Renal mitochondrial impairment is attenuated by AT₁ blockade in experimental Type I diabetes

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Renal mitochondrial impairment is attenuated by AT₁ blockade in experimental Type I diabetes. Am J Physiol Heart Circ Physiol 294: H456–H465, 2008. First published November 16, 2007; doi:10.1152/ajpheart.00926.2007.—To investigate whether ANG II type 1 (AT₁) receptor blockade could protect kidney mitochondria in streptozotocin-induced Type I diabetes, we treated 8-wk-old male Sprague-Dawley rats with a single streptozotocin injection (65 mg/kg ip; STZ group), streptozotocin and drinking water containing either losartan (30 mg·kg⁻¹·day⁻¹; STZ+Los group) or amlodipine (3 mg·kg⁻¹·day⁻¹; STZ+Aml group), or saline (intraperitoneally) and pure water (control group). Four-month-long losartan or amlodipine treatments started 30 days before streptozotocin injection to improve the antioxidant defenses. The number of renal lesions, plasma glucose and lipid levels, and proteinuria were higher and creatinine clearance was lower in STZ and STZ+Aml compared with STZ+Los and control groups. Glycemia was higher in STZ+Los compared with control. Blood pressure, basal mitochondrial membrane potential and mitochondrial pyruvate content, and renal oxidized glutathione levels were higher and NADH/cytochrome c oxidoreductase activity was lower in STZ compared with the other groups. In STZ and STZ+Los groups, mitochondrial H₂O₂ production rate was higher and uncoupling protein-2 content, cytochrome c oxidase activity, and renal glutathione level were lower than in STZ+Los and control groups. Mitochondrial nitric oxide synthase activity was higher in STZ+Aml compared with the other groups. Mitochondrial pyruvate content and H₂O₂ production rate negatively contributed to electron transfer capacity and positively contributed to renal lesions. Uncoupling-protein-2 content negatively contributed to mitochondrial H₂O₂ production rate and renal lesions. Renal glutathione reduction potential positively contributed to mitochondrial electron transfer capacity. In conclusion, AT₁ blockade protects kidney mitochondria and kidney structure in streptozotocin-diabetic diabetes independently of blood pressure and glycemia.

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captopril-treated diabetic rats, suggesting that ACE inhibitors may protect the myocardium by enhancing energy supply (13).

In this study we investigated whether long-term AT1 receptor blockade could protect kidney mitochondria functioning in streptozotocin-induced Type 1 diabetes. A calcium channel blocker, amiodipine, i.e., an antihypertensive agent acting through a mechanism distinct from AT1 blockade that can also provide antioxidant protection, was used for comparison.

**MATERIALS AND METHODS**

**Animal Ethics Committee.** The experiments were approved by the Hospital Aleman Ethics Committee (Buenos Aires, Argentina) and were conducted according to the NIH Guide for the Care and Use of Laboratory Animals. Thirty-two inbred, 8-wk-old, male Sprague-Dawley rats (Laboratory of Experimental Medicine, Hospital Aleman) were randomly divided into four groups that were administered a single streptozotocin injection (65 mg/kg ip; STZ group), streptozotocin and drinking water containing either losartan (30 mg·kg⁻¹·day⁻¹; STZ+Los group) or amiodipine (3 mg·kg⁻¹·day⁻¹; STZ+Amlo group), or saline (intraperitoneally) and pure drinking water (control group). Four-month-long losartan and amiodipine treatments were started 30 days before streptozotocin injection to enhance the antioxidant defenses that would prevent and/or diminish streptozotocin-related increases in oxidant production in the kidney. The pretreatment period was chosen on the basis of previously published work (15–17). Insulin was not administered to the animals. Rats had free access to water and standard rat chow (Cargill, Buenos Aires, Argentina) and were housed in metabolic cages (21 ± 2°C, 12:12-h light-dark cycle) to allow for food consumption determination and urine collection. Stastistic BP was determined by tail plethysmography.

Levels of plasma glucose, cholesterol and triglycerides, serum albumin, urinary protein, and creatinine clearance were determined at the end of the study by performing standard laboratory assays.

