Antioxidant *N*-Acetylcysteine Protects Pancreatic β-Cells Against Aldosterone-Induced Oxidative Stress and Apoptosis in Female *db/db* Mice and Insulin-Producing MIN6 Cells

Hui Min Jin, Dong Chi Zhou, Hui Fang Gu, Qing Yan Qiao, Shun Kun Fu, Xiao Li Liu, and Yu Pan

Division of Nephrology (H.M.J., D.C.Z., H.F.G., Q.Y.Q., S.K.F.), Shanghai Pudong Hospital, Fudan University, Pudong Medical Center, Shanghai, 201900 China; and Division of Nephrology (X.L.L., Y.P.), Shanghai No.3 People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 201900 China

Previous studies have shown that primary aldosteronism is associated with glucose-related metabolic disorders. However, the mechanisms by which aldosterone (ALDO) triggers β-cell dysfunction remains unclear. This study aimed to investigate whether oxidative stress is involved in and whether the antioxidant *N*-acetylcysteine (NAC) or the mineralocorticoid receptor antagonist spironolactone (SPL) could prevent or delay β-cell damage in vivo and in vitro. As expected, 8 weeks after ALDO treatment, 12-week-old female diabetic *db/db* mice exhibited impaired oral glucose tolerance, decreased β-cell mass, and heightened levels of oxidative stress marker (urinary 8-hydroxy-2′-deoxyguanosine). NAC reversed these symptoms completely, whereas SPL treatment did so only partially. After exposure to ALDO, the mouse pancreatic β-cell line MIN6 exhibited decreased viability and increased caspase-3 activity, as well as reduced expression of Bcl-2/Bax and p-AKT, even if mineralocorticoid receptor was completely suppressed with small interfering RNA. NAC, but not SPL, suppressed oxidative stress in MIN6 cells, as revealed by the decrease in inducible NOS levels and expression of the proteins p22-phox and p67-phox. These findings suggest that oxidative stress may be involved in ALDO-induced β-cell dysfunction and that NAC, but not SPL, may protect pancreatic β-cells of mice from ALDO-induced oxidative stress and apoptosis in a manner independent of its receptor. **(*Endocrinology* 154: 4068–4077, 2013)**

Aldosterone (ALDO), a mineralocorticoid hormone, is typically synthesized in the adrenal zona glomerulosa by aldosterone synthase. ALDO exerts its effects through the mineralocorticoid receptor (MR), which is widely expressed in polarized epithelial tissues (ie, kidney, colon, lung, salivary and sweat glands, and liver) and nonepithelial tissues (ie, heart, vasculature, brain, and adipocytes) (1). Recent studies have found that activation of ALDO and MR enhances the generation of oxygen free radicals in tissues and causes systemic inflammation, which, in turn, contributes to impaired insulin metabolic signaling (2). Insulin resistance progresses to overt type 2 diabetes when pancreatic β-cells can no longer secrete sufficient insulin to maintain normoglycemia (3). Elevated plasma ALDO levels are associated with lower pancreatic β-cell function and impairs first-phase insulin secretion in primary aldosteronism (4, 5). Blocking ALDO with spironolactone (SPL), an MR antagonist, significantly reduces ALDO-induced tissue injury and consequent mortality (6, 7). However, in a recent study, Luther et al (8) observed that ALDO decreased glucose-stimulated insulin secretion in mice in vivo and in murine islets in an MR-independent manner. Thus, other strategies can be considered to interfere with the direct effects of ALDO on pancreatic β-cells.

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received February 1, 2013. Accepted August 26, 2013.
First Published Online September 5, 2013

Abbreviations: ALDO, aldosterone; BP, blood pressure; FBG, fasting blood glucose; FBS, fetal bovine serum; iNOS, inducible NOS; MR, mineralocorticoid receptor; NAC, *N*-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; siRNA, small interfering RNA; SPL, spironolactone.

Taking into account that oxidative stress is likely involved, we hypothesize that the antioxidant *N*-acetylcysteine (NAC) may prevent ALDO-induced malfunction of pancreatic islet cells and β-cells. Although NAC has been proven to prevent hyperglycemia- (9), fructose- (10), and sucrose- (11) induced insulin resistance, no studies have been conducted to investigate the relationship between ALDO and NAC in insulin resistance.

Materials and Methods

Animal models and experimental design in vivo

Eight-week-old female *db/db* mice and background control mice (C57BLKS/J) were purchased from SLAC Laboratories. The *db/db* mice were housed under controlled light (12 hours light/12 hours dark) and temperature conditions and had free access to food (normal rodent chow, 1% salt) and distilled water. All procedures were conducted in accordance with the guidelines of the Chinese Council on Animal Care and were approved by Fudan University and the Animal Care Committee of Shanghai Jiao Tong University.

All experiments involving *db/db* mice were performed when the mice were 12 weeks of age, after determining glucose concentrations from fasting blood samples to ensure that the mice were diabetic. Mice were separated into the following treatment groups (n = 6, each): 1) Nontreated *db/db* (0.9% saline); 2) ALDO (Sigma-Aldrich; 20 mg/kg/d); 3) SPL (Merck; 30 mg/kg/d in drinking water) (12); 4) NAC (40 mM in drinking water); 5) ALDO (20 mg/kg/d) + SPL (30 mg/kg/d in drinking water); and 6) ALDO (20 mg/kg/d) + NAC (40 mM in drinking water). ALDO (40% solution) was administered to mice for 56 days through an osmotically driven infusion minipump (Alzet 2ML; Durect Corp.) implanted sc on the back. This pump released a continuous small dose of ALDO into the body at a rate of 2.5 mL/h, which was equal to 20 mg/kg/d (13). No inflammatory infiltration or response was observed in the animals that were implanted with the infusion pump. NAC was dissolved in the drinking water to a final concentration of 40 mM (14), prepared fresh from the chemical (catalog no. A9165; Sigma) and replaced once a week. Treatment lasted 8 weeks in all cases.

Fasting glycemic and plasma insulin levels during oral glucose tolerant test

Mice were subjected to oral glucose tolerant test at days 0, 28, and 56. Briefly, mice were made to fast for 15 hours, and a basal blood sample (20 μL) was collected from the tail vein (t = 0). Blood glucose levels were determined by using the One Touch Basic glucose meter (Lifescan Canada). Then, mice were gavaged with glucose (1.59 g/kg), and additional blood samples were collected at 0, 30, 60, 90, 120, and 150 minutes for glucose measurements. Plasma insulin was determined using a mouse ELISA kit (Millipore Corp.).

Plasma levels of ALDO, urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG), and Na^+/K^+

Plasma samples were collected from C57BL and *db/db* mice that were submitted to treatment with ALDO plus SPL or ALDO plus NAC. Plasma ALDO levels were measured by an ELISA kit (Diagnostic Biochem Canada). Individual mice were placed in metabolic cages for 24-hour urine collection. The urine samples were stored at −80°C until analysis. Excretion of urinary 8-OHdG was measured for 24 hours using an ELISA kit (USCN Life). Concentrations of Na^+ and K^+ in urine were measured using the Automatic Biochemistry Analyzer Beckman LX20 (Beckman).

