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Original Paper

Mineralocorticoid receptor blockade prevents vascular remodelling in a rodent model of type 2 diabetes mellitus

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Abstract

Mineralocorticoid receptors (MRs), which are activated by mineralocorticoids and glucocorticoids, actively participate in mechanisms that affect the structure and function of blood vessels. Although experimental and clinical evidence shows that vascular damage in diabetes is associated with structural alterations in large and small arteries, the role of MR in this process needs further studies. Thus, we tested the hypothesis that MR, through redox-sensitive mechanisms, plays a role in diabetes-associated vascular remodelling. Male, 12–14-weeks-old db/db mice, a model of type 2 diabetes and their non-diabetic counterpart controls (db/+) were treated with spironolactone (MR antagonist, 50 mg/kg/day) or vehicle for 6 weeks. Spironolactone treatment did not affect blood pressure, fasting glucose levels or weight gain, but increased serum potassium and total cholesterol in both, diabetic and control mice. In addition, spironolactone significantly reduced serum insulin levels, but not aldosterone levels in diabetic mice. Insulin sensitivity, evaluated by the HOMA (homoeostatic model assessment)-index, was improved in spironolactone-treated diabetic mice. Mesenteric resistance arteries from vehicle-treated db/db mice exhibited inward hypertrophic remodelling, increased number of smooth muscle cells and increased vascular stiffness. These structural changes, determined by morphometric analysis and with a myography for pressurized arteries, were prevented by spironolactone treatment. Arteries from vehicle-treated db/db mice also exhibited augmented collagen content, determined by Picrosirius Red staining and Western blotting, increased reactive oxygen species (ROS) generation, determined by dihydroethidium (DHE) fluorescence, as well as increased expression of NAD(P)H oxidases 1 and 4 and increased activity of mitogen-activated protein kinases (MAPKs). Spironolactone treatment prevented all these changes, indicating that MR importantly contributes to diabetes-associated vascular dysfunction by inducing oxidative stress and by increasing the activity of redox-sensitive proteins.

Key words: db/db mice, diabetes, mineralocorticoid receptor, spironolactone, vascular remodelling, vascular stiffness.

INTRODUCTION

Diabetes mellitus (DM) is a disease marked by high levels of blood glucose resulting from defects in insulin production (type 1), insulin action (type 2) or both [1]. In 2011, a study estimated 366 million people worldwide with DM and predicted that this number will increase 50.7% in 2030, at an average annual growth of 2.7%, which is 1.7 times the annual growth of the total world adult population [2].

Type 2 DM, the most common form of DM, is characterized by hyperglycaemia, insulin resistance and relative insulin deficiency [3]. These alterations promote severe diabetic complications, such as retinopathy, neuropathy, nephropathy and cardiovascular damage, major causes of disability and death in diabetic patients [4].

Oxidative stress, due to exaggerated NAD(P)H oxidase (NOX)-induced reactive oxygen species (ROS) production and endothelial dysfunction, mainly due to decreased nitric oxide

Abbreviations: AGE, advanced glycation end products; CSA, cross-sectional area; DHE, dihydroethidium; DM, diabetes mellitus; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; HOMA, homoeostatic model assessment; JNK, c-jun n-terminal kinase; KHS, Krebs–Henseleit solution; MAPK, mitogen-activated protein kinase; MR, mineralocorticoid receptor; MRA, mesenteric resistance arteries; NO, nitric oxide; NOX, NAD(P)H oxidase; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; SBP, systolic blood pressure; VSMC, vascular smooth muscle cell.

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(NO) bioavailability, are hallmarks of diabetic vasculopathy [5].

Vascular remodelling, characterized by cell growth and rearrangement of cellular components and extracellular matrix (ECM) proteins, is another feature of DM-associated vascular damage and accounts for an increased incidence of complications in diabetic patients [6]. At the macrovascular level, the intima and media thickening, along with increased stiffness and decreased vasomotion, predict higher risk of adverse cardiovascular events in DM [7].

Potential mechanisms involved in DM-induced vascular remodelling include advanced glycation end products (AGE)- and ROS-associated signalling pathways, mediated by activation of AGE receptors and NOX enzymes [7]. ROS activate signalling proteins that promote cell proliferation, migration, inflammation, ECM deposition, fibrosis and angiogenesis, factors that directly influence vascular remodelling [8]. Mitogen-activated protein kinases (MAPKs), e.g. including extracellular signal-regulated kinase 1/2 (ERK 1/2), p38 MAPK and c-jun N-terminal kinase (JNK), are ROS-sensitive enzymes that regulate vascular cell growth, apoptosis, contraction, migration and inflammation [9,10].

The mineralocorticoid hormone aldosterone mediates maladaptive changes in the cardiovascular system by inducing NOX activation and oxidative stress and by reducing NO bioavailability and endothelium-dependent relaxation [11]. Accordingly, mineralocorticoid receptor (MR) blockade reduces cardiac inflammation, oxidative stress, fibrosis, remodelling, hypertrophy, improves diastolic function and enhances endothelial-mediated relaxation [11–13].

These effects, mediated by MR, are well established in hypertension, heart failure and myocardial fibrosis, but their contribution to DM-associated vascular remodelling requires further studies, performed especially in animal models with insulin resistant and/or type 2 DM.

Thus, we tested the hypothesis that MR, through ROS-sensitive mechanisms, plays a role in DM-associated vascular remodelling, contributing to vascular injury in this disease. To test our hypothesis, we determined the effects of spironolactone in the vascular structural abnormalities found in an experimental model of type 2 DM. Signalling pathways involved in the beneficial effects produced by MR blockade were also determined.

MATERIALS AND METHODS

Animal model

Male, 12-14-weeks-old, db/db mice [B6.BKS(D)-Lepr<db>/J], animal model of type 2 DM and age-matched heterozygote non-diabetic mice [(db/+), The Jackson Laboratory] were used in the study. Mice were kept in the animal facility of the Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, under controlled temperature (22 °C–24 °C) and humidity, 12-h light/dark cycles, fed with standard diet and water *ad libitum*. The study was performed in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation and was approved by the Ethics Committee on Animal Research of the Ribeirao Preto Medical School of the University of Sao Paulo (protocol number 062/2012).

Control and db/db mice were treated for 6 weeks with the MR antagonist spironolactone (50 mg/kg body weight/day) or vehicle (ethanol 1%) via oral per gavage. At the beginning, third week and at the end of treatment, after 12-h fasting, the blood glucose levels were measured with a portable glucose meter (Accu-Chek Active®, Roche Diagnostics). Body weight and systolic blood pressure [(SBP), assessed by a tail-cuff pletysmography (CODA™ High Throughput – Kent Scientific Corporation)] were weekly determined.

After the treatment, blood was collected from anaesthetized mice (2% isoflurane vapourized with oxygen). Still under anaesthesia, animals were killed by cervical dislocation and tissues of interest were collected.

