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DOTTORATO DI RICERCA

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SCIENZE ENDOCRINOLOGICHE, METABOLICHE E ANDROLOGICHE

XXV ciclo

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Il ruolo degli estrogeni nella prevenzione della disfunzione e del danno dei podociti glomerulari nella nefropatia diabetica.

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Background information

Renal corpuscle in the kidney: Mammalian kidneys are a set of two, bean-shaped organs which normally lie on each side of the spine. Urine is produced in two well-defined regions of the kidneys, the renal cortex and the renal medulla. Within these structures lie the renal corpuscles and excretory tubules, together known as *nephrons*. By regulating blood concentration of water and salts, the renal corpuscle maintains blood chemistry at desirable levels.

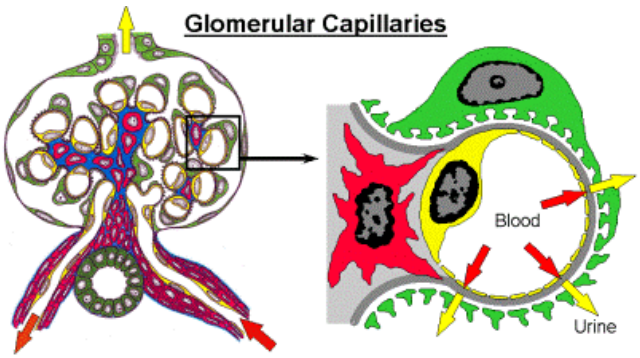
The renal corpuscle is actually the meeting point of two separate structures, the glomerulus and the Bowman's capsule. The glomerulus, a ball of capillaries, sits inside the Bowman's capsule. The Bowman's capsule is a cup-shaped structure arising from the dead end of the nephron's excretory tubule.

The blood vessels within the glomerulus are non-selectively permeable. Any blood solute smaller than 60 nm is able to cross the endothelial capillary wall membrane therefore enabling the glomerulus to act as a filter for blood impurities. As pressure from the heart pumps blood into the glomerulus, solutes such as salts, glucose, and urea, as well as water, are pressed through the tiny openings present in the membrane. Larger particles, such as proteins and blood cells, are unable to penetrate, and so remain suspended in the blood.

Podocytes are specialized cells located in the Bowman's capsule that wrap around the capillaries of the glomerulus and help with the filtration process trapping any large solutes missed by the capillary membrane. The Bowman's capsule retains the filtrate and passes it out of the renal corpuscle. Glomerular

filtrate travels through a series of tubules and finally into a general collecting duct, which receives contributions from a number of nephrons. The Bowman's capsule and the collecting duct are lined with a specialized tissue known as *transport epithelium*. This tissue processes the filtrate into urine. Once processed, the urine is funneled by the collecting duct into the ureter, and then to the urinary bladder. From the roughly 1100-2000 L of blood which pass through the human body daily, the renal corpuscles produce just under 180 L of filtrate. Once processed by the tubules and collecting ducts, this amount of filtrate produces, on average, just under 2L of urine. The remainder of the filtrate is reabsorbed into the blood. This process is regulated by the endocrine system, and serves to keep the blood both chemically-balanced and free of waste products.

Bowman's capsule contains mesangial cells, smooth muscle-like cells,involved in the filtration process. Mesangial cells are located between the capillaries and provide support for glomerular structure. In addition, mesangial cells have the ability to contract since they express myosin and actin.

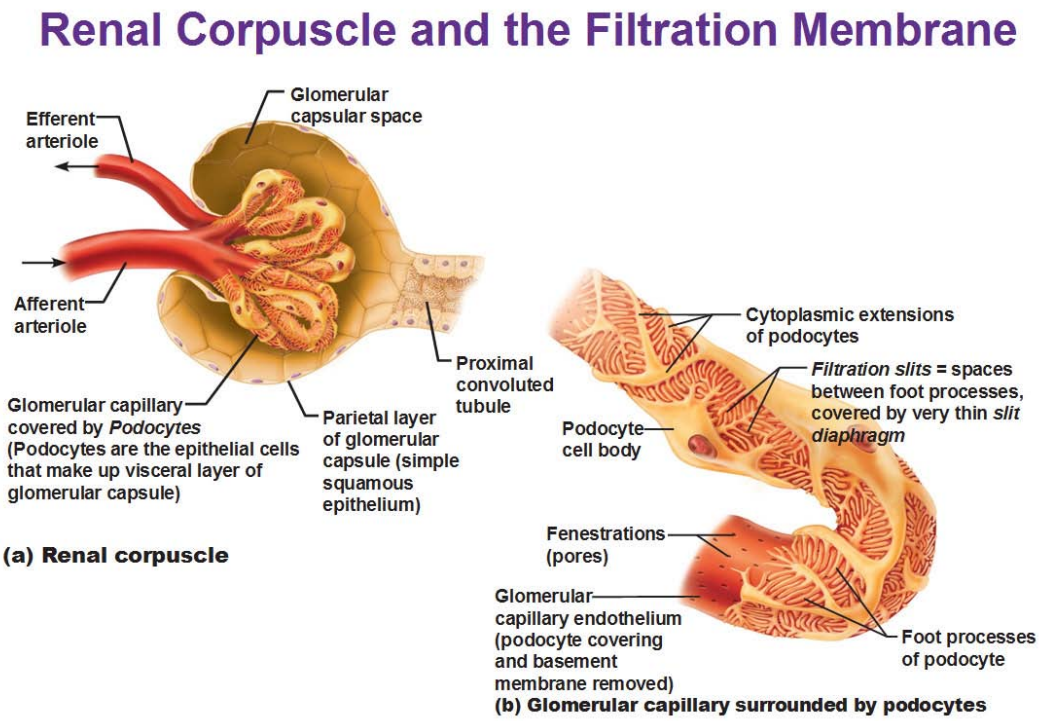


Drawing of a cross section of a GLOMERULUS with arrows showing the flow of blood (red) and urine (yellow)

Drawing of a cross section of one very small blood vessel called a CAPILLARY in a glomerulus showing the filtration of blood (red) into urine (yellow)

Normal podocyte structure

Podocyte gross structure: Podocytes are highly specialized, terminally differentiated epithelial cells, with a quiescent phenotype (1). Podocytes derive embryonically from mesenchymal cells (2). Each mature podocyte has distinct anatomical, and therefore functional, components (3). The cell body is at the center of the cell, and essentially lies in the urinary space and the nucleus is oriented toward the urinary space. From the cell body arise long primary processes, the ends of which contain foot processes. These secondary processes attach to the underlying GBM (Glomerular Basement Membrane) via integrins (4) and dystroglycans (5). Foot processes from neighboring podocytes overlap (interdigitate). The 'filtration slit' formed between adjacent interdigitating podocyte foot processes is a highly specialized gap junction called the slit diaphragm, which forms the major size barrier to protein leakage.