Pancreatic islet isolation and β-cell mass analysis

Pancreatic islets (120–140) were isolated from each mouse with collagenase (Sigma-Aldrich) digestion and purified using different densities of Ficoll solution (29%, 24%, and 15% [wt/vol] mixed with Hanks' balanced salt solution). After centrifugation for 30 minutes, the layer between the 24% and 15% densities was collected and rinsed twice with cold Hanks' balanced salt solution. Islets were handpicked and placed in RPMI 1640 culture medium containing 11 mM glucose, which was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 60 μg/mL penicillin G and 50 μg/mL streptomycin sulfate, for overnight recovery at 37°C and 5% CO_2. The next day, islets were transferred to a similar culture medium, but supplemented with 1% FBS, and in the absence or presence of 0.5 mM palmitate complexed with 0.5% fatty acid-free BSA. Primary islets were collected to isolate total protein for Western blot analysis.

Mice were anesthetized, and the pancreas was immediately dissected and placed in Bouin's fixative. Pancreatic sections (4 μm) were processed as previously described (13). Briefly, the sections were incubated overnight at 4°C with guinea pig anti-insulin antibody. β-Cell mass from the insulin antibody-stained sections was evaluated by point-counting morphometry. Images were acquired using a Nikon (ECLIPSE-E1000) microscope connected to a video camera equipped with a color monitor and ImagePlus software.

Murine MIN6 cell culture

The pancreatic MIN6 β-cell line was a gift from the Institute of Endocrinology of Rui Jin Hospital affiliated with the Shanghai Jiao Tong University School of Medicine (Shanghai, China). MIN6 cells (20–30 passages) were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 U/mL penicillin with 0.1 mg/mL streptomycin. Cells were grown to 75% confluence and passaged every 3 days. MIN6 cells were treated with ALDO (0 nM, 10 nM, 100 nM, 1 μM, and 10 μM) and/or SPL (100 nM) or NAC (1 mM) over 12, 18, and 24 hours.

Assessment of MIN6 β-cell viability and apoptosis pathways

Cells were cultured in medium containing 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich) for an additional 4 hours. Cells were then incubated with solubilization solution (10% SDS and 5% isopropanol in 0.012 M HCl) at 37°C in humidified air with 5% CO_2 overnight. The absorbance of the supernatant was measured at 570 nm using an automated microtiter plate reader. Data are expressed as the mean percentage of viable cells vs control.

The caspase-3 activity assay was used to detect cell apoptosis. In brief, treated cells were washed once with ice-cold PBS and

assayed for caspase-3 activity by using a colorimetric assay (Sigma). Cleavage of caspase-3 colorimetric substrate Ac-DEVD-pNA (Sigma) substrate by caspase-3 releases pNA, which was quantified spectrophotometrically at 405 nm using an ELISA reader. The change in optical density is directly proportional to caspase-3 activity.

Evaluation of MR mRNA expression in islets and MIN6 cells by real-time PCR

Total RNA from islets and MIN6 cells was isolated using Trizol reagent (Invitrogen) according to manufacturer's instruction. Total RNA was quantified by absorbance at 260 nm. Primers for MR (5′-GACCTTGGAGCGTTCTTC-3′ [sense]; 5′GACCGACTATTGTCTTGC3′ [antisense]) were used to detect MR transcripts. mRNA was extracted using the RNAeasy mini kit (QIAGEN Science), and cDNA was synthesized from 1.5 μg RNA with the first-strand cDNA synthesis kit (Amersham). Each sample was run and analyzed in triplicate. β-Actin was used as an internal control. Real-time PCR was performed in an ABI 7500 System (Applied Biosystems) by using the SYBR Premix Ex Taq PCR kit (TaKaRa). Real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method with the SDS Software package (Applied Biosystems).

Western blot analysis

Samples of total protein extracted from nontreated and treated islets or MIN6 cells were subjected to SDS-PAGE (50 μg protein per sample). After removal from the medium, the islets and MIN6 cells were washed 3 times with ice-cold PBS and then lysed using lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride). The protein was boiled for 10 minutes and separated via 10% SDS-PAGE. The separated protein was then transferred onto a nitrocellulose membrane. Next, the membranes were incubated for 2 hours at room temperature in a mixture of blocking buffer (TBST consisting of 10 mmol/L Tris-HCl [pH 7.4], 100 mmol/L NaCl, and 0.1% Tween 20) and 5% nonfat powdered milk to prevent binding to nonspecific sites. Then, the membranes were incubated with the following primary antibodies: MR (Santa Cruz Biotechnology; 1:500), Bax (Santa Cruz; 1:1000), Bcl-2 (Santa Cruz; 1:500), AKT (Santa Cruz; 1:1000), p-AKT (Santa Cruz; 1:500), inducible NOS (iNOS) (Santa Cruz; 1:1000), $p22^{phox}$ (Santa Cruz; 1:1000), $p67^{phox}$ (Santa Cruz; 1:1000) and β-actin (1:1000). After 3 washes with TBST, the second antibody was added (1:2000 dilution of a horseradish peroxidase-conjugated antirabbit IgG), followed by a 2-hour incubation at room temperature. After 3 more washes with TBST, the protein bands were visualized using BeyoECL Plus, and densities were determined with a fluorescence scanner (Bio-Rad Laboratories).

Transfection of MIN6 cells with small interfering RNA (siRNA) against MR

MIN6 cells were transfected with siRNA against MR (Santa Cruz) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. In short, MIN6 cells were plated onto 6-well plates and cultured until they were 80% confluent. The cells were then transfected with 100 nM MR siRNA or control siRNA in a serum-free medium for 6 hours. Growth medium consisting of more than 20% FBS was then added, and the levels of mRNA and protein expression were measured at 48 and 72 hours after transfection.

Statistical analysis

The SAS 8.13 statistical package (SAS Institute) was used for all analyses. Data were presented as mean ± SE for all groups, with the exception of skewed data. Statistical analysis was performed using a one-way ANOVA. For all comparisons, results were considered statistically significant at $P < .05$.

Results

Plasma ALDO level, urinary Na^+:K^+ ratio, and body weight

Table 1 summarizes the data of ALDO levels in plasma, urinary Na^+:K^+ ratio, and body weight. The body weight of *db/db* mice was significantly higher than that of C57BL mice. Administration of ALDO, with or without SPL, increased body weight in all treated *db/db* mice. Mice treated with NAC, however, had a lower body weight when compared with other treated *db/db* mice. ALDO level was somewhat higher in the plasma of *db/db* mice than that in C57BL mice. Administration of SPL alone had no statistical effect on the ALDO level in plasma with 4 weeks of treatment (546 ± 21 vs 554 ± 24; $P > .05$), but ALDO levels increased to 930 ± 22 (vs 650 ± 18; $P < .01$) after 8 weeks (Table 1). On the other hand, NAC treatment was observed to lower the ALDO levels in *db/db* mice after 4- and 8-week treatments. Compared with the urinary Na^+:K^+ ratio in nontreated *db/db* mice, the ratio in mice treated with ALDO alone reduced after 8 weeks and that in mice treated with SPL alone increased. Treatment with NAC alone had no effect on the urinary Na^+:K^+ ratio in *db/db* mice. During the period of observation, ALDO and SPL had no effects on blood pressure (BP) (data not shown).

