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ARTICLE

Aliskiren prevents cardiovascular complications and pancreatic injury in a mouse model of obesity and type 2 diabetes

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Abstract
Aims/hypothesis The effect of renin inhibition on type 2 diabetes is still unclear. The present study was undertaken to examine the efficacy of aliskiren, a direct renin inhibitor, on cardiovascular injuries, glucose intolerance and pancreatic injury in a mouse model of type 2 diabetes.
Methods Groups of *db*/*db* mice, with obesity and type 2 diabetes, were treated with aliskiren (3, 6, 12 and 25 mg kg^{-1} day^{-1}) or hydralazine (80 mg kg^{-1} day^{-1}) for 6 weeks, and the protective effects were extensively compared among groups.
Results All sub-pressor and hypotensive 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 vascular endothelial nitric oxide synthase (eNOS) production. Aliskiren at the highest dose (25 mg kg^{-1} day^{-1}), but not at lower doses, partially reduced glucose intolerance in *db*/*db* mice. Furthermore, the highest dose of aliskiren significantly attenuated the decreases in pancreatic islet insulin content and beta cell mass, and prevented pancreatic islet fibrosis in *db*/*db* mice, being associated with the reduction of 8-hydroxy-2′-deoxyguanosine-positive cells and *Nox2* (also known as *Cybb*) expression in pancreatic islets by aliskiren.
Conclusions/interpretation Our work provides the first evidence that direct renin inhibition with aliskiren protects against cardiovascular complications and pancreatic injury, through the attenuation of oxidative stress. Thus, we propose that aliskiren may be a promising therapeutic agent for type 2 diabetes.

Keywords Aliskiren · Cardiovascular complications · Insulin resistance · Oxidative stress

Abbreviations

ARB	Angiotensin receptor blocker
DHE	Dihydroethidium
eNOS	Endothelial nitric oxide synthase
HOMA-IR	HOMA of insulin resistance
IGTT	Intraperitoneal glucose tolerance test
IITT	Intraperitoneal insulin tolerance test
NOX2	NADPH oxidase 2
8-OHdG	8-Hydroxy-2′-deoxyguanosine
RAS	Renin–angiotensin system
SNAP	*S*-Nitroso-*N*-acetylpenicillamine

Introduction

Diabetes is a major risk factor for cardiovascular mortality and morbidity [1–5]. Emerging evidence indicates that

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ACE inhibitors and angiotensin receptor blockers (ARBs) slow the progression of type 2 diabetic nephropathy [1–5] and reduce the incidence of new-onset type 2 diabetes [6–9]. Thus, the renin–angiotensin system (RAS) is known to play a key role in the pathogenesis of type 2 diabetes and its complications, and ACE inhibitors and ARBs are now considered as first-line agents for treatment of hypertensive patients with type 2 diabetes. However, it remains to be defined whether this therapeutic strategy with conventional RAS blockers leads to optimum inhibition of tissue RAS.

Aliskiren is the first in a new class of orally effective direct renin inhibitors, and blocks the RAS in different pharmacological mechanisms from those of conventional RAS blockers [10, 11]. Aliskiren not only lowers BP in hypertensive animals and patients [12, 13] but also has been recently reported to attenuate cardiovascular and renal injuries in various animal disease models [14–20]. However, there is no report investigating the significance of direct renin inhibition with aliskiren in treatment of type 2 diabetes and its complications.

In this study, using *db*/*db* mice, a useful model of human type 2 diabetes, we examined the efficacy of aliskiren on cardiovascular injuries, glucose intolerance and pancreatic injury in type 2 diabetic mice. We obtained the first evidence that direct renin inhibition lessens cardiovascular complications and pancreatic injury in type 2 diabetes.

Methods

Animals and drugs All procedures were in accordance with institutional guidelines for animal research. Male *db*/*db* mice (C57BLKS/J-*lepr*^{db}/*lepr*^{db}) and male non-diabetic *db*/*m* mice (C57BLKS/J-*lepr*$^{db/+}$) as control were purchased from Charles River Laboratories Japan (Yokohama, Japan). All mice were housed in an animal facility with a 12 h 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).

Treatment protocol The *db*/*db* mice at 6 weeks of age already displayed significant obesity and hyperglycaemia. Six-week-old *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 via ALZET micro-osmotic pumps (Durect, Cupertino, CA, USA). Hydralazine was given to mice in drinking water. The drug treatments were carried out for 6 weeks (from 6 to 12 weeks of age). Body weight was periodically measured. BP was measured by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) [21] 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.

IGTT and IITT For IGTT, mice were deprived of food for 6 h 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, 120 min after the injection. Blood glucose concentrations were measured by a portable glucose meter (Sanwa Kagaku Kenkyusho, Nagoya, Japan).

For IITT, 6 h-food-deprived mice 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 min after injection, to measure blood glucose concentrations.

HOMA of insulin resistance (HOMA-IR) HOMA-IR, a simple assessment of insulin sensitivity, was calculated by using the following formula: [fasting plasma glucose (mmol/l) × insulin (pmol/l)]/405. Plasma insulin levels were quantified by using a commercial ELISA kit (Morinaga, Tokyo, Japan).

Vessel ring preparation and organ chamber experiments Isometric tension studies were performed as previously described [22]. In brief, thoracic aortas from mice were cut into 5 mm rings with special care to preserve the endothelium, and 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_2PO_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 L-phenylephrine (10^{-7} mol/l). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (10^{-9} to 10^{-4} mol/l) or *S*-nitroso-*N*-acetylpenicillamine (SNAP; 10^{-9} to 10^{-4} mol/l) to obtain cumulative concentration–response curves.