Podocyte Molecular structure: Podocytes are polarized cells. They derive their unique shape due to a rich actin cytoskeleton, which serves as the podocyte's 'backbone' (6). The actin cytoskeleton also enables podocytes to continually and dynamically alter shape. The cytoskeleton comprises three distinct ultrastructural elements: (a) microfilaments (7–9 nm diameter), (b) intermediate filaments (10 nm), and (c) microtubules (24 nm). Microfilaments are the predominant cytoskeletal constituents of the foot process, and contain a dense network of F-actin and myosin.

The actin cytoskeleton is linked with other proteins. Tryggvason et al. was the first to discover nephrin, (7) a member of the Immunoglobulin superfamily, as one of the now increasing number of complex slit diaphragm proteins. The cytoplasmic tail of nephrin binds to podocin (8-10). Nephrin also interacts with and localizes to CD2AP (11;12).More recently, Neph-1, another member of the Immunoglobulin superfamily of proteins, has been identified Neph-1 interacts with nephrin, podocin, and FAT1 (13;14).Other slit diaphragm proteins include ZO-1, Neph-2 and -3, and densin. By forming the only connection between adjacent podocytes, the slit diaphragm limits protein leakage by acting as a size barrier, analogous to a sieve. It is tempting to speculate that the slit may also function as a charge barrier, as some of these proteins are phosphorylated.

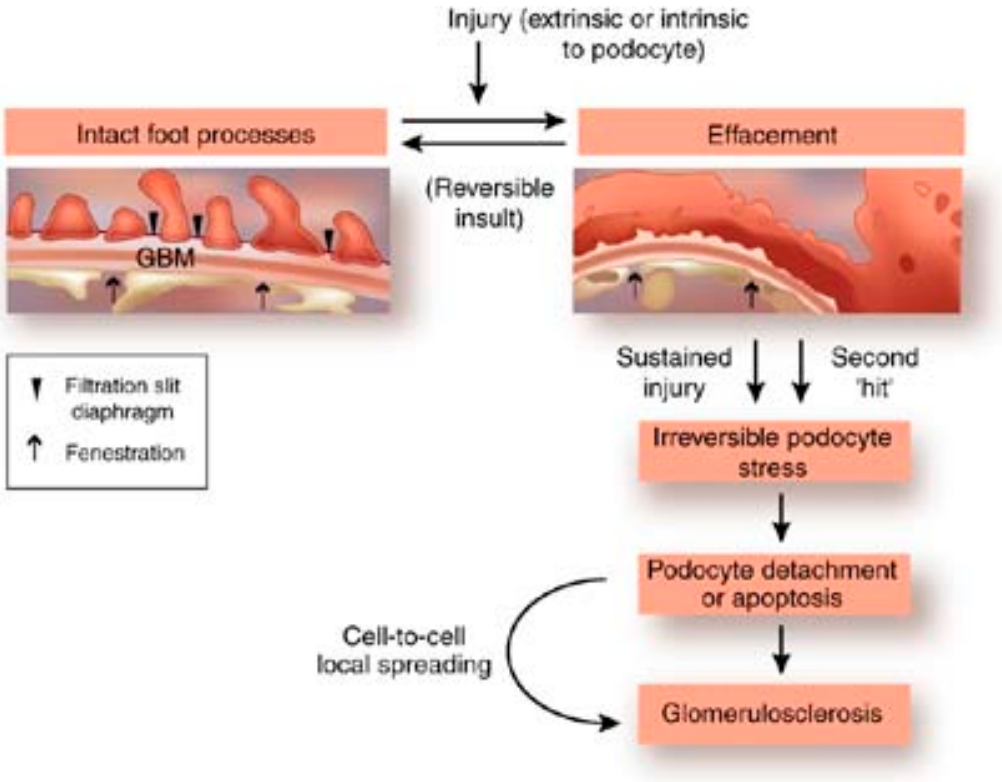
The *apical membrane domain* of podocytes is negatively charged, due to the presence of the surface anionic proteins podocalyxin (16), podoplanin (17), and podoendin (15). This serves two functions. First, the negative charge limits the passage of albumin (also negatively charged). Second, adjacent podocytes

maintain their separation by anion charge. The *basal domain* is required to anchor podocyte to the underlying GBM. *α*3*β*1 integrin (15) and *α*- and *β*-dystroglycans (16) serve this function, and connect the body of the podocyte to certain matrix proteins within the GBM.

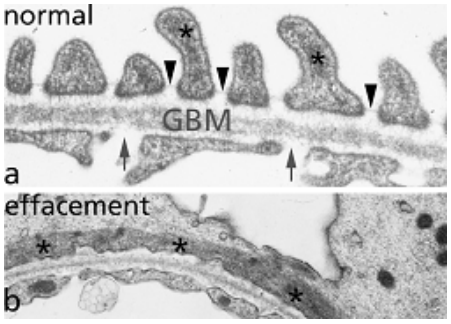
Podocyte function: The complex architecture of constitutive proteins is required for the highly specialized functions of podocytes, which includes a size barrier to protein, charge barrier to protein, maintenance of the capillary loop shape, counteracting the intraglomerular pressure, synthesis and maintenance of the GBM, production and secretion of vascular endothelial growth factor (VEGF). Therefore, it is not surprising that perturbations in one or more of these functions following podocyte injury underlies the signature clinical findings including marked proteinuria, and often a decrease in renal function with elevated creatinine.

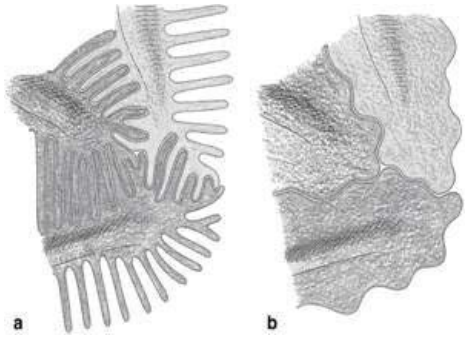
Podocyte injury: Accumulation of protein in the urine (proteinuria) is in part due to podocyte damage and is present in a range of kidney diseases such as glomerulosclerosis, membranous nephropathy, membranoproliferative glomerulonephritis, amyloid and diabetic nephropathy. The focus of my dissertation is diabetic glomerulosclerosis characterized by a significant podocyte injury which ultimately leads to marked proteinuria.

Histologic changes in podocytes following injury: Regardless of the cause of podocyte damage, typical podocyte abnormalities are best seen on electron microscopy and include vacuolization, microcystic, or pseudocystic changes, the presence of cytoplasmic inclusion bodies, and detachment from the GBM (17). In areas of reduced podocyte number, there may be focal areas of denudation of the underlying GBM. Although these changes are common, the characteristic response to podocyte damage/injury is a change in cell shape called effacement. It should be noted that these electron microscopy changes do not typically distinguish one podocyte disease from another, but rather represent a common final pathway of the podocyte's response to injury, proteinuria in the urine.