Metabolic profile

Serum levels of total cholesterol and triglycerides were determined using enzymatic methods (Colestat enzymatic and TG Color GPO/PAP, Wiener Lab). Serum sodium (Na^+) and potassium (K^+) were determined using ion selective electrodes by potentiometry in an automatic biochemistry analyser (BT 3000 plus, Wiener Lab). Serum aldosterone (Aldosterone Rodent ELISA Kit, Abnova) and insulin (Rat/Mouse Insulin ELISA, Millipore) was determined by enzyme immunoassay. Insulin resistance was estimated by the homoeostatic model assessment (HOMA) index, which was calculated by the following formula: fasting plasma insulin (microunits/ml) × fasting glucose (mmol/l)/22.5 [14].

Isolation of blood vessels

Mesenteric arcade and thoracic aorta were removed and placed in Krebs–Henseleit solution (KHS) of the following composition (in mmol/l): 130.0 NaCl, 4.7 KCl, 1.6 $CaCl_2.2H_2O$, 1.18 KH_2PO_4, 1.17 $MgSO_4.7H_2O$, 14.9 $NaHCO_3$, 5.5 glucose and 0.026 EDTA, maintained at 4 °C. The second-order branches of the mesenteric resistance arteries (MRA) were dissected and isolated from the mesenteric bed and, along with the aorta, carefully cleaned from surrounding adipose and connective tissue.

Pressure myography

Mechanical and structural properties of MRA were determined with a pressure myography (Danish Myo Tech, Model 110P and 111P), as previously described [15,16]. Vessel segments were placed between two-glass microcannulae and tied with surgical nylon suture. At this time, the intraluminal pressure was increased to 140 mmHg for adjustments in the artery length and to check if vessels were correctly placed.

The segment was then set to a pressure of 70 mmHg under no-flow conditions and allowed to equilibrate at 37 °C, for 30 min, in calcium-free ($0Ca^{2+}$) KHS containing EGTA 10 mmol/l and gassed with a mixture of 95% O_2 and 5% CO_2. Intraluminal pressure was then reduced to 3 mmHg and a pressure–diameter curve obtained by progressively increasing intraluminal pressure between 3 and 140 mmHg, when internal and external diameters (Di and De respectively) were measured.

Di and De were used to calculate parameters such as: wall thickness, cross-sectional area (CSA), wall:lumen ratio,

incremental distensibility, stress–strain relationship and β-value, as previously described [15–17].

Morphometric analysis

Segments of MRA were fixed in 4% paraformaldehyde for 1 h and then immersed in 30% sucrose solution for 24 h. Thereafter, vessels were transferred to a cryomold containing Tissue-Tek® OCT compound embedding medium, frozen in small blocks and cut in histological sections (10 μm, Cryocut 1800, Leica), which were stained with haematoxylin and eosin for microscopic examination (Olympus, BX50, 40× objective). Colour images obtained were analysed with ImageJ analysis software (National Institute of Health). Tunica media, lumen and outer border were traced to calculate: media CSA, media thickness and media/lumen ratio, as previously described [18]. In addition, the vascular smooth muscle cell (VSMC) nuclei of the media layer in each cross-section were counted.

Determination of collagen content

Collagen content was determined as previously described [15,16]. Frozen transverse sections (10 μm) obtained as described above, were incubated with Picrosirius Red (0.1% Sirius Red F3B in saturated aqueous picric acid) for 30 min for collagen staining. Colour images were captured with a microscope (Olympus, BX50, 40× objective) and quantitative analysis of collagen content both in the adventitia and media layers was performed with the ImageJ analysis software.

Detection of intracellular ROS by dihydroethidium fluorescence

The fluorescent dye dihydroethidium (DHE) was used to determine superoxide anion (O_2^-) production [15]. Aortic rings from control and db/db mice were frozen in Tissue-Tek® OCT compound embedding medium, cut in histological sections (10 μm) and incubated for 10 min at 37 °C in KHS containing 5 μmol/l DHE [19]. Fluorescent intensity was captured by Leica DMI 4000B inverted microscope (×40 objective). The images were processed using LAS AF software (Leica Microsystems) and analysed by the ImageJ analysis software.

Western blot analysis

Protein samples (50 μg) prepared from MRA homogenates were submitted to electrophoresis in 10% polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare). After non-specific binding sites blockade (5% BSA for 1 h at 24 °C), the membranes were incubated with primary antibodies overnight at 4 °C.

Antibodies and dilutions were as follows: collagen type-I (1:250, Santa Cruz Biotechnology); p-ERK 1/2, ERK 1/2, p-SAPK/JNK and SAPK/JNK (1:1000, Cell Signaling); NOX 1 and NOX 4 (1:500, Abcam), p-p38 MAPK and p38 MAPK (1:500, Cell Signaling), proliferating cell nuclear antigen (PCNA; 1:500, Santa Cruz Biotechnology) and β-actin (1:3000, Abcam).

After incubation with secondary antibodies for 1 h at room temperature, signals were revealed by a chemiluminescence system (ECL Plus, GE Healthcare), visualized by autoradiography and quantified by densitometry using the ImageJ analysis software. Results were normalized by the total protein and/or β-actin expression and expressed as units relative to the db/+ vehicle-treated.

Drugs

Spironolactone, EGTA, Sirius Red and paraformaldehyde were obtained from Sigma–Aldrich Chemical. DHE was obtained from Polysciences and isoflurane from BioChimico. All other chemicals (analytical grade or higher) were obtained from Sigma–Aldrich Chemical and Merck.

Data analysis and statistical procedures

Data are represented as mean ± S.E.M. with a probability of $P < 0.05$ used for significance. All parameters and calculations were analysed using two-way ANOVA followed by a Bonferroni post-hoc test. The Prism software, version 5.0 (GraphPad Software Inc.) was used to analyse the results. The notation '*n*' denotes the number of animals used in each experiment.

RESULTS

Weight gain, systolic blood pressure and fasting blood glucose

Weight gain and fasting blood glucose levels were increased in vehicle-treated and spironolactone-treated db/db mice, when compared with their respective db/+ lean control mice, over the treatment period (Figures 1A and 1C). On the other hand, SBP was similar among the groups (Figure 1B). Spironolactone treatment did not affect body weight gain in db/+ or db/db mice.

Basic metabolic profile

Serum aldosterone, insulin, total cholesterol and triglycerides levels were higher in db/db mice compared with non-diabetic controls. Treatment with spironolactone increased K^+ and total cholesterol serum levels in db/db and db/+ mice. No significant changes in serum Na^+ levels were observed (Table 1). Db/db mice presented increased serum insulin levels and lower sensitivity to insulin, assessed by the HOMA-index, compared with db/+ mice. Spironolactone significantly reduced serum insulin levels and improved insulin sensitivity in db/db mice (Table 1).

Structural and mechanical alterations in MRA from diabetic mice are prevented by spironolactone treatment

The external diameter (Figure 2A) was similar in MRA from all experimental groups. On the other hand, the internal diameter (Figure 2B) was decreased in MRA from vehicle-treated db/db mice compared with all the other groups. Wall thickness (Figure 2C), wall:lumen ratio (Figure 2D) and CSA (Figure 2E) were significantly increased in vehicle-treated diabetic mice. These vascular structural alterations in db/db mice were prevented by spironolactone treatment.

