Resveratrol prevents renal lipotoxicity and inhibits mesangial cell glucotoxicity in a manner dependent on the AMPK–SIRT1–PGC1 α axis in *db/db* mice

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Abstract

Aims/hypothesis Many of the effects of resveratrol are consistent with the activation of AMP-activated protein kinase (AMPK), silent information regulator T1 (SIRT1) and peroxisome proliferator-activated receptor (PPAR) γ co-activator 1 α (PGC-1 α), which play key roles in the regulation of lipid and glucose homeostasis, and in the control of oxidative stress. We investigated whether resveratrol has protective effects on the kidney in type 2 diabetes.

Methods Four groups of male C57BLKS/J *db/m* and *db/db* mice were used in this study. Resveratrol was administered

via gavage to diabetic and non-diabetic mice, starting at 8 weeks of age, for 12 weeks.

Results The *db/db* mice treated with resveratrol had decreased albuminuria. Resveratrol ameliorated glomerular matrix expansion and inflammation. Resveratrol also lowered the NEFA and triacylglycerol content of the kidney, and this action was related to increases in the phosphorylation of AMPK and the activation of SIRT1–PGC-1 α signalling and of the key downstream effectors, the PPAR α –oestrogen-related receptor (ERR)-1 α –sterol regulatory element-binding protein 1 (SREBP1). Furthermore, resveratrol decreased the activity of phosphatidylinositol-3 kinase (PI3K)–Akt phosphorylation and class O forkhead box (FOXO)3a phosphorylation, which resulted in a decrease in B cell leukaemia/lymphoma 2 (BCL-2)-associated X protein (BAX) and increases in BCL-2, superoxide dismutase (SOD)1 and SOD2 production. Consequently, resveratrol reversed the increase in renal apoptotic cells and oxidative stress, as reflected by renal 8-hydroxy-deoxyguanosine (8-OH-dG), urinary 8-OH-dG and isoprostane concentrations. Resveratrol prevented high-glucose-induced oxidative stress and apoptosis in cultured mesangial cells through the phosphorylation of AMPK and activation of SIRT1–PGC-1 α signalling and the downstream effectors, PPAR α –ERR-1 α –SREBP1.

Conclusions/interpretation The results suggest that resveratrol prevents diabetic nephropathy in *db/db* mice by the phosphorylation of AMPK and activation of SIRT1–PGC-1 α signalling, which appear to prevent lipotoxicity-related apoptosis and oxidative stress in the kidney.

Keywords Apoptosis · Diabetic nephropathy · Oxidative stress · Resveratrol

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Abbreviations

8-OH-dG	8-Hydroxy-deoxyguanosine
AICAR	5-Aminoimidazole-4-carboxamide-1 β -D-ribofuranoside
AMPK	AMP-activated protein kinase
BAX	BCL-2-associated X protein
BCL	B cell leukaemia/lymphoma 2
CMC	Cellulose sodium salt
CPT-1A	Carnitine palmitoyltransferase-1A
<i>db/db</i> Res	Diabetic mice receiving resveratrol
<i>db/m</i> Res	Non-diabetic mice receiving resveratrol
eNOS	Endothelial nitric oxide synthase
ERR	Oestrogen-related receptor
F4/80	Cell surface glycoprotein F4/80
FOXO	Class O forkhead box
NF- κ B	Nuclear factor κ B
PGC-1 α	PPAR γ co-activator 1 α
PI3K	Phosphatidylinositol-3 kinase
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
si	Small interfering
SIRT1	Silent information regulator T1
SOD	Superoxide dismutase
SREBP1	Sterol regulatory element-binding protein 1

Introduction

Diabetic nephropathy is the most prevalent cause of end-stage renal disease in developed countries, including Korea [1]. Despite progress in pharmacological strategies to modulate diabetes, the number of patients entering renal failure remains extremely high and the development of new classes of therapeutic agents is eagerly anticipated. Lipotoxicity is one of the causal factors of the progression of diabetic and obesity-induced renal damage [2], but the underlying molecular mechanism remains elusive.

Resveratrol is a natural plant polyphenol that may target ageing and obesity-related chronic diseases by preventing inflammation and oxidative stress [3–6]. Resveratrol activates silent information regulator T1 (SIRT1), an NAD⁺-dependent protein deacetylase, and AMP-activated protein kinase (AMPK). It subsequently augments peroxisome proliferator-activated receptor (PPAR) γ co-activator 1 α (PGC-1 α), endothelial nitric oxide synthase (eNOS) and class O forkhead box (FOXO) activation, which is followed by catabolic metabolism, mitochondrial activation, angiogenesis and enhanced cell survival [3–6]. Some of the best-characterised effects of SIRT1 include increased stress resistance and altered metabolism mediated by changes in the activity of the transcriptional factor FOXO [3], suppression of nuclear factor κ B (NF- κ B)-dependent inflammatory

responses [4] and the promotion of gluconeogenesis, fatty acid oxidation and mitochondrial biogenesis, through PGC-1 α [5, 6]. The activation of SIRT1 by resveratrol is substrate dependent and many of its effects are consistent with the modulation of its target genes [7, 8]. Hepatocyte-specific deletion of *Sirt1* impairs PPAR α signalling and decreases fatty acid β -oxidation, whereas the overexpression of *Sirt1* induces the expression of PPAR α targets. SIRT1 interacts with PPAR α , and this is required to activate the PPAR α co-activator PGC-1 α . It has been reported that SIRT1 and PGC-1 α form a stable complex and that SIRT1 regulates the activity and acetylation status of PGC-1 α [9]. AMPK is a fuel-sensing enzyme that is activated by decreases in the cellular energy state, as reflected by an increased AMP/ATP ratio [10]. When activated, AMPK initiates metabolic and genetic processes that generate ATP (fatty acid oxidation), and inhibits other processes that consume ATP. The former occurs via its effects on various transcriptional activators and co-activators, including PGC-1 α and PPAR α [11]. The collective results favour the view that SIRT1 and AMPK play a vital role in the regulation of organic lipid homeostasis, and that pharmacological activation of SIRT1 and AMPK may be important for the prevention of obesity-associated metabolic diseases [8, 10, 11].

