitric oxide synthase (eNOS)<sup>_--</sup> db/db mice. A and B: treatment with angiotensin-converting enzyme (ACE) inhibitor captopril or “triple therapy” (hydralazine, reserpine, hydrochlorothiazide) for 12 wk reduced blood pressure [systolic blood pressure (SBP)] to similar levels as determined by tail-cuff monitoring (A) and carotid catheterization (B). MAP, mean arterial pressure. *P < 0.01 vs. vehicle group. †P < 0.01 vs. corresponding vehicle.‡P < 0.01 vs. corresponding captopril.
the limitations of the tail-cuff methodology and carotid catheterization in that they only provide a snapshot of the level of hypertension rather than continuous monitoring of changes in blood pressure. In vehicle-treated eNOS−/− db/db mice, albuminuria progressively increased during the 12 wk of study and by age 20 wk was 2.574 ± 974 μg/mg albumin/creatinine (n = 5) (Fig. 1C). In contrast, captopril treatment led to decreases in albuminuria even compared with baseline values and reached a nadir of 432 ± 101 μg/mg albumin/creatinine at age 20 wk (P < 0.01, n = 5). As indicated in Fig. 1C, triple therapy largely prevented further increases in albuminuria but did not induce the regression seen with ACEI treatment (1,204 ± 180 μg/mg albumin/creatinine at 20 wk, P > 0.05 vs. baseline values, but P < 0.05 vs. vehicle group or captopril group at 20 wk). In the additional subset of mice used for blood pressure measurements by using carotid catheterization, albuminuria was highest in vehicle-treated eNOS−/− db/db mice and lowest in captopril-treated eNOS−/− db/db mice (vehicle vs. captopril vs. triple therapy: 1,933 ± 155 vs. 516 ± 84 vs. 1,123 ± 112 μg/mg albumin/creatinine at 20 wk, n = 4/group). As in our previous report (23), serum creatinines were elevated in vehicletreated eNOS−/− db/db mice (0.165 ± 0.021 mg/dl; n = 5) while captopril-treated mice had significantly lower serum creatinines, which were not different from nondiabetic values (0.109 ± 0.004 mg/dl; n = 5; P < 0.05 vs. vehicle group) (23).

Captopril significantly decreased the glomerulosclerosis index compared with vehicle-treated mice (0.37 ± 0.03 vs. 1.26 ± 0.29; n = 5, P < 0.01) while triple therapy also reduced glomerulosclerosis but was somewhat less effective than captopril (0.58 ± 0.07, P < 0.01 vs. vehicle group and captopril group, n = 5) (Fig. 2, A and B). In addition to increased glomerulosclerosis, there was increased tubular damage in vehicle-treated eNOS−/− db/db mice, as indicated by increased Kim-1 immunoreactivity (Fig. 2, A and C) and increased tubulointerstitial fibrosis (Fig. 2, A and D), both of which were significantly inhibited by ACEI and partially inhibited by triple therapy.

In eNOS−/− db/db mice, there was an increase in macrophage infiltration, as indicated by increased F/480 immunostaining (Fig. 3A). Both captopril and triple therapy significantly decreased macrophage infiltration, although the inhibitory effect of captopril was more pronounced. In addition, there were selective increases in the macrophage M2 markers arginase-1 and IL4RA in the captopril-treated mice (Fig. 3B). Both captopril and triple therapy treatment also decreased nitrotyrosine staining and 4-HNE staining, markers of oxidative stress.

Fig. 2. Effects of captopril and triple therapy on the progression of diabetic nephropathy in eNOS−/− db/db mice. A: glomerulosclerosis [hematoxylin and eosin (H&E) staining], tubular damage (Kim-1 staining, a marker of tubular injury), and interstitial fibrosis (picrosirius red staining) were significantly reduced by captopril treatment, but less reduced by triple therapy. Original magnification: H&E staining, ×250; Kim-1 staining, ×100; picrosirius red, ×160. B: quantitative glomerulosclerotic index was significantly reduced by captopril treatment, but was only partially reduced by triple therapy. *P < 0.01 vs. vehicle. †P < 0.01 vs. captopril. C: expression of Kim-1 was significantly reduced by captopril treatment, but was less reduced by triple therapy. D: quantitative interstitial fibrosis was significantly reduced by captopril treatment, but was less reduced by triple therapy. *P < 0.01 vs. vehicle. †P < 0.01 vs. captopril.

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Table 1. Of note, renal ACE1 mRNA expression was decreased in eNOS\(^{-/-}\) mice and renin mRNA was significantly decreased by triple therapy and further inhibited by captopril. Macrophages slightly increased by triple therapy.

