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Original Communication

Estrogen receptor subtype ratio change protects against podocyte damage

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ABSTRACT

Women are relatively protected against the development and progression of glomerulosclerosis (GS) prior to menopause. However, the "female advantage" is lost in women who are either diabetic, post-menopausal or both. We showed that 17β-estradiol (E_2) was effective in prevention of diabetic GS development in part through the stabilization of podocyte cytoskeleton and a change in estrogen receptor (ER) subtype ratio. The objective of this study was to examine whether resveratrol (RSV), reported to have estrogen-like action and renoprotective activity against diabetic GS, would affect similar pathways. After *in vitro* treatment with RSV we found a change in the ERα and ERβ expression ratio in favor of ERβ, suppression of heat shock protein 25 (Hsp25) expression and increase in β1-integrin expression, important for maintaining podocyte cytoskeleton. We noted a reduction of insulin-like growth factor 1 receptor (IGFR1) expression, decrease in extracellular signal-regulated kinase (ERK) activation, decrease in reactive oxygen species (ROS), and decrease in cleaved-caspase 3 expression. We found an increase in [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and an increase in matrix metalloproteinases (MMP-2 and MMP-9) activity. Using cre-loxP strategy we developed podocyte-specific ERα knockout mice to show the importance of ERβ. In isolated podocytes, we confirmed reduction of ERα expression in conjunction with a decrease in IGFR1 expression, ERK and increase of MMP-2 similar to that of our *in vitro* treatment with RSV. Taken together these data suggest an important role for ERβ and ER subtype ratio in podocyte stabilization. Therefore RSV or other regulators of ER pathways could offer protection against diabetic and age-related podocyte changes.

KEYWORDS: diabetic glomerulosclerosis, podocytes, estrogen receptor, resveratrol

ABBREVIATIONS

GS, Glomerulosclerosis; E_2, 17β-estradiol; ER, estrogen receptor; RSV, resveratrol; Hsp25, heat shock protein 25; IGFR1, insulin-like growth factor 1 receptor; ERK, extracellular signal-regulated kinase; MMP2 and MMP9, matrix metalloproteinases; ROS, reactive oxygen species; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; T2D, type 2 diabetes; HRT, hormone replacement therapy; (STZ-DM), insulin-like effects in streptozotocin (STZ)-induced diabetes mellitus; DM, diabetes mellitus; AGE/RAGE/NF-κB, AGE/nuclear factor kappa B; Sirt2, Sirtuin 2; AMPK, AMP-activated protein kinase; DN, diabetic nephropathy; HPV, human papilloma virus; FBS, fetal bovine serum; EFS, charcoal stripped serum; DKD, diabetic kidney disease; ECM, extracellular matrix; AGEs, advanced glycation endproducts.

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INTRODUCTION

Among the millions affected by the world-wide epidemic of type 2 diabetes (T2D), women are relatively protected against the development and progression of glomerulosclerosis prior to menopause. However, the "female advantage" is lost in women who are either diabetic, post-menopausal or both. Podocyte damage and apoptosis are thought to be important if not essential in the development and progression of glomerulosclerosis. We have previously shown that in female mice, 17β-estradiol (E_2) is particularly important for maintaining glomerular structure and function [1, 2]. We also recently published that E_2 treatment inhibited type 2 diabetic glomerular disease in db/db mice in an estrogen receptor (ER)-dependent manner by prevention of podocyte injury. In addition, we found that E_2 mediates changes in the podocyte ERα–ERβ ratio and that ER regulation prevented podocyte actin remodeling and apoptosis [3].

The findings on hormone replacement in the Women's Health Initiative (WHI) led to a decline in the number of women who use hormone replacement therapy (HRT) [4]. Women are more commonly utilizing either short-term targeted therapy or nutraceuticals. Therefore we sought to extend our research to other compounds which maintain or restore E_2/ER function to levels that might help prevent or suppress diabetic or age-related kidney disease. Resveratrol (RSV), a polyphenolic compound found in grapes, berries and red wine, has been reported to act as an estrogen agonist or antagonist depending on the cell type (http://lpi.oregonstate.edu/mic). It has also been shown that RSV exhibits insulin-like effects in streptozotocin (STZ)-induced diabetic (STZ-DM) rats and that it ameliorates hyperglycemia, hyperlipidemia and other common diabetic symptoms [5]. Further, RSV can alleviate diabetes mellitus (DM)-induced vasculopathy by attenuating the advanced glycation end products/receptor for AGE/nuclear factor kappa B (AGE/RAGE/NF-κB) signaling pathway [6]. RSV has also been shown to attenuate glomerulosclerosis by an anti-oxidative mechanism and by reducing the expression of Sirtuin 2 (Sirt2) and p38 in diabetic kidneys [7]. Finally, Ding *et al*. demonstrated that RSV attenuates renal hypertrophy in STZ-DM rats by AMP-activated protein kinase (AMPK) activation [8].

To date however downstream consequences of RSV-mediated ER effects have not been explored in diabetic nephropathy (DN). In this study we therefore treated podocytes *in vitro* with RSV and compared the treatment to podocytes treated with E_2. We investigated 1) ER (estrogen receptor) modulation; 2) regulation of Hsp-25 and β1-integrin, two molecules necessary to stabilize podocyte cytoskeleton; and 3) insulin-like growth factor receptor (IGFR1) expression as we have previously shown IGF1 to promote GS in diabetic mice [9]. We also investigated the regulation of ER-mediated downstream pathways such as ERK activation and matrix metalloproteinases (MMP-2 and MMP-9) activity. To complement these studies and show the *in vivo* relevance of ER subtype ratio expression changes, we utilized cre-lox mice and developed a podocyte-specific ERα knockout mouse.

