Azilsartan Improves Glycemic Status and Reduces Kidney Damage in Zucker Diabetic Fatty Rats

Md. Abdul Hye Khan,¹ Jan Neckář,¹,² Jasmine Haines,¹ and John D. Imig¹,³

BACKGROUND
Azilsartan medoxomil (AZL-M), an angiotensin II receptor blocker, demonstrates antihypertensive and organ protective effects in hypertension. We investigated the efficacy of AZL-M to ameliorate metabolic syndrome and kidney damage associated with type 2 diabetes using Zucker diabetic fatty (ZDF) rats.

METHODS
ZDF rats were treated with vehicle or AZL-M for 8 weeks. Zucker diabetic lean (ZDL) rats were used as controls. Urine and plasma samples were collected for biochemical analysis, and kidney tissues were used for histopathological and immunohistopathological examination at the end of the 8-week protocol.

RESULTS
ZDF rats were diabetic with hyperglycemia and impaired glucose tolerance, and AZL-M ameliorated the diabetic phenotype. ZDF rats were hypertensive compared with ZDL rats (181±6 vs. 129±7 mm Hg), and AZL-M decreased blood pressure in ZDF rats (116±7 mm Hg). In ZDF rats, there was marked renal damage with elevated proteinuria, albuminuria, nephrinuria, 2–4-fold higher tubular cast formation, and glomerular injury compared with ZDL rats. AZL-M treatment reduced renal damage in ZDF rats. ZDF rats demonstrated renal inflammation and oxidative stress with elevated urinary monocyte chemoattractant protein 1 expression, renal infiltration of macrophages, and elevated kidney malondialdehyde levels. AZL-M reduced oxidative stress and inflammation in ZDF rats.

CONCLUSIONS
Overall, we demonstrate that AZL-M attenuates kidney damage in type 2 diabetes. We further demonstrate that anti-inflammatory and antioxidative activities of AZL-M contribute to its kidney protective action.

Keywords: azilsartan medoxomil; blood pressure; hypertension; inflammation; kidney injury; oxidative stress; type 2 diabetes.

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Type 2 diabetes is characterized by markedly elevated insulin resistance and chronic hyperglycemia.¹ The prevalence of this common disorder has increased alarmingly in the past decade because of increased obesity and sedentary lifestyle. Indeed, currently >346 million people worldwide are type 2 diabetic, and this disease is becoming a serious medical as well as socioeconomic issue.²,³

Patients with type 2 diabetes are at high risk of developing a number of complications that lead to end-organ damage, including damage to the kidney. The main problem with type 2 diabetes management is serious micro- and macrovascular complications that contribute to diabetic nephropathy.⁴,⁵ The incidence of type 2 diabetes is rapidly increasing, along with an escalating prevalence of kidney diseases resulting from diabetic complications.⁶,⁷ Indeed, diabetic nephropathy accounts for approximately one-third of all cases of end-stage renal disease worldwide.⁸,⁹

The renin-angiotensin system (RAS) is an endocrine system predominantly responsible for the regulation of blood pressure. An important component of the RAS, angiotensin II has many actions beyond its effects on blood pressure, and elevated angiotensin II levels can cause tissue damage in susceptible organs.¹⁰ As a result, therapeutic inhibition of the RAS has been used as a method for reducing target organ damage beyond the expected effects to reduce blood pressure. Recent evidence suggests that the RAS and factors functionally linked to the RAS are activated in type 2 diabetes.¹¹ Several clinical studies have demonstrated that treatment with angiotensin type 1 (AT₁) receptor blockers (ARBs) leads to an improvement in renal and cardiac outcomes in patients with type 2 diabetes.¹²,¹³

With this background, in this study we investigated the beneficial effects of a novel ARB, azilsartan medoxomil (AZL-M), in a rat model of type 2 diabetes. We used Zucker diabetic fatty (ZDF) rats that have a mutation in the gene coding the leptin receptor (fa/fa), which results in obesity, insulin resistance, reduced glucose tolerance, hypertension, and renal and cardiovascular disease. This model develops a phenotype very similar to humans with type 2 diabetes, including the existence of diabetic complications.³,⁵ The

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findings of this study demonstrate that AZL-M markedly reduced hyperglycemia, improved glucose tolerance, preserved endothelial function, and provided kidney protection in type 2 diabetic ZDF rats.