Fig. 3. Captopril treatment and triple therapy reduces renal macrophage infiltration in eNOS\(^{-/-}\) mice. A: renal macrophage infiltration was inhibited by triple therapy and further inhibited by captopril. Macrophages were stained with F4/80 antibody and quantitated with an image-analysis system. \(*P < 0.01\) vs. vehicle. \(\dagger P < 0.01\) vs. captopril. Original magnification: \(\times 160\). B: expression of renal arginase-1 and IL-4 receptor-\(\alpha\) (IL4RA), markers of M2 macrophages, significantly increased by captopril treatment, but only slightly increased by triple therapy.

(Fig. 4, A and B), and decreased expression of mediators of inflammation and cell injury, IL-1\(\alpha\), TNF-\(\alpha\), iNOS, and TGF-\(\beta\) (Fig. 4C).

Because of the profound effect of ACE inhibition upon progression of nephropathy in eNOS\(^{-/-}\) mice, we determined whether there was activation of the intrarenal RAS. Compared with either db/db mice or the lean nondiabetic littermates on the same (BKS) background, there were not significant differences in renal expression of ANG II receptor mRNA (AT\(1_a\), AT\(1_b\), AT\(2\), or Mas) in eNOS\(^{-/-}\) mice (Table 1). Of note, renal ACE1 mRNA expression was decreased in both db/db and eNOS\(^{-/-}\) mice compared with their lean littermates, and renin mRNA was significantly decreased in eNOS\(^{-/-}\) mice. In contrast, renal angiotensinogen mRNA expression was significantly increased in db/db and was further increased in eNOS\(^{-/-}\) mice (Table 1). Similar increases in angiotensinogen were seen in isolated glomeruli (Table 2). It was also noteworthy that glomerular mRNA expression of the Ang 1–7 receptor Mas was decreased in both db/db and eNOS\(^{-/-}\) db/db mice. Glomerular mRNA levels of other components of RAS were comparable among lean nondiabetic littermates, wild-type db/db mice, and eNOS\(^{-/-}\) db/db mice (Table 2).

DISCUSSION

eNOS\(^{-/-}\) db/db mice manifest characteristics reminiscent of type II diabetes in humans, namely, obesity, hyperglycemia, moderate hypertension, progressive albuminuria, and decline in glomerular filtration rate (23). As discussed in a recent report from the Animal Models of Diabetic Complications Consortium (4), this is currently the best available model for studying progressive diabetic nephropathy seen in type II diabetes. The current studies investigated the role of blood pressure and the RAS in the progressive renal injury seen in this model. The results emphasize the important role of elevated blood pressure in the progression of diabetic glomerular and tubulointerstitial injury. Previous studies indicated that blood pressures are moderately increased in eNOS null mice on the BKS background, and eNOS\(^{-/-}\) mice on the same background have a similar degree of hypertension (23). However, nondiabetic eNOS null mice do not present the same extent or type of glomerular lesions as are seen in eNOS\(^{-/-}\) db/db mice (23). Therefore, these studies suggest that the combination of persistent hyperglycemia, perhaps resulting in altered glycation of glomerular basement membranes, coupled with glomerular hyperfiltration and increased glomerular capillary pressures, are important mediators of the progressive renal injury seen in diabetic nephropathy. That reduction of systemic blood pressure with the triple therapy regimen largely prevented further increases in albuminuria and led to a decreased glomerulosclerosis index emphasizes the importance of increased systemic blood pressure in the progression of diabetic renal injury.

These studies also indicate that RAS inhibition does indeed provide additional benefits beyond lowering blood pressure to prevent progression of diabetic nephropathy in eNOS\(^{-/-}\) db/db mice by the end of the 12-wk treatment period. Captopril treatment not only presented further increases in albuminuria but further decreased albuminuria to levels seen in nondiabetic eNOS\(^{-/-}\) mice (23). Captopril treatment also decreased the glomerular injury index to levels seen in nondiabetic eNOS\(^{-/-}\) mice (23). Although clinical studies with RAS inhibition have suggested additional benefits beyond blood pressure control in retarding nephropathy progression (17), there has continued to be controversy about this question (2, 3, 8, 9). The findings in the current study clearly indicate that in this mouse model of type II diabetes, there is additional protection against progressive nephropathy conferred by RAS blockade.

The current studies also differ sharply from recent studies in a type I model of diabetic nephropathy (eNOS\(^{-/-}\) mice+streptozotocin), in which the investigators failed to detect a significant effect on progressive glomerulopathy with either the ACE inhibitor enalapril or the angiotensin receptor blocker telmisartan (12). Of note, they also failed to see a sustained effect to lower blood pressure with these compounds, even with higher doses of the ACEI. Although this model utilized a high-dose streptozotocin protocol, which raises the possibility of persistent drug-mediated vascular or renal injury, Nakagawa and coworkers (11, 12) have shown that reduction of blood pressure with hydrozine treatment with spironolactone or control of blood sugar with insulin all lessened the progression of renal injury in this model. Therefore, it appears that the failure of RAS blockade in this model may be due to an inability of the mice to lower blood pressure. The underlying reason for such a finding remains uncertain. The beneficial effects of ACE inhibition in the present studies suggest that the renoprotective benefit of RAS blockade are not only due to increasing endothelial nitric oxide bioavailability (18).
Studies in experimental animals and in humans have suggested that there is decreased eNOS expression and/or activity with progressive diabetic nephropathy (16), and polymorphisms in the eNOS gene have been associated with increased incidence or accelerated progression of diabetic nephropathy (7, 13, 19, 21). We investigated whether eNOS deficiency induced alterations in the intrarenal RAS by studying \textit{db/db} mice with or without eNOS deficiency, but aside from increased angiotensinogen expression, there was not a pattern consistent with a selective increase in the intrarenal RAS by eNOS deficiency. The effects on glomerular