Accumulated experimental evidence suggests that PPAR α activation attenuates or inhibits several mediators of vascular injury, including lipotoxicity, inflammation, reactive oxygen species (ROS) generation, endothelial dysfunction, angiogenesis and thrombosis in type 2 diabetes and high-fat-diet-induced renal damage [12, 13], which are all associated with AMPK activation and eNOS production [14–16]. PPARs activate PGC-1 α/β and its key downstream effector oestrogen-related receptor (ERR)-1 α , which induces mitochondrial biogenesis and enhanced mitochondrial antioxidative capacity to provide relief from oxidative stress [17, 18]. Additionally, PPAR α activation can suppress the sterol regulatory element-binding protein 1 (SREBP1) pathway through the reduction of liver X receptor (LXR)/retinoid X receptor (RXR) formation in the liver, which plays a crucial role in the regulation of fatty acid metabolism [19]. Notably, FOXO3a is a direct transcriptional regulator of a group of oxidative protection genes and this regulation requires PGC-1 α . FOXO3A and PGC-1 α interact directly and cooperatively and their interaction regulates mitochondrial oxidative stress [20].

We hypothesised that resveratrol can potentially prevent renal cell apoptosis and oxidative stress, which are the main causes of renal damage in diabetic nephropathy, via activation of AMPK–SIRT1–PGC-1 α and the consequent effects on its target molecules PPAR α –ERR-1 α and the phosphatidylinositol-3 kinase (PI3K)–Akt–FOXO3a pathway.

Methods

Experimental methods Male 6-week-old C57BLKS/J *db/m* and *db/db* mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Four groups of male C57BLKS/J *db/m* and *db/db* mice were used in this study. Resveratrol (Sigma-Aldrich, St Louis, MO, USA) dissolved with 0.5% carboxymethyl cellulose sodium salt (CMC), 20 mg/kg⁻¹ day⁻¹, was administered via gavage to diabetic mice (*db/db* Res, *n*=8) and non-diabetic mice (*db/m* Res, *n*=8) starting at 8 weeks of age, for 12 weeks [21]. The diabetic *db/db* group (*n*=8) and the non-diabetic *db/m* control group (*n*=8) received only 0.5% CMC. The mice were placed in individual metabolism cages (Nalgene, Rochester, NY, USA) with access to water and food ad libitum. After 12 weeks of treatment, the systolic BP was determined by the non-invasive tail-cuff system in conscious mice (IITC Life Science, Woodland Hill, CA, USA) after a 5 day accommodation period. At week 20, all animals were anaesthetised by intraperitoneal injection of a mixture of Rompun, 10 mg/kg (Bayer Korea, Ansan, Gyeonggi-Do, Korea) and Zoletil, 30 mg/kg (Virbac, Carros, France). The mice were killed and the kidneys removed. Immediately following removal, each kidney was divided into the cortex and medullar. All our experiments, including western blots, were performed with renal cortex. The kidneys were rapidly dissected and stored in buffered formalin (10%) for subsequent immunohistochemical analyses. Blood was collected from the left ventricle and the plasma was stored at -70°C for subsequent analyses. HbA_{1c} was determined from the red cell lysates by HPLC (Bio-Rad, Richmond, CA, USA). The total cholesterol and triacylglycerol concentrations were measured by an autoanalyser (Hitachi 917, Tokyo, Japan) using commercial kits (Wako, Osaka, Japan). NEFA levels were measured with a JCA-BM1250 automatic analyser (JEOL, Tokyo, Japan). The experiments were performed in accordance with our institutional animal care guidelines, and all the procedures complied with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85–23, revised 1996).

Assessment of renal function and the lipid contents At week 20, the animals were housed in metabolism cages (Nalgene) for 24 h to collect urine for subsequent measurements of the albumin concentrations by an immunoassay (Bayer, Elkhart, IN, USA). Plasma and urine creatinine concentrations were measured, using HPLC (Beckman Instruments, Fullerton, CA, USA). The kidney lipids were extracted using the method of Bligh and Dyer with slight modifications, as previously described [22].

Light microscopy study The mesangial matrix and glomerular tuft areas were quantified for each glomerular cross-section, using sections stained with periodic acid–Schiff's reagent. More than 30 glomeruli, cut through the vascular pole, were counted per kidney and the average was used for analysis.

Immunohistochemistry for TGF-β1, type IV collagen, PPARα, cell surface glycoprotein F4/80, 8-hydroxy-deoxyguanosine and TUNEL assay We performed immunohistochemistry for type IV collagen, TGF-β1, PPARα, cell surface glycoprotein F4/80 (F4/80), 8-hydroxy-deoxyguanosine (8-OH-dG) and active caspase-3. We also performed a TUNEL assay. See the electronic supplementary material (ESM) [Methods](#) for further details.

