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Kumamoto University
Direct Renin Inhibition Ameliorates Cardiovascular Complications and Pancreatic Injury in Obese and Type 2 Diabetic Mice

(直接レニン抑制は肥満および2型糖尿病マウスにおける心血管合併症、膵臓障害を抑制する)

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1. Summary

**Background and Purpose** It is still unclear about the effect of direct renin inhibition on type 2 diabetes and its complications. The present study was undertaken to examine the efficacy of aliskiren, a direct renin inhibitor, on cardiovascular injuries, glucose intolerance and pancreatic injury in obese and type 2 diabetic mice.

**Methods** Aliskiren (3, 6, 12 and 25 mg kg\(^{-1}\) day\(^{-1}\)) or hydralazine (80 mg kg\(^{-1}\) day\(^{-1}\)) were administrated to obese and type 2 diabetic db/db mice for 6 weeks. The protective effects were compared among these groups.

**Results** Sub-pressor (3 mg kg\(^{-1}\) day\(^{-1}\)) and hypotensive (6, 12 and 25 mg kg\(^{-1}\) day\(^{-1}\)) doses of aliskiren significantly attenuated cardiac fibrosis, macrophage infiltration and coronary remodelling, and improved vascular endothelial function in db/db mice. These protective effects of aliskiren were attributed to the attenuation of cardiac p22\(^{phox}\)-related NADPH oxidase-induced superoxide and the restoration of downregulation of vascular endothelial nitric oxide synthase. Aliskiren, at the maximum dose (25 mg kg\(^{-1}\) day\(^{-1}\)) partially reduced glucose intolerance in db/db mice. Furthermore, the maximum dose of aliskiren significantly attenuated the decreases of pancreatic islet insulin content and beta cell mass, and prevented pancreatic islet fibrosis in db/db mice. These beneficial effects of aliskiren on pancreatic injury were associated with the reduction of 8-hydroxy-2'-deoxyguanosine-positive cells and Nox2 expression in pancreatic islets.

**Conclusions** This study provides the first evidence that direct renin inhibition with aliskiren protects against cardiovascular complications and pancreatic injury through the attenuation of oxidative stress in type 2 diabetic animal model. Furthermore, our data also indicate the
potential beneficial effect of aliskiren on diabetes itself. Therefore, aliskiren may be a promising therapeutic agent for type 2 diabetes and its cardiovascular complications.
2. Thesis

① Related Paper


② Other Papers


3. Acknowledgements

My deepest gratitude goes foremost to Professor Shokei Kim-Mitsuyama, my supervisor, for his constant encouragement and guidance. He has walked me through all the stages of the research and the writing of this thesis.
4. Abbreviations

ARB Angiotensin receptor blocker
ACE Angiotensin converting enzyme
AVOID Aliskiren in the evaluation of proteinuria in diabetes
ALTITUDE Aliskiren trial in type 2 diabetes using cardiorenal disease endpoints
AUC Areas under the curve
CVD Cardiovascular disease
DHE Dihydroethidium
eNOS Endothelial nitric oxide synthase
HOMA-IR Homeostasis model assessment of insulin resistance
IGTT Intraperitoneal glucose tolerance test
IITT Intraperitoneal insulin tolerance test
NADPH Reduce nicotinamide adenine dinucleotide phosphate
NOX2 NADPH oxidase 2
8-OHdG 8-Hydroxy-2'-deoxyguanosine
RAS Renin–angiotensin system
SNAP S-Nitroso-N-acetylpenicillamine
5. Background and Purpose of the Research

5-1. Diabetes, Diabetic Cardiovascular Complications and Renin-Angiotensin System Blockade

Diabetes mellitus affects more than 170 million people, globally.\(^1\) With the increasing prevalence of diabetes worldwide, this figure is predicted to rise from 4.0%, in 1995, to 5.4% by the year 2025, by which time it is estimated that there will be 300 million adults with diabetes.\(^2\) Type 2 diabetes is the predominant form and an independent risk factor for cardiovascular disease (CVD).\(^3\) Up to 80% of patients with diabetes die prematurely from cardiovascular complications. Thus, the growing number of people with type 2 diabetes will lead to greater morbidity and mortality from CVD, and preventing or delaying diabetes in subjects at high risk, or subjects with diabetes, are essential management strategies.\(^3\)

The management of patients with diabetes improved significantly over the past three decades, and the outcomes of those patients markedly improved simultaneously with better diagnosis and treatment of diabetic complications. However, current interventions can only delay rather than prevent the eventual development of diabetic complications, notwithstanding the fact that numerous clinical studies have shown that antihypertensive therapy with conventional renin-angiotensin system (RAS) blockade in individuals with diabetes significantly reduced the risks of cardiovascular events.\(^2\) Following the wide utility of RAS blockers in individuals with diabetes, the beneficial effects of RAS blockade was not only found on diabetic cardiovascular complications, but also on diabetes itself.\(^4-6\) Results from the recent large randomized controlled trials of cardiovascular drugs suggest that RAS
blockade also reduced the incidence of new onset diabetes in addition to their proven cardiovascular benefits. The mechanisms underlying this beneficial effect of RAS blockade remain to be established. There are some suggested protective mechanism of RAS blockade in diabetes onset and progression. They include the effects that increase insulin secretion, preserve the islet cell morphology, and decrease oxidative stress in the pancreatic islets. These beneficial effects may improve insulin sensitivity, and eventually prevent new-onset diabetes.

5-2. Animal Model of Type 2 Diabetes: db/db mouse

db/db mouse is the best characterized and most intensively investigated mouse model of human type 2 diabetes. db/db mouse was identified, initially, in 1966 in Jackson Labs as an obese mouse that was hyperphagic. The diabetic gene (db) is transmitted as an autosomal recessive trait. The db gene encodes for a G-to-T point mutation of the leptin receptor, leading to abnormal splicing and defective signaling of the adipocyte-derived hormone leptin. A deficiency of leptin signaling in the hypothalamus leads to persistent hyperphagia and obesity with consequently high leptin and insulin levels. The recognition of diabetes was initially recognized in mice from the C57BLKS/J strain, and the natural history of diabetes has been described primarily in this strain. Initially, increased insulin secretion might compensate for the peripheral insulin resistance. Hyperglycemia develops when enhanced insulin secretion ceases to compensate for insulin resistance. Subsequently, insulin levels decrease rapidly as beta-cells exhibit a severe secretory defect, resulting in a progressive increase in hyperglycemia. Therefore, the metabolic features of db/db mice are
similar to the pathogenesis of type 2 diabetes in humans. \textit{db/db} mouse exhibits characteristics of a diabetic cardiomyopathy at 10–14 weeks of age, with decreased contractile performance and altered cardiac metabolism.\textsuperscript{13, 14} It is, thus, regarded as a useful model utilized to investigate the cardiovascular injuries in type 2 diabetes.\textsuperscript{15, 16}