Measurement of tissue superoxide and NADPH oxidase activity Hearts and aortas removed from mice were immediately frozen in Tissue-Tek OCT embedding medium

(Sakura Finetek, Tokyo, Japan). Dihydroethidium (DHE) was used to evaluate tissue superoxide levels in situ, as described in detail previously [23]. 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 measurement of cardiac NADPH oxidase activity, cardiac tissues were homogenised with an Ultraturrax T8, centrifuged and NADPH oxidase activity of the resulting supernatant fraction measured by lucigenin chemiluminescence in the presence of 10 μmol/l NADPH and 10 μmol/l lucigenin as electron acceptor, as described previously [23]. Protein concentrations were measured by the method of Bradford.

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 [24]. For pancreatic sections, fibrosis in and around the islets was quantified as described previously [25].

For 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 [21]. For $p22^{phox}$ immunohistochemistry, cardiac paraffin sections (0.01 mol/l citric acid pH 6.0-antigen unmasked) were incubated overnight with rabbit anti-$p22^{phox}$ primary antibody (×300; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For 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 min. For NADPH oxidase 2 (NOX2) immunohistochemistry, pancreatic paraffin sections (0.01 mol/l citric acid pH 6.0-antigen unmasked) were incubated overnight with mouse anti-NOX2 primary antibody (×100; BD Transduction Laboratories, San Jose, CA, USA), followed by incubation with HRP-conjugated anti-mouse secondary antibody (ready-to-use; Dako North American) for 30 min. 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 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 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 mouse anti-eNOS primary antibody (×200; BD Transduction Laboratories) followed by incubation with FITC-conjugated anti-mouse IgG secondary antibody (×200; Invitrogen, Tokyo, Japan) for 1 h.

Quantitative analysis of cardiac CD68- and $p22^{phox}$-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/the area of the whole pancreatic area] × pancreas weight, as described previously [26].

Preparation of cardiac and aortic protein extracts and western blot analysis The detailed method was previously described [27]. Briefly, after left ventricular or aortic protein extracts were subjected to SDS-PAGE and electric transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. Antibodies used were as follows: anti-phospho-eNOS (×2,000; BD Transduction Laboratories), anti-$p22^{phox}$ (×1,000; Santa Cruz Biotechnology) and GAPDH (×5,000; Santa Cruz Biotechnology).

Quantitative real-time PCR Total RNA was extracted from heart, pancreas and kidney, according to the manufacturer's instruction. One microgram of RNA sample 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*, *$p22^{phox}$* (also known as *Cyba*), *$p47^{phox}$* (also known as *Ncf1*), *$p67^{phox}$* (also known as *Ncf2*), renin and β-actin (see Electronic supplementary material [ESM] Table 1). Amplification conditions included 10 s at 95°C and 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

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

Mice/treatment	Body weight (g)	Left ventricle (mg)	Pancreas (g)	Liver (g)	Visceral fat (g)
db/*m* (*n*=9)	24.71±0.33**	82.92±3.7	0.22±0.17*	1.19±0.02**	0.73±0.04**
db/*db*					
Vehicle (*n*=13)	46.67±0.65	89.90±2.50	0.27±0.15	2.72±0.11	4.86±0.16
Hydralazine (*n*=6)	46.80±0.87	95.97±2.22	0.29±0.13	2.60±0.09	4.78±0.17
Aliskiren (mg $kg^{-1} day^{-1}$)					
3 (*n*=6)	45.56±0.54	83.90±1.95	0.29±0.20	2.81±0.05	4.54±0.21
6 (*n*=6)	44.52±0.35	82.30±1.25	0.28±0.17	2.67±0.08	4.41±0.09
12 (*n*=8)	46.84±0.81	83.55±1.84	0.24±0.18	2.64±0.06	4.60±0.16
25 (*n*=8)	45.82±0.83	83.73±1.14	0.24±0.26	2.60±0.09	4.64±0.20

Values are means±SEM

*$p<0.05$, **$p<0.01$ vs vehicle

was confirmed by melting point analysis. Each threshold cycles (C_t) value was normalised to β-actin C_t value and a control sample. The delta–delta C_t method, according to the instruction of Thermal Cycler Dice Real Time System, was used for relative quantification.

Statistical analysis Statistical analysis was performed 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 $p<0.05$.

Results

Body weight and organ weights of db/m and db/db mice As shown in Table 1, body weight and the weights of pancreas, liver and visceral fat were significantly greater in 12-week-old *db*/*db* mice than the same age of *db*/*m* mice, and these variables were not significantly affected by 6 weeks of treatment with hydralazine or aliskiren.

Effect of aliskiren on BP in db/db mice As shown in Fig. 1, aliskiren, at a dose of 3 mg $kg^{-1} day^{-1}$, did not significantly lower 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 $kg^{-1} day^{-1}$.

Aliskiren ameliorated 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, at 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. 3b) were similar between the lowest dose (3 mg $kg^{-1} day^{-1}$) and the highest dose (25 mg $kg^{-1} day^{-1}$) of aliskiren. Cardiac interstitial (Fig. 2b) and perivascular (Fig. 3c) fibrosis were attenuated by aliskiren in a dose-dependent manner. On the other hand, hydralazine treatment did not ameliorate these cardiac injuries in *db*/*db* mice.