Effect of ALDO and/or SPL and NAC on glucose metabolism in *db/db* and C57BL mice

Compared with control mice, 8-week old *db/db* diabetic mice exhibited significantly higher blood glucose levels, which consistently increased throughout the study period (Table 1). Fasting blood glucose (FBG) was further increased in ALDO-treated *db/db* mice compared with the nontreated *db/db* mice. Decreasing FBG was observed at 4 weeks of treatment with SPL alone, but after another 4 weeks of treatment, FGB level increased noticeably. However, treatment of *db/db* mice with NAC significantly improved the glucose tolerance observed at 4 and 8 weeks, resulting in significantly lower FGB and fasting insulin. The mean value of the area under the curve (AUC) for the glucose tolerance test was also significantly lower in

Table 1. Plasma ALDO Level, Urinary Na^+:K^+ Ratio, and Body Weight at 4 and 8 Weeks of Treatment

	C57BL Nontreated	*db/db* Nontreated	*db/db* ALDO	*db/db* SPL	*db/db* NAC	*db/db* ALDO+SPL	*db/db* ALDO+NAC
Body weight (g)							
8 wk	26.1 ± 1.9	38.3 ± 2.5[a]	40.7 ± 3.6[b]	39.5 ± 2.6	32.2 ± 3.3[c,d]	39.1 ± 5.2[e]	35.8 ± 5.1[c,f]
Plasma ALDO (pg/mL)							
4 wk	120 ± 12	554 ± 24[a]	1220 ± 34[d]	546 ± 21	487 ± 33[c,d]	712 ± 25[c,g]	723 ± 31[c,f,g,h]
8 wk	118 ± 13	650 ± 18[a]	1340 ± 32[d]	930 ± 22[d]	465 ± 23[c,d]	1600 ± 46[g,h]	643 ± 17[c,g,h,i]
Urinary Na^+:K^+ ratio							
8 wk	0.61 ± 0.01	0.54 ± 0.02[a]	0.33 ± 0.03[d]	0.82 ± 0.02[c,d]	0.60 ± 0.02[c]	0.76 ± 0.04[g]	0.40 ± 0.02[e,g,h,i]
Fasting plasma glucose (mM)							
4 wk	6.8 ± 1.5	12.1 ± 2.9[a]	19. 3 ± 3.8[d]	9.4 ± 3.3[c,d]	7.4 ± 5.9[c,d]	9.5 ± 5.5[e,g]	7.5 ± 5.7[c,g,h]
8 wk	6.5 ± 1.7	15.1 ± 4.0[a]	20.1 ± 3.6[d]	19.6 ± 4.5[b]	9.5 ± 6.4[c,d]	19.4 ± 3.8[g]	12.1 ± 5.4[c,g,h,i]
Fasting plasma insulin (ng/mL)							
4 wk	2.4 ± 0.9	4.6 ± 1.2[a]	3.5 ± 3.9[d]	3.4 ± 1.3[b]	3.1 ± 1.5[b,e]	5.4 ± 6.1[c,g,h]	5.1 ± 5.6[c,f,g,h]
8 wk	2.3 ± 1.1	4.6 ± 1.4[a]	15.4 ± 1.8[d]	14.3 ± 3.4[d]	3.5 ± 1.2[c,d,f,g]	13.2 ± 1.2[g]	4.5 ± 1.2[c,g,h,i]
AUC (glucose)							
4 wk	2100 ± 21	5002 ± 46[a]	5500 ± 35[b]	4230 ± 36[d,e]	3150 ± 29[c,d]	4100 ± 72[e,g]	3500 ± 41[c,f,g,h]
8 wk	2050 ± 23	5300 ± 51[a]	5950 ± 43[d]	5840 ± 56[d]	4330 ± 52[c,d]	5670 ± 63[g]	4930 ± 71[c,g,h,i]
Urinary 8-OHdG (ng/d)							
4 wk	12 ± 1	151 ± 12[a]	244 ± 22[d]	141 ± 13[b,c]	23 ± 9[c,d]	235 ± 31[c,g,h]	180 ± 18[c,g,h,i]
8 wk	15 ± 2	162 ± 11[a]	291 ± 12[d]	153 ± 14[c,d]	51 ± 11[c,d]	213 ± 41[c,g,h]	170 ± 31[c,g,h,i]

[a] $P < .01$ vs C57BL nontreated; [b] $P < .05$ and [d] $P < .01$ vs nontreated *db/db*; [e] $P < .05$ and [c] $P < .01$ vs ALDO-treated *db/db*; [f] $P < .05$ and [i] $P < .01$ vs ALDO + SPL treated *db/db*; [h] $P < .01$ vs SPL treated *db/db*; [g] $P < .01$ vs NAC treated *db/db*.

AUC, area under the curve. n = 6, values as means ± SE.

the NAC-treated *db/db* mice than that in mice treated with ALDO alone, SPL alone, and ALDO + SPL. Treatment with ALDO alone, SPL alone, and ALDO + SPL all showed increased fasting plasma insulin levels at 8 weeks. In contrast, treatment with NAC alone and ALDO + NAC was observed to decrease fasting plasma insulin levels.

Effect of ALDO and/or SPL and NAC on excretion of urinary 8-OHdG in *db/db* and C57BL mice

As shown in Table 1, ALDO treatment increased 8-OHdG excretion in the 4-week and 8-week periods. In comparison, 8-OHdG excretion was lower with SPL treatment and was even more reduced with NAC treatment. 8-OHdG excretion was also significantly lower in the ALDO + NAC treatment group than that in the ALDO + SPL group.

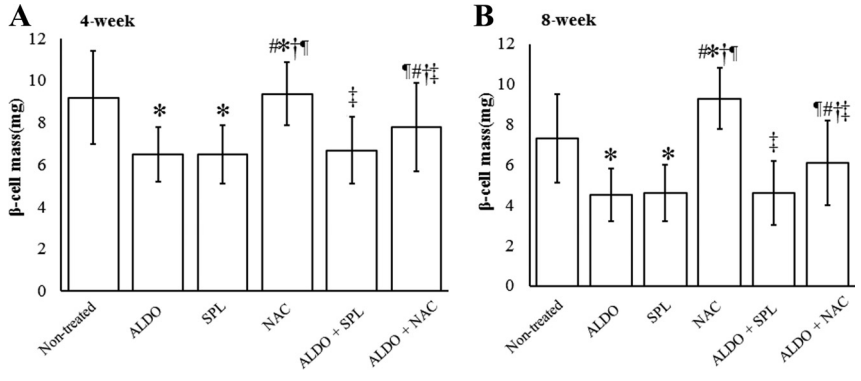

Figure 1. Islet β-cell mass in *db/db* mice after 4 (A)- and 8-week (B) treatment. *, $P < .05$ vs nontreated *db/db* mice; #, $P < .05$ vs ALDO-treated *db/db* mice; †, $P < .01$ vs SPL-treated *db/db* mice; ‡, $P < .01$ vs NAC-treated *db/db* mice; $P < .05$ vs ALDO + SPL-treated *db/db* mice. Values are means ± SE (n = 3/group).

Effects of ALDO, SPL, and NAC on β-cell mass of *db/db* mice

The mass of islet β-cells was determined in pancreas sections immunostained for insulin. After 4 weeks (Figure 1A) and 8 weeks (Figure 1B) of treatment, the β-cell mass in ALDO-treated *db/db* mice was significantly lower than that in nontreated *db/db* mice. NAC treatment appeared to maintain the β-cell mass better than treatment with ALDO or SPL alone. Treatment with ALDO + NAC also appeared to reduce the β-cell mass less than treatment with ALDO + SPL (n = 5).