MATERIALS AND METHODS

Podocyte cell lines

We utilized podocytes isolated from C57BL/6J female db/db mice treated with either *in vivo* placebo or 17β-estradiol [3]. Mice were sacrificed at 24 weeks of age and glomerular podocytes were isolated, propagated, and immortalized using human papilloma virus (HPV) as previously described [3]. Briefly, db/db female mice were purchased from Jackson Laboratories. Mice were kept in a conventional grade facility, housed in groups of 3-5 under a 12 h light/dark cycle (light from 7 am to 7 pm) at 22 ± 1 °C, with unrestricted access to food. Mice were anesthetized by a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) intra-peritoneal injection. The protocol has been approved by the Institutional Animal Care and Use Committee at the University Of Miami Miller School Of Medicine. All efforts were made to minimize animal discomfort and suffering. Glomerular cells isolated from these mice exhibit a decreased ER expression and function [3]. Cells were grown and maintained in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS). The stock RSV was re-suspended in ethanol. For RSV treatment,

podocyte cell lines were exposed for 24 h in phenol red-free medium and 10% charcoal-stripped serum (EFS) and treated with different doses of RSV (Sigma) (10-50 uM) daily for an additional 48 hours in 0.1% bovine serum albumin (BSA).

Western blot analysis and immunoprecipitation

Podocyte cell lysates were extracted, protein assay performed (Pierce Biotechnology, Rockford, IL) and samples were resolved by electrophoresis on 10% polyacrylamide gels as previously described (3). Electrotransfer of proteins from the gel to the nitrocellulose was performed. The blots were then exposed to the following antibodies: Hsp25 (Enzo Life Sciences International, Plymouth Meeting, PA), β1-integrin, ERK1/2 and phospho-ERK1/2, IGFR1, ERα (H184) and (MC-20) combined, and ERβ for the immunoprecipitated samples (Santa Cruz Biotechnology, Santa Cruz, CA), (Chemicon International, Temecula, CA), cleaved-caspase 3 (Cell Signaling Technology, Denvers, MA) and MMP2 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight followed by secondary antibodies. Expression of proteins were determined by exposing the nitrocellulose blots to a chemiluminescence solution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 min, followed by exposure to X-OMAT AR film (Eastman Kodak Co., Rochester, NY). The films were scanned for densitometric analysis using ImageJ software (National Institutes of Health) as previously described [3]. Blots were treated with β-actin antibody (loading control) (Sigma-Aldrich, St. Louis, MO) after stripping to ensure equal loading of gels.

For ERβ immunoprecipitation, 20 ug of protein was incubated with an antibody against ERβ for 2 h at 4 °C, followed by the addition of protein G-agarose overnight. The resulting protein-antibody conjugate was centrifuged at 4 °C and washed four times with lysis buffer. The final pellet was re-suspended in sample buffer and the mixture was boiled for 5 min before it was loaded on a 10% SDS gel. Gels were analyzed by densitometry using ImageJ densitometry program (ver. 1.17; available by ftp at zippy.nimh.nih.gov/ or at https://imagej.nih.gov/nih-image/, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD), to determine protein expression.

MMP-2 and MMP-9 activity

Podocyte supernatants were collected after 48 hours and then centrifuged at 13,000 g for 15 minutes at 4 °C. Pellets were discarded and the supernatant collected and analysis performed. Protein concentration from cell lysates was determined as described for Western analysis and supernatants were loaded according to the protein assay. MMP-2 and MMP-9 activity were assessed using 10% zymogram gels (Invitrogen Corp., Carlsbad, CA) as described previously [10]. Gels were analyzed by densitometry using ImageJ densitometry program, as described above, to determine relative MMP-2 and MMP-9 activity.

ROS measurement

5,000 cells per well in triplicate were plated after treatment in a 96-well plate. Reactive oxygen species were measured using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate according to manufacturer's directions (carboxy-H_2DCFDA, Molecular Probes, Eugene, OR).

MTT assay

E_2 or RSV-treated podocytes were plated in 6 well plates. After treatment they were trypsinized and plated with a concentration of 7,500 cells in 96-well tissue culture plates with a final volume of 100 ul. Briefly, 10 ul of AB solution from Colorimetric (MTT) kit for cell survival and proliferation (Chemicon International, Temecula, CA) was added to each well and the plate incubated at 37 °C for cleavage of MTT to occur. After 4 hours the MTT formazan produced in wells containing live cells appeared to be black. Isopropanol was added to each well and mixed to dissolve MTT formazan to give a homogeneous blue solution suitable for absorbance measurement at 570 nm.

Breeding and genotyping of double transgenics

For selective deletion of ERα in glomerular podocytes, transgenic mice that express Cre-recombinase specifically in podocytes were crossed with "floxed" mice, which contain loxP sites upstream of the ERα gene. 2.5P-Cre mice (podocin$^{Cre/Cre}$) with the Cre-recombinase cassette under the regulation of a fragment of the human NPHS2 promoter of the podocin gene, leading to

podocyte-specific expression of the Cre-recombinase, were crossed with homozygous floxed ERα mice. The ERα gene is floxed around exon 3 which encodes the first zinc finger of the DNA-binding domain. The F1 podocin$^{Cre/-}$/ER$^{flox/-}$ bitransgenic mice is crossed to homozygous ER$^{flox/flox}$/podocin$^{-/-}$ mice, generating homozygous podocin$^{Cre+/-}$-ER$^{flox/flox}$ mice. Genotyping for Cre positive mice was performed as previously described [11] using primers that recognize a 268 bp fragment of the Cre coding sequence. Genotyping of the floxed mice was performed by using primer pairs obtained from Dr. Korach.