\textit{5-3. Direct Renin Inhibitor: Aliskiren}

\textit{5-3-1. Direct Renin Inhibitor}

Blocking RAS at its origin by inhibiting renin has been expected for over 50 years. The first synthetic renin inhibitor was pepstatin, which was effective at inhibiting renin activity and reducing blood pressure in animals and people,\textsuperscript{17} though, it has to be administered parenterally. In the last 30 years, several drug companies developed renin inhibitors that could be administered orally, for example, enalkiren, CGP38560A, remikiren, zankiren. However, they had a bioavailability of less than 2\% with a short half-life, and weak blood pressure-lowering activity.\textsuperscript{17} Crystal structure analyses of renin inhibitor complexes and computational molecular modeling were later utilized to design selective nonpeptide direct renin inhibitor without the extended peptide-like backbone of previous inhibitors and with improved pharmacokinetic properties.\textsuperscript{18} This led to the discovery of aliskiren, a highly potent and selective inhibitor of human renin in vitro, and in vivo.\textsuperscript{18} Aliskiren is the first orally active inhibitor of renin to be approved for clinical utilization as an antihypertensive agent, and represents the first in a novel class of renin inhibitors with the potential for treatment of hypertensive cardiovascular diseases.
5-3-2. Aliskiren and Diabetes

In comparison with numerous clinical trials evaluating the effect of aliskiren in hypertension, few clinical trials are designed to investigate the effect of aliskiren in diabetes. Aliskiren in the evaluation of proteinuria in diabetes (AVOID) is a multinational, randomized and double-blind study investigating the effect of aliskiren (150 mg daily for 3 months, followed by an increase in dosage to 300 mg daily for a further 3 months) or placebo, in addition to losartan on reduction in the ratio of albumin to creatinine.19 This trial was completed with the result displaying that treatment with 300 mg of aliskiren daily, as compared with placebo, reduced the mean urinary albumin-to-creatinine ratio by 20% (95% confidence interval, 9 to 30; P<0.001), with a reduction of 50% or greater in 24.7% of the patients who received aliskiren, as compared with 12.5% of those who received placebo (P<0.001).19 There is another double-blind, multicentre trial assessing the antihypertensive efficacy and safety of the combination of aliskiren and ramipril, an angiotensin converting enzyme (ACE) inhibitor, in patients with diabetes and hypertension.20 The investigators concluded that the combination of aliskiren with ramipril provided a greater reduction in mean sitting diastolic blood pressure than either drug alone in patients with diabetes and hypertension.20 Aliskiren trial in type 2 diabetes using cardiorenal disease endpoints (ALTITUDE) is to determine whether aliskiren, 300 mg administered once daily, reduces cardiovascular and renal morbidity and mortality compared with placebo when added to conventional treatment (including ACE inhibitor or angiotensin receptor blocker (ARB)).21 With a primary endpoint of time to diabetic complications, 4 years later, ALTITUDE would assist with the determination of whether or not aliskiren with conventional treatment reduces
cardiovascular and renal morbidity and mortality in high-risk patients with type 2 diabetes.

Experimental research of aliskiren in animal models provided further detailed efficacy of this new drug for the potential treatment of diabetes and its complications. Aliskiren was proven to be capable of: (1) ameliorate cardiac hypertrophy and improve diastolic filling in rat transgenic for human renin and angiotensinogen genes; (2) restore nitric oxide bioavailability and reduce atherosclerosis in hyperlipidemic rabbit; (3) reduce myocardial remodeling, nicotinamide adenine dinucleotide phosphate (NADPH) oxidative activity and 3-nitrotyrosine in transgenic TG(mRen-2)27 rat overexpression mouse renin; (4) attenuate insulin resistance and pancreatic remodeling in transgenic TG(mRen-2)27 rat; and (5) improve skeletal muscle glucose transport in transgenic TG(mRen-2)27 rat. In streptozotocin-induced type 1 diabetic animal models, aliskiren was proven to be capable of preventing albuminuria, suppressing renal transforming growth factor-β and collagen I in a diabetic TG(mRen-2)27 rat, and suppressing cardiomyocyte apoptosis, superoxide and interstitial fibrosis in a diabetic Sprague Dawley rat. However, there is no experimental research investigating the significance of direct renin inhibition with aliskiren in treatment of type 2 diabetes and its complications.

5.4. Purpose of the Study

Having regard for the different physiopathology of type 1 and type 2 diabetes and the different pharmacological property of aliskiren from other conventional RAS blockers, in this study, we examined the efficacy of aliskiren on cardiovascular injuries, glucose intolerance and pancreatic injury in type 2 diabetic animal models of db/db mice, and provided the first
experimental evidence reinforcing the potential treatment of type 2 diabetes and its complications with aliskiren.
6. Methods

6-1. Protocol of The Experiment

All procedures were in accordance with institutional guidelines for animal research. Male 
\( \text{db/db} \) mice (C57BLKS/J-lepr\(^{db}/lepr^{db}\)) and male non-diabetic \( \text{db/m} \) mice 
(C57BLKS/J-lepr\(^{db/\text{a}}\)) as control were purchased from Charles River Laboratories Japan 
(Yokohama, Japan). All mice were housed in an animal facility with a 12-hour 
light–darkness cycle and were given standard chow and water ad libitum. Aliskiren was a 
kind gift from Novartis (Basel, Switzerland). Hydralazine was purchased from 
Sigma-Aldrich (St. Louis, MO, USA).

