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 1 diabetes, we treated 8-week-old male Sprague-Dawley rats with a single streptozotocin injection (65 mg/kg; STZ group), streptozotocin and drinking water containing either losartan (30 mg·kg⁻¹·day⁻¹; STZ+Los group) or amloidipine (3 mg·kg⁻¹·day⁻¹; STZ+Amlo group), or saline (intraperitoneally) and pure water (control group). Four-month-long losartan or amloidipine 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+Amlo 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+Amlo groups, mitochondrial H₂O₂ production rate was higher and uncoupling protein-2 content, cytochrome c oxidase activity, and renalin oxidized glutathione levels were lower than in STZ+Los and control groups. Mitochondrial nitric oxide synthase activity was higher in STZ+Amlo compared with the other groups. Mitochondrial nitric oxide synthase 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-induced diabetes independently of blood pressure and glycemia.

Reactive oxygen species (ROS) are continuous by-products of normal aerobic metabolism that can oxidize nucleic acids, lipids, and proteins, leading to the modification and/or loss of their biological functions. Mitochondria are relevant cellular sources of ROS and, consequently, are themselves major oxidation targets. This eventually leads to mitochondrial dysfunction, i.e., a defective capacity to generate ATP accompanied by increased ROS generation. Experimental and clinical evidence indicates that mitochondrial dysfunction could be a contributing factor to the pathogenesis and complications of diabetes mellitus. Renal function is highly dependent on mitochondrial energy; hence, the kidneys are especially susceptible to mitochondrial decay. Recent data suggest that the kidneys are main targets of mitochondrial impairment at the onset of, as well as throughout, streptozotocin-induced diabetes. The effects of insulin treatment on mitochondria are controversial. Several studies have shown that insulin is unable to restore normal mitochondrial function in the kidney, as well as in other organs, whereas other results indicate that insulin attenuates kidney, brain, and cardiac mitochondria alterations in streptozotocin-diabetic rats. Consistent with a marked increase in oxidative damage to mitochondria, an elevation in the mitochondrial production of ROS (mtROS), accompanied by changes in mitochondrial lipid oxidation and mitochondrial antioxidant defense levels, was observed in the pancreas, kidney, brain, and liver from streptozotocin-diabetic rats.

Chronic inhibition of the renin-angiotensin system (RAS) can delay the onset and progression of nephropathy in diabetic patients, as well as development of type 2 diabetes in patients with hypertension and high cardiovascular risk, and the benefits of RAS inhibition in diabetes seem to be unrelated to the lowering of arterial blood pressure (BP), since other antihypertensive treatments do not exhibit the same protective effects as RAS inhibition. This has led to recommendations for the use of RAS blockers as first-line drug therapy for kidney protection in diabetic patients, even in the absence of hypertension. The support of clinical data, the cellular mechanisms underlying the protective effects of this therapeutic strategy are poorly understood. Previously, we showed that treatment with an angiotensin I-converting enzyme (ACE) inhibitor (enalapril) attenuated both the oxidation of mitochondrial components and the structural changes in the kidneys of streptozotocin-diabetic rats. In addition, chronic administration of enalapril or losartan, an angiotensin II (ANG II) type 1 (AT₁) receptor blocker, protected kidney mitochondria from the effects of aging. A more recent study showed that the expression of genes related to mitochondrial energy production were upregulated in

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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 Alemán 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-week-old, male Sprague-Dawley rats (Laboratory of Experimental Medicine, Hospital Alemán) 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. Stycotic 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.

Three months after streptozotocin administration, the rats were anesthetized with pentobarbital (40 mg/kg ip), blood was drawn from the thoracic aorta, and a systemic perfusion was performed with 0.9% (wt/vol) NaCl before kidney excision. One kidney was used for mitochondrial isolation, and the other was fixed in phosphate-buffered 10% (vol/vol) formaldehyde (pH 7.2) and embedded in paraffin. Sections (3 μm) were cut and stained with hematoxylin-eosin and Masson’s trichrome. Unless otherwise stated, all reagents were from Sigma Chemical (St. Louis, MO). All the biochemical determinations were conducted by operators that were blinded to the study groups.