Western blots The total protein of the renal cortical tissues was extracted with a Pro-Prep Protein Extraction Solution (Intron Biotechnology, Gyeonggi-Do, Korea) according to the manufacturer's instructions. Western blot analysis was performed to further confirm the responses using epitope-specific antibodies. Blots were carried out for SIRT1, PPARα, PGC-1α, ERR-1α, SREBP1, carnitine palmitoyltransferase-1A (CPT-1A), total AMPK, phosphorylated (phospho)-AMPK Thr¹⁷², PI3K, total Akt, phospho-Akt Ser⁴⁷³, total FOXO3a, phospho-FOXO3a Ser²⁵³, active caspase-3, B cell leukaemia/lymphoma 2 (BCL-2), superoxide dismutase (SOD)1 and SOD2. We also measured PI3K activity. See ESM [Methods](#) for further details.

Table 1 Biochemical and physical characteristics of study groups

Characteristic	<i>db/m</i> control	<i>db/m</i> Res	<i>db/db</i> control	<i>db/db</i> Res
Body weight (g)	30±2	30±2	40±5***	41±4***
Kidney weight (g)	0.18±0.01	0.18±0.03	0.22±0.03*	0.22±0.02*
FBS (mmol/l)	9.38±2.94	9.99±1.94	26.92±2.72***	25.36±5.05***
HbA _{1c} (%)	4.2±0.2	4.1±0.1	12.2±2.1***	13.4±0.9***
HbA _{1c} (mmol/mol)	(22.4±0.7)	(21.3±0.6)	(109.8±5.3***)	(123.0±2.7***)
24 h albumin (μg)	8.6±4.2	4.5±1.2	117.8±54.2***	25.7±17.2
Serum Cr (mmol/l)	6.98±0.70	7.16±0.88	7.24±0.97	7.07±0.79
Cr clearance (ml/min)	0.34±0.21	0.30±0.33	0.67±0.29***	0.41±0.21
Systolic BP (mmHg)	98.6±4.5	96.6±5.2	101.1±5.2	99.3±4.5
Food intake (g)	12.0±0.3	11.7±0.7	23.6±4.9***	28.6±1.1***

Cr, creatinine; FBS, fasting blood sugar

p*<0.05 and **p*<0.001 compared with other groups

Fig. 1 Changes in glomerular phenotypes in resveratrol-treated *db/db* mice. Glomerular mesangial fractional area, pro-fibrotic TGF- β 1 and type IV collagen, and F4/80-positive cell infiltration in the cortical glomerulus of *db/m* and *db/db* mice, with or without resveratrol treatment. Representative sections stained with periodic acid–Schiff reagent are shown for estimation of the mesangial fractional area (%) (a), together with quantitative analysis by group (b). Immunohistochemical staining and quantitative analyses are shown for TGF- β 1 (a, c), type IV collagen (a, d) and F4/80-positive cells (a, e). Col IV, type IV collagen; Cont, control; PAS, periodic acid–Schiff stain. ** $p < 0.01$ vs the *db/m* control, *db/m* Res and *db/db* Res mice

Immunoprecipitation for acetylated PGC-1 α assay Acetylated-lysine PGC-1 α was analysed by immunoprecipitation of PGC-1 α from the renal cortex protein lysates with anti-PGC-1 α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by western blotting using an acetyl-lysine antibody (Cell Signaling, Denver, MA, USA).

Assessment of renal oxidative stress To evaluate the oxidative DNA damage and lipid peroxidation, the 24 h urinary 8-OH-dG (OXIS Health Products, Portland, OR, USA) and 24 h urinary 8-epi-prostaglandin F_{2 α} (8-epi-PGF_{2 α} ; OxisResearch, Foster City, CA, USA) concentrations were measured, respectively.

Cell culture To find the effects of high glucose and resveratrol on NMS2 mesangial cells [23] with regard to the activation of the AMPK–SIRT1–PGC-1 α pathway and apoptosis, mesangial cells were grown in 5 mmol/l D-glucose (low glucose), 30 mmol/l D-glucose (high glucose) or 5 mmol/l D-glucose plus 25 mmol/l mannitol (as an osmotic control for 30 mmol/l D-glucose). After reaching 80% confluency, the mesangial cells were exposed to low glucose, high glucose or mannitol control, with or without the additional 48 h application of resveratrol (1, 10 or 50 ng/ml), 5-aminoimidazole-4-carboxamide-1 β -D-ribofuranoside (AICAR), 0.5 mmol/l, or metformin (1 mmol/l). The small

interfering (si)RNA targeted to *Ampk α 1* (also known as *Prkaa1*), *Ampk α 2* (also known as *Prkaa2*), *Sirt1* and scrambled siRNA (siRNA control) were complexed with transfection reagent (G-Fectin; Genolution, Seoul, Korea), according to the manufacturer's instructions. We also performed a TUNEL assay to evaluate the effects of siRNAs on mesangial cell apoptosis. See ESM Methods for further details.