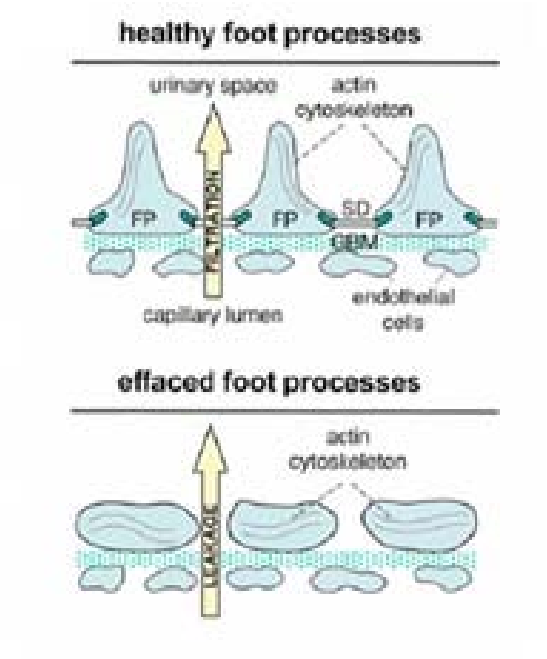
Podocyte effacement: Podocyte foot process effacement consists of gradual simplification of the inter-digitating foot process pattern, resulting in the formation of a cell that looks flat and elongated (b). This is not fusion of neighboring cells but rather retraction, widening, and shortening of each podocyte process. The frequency of filtration slits is reduced (18), giving the appearance of a continuous cytoplasmic sheet covering the GBM (b). Effacement is not specific to one disease, but rather is synonymous with podocyte injury of many forms. Studies have shown that effacement is initiated by changes in the podocyte's cytoskeleton.





Actin cytoskeleton: the backbone of podocyte shape: Foot processes are highly dynamic structures rich in actin filaments (19;20). Microtubules and vimentin-type intermediate filaments are distributed in the cell body and primary processes. In the major processes, the cytoskeleton is composed mainly of microtubules, interwoven with intermediate filament proteins. In contrast to the cytoskeletal proteins in the cell body and major processes, foot processes have an elaborate microfilamant-based contractile apparatus composed of actin, myosin-II, *α*-actinin, talin, and vinculin.

The actin cytoskeleton ultimately determines the podocyte's shape. Proteins regulating or stabilizing the actin cytoskeleton are therefore critical in the normal function of the podocyte, and any alterations in the actin itself, or in actin-regulating proteins (as explained in our study) might lead to changes in podocyte shape, and therefore function.



Podocyte apoptosis: a life or death decision

When podocytes begin to change shape, they start a process of programmed cell death, also called apoptosis. There is emerging experimental and clinical evidence showing that apoptosis is a major cause of reduced podocyte number, leading to proteinuria and/or glomerulosclerosis (21).

It has been well established that diabetes is associated with reduced podocyte number, which correlates with the onset and magnitude of proteinuria.Previous publications have shown (22-24) that hyperglycemia directly induces apoptosis in cultured podocytes, thereby providing an additional possible explanation for reduced podocyte number in this disease.

Introduction to the study:

The female-to-male incidence ratio of end-stage renal disease caused by diabetic glomerulosclerosis (GS) is higher in post-menopausal than in pre-menopausal women compared with men of the same age (25). In addition, hormone replacement therapy results in decreased proteinuria and improvement of the creatinine clearance in post-menopausal women with diabetes mellitus (26-28).Taken together, these data suggest an important role for estrogen deficiency in progression of diabetic glomerulosclerosis.

The glomerulus is an estrogen target tissue, and estrogen 17β-estradiol (E_2) prevents the onset or slows the development and/or progression of GS in mouse models of progressive kidney disease (26-29). Estrogens are steroid hormones naturally produced in human and other mammals with greater abundance in females. There are two estrogen receptors ERα and ERβ, known to mediate estrogen signaling; and they function as ligand-dependent transcription factors. After crossing the cellular membrane, estrogens bind to the receptors in the nucleus; the receptors dimerize and bind to specific response elements known as estrogen response elements (EREs) located in the promoters of target genes.

Since proteinuria is associated with podocyte injury, the objective of this project was therefore to study the role of estrogen in preventing loss of podocytes in diabetic female mice with glomeruloslerosis and to clarify the molecular mechanisms responsible for estrogen protection. To achieve this goal we

isolated, propagated and characterized podocyte cell lines from diabetic mice (db/db) that developed glomerulosclerosis.

ERs, found to be localized on podocytes by histochemical studies (30), are regulated by the levels of E_2 (26;31-33). From our previous studies we found that E_2 mediates changes in the podocyte ERα–ERβ ratio. Neither ERα copy number nor protein expression was regulated by E_2 in podocytes. Although we found no change in ERβ mRNA copy number, the ERβ protein expression increased after E_2 treatment, suggesting that there was a post-translational regulation, such as protein stabilization (34-36) (Fig.3).

In our study we show how estrogen is important as a treatment in preventing diabetic glomerulosclerosis by protecting actin filaments and podocyte dysfunction. Effacement that occurs in diabetic glomerulosclerosis is due to a change in actin cytoskeleton resulting in a phenotypic change of the podocytes.

In eukaryotic cells, actin exists in two forms: the filamentous f-actin and the globular g-actin. The morphologic change of podocytes is associated to a change in percentage between filamentous actin (f-actin) and globular actin (g-actin). Recent data suggest that Hsp25 and Rac1 expressed in podocytes are involved with this process. Hsp25 a low- molecular-weight heat shock protein, is an actin-associated protein which regulates actin polymerization. In addition, Hsp25 is involved in actin capping, by binding to the growing ends of actin filaments and inhibiting filament extension (37).

Finally, Rho GTPases are molecular switches that control a wide variety of signal transduction pathways in all eukaryotic cells. They are known principally for their pivotal role in regulating the actin cytoskeleton. Rac1 stimulates actin polymerization and membrane protrusion. Cdc42 controls cell polarity and RhoA promotes assembly of actin-myosin filaments and cell contraction. In our study we focused our attention on podocyte Rac1 function and its relationship to Hsp to prevent changes in podocytes phenotype and apoptosis.

Purpose of the study:

In our previous publication (Catanuto et al. KI, 2009) (38) we found that estrogen protects the glomerulus against diabetic glomerulosclerosis by preventing the diabetic associated increase in albumin excretion, glomerular volume, and collagen type IV deposition (Fig.1). To study the mechanisms associated with the estrogen-mediated protection, we isolated, characterized and immortalized (Fig.2) podocytes from placebo and estrogen treated diabetic mice.We found that estrogen protects the podocyte phenotype by changing the ERα–ERβ ratio (Fig.3) modulating apoptotic and anti-inflammatory signaling pathways (data not shown) and metalloproteinases (Fig.4).

In the current study: ***"In vivo* 17β-estradiol treatment contributes to podocyte actin stabilization in female db/db mice"** we determined the mechanisms by which estrogen maintains and protects podocyte structure and function through stabilization of the actin cytoskeleton and prevention of podocyte apoptosis.