Table 1 Basic metabolic parameters in db/db and db/+ mice after spironolactone or vehicle treatment

Parameter	db/+ vehicle	db/+ spironolactone	db/db vehicle	db/db spironolactone
Aldosterone (pg/ml)	170.00 ± 28.75	216.00 ± 45.75	492.80 ± 39.11*	508.00 ± 55.19*
HOMA-index	2.07 ± 0.25	3.81 ± 0.31	243.70 ± 39.80*	83,63 ± 22,63*†
Insulin (ng/ml)	0.30 ± 0.03	0.55 ± 0.03	14.40 ± 1.19*	5.99 ± 1.54*†
Potassium (mEq/l)	4.83 ± 0.08	5.06 ± 0.11†	5.00 ± 0.31	6.12 ± 0.25*†
Sodium (mEq/l)	147.00 ± 0.76	146.50 ± 0.68	145.00 ± 1.52	145.50 ± 3.02
Total cholesterol (mmol/l)	1.86 ± 0.16	2.20 ± 0.05†	3.15 ± 0.43*	4.39 ± 0.21*†
Triglycerides (mmol/l)	0.87 ± 0.04	0.95 ± 0.07	1.70 ± 0.23*	1.66 ± 0.17*

Results are expressed as mean ± S.E.M. *$P < 0.05$ compared with respective db/+ group, †$P < 0.05$ compared with respective vehicle group; $n = 5$–7.

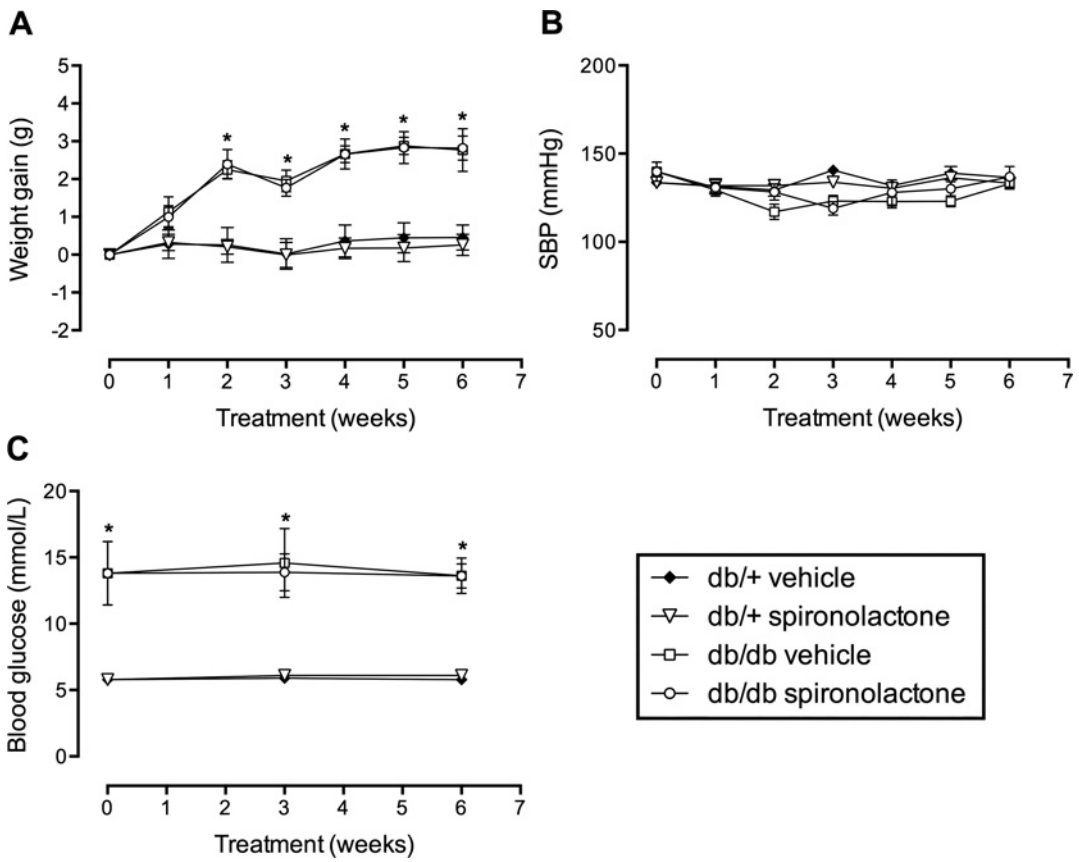

Figure 1 Weight gain (A), SBP (B) and fasting blood glucose levels (C) in db/db and db/+ mice during the treatment with spironolactone or vehicle for 6 weeks

Data are expressed as mean ± S.E.M. *$P < 0.05$ compared with respective db/+ group; $n = 5$–7.

The mechanical parameters of MRA are presented in Figure 3. The incremental distensibility was decreased in MRA from vehicle-treated db/db mice compared with the other experimental groups (Figure 3A). The leftward shift of the stress–strain relationship, shown in Figure 3(B), confirms decreased elasticity in arteries from db/db mice. Spironolactone treatment improved incremental distensibility and partially restored stress–strain relationship in MRA from diabetic mice with values close to those observed in control arteries. Moreover, the MR antagonist reduced the β-value in arteries from diabetic animals, confirming improvement in arterial elasticity (db/+ vehicle: 3.24 ± 0.05; db/+ spironolactone: 3.34 ± 0.07; db/db vehicle: 5.10 ± 0.27; db/db spironolactone: 3.74 ± 0.10; $n = 5$; $P < 0.05$).

Spironolactone treatment prevents morphometric alterations and cell proliferation in MRA from diabetic mice

As demonstrated in Table 2, OCT-embedded, haematoxylin and eosin-stained MRA from vehicle-treated db/db mice