**METHODS**

**Chemicals**

AZL-M (also known as TAK-491) is chemically known as (5-methyl-2-oxo-1,3-dioxol-4-yl) methyl 2-ethoxy-1-[(2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl]-1H-benimidazole-7-carboxylate monopotassium salt. Takeda Pharmaceuticals (Deerfield, IL) provided AZL-M. All chemicals used in this study were purchased from Sigma Aldrich (St Louis, MO) unless otherwise mentioned.

**Animal groups**

The Medical College of Wisconsin Institutional Animal Care and Use Committee, according to the National Institutes of Health Guidelines for Care and Use of Laboratory Animals, approved all animal studies. Studies were conducted using male (aged 6–8 weeks) obese ZDF (fa/fa) and Zucker diabetic lean (ZDL (+/?)) rats obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in the Biomedical Resource Center at Medical College of Wisconsin with a 12-hour light–12-hour dark cycle and free access to tap water and rodent chow. ZDL rats (n = 6) were used as the control group. ZDF rats were divided into two groups (n = 6), and the vehicle-treated ZDF group received vehicle, whereas the second group of ZDF received AZL-M (3 mg/d) orally once a day as a bolus dose for 8 weeks. The dose of AZL-M was recommended by Takeda Pharmaceuticals based on their studies. Before the start of the experimental protocol, rats were trained for 5 days for tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA). All rats were weighed, and systolic blood pressure was measured by tail-cuff plethysmography at baseline and then at 4 and 8 weeks of the treatment protocol.

**Glucose tolerance test**

Intraperitoneal glucose tolerance testing was carried out at the end of the 8-week treatment protocol in rats that were fasted overnight and injected with glucose (2g/kg). Blood samples were collected from the tail vein before and at different time points after glucose injection. The tail vein blood glucose levels were measured using a LifeScan glucometer (Miltipas, CA). Glucose area under the curve was calculated as previously described.14

**Urine and plasma biochemical analysis**

At the end of the 8-week experimental period, urine was collected from rats housed in metabolic cages for 24 hours. The following day rats were anesthetized with isoflurane, and blood samples were collected from the abdominal aorta. Serum and urinary biochemical analysis was done using colorimetric and enzyme-linked immunosorbent assays. Triglyceride, cholesterol, protein, and creatinine assay kits were from Cayman (Ann Arbor, MI); albumin and nephrin assay kits were from Exocell (Philadelphia, PA); and monocyte chemoattractant protein 1 (MCP-1) assay kit was from BD Biosciences (San Jose, CA). To determine the kidney tissue malondialdehyde (MDA) level, the rat kidney was homogenized with buffer containing 1.5% potassium chloride to obtain a 1:10 (w/v) whole kidney homogenate. MDA was measured using colorimetric method after reaction with thiobarbituric acid. Kidney tissue MDA was measured in the kidney using a commercially available kit (Cayman Chemical).

**Vascular reactivity study**

Second-order mesenteric resistance arteries were collected at the end of the experimental protocol to determine vascular function. Arterial segments were mounted between 2 glass cannulas in a pressure myograph system (Danish Myo Technology model 111P; DMT, Aarhus, Denmark). Mesenteric arteries were oxygenated in 95% oxygen/5% carbon dioxide Krebs physiological salt solution at pH 7.4 and 37 °C. Under no-flow conditions, the pressure within the vessel was increased in 10–mm Hg increments from 20 to 65 mm Hg. The blood vessel was then equilibrated at 65 mm Hg for 30 minutes, and control lumen diameter was calculated as the mean diameter during the last minute of the 30-minute equilibration period. Mesenteric resistance arteries were constricted with the thromboxane mimetic U-46619, and diameter of the constricted artery was calculated as the mean during the last minute of the 15-minute period. After U46619 constriction, vessel diameter responses to graded doses of ace