MIN6 cell viability and caspase-3 activity after exposure to ALDO, SPL, and NAC

Cell viability and caspase-3 activity were used to test the potential effects of ALDO, SPL, and NAC on MIN6 β-cell apoptosis. As shown in Figure 2A, cell viability was decreased to 65.2% ± 9.1% ($P < .01$ vs 0 nM), 47.2% ± 6.1% ($P < .01$ vs 10 nM), 45.4% ± 5.3%, and 45.1% ± 5.1% after treatment with 10 nM, 100 nM, 1 μM, and 10 μM ALDO for 24 hours, respectively. One hundred nanomoles ALDO produced the maximal decrease in cell viability. Figure 2B indicates that both ALDO alone and SPL alone could inhibit cell viability. In contrast, NAC increased cell viability ($P < .01$ vs ALDO alone and SPL alone). Furthermore, cell viability was higher in the ALDO + NAC treatment group

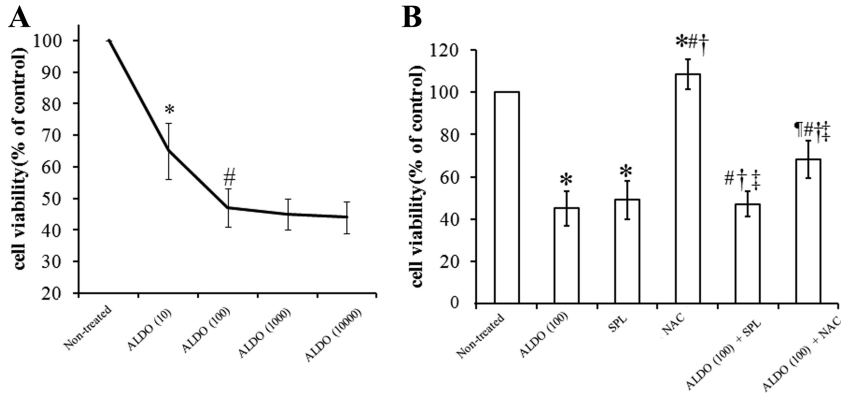

Figure 2. ALDO dose (nM)-response curve on MIN6 cell viability and the effect of ALDO and/or SPL and NAC on MIN6 cell viability. A, ALDO dose (nM)-response curve on MIN6 cell viability. MIN6 cell vitality was assessed with a MTT. One hundred nanomoles ALDO produced the maximal decrease in cell viability. *, $P < .05$ vs nontreated MIN6 cells; #, $P < .01$ vs ALDO-treated MIN6 cells. B, The effect of ALDO and/or SPL and NAC on MIN6 cells viability after exposure of 24 hours. *, $P < .05$ vs nontreated MIN6 cells; #, $P < .05$ vs ALDO-treated MIN6 cells; †, $P < .01$ vs SPL-treated MIN6 cells; ‡, $P < .01$ vs NAC-treated MIN6 cells; ¶, $P < .05$ vs ALDO + SPL-treated MIN6 cells. Values are means ± SE (n = 3/group).

than that in the ALDO + SPL group ($P < .01$).

Figure 3 presents the caspase-3 activity, as determined through ELISA. Treatment with 100 nM ALDO for 24 hours significantly increased the rate of apoptosis to twice the rate in nontreated cells. Simultaneous treatment with SPL (100 nM) also increased caspase-3 activity. In contrast, treatment with NAC inhibited caspase-3 activity. Caspase-3 activity was also lower with ALDO + NAC treatment than with ALDO + SPL treatment. No significant difference was observed in the caspase-3 activities of the groups treated with ALDO alone and SPL alone.

NAC induces Bcl-2 expression and AKT phosphorylation in ALDO-treated islet and MIN6 cells

To further determine whether NAC inhibits ALDO-induced β-cell apoptosis, we assessed the expression level of proapoptotic genes by determining the levels of Bax and pAKT/AKT. As shown in Figure 4B (isolated islets) and Figure 5B (MIN6 cells), Bcl-2/Bax expression decreased in the group treated with ALDO ($P < .01$ vs nontreated group). However, Bcl-2/Bax increased significantly after treatment with NAC. Further study demonstrated that phosphorylation of AKT kinase (Figures 4C and 5C) was lower after exposure to ALDO, and the ratio of pAKT:AKT was 70% lower than that of the nontreated group ($P < .01$). However, the ratio of pAKT:AKT obtained with NAC treatment was higher than that obtained with ALDO treatment ($P < .01$).

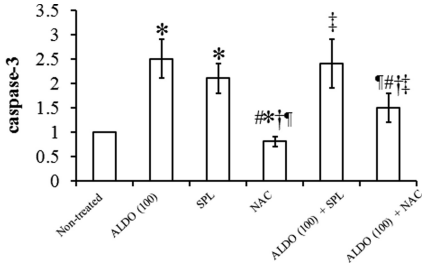

Figure 3. Effect of ALDO and/or SPL and NAC on caspase-3 activity in MIN6 cells. Caspase-3 activity in MIN6 cell was assessed through ELISA. MIN6 β-cells were exposed to ALDO and/or SPL, NAC over 24 hours. *, $P < .05$ vs nontreated MIN6 cells; #, $P < .05$ vs ALDO-treated MIN6 cells; †, $P < .01$ vs SPL-treated MIN6 cells; ‡, $P < .01$ vs NAC-treated MIN6 cells; ¶, $P < .05$ vs ALDO + SPL-treated MIN6 cells. Values are means ± SE (n = 3/group) (ANOVA followed by Tukey's post hoc test).

Accumulation of reactive oxygen species due to ALDO-induced β-cell damage in islet and MIN6 cells

To determine the potential mechanism of ALDO-induced β-cell damage, we examined iNOS expression and nitric oxide (NO) production (determined by the nitrate reductase method). As shown in Figure 4D (isolated islets) and Figure 5D (MIN6 cells), iNOS protein expression increased after ALDO treatment ($P < .01$), but this expression was inhibited by NAC. Furthermore, compared with nontreated controls ($P < .01$), cells treated with ALDO showed significantly higher NO release (Figures 4E and 5E).

ALDO-induced overexpression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in islet and MIN6 cells

Elevated levels of NADPH oxidases such as p22phox and p67phox are one of the major factors contributing to oxidative stress. Given that oxidative stress may lead to β-cell dysfunction, we sought to determine whether ALDO induces overexpression of NADPH oxidase. As shown in Figure 4 (isolated islets) and Figure 5 (MIN6 cells), the expression of p22phox (Figures 4F and 5F) and p67phox (Figures 4G and 5G) increased following ALDO treatment ($P < .01$). However, this effect was diminished by NAC treatment.