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 components 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 μM rhodamine-123, and 0.2 mg mitochondrial protein in the absence [basal mitochondrial membrane potential (mtpMP)] or presence of glutamate (10 mM) and malate (5 mM) at 28°C. mtpMP 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 (CytcOx), 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 0.85% (wt/vol) metaphosphoric acid and detected electrochemically at 0.60 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/GSH couple (EₕGSSG/GSH) was calculated, using the Nernst equation, for pH 7.0 and 37°C: EₕGSSG/GSH = −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) one-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 ± 1.2 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
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 ± 0.25</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>138 ± 1</td>
<td>129 ± 1</td>
<td>122 ± 1</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 ± 31.1</td>
<td>23.6 ± 3.5</td>
</tr>
<tr>
<td>Urinary protein, mg/24 h</td>
<td>108.3 ± 4.0</td>
<td>21.4 ± 2.6</td>
<td>90.0 ± 5.8</td>
<td>5.7 ± 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 amlodipine (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. STZ + Los. group, plasma triglycerides were three times higher than in the control group and ~35% lower than in the STZ and STZ + Amlo groups. In the STZ and STZ + Amlo groups, plasma cholesterol was between 80 and 90% higher, and creatinine clearance between 55 and 75% lower, than in the STZ + Los and control groups (P < 0.05). In the STZ and STZ + Amlo groups, urinary protein was ~5 and 18 times higher than in the STZ + Los and control groups, respectively. Serum albumin was ~20% higher in the control group relative to the other groups (P < 0.05).

Losartan and amlodipine effects on kidney mitochondria. Mitochondrial functioning was evaluated by determining mtMP, H2O2 production, and mtNOS, Mn-SOD, UCP-2, and to the other groups (Los and control groups, respectively) higher than in the STZ group (P < 0.05; Fig. 1B). In the STZ group, NADH/cytcOxRed 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/cytcOxRed activity was 58 and 45% lower than in the control group, respectively (P < 0.05; Fig. 1C). CytcOx activity was similar in the STZ and control groups. In the STZ and STZ + Amlo groups, CytcOx 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 amlodipine 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, the GSH/GSSG ratio was ~94% lower than in the STZ + Los, STZ + Amlo, and control groups (P < 0.05). In the STZ group, E(GSSG/GSH) was lower (P < 0.05) than in the STZ + Los (~32%), STZ + Amlo (~24%), and control groups (~34%). In the STZ + Amlo group, E(GSSG/GSH) 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
$E_{\text{GSSG}/\text{GSH}}$ positively contributed to electron transfer through complexes I to III ($P < 0.04$).

Losartan and amiodipine effects on renal histology. Table 2 shows the quantification of the results in Fig. 3–5. In the STZ group, glomerular and tubulointerstitial α-SMA immunolabeling was markedly higher than in the control group (Fig. 3, A vs. D and E vs. H). Accordingly, Col-III immunolabeling was higher 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 α-SMA immunolabeling was significantly lower than in the STZ group, but only STZ+Los α-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 α-SMA immu-
Histological and immunohistochemical parameters in kidneys of streptozotocin-diabetic rats

Los group (Fig. 4, Col-III glomerular and tubulointerstitial immunolabeling in the
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 sig-
nificantly lower than in the STZ group and similar to that 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 thinner than in the control (Fig. 5,
bottom right) and STZ+Los groups (Fig. 5, top right). In the

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>2.6±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, ×10^{-3} μm²</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, ×10^{-3} μm²</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, ×10^{-3} μm²</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.
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+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/cyt-cOxRed 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 ...
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 H₂O₂ production rate. This is not at variance with amlodipine being able to lower pyruvate content while failing to lower H₂O₂ 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 H₂O₂ 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/H11001Amlo group. mtNOS seems to modulate kidney 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 $E_{\text{GSSG/GSH}}$ 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 AT₁ 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 β-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 glycemia 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 unrelated 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 or amlodipine 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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