Statistical analysis The data are expressed as the means $\pm$ SD. Differences between the groups were tested for statistical

Fig. 2 Phospho-AMPK Thr¹⁷², total AMPK, SIRT1, acetylated PGC-1 α , total PGC-1 α , PPAR α , ERR-1 α , SREBP1 and intra-renal lipid levels in the renal cortex of the *db/m* and *db/db* mice, with or without resveratrol treatment. Protein lysates (40 μ g) from renal cortex were separated by SDS-PAGE and analysed by western blotting. Representative western blotting (a) is shown for phospho-AMPK Thr¹⁷², total AMPK, SIRT1, PGC-1 α , total PGC-1 α and β -actin. Quantitative analyses are shown for phospho-AMPK Thr¹⁷²/total AMPK (b), SIRT1/ β -actin (c), PGC-1 α / β -actin (d) and acetylated Lys-PGC-1 α / β -actin (e). (f) Representative immunohistochemical staining for PPAR α and quantitative analysis of the results. (g) Western blot of PPAR α and ERR-1 α , SREBP1 and β -actin and the corresponding quantitative analyses. (h–j) Quantitative analyses of intra-renal total cholesterol (h), NEFA (i) and triacylglycerol (j) concentrations. ** $p < 0.01$ vs the *db/m* control, *db/m* Res and *db/db* Res mice; ††† $p < 0.001$ vs the *db/m* control and *db/m* Res mice; and ‡ $p < 0.05$ vs the *db/db* Res mice. Cont, control; pAMPK, phospho-AMPK Thr¹⁷²

significance by ANOVA with Bonferroni correction, using SPSS version 11.5 (SPSS, Chicago, IL, USA). A p value <0.05 was considered to show a statistically significant difference.

Results

Physical and biochemical characteristics of mice The diabetic *db/db* mice were heavier than the non-diabetic *db/m*

mice (Table 1). Kidney weight, blood glucose and HbA_{1c}, together with the amount of food eaten, were significantly higher for the *db/db* mice than for the *db/m* mice (Table 1). There were no differences in serum creatinine level and systolic BP between the study groups. There was significantly increased albuminuria and creatinine clearance in the *db/db* mice compared with the *db/m* and *db/m* Res mice. Despite the same degree of hyperglycaemia in the *db/db* Res and *db/db* mice, resveratrol treatment restored the albuminuria and creatinine clearance to the levels of the *db/m* and *db/m* Res mice (Table 1).

Effects of resveratrol on renal phenotype, TGF- β 1, type IV collagen and F4/80 There were no differences in the fractional mesangial areas between the *db/m* and *db/m* Res mice (Fig. 1a). In contrast, there was a marked (2.6-fold) increase in the mesangial area in the *db/db* mice compared with the *db/m* mice ($p < 0.01$). Consistent with the changes of the mesangial fractional area, the levels of the pro-fibrotic growth factor TGF- β 1 and associated extracellular matrix type IV collagen and inflammatory cell glomerular infiltration were significantly increased in the *db/db* mice

compared with the *db/m* and *db/m* Res mice (Fig. 1a–e). All of the diabetes-induced renal phenotypic changes and inflammation seen in the *db/db* mice were reversed by resveratrol treatment. The significantly increased production of type IV collagen in *db/db* mice was reversed by resveratrol treatment (ESM Fig. 1 and ESM Results).

Renal levels of phospho-AMPK Thr¹⁷² and total AMPK, SIRT1, total and acetylated-Lys-PGC-1 α , PPAR α –ERR-1 α –PGC-1 α signalling, and intra-renal triacylglycerol and NEFA Western blot analysis showed that diabetes markedly decreased the phospho-AMPK Thr¹⁷²/total AMPK ratio and the SIRT1 protein levels in *db/db* mice compared with *db/m* and *db/m* Res mice (Fig. 2). Resveratrol treatment restored the phospho-AMPK Thr¹⁷²/total AMPK, SIRT1 and total and acetylated Lys-PGC-1 α levels in *db/db* mice to the levels of *db/m* and *db/m* Res mice (Fig. 2a–e; $p < 0.01$). Interestingly, the PPAR α level was lower in the *db/db* mice compared with that in the *db/m* and *db/m* Res mice, as assessed by immunohistochemical and western blot analyses (Fig. 2f, g). The levels of ERR-1 α and SREBP1 in the kidney were evaluated to determine the changes in the

Fig. 3 PI3K activity, PI3K, phospho-Akt Ser⁴⁷², total Akt, phospho-FOXO3a Ser²⁵³ and total FOXO3a in the renal cortex of the *db/m* and *db/db* mice with or without resveratrol treatment. Protein lysates (40 μ g) from renal cortex were separated by SDS-PAGE and analysed by western blotting. Representative western blot showing PI3K, phospho-Akt Ser⁴⁷², total Akt, phospho-FOXO3a Ser²⁵³, total FOXO3a and β -actin levels (a). (b) PI3K activity. (c–f) Quantitative analyses of the results from the blot: (c) PI3K/ β -actin; (d) phospho-Akt Ser⁴⁷²/total Akt; (e) phospho-FOXO3a Ser²⁵³/ β -actin; and (f) phospho-FOXO3a/total FOXO3a. * $p < 0.05$ and ** $p < 0.01$ vs *db/m* control, *db/m* Res and *db/db* Res mice. Cont, control; pAkt, phospho-Akt Ser⁴⁷²; pFOXO3a, phospho-FOXO3a Ser²⁵³

PPAR α target proteins. There was a decrease in ERR-1 α and an increase in SREBP1 levels in *db/db* mice, both of which were restored with resveratrol treatment (Fig. 2g). Consistent with the PPAR α –ERR-1 α –SREBP1 changes in the *db/db* mice, direct measurement of the intra-renal lipid concentrations showed increases in triacylglycerol and NEFA concentrations, but not in the total cholesterol concentration, in this group (Fig. 2 h–j). Intra-renal triacylglycerol and NEFA concentrations were decreased by resveratrol treatment ($p<0.05$, Fig. 2i, j).