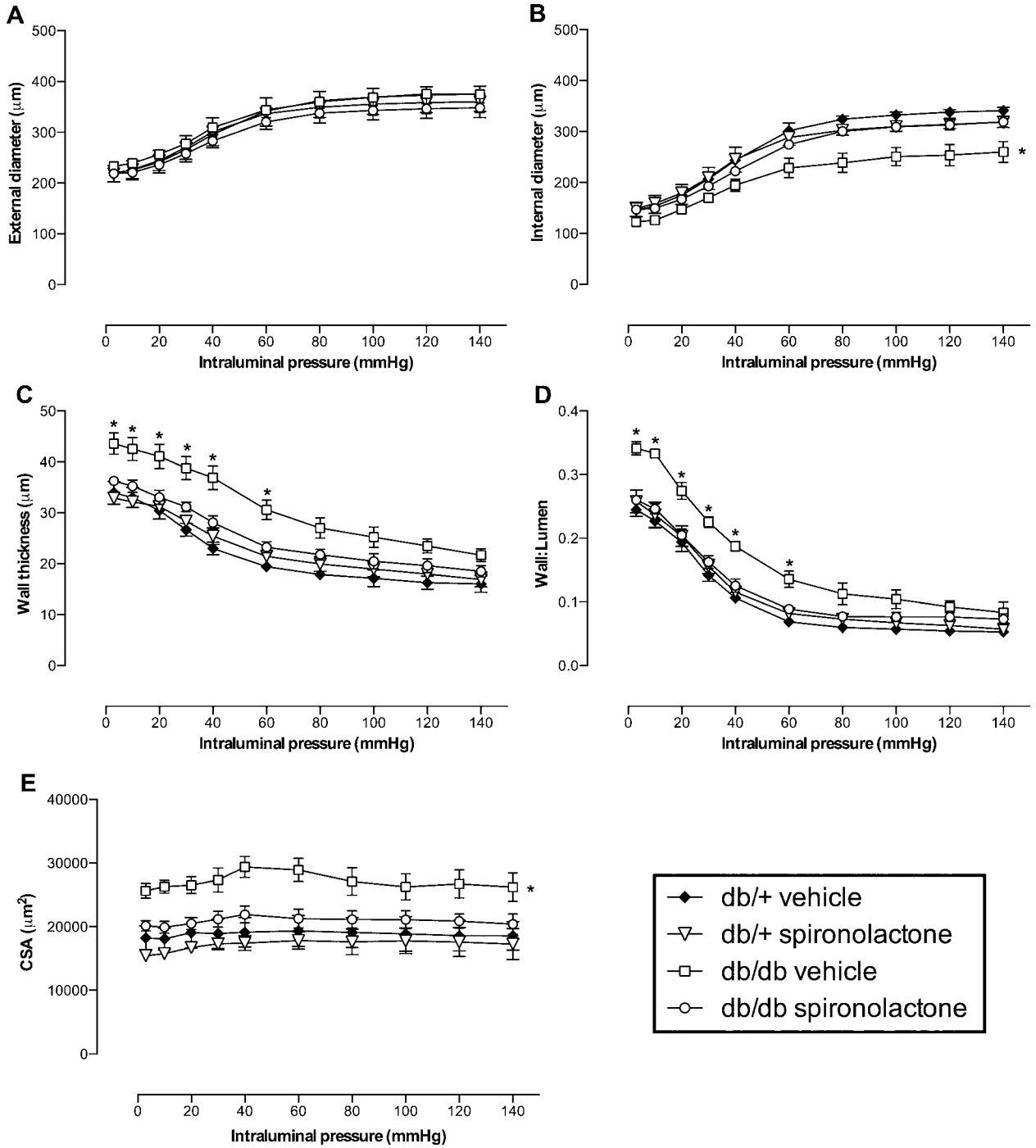

Figure 2 **External diameter (A), internal diameter (B), wall thickness (C), wall:lumen ratio (D) and CSA (E) in MRA from db/db and db/+ mice treated with spironolactone or vehicle**

Values were obtained in arteries gradually pressurized in passive conditions (0Ca^{2+} KHS). Data are expressed as mean ± S.E.M. $^{*}P < 0.05$ compared with all other groups; $n = 5–7$.

exhibited significant morphometric alterations that strongly support the presence of vascular remodelling in diabetic animals and confirm results obtained in the pressure myography analysis. The increased number in media VSMC nuclei indicates cell proliferation in vessels from diabetic mice, which was reinforced by increased PCNA expression in MRA from vehicle-treated db/db mice (Figure 4). Spironolactone treatment effectively reduced these vascular alterations in diabetic mice.

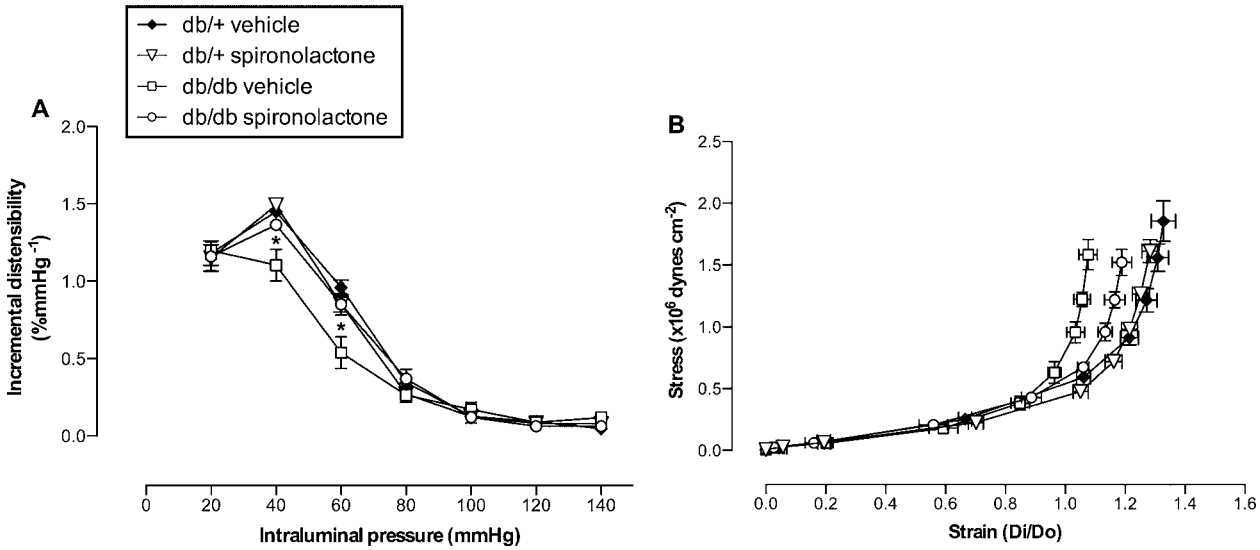

Figure 3 Incremental distensibility (A) and stress–strain relationship (B) in MRA from db/db and db/+ mice treated with spironolactone or vehicle

Arteries were gradually pressurized in passive conditions (0Ca^{2+} KHS). Data are expressed as mean ± S.E.M. *$P < 0.05$ compared with all other groups; $n = 5$–7. Do: external diameter at 3 mmHg and Di: internal diameter at a given intraluminal pressure.

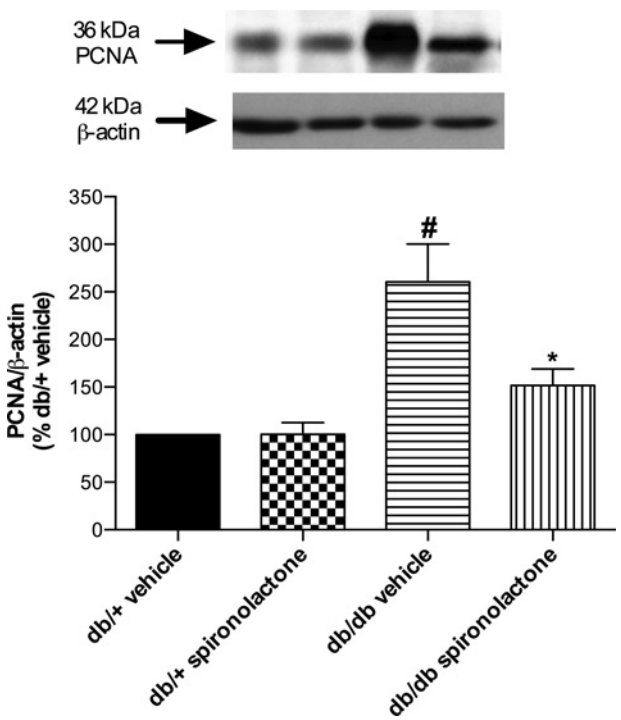

Figure 4 Representative Western blotting and densitometric analysis of PCNA expression in MRA from db/db and db/+ mice treated with spironolactone or vehicle

The loading control β-actin is also shown. Data are expressed as mean ± S.E.M. *$P < 0.05$ compared with db/db vehicle; #$P < 0.05$ compared with db/+ vehicle group; $n = 5$.