**Histopathological analysis**

The kidney was excised and immersion-fixed in 10% neutral buffered formalin and paraffin embedded. The embedded kidney section was cut into 4-μm slices for use in histology. Formalin-fixed paraffin-embedded tissue slices were deparaffinized, rehydrated, and stained with Periodic Acid-Schiff. Kidney glomerulosclerosis and mesangial matrix expansion were blindly scored from kidney sections to determine glomerular damage using the following numeric scale: 0 = no damage, +1 = very mild, +2 = mild, +3 = moderate, and +4 = severe. Two observers in a blinded fashion conducted histological analysis at a magnification of ×200 using Nikon NIS Elements Software (Nikon Instruments, Melville, NY). The kidney tubules containing proteinaceous casts were determined at magnification of ×100 using Nikon NIS Elements Software, and the percentage of area positive for proteinaceous cast was calculated from the mean of 8 cortical and 5 medullary fields for each animal.
Immunohistopathological analysis

Kidney sections were embedded and cut into 4-µm slices for use in immunohistochemistry. Formalin-fixed paraffin-embedded kidney slices were deparaffinized, rehydrated, and subjected to immunohistochemistry. Kidney sections were immunostained with anti-CD68 (1:100; Serotec, Raleigh, NC) to determine macrophage/monocyte infiltration. Biotinylated rat antimouse secondary antibody (1:200) was used for development with avidin-biotinylated HRP complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) followed by counterstaining with hematoxylin and mounted for image capturing. Stained sections were visualized by light microscopy at ×400 magnification, and digital images of the stained kidney were taken for analysis using Nikon NIS Elements Software. Macrophage/monocyte infiltration was determined by point counting CD68-positive cells by 2 experienced blinded reviewers. The number of positive cells per picture was divided by the metric area of the image to obtain the number of positive cells per millimeter squared.

Statistical analysis

Data are expressed as mean ± SE and were analyzed using 1-way analysis of variance followed by Tukey’s post hoc test for multiple group comparisons using Prizm version 4.0 software by GraphPad Software (La Jolla, CA). Statistical significance was assumed at P < 0.05.

RESULTS

AZL-M on the metabolic profile of ZDF rats

At the end of the experimental period, ZDF rats were diabetic (fasting blood glucose = 287 ± 45 mg/dl) and ZDL rats were normoglycemic (fasting blood glucose = 99 ± 6 mg/dl). AZL-M lowered fasting blood glucose in ZDF rats (200 ± 46 mg/dl). ZDF rats also had impaired glucose tolerance with a higher glucose area under the curve compared with ZDL rats at the end of the 8-week protocol period, and AZL-M treatment improved glucose tolerance in ZDF rats (Figure 1). At the same time, ZDF rats (433 ± 14 g) had higher body weight compared with ZDL rats (329 ± 16 g), and AZL-M treatment did not affect the body weight of ZDF rats (447 ± 13 g). ZDF rats also had dyslipidemia with elevated levels of cholesterol, triglyceride, and free fatty acid compared with ZDL rats, and none of these parameters were altered by AZL-M treatment (Table 1).

AZL-M reduces blood pressure and improves vascular function in ZDF rats

As shown in Figure 2a, at the end of the 8-week experimental protocol, ZDF rats were hypertensive compared with ZDL rats, and AZL-M treatment blunted the increase in blood pressure of ZDF rats. In this study, we determined the mesenteric resistance artery response to acetylcholine in vehicle- and AZL-M–treated ZDF and ZDL rats. Among the experimental groups, there were no differences in the