Statistics

In vitro assays were performed in triplicate. One-way ANOVA (analysis of variance) and the Dunnett multiple-comparison post hoc test or Student's t test were performed for the statistical analysis. (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Statistical significance was set at $P < 0.05$.

RESULTS

ERα, ERβ, Hsp25, and β1-integrin expression

We previously showed that isolated podocytes from E_2-treated mice exhibited higher expression of estrogen receptors and a direct protective effect of E_2. We therefore compared the effects of E_2 and RSV (10 uM), another compound shown to regulate ER [12], on ERα and ERβ protein expression and subsequent ER subtype ratios. Western blot analysis of podocyte lysates revealed decreased expression of ERα after *in vitro* treatment with RSV (66.78 ± 7.58) and *in vivo* with E_2 (40.00 ± 9.26) compared with vehicle control (99.89 ± 0.200) (Fig. 1A) but an increased expression of ERβ after *in vitro* treatment with RSV (181.0 ± 13.92) and E_2 (135.3 ± 10.63) versus the vehicle control (100 ± 2.84), showing a change in the subtype ratio (Fig. 1B).

Once we established a similar change in ER subtype ratio expression between E_2 and RSV, we determined if podocyte cytoskeleton was stabilized. Podocyte Hsp25 protein expression was decreased after RSV (83.55 ± 4.69) and E_2 (65.08 ± 8.85) treatment compared with podocytes treated with vehicle control (100 ±1.65) (Fig. 1C). β1-integrin expression, however, was increased in RSV treated podocytes (157.8 ± 20.71) compared to vehicle treated cells (99.83 ± 0.167). This was similar to the expression levels found in podocytes isolated from mice treated *in vivo* with E_2 (134 ± 3.49) compared to vehicle treated cells (99.83 ± 0.167) (Fig. 1D).

IGFR1R, ERK2, MMP-2 and MMP-9 activity, ROS, MTT, and cleaved-caspase 3

We and others have shown an increase in IGFR and downstream IGFR1 signaling in the kidneys of diabetic mice and glomerular cells [9, 13, 14]. We also showed that glomerular mesangial cell IGFR was modulated by E_2 or estrogen deficiency and decreased ER expression [2, 13]. Therefore we investigated the effect of E_2 on podocyte expression of IGFR and compared these effects to RSV. We found that protein expression decreased following podocyte exposure to *in vitro* RSV (10 uM) (68.45 ± 10.95) or *in vivo* E_2 (41.73 ± 7.16) treatment compared with placebo db/db mice (100 ± 1.09, Fig. 2A).

Since ERK activation is stimulated by IGFR activation, and we have shown that ERK regulated ER [15], we also investigated whether there was a parallel decrease in ERK activation. Only ERK2 activation was altered in podocytes after *in vitro* RSV (10 uM) (54.50 ± 9.67) or *in vivo* E_2 (70.13 ± 7.59) treatment compared with vehicle db/db podocytes (100 ± 1.34, Fig. 2B). Finally we assessed MMP-2 (Fig. 2C) and -9 activity (Fig. 2D). In glomerular cells, including podocytes, MMPs are regulated by E_2 [16, 17]. As expected podocytes treated *in vitro* with RSV (10 uM) (148.3 ± 16.75) or *in vivo* with E_2 (138.6 ± 11.13) have higher activity compared to control (99.67 ± 0.21, Fig. 2C). In addition, MMP-9 was regulated in a similar manner with higher activity after *in vitro* treatment with RSV (50 uM) (215.5 ± 4.95) and E_2 (361 ± 11.97) versus vehicle control (99.5 ± 0.71, Fig. 2D).

ER changes are associated with apoptosis [18, 19], oxidant stress [20, 21], and cell cycle [22] regulation. We found that cleaved caspase 3 expression was decreased following both RSV *in vitro* treatment (50 uM) (71.5 ± 2.1) and E_2 treatment (29 ± 15.56) versus control (99.5 ± 0.71, Fig. 2E). RSV (10 uM) (13.7 ± 2) or E_2 (13 ± 2.6)

decreased ROS compared to vehicle control [23]. While MTT was increased by 131% for RSV and 216% for E_2 compared to the control.

ERα, ERβ, IGFR1, ERK1, ERK2, and MMP-2 expression in cre-lox mice

As previously described, we used immortalized podocyte cell lines isolated and characterized in our laboratory from db/db mice [3]. We also isolated podocytes from mice that were crossed to produce a podocyte-specific ERα knockdown without regulating ERβ expression levels. Thus, influencing ERα:ERβ subtype ratio in podocytes isolated from wild-type (Wt) mice was confirmed by western blot analysis. ERα expression was decreased in podocytes isolated from female cre-lox mice (58.33 ± 8.024) compared to Wt female mice (100 ± 10.75, Fig. 3A). No difference in ERβ expression