![Fig. 4. Captopril treatment and triple therapy reduce renal oxidative stress and cytokine production in eNOS\textsuperscript{-/-} \textit{db/db} mice. A: oxidative stress, as indicated by nitrotyrosine and 4-hydroxynonenal (4-HNE) staining, was significantly reduced by captopril treatment, but was less reduced by triple therapy. Original magnification: \(\times 100\). B: Western blot shows the levels of nitrotyrosylated proteins were significantly reduced by captopril treatment, but were less reduced by triple therapy. C: quantitative PCR shows reduced expression of renal transforming growth factor (TGF)-\(\beta\), TNF-\(\alpha\), IL-1\(\alpha\), and inducible nitric oxide synthase (iNOS) after captopril treatment or triple therapy. *\(P < 0.01\) vs. vehicle. †\(P < 0.05\) vs. vehicle. ‡\(P < 0.05\) vs. captopril.}
Mas oncogene expression is intriguing and suggest alterations in Ang 1–7 signaling, but further studies will be required to determine whether this mediates any of the observed pathophysiologic alterations.

In summary, in this model of type II diabetes and nephropathy, although decreasing blood pressure without inhibiting RAS decreased development of nephropathy, RAS inhibition led to more profound reductions in albuminuria, glomerulosclerosis, markers of tubulointerstitial injury, and macrophage infiltration. Therefore, studies in this mouse model of type II diabetes suggest that while there is an important role for blood pressure control, RAS blockade provides additional benefits in slowing the progression of diabetic nephropathy.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.-Z.Z. and R.C.H. drafted manuscript; R.C.H. edited and revised manuscript; M.-Z.Z., T.T., and R.C.H. approved final version of manuscript; R.C.H. drafted manuscript.

REFERENCES


Table 1. mRNA levels of renin-angiotensin system in kidneys

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<th>BKS</th>
<th>db/db</th>
<th>eNOS KO db/db</th>
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<tr>
<td>AT1a</td>
<td>69 ± 10</td>
<td>54 ± 3</td>
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<tr>
<td>AT1b</td>
<td>0.75 ± 0.03</td>
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<td>AT2</td>
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<td>0.89 ± 0.07</td>
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<td>Renin</td>
<td>104 ± 8</td>
<td>82 ± 9</td>
<td>16 ± 2†</td>
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<td>AGT</td>
<td>6.36 ± 0.24</td>
<td>9.90 ± 0.55*</td>
<td>13.80 ± 0.85*†</td>
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<tr>
<td>ACE1</td>
<td>279 ± 29</td>
<td>40 ± 9*</td>
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<td>ACE2</td>
<td>4.25 ± 0.37</td>
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<td>Mas</td>
<td>1.67 ± 0.21</td>
<td>1.81 ± 0.07</td>
<td>2.12 ± 0.13</td>
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Values are means ± SE. eNOS, endothelial nitric oxide synthase; KO, knockout. AGT, angiotensinogen; ACE, angiotensin-converting enzyme. *P < 0.01 vs. BKS. †P < 0.05 vs. db/db.

Table 2. mRNA levels of renin-angiotensin system in glomeruli

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<th>db/db</th>
<th>eNOS KO db/db</th>
</tr>
</thead>
<tbody>
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<td>AT1a</td>
<td>420 ± 59</td>
<td>411 ± 122</td>
<td>324 ± 41</td>
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<tr>
<td>AT1b</td>
<td>0.45 ± 0.01</td>
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<tr>
<td>AT2</td>
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<td>0.19 ± 0.02</td>
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<tr>
<td>Renin</td>
<td>377 ± 133</td>
<td>250 ± 77</td>
<td>277 ± 79</td>
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<tr>
<td>AGT</td>
<td>19.11 ± 2.14</td>
<td>28.55 ± 0.96*</td>
<td>38.56 ± 1.69*†</td>
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<tr>
<td>ACE1</td>
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<tr>
<td>ACE2</td>
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<tr>
<td>Mas</td>
<td>16.11 ± 3.99</td>
<td>10.78 ± 0.34*</td>
<td>11.83 ± 0.86*</td>
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</table>

Values are means ± SE. *P < 0.05 vs. BKS. †P < 0.05 vs. db/db.