Renal levels of PI3K, phospho-Akt Ser⁴⁷³ and pFOXO3a
We determined the levels of intra-renal PI3K and phospho-Akt Ser⁴⁷³ using western blot analysis and PI3K activity by ELISA. The PI3K level and activity, as well as the phospho-Akt Ser⁴⁷³/total Akt ratio were significantly increased in *db/db* mice compared with *db/m* and *db/m* Res mice (Fig. 3a–d, $p<0.01$ [PI3K activity and level] and $p<0.05$ [phospho-Akt Ser⁴⁷³/total Akt ratio]). Resveratrol treatment of *db/db* mice decreased the PI3K level and phospho-Ser⁴⁷³ Akt/total Akt ratio compared with the levels of the *db/db* mice. An increased level of phospho-FOXO3a Ser²⁵³ was noted in *db/db* mice compared with *db/m* and *db/m* Res mice (Fig. 3e; $p<0.05$). Treatment with resveratrol

markedly decreased the level of phospho-FOXO3a Ser²⁵³ in *db/db* mice, which resulted in an increase in total FOXO3a, according to the western blot analysis (Fig. 3a, f).

Renal levels of pro-apoptotic BAX, anti-apoptotic BCL-2, cleaved caspase-3 and TUNEL-positive cells
It is well known that FOXO3a activation has an anti-stress and anti-apoptotic action via the enhancement of BCL-2 activity and the downregulation of pro-apoptotic BCL-2-associated X protein (BAX) activity. Consistent with the changes in FOXO3a, the BAX level on western blot was increased in samples from *db/db* mice compared with those from *db/m* and *db/m* Res mice (Fig. 4a). However, the BCL-2 protein level was decreased in *db/db* mice compared with the *db/m* and *db/m* Res mice (Fig. 4a). Consequently, the BAX/BCL-2 ratio was significantly increased in *db/db* mice, resulting in an increase in cleaved caspase-3 (Fig. 4a, c, $p<0.01$), which is a critical executioner of apoptosis. In contrast, resveratrol treatment in *db/db* mice resulted in increased BCL-2 protein and decreased BAX protein, which resulted in a threefold increase in the ratio of BAX/BCL-2 ($p<0.01$) and, ultimately, a decrease in cleaved caspase-3 (Fig. 4a, c). Furthermore, there was a significant increase in the number of TUNEL-positive cells in the glomeruli of *db/db* mice

Fig. 4 Pro-apoptotic BAX, anti-apoptotic BCL-2 and cleaved caspase-3 levels in the renal cortices of *db/m* and *db/db* mice with or without resveratrol treatment. Protein lysates (40 μ g) from renal cortex were separated by SDS-PAGE and analysed by western blotting. Representative western blot analysis of the BAX, BCL-2, cleaved caspase-3 and β -actin levels (a) and the quantitative analyses of the results of the BAX/BCL-2 ratio (b) and cleaved caspase-3 (c) are shown. Representative immunohistochemical staining for TUNEL-positive cells and the quantitative analyses of the results are shown (d). ** $p<0.01$ vs *db/m* control, *db/m* Res and *db/db* Res mice. Cont, control

Fig. 5 Intra-renal SOD1, SOD2 and 8-OH-dG levels, and 24 h urinary 8-OH-dG and isoprostane concentrations in *db/m* and *db/db* mice with or without resveratrol treatment. Protein lysates (10 μ g) from renal cortex were separated by SDS-PAGE and analysed by western blotting. Representative western blot analysis of the SOD1 and SOD2 (a) and the quantitative analyses of the results are shown: (b) SOD1; and (c) SOD2. Representative immunohistochemical staining for 8-OH-dG and the quantitative analyses of the results are shown (d). The 24 h urinary 8-OH-dG (e) and isoprostane (f) concentrations of the study mice are shown. * $p<0.05$ and ** $p<0.01$ vs the *db/m* control, *db/m* Res and *db/db* Res mice. Cont, control

compared with *db/m* and *db/m* Res mice. The number of TUNEL-positive cells in *db/db* mice was decreased after resveratrol treatment (Fig. 4d).

Effects of resveratrol on SOD1, SOD2, and renal and 24 h urinary 8-OH-dG and isoprostane concentrations We investigated the changes in SOD1 and SOD2 content associated with FOXO3a activation. SOD1 and SOD2 levels were lower in *db/db* mice than in *db/m* and *db/m* Res mice (Fig. 5a–c). In contrast, SOD1 and SOD2 levels were significantly increased in *db/db* Res mice compared with *db/db* mice. Increases in the renal oxidative DNA damage and lipid peroxidation, as reflected by the renal and urinary 8-OH-dG and urinary 8-isoprostane concentrations, respectively, were observed in *db/db* mice and were compared with the levels in *db/m* and *db/m* Res mice (Fig. 5e–f). However, 12 weeks of resveratrol treatment decreased not only the renal 8-OH-dG level and the urinary 8-OH-dG concentration, but also the urinary 8-isoprostane concentration in *db/db* mice. Taken together, these findings suggest that oxidative stress in the *db/db* mice could be ameliorated by resveratrol treatment.