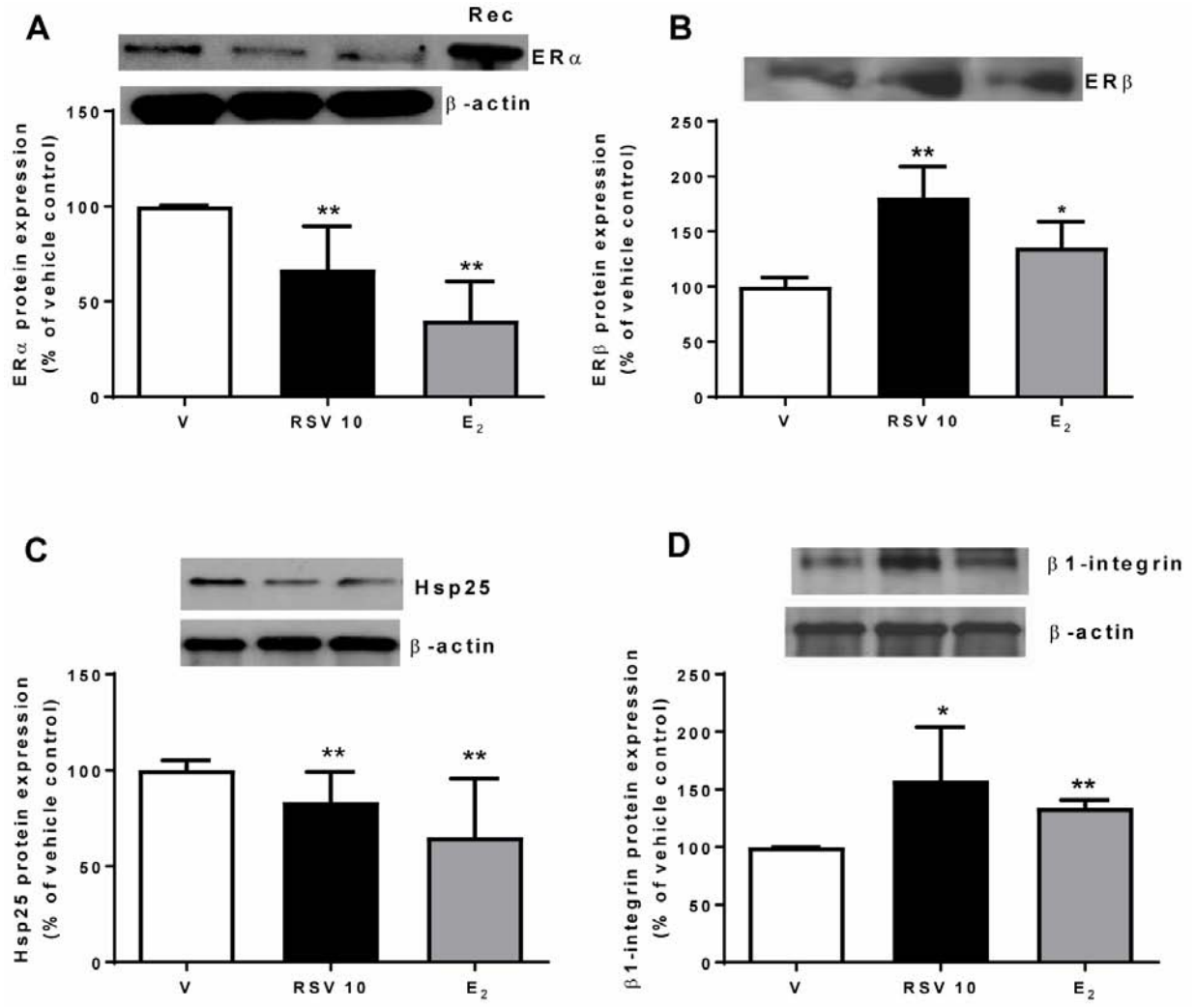

Fig. 1. Podocytes isolated from female diabetic db/db mice treated *in vitro* with RSV and *in vivo* with E_2 have (A) decreased expression of ERα (B) increased expression of ERβ (C) decreased expression of Hsp25 and (D) increased expression of β1-integrin compared to podocytes isolated from placebo db/db treated mice (vehicle control, V). A. ERα expression was detected at a molecular mass of 67 kDa. **B.** Immunoprecipitation of ERβ using 20 ug of lysate. ERβ expression was detected at a molecular mass of 52 kDa. **C.** Hsp25 expression was detected at a molecular mass of 25 kDa. **D.** Expression of β1-integrin was detected at 135 kDa. All data are graphed as mean ± SEM and expressed as a percent of vehicle cells. Inserts show representative Western blots. β-Actin is shown below as loading control. $*P < 0.05$, $**P < 0.005$ compared to placebo, n = 3/group. Rec = recombinant in Fig. 1A.