Study:

In vivo 17β-estradiol treatment contributes to podocyte actin stabilization in female db/db mice

Abstract:

We recently showed that 17β-estradiol (E_2) treatment ameloriated type 2 diabetic glomerulosclerosis in mice in part by protecting podocyte structure and function. Progressive podocyte damage is characterized by foot process effacement, vacuolization, detachment of podocytes from the glomerular basement membrane and apoptosis. In addition, podocytes are highly dependent on the preservation of their actin cytoskeleton to ensure proper function and survival. Since E_2 administration prevented podocyte damage in our study on diabetic db/db mice, and has been shown to regulate both actin cytoskeleton and apoptosis in other cells types and tissues, we investigated whether actin remodeling and apoptosis were prevented in podocytes isolated from E_2 treated diabetic db/db mice as compared to placebo. We performed G-actin/F-actin assays, western analysis for Hsp25 expression, Rac1 activity and apoptosis assays on previously characterized podocytes isolated from both *in vivo* treated placebo and E_2 female db/db mice. We found that *in vivo* E_2 protects against a phenotype change in the cultured podocytes with an increase in F-actin versus G-actin, decrease in Hsp25 expression and transcriptional activation, increase of Rac1 activity and decrease apoptotic intermediates. Based on these results we

conclude that E_2 treatment protects against podocyte damage and may prevent/reduce diabetes-induced kidney disease.

Introduction:

Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease (ESRD) and is increasing due to the epidemic of obesity and diabetes (25). Tight glycemic control and multiple therapeutic agents have been reported to slow, but not prevent the progression of DKD. Importantly, estrogen deficiency contributes to the development and progression of DKD in women (25). Multiple experimental studies have suggested that 17β-estradiol (E_2) treatment protects the glomerulus against injury and therefore prevents DKD (27;38-41). We recently showed that E_2 treatment prevented type 2 diabetic glomerular disease in db/db mice in part by protecting podocytes against oxidant-induced injury (38). In addition isolated podocytes from E_2 treated mice exhibited higher expression of estrogen receptors suggesting that a direct protective effect of E_2 may occur.

Progressive podocyte damage is characterized by foot process (FP) effacement, vacuolization, detachment of podocytes from the glomerular basement membrane and apoptosis (21;42-46). Podocytes are highly dependent on the preservation of their actin cytoskeleton to ensure proper function and survival. In eukaryotic cells, actin exists in two forms: the filamentous F-actin and the globular G-actin. One of the proteins involved in actin capping, heat shock protein (Hsp27/25),

binds to the growing ends of actin filaments and inhibits filament extension (37). Actin capping changes the F-actin: G-actin ratio in favor of G-actin, which may promote podocyte foot process effacement and proteinuria. In addition, the small Rho-GTPase, (47) Rac1, stimulates actin polymerization and membrane protrusion (47) and may have an important role in protecting podocytes (48).

Because E_2 administration prevented podocyte damage in our study on diabetic db/db mice (38), and has been shown to regulate both actin cytoskeleton and apoptosis in other cells types and tissues (49;50), we hypothesized that *in vivo* E_2 protects against a phenotype change in podocytes by preventing actin remodeling and apoptosis.

Materials and Methods:

Animal Model and Cell lines: We used C57BL/6Jdb/db mice. These mice developed diabetic glomerulosclerosis similar to the one seen in patients. They are infertile due to a difect in the axis hipotalamous-pituitary-ovaries.These mice also have a decrease estrogen receptor similar to menopausal women.

We treated female db/db mice, from 7 to 24 weeks of age with either placebo or 17β-estradiol (E_2) (0.05 mg) (Innovative Research of America, Sarasota, Fl, USA). The dose of E_2 was chosen to obtain blood levels similar to that found during estrous (51). All the db/db mice were obese and had stable diabetes at the initiation of treatment. There were no differences in body weight, kidney weight, or in the kidney weight–body weight ratio between any of the studied groups. Mice were sacrificed at 24 weeks. Uterine weight was assessed as a

measure of estrogen replacement efficiency. Uterine weight increased with E_2. This is correlated with an increase in the 17β-estradiol levels.

Their left kidney was perfused with a buffer solution containing collagenase and RNase inhibitors for microdissection of glomeruli. The glomerular podocytes were isolated, propagated and immortalized using HPV as previously described (38). Both immortalized and primary cells retained characteristic podocyte markers found *in vivo* including nephrin, WT1, and TRPC6 (38). Cells were grown and maintained in DMEM:F12 medium supplemented with 10% fetal bovine serum (FBS). In those experiments where tranfection with Hsp25 was performed, podocyte cell lines were placed for 24 hours in phenol red free medium and 10% charcoal stripped serum.

Immunofluorescence with Rhodamine Phalloidin. Kidney sections (4µm thick) embedded in OCT were fixed in 2% paraformaldhyde for 10 minutes at room temperature, permeabilized with 1% Triton X-100 and blocked with 5% BSA (Bovine serum albumin).The kidney sections from three mice/group were incubated with rodamine phalloidin (1:1000) (Invitrogen, Carlsbad, CA, USA) overnight at 4°C to detect actin filaments and observed under the LSM700 confocal microscope after washing with PBS (Phosphate Buffered Saline). A percent ratio of color intensity per glomerular area was determined using image J software.

Percent F-actin versus G-actin: F and G-actin were measured using the *In Vivo Assay Kit* (Cytoskeleton, Inc. Denver, CO, USA) according to manufacturer's directions. Briefly, podocyte cell lines were lysed in a detergent-

based buffer that stabilized and maintained the G- and F- forms of cellular actin. Only G-actin was solubilized by the buffer and following a centrifugation step F-actin was pelleted while G-actin remained in the supernatant. The F-actin was depolymerizated and aliquots of supernatant and pellet were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis. Total cellular actin (100%) was measured as G-actin+F-actin. After film development, the blots were washed with India ink (1ul/ml) for 2 hr, to visualize the protein bands and confirm loading equivalency (52).

Western Blot Analysis: Podocyte cell line lysates were extracted and a protein concentration assay was performed using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were loaded in precast SDS polyacrylamide gels (invitrogen) after boiling the samples with Laemmli buffer and β-mercaptoethanol. Following electrophoresis, the proteins were transferred to nitrocellulose membrane and the blots were exposed to the following antibodies: Actin (cat#.AAN01, Cytoskeleton, Denver, CO, USA), Hsp25 (Enzo Life Sciences International, Plymouth Meeting, PA, USA), Rac1 (Cell Biolabs, Inc. San Diego,CA,USA), Caspase-9 (Cell Signaling Technology, Inc. MA, USA), or Apaf-1 (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody was washed and the secondary antibodies were added for 1hour followed by chemiluminescence solution and exposure to autoradiograph film. The films were scanned for densitometric analysis using Image J software from NIH as previously described (38). Blots were treated with β-actin antibody

(loading control) (Sigma-Aldrich, St.Louis, MO) after stripping to ensure equal loading of gels.

Real-time Polymerase Chain Reaction (PCR): Real-time PCR was performed on podocyte cell lines isolated RNA as previously described to amplify Hsp25 fragment (385pb) using a Syber Green kit (Quiagen, Valencia, CA, cat#204243) with specific primers (sense 5'-AGC GCC GCG TGC CCT TCT C-3'; antisense, 5'-TGC CTT TCT TCG TGC TTG CCA GTG-3') and RT (Reverse Transcriptase) mix for 40 cycles. The product of the PCR was then run on a 2% acrylamide gel (53). The TaqMan ribosomal RNA control reagents kit was used to detect 18S ribosomal RNA gene, which represented an endogenous control. Each sample was normalized to the 18S transcript content as previously described (54).