MR mRNA and protein expression in islets and MIN6 cells

MR protein and mRNA expression levels were determined using Western blot (Figure 6, A–C) and real-time PCR (Figure 6, D and E). The isolated islets of C57BL and MIN6 cells cultured at 0 and 24 hours in a nonstimulated

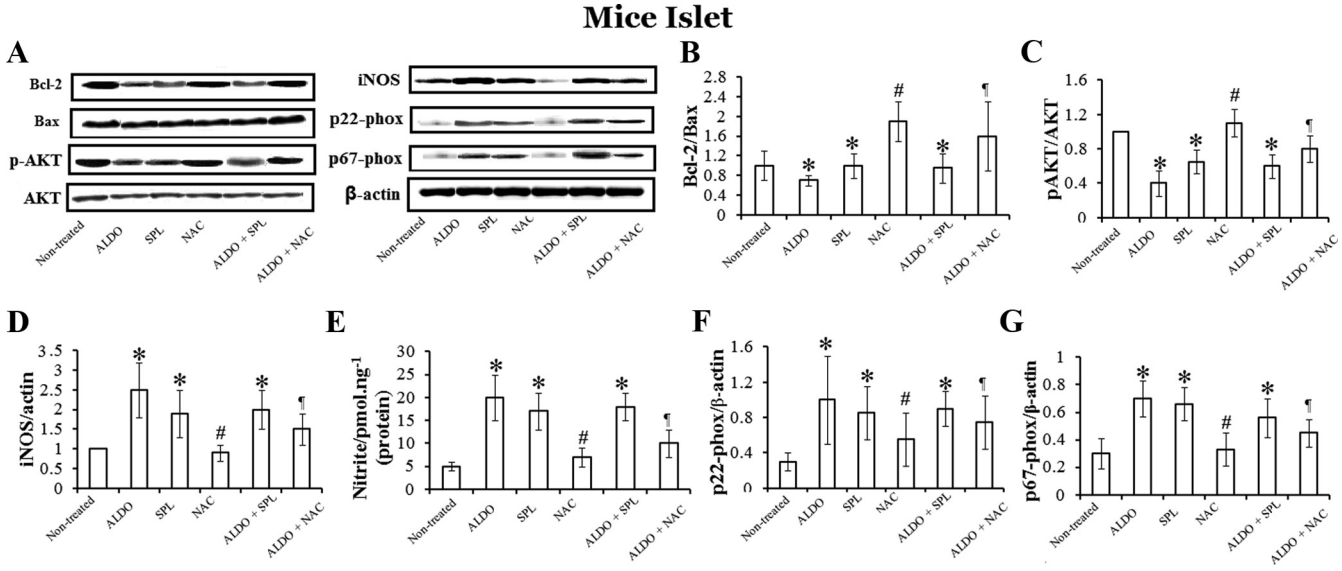

Figure 4. Effects of ALDO and/or SPL and NAC on the expression of Bcl-2/Bax (B), p-AKT/total AKT protein (C), iNOS (D), nitrite (E), and NADPH oxidase p22phox (F) and p67phox (G) in *db/db* mice islet. Diabetic *db/db* mice received ALDO (20 mg/kg/d), SPL (30 mg/kg/d in drinking water), NAC (40 mM), ALDO + SPL, and ALDO + NAC for 4 weeks. ALDO treatment increased the levels of p22phox, p67phox, and iNOS expression and decreased the expression of Bcl-2/Bax and pAKT/AKT. However, the SPL had no effect; NAC inhibited the effects of ALDO. *, $P < .05$ vs nontreated *db/db* mice; #, $P < .01$ vs ALDO alone treated *db/db* mice; $P < .05$ vs Aldo + SPL-treated *db/db* mice (n = 3/group). Data are presented as mean ± SE.

state showed low MR expression at both mRNA and protein levels at 4 and 8 weeks. In contrast, high MR expression was observed at the same point in nontreated *db/db* mice.

ALDO-induced dysfunction in β-cells is not mediated by MR

To determine whether the effects of ALDO are mediated through MR in MIN6 cells, siRNA specific for MR

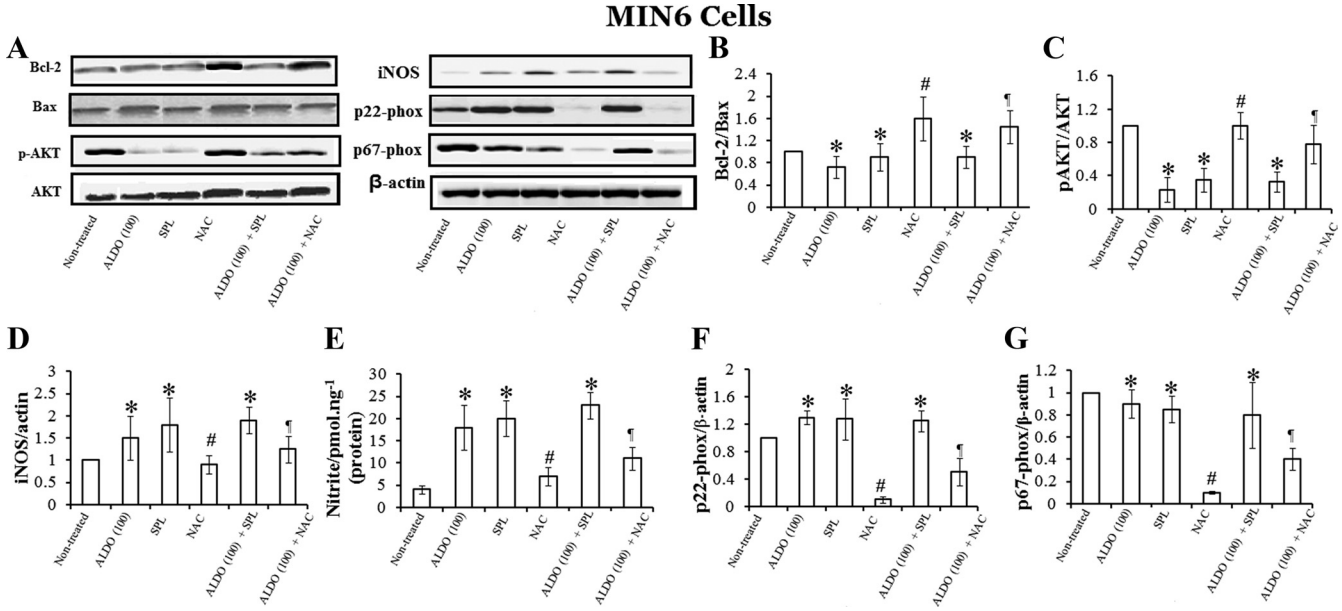

Figure 5. Effects of ALDO and/or SPL and NAC on the expression of Bcl-2/Bax (B), p-AKT/total AKT protein (C), iNOS (D), nitrite (E), and NADPH oxidase p22phox (F) and p67phox (G) in MIN6 cells. MIN6 β-cells were exposed to nontreatment, ALDO (100 nM), SPL (100 nM), NAC (1 mM), ALDO + SPL, and ALDO + NAC over 24 hours. ALDO increased the levels of iNOS expression and decreased the expression of Bcl-2/Bax and pAKT/AKT. Furthermore, ALDO increased the expression of oxidase p22phox and p67phox. However, the effects of ALDO were inhibited by NAC, but not by the ALDO antagonist. *, $P < .05$ vs nontreated MIN6 cells; #, $P < .01$ vs ALDO alone-treated MIN6 cells; $P < .05$ vs Aldo + SPL-treated MIN6 cells (n = 3/group). Data are presented as mean ± SE.