Spironolactone decreases collagen deposition in MRA from db/db mice

As shown in Figure 5(A), MRA from vehicle-treated diabetic animals exhibited greater collagen deposition than arteries from the other groups. Spironolactone reduced collagen deposition, both in the adventitia (Figure 5B) and media (Figure 5C) layers, in db/db arteries.

Western blot analysis showed increased mature collagen type I expression in MRA from vehicle-treated db/db mice. Spironolactone treatment effectively decreased collagen I expression to levels observed in control mice (Figure 6).

Mineralocorticoid receptor blockade reduces vascular ROS generation in db/db mice

Basal levels of ROS, analysed by DHE fluorescence intensity, were increased in the aortic wall of vehicle-treated db/db mice. On the other hand, aortas from spironolactone-treated db/db mice displayed a smaller fluorescence intensity, almost similar to that observed in control arteries (Figures 7A and 7B). In agreement with the DHE fluorescence data, expression of NOX1 and NOX4, important sources of ROS in the vasculature, was decreased in MRA from spironolactone-treated db/db mice, with expression levels similar to those observed in MRA from control mice (Figures 8A and 8B). These results indicate that MR blockade by spironolactone effectively reduces oxidative stress in vessels from diabetic mice.

Spironolactone decreases MAPK phosphorylation in MRA from db/db mice

Phosphorylated MAPK proteins expression, ERK1/2 (Thr^{202}/Tyr^{204}; Figure 9A), JNK (Thr^{183}/Tyr^{185}; Figure 9B) and p38 MAPK (Thr^{180}/Tyr^{182}; Figure 9C), determined by Western blot analysis, was increased in MRA from vehicle-treated db/db mice. Treatment with spironolactone decreased MAPKs phosphorylation in db/db mice without affecting expression in MRA from control mice. Representative images are presented in Figure 9(D).

Table 2 **Morphometric parameters in media layer of MRA from db/db and db/+ mice treated with spironolactone or vehicle**
Data were obtained from OCT-embedded, haematoxylin and eosin stained MRA sections.

Parameter	db/+ vehicle	db/+ spironolactone	db/db vehicle	db/db spironolactone
Media CSA (mm^2)	0.034 ± 0.002	0.037 ± 0.002	0.057 ± 0.006*	0.039 ± 0.006†
Media thickness (μm)	24.6 ± 1.6	28.0 ± 1.9	37.8 ± 2.9*	27.0 ± 2.4†
Media:lumen ratio	0.060 ± 0.006	0.061 ± 0.006	0.093 ± 0.009*	0.053 ± 0.005†
Media VSMC nuclei (unit/section)	8.1 ± 1.6	9.8 ± 0.1	16.25 ± 0.5*	9.5 ± 1.4†

Results are expressed as mean ± S.E.M. *$P < 0.05$ compared with db/+ vehicle group, †$P < 0.05$ compared with db/db vehicle group; $n = 5$–7.

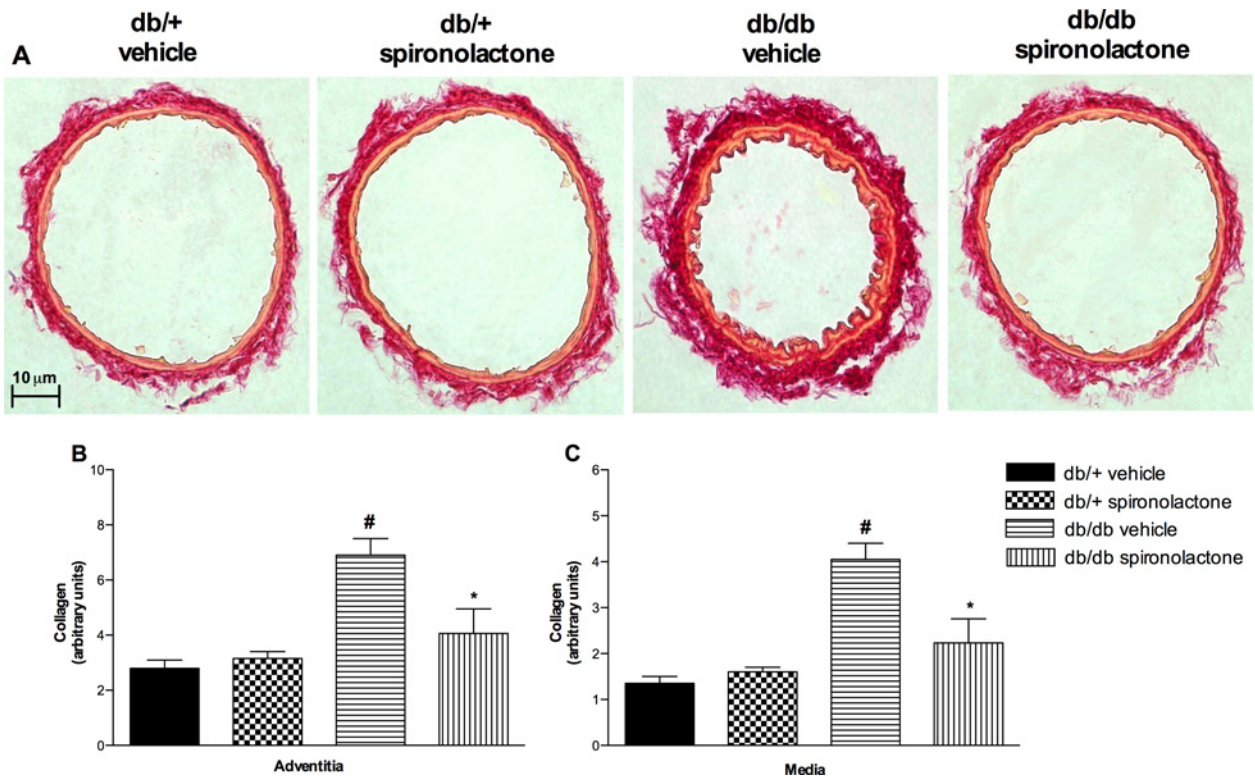

Figure 5 **Representative images (A) and quantitative analysis of collagen content in the adventitia (B) and media (C) layers of MRA from db/db and db/+ mice treated with spironolactone or vehicle**
The transverse sections were stained with Picrosirius Red and the images captured with a light microscope (40× objective). Data are expressed as mean ± S.E.M. *$P < 0.05$ compared with db/db vehicle; #$P < 0.05$ compared with db/+ vehicle group; $n = 5$–7.

DISCUSSION

Major findings in the present study demonstrate that vascular remodelling in diabetic db/db mice is prevented by MR blockade through processes associated with reduced vascular oxidative stress and decreased MAPK signalling. These phenomena were associated with smaller alterations in the vascular mechanical and structural properties, reduced NOX expression, ROS generation and collagen deposition. These data indicate that MR antagonists, such as spironolactone, protect against vasculopathy in DM and may represent an adjuvant therapy in the management of this disease.