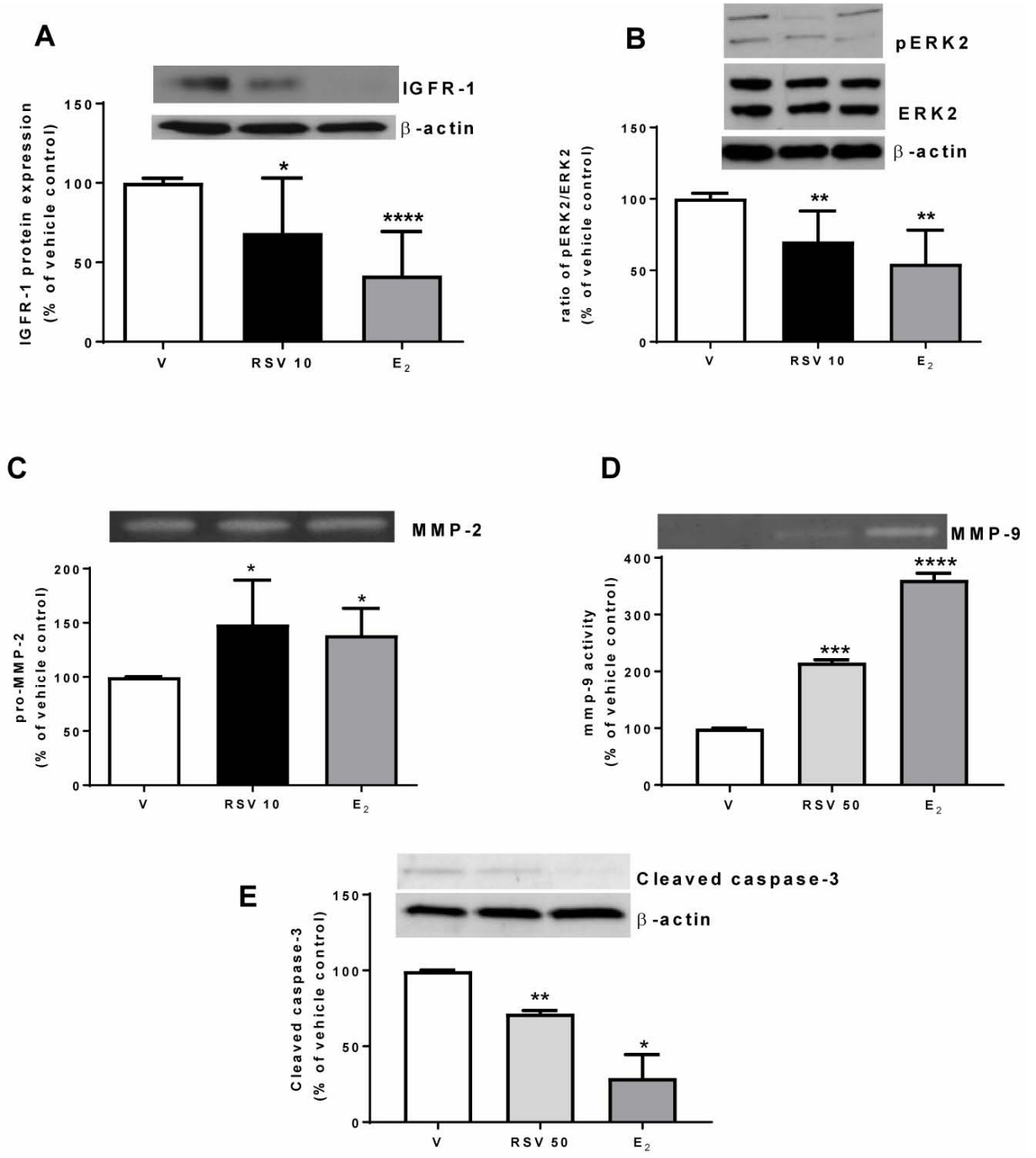

Fig. 2. Podocytes isolated from female diabetic db/db mice treated *in vitro* with RSV and *in vivo* with E_2 have (A) decreased expression of IGF1R (B) and ERK2 (C) increased expression of MMP-2 and (D) MMP-9 and (E) decreased Cleaved caspase 3 expression compared to podocytes isolated from placebo db/db treated mice (vehicle control-V). A. IGFR1 expression was detected at a molecular mass of 120 kDa. **B.** pERK2 and ERK2 were detected at a molecular mass of 42 kDa (lower band). **C.** Insert of representative zymogram shows the proMMP-2 active band at 68 kDa. **D.** Insert of representative zymogram shows the proMMP-9 active band at 92 kDa. **E.** Cleaved caspase-3 was detected between 15 and 20 kDa. All data are graphed as mean ± SEM and expressed as a percent of vehicle cells. Inserts show representative Western blots. β-Actin is shown below as loading control. **P* < 0.05, ***P* < 0.005, ****P* < 0.001, *****P* < 0.0001 compared to placebo, n = 3/group.