In vitro studies In diabetic *db/db* mice, mesangial matrix expansion, with an increased number of apoptotic mesangial

cells, is one of the characteristic phenotypes of oxidative stress. As resveratrol reversed the diabetes-induced detrimental renal effects in *db/db* mice, we evaluated the effects of several doses of resveratrol on high-glucose-induced oxidative stress, and the effects of apoptosis related to AMPK–SIRT1–PGC-1 α signalling and its downstream effector, the

Fig. 6 The effect of resveratrol on intracellular signalling and apoptosis in the mesangial cells cultured in low glucose (5 mmol/l D-glucose) or high glucose (30 mmol/l D-glucose) with or without resveratrol at different concentrations (1 μ g/ml, 10 μ g/ml or 50 μ g/ml, as shown). Phospho-AMPK Thr¹⁷², total AMPK, SIRT1 and total PGC-1 α levels were assessed in the cultured mesangial cells. Protein lysates (10 μ g) were separated by SDS-PAGE and analysed by western blotting. Representative western blot analysis of the phospho-AMPK Thr¹⁷², total AMPK, SIRT1, PGC-1 α and β -actin levels and the quantitative analyses of the results are shown (a). (b) Representative western blot analysis of PPAR α , ERR-1 α , SREBP1, CPT-1A, PI3K, phospho-Akt Ser⁴⁷², total Akt, phospho-FOXO3a Ser²⁵³ and total FOXO3a levels in the cultured mesangial cells, and quantitative analyses of the results. (c) Representative western blot analysis of SOD1 and SOD2 in cultured mesangial cells and the quantitative analyses of the results. (d) Representative pictures of TUNEL-positive mesangial cells (original magnification $\times 400$) and quantitative analyses of the results. $n=4$; * $p<0.05$ and ** $p<0.01$ compared with low glucose, +1/+10/+50, +1/+10/+50 μ g/ml resveratrol; HG, high glucose; LG, low glucose; pAkt, phospho-Akt Ser⁴⁷²; pAMPK, phospho-AMPK Thr¹⁷²; pFOXO3a, phospho-FOXO3a Ser²⁵³

PPAR α –ERR-1 α –FOXO3a axis, in cultured mesangial cells. High glucose (30 mmol/l D-glucose) induced significant decreases in the activation of phospho-AMPK Thr¹⁷²–SIRT1–PGC-1 α signalling (Fig. 6a) and the levels of PPAR α –ERR-1 α (Fig. 6b). Consistent with the change in

PPAR α –ERR-1 α , high glucose increased the level of the lipogenic enzyme SREBP1 and decreased the lipolytic enzyme CPT-1A (Fig. 6b). High glucose additionally increased PI3K–Akt phosphorylation and subsequent FOXO3a phosphorylation (Fig. 6b), which related to decreases in SOD1 and

SOD2 content, and an increase in the number of apoptotic mesangial cells (Fig. 6c, d). In contrast, resveratrol, 1–50 ng/ml, prevented high-glucose-induced oxidative stress and apoptosis related to the activation of AMPK–SIRT1–PGC-1 α signalling and the PPAR α –ERR-1 α pathway. When compared with high glucose, the addition of resveratrol in low glucose did not affect the intracellular signalling and mesangial cell apoptosis. Mannitol was without any effect on the intracellular signalling (data not shown). To validate the pro-apoptotic effect of resveratrol as AMPK dependent, we performed additional experiments using the AMPK activators AICAR and metformin, and siRNAs for *Ampk α 1*, *Ampk α 2* and *Sirt1* in cultured mesangial cells. Similar to resveratrol, AICAR and metformin also activated AMPK–SIRT1–PGC-1 α signalling in high-glucose conditions (Fig. 7a). In contrast, transfection with *Ampk α 1*, *Ampk α 2* and *Sirt1* siRNAs suppressed resveratrol-induced AMPK–SIRT1–PGC-1 α signalling, respectively, compared with resveratrol treatment only in high-glucose media (Fig. 7b, c). Moreover, transfection with *Ampk α 1*, *Ampk α 2*, *Sirt1* and *Pgc-1 α* (also known as *Ppargc1a*) siRNAs prevented the resveratrol-induced anti-apoptotic effect in mesangial cells in high-glucose media (Fig. 7d).

Discussion

Pharmacologically targeting transcriptional networks to regulate or modulate the gene-expression programmes that favour energy expenditure is an attractive concept to combat metabolic diseases, particularly the complications of diabetes. Both AMPK and SIRT1 have emerged as interesting targets as they are heavily involved in catabolic metabolism, mitochondrial activation, angiogenesis and enhanced cell survival [3–6]. It is well known that the effects of resveratrol are mediated by both SIRT1 and AMPK [24]. However, the effects of resveratrol in the kidneys of an animal model of type 2 diabetes are not well known. The results of the present study demonstrate that diabetic nephropathy in *db/db* mice is associated with increases in renal lipid accumulation, apoptotic renal cell injury and oxidative stress that relate to the inactivation of AMPK–SIRT1–PGC-1 α signalling and the deregulation of their target molecules, PPAR α –ERR-1 α and PI3K–Akt–FOXO3a. Diabetic nephropathy was ameliorated by resveratrol treatment via the activation of AMPK–SIRT1–PGC-1 α signalling and the subsequent activation of PPAR α –ERR-1 α and FOXO3a, which reversed renal lipid accumulation, apoptotic renal cell injury and oxidative stress.