The \( \text{db/db} \) mice at 6 weeks of age already displayed significant obesity and 
hyperglycaemia. Six-week-old \( \text{db/db} \) mice were assigned to six groups, and were given (1) 
vehicle, (2) aliskiren (3 mg kg\(^{-1}\) day\(^{-1}\)), (3) aliskiren (6 mg kg\(^{-1}\) day\(^{-1}\)), (4) aliskiren (12 mg 
kg\(^{-1}\) day\(^{-1}\)), (5) aliskiren (25 mg kg\(^{-1}\) day\(^{-1}\)) or (6) hydralazine (80 mg kg\(^{-1}\) day\(^{-1}\)). Vehicle 
(saline) or aliskiren was s.c. infused into mice by way of ALZET micro-osmotic pumps 
(Durect, Cupertino, CA, USA). Hydralazine in drinking water was given to mice. The 
drug treatments were carried out for 6 weeks (from 6 to 12 weeks of age), and body weight 
was periodically measured. BP was measured by tail-cuff plethysmography (BP-98A; 
Softron, Tokyo, Japan) \(^{29}\) before, and 2, 4 and 6 weeks after the start of drug treatment. 
After 5 weeks of drug treatment (at 11 weeks of age), an intraperitoneal glucose tolerance test 
(IGTT) and an intraperitoneal insulin tolerance test (IITT) were performed to evaluate the 
effect of each treatment on glucose tolerance and insulin sensitivity, respectively. After 6
weeks of drug treatment, 12-week-old mice were anaesthetised with ether, and the heart, aorta and pancreas were rapidly excised to perform biochemical and histological examinations, as described below in detail.

6-2. Insulin and Glucose Tolerance Testing

With regard to the IGTT, mice were deprived of food for 6 hrs. and then given glucose (1 mg/g; Wako, Osaka, Japan) by i.p. injection. Caudal vein bloods were taken from mice before, and 30, 60 and 120 minutes after the injection. Blood glucose concentrations were measured by a portable glucose meter (Sanwa Kagaku Kenkyusho, Nagoya, Japan).

With regard to the IITT, the mice deprived of food for 6 hrs. were i.p. injected with insulin (2 U/kg; Eli Lilly Japan, Kobe, Japan), and caudal vein bloods were collected before, and 20, 40 and 60 minutes after injection to measure blood glucose concentrations.

6-3. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)

HOMA-IR, a simple assessment of insulin resistance, was calculated by using the following formula: \( \frac{\text{fasting plasma glucose (mmol/l)} \times \text{insulin (pmol/l)}}{405} \). Plasma insulin levels were quantified by using a commercial ELISA kit (Morinaga, Tokyo, Japan).

6-4. Organ Chamber Experiments

Thoracic aortas from mice were carefully cut into 5 mm rings to preserve the endothelium, and then mounted in organ baths filled with modified Tyrode buffer (pH 7.4; NaCl 121 mmol/l, KCl 5.9 mmol/l, CaCl\(_2\) 2.5 mmol/l, MgCl\(_2\) 1.2 mmol/l, NaH\(_2\)PO\(_4\) 1.2 mmol/l, NaHCO\(_3\) 15.5 mmol/l, and d-glucose 11.5 mmol/l) aerated with 95% O\(_2\) and 5% CO\(_2\) at 37°C.
The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. A resting tension of 1 g was maintained throughout the experiment. Vessel rings were precontracted with 1-phenylephrine ($10^{-7}$ mol/l). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine ($10^{-9}$ mol/l to $10^{-4}$ mol/l) or $S$-nitroso-$N$-acetylpenicillamine (SNAP) ($10^{-9}$ mol/l to $10^{-4}$ mol/l) to obtain cumulative concentration–response curves.

6-5. Dihydroethidium (DHE) staining and NADPH Oxidase Activity

Hearts and aortas removed from mice were immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Tokyo, Japan). Sections were thawed at room temperature, rehydrated with 1x PBS, and incubated for 30 minutes in the dark with the DHE (Sigma; 5 µmol/L). DHE fluorescence was visualized by fluorescent microscopy using an excitation wavelength of 520 to 540 nm and a rhodamine emission filter. Detector and laser settings were kept constant across all samples within individual experiments, and control and experimental samples were always processed in parallel. DHE fluorescence of cardiac sections was quantified by Image-Pro Plus v6 analysis software (Media Cybernetics, Bethesda, MD, USA). The mean fluorescence was quantified and expressed relative to values obtained from control mice.

For the measurement of cardiac NADPH oxidase activity, cardiac tissues were homogenised with an Ultraturrax T8, and centrifuged, and the NADPH oxidase activity of the resulting supernatant fraction was measured by lucigenin chemiluminescence in the presence of 10 µmol/l NADPH and 10 µmol/l lucigenin as electron acceptor, as described previously.30
Protein concentrations were measured by the Bradford method.

6-6. Histological and Immunohistochemical Analysis

Hearts and pancreases were fixed in 4% (wt/vol.) paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with Sirius Red F3BA (0.5% wt/vol. in saturated aqueous picric acid; Aldrich Chemical Company, St. Louis, MO, USA) for the measurement of collagen volume fraction. For cardiac sections, coronary arterial thickness, perivascular fibrosis and interstitial fibrosis were quantified, as described previously. For pancreatic sections, fibrosis in and around the islets was quantified, as described previously.9

With regard to CD68 immunohistochemistry, frozen cardiac sections were incubated overnight with rat anti-mouse primary antibody (×500; Serotec, Raleigh, NC, USA) followed by anti-rat secondary antibody (BioSource, Camarillo, CA, USA), as described previously.29 With regard to p22phox immunohistochemistry, cardiac paraffin sections (0.01 mol/l citric acid pH 6.0-antigen unmasked) were incubated overnight with rabbit anti-p22phox primary antibody (×300; Santa Cruz Biotechnology, Santa Cruz, CA, USA). With regard to insulin immunohistochemistry, pancreatic sections (1 mmol/l EDTA pH 8.0-antigen unmasked) were incubated overnight with rabbit anti-insulin primary antibody (×100; Santa Cruz Biotechnology). Both were followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (ready-to-use; Dako North American, Carpinteria, CA, USA) for 30 minutes. With regard to NADPH oxidase 2 (NOX2) immunohistochemistry, pancreatic paraffin sections (0.01 mol/l citric acid pH 6.0-antigen unmasked) were incubated overnight with the mouse anti-NOX2 primary antibody (×100;
BD Transduction Laboratories, San Jose, CA, USA), followed by incubation with the HRP-conjugated anti-mouse secondary antibody (ready-to-use; Dako North American) for 30 minutes. A Vector MOM kit (Vector Laboratories, Burlingame, CA, USA) was used for 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunohistochemistry. Pancreatic paraffin sections (0.01 mol/l citric acid pH 2.0-antigen unmasked) were incubated with the mouse anti-human 8-OHdG primary antibody (10 μg/ml; Japan Institute for the Control of Aging, Shizuoka, Japan). The subsequent steps were according to the instructions of the kit. The above-mentioned reactions were finally visualised with 3,3’-diaminobenzidine (DakoCytomation, Carpinteria, CA, USA) and counterstained with haematoxylin.