**Isolation of mitochondria.** Kidney mitochondria were isolated by differential centrifugation, as previously described (18). The purity of the mitochondrial preparation was confirmed by the insubstantial presence of marker enzyme activities for endoplasmic reticulum (glucose-6-phosphatase, 0.10%), plasma membrane (5'-nucleotidase, 0.11%), lysosomes (acid phosphatase, 2.8%), and peroxisomes (catalase, 0.30%). The absence of fragments from other subcellular compartments was assessed using electron microscopy. For nitric oxide synthase (NOS) determination, mitochondria were further purified in a Percoll gradient. Protein content was assayed by the Bradford reagent.

**Mitochondrial membrane potential and H2O2 production.** Mitochondria were incubated in 150 mM sucrose, 5 mM MgCl2, 5 mM potassium phosphate, 20 mM K-HEPES (pH 7.4), 0.24 mM, 0.02 mg mitochondrial protein in the absence [basal mitochondrial membrane potential (mtMP)] or presence of glutamate (10 mM) and malate (5 mM) at 28°C. mtMP was quantified by calculating the ratio of rhodamine-123 fluorescence at 520 and 497 nm (excitation) and at 529 nm (emission) (19). Mitochondrial H2O2 production rate was evaluated using scopoletin fluorescence as described previously (19).

**Mitochondrial enzyme activities.** Mitochondrial NOS (mNOS), Mn-superoxide dismutase (Mn-SOD), NADH/cytochrome c oxidoreductase (NADH/cytcOxRed), cytochrome c oxidase (CycOx), and citrate synthase activities were determined as previously described (19, 65). NADH/cytcOxRed activity was used to evaluate electron transfer through complexes I to III.

**Western blot analysis of uncoupling protein-2.** Proteins in mitochondrial fractions were separated on SDS-12.5% polyacrylamide gels, followed by liquid electroblotting transfer to polyvinylidene difluoride membranes and incubation in the presence of uncoupling protein-2 (UCP-2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein amounts loaded into gels were normalized by staining membranes with Ponceau red.

**Mitochondrial pyruvate content.** Mitochondria (0.2 mg protein) were added with 1 volume of 10% (wt/vol) metaphosphoric acid. After mixing, extracts were centrifuged at 12,000 g for 10 min and supernatants were subjected to HPLC separation using a C-18 column (15 cm × 4.6 mm, 3-µm particle size). Pyruvate was eluted with 0.8% (wt/vol) metaphosphoric acid and detected electrochemically at 0.600 V.

**Reduced and oxidized glutathione levels.** Kidney homogenates were added with 1.0 M HClO4-2 mM EDTA. After 20 min of centrifugation at 29,000 g, supernatants were filtered through 0.2-µm membranes, diluted to 1:20 (vol/vol) with mobile phase, and subjected to HPLC separation using a LC-18 column (25 cm × 4.6 mm, 5-µm particle size). Reduced (GSH) and oxidized glutathione (GSSG) were eluted with 20 mM sodium phosphate (pH 2.7) and detected electrochemically at 0.800 V (72). The reduction potential of GSSG/2GSH couple (E0GSSG/2GSSH) was calculated, using the Nernst equation, for pH 7.0 and 37°C: E0GSSG/2GSSH = −240 mV - 30.75 mV log[GSH]/[GSSG] (74).

**Immunolabeling.** Renal α-smooth muscle actin (α-SMA) was used as an early marker of the fibrotic process, and collagen III (Col-III) was used to assess the magnitude of extracellular matrix deposition. α-SMA and Col-III were detected with anti-mouse monoclonal α-SMA and Col-III antibodies (BioGenex, San Ramon, CA). Immunolabeling was revealed with Vectastain ABC methodology (Vector Laboratories, Burlingame, CA).

**Histological evaluation.** Histological sections were analyzed with a Nikon E400 light microscope (Nikon Instrument Group, Melville, NY). Image-Pro Plus 4.5.1.29 (Media Cybernetics, Silver Spring, MD) was used to quantify areas. Ten consecutive cortical areas per rat were examined. Vessel wall thickness was calculated by subtracting luminal areas from total areas (excluding the adventitia).

**Statistics.** Values are means ± SE. Statistical analyses included 1-way ANOVA, followed by F-test (Statview SE+Graphics version 1.03; Abacus Concepts, Berkeley, CA), and 2) matrix of correlation coefficients and multiple regression analysis tests (GraphPad Prism; GraphPad Software, San Diego, CA).