Aliskiren attenuated cardiac superoxide, NADPH oxidase activity and the expression of NADPH oxidase subunit $p22^{phox}$ in db/db mice As shown in Fig. 4, cardiac superoxide levels

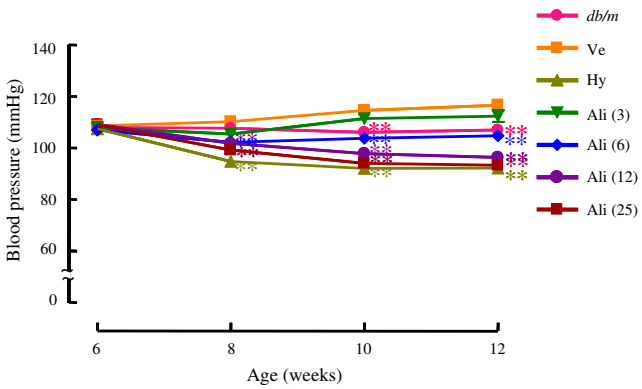

Fig. 1 BP of *db*/*m* mice and *db*/*db* mice during 6 weeks of treatment with aliskiren and hydralazine. Red circles, *db*/*m* mice; orange squares, vehicle-treated *db*/*db* mice; gold triangles, hydralazine (80 mg $kg^{-1} day^{-1}$)-treated *db*/*db* mice; inverted green triangles, aliskiren (3 mg $kg^{-1} day^{-1}$)-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)

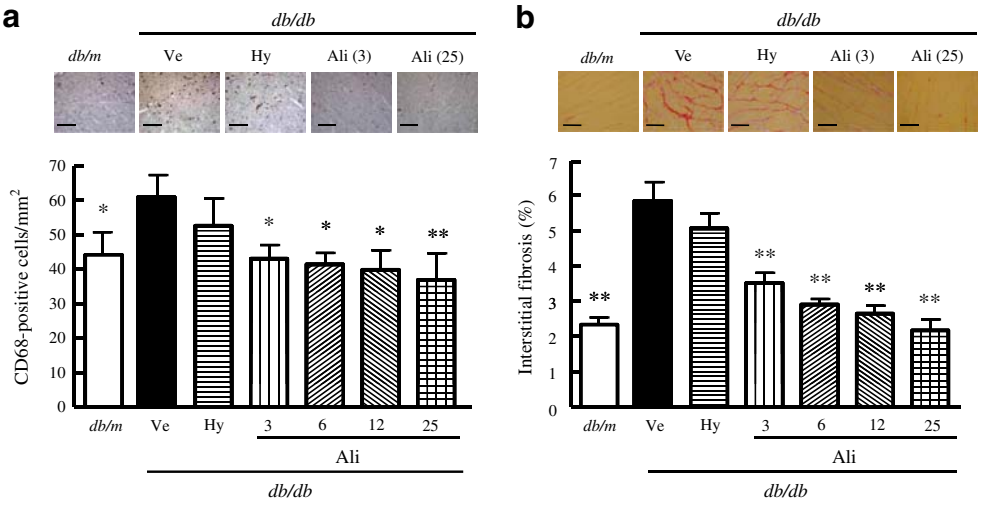

Fig. 2 Cardiac macrophage infiltration (**a**) and interstitial fibrosis (**b**) in *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg^{-1} day^{-1}, respectively. **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)

(p<0.01) and NADPH oxidase activity (p<0.01) were significantly higher in *db*/*db* mice than *db*/*m* mice. All doses of aliskiren significantly reduced cardiac superoxide and NADPH oxidase activity in *db*/*db* mice.

As shown in Fig. 5, cardiac mRNA expressions of *Nox2*, *Nox4*, *p47^{phox}* and *p67^{phox}* were not different between *db*/*db* mice and *db*/*m* mice, and these mRNA expressions of *db*/*db* mice were not significantly affected by any drug treatment. On the contrary, as shown 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* mice was significantly reduced by aliskiren at all doses. Cardiac p22^{phox} protein levels in *db*/*db* mice were also higher than in *db*/*m* mice (p<0.05), and were significantly reduced by the lowest (3 mg kg^{-1} day^{-1}) or the highest (25 mg kg^{-1} day^{-1}) dose 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 *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.

Aliskiren preserved vascular endothelial function in db/db mice As shown 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, at all doses tested, significantly preserved vascular endothelial function in *db*/*db* mice. However, hydralazine failed to restore vascular endothelial function in *db*/*db* mice.

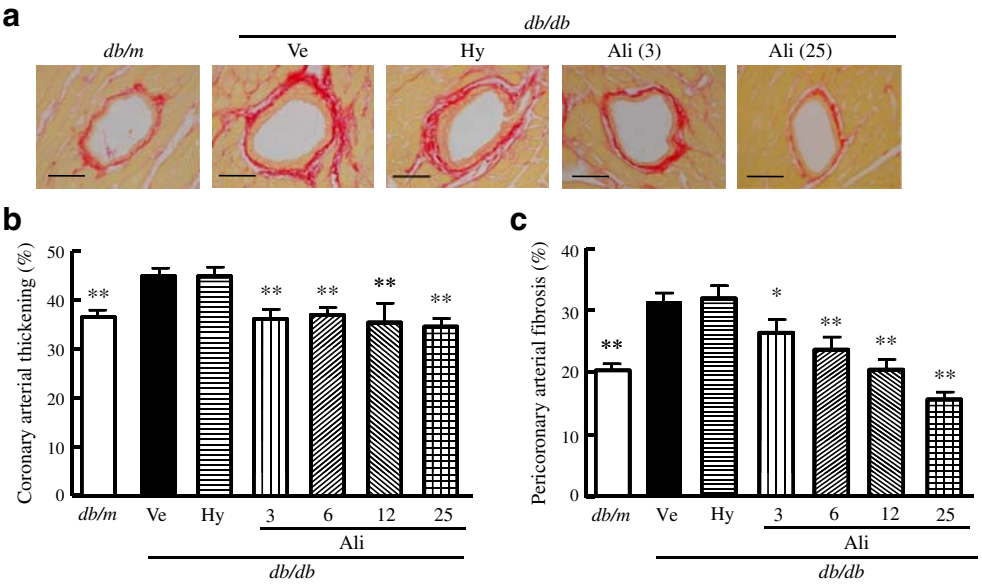

Fig. 3 **a** Representative photomicrographs of Sirius Red-stained cardiac sections. Scale bar, 50 μm. Coronary arterial thickening (**b**) and peri-coronary arterial fibrosis (**c**) in hearts of *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; 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)