For endothelial nitric oxide synthase (eNOS) immunofluorescence, frozen aortic sections were incubated overnight with the mouse anti-eNOS primary antibody (×200; BD Transduction Laboratories) followed by incubation with the FITC-conjugated anti-mouse IgG secondary antibody (×200; Invitrogen, Tokyo, Japan) for 1 hour.

Quantitative analysis of cardiac CD68- and p22phox-positive cells was done by counting the cell numbers in sections. Cells positive for 8-OHdG staining were identified by the presence of a dark brown nuclear stain and were expressed as a percentage of the total number of islet cells. Intensity of fluorescence for eNOS and the intensity of peroxidase staining for insulin and NOX2 were quantified with Image-Pro Plus v6 analysis software.

Beta cell mass was determined on insulin-stained sections and was estimated by the following formula: beta cell mass (g)=[area of islets/area of the whole pancreatic area]×pancreas weight, as described previously.32
6-7. SDS-PAGE, Preparation of Cardiac and Aortic Protein Extracts and Western Blot Analysis

The detailed method was previously described. Briefly, after left ventricular or aortic protein extracts were subjected to SDS-PAGE and electric transfer to the polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. Antibodies used were as follows: anti-phospho-eNOS (×2,000; BD Transduction Laboratories), anti-p22phox (×1,000; Santa Cruz Biotechnology) and GAPDH (×5,000; Santa Cruz Biotechnology).

6-8. Quantitative Real-Time PCR

Total RNA was extracted from the heart, pancreas and kidney, according to the manufacturer’s instructions. One microgram sample of RNA was reverse-transcribed to first-strand cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Tokyo, Japan), according to the manufacturer’s recommended protocol. A Thermal Cycler Dice Real Time System (Takara Bio, Shiga, Japan) was used for two-step RT-PCR. cDNA was amplified using SYBR Premix Ex TagTM with specific oligonucleotide primers for target sequences of Nox2 (also known as Cybb), Nox4, p22phox (also known as Cyba), p47phox (also known as Ncf1), p67phox (also known as Ncf2), renin and β-actin (see Table 3). Amplification conditions included 10 s at 95°C, runs for 40 cycles at 95°C for 5 s, and 60°C for 30 s, and then dissociation for 15 s at 95°C and 30 s at 60°C on the Thermal Cycler Dice Real Time System. Specificity of the SYBR Premix Ex TagTM assays was confirmed by melting point analysis. Each threshold cycle’s (Ct) value was normalised to β-actin Ct value and a control
sample. The delta–delta Cₜ method, according to the instruction of Thermal Cycler Dice Real Time System, was used for relative quantification.

6-9. Statistical Analysis

Statistical analysis was performed by using SPSS 11.5.0 (SPSS, Chicago, IL, USA). Data are presented as means±SEM. Statistical significance was determined with one-way ANOVA followed by least square differences analysis. Differences were considered statistically significant at a value of $p<0.05$. 
7. Results

7-1. Body Weight, Organ Weights, Blood Pressure, Blood Glucose and Plasma Insulin Concentrations

As illustrated in Table 1, body weight and the weights of the pancreas, liver and visceral fat were significantly greater in 12-week-old *db/db* mice than in *db/m* mice of the same age, and these variables were insignificantly affected by 6 weeks of treatment with hydralazine nor aliskiren.

As illustrated in Fig. 1, aliskiren, at a dose of 3 mg kg\(^{-1}\) day\(^{-1}\), did not significantly reduce BP of *db/db* mice throughout the treatment. Aliskiren, at doses of 6, 12 and 25 mg kg\(^{-1}\) day\(^{-1}\), significantly reduced BP of *db/db* mice in a dose-dependent manner throughout the treatment. Hydralazine treatment reduced BP of *db/db* mice to a degree comparable to aliskiren at 25 mg kg\(^{-1}\) day\(^{-1}\).

Table 2 illustrated blood glucose and plasma insulin levels before, and 5 weeks of after the start of the drug treatment. *db/db* mice at 6 weeks of age (before the start of drug treatment) exhibited hyperglycaemia and hyperinsulinaemia.

7-2. Effect of Aliskiren On Cardiac Injuries In *db/db* Mice

Figures 2 and 3 indicate that cardiac interstitial macrophage infiltration, interstitial fibrosis, coronary arterial thickening and peri-coronary arterial fibrosis were significantly greater in *db/db* mice than in *db/m* mice. Aliskiren, with all doses examined, significantly ameliorated these cardiac injuries in *db/db* mice. The magnitude of amelioration of cardiac macrophage infiltration (Fig. 2a) and coronary arterial thickening (Fig. 3a) was similar between the
minimum dose (3 mg kg\(^{-1}\) day\(^{-1}\)) and the maximum dose (25 mg kg\(^{-1}\) day\(^{-1}\)) of aliskiren. Cardiac interstitial (Fig. 2b) and perivascular (Fig. 3b) fibrosis were attenuated by aliskiren in a dose-dependent manner. In contrast, hydralazine treatment did not ameliorate these cardiac injuries in \(db/db\) mice.

7-3. Effect of Aliskiren On Cardiac Superoxide, NADPH Oxidase Activity and The Expression of NADPH Oxidase Subunit \(p22^{phox}\) In \(db/db\) Mice

As illustrated in Fig. 4, cardiac superoxide levels \((p<0.01)\) and NADPH oxidase activity \((p<0.01)\) were significantly greater in \(db/db\) mice than in \(db/m\) mice. All doses of aliskiren significantly reduced cardiac superoxide and NADPH oxidase activity in \(db/db\) mice.

As illustrated in Fig. 5, cardiac mRNA expressions of \(Nox2\), \(Nox4\), \(p47^{phox}\) and \(p67^{phox}\) were comparable between \(db/db\) mice and \(db/m\) mice, and these mRNA expressions of \(db/db\) mice were insignificantly affected by any drug treatment. Contrarily, as illustrated in Fig. 6a, cardiac mRNA expression of \(p22^{phox}\) was significantly greater in \(db/db\) mice than in \(db/m\) mice \((p<0.05)\). \(p22^{phox}\) mRNA expression in \(db/db\) mouse was significantly reduced by aliskiren with all doses. Cardiac \(p22^{phox}\) protein levels in \(db/db\) mice were also greater than in \(db/m\) mice \((p<0.05)\), and were significantly reduced by the minimum (3 mg kg\(^{-1}\) day\(^{-1}\)) and the maximum (25 mg kg\(^{-1}\) day\(^{-1}\)) doses of aliskiren \((p<0.01)\) (Fig. 6b). Furthermore, the number of cardiac \(p22^{phox}\) protein-positive cells was significantly greater in \(db/db\) mice than in \(db/m\) mice \((p<0.05)\), and aliskiren significantly reduced the number of cardiac \(p22^{phox}\)-positive cells in \(db/db\) mice \((p<0.01)\) (Fig. 6c).