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**Figure 1.** Azilsartan medoxomil (AZL-M) improved glucose tolerance in Zucker diabetic fatty (ZDF) rat. Blood glucose levels at different time points of intraperitoneal glucose tolerance test (a) and glucose area under the curve (b) in Zucker diabetic lean (ZDL) and ZDF rats after 8 weeks of vehicle or AZL-M treatment. Values are mean ± SEM. n = 6 rats per group; *P < 0.05 vs. ZDL + Vehicle; **#P < 0.05 vs. ZDF + Vehicle.
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Table 1. Lipid profile of Zucker diabetic lean (ZDL) and Zucker diabetic fatty (ZDF) rats in different experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ZDL + vehicle</th>
<th>ZDF + vehicle</th>
<th>ZDF + AZL-M</th>
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<tr>
<td>Cholesterol, mM</td>
<td>3.0±0.3</td>
<td>4.0±0.6</td>
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<td>Triglyceride, mg/dl</td>
<td>476±23</td>
<td>718±71*</td>
<td>796±62</td>
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<tr>
<td>Free fatty acid, µM</td>
<td>300±56</td>
<td>2.170±148*</td>
<td>1.922±80</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. n = 6 rats per group. Abbreviation: AZL-M, azilsartan medoxomil. *P < 0.05 vs. ZDL + vehicle.

AZL-M reduces renal inflammation and oxidative stress in ZDF rats

As depicted in Figure 3, ZDF rats exhibited marked renal oxidative stress with 50% higher level of MDA in the kidney compared with ZDL rats. AZL-M reduced renal oxidative stress by markedly reducing kidney MDA level in ZDF rats. ZDF rats also exhibited marked inflammation with 40% higher urinary excretion of MCP-1 and 3 times higher infiltration of macrophage in the kidney compared with ZDL rats. AZL-M treatment reduced renal inflammation in ZDF rats with 30%–60% reductions in urinary excretion of MCP-1 and kidney macrophage infiltration.

AZL-M reduces renal damage in ZDF Rats

Marked renal injury with 5–10-fold elevated proteinuria and albuminuria was observed in ZDF rats compared with ZDL rats. AZL-M markedly reduced renal injury in ZDF rats by reducing proteinuria and albuminuria (Figure 4a, b). Renal injury in ZDF rats was further characterized by marked tubular cast formation compared with ZDL rats, and renal cortical and medullary cast area was reduced by AZL-M (Figure 4c–e). In addition to tubular cast formation, ZDF rats had elevated nephropria along with marked glomerular injury compared with ZDL rats, and AZL-M reduced these renal injury indices (Figure 5a–c).

Figure 2. Azilsartan medoxomil (AZL-M) lowered blood pressure and improved endothelial function in Zucker diabetic fatty (ZDF) rat. Systolic blood pressure at the start of study and on week 4 and 8 of AZL-M treatment (a) and concentration-dependent mesenteric resistance artery dilatation to acetylcholine after 8 weeks of vehicle or AZL-M treatment (b) in Zucker diabetic lean (ZDL) and ZDF rats. Values are mean ± SEM. n = 6 rats per group; *P < 0.05 vs. ZDL + Vehicle; #P < 0.05 vs. ZDF + Vehicle.
Figure 3. Azilsartan medoxomil (AZL-M) reduced renal inflammation in Zucker diabetic fatty (ZDF) rat. (a) Monocyte chemoattractant protein-1 (MCP-1) excretion in Zucker diabetic lean (ZDL) and ZDF rats after 8 weeks of vehicle or AZL-M treatment. (b) Renal macrophage infiltration and representative photomicrographs showing macrophage infiltration in the kidney (×200; white arrows) of ZDL and ZDF rats after 8 weeks of vehicle or AZL-M treatment. Values are mean ± SEM. n = 6 rats per group. *P < 0.05 vs. ZDL + vehicle; #P < 0.05 vs. ZDF + vehicle.

DISCUSSION

Type 2 diabetes is a debilitating chronic disease that is increasing at an alarming rate to an epidemic level. In type 2 diabetes, chronic hyperglycemia promotes the development of micro- and macrovascular disease. Approximately 30%-40% of all patients develop microvascular disease and diabetic nephropathy. Macrovascular diseases in diabetic individuals affect the coronary, carotid, and peripheral arteries and thereby increase the risk of cardiovascular disease.