Transfection using Hsp25 Promoter: Podocyte cell lines were plated in 10% charcoal/dextran-treated fetal bovine serum (<5 pg/ml estrogens) in 24 well plates. Cells were transfected at 70% confluency using TransIT-LT1 (Mirus, Madison, WI, USA) with Hsp25-promoter-luciferase reporter gene construct (kind gift of Dr. Gaestel Matthias, Germany, 0.5 µg/well) and β -galactosidase gene (pRSV-βgal, 0.5 µg/well) to control for transfection efficiency. After 24 hours cells were harvested and luciferase and β-galactosidase assays were performed. Briefly, cells were lysed with 100ul of reporter lysis buffer (Promega) and lysate transferred to a microcentrifuge tube, spun for 10 minutes at 12,000rpm 4°C and supernatant transferred to a clean tube. The luciferase and β-galactosidase assays were performed as previously described (26).

Rac1 GTPase Activity Pull-Down Assays

Rac1 activity was assessed by pull-down assays using the Rac/Cdc42 binding domain (PBD) of p21-activated kinase coupled to GST (PBD-GST) (according to Rosenblatt et al.) (55). Active (GTP-bound) GTPases bind to their respective effector proteins. Podocyte cell lysates containing 400ug total protein were immediately added to 100 µl glutathione sepharose beads and rotated gently for 30 min at 4°C. Sepharose beads were pelleted by centrifugation, and complexes were washed four times with 1× lysis buffer not containing PBD-GST. GTP-bound Rac1/Cdc42 was eluted with SDS sample buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and exposed to a Rac1 antibody and visualized by chemiluminescence. In some experiments, podocytes were treated with either vehicle control (DMSO used to dilute EHT) or 10 and 20uM of EHT 1864, a small molecule Rac1 inhibitor. (EHT 1864: Sigma, E1657)

Immunohistochemestry: Chamber slides containing podocytes were exposed to an antibody against Cleaved-Caspase-9 (1:1000) (Cell Signaling Technology, Inc. Denvers, MA, USA) to detect apoptosis followed by application of the seconday antibody Alexa Fluor 488 goat anti-rabbit (1:500) (Invitrogen) for 2 hours at room temperature. Slides were mounted with DAPI (Vector Laboratories, Inc. Burlingame, CA, USA) to identify the nuclei and images examined under a fluorescence microscope with 630X magnification. 10 fields per slide were examined. 3 slides per group.

Statistics: Data are presented as mean ±s.e.m. Statistical differences were assessed using Student's *t*-test (Prism, GraphPad 5, San Diego, CA, USA). A *P*-value of <0.05 was considered significant.

Results:

Mouse model: As previously published db/db mice receiving placebo pellets had increased albumin excretion which was abrogated by E_2 treatment (38). E_2-treated mice also exhibited decreased glomerular volume and type IV collagen deposition compared with that of the placebo-treated control. The current study employed previously characterized immortalized podocyte cell lines isolated and characterized in our laboratory (38).

Actin expression and percent of F-actin and G-actin: We found that F-actin appeared in the characteristic pattern described for glomerular podocytes in the E_2 treated sections (56) compared to F-actin in glomeruli of placebo treated mice (Fig.5). Glomeruli isolated from E_2 treated mice had a higher % intensity of staining (1.3 ± 0.13, **p<0.05) compared to those glomeruli isolated from placebo treated mice (0.74 ± 0.08). Based on the staining of the glomeruli, we performed experiments to determine the expression of F and G-actin in isolated podocytes (Fig.6). There was a greater percentage of F-actin than G-actin (~76% versus 24%) in podocyte lysates isolated from db/db mice treated with E_2 compared to lysates from placebo treated mice (~50% of F versus 50% G-actin).

Hsp25 expression, amplification and transcriptional activation. Western blot analysis revealed that lysates from podocytes treated *in vivo* with E_2

(Fig.7A) express less Hsp25 protein compared to those isolated from placebo treated mice (***p<0.005). In parallel, levels of Hsp25 mRNA were decreased in those podocytes previously treated with E_2 (Fig.37B). 18S content was equal between samples with an average ct of 31 for placebo and 31.5 for E_2 podocytes. Finally, we also transfected podocytes with an Hsp25-promoter-luciferase reporter plasmid. Hsp transcriptional activation was also repressed in podocytes from the E_2 treated mice (Fig.7C), (***p<0.005).

Increase of Rac1 activity by E_2 and treatment with EHT 1864: Since Rac1 has been shown to participate in actin polymerization and stabilization (57), we investigated Rac1 activity in podocytes. We found that Rac1 activity was increased in podocytes isolated from *in vivo* E_2 treated mice compared to those of placebo treated mice (0.7±0.3 vs 0.4±0.2,*p<0.05). The increase of Rac1 activity was blocked in a dose dependent manner (Fig.8A) following 24 hours treatment with EHT 1864 (55;58). After 72 hours of EHT 1864 treatment, podocytes isolated from E_2 treated mice exhibited: 1) a change in the **baseline** percent of F-actin (76%) and G-actin (24%) to a higher percent of G-actin (F-actin 68% and G-actin 32%) (Fig.8B); and 2) increased Hsp25 expression (Fig.8C).

Apoptotic intermediates: We found an E_2-induced reduction of Apaf-1 (30% decrease). Since Apaf-1 activates Caspase-9, we were not surprised by the decrease in cleaved Caspase-9 (Fig.9A and B). In addition, Caspase-9 expression was also

(Fig.5C, ***p<0.005) reduced in podocytes isolated from in vivo E_2 treated mice.

Discussion:

We report here a phenotypic change observed in podocytes isolated from db/db diabetic mice, characterized by dysregulation of the percent of filamentous actin (F-actin) and globular actin (G-actin) that is prevented by *in vivo* E_2 treatment. We also confirmed a diabetic-induced increase of Hsp25, a regulator of actin cytoskeleton (37). E_2 treatment reduced Hsp25 mRNA and protein expression, and repressed Hsp25 promoter activity. These data support the hypothesis that repression of Hsp25 prevented actin capping and allowed for increased ability of actin to polymerize and maintain the filamentous form thereby helping to stabilize the podocyte phenotype.

These data are in agreement with other studies. Dai *et al.,* reported that short term glucose treatment of podocytes *in vitro* induced phosphorylation Hsp25 (59). In a streptozotocin rat model of diabetes, the phosphorylated form of Hsp25 increased in diabetic podocytes compared with controls. A recent study showed that Hsp27 was upregulated in glomerular podocytes isolated from patients with DN (diabetic nephropathy) (60). Additional studies have also reported an increase in the phosphorylated form of Hsp25 in diabetic glomeruli (59;61). An E_2-mediated repression of Hsp25 was reported in neurons (62). In addition, the presence of a half palindrome estrogen response element and two specificity protein-1 sites in the Hsp promoter (63) coupled with the increase in estrogen receptor (ER)β expression in our cells, suggest that our results may be ER-dependent. This effect however may be tissue specific since Hsp27 (human equivalent form to mice Hsp25) expression is augmented in an Erβ-dependent manner in aortic

vessel walls (64). Our future studies will include a more in depth look at ER subtype dependent regulation of Hsp25/27 in diabetic podocytes.