By contrast, hydralazine treatment failed to ameliorate any of these variables in \(db/db\)
mice.

7-4. Effect of Aliskiren On Vascular Endothelial Function, Aortic Superoxide and Vascular Endothelial Production of eNOS In db/db Mice

As illustrated in Fig. 7a, compared with db/m mice, vascular endothelium-dependent relaxation by acetylcholine was significantly impaired in db/db mice \((p<0.01)\). Aliskiren, with all doses examined, significantly preserved vascular endothelial function in db/db mice, however, hydralazine failed to restore vascular endothelial function in db/db mice.

As illustrated in Fig. 7b, vascular endothelium-independent relaxation by SNAP was unimpaired in db/db mice and was unaffected by any drug treatment.

As illustrated in Fig. 8a, aortic superoxide was significantly greater in db/db mice than in db/m mice \((p<0.01)\). Aliskiren, with all doses examined, significantly reduced aortic superoxide in db/db mice \((p<0.01)\), however, Hydralazine treatment unaffected aortic superoxide in db/db mice.

As illustrated in Fig. 8b, the vascular endothelial production of eNOS was significantly reduced in db/db mice in comparison with db/m mice \((p<0.01)\). Collectively, the maximum dose \(25 \text{ mg kg}^{-1} \text{ day}^{-1}\) and the minimum dose \(3 \text{ mg kg}^{-1} \text{ day}^{-1}\) of aliskiren significantly restored vascular eNOS production in db/db mice. Hydralazine unaffected eNOS production in db/db mice. The western blot analysis of aortic phospho-eNOS (Fig. 8c) displayed that phospho-eNOS levels in db/db mice were attenuated in comparison with db/m mice, and were increased by aliskiren treatment, however, not by hydralazine treatment.
7-5. Effect of Aliskiren On Glucose Tolerance, Pancreatic Islet Insulin Content, Beta Cell Mass and Islet Fibrosis In db/db Mice

IGTTs (Fig. 9a) indicated that aliskiren at the maximum dose (25 mg kg\(^{-1}\) day\(^{-1}\)) significantly decreased blood glucose concentrations 30 minutes after the glucose injection. There was a trend for aliskiren to reduce the value of area under the curve (AUC) in the IGTT, however, the difference was statistically insignificant (Fig. 9b). All reduced doses of aliskiren (3, 6 and 12 mg kg\(^{-1}\) day\(^{-1}\)) failed to improve glucose tolerance in db/db mice (data not provided). Hydralazine treatment did not improve glucose tolerance. IITTs (Fig. 9c) evidenced that plasma glucose levels 20 minutes after the insulin injection were reduced in aliskiren-treated (at 25 mg kg\(^{-1}\) day\(^{-1}\)) db/db mice than in vehicle-treated db/db mice. The increased HOMA-IR in db/db mice was significantly attenuated by aliskiren treatment \((p<0.05)\), however, not by hydralazine treatment (Fig. 9d).

As illustrated in Fig. 10a,b, pancreatic insulin content \((p<0.01)\) and beta cell mass \((p<0.05)\) were less in db/db mice than in db/m mice. Aliskiren at 25 mg kg\(^{-1}\) day\(^{-1}\) prevented the decrease in islet insulin content \((p<0.05)\) and beta cell mass \((p<0.05)\) in db/db mice. As illustrated in Fig. 10c, islet fibrosis in db/db mice was much greater than in db/m mice \((p<0.01)\), and was significantly reduced by aliskiren (25 mg kg\(^{-1}\) day\(^{-1}\)). However, reduced doses of aliskiren (3, 6 and 12 mg kg\(^{-1}\) day\(^{-1}\)) insignificantly improved these pancreatic changes (data not provided). Hydralazine treatment did not improve these pancreatic changes in db/db mice.
7-6. Effect of Aliskiren on 8-OHdG-positive Cells and Expression of Nox2 In Pancreatic Islets In db/db Mice

As illustrated in Fig. 11a, the ratio of 8-OHdG-positive cells was significantly greater in db/db mice than in db/m mice (p<0.01), and in db/db mice was significantly reduced by aliskiren (25 mg kg⁻¹ day⁻¹) (p<0.05). As illustrated in Fig. 11b,c, mRNA expression and protein levels of Nox2 were significantly greater in db/db mice than in db/m mice. Aliskiren (25 mg kg⁻¹ day⁻¹) significantly and similarly reduced the increased mRNA expression and protein levels of Nox2 in db/db mice, while hydralazine did not reduce them. Reduced doses (3, 6 and 12 mg kg⁻¹ day⁻¹) of aliskiren failed to improve these variables.

7-7. Effect of Each Treatment On Renal Renin mRNA Expression In db/db Mice

As illustrated in Fig. 12, treatment of db/db mice with aliskiren significantly increased renal renin mRNA expression in a dose-dependent manner.
8. Discussion

We provided the first experimental evidences, in this work, that direct renin inhibition with aliskiren beneficially affects cardiovascular injuries, glucose intolerance and pancreatic injury in obese type 2 diabetic mice, thereby highlighting aliskiren as a promising therapeutic agent for type 2 diabetes and its complications.