One of the leading causes of mortality in diabetic patients is diabetic nephropathy, which occurs in 20%-40% of patients. Diabetic nephropathy is one of the most serious complications of diabetes, and its incidence is increasing dramatically worldwide. Characteristic features of diabetic nephropathy include persistent albuminuria, a progressive decline in renal function, glomerular mesangial expansion followed by glomerulosclerosis, and interstitial fibrosis. Although no efficient therapy is yet available to treat diabetic nephropathy, the treatment strategy involves reduction of risk factors such as hypertension to reduce cardiovascular morbidity and mortality and also to reduce the development of kidney disease. Indeed, hypertension accelerates the progression of diabetic nephropathy, whereas lowering blood pressure reduces renal damage. Clinical studies have shown that treatment with ARBs leads to an improvement in renal outcomes in patients with type 2 diabetes.

Consequently, in this study we investigated the effects of a newly developed ARB, AZL-M, in a rat model of type 2 diabetes. We used ZDF rats to investigate the effects of AZL-M on diabetes and associated kidney damage. In this study, ZDF rats developed marked hyperglycemia, dyslipidemia, hypertension, and kidney damage. These observations were in agreement with earlier reports on the characteristic features of type 2 diabetic ZDF rats. Indeed, the ZDF rat is a recognized model of metabolic syndrome associated with development and progression of renal damage, obesity, insulin resistance, hyperglycemia, and hyperlipidemia. Because of the presence of these pathophysiological features of type 2 diabetes, the ZDF rat is an attractive experimental model to investigate end-organ damage, particularly kidney damage, which is similar to that observed in human metabolic syndrome and type 2 diabetes.

Interestingly, in this study we demonstrate that AZL-M treatment reduced kidney injury in ZDF rats, with marked reductions in proteinuria, albuminuria, nephrinuria, tubular...
cast formation, and glomerular injury. In accordance with our findings, clinical studies have shown that treatment with ARBs leads to an improvement in renal outcomes in patients with type 2 diabetes. Apart from clinical studies, the renal protective effects of ARBs in diabetes have also been demonstrated in animal models of diabetes. Similar to our findings with AZL-M in ZDF rats, another ARB, olmesartan, suppressed the progression of diabetic nephropathy in ZDF rats. This same study also demonstrated that the olmesartan-mediated amelioration of renal disease was not accompanied by the lowering of plasma glucose, suggesting that the renoprotective effect of olmesartan on diabetic nephropathy was not dependent on glucose metabolism. However, in our study we demonstrate that the kidney protective effect of AZL-M was associated with marked reduction of fasting blood glucose and improvement of glucose tolerance in ZDF rats. A similar observation has been made for irbesartan, which normalized hyperglycemia and delayed the onset of mortality associated with diabetic nephropathy in obese Zucker rats. Our findings with AZL-M, along with the earlier observations on irbesartan, support the view that some ARBs possess pleiotropic effects beyond blood pressure reduction. These pleiotropic effects of some ARBs on glucose metabolism have been attributed to their ability to enhance peroxisome proliferator-activated receptor-γ (PPARγ) activity. For instance, irbesartan, an ARB that acts as a PPAR-γ modulator, provides beneficial effects in cardiometabolic syndrome. A similar PPAR-γ modulator activity has also been reported for AZL-M, and such activity could be responsible for the antidiabetic effects of AZL-M in ZDF rats.

The blood pressure lowering effect of AZL-M, in addition to its action on blood glucose, could contribute to the reduction in renal damage. Indeed, in diabetic patients hypertension is considered a prominent risk factor for end-organ failure. It has been suggested that hypertension accelerates the progression of diabetic nephropathy, whereas lowering of blood pressure reduces renal damage. Accordingly, the current strategy for treating patients with...
Azilsartan reduces kidney damage in Zucker diabetic fatty rat

Diabetes recommends the use of well-tolerated antihypertensive agents. In accordance with this notion, we demonstrate a marked antihypertensive effect of AZL-M in ZDF rats. AZL-M also improved vascular function in ZDF rats. The antihypertensive effect and the ability to preserve vascular endothelial function are factors that contributed to the kidney-protective effect of AZL-M in ZDF rats.