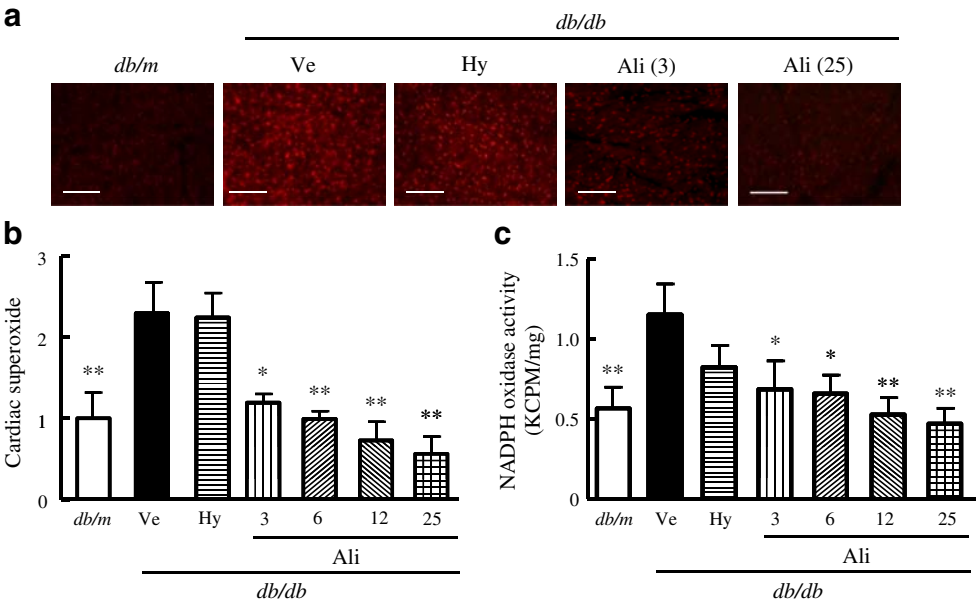

Fig. 4 **a** Representative photomicrographs of DHE-stained cardiac sections. Scale bar, 100 μm. Cardiac superoxide levels (**b**) and NADPH oxidase activity (**c**) in *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg^{-1} day^{-1}, respectively. KCPM, kilocounts/min. *p<0.05, **p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13)

As shown in Fig. 7b, vascular endothelium-independent relaxation by SNAP was not impaired in *db*/*db* mice and was not affected by any drug treatment.

Aliskiren attenuated aortic superoxide and restored vascular endothelial production of eNOS in db/db mice As shown in Fig. 8a, aortic superoxide was significantly greater in *db*/*db* mice than *db*/*m* mice (p<0.01). Aliskiren, at all doses tested, significantly reduced aortic superoxide in *db*/*db* mice (p<0.01). Hydralazine treatment did not affect aortic superoxide in *db*/*db* mice.

As shown in Fig. 8b, vascular endothelial production of eNOS was significantly reduced in *db*/*db* mice compared with *db*/*m* mice (p<0.01). Both the highest dose (25 mg kg^{-1} day^{-1}) and the lowest dose (3 mg kg^{-1} day^{-1}) of aliskiren significantly restored vascular eNOS production in *db*/*db* mice. Hydralazine did not affect eNOS production in *db*/*db* mice. Western blot analysis of aortic phospho-eNOS (Fig. 8c) showed that phospho-eNOS levels in *db*/*db* mice were attenuated compared with *db*/*m* mice and were apparently increased by aliskiren treatment but not by hydralazine treatment.

Aliskiren partially improved glucose tolerance, increased pancreatic islet insulin content and beta cell mass, and attenuated islet fibrosis in db/db mice Table 2 shows blood

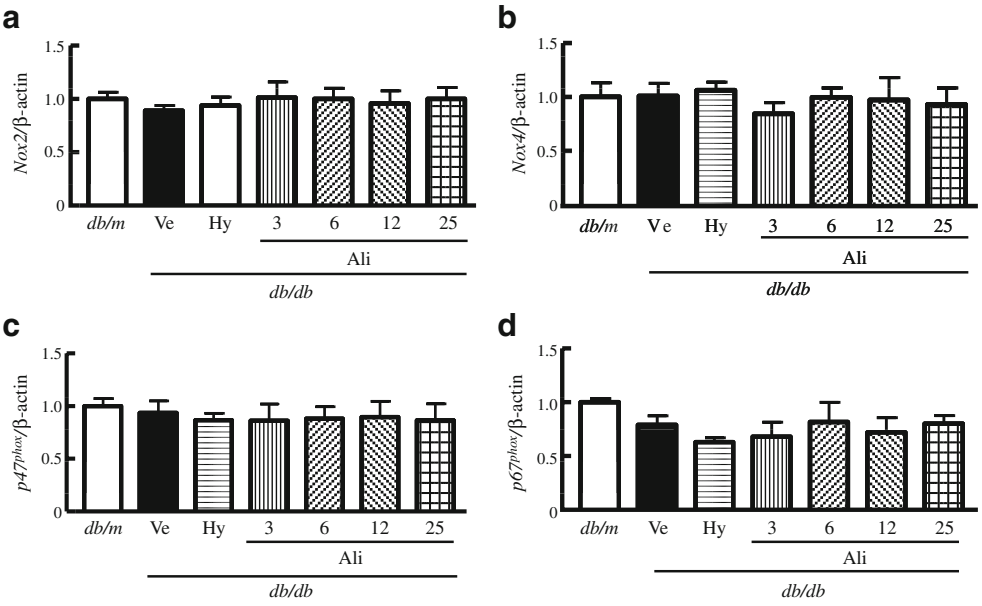

Fig. 5 mRNA expression of *Nox2* (**a**), *Nox4* (**b**), $p47^{phox}$ (**c**) and $p67^{phox}$ (**d**) in hearts of *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg^{-1} day^{-1}, respectively. mRNA levels of each subunit in individual sample were corrected for β-actin mRNA levels. Values are means±SEM (n=6–13)