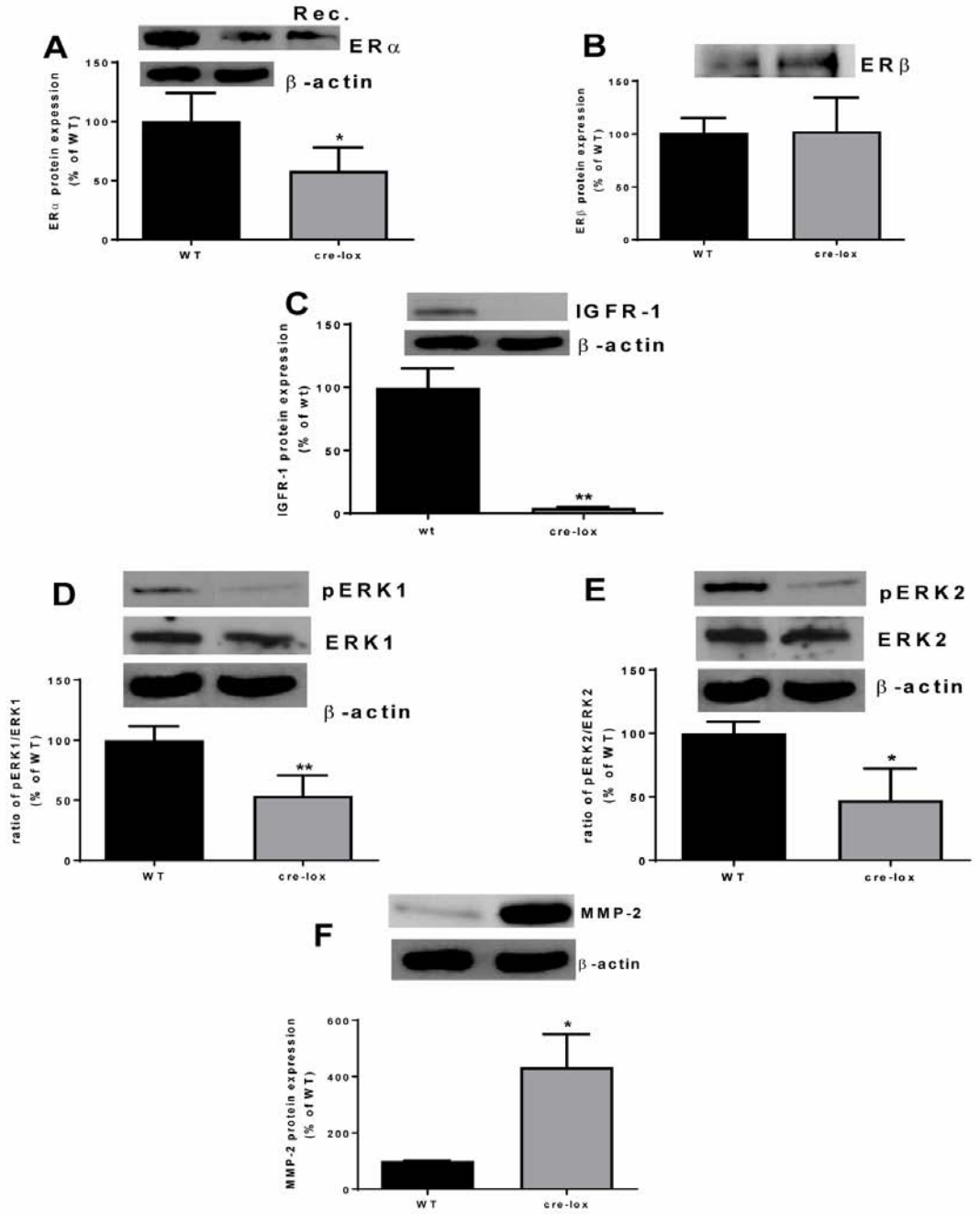

Fig. 3. Podocytes isolated from female cre-lox mice have (A) decreased expression of ERα and (B) no change in ERβ expression, (C) decreased protein expression of IGFR1, (D) decreased phosphorylated form of ERK1, (E) ERK2 and (F) MMP-2 compared to podocytes isolated from Wt mice. Cell lysates were collected from podocytes isolated from both Wt mice (black bars) and cre-lox mice (gray bars). **A.** ERα expression was detected at a molecular mass of 67 kDa. **B.** Immunoprecipitation of ERβ using 20 ug of lysate. ERβ expression was detected at a molecular mass of 52 kDa. **C.** Representative Western blots showing podocyte expression of IGFR1 from Wt and cre-lox detected at 120 kDa. **D.** Representative Western blot showing expression of pERK1 and ERK1 around 44 kDa. **E.** Representative Western blot showing expression of pERK2 and ERK2 at 42 kDa **F.** Representative Western blot showing expression of MMP-2 at 90 kDa. All data are graphed as mean ± SEM and expressed as a percent of vehicle cells. Inserts show representative Western blots. β-Actin is shown below as loading control. **P* < 0.05, ***P* < 0.005 compared to placebo, n = 3 group. Rec = recombinant in Fig. 3A.

was observed in podocytes isolated from female cre-lox mice compared to Wt mice (Fig. 3B). IGFR1 expression was reduced in podocytes isolated from cre-lox knockouts (4.3 ± 0.5276) compared to Wt mice (99.75 ± 7.598, Fig. 3C). In parallel, podocytes isolated from female cre-lox mice had a lower protein expression of the phosphorylated form of ERK1 and ERK2 (about 2 fold) compared with podocytes isolated from Wt mice (Fig. 3D and 3E). As expected, podocytes isolated form female cre-lox mice showed a 4-fold increase in the expression of MMP-2 (Fig. 3F).

DISCUSSION

Multiple studies have reported the significance of ER regulation in diabetic and age-related glomerulosclerosis [3, 16, 23]. Since the safety of HRT is still an ongoing subject of debate [24] the search for estrogen supplementation alternatives has been sought. We have previously shown the importance of the ER subtype ratio change in maintenance of podocyte stability in a model of diabetic glomerular disease [3]. To date however, the effects of this ratio change have not been studied with other compounds that may have similar effects. Resveratrol, a popular nutraceutical shown to act as an estrogen agonist/antagonist, may be one such alternative [25]. In fact, our study is the first to show that RSV can regulate expression of the podocyte ER subtype ratio and act to stabilize podocytes. Furthermore, we also confirm the protective role of podocyte ERβ expression against age-related glomerular changes *in vivo*.

We found that RSV mediated changes in the podocyte ERα:ERβ ratio by decreasing ERα protein expression. This is similar to our previous findings in podocytes isolated from db/db mice after E_2 exposure [3]. In estrogen receptor-positive breast cancer cells, RSV acts as an estrogen agonist in the absence of the endogenous estrogen E_2, but acts as an estrogen antagonist in the presence of E_2. Since the subtype ratio change in podocytes appears to be beneficial, this is an important finding and suggests a benefit of RSV in the glomerulus not previously investigated.

RSV and E_2 decreased expression of Hsp25 and increased β1-integrin expression, molecules important for podocyte stabilization. Reduced expression of Hsp25 could decrease actin capping, promote filament extension and protect against podocyte foot process effacement and proteinuria. In addition, β1-integrin has a primary and essential function in establishing and maintaining the characteristic podocyte cytoskeleton. Podocytes are highly dependent on the preservation of their actin cytoskeleton to ensure proper function and survival. Increased albumin excretion, one of the hallmarks of diabetic kidney disease (DKD), results from damage to podocytes [26-29].

Although we have previously reported that E_2 is protective against podocyte damage [3], the mechanism(s) have not been fully elucidated. Dysregulation of the IGF1 signaling pathway has been implicated in a variety of diseases including DKD [14, 30]. Studies on glomerular cells by our group and others have shown that IGF is produced and IGFR is expressed on podocytes, mesangial and endothelial cells [31-34]. Therefore we postulated that RSV and E_2 could reduce expression levels of IGFR1 and thereby regulate downstream events that could protect against podocyte damage. In fact, ERK activation, which has been shown to be downstream of IGF activation, also decreased. These data are in agreement with our previous study that showed E_2 replacement in aged female mice exposed to cigarette smoke reduced IGFR mRNA and protein expression [13] and prevented smoking-associated glomerular changes.