**RESULTS**

**Animal outcome.** Initial rat body weight (control: 240.0 ± 21.7 g) and initial and final food consumption (control: 17.8 ± 2.1 and 30.9 ± 6.7 g/day, respectively) showed no significant differences among groups. End-of-study values of biological parameters are shown in Table 1. In the STZ and STZ+Amlo groups, body weight was significantly lower than in STZ+Los and control groups. In the STZ group, systolic BP was higher than in the other groups; in the STZ+Amlo group, systolic BP was 5% lower than in the STZ+Los group (P < 0.05). In the STZ and STZ+Amlo groups, plasma glucose and triglycerides were four times higher than in the control group. In the STZ+Los group, plasma glucose was three times higher than in the control group and 28 and 20% lower than in the STZ and STZ+Amlo groups, respectively (P < 0.05). In the STZ+Los
Losartan protects mitochondria in streptozotocin-diabetes

Table 1. Biological parameters in streptozotocin-diabetic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>STZ</th>
<th>STZ + Los</th>
<th>STZ + Amlo</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>339 ± 10*</td>
<td>493 ± 7</td>
<td>348 ± 7*</td>
<td>524 ± 10</td>
</tr>
<tr>
<td>Two-kidney weight, g</td>
<td>3.69 ± 0.08*</td>
<td>3.08 ± 0.05</td>
<td>3.43 ± 0.04 §§</td>
<td>2.83 ± 0.05</td>
</tr>
<tr>
<td>Two kidney weight/body weight, g/kg</td>
<td>10.92 ± 0.32</td>
<td>6.22 ± 0.1</td>
<td>9.92 ± 0.2%</td>
<td>5.42 ± 0.1</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>138 ± 11</td>
<td>129 ± 1</td>
<td>122 ± 18</td>
<td>125 ± 1</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>468 ± 14*</td>
<td>337 ± 10‡</td>
<td>423 ± 21 §§</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>72.0 ± 3.5*</td>
<td>39.4 ± 2.3</td>
<td>70.2 ± 4.5*</td>
<td>37.8 ± 2.4</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
<td>103.2 ± 5.5*</td>
<td>64.2 ± 4.5‡</td>
<td>97.8 ± 3.11‡</td>
<td>23.6 ± 5.3</td>
</tr>
<tr>
<td>Urinary protein, mg/24 h</td>
<td>108.3 ± 4.0*</td>
<td>21.4 ± 2.6‡</td>
<td>97.0 ± 5.8‡</td>
<td>5.74 ± 1.3</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>0.87 ± 0.02*</td>
<td>1.92 ± 0.03‡</td>
<td>0.92 ± 0.03*</td>
<td>2.62 ± 0.05</td>
</tr>
<tr>
<td>Serum albumin, g/dl</td>
<td>2.55 ± 0.04‡</td>
<td>2.70 ± 0.03§</td>
<td>2.67 ± 0.05</td>
<td>3.17 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 8 rats per group) in streptozotocin-diabetic rats (STZ) treated during 4 mo with losartan (STZ + Los) or amlopidine (STZ + Amlo) and in control rats. *P < 0.001 vs. STZ + Los and control. †P < 0.001 vs. STZ + Los, STZ + Amlo, and control. §P < 0.001 vs. control. ¶P < 0.05 vs. STZ + Los.

Losartan and amlopidine effects on kidney mitochondria. Mitochondrial functioning was evaluated by determining mtMP, H2O2 production, and mtNOS, Mn-SOD, UCP-2, and respiratory chain activities. MtMP drives vital mitochondrial functions, i.e., ATP synthesis, cytosolic calcium pumping to the matrix, mitochondrial protein import, and active metabolite transport (69). H2O2 production accounts quantitatively for most of the mitochondrial superoxide production, which depends mainly on optimal electron transport and O2 reduction (12). MtNOS generates NO, which directly modulates mitochondrial respiration and signaling (9). The antioxidant enzyme Mn-SOD is both labile to oxidative conditions and highly relevant to prevent mtROS damage to other mitochondrial components (12). Finally, UCP-2, by regulating mitochondrial energy handling, can attenuate excessive mitochondrial superoxide production (5). The fact that whole kidney homogenates were used to isolate mitochondria does not allow identification of a particular kidney section, i.e., epithelium, cortex, or medulla, as the major source of mitochondria. However, it seems reasonable to assume that the mitochondria preparation used provides an approximation to tubular cell mitochondria, considering that 1) rat kidneys consist of ~80% medullar and 20% cortical tissue, 2) total medullary tubules amount to ~85% of medullary volume, and 3) of all the cell types that comprise the kidney, medullar tubular cells have the highest number of mitochondria (32).