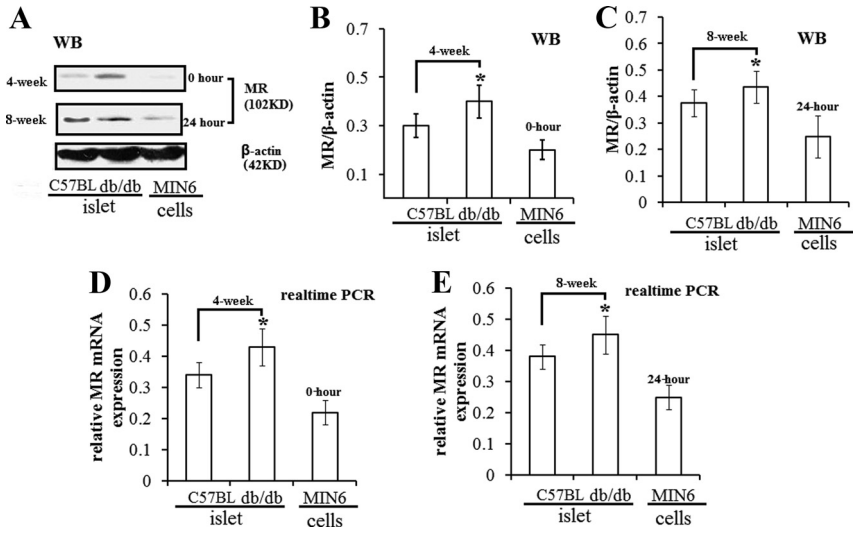

Figure 6. Presence of MR in mice islet and MIN6 β-cells. MR protein expression (A–C) and expression levels of MR mRNA (D and E) relative to β-actin were high in *db/db* mice islets but weak in C57BL mice islets and MIN6 β-cells. *, $P < .01$ vs C57BL mice islet (n = 5/group). Values are means ± SE (ANOVA followed by Tukey's post hoc test). WB, Western blot.

was used to silence endogenous MR expression. As shown in Figure 7, Western blot analysis revealed that, unlike control siRNA, siRNA specific for MR significantly reduced MR expression. Treatment with 100 nM ALDO or 1 mM NAC for 24 hours could not alter the expression of MR after the inhibition of MR expression with MR siRNA. However, MR siRNA-inhibited MR expression could not prevent ALDO-induced activation of iNOS and the decrease in cell viability. Treatment with 1 mM NAC for 24 hours significantly inhibited the expression of iNOS and increased cell viability ($P < .01$). These results suggest that activation of iNOS pathways may not be completely involved in ALDO/MR signal transduction in MIN6 cells.

Discussion

Diabetes mellitus is a worldwide epidemic affecting more than 150 million people worldwide, a number expected to double by 2025 (15). The cardiovascular consequences of diabetes mellitus, especially coronary heart disease, make prevention of diabetes and its vascular complications of paramount public health significance. Recent studies demonstrate that higher levels of plasma aldosterone, a physiological ligand of the MR, precede the development of glucose intolerance and insulin resistance in patients with metabolic syndrome (5, 16, 17) and that aldosterone may directly interfere with insulin signaling by down-regulating insulin receptor substrate-1 in vascular smooth muscle cells (19). In this study, we also found that ALDO levels were significantly elevated in female diabetic *db/db* mice compared with the levels in nondiabetic C57BL mice. Additional exogenous ALDO treatment increased ALDO levels further and impaired glucose tolerance and plasma insulin levels, which was consistent with the findings of a previous report (8). This may also be suitable for the male mice because previous studies have reported similar weight and glucose levels of diabetic male and female mice (20), and male and female *db/db* mice developed similar levels of type 2 diabetes, which indicated no difference between female and male *db/db* mice to occur in type 2 diabetes. Thus, blocking the action of ALDO with an MR antagonist (ie, SPL) is thought to improve systemic insulin sensitivity, insulin signaling, and insulin-stimulated glucose uptake (21), which would provide clinical benefits for diabetic patients. In this study, we observed that SPL was effective in reducing FBG concentration and glucose-induced insulin release and proinsulin biosynthesis after 4 weeks of treatment. However, an 8-week treatment with SPL reversed the above phenomenon, which may be in association with a higher blood level of ALDO after 8-week SPL treatment (12). This also indicates limited effects of SPL for diabetic patients. In addition, ALDO, through effects on the MR, regulates sodium balance by increasing the expression of the epithelial Na^+ channel and the Na^+/K^+-ATPase pump found on the apical and basolateral membranes, respectively, of distal epithelial cells (22). Once ALDO binds to MR on renal epithelial cells, urinary Na+ reabsorption and K+ excretion occur, and therefore the urinary Na+:K+ ratio becomes lower. Thus, MR blockade leads to increased urinary excretion of Na^+:K^+ in rats and humans (12, 23), which was also confirmed in this study after SPL treatment. Some studies show that decrease in the 24-hour urinary sodium excretion is associated with decrease in BP (24). Stolarz-Skrzypek et al (25) find that changes in systolic BP over time align with changes in sodium excretion, but this association does not translate into increased risk of hypertension-related complications. Consistent with these findings, we also found that BP remained constant after SPL treatment.

Both functional defects and reduced β-cell mass contribute to the failure of β-cells to compensate for insulin resistance in type 2 diabetes, but apoptosis is the main cause of β-cell failure (26). In vitro and in vivo studies show that ALDO can stimulate cardiac ventricular myocyte apoptosis (27) and that it promotes apoptosis of prox-