We, along with others, have also reported that IGF1 can promote sclerosis by either increasing synthesis, or decreasing the degradation of glomerular extracellular matrix (ECM) [14, 35]. Decreased MMP-2 levels have been found in the glomeruli of patients with type 2 diabetes and progressive nephropathy [36]. Based on these *in vivo* data in humans, and rodents [14, 37] it was not surprising that E_2 and RSV could alter MMP-2 and MMP-9 activity in part by regulation of IGFR expression. Finally, IGFR expression is sensitive to oxidant stress. ROS stimulates the synthesis of IGF1 in vascular smooth muscle cells [38]. It is well established that estrogen deficiency is associated with increased oxidant stress [39], which promotes age-related diseases in the renal vasculature [40, 41]. Oxidant stress occurs when

free radicals, single reactive oxygen species (ROS) and other reactive intermediates, such as advanced glycation endproducts (AGEs) of lipid peroxidation products overwhelm antioxidant systems [42, 43]. Of note, Robb *et al*. showed that RSV, through ERβ, induced the mitochondrial antioxidant MnSOD [44]. It is likely, therefore, that treatments with either E_2 or RSV that reduce ROS [44, 45] may also be beneficial by reducing IGFR expression.

We also investigated the expression of cleaved caspase 3, a death protease and marker of apoptosis. In some cell types and in cancer, IGF1 signaling has been shown to regulate apoptosis [46]. Our data suggest a correlation between IGFR expression and cleaved caspase 3, although further experiments are underway to determine if the reduction of IGFR signaling directly regulates caspase 3, or if alternative pathways stimulated by RSV and E_2, such as reduction in oxidant stress were responsible [47, 48].

To our knowledge no one has reported the consequences of targeted knockout of ERα on podocytes. Podocytes express both ER subtypes, and it has been postulated that each subtype has opposing roles in regulating estrogen action, especially in the breast and the brain [49-51]. However, the precise differential roles of estrogen receptor subtypes ERα and ERβ in many organs remain unresolved. Therefore we used cre-lox technology and bred mice to obtain podocyte-specific ERα knock out mice and isolated a small number of podocytes from the glomeruli. We confirmed the decrease of ERα expression by Western analysis and found that the presence of ERβ resulted in reduced IGFR expression, decreased ERK activation and increased MMP-2 activity, similar to that seen after reduction of ERα by pharmacologic means. Not surprisingly, we were unable to observe any age-associated changes in albumin excretion, most likely due to the presence of ERβ.

CONCLUSION

These data further support the importance of ER subtype regulation of podocyte stabilization *in vivo*. Since the balance between the expression of ER subtypes may play an important role in the paradoxical characterization of estrogens as both beneficial and harmful, it is likely that increased ERβ expression/activation in podocytes induced by E_2 or RSV is protective. These data also underscore the importance of finding alternative compounds to maintain or restore E_2/ER function to levels that might restore the “female advantage” and thereby prevent or suppress diabetic or age-related kidney disease.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

REFERENCES

1. Elliot, S. J., Karl, M., Berho, M., Potier, M., Zheng, F., Leclercq, B., Striker, G. E. and Striker, L. J. 2003, Am. J. Pathol., 162, 1441-8.
2. Karl, M., Berho, M., Pignac-Kobinger, J., Striker, G. E. and Elliot, S. J. 2006, Am. J. Pathol., 169, 351-61.
3. Catanuto, P., Fornoni, A., Pereira-Simon, S., Wu, F., Burnstein, K. L., Xia, X., Conti, F., Lenzi, A. and Elliot, S. 2012, Endocrinology, 153, 5888-95.
4. Pickar, J. H., Archer, D. F., Kagan, R., Pinkerton, J. V. and Taylor, H. S. 2017, Expert Opin. Drug Saf., 16, 941-54.
5. Chang, C. C., Wu, Y. T., Huang, J. P., Yen, T. H. and Hung, L. M. 2011, J. Biomed. Sci., 18, 47-57.
6. Chen, K. H., Hung, C. C., Hsu, H. H., Jing, Y. H., Yang, C. W. and Chen, J. K. 2011, Chem. Biol. Interact., 190, 45-53.
7. Yacoub, R., Lee, K. and He, J. C. 2014, Front Endocrinol. (Lausanne), 5, 166.
8. Ding, D. F., You, N., Wu, X. M., Xu, J. R., Hu, A. P., Ye, X. L., Zhu, Q., Jiang, X. Q., Miao, H., Liu, C. and Lu, Y. B. 2010, Am. J. Nephrol., 31, 363-74.