Figure 1 shows the effects of losartan and amlopidine treatments on several mitochondrial parameters. Mitochondrial protein recovery (5.7 ± 0.53 mg protein/g wet tissue) and citrate synthase activity (220 ± 14 μmol CoA·min⁻¹·mg protein⁻¹) were similar among groups. To better account for differences in mitochondrial yield, mitochondrial parameters were normalized to citrate synthase activity. In the STZ group, basal mtMP was 40, 30, and 46% higher than in the STZ + Los, STZ + Amlo, and control groups, respectively (P < 0.05; Fig. 1A). Malate/glutamate-supported mtMP was similar among groups (654 ± 50 mV·mmol CoA⁻¹·min⁻¹·mg protein⁻¹). In the STZ and STZ + Amlo groups, H2O2 production rate was ~40% higher in the STZ + Los and control groups (P < 0.05; Fig. 1B). In the STZ group, NADH/cytOxRed activity was ~35% lower than in the STZ + Los and STZ + Amlo groups and 72% lower than in the control group (P < 0.05; Fig. 1C). In the STZ + Los and STZ + Amlo groups, NADH/cytOxRed activity was 58 and 45% lower than in the control group, respectively (P < 0.05; Fig. 1C). Cytox activity was similar in the STZ and control groups. In the STZ and STZ + Amlo groups, Cytox activity was approximately 27% and 18% lower (P < 0.05), respectively, than in the STZ + Los and control groups.

In the STZ + Amlo group, mtNOS activity was 13, 27, and 3 times higher than in the STZ, STZ + Los, and control groups, respectively (Fig. 1E). In the STZ + Los and control groups, Mn-SOD activity was approximately three and two times higher than in the STZ and STZ + Amlo groups, respectively (Fig. 1F). UCP-2 content was similar in the STZ + Los and control groups and was lower in the STZ and STZ + Amlo groups (Fig. 1G). In the STZ group, mitochondrial pyruvate content was higher than in the STZ + Los, STZ + Amlo, and control groups (52, 110, and 175%, respectively; P < 0.05; Fig. 1H). Multiple regression analysis (R² = 0.795; P < 0.008) showed that mitochondrial pyruvate content (P < 0.01) and H2O2 production rate (P < 0.02) negatively contributed to electron transfer through complexes I to III, and UCP-2 content negatively contributed to mitochondrial H2O2 production rate (P < 0.04).

Losartan and amlopidine effects on renal glutathione. In the STZ + Los and control groups, GSH content was 3 and 4 times higher, respectively, than in the STZ + Amlo group and 8 and 12 times higher, respectively, than in the STZ group (P < 0.05; Fig. 2). In the STZ + Amlo group, GSH content was three times higher than in the STZ group (P < 0.05). In the STZ + Los and STZ + Amlo groups, renal GSSG content was 51 and 80% lower, respectively (P < 0.05) than in the STZ group. In the control group, GSSG content was four times higher than in the STZ + Amlo group (P < 0.05). In the STZ group, the GSH/ GSSG ratio was ~94% lower than in the STZ + Los, STZ + Amlo, and control groups (P < 0.05). In the STZ group, E₆₅₅₂/₂₆₅₈ was lower (P < 0.05) than in the STZ + Los (~32%), STZ + Amlo (~24%), and control groups (~34%). In the STZ + Amlo group, E₆₅₅₂/₂₆₅₈ was ~10% lower than in the control and STZ + Los groups (P < 0.05; Fig. 2). Multiple regression analysis (R² = 0.795; P < 0.008) showed that...