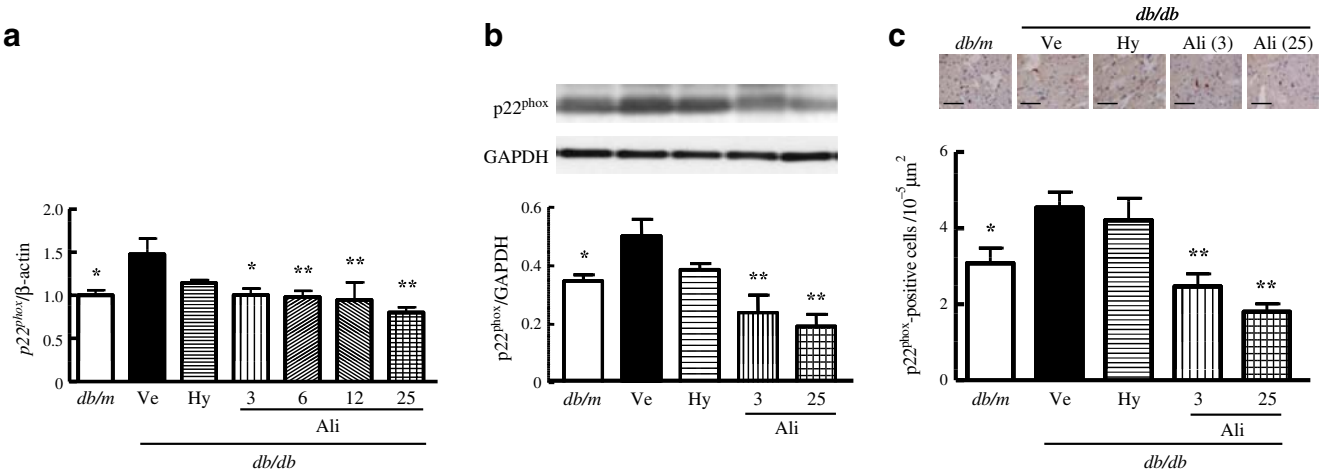

Fig. 6 *p22phox* mRNA expression (**a**), p22phox protein levels (**b**) and p22phox protein-positive cells (**c**) in hearts of *db/m* mice and *db/db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg^{-1} day^{-1}, respectively. **b**, **c** Include representative western blots of p22phox (**b**) and photomicrographs of cardiac sections immunostained with anti-p22phox antibody (**c**). Scale bar, 50 µm. **a**–**c** *p<0.05, **p<0.01 vs vehicle-treated. Values are means±SEM (n=6–13)

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

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

As shown in Fig. 10a, b, pancreatic insulin content (p<0.01) and beta cell mass (p<0.05) were less in *db/db* mice than *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 shown in Fig. 10c, islet fibrosis in *db/db* mice was much greater than *db/m* mice

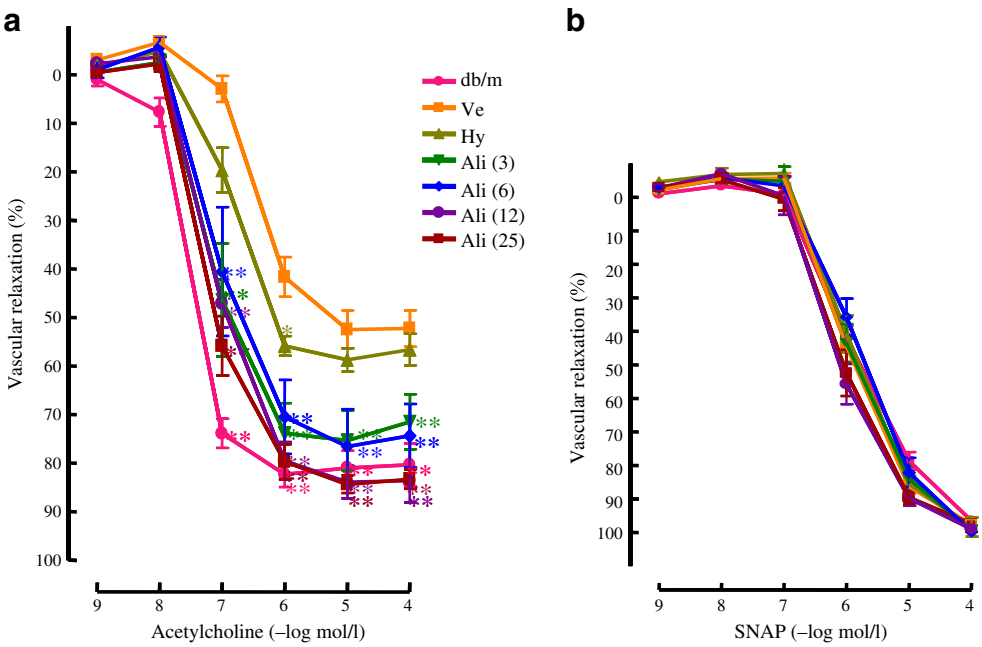

Fig. 7 Vascular endothelium-dependent relaxation by acetylcholine (**a**) and endothelium-independent relaxation by SNAP (**b**) in aortas of *db/m* mice and *db/db* mice. Red circles, *db/m* mice; orange squares, vehicle-treated *db/db* mice; gold triangles, hydralazine (80 mg kg^{-1} day^{-1})-treated *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)