It is notable that AMPK and SIRT1 regulated each other and share many common target molecules, such as PGC-1 α , PPARs, FOXOs, and NF- κ B. Moreover, AMPK and SIRT1 have clinical relevance with regard to metabolic syndrome

Fig. 7 Effect of AICAR and metformin, AMPK activators, and *Ampk α 1*, *Ampk α 2* and *Sirt1* siRNA on the resveratrol-stimulated AMPK–SIRT1–PGC-1 α signalling in mesangial cells. The cultured mesangial cells in high glucose were stimulated by AICAR and metformin. Protein lysates (10 μ g) from the cultured mesangial cells were separated by SDS-PAGE and analysed by western blotting. **(a)** Representative western blot analysis of phospho-AMPK Thr¹⁷², SIRT1, PGC-1 α and β -actin levels and the quantitative analyses of the results. * p <0.05 compared with low glucose, high glucose, high glucose+AICAR and high glucose+metformin. **(b)** The cultured mesangial cells were transfected with 50 nmol/l control siRNA, or 50 nmol/l *Ampk α 1*, *Ampk α 2* or *Sirt1* siRNA using transfection reagent (G-Fectin). Approximately 48 h after transfection, the levels of phospho-AMPK Thr¹⁷², total AMPK, SIRT1 and PGC-1 α signalling in low-glucose media were analysed. Representative western blot analysis and quantitative analyses of the results are shown. ** p <0.01 compared with siRNA control. **(c)** The cultured mesangial cells were transfected with 50 nmol/l control siRNA or 50 nmol/l *Ampk α 1*, *Ampk α 2* or *Sirt1* siRNA and stimulated with resveratrol in high-glucose media. Approximately 48 h after stimulation, the levels of phospho-AMPK Thr¹⁷², total AMPK, SIRT1 and PGC-1 α signalling in high-glucose media were analysed. Representative western blot analysis of the phospho-AMPK Thr¹⁷², total AMPK, SIRT1, PGC-1 α and β -actin levels and the quantitative analyses of the results are shown. * p <0.05 and ** p <0.01 compared with other groups. **(d)** The cultured mesangial cells were transfected with 50 nmol/l control siRNA or 50 nmol/l *Ampk α 1*, *Ampk α 2*, *Sirt1* or *Pgc-1 α* siRNA and stimulated with resveratrol in high-glucose media. Representative pictures of TUNEL-positive mesangial cells (original magnification $\times$ 400) and the quantitative analyses of the results are shown. n =4; ** p <0.01 compared with other cell groups. Cont, control; HG, high glucose; LG, low glucose; Met, metformin; pAMPK, phospho-AMPK Thr¹⁷²

and type 2 diabetes, as their effects on target molecules lead to insulin resistance, mitochondrial dysfunction, oxidative and endoplasmic reticulum stress, and lipotoxicity associated with ectopic lipid accumulation [12]. SIRT1 also responds to changes in the availability of nutrients, such as in conditions of energy-intake restriction or starvation, and energy expenditure, through a forkhead-dependent pathway [25] and PGC-1 α [9]. Thus, it has been suggested that the activation of AMPK and SIRT1 allows for the concurrent deacetylation and phosphorylation of their target molecules and decreases the susceptibility to diabetes-associated disorders. The current study demonstrates that the activation of AMPK and SIRT1 by resveratrol may prevent renal lipid accumulation and cell injury related to the activation of PGC-1 α . This in vitro study demonstrated that the high-glucose-induced suppression of AMPK–SIRT1–PGC-1 α signalling was activated by AMPK activators, such as resveratrol, AICAR and metformin. Additionally, increased AMPK–SIRT1–PGC-1 α production mediated by resveratrol in high-glucose media was suppressed with the introduction of *Ampk α 1*, *Ampk α 2* and *Sirt1* siRNAs. When any of one of AMPK, SIRT1 and PGC-1 α was knocked down in mesangial cells, levels of the others were also reduced. These results suggest that AMPK and SIRT1 activate each other and jointly regulate the downstream PGC-1 α in the mesangial cells, as in the skeletal muscles [11, 26]. Consistent with our results, several recent studies have demonstrated that

AMPK activation by adiponectin [27] or AICAR [28] protected renal podocytes and diminished albuminuria in diabetic animals by decreasing NADPH oxidase 4 (NOX4)-dependent oxidative stress and apoptosis. However, Kitada et al demonstrated that resveratrol protects against diabetic nephropathy through the direct scavenging of ROS, normalisation of the Mn-SOD function and glucose–lipid metabolism via AMPK/SIRT1-independent mechanisms [29]. The discrepancy between our results and previous studies regarding AMPK/SIRT1 signalling might reflect differences in the dose of resveratrol administered (0.3% vs 20 mg kg⁻¹ day⁻¹), the part of the renal tissue examined (whole kidney vs renal cortex) and the renal cell type studied (proximal epithelial cell vs mesangial cell).

PGC-1 α exerts a wide array of effects and it directly co-activates multiple transcription factors, including nuclear receptors such as the PPARs [30, 31], glucocorticoid receptors [24], oestrogen receptors and ERRs [32, 33], and non-nuclear receptor transcription factors, such as myocyte enhancer factor-2 [34] and the family of FOXO transcription factors [35]. By simultaneously co-activating these transcription factors, PGC-1 α can quickly and coordinately modulate a transcriptional programme that governs the energy metabolism. Although PGC-1 α co-activation can change in response to different stimuli or in a tissue-specific manner, in the current study, PGC-1 exerted, in part, various renal effects by enhancing the production of PPAR α and its target molecule ERR-1 α , in addition to co-activating FOXO3a in relation to the PI3K–Akt axis.