**Losartan and amlodipine effects on renal histology.**

Table 2 shows the quantification of the results in Fig. 3–5. In the STZ group, glomerular and tubulointerstitial $\alpha$-SMA immunolabeling was markedly higher than in the control group (Fig. 3, A vs. D and E vs. H). Accordingly, Col-III immunolabeling was significantly lower in the STZ than in the control group (Fig. 4, A vs. D and E vs. H). In the STZ+Los and STZ+Amlo groups (Fig. 3, B and C, respectively), glomerular $\alpha$-SMA immunolabeling was significantly lower than in the STZ group, but only STZ+Los $\alpha$-SMA immunolabeling was similar to that of the control group. In the STZ+Los group (Fig. 3F), but not in the STZ+Amlo group (Fig. 3G), tubulointerstitial $\alpha$-SMA immu-

$E_{GSSG/GSH}$ positively contributed to electron transfer through complexes I to III ($P < 0.04$).

**Losartan and amlodipine effects on renal histology.**

Table 2 shows the quantification of the results in Fig. 3–5. In the STZ group, glomerular and tubulointerstitial $\alpha$-SMA immunolabeling was markedly higher than in the control group (Fig. 3, A vs. D and E vs. H). Accordingly, Col-III immunolabeling was significantly lower in the STZ than in the control group (Fig. 4, A vs. D and E vs. H). In the STZ+Los and STZ+Amlo groups (Fig. 3, B and C, respectively), glomerular $\alpha$-SMA immunolabeling was significantly lower than in the STZ group, but only STZ+Los $\alpha$-SMA immunolabeling was similar to that of the control group. In the STZ+Los group (Fig. 3F), but not in the STZ+Amlo group (Fig. 3G), tubulointerstitial $\alpha$-SMA immu-
nolabeling was significantly lower than in the STZ group. Col-III glomerular and tubulointerstitial immunolabeling in the STZ+Los group (Fig. 4, B and F, respectively), but not in the STZ+Amlo group (Fig. 4, C and G, respectively), were significantly lower than in the control group. In the STZ (Fig. 5, top left) and STZ+Amlo groups (Fig. 5, bottom left), the walls of intrarenal arteries and arterioles were significantly thicker than in the control group (Fig. 5, bottom right) and STZ+Los groups (Fig. 5, top right). In the STZ and STZ+Amlo groups, a significant increase in glomerular sizes relative to that in the control and STZ+Los groups was observed (Table 2). Renal enlargement, glomerular hypertrophy, and mesangial expansion, accompanied by proteinuria, support the suitability of the rat streptozotocin-induced diabetes model, since the latter alterations are analogous to those that characterize glomerulopathy in early to moderately advanced diabetic patients. Multiple regression analysis ($R^2 = 0.767; P < 0.002$) showed that mitochondrial pyruvate content positively contributed ($P < 0.01$), and $E_{\text{GSSG}/2\text{GSH}}$ ($P < 0.03$) negatively contributed, to tubulointerstitial α-SMA immunolabelling. Also, mitochondrial H$_2$O$_2$ production rate positively influenced tubulointerstitial Col-III immunolabelling ($R^2 = 0.748; P < 0.009$).

**DISCUSSION**

In hyperglycemic streptozotocin-diabetic rats, losartan and amlopidine were equally potent antihypertensive agents, but only losartan was able to 1) improve mitochondrial functioning parameters, 2) prevent the increase of mtROS generation, 3) attenuate kidney antioxidant derangement, and 4) prevent structural and functional kidney decay. These results indicate that the renal and mitochondrial benefits of AT$_1$ receptor blockade occurred in the presence of hyperglycemia and exceeded those attributable to BP changes.

Streptozotocin-induced diabetes is an experimental model in which pancreatic β-cell destruction is accompanied by direct damage to other tissues containing cells that express glucose transport protein-2 (GLUT-2), e.g., kidney and liver (24, 54, 78, 79). Thus kidney injury associated with streptozotocin treatment could result from the combined action of streptozotocin-initiated damage and diabetes-mediated damage. Based on evidence showing that the cytotoxic effect of streptozotocin is largely mediated by ROS (27, 29, 35, 77) and that administration of losartan or amlopidine improves antioxidant defenses in several organs, including the kidney (15–17, 66), pretreatment of the rats with losartan or amlopidine was intended to optimize antioxidant defenses to minimize direct streptozotocin-induced oxidative damage to the kidney. However, antioxidant defenses were not assessed at the time of streptozotocin injection, and we can only assume that the protection furnished by losartan or amlopidine was against diabetes-related kidney injury.