Rho GTPases are also known for their essential role in regulating the actin cytoskeleton (65;66). In particular, Rac1 stimulates actin polymerization and membrane protrusion. The E_2 induced Rac1 activity observed in our model could potentially aid in the stabilization of actin. This seems likely since treatment with EHT 1864, the small molecule inhibitor of Rac1, reverted the podocytes to a phenotype more representative of the placebo treated cells including an increase of G-actin and Hsp25 expression. To our knowledge there have been no reports of a direct effect of Rac1 on Hsp25 expression.

Increased albumin excretion, one of the hallmarks of DKD, results from damage to podocytes (21;42-45). The ability of the kidney to replace damaged or lost podocytes is limited since podocytes have a reduced potential to regenerate (43;46) therefore reducing apoptosis is an important mechanism for preserving cell number. Intermediates in the apoptotic signaling pathway were modulated by estrogens as shown for other cell types incuding glomerular mesangial cells (34;67;68). In addition Apaf-1 which binds to procaspase-9 and induces processing of caspase was also reduced suggesting the entire apoptosis cascade may be affected by estrogen treatement (69). Although it is well established that estrogens can inhibit apoptosis in a variety of cells and tissues, the mechanisms underlying this effect are not clearly understood.

We and others have proposed that the regulation of signaling pathways such as PI3K/AKT and p38 by estrogens could protect against podocyte

apoptosis (70;71). Data generated in our laboratory showed that E_2 treatment protects podocytes from apoptosis induced *in vitro* by TGF-β and TNF-α (67). This effect may be mediated by activation of the PI3K-AKT signaling cascade, since podocytes isolated from diabetic mice treated with E_2 have increased levels of AKT phosphorylation (unpublished data). Moreover, we found that glomeruli of db/db mice isolated at onset of albuminuria (12 weeks-old), show reduced AKT phosphorylation compared to db/+ mice (71). In addition, podocytes isolated from db/db mice with diabetes at the onset of albuminuria, even if cultured in normal-glucose medium, showed impaired insulin-dependent AKT phosphorylation, which is associated with enhanced susceptibility to cell death (71).

Finally, we previously showed an increase in glomerular and podocyte ERβ expression, suggesting that the ratio of ERα to ERβ may be responsible for many of the E_2-mediated actions discussed above. These studies are ongoing in our laboratory and will be the topic of future studies. In summary, we propose that in vivo E_2 treatment prevents the deleterious events that lead to actin cytoskeleton rearrangement and an apoptotic cascade in podocytes in a Rac1 dependent manner (Fig. 10).

Figure Legends:

Fig.5 *In vivo*17β-estradiol (E_2) treatment protects against actin filament disruption in female diabetic db/db mice. 4μm thick frozen sections were stained with rhodamine phallodoidin and visualized with confocal microscopy as

described in methods. Representative photomicrographs show rhodamine-phalloidin staining and phase contrast of glomeruli from placebo-treated and E_2-treated db/db mice. 630x original magnification. 10 fields per slide were examined. N=3 per group. Data are graphed as the percent ratio color intensity per area.

Fig.6 Podocytes isolated from female diabetic db/db mice treated *in vivo* with 17β-estradiol (E_2) have a higher percent of filamentous actin (F-actin) compared to podocytes isolated from placebo-treated mice. Cell lysates were collected from podocytes isolated from both placebo and E_2 treated db/db mice. (A) Representative of 5 western blots of F-actin and G-actin. (B) Lower panel is an ink stained blot for loading control. The arrow indicates the band of interest at the molecular weight of actin. (C) Data are graphed as mean ± SEM of percent of F and G-actin White bars represent podocytes isolated from placebo treated female db/db mice and black bars represent podocytes isolated from E_2 treated female db/db mice. ***$p<0.005$ compared to G-actin of estrogen treated cell lysates. N=5 experiments. Duplicate cell lines of each treatment.

Fig.7 Podocytes isolated from female diabetic db/db mice treated *in vivo* with 17β-estradiol (E_2) have decreased expression of Hsp25, decreased mRNA expression, and repressed transcriptional activation compared to podocytes isolated from placebo treated mice. Cell lysates were collected from podocytes isolated from both placebo (white bars) and E_2 (black bars) treated db/db mice. Western blot analysis was performed. (A) Representative western blot showing podocyte expression of Hsp25 from

placebo or E_2 -treated cells. Hsp25 expression was detected at molecular weight of 25 kDa. β-actin western blot is shown below as loading control. Data are graphed as mean ± SEM of Hsp25 and expressed as a percent of placebo cells. ***p<0.005 compared to placebo, (B) mRNA expression of Hsp25 was measured and a representative of 3 gels showing podocyte amplification of Hsp25 from placebo or estrogen treated cells. (C) Placebo and estrogen podocyte cell lines were co-transfected with an Hsp25-promoter-luciferase reporter gene construct and the β-galactosidase gene to control for transfection efficacy. Cells were subsequently harvested and luciferase activity was assessed and normalized to galactosidase activity. Data are the mean ± SEM of 3 experiments, ***p<0.005 compared to vehicle (white bar) and E_2 treated (black bar) control cells. N= 3 experiments. Duplicate cell lines of each treatment were used.

Fig.8 Inhibition of increased Rac1 activity in podocytes isolated from female diabetic db/db mice treated *in vivo* with 17β-estradiol (E_2) increases the percentage of G-actin and Hsp25 protein expression. Cell lysates were collected from podocytes isolated from E_2 treated db/db mice and pull down assay perfomed followed by western analysis as described in material and methods. (A) Dose response of Rac1 activity inhibition by the Rac1 inhibitor EHT 1864 after 24 hours. Data are graphed as the mean ± SEM of the ratio between Rac1-GTP and total (input) protein. N=3 experiments. (B) Representative western blot showing F-actin and G-actin in E_2 treated cells after

72 hours EHT 1864 treatment. Higher percent of G-actin after treatment with EHT 1864 at 72 hours in E_2 treated cells. N=3 experiments. Ink staining is shown below for loading control. The arrow indicates the band of interest at the molecular weight of actin. (C) Representative western blot showing the protein expression of Hsp25 after 72 hours treatment with EHT 1864 in E_2 treated cells. V=Vehicle treated cells (DMSO). N=5 experiments. β-actin western blot is shown below as loading control. Data are graphed as the mean ± SEM. *p<0.05.