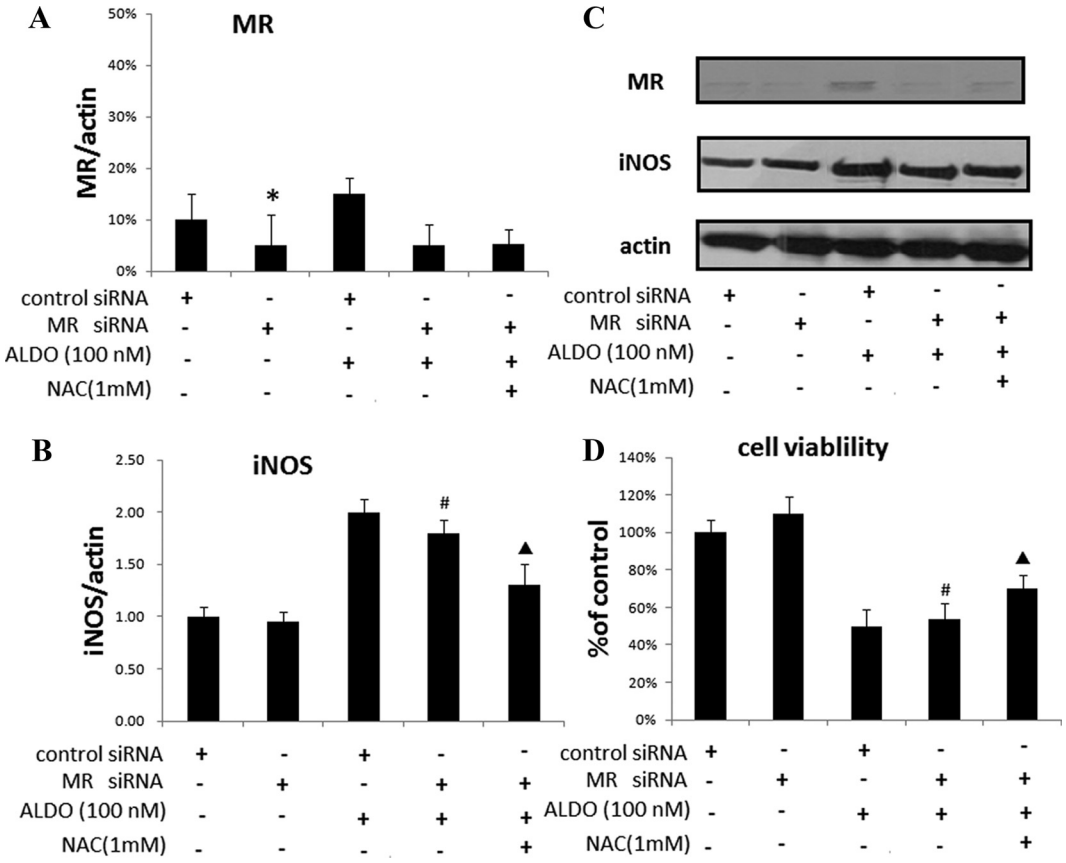

Figure 7. Effect of ALDO on the expression of MR (A), iNOS (B), and cell viability (D) in MIN6 cells with MR siRNA transfection. siRNA specific for MR was employed to silence endogenous MR expression. Both ALDO and NAC showed no effect on MR expression after MR siRNA transfection. However, MR siRNA transfection could not inhibit ALDO-induced activation of iNOS and decrease of cell viability. *, $P < .05$ vs control; #, $P < .05$ vs MR siRNA; ▲, $P < .05$ vs MR siRNA + ALDO (100 nM) (n = 3/group). Data are presented as mean ± SE.

imal tubular cells (28), mesangial cells (29), and podocytes (30). In the present study, we provided further proof-of-principle that ALDO exerted deleterious effects on insulin release from β-cells and directly induced β-cell apoptosis. Moreover, we observed that ALDO activated the prototypic apoptotic marker, caspase-3, and down-regulated Bcl-2/Bax expression, suggesting that mitochondrial dysfunction may be involved in ALDO-induced β-cell or pancreatic islet apoptosis.

The underlying mechanisms by which ALDO triggers β-cell apoptosis are not well understood. Oxidative stress might be involved in the initiation and propagation of β-cell apoptosis (31, 32). Activation of NADPH oxidase and production of superoxide contribute to apoptosis in various cell types (32). ALDO has been shown to increase expression of renal cortical NADPH oxidase and generation of reactive oxygen species, further advancing glomerular injury (33). In the present study, the up-regulation of oxidative stress markers, namely, iNOS and nitrite, in MIN6 cells was similar to that previously observed in renal tissues, indicating that local pancreatic oxidative damage may play an important role in ALDO-induced pancreatic tissue or islet apoptosis.

Under diabetic conditions, the expression of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase is known to be very low in islet cells. Additionally, the pancreas is known to have a relatively weak intrinsic defense system against oxidative stress (34). Previous studies have shown that antioxidant treatment could exert beneficial effects in diabetes by suppressing β-cell apoptosis, as well as preserving insulin content, and protecting insulin mRNA against glucose toxicity in C57BL/KsJ-*db/db* mice (35). NAC is a potent mucolytic agent, and its antioxidant activities are due to its ability to stimulate the synthesis of reduced glutathione and the scavenger hydrogen peroxide. Although NAC and vitamins C and E all have antioxidant activities, NAC alone is capable of ameliorating glucose intolerance (36). Several studies have found that NAC could decrease intracellular hydrogen peroxide levels when β-cells are exposed to high glucose or free fatty acids (36, 37); however, the effectiveness of the antioxidant in reversing ALDO-

mediated pancreatic β-cell damage is less clear. We have demonstrated, for the first time, that NAC also possesses the ability to reduce ALDO-induced pancreatic islet or β-cell oxidative stress and apoptosis, as well as to increase β-cell mass and improve glucose homeostasis.

Although urinary 8-OHdG was observed to be partially decreased after SPL treatment (38), SPL had no effect on the expression of Bcl-2/Bax, p-AKT/total AKT protein, iNOS, NADPH oxidase, p22phox, and p67phox in *db/db* mice islets or MIN6 cells. This indicates that SPL has limited antioxidant activity. Whether the active metabolite of SPL, Canrenone (not available for therapeutic use), exerts these antioxidant effects can only be confirmed after further experimentation. Consistent with the results of a previous study (8), our results showed that inhibition of MR expression with MR siRNA could block neither the ALDO-induced activation of iNOS nor the decrease in cell viability, suggesting MR-independent activation of iNOS pathways in MIN6 cells. However, further study is needed to determine whether ALDO-induced β-cell injury is mediated by another receptor, GPR30 (18).

In summary, the fact that aldosteronism is commonly associated with glucose-related metabolic imbalances has boosted interest in the role of ALDO in pancreatic insulin-producing β-cells. ALDO enhances oxidative stress and apoptosis in pancreatic islets and β-cells. These findings have further widened our knowledge of the mechanisms by which ALDO triggers β-cell dysfunction. Decrease in pancreatic oxidative stress by NAC may improve β-cell function and reduce β-cell apoptosis.

Acknowledgments

Address all correspondence and requests for reprints to: Yu Pan, Division of Nephrology, Shanghai No. 3 People's Hospital, Shanghai Jiao Tong University School of Medicine, 280 Mo He Road, Shanghai 201900, China. E-mail: panyu1980@163.com.

This study was supported and funded by the Outstanding Leaders Training Program of the Pudong Health Bureau of Shanghai (Grant PWRl 2012-05), by the Shanghai Committee on Science and Technology (Grant 08411962000), by the Shanghai Municipal Education Commission (Grant 10YZ35), and by the Shanghai Baoshan District Committee on Science and Technology (Grant 09-E-2).

Disclosure Summary: The authors have no competing interests to declare. All of the authors have approved the final version of the manuscript and agreed to submit the manuscript for publication.