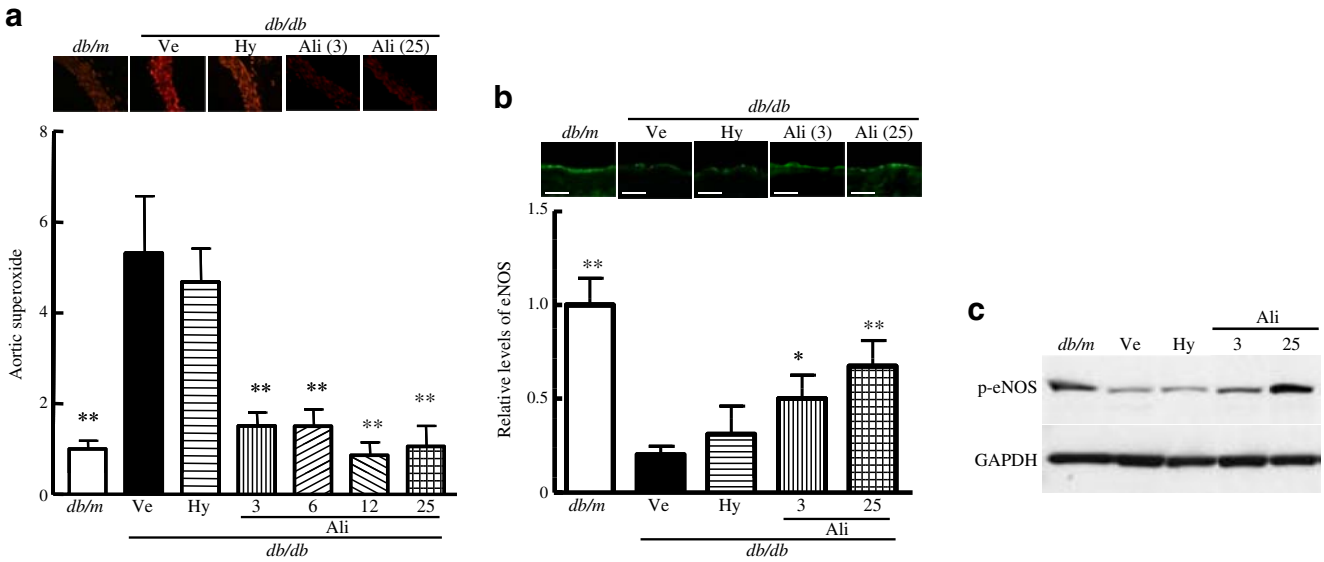

Fig. 8 Superoxide (**a**), eNOS (**b**) and phospho-eNOS (**c**) levels in aortas of *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg^{-1} day^{-1}, respectively. **a** Upper panels are representative photomicrographs of DHE-stained aortic sections. Scale bar, 50 μm. **b** Upper panels are aortic sections immunostained with eNOS antibody. Scale bar, 20 μm. 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 but not by hydralazine. *p<0.05, **p<0.01 vs vehicle-treated

(p<0.01), and was significantly reduced by aliskiren (25 mg kg^{-1} day^{-1}). However, lower doses of aliskiren (3, 6 and 12 mg kg^{-1} day^{-1}) did not significantly improve these pancreatic changes (data not shown). Hydralazine treatment did not improve these pancreatic changes in *db*/*db* mice.

Aliskiren reduced 8-OHdG-positive cells and expression of Nox2 in pancreatic islets in db/db mice As shown in Fig. 11a, the ratio of 8-OHdG-positive cells was significantly higher in *db*/*db* mice than *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 shown in Fig. 11b, c, mRNA expression and protein levels of *Nox2* were significantly higher 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. Lower doses (3, 6 and 12 mg kg^{-1} day^{-1}) of aliskiren failed to improve these variables.

Effect of each treatment on renal renin mRNA expression in *db/db* mice As shown in Fig. 12, treatment of *db*/*db* mice with aliskiren significantly increased renal renin mRNA expression in a dose-dependent manner.

Discussion

The major finding of this study was that direct renin inhibition with aliskiren exerts beneficial effects on cardiovascular injury, 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.

Currently, the impact of a renin inhibitor on diabetes and its complications remains to be defined. Recent reports [28, 29]

Table 2 Blood glucose and plasma insulin before and 5 weeks after start of drug treatment

Mice/treatment	Blood glucose (mmol/l)		Plasma insulin (pmol/l)	
	Before	After	Before	After
db/*m* (n=9)	13.5±0.6*	11.5±0.6*	179±31*	149±20*
db/*db*				
Vehicle (n=13)	20.8±0.9	37.0±2.2	1,632±214	845±117
Hydralazine (n=6)	18.7±2.0	35.3±2.6	1,702±88	806±166
Aliskiren (n=8)[a]	21.2±1.8	32.2±2.2	1,720±170	681±74