Spironolactone, a non-selective MR antagonist in clinical use for several decades, is moderately (~20-fold) more potent than eplerenone (a newer MR antagonist) in competing for MR and binds also to androgen and progesterone receptors [20]. The anti-hypertensive effect of spironolactone is significantly greater than that of eplerenone in patients with primary aldosteronism-associated hypertension [21], demonstrating the importance of MR blockade in this pathological condition. In addition, MR antagonists effectively decrease mortality and morbidity in patients with left-ventricular dysfunction and heart failure after an acute myocardial infarction [22]; prevent salt-induced vascular stiffness, increased media:lumen ratio, collagen content and

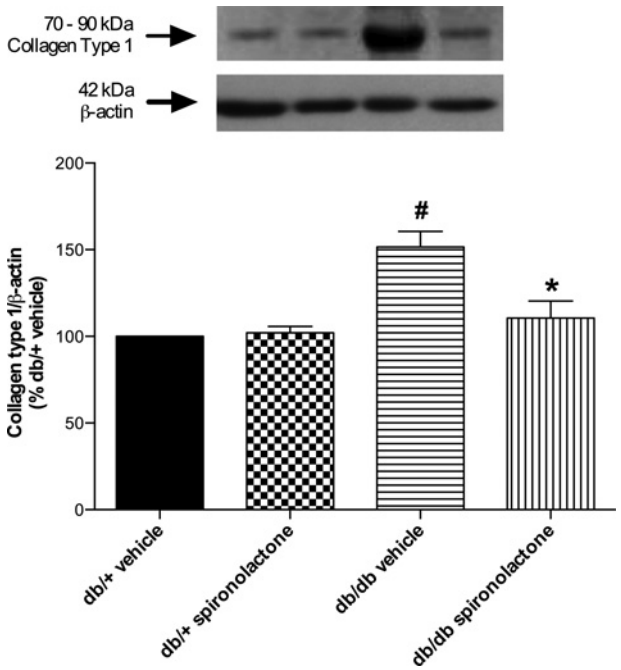

Figure 6 Representative Western blotting images and densitometric analysis of mature collagen type I expression in MRA from db/db and db/+ mice treated with spironolactone or vehicle

The loading control β-actin is also shown. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$ compared with db/db vehicle; $\#P < 0.05$ compared with db/+ vehicle group; $n = 6$.

vascular hypertrophy in Zucker diabetic fatty rats [23]; correct structural and functional abnormalities in mesenteric small arteries from angiotensin II- and aldosterone-infused Sprague–Dawley rats [24,25], indicating that MR blockade attenuates damage in the vascular system [20].

However, the vascular effects of MR blockade in diabetic conditions need to be better comprehended. Thus, the present study addressed the role of MR activation in DM-associated vascular dysfunction or more specifically in DM-associated vascular remodelling, which increases cardiovascular risk and end-organ damage [26]. In this context, we determined structural changes in MRA from db/db and db/+ mice treated with spironolactone or vehicle for 6 weeks.

Serum K^+ was increased in both db/db and db/+ mice treated with spironolactone, which was expected due to the K^+-sparing ability of this drug [20], i.e. MR blockade inhibits Na^+ reabsorption in the distal tubule, increasing the urinary excretion of Na^+ whereas retaining K^+. This clearly shows the efficacy of the treatment. Increased potassium levels were also observed in other studies that evaluated the effects of spironolactone [27–29].

Our data show that db/db mice were more sensitive to spironolactone-induced hyperkalaemia. This phenomenon may be associated with the hyperosmolality of extracellular fluid produced by hyperglycaemia (as is the case for db/db mice), which drives potassium passively out of the cells, favouring the development of hyperkalaemia [30]. Treatment with spironolactone, a potassium-sparing drug, might have potentiated hyperkalaemia in diabetic animals.

As expected, db/db mice exhibited increased body weight and blood glucose, in agreement with previous studies in diabetic mice at the same age [17,19]. Spironolactone treatment did not interfere with body weight gain in the different groups. Other studies have obtained similar results in groups of animals treated with this drug [24,31,32]. However, Armani et al. [33] recently showed that spironolactone, at lower doses (20 mg/kg/day), attenuated body weight gain in a model of high fat diet-induced obesity, indicating that spironolactone may have different pharmacological effects depending on the administered dose.

Spironolactone may increase serum aldosterone levels by reducing sodium concentration in the renal tubules, thus stimulating a compensatory mechanism that activates the renin–angiotensin system [34]. In our study, spironolactone did not change the increased serum aldosterone levels in db/db mice, indicating that beneficial vascular effects of this drug are not due to variations in aldosterone levels. It is possible that spironolactone did not increase serum aldosterone in db/db mice considering that baseline levels of this hormone were already very high in the diabetic group. Likewise, aldosterone levels were unaffected by spironolactone-treatment in other animal studies [24,31,35].

Serum levels of insulin were increased in db/db mice, as already reported [36]. Spironolactone reduced serum insulin levels in diabetic mice, as well as improving insulin sensitivity, as determined by the HOMA index. Several factors are involved in insulin resistance, including oxidative stress. Aldosterone increases systemic and vascular insulin resistance, which is prevented by treatment with a MR antagonist and an antioxidant agent, as well, suggesting that aldosterone via MR and oxidative stress induces insulin resistance [37]. Together, these data indicate that MR blockade may represent an important pharmacological tool for the treatment of hyperinsulinaemia in DM.

Similarly to our study, Wong et al. [19] reported increased total cholesterol and triglycerides in db/db mice. Spironolactone treatment increased total cholesterol in control mice and further augmented cholesterol levels in diabetic mice. In the medical literature, there is little evidence of unfavourable effects of spironolactone on the lipid profile. Nakhjavani et al. [38] noted a significant decline in high-density lipoprotein (HDL) and an increase in low-density lipoprotein (LDL) levels in women with hirsutism after a 3-month treatment with spironolactone. These authors suggested that agonist activity of spironolactone at progesterone receptors may cause progestin-like effects on blood lipids, which can adversely affect the lipid profile.

Inward hypertrophic remodelling, characterized by decreased internal diameter and increased CSA, wall thickness and wall/lumen ratio was observed in MRA from vehicle-treated db/db mice. Katz et al. [39] reported similar results in coronary arterioles from 16-weeks-old db/db mice, associating the structural changes with reduced coronary artery wall stiffness. However, Souza-Smith et al. [17] reported outward hypertrophic remodelling in MRA from db/db mice and speculated that differences in the remodelling processes could be derived from varying degrees of insulin sensitivity, obesity and/or chronic exposure to hyperglycaemia. Regardless of the type of remodelling, it is important to note that structural vascular changes increase the risk of