References

1. **Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombès M.** The mineralocorticoid receptor: insights into its molecular and (patho) physiological biology. *Nucl Recept Signal*. 2007;5:e012.
2. **Whaley-Connell A, Johnson MS, Sowers JR.** Aldosterone: role in the cardiometabolic syndrome and resistant hypertension. *Prog Cardiovasc Dis*. 2010;52(5):401–409.
3. **Kahn SE, Hull RL, Utzschneider KM.** Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*. 2006;444(7121):840–846.
4. **Mosso LM, Carvajal CA, Maiz A, et al.** A possible association between primary aldosteronism and a lower β-cell function. *J Hypertens*. 2007;25:2125–2130.
5. **Fischer E, Adolf C, Pallauf A, et al.** Aldosterone excess impairs first phase insulin secretion in primary aldosteronism. *J Clin Endocrinol Metab*. 2013;98(6):2513–2520.
6. **Guo C, Martinez-Vasquez D, Mendez GP, et al.** Mineralocorticoid receptor antagonist reduces renal injury in rodent models of types 1 and 2 diabetes mellitus. *Endocrinology*. 2006;147(11):5363–5373.
7. **Pitt B, Zannad F, Remme WJ, et al.** The effect of spironolactone on morbidity and mortality in patients with severe heart failure. *N Engl J Med*. 1999;341(10):709–717.
8. **Luther JM, Luo P, Kreger MT, et al.** Aldosterone decreases glucose-stimulated insulin secretion in vivo in mice and in murine islets. *Diabetologia*. 2011;54(8):2152–2163.
9. **Haber CA, Lam TK, Yu Z, et al.** N-acetylcysteine and taurine prevent hyperglycemia-induced insulin resistance in vivo: possible role of oxidative stress. *Am J Physiol Endocrinol Metab*. 2003;285(4):E744–E753.
10. **Song D, Hutchings S, Pang CC.** Chronic N-acetylcysteine prevents fructose-induced insulin resistance and hypertension in rats. *Eur J Pharmacol*. 2005;508(1):205–210.
11. **Lautt WW, Schafer J, Macedo MP, Legare DJ.** Bethanechol and N-acetylcysteine mimic feeding signals and reverse insulin resistance in fasted and sucrose-induced diabetic rats. *Can J Physiol Pharmacol*. 2011;89(2):135–142.
12. **Homma T, Fujisawa M, Arai K, Ishii M, Sada T, Ikeda M.** Spironolactone, but not eplerenone, impairs glucose tolerance in a rat model of metabolic syndrome. *J Vet Med Sci*. 2012;74(8):1015–1022.
13. **Park YM, Park MY, Suh YL, Park JB.** NAD(P)H oxidase inhibitor prevents blood pressure elevation and cardiovascular hypertrophy in aldosterone-infused rats. *Biochem Biophys Res Commun*. 2004;313(3):812–817.
14. **Oprescu AI, Bikopoulos G, Naassan A, et al.** Free fatty acid-induced reduction in glucose-stimulated insulin secretion: evidence for a role of oxidative stress in vitro and in vivo. *Diabetes*. 2007;56(12):2927–37.
15. **King H, Aubert RE, Herman WH.** Global burden of diabetes, 1995–2025: prevalence, numerical estimates, and projections. *Diabetes Care*. 1998;21(9):1414–1431.
16. **Colussi G, Catena C, Lapenna R, Nadalini E, Chiuch A, Sechi LA.** Insulin resistance and hyperinsulinemia are related to plasma aldosterone levels in hypertensive patients. *Diabetes Care*. 2007;30(9):2349–2354.
17. **Krug AW, Ehrhart-Bornstein M.** Aldosterone and metabolic syndrome: is increased aldosterone in metabolic syndrome patients an additional risk factor? *Hypertension*. 2008;51:1252–1258.
18. **Gros R, Ding Q, Sklar LA, et al.** GPR30 expression is required for the mineralocorticoid receptor-independent rapid vascular effects of aldosterone. *Hypertension*. 2011;57(3):442–451.
19. **Sherajee SJ, Fujita Y, Rafiq K, et al.** Aldosterone induces vascular insulin resistance by increasing insulin-like growth factor-1 receptor and hybrid receptor. *Arterioscler Thromb Vasc Biol*. 2012;32(2):257–263.
20. **Nuno DW, Harrod JS, Lamping KG.** Sex-dependent differences in Rho activation contribute to contractile dysfunction in type 2 diabetic mice. *Am J Physiol Heart Circ Physiol*. 2009;297(4):H1469–H1477.
21. **Lastra G, Whaley-Connell A, Manrique C, et al.** Low-dose spirono-

lactone reduces reactive oxygen species generation and improves insulin-stimulated glucose transport in skeletal muscle in the TG-(mRen2)27 rat. *Am J Physiol Endocrinol Metab*. 2008;295(1): E110–E116.
22. **Salyer SA, Parks J, Barati MT, et al.** Aldosterone regulates Na+, K+ATPase activity in human renal proximal tubule cells through mineralocorticoid receptor. *Biochim Biophys Acta*. 2013;1833: 2143–2152.
23. **Shimizu MH, Coimbra TM, de Araujo M, Menezes LF, Seguro AC.** N-acetylcysteine attenuates the progression of chronic renal failure. *Kidney Int*. 2005;68(5):2208–2217.
24. **He FJ, MacGregor G.** Effect of longer-term modest salt reduction on blood pressure. *Cochrane Database Syst Rev*. 2004;(3):CD004937.
25. **Stolarz-Skrzypek K, Kuznetsova T, Thijs L, et al.** Fatal and nonfatal outcomes, incidence of hypertension, and blood pressure changes in relation to urinary sodium excretion. *JAMA*. 2011;305(17):1777–1785.
26. **Thomas HE, McKenzie MD, Angstetra E, Campbell PD, Kay TW.** β-Cell apoptosis in diabetes. *Apoptosis*. 2009;14(12):1389–1404.
27. **De Angelis N, Fiordaliso F, Latini R, et al.** Appraisal of the role of angiotensin II and aldosterone in ventricular myocyte apoptosis in adult normotensive rat. *J Mol Cell Cardiol*. 2002;34(12):1655–1665.
28. **Patni H, Mathew JT, Luan L, Franki N, Chander PN, Singhal PC.** Aldosterone promotes proximal tubular cell apoptosis: role of oxidative stress. *Am J Physiol Renal Physiol*. 2007;293(4):F1065–F1071.
29. **Mathew JT, Patni H, Chaudhary AN, et al.** Aldosterone induces mesangial cell apoptosis both in vivo and in vitro. *Am J Physiol Renal Physiol*. 2008;295(1):F73–F81.
30. **Lee SH, Yoo TH, Nam BY, et al.** Activation of local aldosterone system within podocytes is involved in apoptosis under diabetic conditions. *Am J Physiol Renal Physiol*. 2009;297(5):F1381–F1390.
31. **Evans JL, Goldfine ID, Maddux BA, Grodsky GM.** Are oxidative stress-activated signaling pathways mediators of insulin resistance and β-cell dysfunction? *Diabetes*. 2003;52(1):1–8.
32. **Drews G, Krippeit-Drews P, Düfer M.** Oxidative stress and β-cell dysfunction. *Pflugers Arch*. 2010;460(4):703–718.
33. **Nishiyama A, Yao L, Nagai Y, et al.** Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. *Hypertension*. 2004;43(4):841–848.
34. **Tiedge M, Lortz S, Drinkgern J, Lenzen S.** Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes*. 1997;46(11):1733–1742.
35. **Kaneto H, Kajimoto Y, Miyagawa J, et al.** Beneficial effects of antioxidants in diabetes: possible protection of pancreatic β-cells against glucose toxicity. *Diabetes*. 1999;48(12):2398–2406.
36. **Kaneto H, Fujii J, Myint T, et al.** Reducing sugars trigger oxidative modification and apoptosis in pancreatic β-cells by provoking oxidative stress through the glycation reaction. *Biochem J*. 1996; 320(Pt 3):855–863.
37. **Wang X, Li H, De Leo D, et al.** Gene and protein kinase expression profiling of reactive oxygen species-associated lipotoxicity in the pancreatic β-cell line MIN6. *Diabetes*. 2004;53(1):129–140.
38. **Pessôa BS, Peixoto EB, Papadimitriou A, Lopes de Faria JM, Lopes de Faria JB.** Spironolactone improves nephropathy by enhancing glucose-6-phosphate dehydrogenase activity and reducing oxidative stress in diabetic hypertensive rat. *J Renin Angiotensin Aldosterone Syst*. 2012;13(1):56–66.