Values are means±SEM
[a] 25 mg kg^{-1} day^{-1}
*p<0.01 vs vehicle

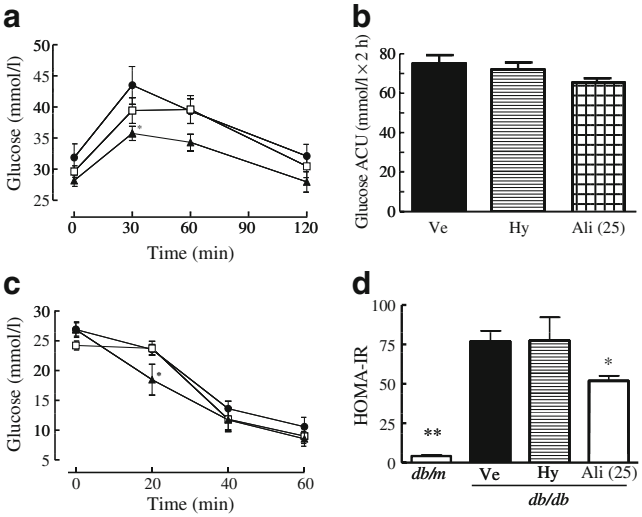

Fig. 9 IGTT (**a**), IGTT AUC (**b**), IITT (**c**) and HOMA-IR (**d**) in *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (25), aliskiren (25 mg kg^{-1} day^{-1})-treated. **a**, **c** Black circles, vehicle-treated; white squares, hydralazine (80 mg kg^{-1} 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)

show 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 shown to prevent renal injury in streptozotocin-induced type 1 diabetic transgenic TG (mRen-2)27 rats [14, 30]. 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 [31]. However, to the best of our knowledge, all previous studies [14, 30, 31] investigating the effects of aliskiren on diabetes are limited to type 1 diabetic models. Taken 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 [32, 33] 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.

In this study, aliskiren at various doses ranging from 3 to 25 mg kg^{-1} day^{-1} was given to *db*/*db* mice, since previous reports [16, 27] indicate that such doses of aliskiren in vivo specifically and significantly inhibits plasma renin in mice. In accord with the previous report [16], aliskiren, at 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, at all doses tested in this study, markedly prevented cardiovascular injuries in *db*/*db* mice. Of note are the observations that aliskiren at the lowest dose (3 mg kg^{-1} day^{-1}), without apparently lowering BP, markedly prevented cardiac injury, as shown 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 lowering with hydralazine treatment did not significantly ameliorate cardiovascular injuries in *db*/*db* mice. Collectively, our current findings provide the first

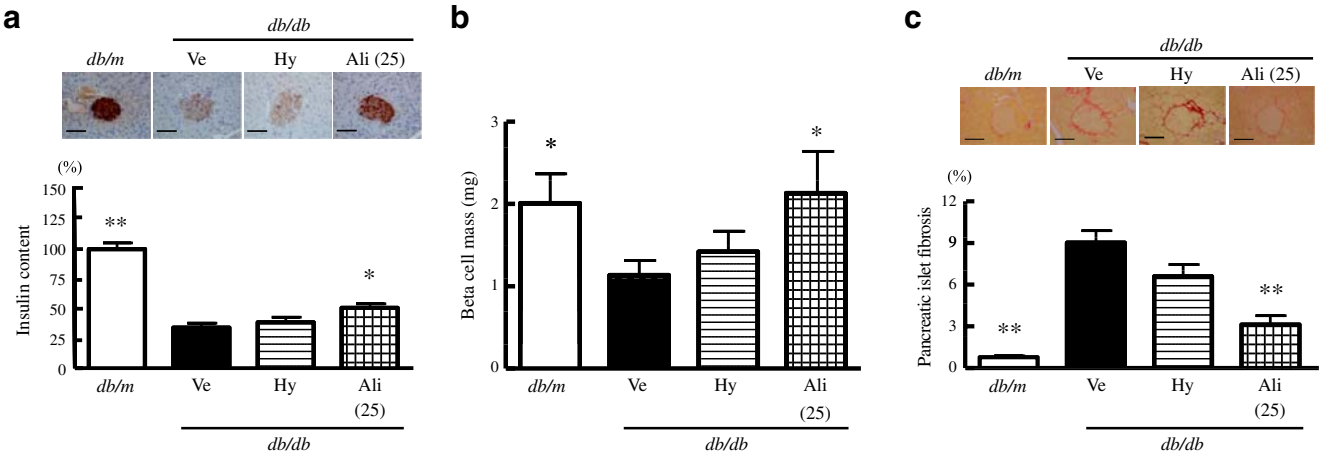

Fig. 10 Insulin content (**a**), beta cell mass (**b**) and fibrosis (**c**) in pancreatic islets of *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (25), aliskiren (25 mg kg^{-1} day^{-1})-treated. **a** Upper panels are representative photomicrographs 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)