The effect of renin inhibitor on diabetes and its complications is still unclear. Recent reports indicate that a hypotensive dose of aliskiren attenuates insulin resistance, pancreatic oxidative stress and remodelling, and improves skeletal muscle glucose transport in the transgenic TG(mRen-2)27 rat, which overexpresses the mouse renin transgene and is hypertensive. However, importantly, the transgenic TG(mRen-2)27 rat does not display hyperglycaemia and is not a diabetic model. A hypotensive dose of aliskiren is also evidenced to prevent renal injury in streptozotocin-induced type 1 diabetic transgenic TG(mRen-2)27 rats. Furthermore, aliskiren was recently reported to suppress cardiomyocyte apoptosis and interstitial fibrosis in streptozotocin-induced type 1 diabetic rats, although the role of BP was not examined in that report. However, to the best of our knowledge, all previous studies investigating the effects of aliskiren on diabetes are limited to type 1 diabetic models. Together with the fact that the underlying pathogenic mechanisms markedly differ between type 2 and type 1 diabetes, these findings encouraged us to examine for the first time, the efficacy of aliskiren on type 2 diabetic db/db mice. db/db mice are characterised by obesity, insulin resistance, severe hyperglycaemia, pancreatic injury and cardiovascular complications, and, therefore, are regarded as a useful model of human type 2 diabetes.
The various doses of aliskiren ranging from 3 to 25 mg kg\(^{-1}\) day\(^{-1}\), was given to db/db mice in this study, as previous reports\(^3\),\(^3\),\(^3\) indicate that such doses of aliskiren in vivo specifically and significantly inhibits plasma renin in mice. In accordance with the previous report,\(^3\) aliskiren, with all doses used in this study, significantly increased renal renin mRNA in a dose-dependent manner (Fig. 12), confirming the significant suppression of RAS by aliskiren in vivo. Aliskiren, with all doses examined in this study, markedly prevented cardiovascular injuries in db/db mice. Notably, are the observations that aliskiren at the minimum dose (3 mg kg\(^{-1}\) day\(^{-1}\)), without reducing BP, markedly prevented cardiac injury, as evidenced by the attenuation of cardiac macrophage infiltration, cardiac interstitial fibrosis and coronary arterial thickening, and also ameliorated vascular endothelial dysfunction in db/db mice. Furthermore, BP reduction with hydralazine treatment insignificantly ameliorated cardiovascular injuries in db/db mice. Collectively, our current findings provide the first evidence that direct renin inhibition with aliskiren causes the prevention of cardiovascular complications in type 2 diabetic mice, and these beneficial effects are at least partially independent of BP reduction. However, it cannot be excluded that BP reduction by aliskiren may be partially responsible for the above-mentioned cardiovascular protection in db/db mice, as the magnitude of amelioration of cardiac interstitial fibrosis and coronary perivascular fibrosis and vascular endothelial dysfunction by aliskiren was dose-dependent.

It is well established that oxidative stress is involved in the progression of cardiovascular injuries,\(^3\),\(^8\) glucose intolerance and pancreatic injuries.\(^3\),\(^9\)-\(^4\) Therefore, in this study, we examined the potential contribution of oxidative stress to the protective effects of aliskiren against type 2 diabetes. Additionally in this study, aliskiren, with all doses examined,
markedly attenuated cardiac superoxide levels, which was associated with the inhibition of cardiac NADPH oxidase activity. To further elucidate the underlying molecular mechanism, we examined the effects of aliskiren on cardiac NADPH oxidase subunits, including Nox2, Nox4, p22phox, p47phox and p67phox. Interestingly, of all these subunits, only p22phox mRNA expression is significantly increased in the cardiac tissue of db/db mice in comparison with control db/m mice. Notably, aliskiren significantly attenuated the increase in cardiac p22phox protein and p22phox-expressing cell numbers, as well as the increased p22phox mRNA expression in db/db mice. These results provide the evidence that the suppression of p22phox-related NADPH oxidase-mediated oxidative stress by aliskiren contributed to the above-mentioned cardiac protection in type 2 diabetes. In this work, we also examined the effect of aliskiren on eNOS, as eNOS counteracts superoxide by way of the production of nitric oxide, and protects against the impairment of vascular endothelial function.42-44 We discovered that aliskiren significantly restored the attenuation of eNOS and phospho-eNOS protein levels in db/db mice. Thus, the upregulation of eNOS by aliskiren appears to be responsible for the improvement of vascular endothelial function in type 2 diabetic mice.

The effect of aliskiren on glucose intolerance, insulin resistance and pancreatic injury in type 2 diabetes was also evaluated in this work. We found that aliskiren at the maximum dose (25 mg kg\(^{-1}\) day\(^{-1}\)) exerted partially beneficial effects on glucose tolerance and insulin sensitivity in type 2 diabetic mice, as evidenced by the results of IGTT, IITT and HOMA-IR. However, in contrast to the prevention of cardiovascular injuries by aliskiren, with all doses examined, reduced doses (3, 6, and 12 mg kg\(^{-1}\) day\(^{-1}\)) of aliskiren failed to improve glucose tolerance in db/db mice. These results suggest that the suppression of glucose intolerance
by aliskiren may require the utilization of higher doses than the prevention of cardiovascular injury by aliskiren. To further elucidate the effects of aliskiren on type 2 diabetes, we examined the effect of aliskiren on pancreatic injuries in db/db mice, as the impairment of pancreatic function in type 2 diabetic animals is ameliorated by treatment with conventional RAS blockers.\textsuperscript{4, 9} We discovered that aliskiren significantly attenuated the decrease in pancreatic islet insulin content and beta cell mass and significantly lessened pancreatic islet fibrosis in db/db mice, and, furthermore, these beneficial effects of aliskiren were associated with the attenuation of pancreatic oxidative stress, as evidenced by the reduction of islet 8-OHdG-positive cells and of NADPH oxidase subunit Nox2. Thus, our current work provides the first evidence that aliskiren protects against the damage of pancreatic islets in type 2 diabetic mice through attenuation of oxidative stress. Collectively, aliskiren appears to exert beneficial effects on glucose tolerance. However, further study on the effect of extended periods of aliskiren treatment on glucose tolerance is required to define the significance of renin inhibition in type 2 diabetes.

Prospectively, it would be interesting to compare the effect of renin inhibition with other conventional RAS blockers, such as ACE inhibitors or ARBs, in future study. Virtually, the effects of an ACE inhibitor and an ARB on glucose tolerance in type 2 diabetic animals, including db/db mice, have been controversial, and depend on the severity of the diabetes, the commencement and duration of drug treatment.\textsuperscript{4, 9, 32, 45} Furthermore, a recent clinical trial\textsuperscript{19} established that the addition of aliskiren to losartan, an ARB, provided additional reduction of urinary albumin excretion in type 2 diabetic patients, suggesting that the combination of aliskiren and an ARB may be a practical therapeutic strategy for diabetic
nephropathy. Further experimental and clinical studies are required to define the precise
efficacy of aliskiren, independently, or in combination with a conventional RAS blocker for
treatment of hypertension with type 2 diabetes.
9. Conclusions

The present study provided the first experimental evidence that direct renin inhibition with aliskiren prevents cardiovascular complications and pancreatic injury in obese type 2 diabetic mice. These beneficial effects are, at least partially, attributed to the amelioration of oxidative stress. Furthermore, aliskiren appears to exert a partially beneficial effect on glucose tolerance, however, future studies on the effect of extended periods of aliskiren treatment are necessary to elucidate it. We propose that aliskiren may be a promising therapeutic agent for type 2 diabetes and its complications.
### 10. Tables