The kidney-protective effect of RAS inhibitors, including ARBs, results not just from ARBs’ actions on the hemodynamic effects of angiotensin II but also from their action on nonhemodynamic effects of angiotensin II. Several earlier studies demonstrated that angiotensin II induced proteinuria, renal dysfunction, inflammation, and oxidative stress, leading to progressive kidney damage. These same events can also happen in diabetes, as an activated RAS is strongly implicated in the pathophysiology of diabetes. Accordingly, in this study with diabetic ZDF rats, we demonstrate marked renal oxidative stress with elevated kidney MDA content. Similar to our finding, earlier reports have demonstrated that ZDF rats develop oxidative stress with increased renal lipid peroxidation, decreased catalase, and superoxide dismutase activities in the kidney. We also demonstrate that AZL-M treatment reduced oxidative stress in the ZDF rats. Earlier studies demonstrated that such antioxidative effects of ARBs are associated with end-organ protection. For instance, the ARB valsartan reduced oxidative stress by reducing the level of MDA and by increasing the activity of superoxide dismutase in high glucose–treated glomerular epithelial cells. Apart from oxidative stress, ZDF rats also demonstrate marked inflammation with elevated urinary MCP-1 excretion and macrophage infiltration in the kidney. AZL-M treatment reduced renal inflammation by reducing urinary MCP-1 and kidney macrophage infiltration in ZDF rats. In an earlier study with ZDF rats, a similar increase in MCP-1 and kidney infiltration of macrophages was reported in ZDF rats, and the ARB olmesartan markedly reduced these inflammatory markers along with marked kidney protection. We found that AZL-M caused stronger effect in reducing urinary MCP-1 than renal macrophage infiltration in ZDF rats, and this suggests that AZL-M has additional anti-inflammatory effects independent of its ability to reduce renal macrophage infiltration.

As discussed in the preceding paragraph, our data with AZL-M, along with that of several earlier studies, indicates an important role of inflammation and oxidative stress in diabetic renal injury and demonstrates the effectiveness of ARBs in reducing these conditions. It is important to note that these antioxidative and anti-inflammatory effects of ARBs could be related to their ability to inhibit angiotensin II signaling. In addition to the ability to regulate angiotensin II signaling, ARBs, including AZL-M, have PPARγ

Figure 5. Azilsartan medoxomil (AZL-M) reduced nephrineuria and glomerular injury in Zucker diabetic fatty (ZDF) rats. (a) Nephrineuria in Zucker diabetic lean (ZDL) and ZDF rats after 8 weeks of vehicle or AZL-M treatment. (b) Representative photomicrographs of Periodic Acid–Schiff staining (×200) depicting glomerular injury with mesangial expansion (arrows) and other changes related to glomerular sclerosis in the kidney in ZDL and ZDF rats after 8 weeks of vehicle or AZL-M treatment. (c) Semiquantitative scoring of glomerular injury in ZDL and ZDF rats after 8 weeks of vehicle or AZL-M treatment. Values are mean ± SEM. n = 6 rats per group. *P < 0.05 vs. ZDL + vehicle; #P < 0.05 vs. ZDF + vehicle.
agonistic activity that could contribute to reducing inflammation, oxidative stress, organ damage. In summary, our data demonstrate that ZDF rats, a model of type 2 diabetes, develop renal damage similar to human diabetic nephropathy. We also demonstrate that a new ARB, AZL-M, markedly reduced diabetic kidney damage in ZDF rats and such protection was accompanied by improved glycemic status, improved vascular functions, reduced blood pressure, and reduced oxidative stress and inflammation.

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DISCLOSURE

The authors declared no conflict of interest.

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