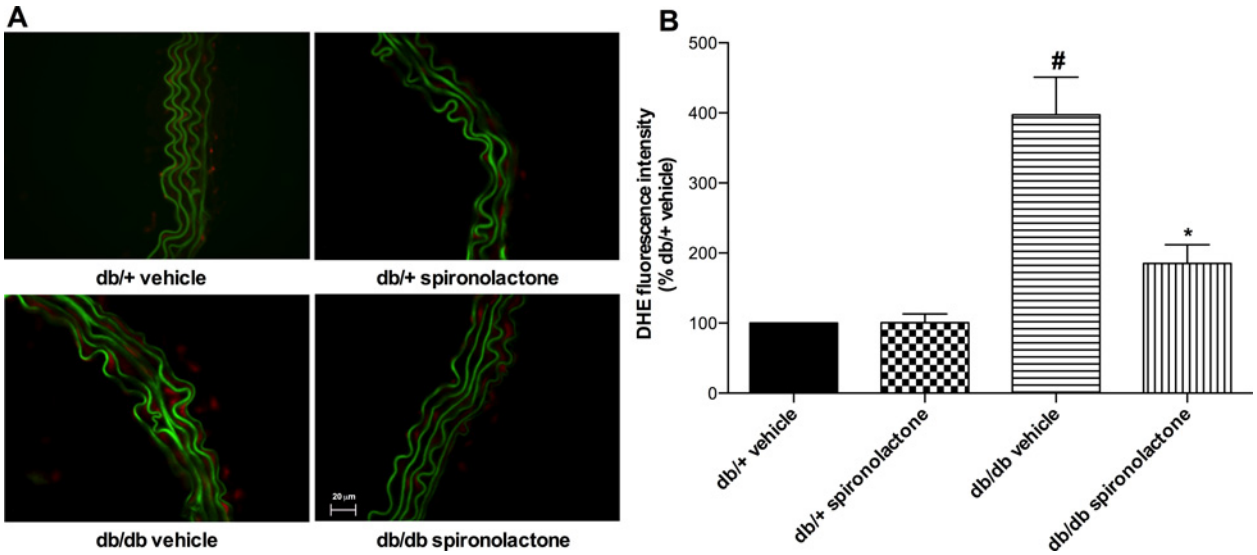

Figure 7 **Representative images (A) and quantification (B) of DHE fluorescence intensity in mouse aortas from db/db and db/+ mice treated with spironolactone or vehicle**

The images of transverse sections were captured with a fluorescence microscope (40× objective). Data are expressed as mean ± S.E.M. $^{*}P < 0.05$ compared with db/db vehicle; $\#P < 0.05$ compared with db/+ vehicle group; $n = 5$–6.

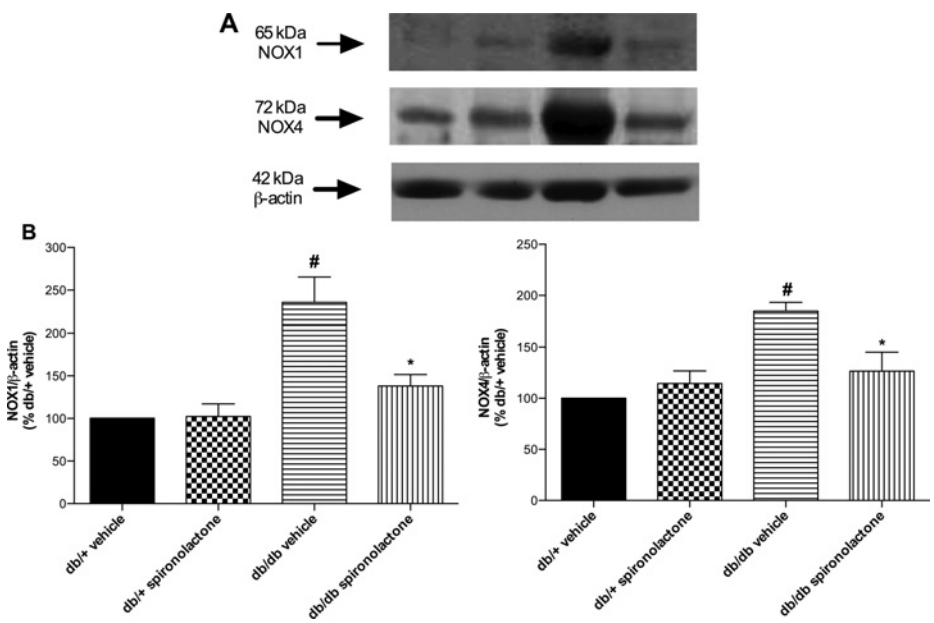

Figure 8 **Representative Western blot images (A) and densitometric analysis (B) of NOX1 and NOX4 protein expression in MRA from db/db and db/+ mice treated with spironolactone or vehicle**

Loading control with β-actin is also shown. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$ compared with db/db vehicle; $\#P < 0.05$ compared with db/+ vehicle group; $n = 5$–6.

adverse cardiovascular events and indicate unfavourable prognosis in type 2 DM [7].

The hypertrophic remodelling in MRA from diabetic mice was confirmed by morphometric analysis of haematoxylin and eosin-stained vascular segments. Proliferation and migration of VSMC play a fundamental role in the media growth of a blood vessel [18]. MRA from vehicle-treated db/db mice exhibited an increased number of nuclei, indicating cell proliferation in these vessels. Indeed, hypertrophic remodelling is a growth-related process and may involve increased number of cells (hyperplasia), increased size of cells (cell hypertrophy), increased deposition of fibrillar or non-fibrillar intercellular matrix or various combinations of these [40]. Analysis of PCNA expression further confirmed increased cell proliferation in MRA from diabetic animals. Although increased levels of PCNA have been used to indicate proliferation or neoplastic transformation, it is important to mention that