The lower body weights in the STZ and STZ+Amlo groups, relative to the control and STZ+Los groups, may have resulted from the insulin deficit elicited by streptozotocin. The fact that serum albumin was lower in the STZ, STZ+Los, and STZ+Amlo groups relative to the controls, whereas food

**Table 2. Histological and immunohistochemical parameters in kidneys of streptozotocin-diabetic rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>STZ</th>
<th>STZ+Los</th>
<th>STZ+Amlo</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular α-SMA, %</td>
<td>13.0±1.3†‡</td>
<td>2.26±0.68</td>
<td>6.90±0.58‡</td>
<td>1.49±0.45</td>
</tr>
<tr>
<td>Glomerular Col-III, %</td>
<td>18.1±0.7‡‡</td>
<td>10.3±1.5</td>
<td>14.1±0.7§</td>
<td>8.6±0.5</td>
</tr>
<tr>
<td>Tubulointerstitial α-SMA, %</td>
<td>3.16±0.53‡‡</td>
<td>0.34±0.05</td>
<td>1.90±0.28‡‡</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>Tubulointerstitial Col-III, %</td>
<td>3.78±0.49§‡</td>
<td>1.46±0.23</td>
<td>4.12±0.09‡</td>
<td>1.90±0.24</td>
</tr>
<tr>
<td>Intrarenal artery wall thickness, $\times 10^{-3}$ μm$^2$</td>
<td>9.7±0.3‡‡</td>
<td>7.4±0.6</td>
<td>11.6±0.8‡‡</td>
<td>5.5±0.4</td>
</tr>
<tr>
<td>Intrarenal arteriole wall thickness, $\times 10^{-3}$ μm$^2$</td>
<td>1.4±0.1‡‡</td>
<td>1.1±0.1</td>
<td>1.3±0.1‡‡</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Glomerular area, $\times 10^{-3}$ μm$^2$</td>
<td>21.2±0.6‡</td>
<td>17.9±1.1</td>
<td>19.6±1.5§</td>
<td>13.1±1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 8$ rats per group) in STZ, STZ+Los, STZ+Amlo, and control rats. Percentages indicate areas relative to total glomerular or tubulointerstitial areas. *$P < 0.01$ vs. STZ+Los. †$P < 0.05$ vs. STZ+Los. ‡$P < 0.01$ vs. control. §$P < 0.05$ vs. control.

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consumption showed no differences among groups, suggests that the lowering of albuminemia resulted from diabetes-related decreases in albumin synthesis and/or increased urinary loss. Importantly, in the three streptozotocin-treated groups, albuminemia remained above values reported for severe malnutrition (31). In the STZ/H11001 Los group, body weight preservation within control values may result from the ability of AT1 receptor blockade to improve glucose transport in peripheral tissues (11, 34). In agreement with other studies (8, 49, 61), losartan prevented diabetes-related changes in lipid metabolism.

Mitochondrial functioning was evaluated by determining mtMP, H2O2 production, and mtNOS, Mn-SOD, UCP-2, and respiratory chain activities. The observed increments in basal mtMP and H2O2 production and the decreases in NADH/Cytc-OxRed and CytcOx, mtNOS, and Mn-SOD activities revealed kidney mitochondrial dysfunction, confirming previous results in different streptozotocin-diabetic rat organs (26, 39, 44, 53, 73). Losartan and amlodipine partially prevented NADH/cytc-OxRed activity changes, but only losartan preserved CytcOx activity. In the absence of ADP, mitochondrial superoxide and H2O2 productions are directly related to mtMP. A threshold value for mtMP seems to exist, above which superoxide formation steeply increases (42).