**10-1. Table 1** Body Weight and The Weights of Left Ventricle, Pancreas, Liver and Visceral Fat In db/m Mice and db/db Mice Treated With Hydralazine or Aliskiren for 6 Weeks

<table>
<thead>
<tr>
<th>Mice/treatment</th>
<th>Body weight (g)</th>
<th>Left ventricle (mg)</th>
<th>Pancreas (g)</th>
<th>Liver (g)</th>
<th>Visceral fat (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>db/m (n=9)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (n=13)</td>
<td>46.67±0.65</td>
<td>89.90±2.50</td>
<td>0.27±0.15</td>
<td>2.72±0.11</td>
<td>4.86±0.16</td>
</tr>
<tr>
<td>Hydralazine (n=6)</td>
<td>46.80±0.87</td>
<td>95.97±2.22</td>
<td>0.29±0.13</td>
<td>2.60±0.09</td>
<td>4.78±0.17</td>
</tr>
<tr>
<td>Aliskiren (mg kg⁻¹ day⁻¹)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (n=6)</td>
<td>45.56±0.54</td>
<td>83.90±1.95</td>
<td>0.29±0.20</td>
<td>2.81±0.05</td>
<td>4.54±0.21</td>
</tr>
<tr>
<td>6 (n=6)</td>
<td>44.52±0.35</td>
<td>82.30±1.25</td>
<td>0.28±0.17</td>
<td>2.67±0.08</td>
<td>4.41±0.09</td>
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<tr>
<td>12 (n=8)</td>
<td>46.84±0.81</td>
<td>83.55±1.84</td>
<td>0.24±0.18</td>
<td>2.64±0.06</td>
<td>4.60±0.16</td>
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<td>25 (n=8)</td>
<td>45.82±0.83</td>
<td>83.73±1.14</td>
<td>0.24±0.26</td>
<td>2.60±0.09</td>
<td>4.64±0.20</td>
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</tbody>
</table>

Values are means±SEM

*p<0.05, **p<0.01 vs vehicle
### 10-2. Table 2  Blood Glucose and Plasma Insulin Before and 5 Weeks After Start of Drug Treatment

<table>
<thead>
<tr>
<th>Mice/treatment</th>
<th>Blood glucose (mmol/l)</th>
<th>Plasma insulin (pmol/l)</th>
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</thead>
<tbody>
<tr>
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<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>db/m (n=9)</td>
<td>13.5±0.6*</td>
<td>11.5±0.6*</td>
</tr>
<tr>
<td>db/db</td>
<td>20.8±0.9</td>
<td>37.0±2.2</td>
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<tr>
<td>Vehicle (n=13)</td>
<td>18.7±2.0</td>
<td>35.3±2.6</td>
</tr>
<tr>
<td>Hydralazine (n=6)</td>
<td>21.2±1.8</td>
<td>32.2±2.2</td>
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<tr>
<td>Aliskiren (n=8)a</td>
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</table>

Values are means±SEM  
*25 mg kg⁻¹ day⁻¹  
*p<0.01 vs vehicle
### Table 3 The Sequence of Primers Used In Real-time PCR assays

<table>
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<tr>
<th>Target/primer</th>
<th>Sequence</th>
<th>Acc No.</th>
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</thead>
<tbody>
<tr>
<td><strong>p22phox</strong></td>
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<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TGGCTACTGCTGGACGTTTCAC-3'</td>
<td>NM_007806.3</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTCCAGCAGACAGATGAGCACA-3'</td>
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</tr>
<tr>
<td><strong>p47phox</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GCCTGGCTGGTCTATGTCA-3'</td>
<td>NM_010876</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AGGCAAATGTGGATGCTGGAA-3'</td>
<td></td>
</tr>
<tr>
<td><strong>p67phox</strong></td>
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<tr>
<td>Forward</td>
<td>5'-ACTACTGCCTGACTCTGTGGTGTGA-3'</td>
<td>NM_010877.4</td>
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<tr>
<td>Reverse</td>
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<tr>
<td><strong>Nox2</strong></td>
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<td>5'-TTGAAACCACACCTAAGCCATCTG-3'</td>
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<td>Reverse</td>
<td>5'-AACCTGAGGCTTGAGACACCTGGTA-3'</td>
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<tr>
<td><strong>Nox4</strong></td>
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<td>5'-ATTTGGATAGGCTGCAACCAAC-3'</td>
<td>NM_015760.4</td>
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<tr>
<td>Reverse</td>
<td>5'-CACATGAGGTTATAAGGTTTGTGAGCA-3'</td>
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<tr>
<td><strong>Renin</strong></td>
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<td>5'-GTAGCGACCACCGACATTATC-3'</td>
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<td>5'-AGTGTACCACCTACCGCAACAG-3'</td>
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<td><strong>β-Actin</strong></td>
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<tr>
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Acc No., GenBank accession number
**11. Figures and Figure Legends**

11-1. Figure-1 BP of db/m Mice and db/db Mice During 6 Weeks of Treatment With Aliskiren and Hydralazine.

Figure 1

Red circles, db/m mice; orange squares, vehicle-treated db/db mice; gold triangles, hydralazine (80 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; inverted green triangles, aliskiren (3 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; blue diamonds, aliskiren (6 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; purple circles, aliskiren (12 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; brown squares, aliskiren (25 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice. **p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13).
**11-2. Figure 2** Cardiac Macrophage Infiltration and Interstitial Fibrosis In db/m Mice and db/db Mice.

Figure 2

<table>
<thead>
<tr>
<th></th>
<th>db/m</th>
<th>Ve</th>
<th>Hy</th>
<th>Ali (3)</th>
<th>Ali (25)</th>
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</thead>
<tbody>
<tr>
<td>db/m</td>
<td>Ve</td>
<td>Hy</td>
<td>Ali (3)</td>
<td>Ali (25)</td>
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<tr>
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<td>Ve</td>
<td>Hy</td>
<td>Ali (3)</td>
<td>Ali (25)</td>
<td></td>
</tr>
</tbody>
</table>

**a** includes representative photomicrographs of cardiac sections immunostained with anti-CD68 antibody. Scale bar, 200 μm. **b** includes a representative Sirius Red-stained cardiac sections. Scale bar, 50 μm. *p<0.05, **p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13).