We and others have demonstrated that PPAR α activation by PPAR α agonist prevents and improves diabetes- or high-fat-diet-induced renal injury by increasing the production of lipolytic enzymes and reducing lipid accumulation, oxidative stress and renal cell apoptosis, while inhibiting the development of albuminuria and glomerular fibrosis [12, 13, 36]. ERR-1 α activation by PGC-1 α and PPAR α induces genes with roles in lipid transport [36], oxidative phosphorylation [17, 37], fatty acid oxidation [38, 39], the tricarboxylic acid cycle [30, 34, 35], mitochondrial biogenesis [17, 39] and oxidative stress defence [18]. In the current study, resveratrol increased PPAR α –ERR-1 α production and decreased SREBP1, and this was closely associated with decreased lipid content in the kidney. We also showed that high-glucose treatment of mesangial cells inhibits the expression of genes involved in lipolysis, such as those encoding PPAR α and CPT-1A. Additionally, lipogenic genes, such as that encoding SREBP1, were induced by high-glucose treatment. Resveratrol treatment in the presence of high glucose reversed these changes, suggesting that the renoprotective impact of resveratrol may be mediated by shifting the gene-expression profile of the cells to a state that favours lipolysis.

The *db/db* mouse, which lacks signalling through the leptin receptor, is an excellent model of type 2 diabetes

because it develops hyperphagia, obesity, hyperleptinaemia and overt hyperglycaemia [40]. In this study, *db/db* mice, with or without resveratrol treatment, ate about twice the amount eaten by the *db/m* mice. Leptin increases glucose uptake and type 1 collagen in *db/db* mesangial cells through a TGF- β –PI3K-dependent pathway [41]. In addition, transgenic mice with leptin overexpression demonstrated an increase in collagen type IV and fibronectin mRNA in the kidney [42]. Recently, it has been demonstrated that the reduced AMPK and SIRT1 function in *db/db* mice causes dysregulation of adipogenic, lipogenic and lipo-oxidative genes, including leptin, to favour an obese phenotype [42]. These results suggest that resveratrol treatment might partially improve leptin-related TGF- β –PI3K and lipid dysregulation through the activation of AMPK–SIRT1–PGC-1 α in the *db/db* mouse kidney.

In the current study, we found that resveratrol treatment increased the production of AMPK and SIRT1 and decreased PI3K–Akt signalling in the kidneys of *db/db* mice and the mesangial cells in high-glucose media. In contrast, Schenk et al demonstrated that restriction of energy intake induced SIRT1 activation in mice, resulting in enhanced skeletal muscle insulin sensitivity, via PI3K modulation, and suggested that insulin-stimulated PI3K signalling is essential for the functional improvement of glucose transport in the skeletal muscle, and that SIRT1 is a key orchestrator of adaptation mediated by restriction of energy intake [43]. The discrepancy in effects in this study and the previous energy-restriction study might be because of differences in plasma insulin concentration and target organ. Restriction of energy intake augments insulin-stimulated PI3K activity in response to a physiological insulin concentration (60 μ U/ml) [29]. In contrast, at the supraphysiological concentration of insulin, such as in *db/db* (more than 200 μ U/ml) mice, PI3K would be activated in the kidney [44].

Regarding FOXO signalling in diabetic nephropathy, a novel mechanism of diabetic nephropathy has been suggested [39]. Diabetic conditions increase TGF- β abundance in the mesangial cells and TGF- β activates p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERKs), PI3K–Akt and Smads [45]. Among these, PI3K–Akt activation by TGF- β induces FOXO3a phosphorylation that results in decreased BCL-2 interacting mediator of cell death (BIM) and Mn-SOD. In contrast, another study has demonstrated that the PI3K–Akt pathway acts as a survival and anti-apoptotic signal in the renal cells [46]. Overall, the data from the present study indicate that diabetes increases PI3K–Akt phosphorylation and FOXO3a phosphorylation, and this is accompanied by significant changes in the expression of key FOXO3a target genes, as reflected by the decreases in anti-apoptotic BCL-2 and the antioxidants SOD1 and SOD2, and the increase in the expression of the pro-apoptotic gene *Bax*. Consequently, FOXO3a inactivation resulted in apoptotic

renal cell death and oxidative stress. However, it is interesting that 12 weeks of treatment with resveratrol led to a protective increase in FOXO3a in the kidney, where it contributed to protection against apoptotic cell death and oxidative stress.

In conclusion, our results demonstrate that activation of AMPK and SIRT1 was associated with PGC-1 α -ameliorated lipotoxicity in the kidney, which could be related to apoptosis and oxidative stress in diabetic nephropathy. Resveratrol, as an activator of AMPK–SIRT1–PGC-1 α signalling, affected at least two pathways in the development of diabetic nephropathy. One pathway enhanced the PPAR α –ERR-1 α –SREBP1-mediated removal of lipids that had accumulated in the kidney. The other pathway decreased apoptotic renal cell death and oxidative stress related to FOXO3a activation. These signalling events may improve glomerular matrix accumulation, inflammation and albuminuria. Our data further substantiate the suggestion that high-glucose-induced mesangial cell damage translates into lower AMPK–SIRT1–PGC-1 α activity and exacerbated oxidative stress and apoptotic cell damage in the setting of diabetes. All of the changes in AMPK–SIRT1–PGC-1 α signalling were reversed by resveratrol treatment. These results suggest that resveratrol may be a novel therapeutic agent for type 2 diabetic nephropathy.

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Contribution statement MYK, JHL, HHY, YAH, KSY, HSP, SC, SHK, SJS, BSC, HWK, YSK, JHL, YSC and CWP designed and performed the studies and analysed data. CWP directed the study, interpreted the data and wrote the paper. All authors critically revised and approved the final version.

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