The augmentation of basal mtMP observed in the STZ group may be a consequence of overactivation of mitochondrial electron transport, secondary to an overabundance of tricarboxylic acid cycle-derived respiratory substrates that resulted from an excessive availability of pyruvate, as was described in endothelial cells (59). Facilitative glucose transport is augmented in diabetic kidneys as a result of changes in both gene expression and cellular localization of glucose transporters (50). Glycolytic capacity is minimal in kidney proximal tubules, which depend exclusively on mitochondria for ATP production. Medullary nephron segments are highly glycolytic, resulting in high lactate generation. However, medullary anaerobic glycolysis does not seem to provide enough ATP to fully support sodium transport. Notably, the medullary thick ascending limb exhibits the highest mitochondrial density along the mammalian nephron (32). These last two observations suggest that medullary thick ascending limb mitochondria may furnish the ATP that anaerobic glycolysis cannot provide. In this context, lactate, once considered merely a by-product of glycolysis, can be taken up by mitochondria and converted into

Fig. 3. α-Smooth muscle actin (α-SMA) immunolabeling in kidneys from STZ, STZ+Los, STZ+Amlo, and control rats. Photomicrographs (×400) show differences regarding α-SMA immunolabeling in the glomerular area of STZ (A) STZ+Los (B), STZ+Amlo (C), and control rats (D) and in the tubulointerstitium of STZ (E), STZ+Los (F), STZ+Amlo (G), and control rats (H).

Fig. 4. Collagen III (Col-III) immunolabeling in kidneys from STZ, STZ+Los, STZ+Amlo, and control rats. Photomicrographs (×400) show differences regarding Col-III immunolabeling in the glomerular area of STZ (A) STZ+Los (B), STZ+Amlo (C), and control rats (D) and in the tubulointerstitium of STZ (E), STZ+Los (F), STZ+Amlo (G), and control rats (H).
pyruvate, which is fully oxidized by the respiratory chain (7). Evidence supporting the existence of this so-called “lactate shuttle” in skeletal muscle, liver, heart, and brain (30) prompted us to consider that a lactate shuttle might exist in the kidney. Thus, in hyperglycemic rats, excess renal glucose reabsorption might result in increased medullar lactate production, increased mitochondrial lactate uptake, and, finally, higher mitochondrial pyruvate content. Supporting this hypothesis, in STZ kidneys, mitochondrial pyruvate content was higher than in controls, and both losartan and amlodipine corrected this augmentation, notwithstanding that rats remained hyperglycemic. This increase in mitochondrial pyruvate content in the STZ group may contribute to the higher basal mtMP and H$_2$O$_2$ production rate. This is not at variance with amlodipine being able to lower pyruvate content while failing to lower H$_2$O$_2$ production rate, since amlodipine cannot correct ANG II-induced mtROS production. The conversion of pyruvate into a tricarboxylic acid cycle substrate, which is normally catalyzed by mitochondrial pyruvate dehydrogenase, can also be catalyzed by pyruvate carboxylase. This is relevant considering that 1) in diabetes, pyruvate dehydrogenase complex activity is depressed in several tissues, including the kidney (36); and 2) the amount of pyruvate entering into the tricarboxylic acid cycle by the pyruvate carboxylase route was two times higher than that entering through the pyruvate dehydrogenase route in the liver of streptozotocin-diabetic rats (14).

Drawing from current evidence and supported by the present results, two mechanisms can be envisioned that may contribute to increased kidney mtROS production in diabetes. The first one, described above as a result of the lactate shuttle, involves hyperglycemia-induced stimulation of mtROS formation through substrate regulation of the respiratory chain (59). The second mechanism involves ANG II and is conceptually supported by 1) ANG II-mediated stimulation of mtROS production and mtMP reduction in rat cultured vascular smooth muscle cells and aorta in vivo (41), 2) ANG II-induced mtROS generation in myocytes isolated from dilated cardiomyopathy human hearts (58), and 3) diabetes-related increases in ANG II generation (71) and systemic and local RAS overstimulation (48) in both animals and humans (1, 55, 57, 82). Thus hyperglycemia and excess ANG II production/responsiveness may be accountable for increased mtROS in diabetes. A causative link among RAS activation, ANG II, and mitochondrial function alterations (10, 43, 76) is in line with the observed divergent effects of losartan and amlodipine.