9. Elliot, S. J., Striker, L. J., Hattori, M., Yang, C. W., He, C. J., Peten, E. P. and Striker, G. E. 1993, Endocrinology, 133, 1783-8.
10. Marin-Castano, M. E., Striker, G. E., Alcazar, O., Catanuto, P., Espinosa-Heidmann, D. G. and Cousins, S. W. 2006, Invest. Ophthalmol. Vis. Sci., 47, 4098-112.
11. Moeller, M. J., Sanden, S. K., Soofi, A., Wiggins, R. C. and Holzman, L. B. 2003, Genesis, 35, 39-42.
12. Bhat, K. P. and Pezzuto, J. M. 2002, Ann. NY Acad. Sci., 957, 210-29.
13. Elliot, S. J., Karl, M., Berho, M., Xia, X., Pereria-Simon, S., Espinosa-Heidmann, D. and Striker, G. E. 2006, J. Am. Soc. Nephrol., 17, 3315-24.
14. Lupia, E., Elliot, S. J., Lenz, O., Zheng, F., Hattori, M., Striker, G. E. and Striker, L. J. 1999, Diabetes, 48, 1638-44.
15. Karl, M., Potier, M., Schulman, I. H., Rivera, A., Werner, H., Fornoni, A. and Elliot, S. J. 2005, Endocrinology, 146, 889-900.
16. Catanuto, P., Doublier, S., Lupia, E., Fornoni, A., Berho, M., Karl, M., Striker, G. E., Xia, X. and Elliot, S. 2009, Kidney Int., 75, 1194-201.
17. Potier, M., Elliot, S. J., Tack, I., Lenz, O., Striker, G. E., Striker, L. J. and Karl, M. 2001, J. Am. Soc. Nephrol., 12, 241-51.
18. Alamolhodaei, N. S., Tsatsakis, A. M., Ramezani, M., Hayes, A. W. and Karimi, G. 2017, Food and Chemical Toxicology, 103, 223-32.
19. Chimento, A., Sirianni, R., Saturnino, C., Caruso, A., Sinicropi, M. S. and Pezzi, V. 2016, Mini Rev. Med. Chem., 16, 699-709.
20. Hall, S., Dixit, M. and Arany, I. 2017, In Vivo, 31, 551-5.
21. Madrigal-Perez, L. A. and Ramos-Gomez, M. 2016, Int. J. Mol. Sci., 17, 368.
22. Britton, R. G., Kovoor, C. and Brown, K. 2015, Ann. NY Acad. Sci., 1348, 124-33.
23. Liu, F. C., Tsai, Y. F., Tsai, H. I. and Yu, H. P. 2015, Mediators Inflamm., 2015, 643763.
24. Sassarini, J. and Lumsden, M. A. 2015, Age Ageing, 44, 551-8.
25. Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C. and Klinge, C. M. 2000, Endocrinology, 141, 3657-67.
26. Asanuma, K. and Mundel, P. 2003, Clin. Exp. Nephrol., 7, 255-9.
27. Barisoni, L. and Mundel, P. 2003, Am. J. Nephrol., 23, 353-60.
28. Ly, J., Alexander, M. and Quaggin, S. E. 2004, Curr. Opin. Nephrol. Hypertens, 13, 299-305.
29. Shankland, S. J. 2006, Kidney Int., 69, 2131-47.
30. Bach, L. A. and Hale, L. J. 2015, Am. J. Kidney Dis., 65, 327-36.
31. Aron, D. C., Rosenzweig, J. L. and Abboud, H. E. 1989, J. Clin. Endocrinol. Metab., 68, 585-91.
32. Conti, F. G., Elliot, S. J., Striker, L. J. and Striker, G. E. 1989, Biochem. Biophys. Res. Commun., 163, 952-8.
33. Conti, F. G., Striker, L. J., Elliot, S. J., Andreani, D. and Striker, G. E. 1988, Am. J. Physiol., 255, F1214-9.
34. Conti, F. G., Striker, L. J., Lesniak, M. A., MacKay, K., Roth, J. and Striker, G. E. 1988, Endocrinology, 122, 2788-95.
35. Hirschberg, R. 1996, Miner Electrolyte Metab., 22, 128-32.
36. Del Prete, D., Anglani, F., Forino, M., Ceol, M., Fioretto, P., Nosadini, R., Baggio, B. and Gambaro, G. 1997, Diabetologia, 40, 1449-54.
37. Mankhey, R. W., Wells, C. C., Bhatti, F. and Maric, C. 2007, Am. J. Physiol. Regul. Integr. Comp. Physiol., 292, R769-77.
38. Delafontaine, P. and Ku, L. 1997, Cardiovasc Res., 33, 216-22.
39. Helmersson, J., Mattsson, P. and Basu, S. 2002, Clin. Sci. (Lond.), 102, 39-43.
40. Vlassara, H., Torreggiani, M., Post, J. B., Zheng, F., Uribarri, J. and Striker, G. E. 2009, Kidney Int. Suppl., S3-11.
41. Yubero-Serrano, E. M., Woodward, M., Poretsky, L., Vlassara, H., Striker, G. E. and Group AG-lS. 2015, Clin. J. Am. Soc. Nephrol., 10, 759-66.
42. Cadenas, E. and Davies, K. J. 2000, Free Radic. Biol. Med., 29, 222-30.
43. Lee, H. C. and Wei, Y. H. 2007, Exp. Biol. Med. (Maywood), 232, 592-606.
44. Robb, E. L. and Stuart, J. A. 2014, Phytother. Res., 28, 120-31.

45. Robb, E. L. and Stuart, J. A. 2014, Phytochemistry, 98, 164-73.
46. Kasprzak, A., Kwasniewski, W., Adamek, A. and Gozdzicka-Jozefiak, A. 2017, Mutation Research/Reviews in Mutation Research, 772, 78-104.
47. Pereira-Simon, S., Xia, X., Catanuto, P. and Elliot, S. 2012, Endocrinology, 153, 5491-9.
48. Wasik, A. and Antkiewicz-Michaluk, L. 2017, Pharmacol. Rep., 69, 851-60.
49. Kumar, S., Lata, K., Mukhopadhyay, S. and Mukherjee, T. K. 2010, Biochim. Biophys. Acta, 1800, 1127-35.
50. Liu, M. M., Albanese, C., Anderson, C. M., Hilty, K., Webb, P., Uht, R. M., Price, R. H. Jr., Pestell, R. G. and Kushner, P. J. 2002, J. Biol. Chem., 277, 24353-60.
51. Paech, K., Webb, P., Kuiper, G. G. J. M., Nilsson, S., Gustafsson, J-Å., Kushner, P. J. and Scanlan, T. S. 1997, Science, 277, 1508-10.