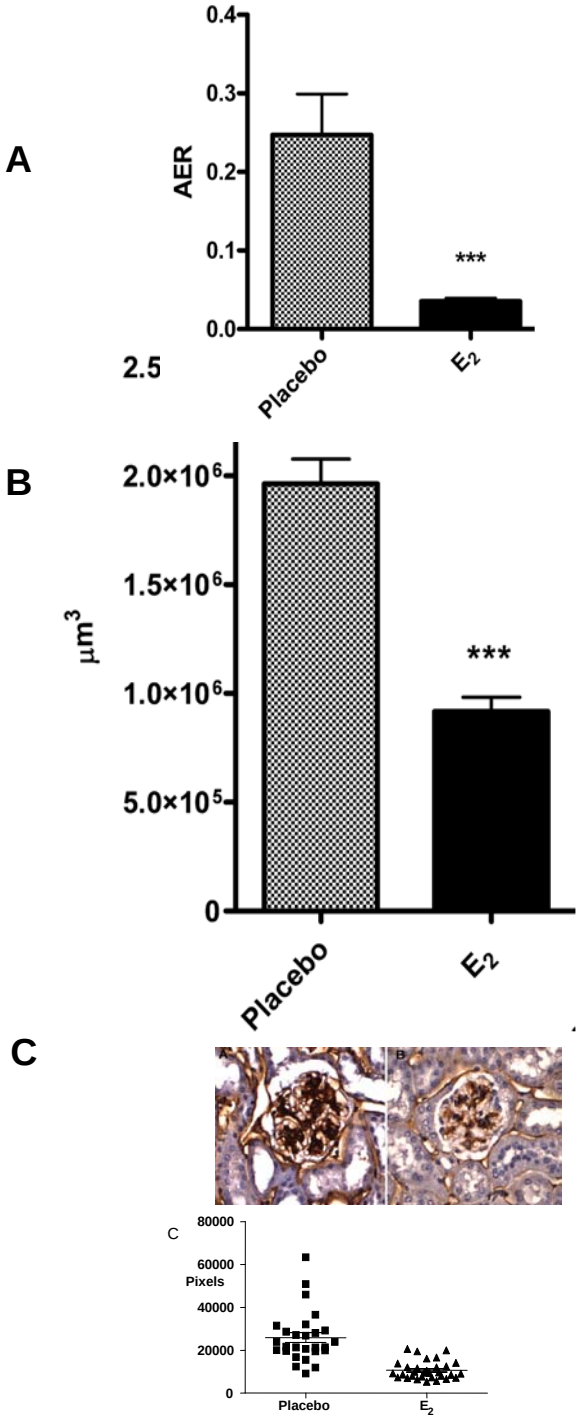
Fig.9 Immunofluorescence staining of Cleaved Caspase-9 and expression of Caspase-9 decreased in podocytes isolated from female diabetic db/db mice *in vivo* treated with 17β-estradiol (E_2). (A, B) Immunofluorescence staining of Cleaved Caspase-9 was performed on podocytes according to methods described. Dapi staining of nuclei appeared blue, and FITC staining of Cleaved Caspase-9 appeared green. Original magnification 400x. (C) Representative western blot showing the expression of Caspase-9 in podocytes. Cell lysates were collected from podocytes isolated from *in vivo* treated placebo (white bar) and E_2 (black bar) db/db mice and analyzed by western blot for Caspase-9 protein. β-actin western blot is shown below as loading control. Data are graphed as the mean ± SEM ***p<0.005, N=5 experiments of duplicate cell lines.

Fig.10 Schematic design depicting possible estrogen contribution to actin stabilization. Estrogen action stabilizes podocyte F-actin though an increase of Rac1 activity and a decrease of Hsp25 protein and transcriptional activation. Rac1 activity promotes the decrease of Hsp25 expression. In addition, Apaf-1,

which activates Caspase-9, and Caspase-9 expression are decreased. These factors may combine to reduce podocyte apoptosis. (Dotted line =potential effects).

Fig.1 Figures

Estrogen (E_2) decreases urinary albumin excretion (A), glomerular volume (B), and collagen type IV deposition (C) in female diabetic db/db mice compared to placebo.



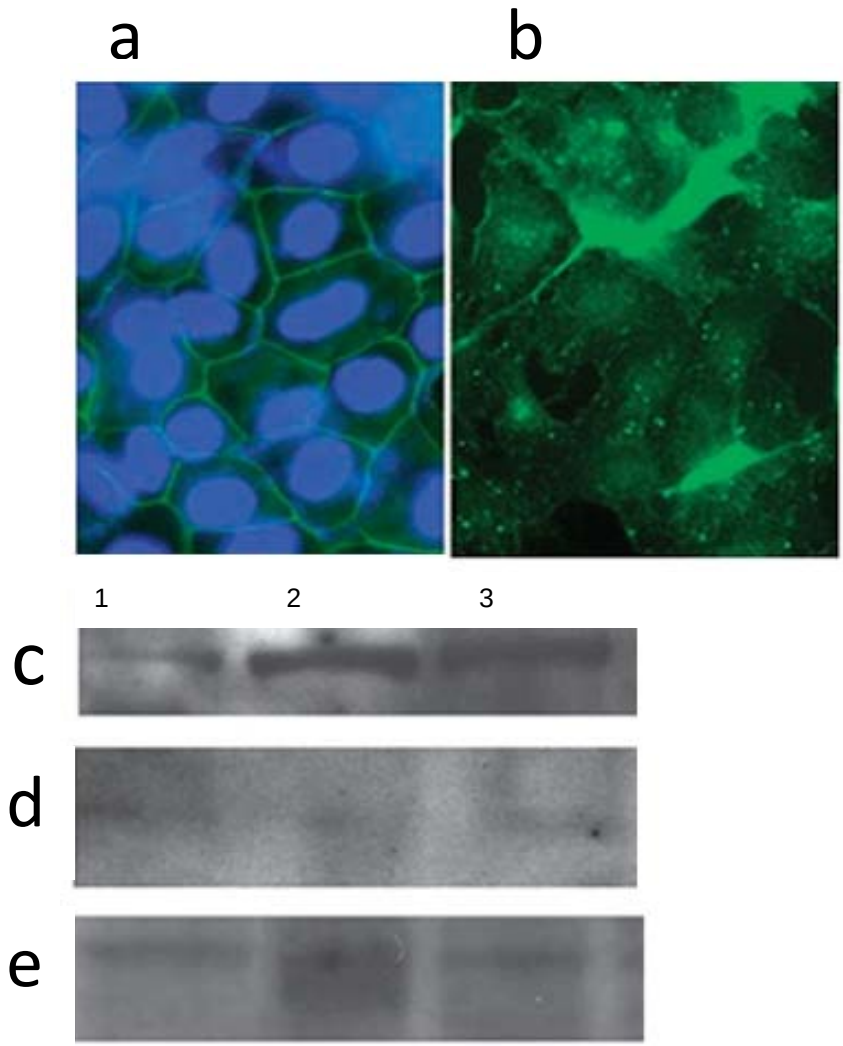
A. Urine albumin excretion was reduced at the time of killing (24 weeks) in db/db diabetic mice treated with E_2. Data are expressed as mg albumin/mg creatinine. ***P<0.0001, compared with placebo-treated group, n=5–7 mice per group.

B. The glomerular volume of E_2 treated mice is decreased compared with placebo db/db diabetic mice.*** P<0.0001; n=5–7 mice per group.