Ve, vehicle-treated; Hy, hydralazine (80 mg kg\(^{-1}\) day\(^{-1}\))-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg\(^{-1}\) day\(^{-1}\), respectively.
11-3. Figure-3 Coronary Arterial Thickening and Peri-coronary Arterial Fibrosis In Hearts of db/m Mice and db/db Mice.

**Figure 3**

<table>
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<tr>
<th>db/m</th>
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<th>Hy</th>
<th>Ali (3)</th>
<th>Ali (25)</th>
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<td><img src="image4.png" alt="Image" /></td>
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</table>

**a** Representative photomicrographs of Sirius Red-stained cardiac sections. Scale bar, 50 μm.

Coronary arterial thickening (b) and peri-coronary arterial fibrosis (c) in hearts of db/m mice and db/db mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg⁻¹ day⁻¹)-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg⁻¹ day⁻¹, respectively.

* p<0.05, ** p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13).
11-4. Figure-4 Cardiac Superoxide Levels and NADPH Oxidase Activity In db/m Mice and db/db Mice.

**Figure 4**

**a**

Representative photomicrographs of DHE-stained cardiac sections. Scale bar, 100 μm.

Cardiac superoxide levels (b) and NADPH oxidase activity (c) in db/m mice and db/db mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg$^{-1}$ day$^{-1}$)-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg$^{-1}$ day$^{-1}$, respectively. KCPM, kilocount per minute. *$p<0.05$, **$p<0.01$ vs vehicle-treated. Values are means±SEM ($n$=6–13).
mRNA expression of Nox2 (a), Nox4 (b), p47\textsuperscript{phox} (c) and p67\textsuperscript{phox} (d) in hearts of \(db/m\) mice and \(db/db\) mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg\textsuperscript{-1} day\textsuperscript{-1})-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg\textsuperscript{-1} day\textsuperscript{-1}, respectively. mRNA levels of each subunit in individual sample were corrected for \(\beta\)-actin mRNA levels. Values are means±SEM (\(n=6–13\)).
Figure 6  

**11-6. Figure-6**  

p22\textsuperscript{phox} mRNA Expression, p22\textsuperscript{phox} Protein Levels and p22\textsuperscript{phox} Protein-positive Cells In Hearts of db/m Mice and db/db Mice.

\( p22\textsuperscript{phox} \) mRNA expression (a), \( p22\textsuperscript{phox} \) protein levels (b) and \( p22\textsuperscript{phox} \) protein-positive cells (c) in hearts of db/m mice and db/db mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg\(^{-1}\) day\(^{-1}\))-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg\(^{-1}\) day\(^{-1}\), respectively. b, c include representative western blots of p22\textsuperscript{phox} (b) and photomicrographs of cardiac sections immunostained with anti-p22\textsuperscript{phox} antibody (c). Scale bar, 50 \( \mu \)m. a, b, c *p<0.05, **p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13).
Vascular endothelium-dependent relaxation by acetylcholine (a) and endothelium-independent relaxation by SNAP (b) in aortas of db/m mice and db/db mice. Red circles, db/m mice; orange squares, vehicle-treated db/db mice; gold triangles, hydralazine (80 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; inverted green triangles, aliskiren (3 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; blue diamonds, aliskiren (6 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; purple circles, aliskiren (12 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice; brown squares, aliskiren (25 mg kg\(^{-1}\) day\(^{-1}\))-treated db/db mice. a \(*p<0.05, **p<0.01\) vs vehicle-treated. Values are means±SEM (n=6–13)
Superoxide (a), eNOS (b) and phospho-eNOS (c) levels in aortas of db/m mice and db/db mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg\(^{-1}\) day\(^{-1}\))-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg\(^{-1}\) day\(^{-1}\), respectively. a Upper panels are representative photomicrographs of DHE-stained aortic sections. Scale bar, 50 \(\mu\)m. b Upper panels are aortic sections immunostained with eNOS antibody. Scale bar, 20 \(\mu\)m. Values are means±SEM (n=6–13). c Western blot analysis of phospho-eNOS (p-eNOS) and GAPDH was performed on the pooled aortic tissues from three or four mice. Phospho-eNOS levels in db/db mice were decreased compared with db/m mice, and were apparently increased by aliskiren, however, not by hydralazine. *p<0.05, **p<0.01 vs vehicle-treated.
IGTT (a), IGTT AUC (b), IITT (c) and HOMA-IR (d) in db/db mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg$^{-1}$ day$^{-1}$)-treated; Ali (25), aliskiren (25 mg kg$^{-1}$ day$^{-1}$)-treated; AUC, areas under the curves. a, c Black circles, vehicle-treated; white squares, hydralazine (80 mg kg$^{-1}$ day$^{-1}$)-treated; black triangles, aliskiren (25 mg kg$^{-1}$ day$^{-1}$)-treated. *$p<0.05$, *$p<0.01$ vs vehicle-treated. Values are means±SEM (n=6–13)
11-10. Figure-10 Insulin Content, Beta Cell Mass and Fibrosis In Pancreatic Islets of db/m Mice and db/db Mice.

**Figure 10**

Insulin content (a), beta cell mass (b) and fibrosis (c) in pancreatic islets of db/m mice and db/db mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg$^{-1}$ day$^{-1}$)-treated; Ali (25), aliskiren (25 mg kg$^{-1}$ day$^{-1}$)-treated. a Upper panels are representative photomicrographs of pancreatic sections immunostained with anti-insulin antibody. Scale bar, 100 μm. c Upper panels are Sirius Red-stained pancreatic sections. Scale bar, 100 μm. *p<0.05, **p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13).
8-OHdG-positive cells (a), Nox2 mRNA expression (b) and NOX2 protein levels (c) in pancreatic islets of db/m mice and db/db mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg$^{-1}$ day$^{-1}$)-treated; Ali (25), aliskiren (25 mg kg$^{-1}$ day$^{-1}$)-treated. a, c Upper panels are representative photomicrographs of pancreatic sections immunostained with anti-8-OHdG antibody and anti-NOX2 antibody, respectively. Scale bar, 100 μm. *p<0.05, **p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13).
Renal renin mRNA expression of $db/m$ mice and $db/db$ mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg$^{-1}$ day$^{-1}$)-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg$^{-1}$ day$^{-1}$, respectively. Renin mRNA levels in individual sample were corrected for $\beta$-actin mRNA levels. **$p<0.01$ vs $db/m$. Values are means±SEM ($n=6$–$13$).
12. References


23. Imanishi T, Tsujioka H, Ikejima H, Kuroi A, Takarada S, Kitabata H, Tanimoto T,