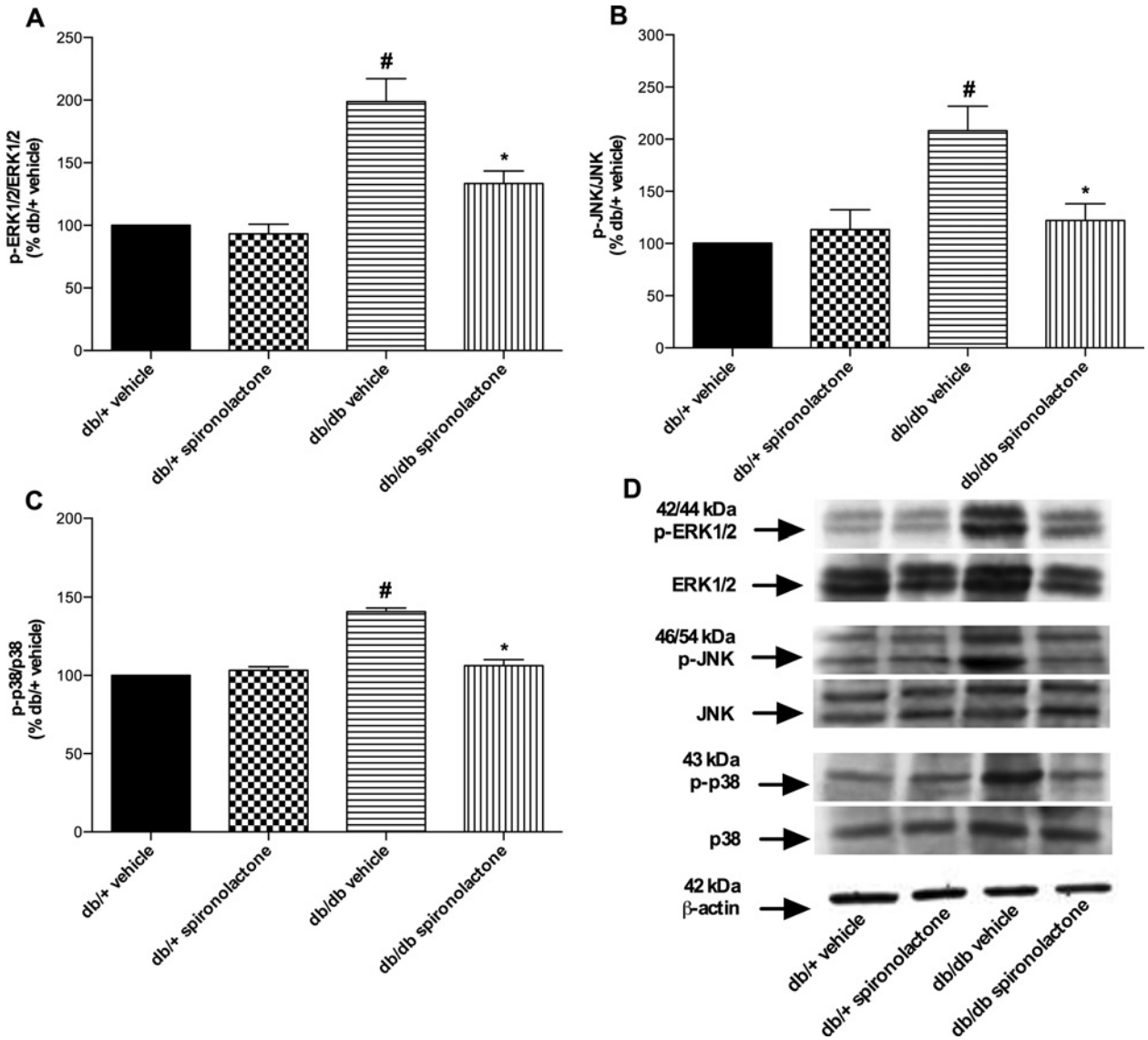

Figure 9 Densitometric analysis of MAPK protein activation: ERK1/2 (A), JNK (B) and p38 MAPK (C) in MRA from db/db and db/+ mice treated with spironolactone or vehicle; representative blots and β-actin are also shown (D)

Results are expressed as mean ± S.E.M. $^{*}P < 0.05$ compared with db/db vehicle; #$P < 0.05$ compared with db/+ vehicle group; $n = 5$–6.

changes in PCNA expression are also associated with other vital cellular processes, such as chromatin remodelling, DNA repair, sister-chromatid cohesion and cell cycle control [41].

Spironolactone significantly prevented these vascular changes, suggesting that MR activation plays an important role in type 2 DM-associated vascular remodelling. Indeed, MR activation by aldosterone has been shown to contribute to vascular growth and remodelling through increased protein synthesis, inflammation and fibrosis [42]. However, these effects are better described in cultured VSMC [43] and in experimental models of arterial hypertension [44] and need to be studied in more detail in animal models of DM.

In addition to preventing structural changes in MRA from db/db mice, spironolactone also improved the vascular mechanical properties, i.e. reduced arterial stiffness, evidenced by an increase in the vascular distensibility, reversion of the leftward shift in the stress–strain relationship and reduction in the β value. Distensibility or compliance corrected for vascular lumen size, depends on the medial mass and intrinsic elastic properties of the vascular wall, and leftward deviations of the stress–strain relationship functionally describe a stiffer, less compliant vessel [17]. In addition, an increased β-value implies in an increased incremental elastic modulus or increased arterial stiffness [14].

Collagen deposition, which contributes to the remodelling process and changes in vascular stiffness, was reduced in MRA from spironolactone-treated db/db mice, as shown by Picrosirius Red staining and Western blotting analyses. Collagen might be located in the intracellular gaps between VSMC, thus explaining

the increased wall thickness in MRA from diabetic mice [19]. The role of collagen in inward hypertrophic remodelling accompanied by impaired distensibility of the MRA from hypertensive rats has been previously demonstrated [16].

ROS regulate vascular function by modulating cell growth, apoptosis, migration, inflammation, secretion and ECM protein production. However, oxidative stress may lead to accelerated proliferation and hypertrophy, further contributing to vascular injury and vascular remodelling [10]. Spironolactone reduced vascular ROS generation in db/db mice, indicating that MR activation-induced ROS may be important players in vascular remodelling observed in this model of type 2 DM. In agreement with these data, NOX1 and NOX4 expression was also restored to control levels in arteries from spironolactone-treated db/db mice. Considering that the NOX family enzymes are a major source of ROS in the vascular system, these results indicate that the beneficial effects of spironolactone may be due to inhibition of NOX activity. The NOX4 isoform is abundantly expressed in both endothelial cells and vascular smooth muscle, whereas NOX1 has been described in VSMC [45]. NOX inhibition is considered a potential therapeutic approach to reduce ROS-derived vascular damage in DM and, as our results indicate, spironolactone appears to be an alternative approach in this context.

Of importance, ROS induce activation of MAPK signalling pathways, possibly by modifying critical amino acid residues in these proteins [46]. The oxidative modification of signalling proteins by ROS, including activation of MAPK pathways, leads to further vascular damage, by enhancing growth, fibrotic and inflammatory processes [10]. Vessels from diabetic animals exhibited increased levels of active redox sensitive-MAPK proteins, ERK1/2, JNK and p38 MAPK. These intracellular signalling proteins are involved in the regulation of profibrotic factors and, hence, in ECM deposition. Increased MAPK phosphorylation was described in other studies where hypertrophic remodelling was reported [16]. By reducing ROS generation, spironolactone may decrease MAPK phosphorylation, reducing the ability of these proteins to induce vascular remodelling in diabetic animals.

In conclusion, the present results demonstrate that spironolactone has beneficial effects in resistance arteries from diabetic mice, reducing vascular remodelling possibly by mechanisms that involve a decrease in oxidative stress and MAPK activation. These indicate that MR activation plays an important role in DM-associated vascular damage.

CLINICAL PERSPECTIVES

- Besides its well-known physiological effects, MR activation, especially by aldosterone, produces cardiovascular system injuries that are part of the pathogenesis of various cardiovascular diseases. Therefore, the characterization of the mechanisms by which MR contribute to diabetic vascular complications will advance knowledge and are essential for the development of new effective anti-diabetic therapies.
- The results of the present study show that MR activation induces oxidative stress and MAPK signalling, resulting in vascular remodelling and fibrosis in diabetic animals. These effects are reduced by MR blockade using spironolactone.
- Spironolactone or other MR antagonists can further improve survival and quality of life in diabetic patients. However, adverse effects of these drugs, such as hyperkalaemia and changes in lipid profile, should be considered.

AUTHOR CONTRIBUTION

All authors contributed to the work presented in the present paper. Marcondes Silva designed and performed all experiments, analysed, interpreted and organized the data and wrote the paper. Stefany Cau, Rheure Lopes, Carla Manzato, Karla Neves, Thiago Bruder-Nascimento, Augusto Montezano, Aurelie Cat and Fabiola Mestriner designed and performed specific experiments and helped in the interpretation of results. Rita Tostes and Rhian Touyz coordinated and mentored the project execution. All authors approved the paper for publication.

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