Losartan, but not amlodipine, prevented UCP-2 content and Mn-SOD activity decline in streptozotocin-induced diabetes. This is in agreement with results observed in spontaneously hypertensive rats (19). The increased expression of UCP-2 and Mn-SOD activity associated with both lower mitochondrial H$_2$O$_2$ production rates and kidney structure preservation are consistent with findings in endothelial cells (59, 62). Kidney neuronal NOS activity declines in diabetes models with no insulin administration (40, 81). Kidney mtNOS seems to be a gene variant of neuronal NOS (23). These evidences are compatible with the present results showing that mtNOS activity is much lower in the STZ groups, with the exception of the STZ+Amlo group. mtNOS seems to modulate kidney mitochondrial function efficiency (20); therefore, losartan’s failure to preserve mtNOS activity suggests that in this diabetes model, the influence of mitochondrial nitric oxide on kidney mitochondrial functioning is of relative importance.

The calculated $E_{GSSG/GSH}$ is an index of the antioxidant capacity of the glutathione pool that depends on both the GSSG/GSH ratio and GSH absolute concentration. In the STZ and STZ+Amlo groups, the antioxidant capacity of GSSG/2GSH couple was significantly diminished, i.e., the glutathione pool was relatively more oxidized than in the control and STZ+Los groups. However, losartan treatment effectively blunted the derangement of the kidney glutathione pool, thus supporting a protective effect of AT$_1$ receptor blockers against diabetes-induced oxidant stress (2). The relative contribution
of mtROS to increased tissue oxidation cannot be assessed using the present results. It is important to emphasize that NAD(P)H-oxidase activation is mainly responsible for ANG II-induced nonmitochondrial superoxide generation (28). An effect on mitochondria mediated by an increase in NAD(P)H-oxidase-dependent ROS generation cannot be ruled out and could coexist with NAD(P)H-oxidase-independent increases of mtROS. The existence of intracellular ANG II receptors (4, 21, 22, 64) supports the speculation that the antioxidant effect of losartan was mediated, at least in part, by inhibiting the direct interaction of ANG II with mitochondria.

As previously reported (17, 46, 47), streptozotocin-induced diabetes was associated with undesirable structural and functional kidney changes. Kidney damage was indicated by the increases of kidney size and glomerular areas, which are known to accompany hyperfiltration in diabetes (63). Multiple regression analysis showed that mitochondrial pyruvate content and H$_2$O$_2$ production rate positively contributed, and UCP-2 content negatively contributed, to renal interstitial fibrosis, supporting a role for mtROS in kidney damage associated with streptozotocin-induced diabetes.

Given the type of study design used, it is difficult to conclude whether the mitochondrial changes are causal to the observed structural and functional changes or, rather, consequences of other beneficial effects of losartan, such as restoration of $\beta$-adrenergic-dependent activation of glucose transporters (67) or modulation of PKC-dependent glucose uptake (52). In addition, it is important to indicate that an effect of losartan mediated by the induced decrease in hyperglycemia cannot be disregarded. However, the facts that 1) losartan lowered blood glucose levels to 337 mg/dl, a value that defines marked diabetes in rats, and 2) values closer to normal glyce- mia were unable to restore normal function in rat kidney, liver, and heart mitochondria in streptozotocin-mediated diabetes (3, 39) suggest that it is unlikely that the observed mitochondrial actions of losartan may be ascribed to the observed modest decrease of hyperglycemia. Since glucose delivery to the kidney is not impaired in diabetes, mitochondrial decay in this organ seems to be related to a lack of metabolic fuel and, on the contrary, possibly results from excess glucose. Thus the observed alterations in kidney mitochondria appear unrelated to the impaired nutrient availability in other tissues.

The present treatment design, i.e., administering losartan oramlodipine before the onset of diabetes, has the limitation of not representing a genuine therapeutic approach. However, the protective mitochondrial effects displayed by losartan may provide a potential mechanism to explain the reduction of type 2 diabetes incidence observed in hypertensive patients treated with RAS inhibitors (ACE inhibitors or AT$_1$ receptor blockers) compared with other antihypertensive agents (75).

In summary, in streptozotocin-induced diabetes, kidney structural and functional protection by AT$_1$ blockade would be mediated, at least partly, by attenuation of mitochondrial functioning impairment. These benefits were independent of both systolic BP reduction and blood glucose levels and point to ANG II as a relevant contributor to mitochondrial dysfunction in diabetes.

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