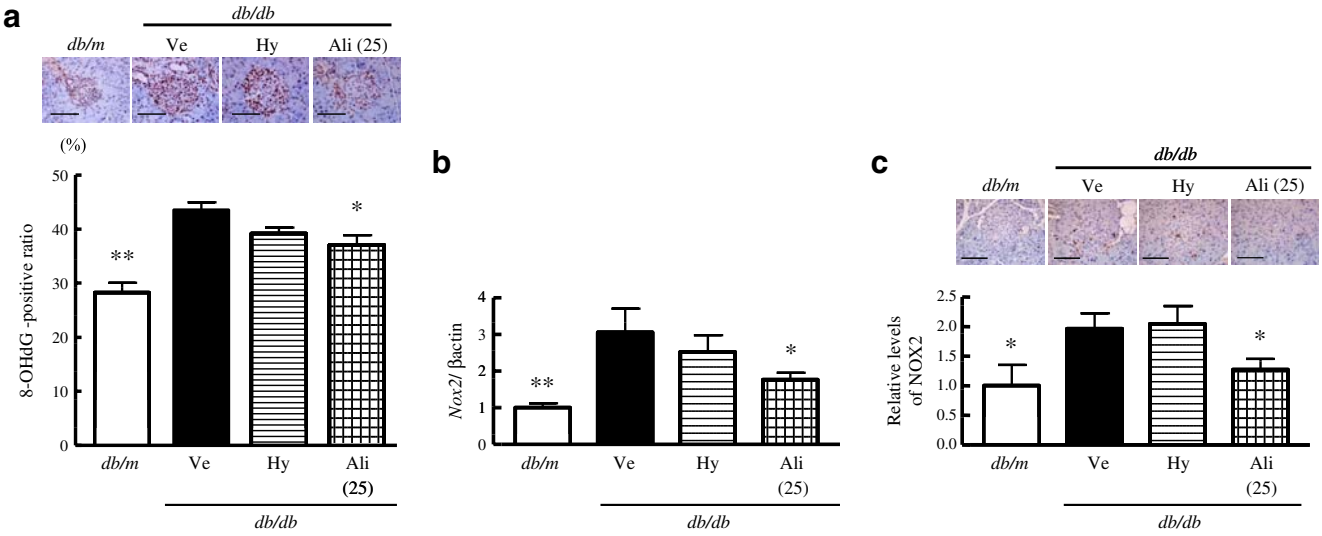

Fig. 11 8-OHdG-positive cells (**a**), *Nox2* mRNA expression (**b**) and NOX2 protein levels (**c**) in pancreatic islets of *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (25), aliskiren (25 mg kg^{-1} day^{-1})-treated. **a, c** Upper panels 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)

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 lowering. However, it cannot be excluded that BP lowering by aliskiren might be partially responsible for the above-mentioned cardiovascular protection in *db*/*db* mice, since the magnitude of amelioration of cardiac interstitial fibrosis and coronary perivascular fibrosis and vascular endothelial dysfunction by aliskiren was dose-dependent.

Oxidative stress is well established to be involved in the progression of cardiovascular injuries [34, 35], glucose intolerance and pancreatic injuries [36–38]. Therefore, in this study, we examined the potential contribution of oxidative stress to the protective effects of aliskiren against type 2 diabetes. In this study, aliskiren at 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*, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$. Interestingly, of all these subunits, only $p22^{phox}$ mRNA expression is significantly increased in cardiac tissue of *db*/*db* mice compared with control *db*/*m* mice. Of note, aliskiren significantly attenuated the increase in cardiac $p22^{phox}$ protein and $p22^{phox}$-expressing cell numbers as well as the increased $p22^{phox}$ mRNA expression in *db*/*db* mice. These results provide the evidence that the suppression of $p22^{phox}$-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, since eNOS counteracts superoxide via the production of nitric oxide, and protects against the impairment of vascular endothelial function [22, 39, 40]. We found 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.

In the present study, we also examined the effect of aliskiren on glucose intolerance, insulin resistance and pancreatic injury in type 2 diabetes. We found that aliskiren at the highest dose (25 mg kg^{-1} day^{-1}) exerted partially bene-

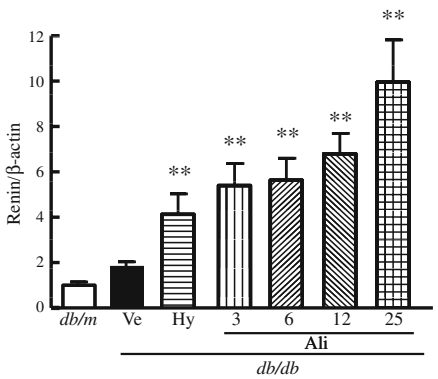

Fig. 12 Renal renin mRNA expression of *db*/*m* mice and *db*/*db* mice. Ve, vehicle-treated; Hy, hydralazine (80 mg kg^{-1} day^{-1})-treated; Ali (3), Ali (6), Ali (12) and Ali (25), treated with aliskiren at 3, 6, 12 and 25 mg kg^{-1} day^{-1}, respectively. Renin mRNA levels in individual sample were corrected for β-actin mRNA levels. **$p<0.01$ vs *db*/*m*. Values are means±SEM (n=6–13)

ficial effects on glucose tolerance and insulin sensitivity in type 2 diabetic mice, as shown by the IGTT, IITT and HOMA-IR results. However, in contrast to the prevention of cardiovascular injuries by aliskiren at all doses examined, lower 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 need the use 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, because the impairment of pancreatic function in type 2 diabetic animals is ameliorated by treatment with conventional RAS blockers [25, 41]. We found 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 these beneficial effects of aliskiren were associated with the attenuation of pancreatic oxidative stress, as shown 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 seems to exert beneficial effects on glucose tolerance. However, further study on the effect of longer periods of aliskiren treatment on glucose tolerance is required to define the significance of renin inhibition in type 2 diabetes.

The present study did not address comparison of aliskiren with conventional RAS blockers in diabetes. Importantly, 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 severity of diabetes, the time of start of drug treatment and the duration of drug treatment [25, 26, 41, 42]. Furthermore, a recent clinical trial [43] showed that the addition of aliskiren to losartan, an ARB, provided additive reduction of urinary albumin excretion in type 2 diabetic patients, suggesting that the combination of aliskiren plus an ARB may be a useful therapeutic strategy for diabetic nephropathy. Further experimental and clinical studies are needed to define the precise efficacy of aliskiren alone or in combination with a conventional RAS blocker in treatment of hypertension with type 2 diabetes.

In conclusion, our present study has provided the first evidence that direct renin inhibition with aliskiren prevents cardiovascular complications and pancreatic injury in obese type 2 diabetic mice, at least partially through the amelioration of oxidative stress. Furthermore, aliskiren seems to exert a partially beneficial effect on glucose tolerance, although future studies on the effect of longer 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.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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