C. Representative kidney sections of diabetic db/db mice: (a) placebo, (b) E_2, at the time of killing. Original magnification 400X. N=3 sections per group. (c) A representative scatter plot shows the number of pixels on stained sections as measured using the NIH Image J

Fig.2

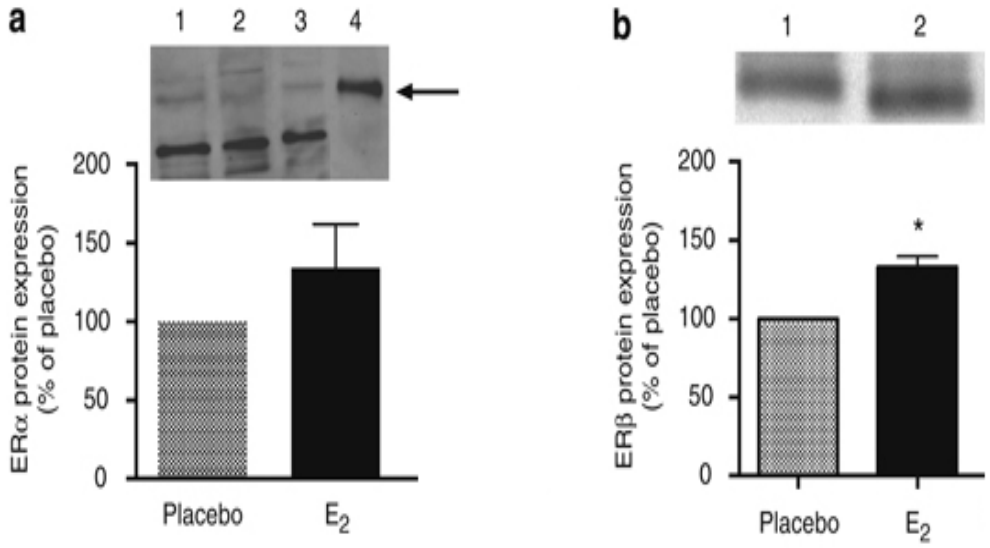
Podocytes isolated from diabetic db/db mice retain expression of in vivo markers.



Immunofluorescence staining of (a) ZO-1 and (b) nephrin. Cell lysates were collected and western blot analysis was performed for (c) nephrin protein expression, (d) TRPC expression, and (e) WT-1 expression. Cells isolated from a placebo-treated non-transfected mouse (lane 1), and transfected (lane 2), cells isolated from E_2 (lane 3) treated mice.

Fig.3

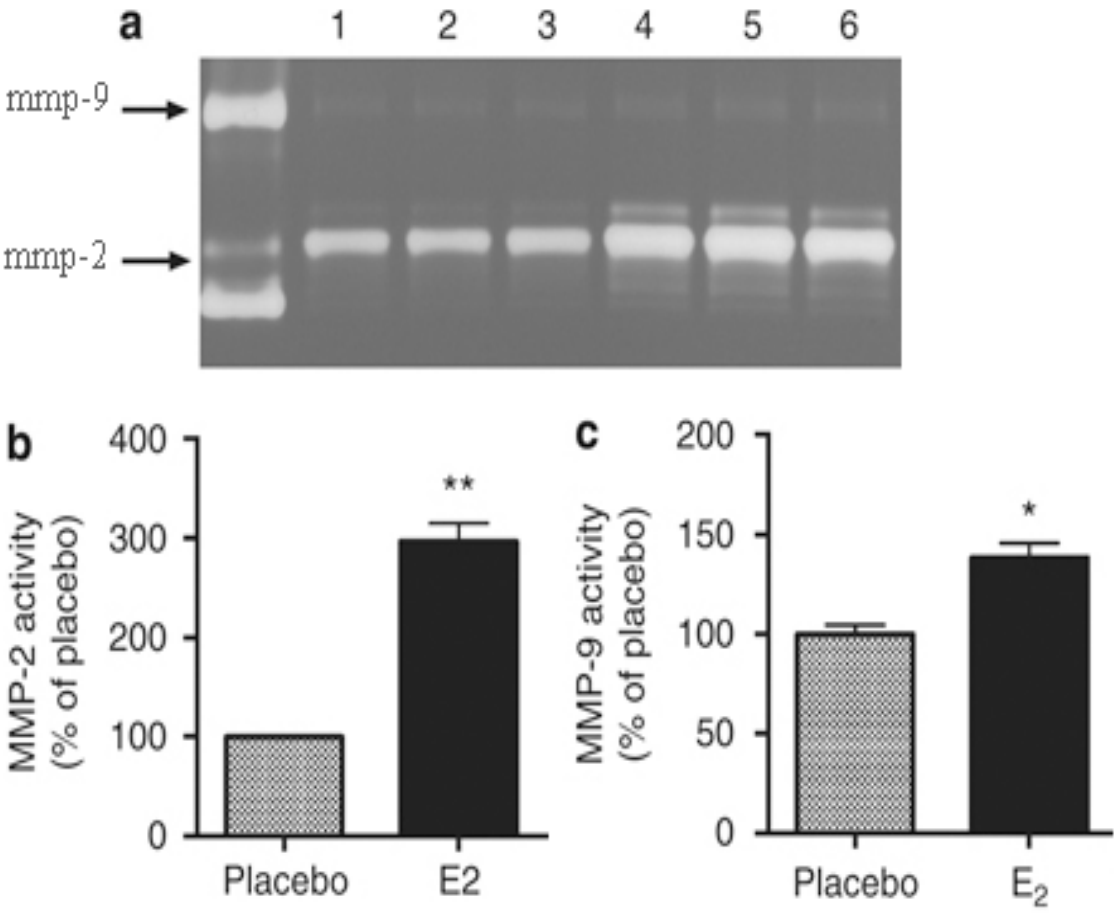
Estrogen receptor β expression is increased in podocytes isolated from E_2 treated mice.



Podocyte cell lysates were collected and western blot analysis was performed as described in Methods (Catanuto et al.KI, 2009). (a) There was no change in ERα protein expression in podocytes isolated from mice treated with E_2 (lane 3) compared with placebo-treated mice (lane 2). Immortalization did not alter ERα protein expression (lane 1). Recombinant ERα protein (lane 4), n=2. Data are graphed as the mean±s.e.m % of placebo ERα protein expression. (b) Estrogen receptor-β was increased in podocytes isolated from mice treated with E_2 (lane 2) compared with placebo-treated mice (lane 1). Data are graphed as the mean±SEM % of placebo ERβ protein expression. N=3, **P<0.005. Arrows denote specific bands.

Fig.4

MMP-2 and MMP-9 activity is increased in podocytes isolated from E_2 treated db/db diabetic mice.



Cell lysates were collected in triplicate and normalized to cell number as described (Catanuto at al, KI, 2009). Lysates were electrophoresed on zymogram gels as described in Methods (Catanuto et el., KI, 2009) MMP-2 and MMP-9 were increased in podocytes isolated from mice treated with E_2 (lanes 4–6) compared with placebo-treated mice (lanes 1–3). N=2, *P<0.05, **P<0.005 E_2 compared with placebo.

Fig.5

***In vivo* 17β-estradiol (E_2) treatment protects against actin filament disruption in female diabetic db/db mice.**

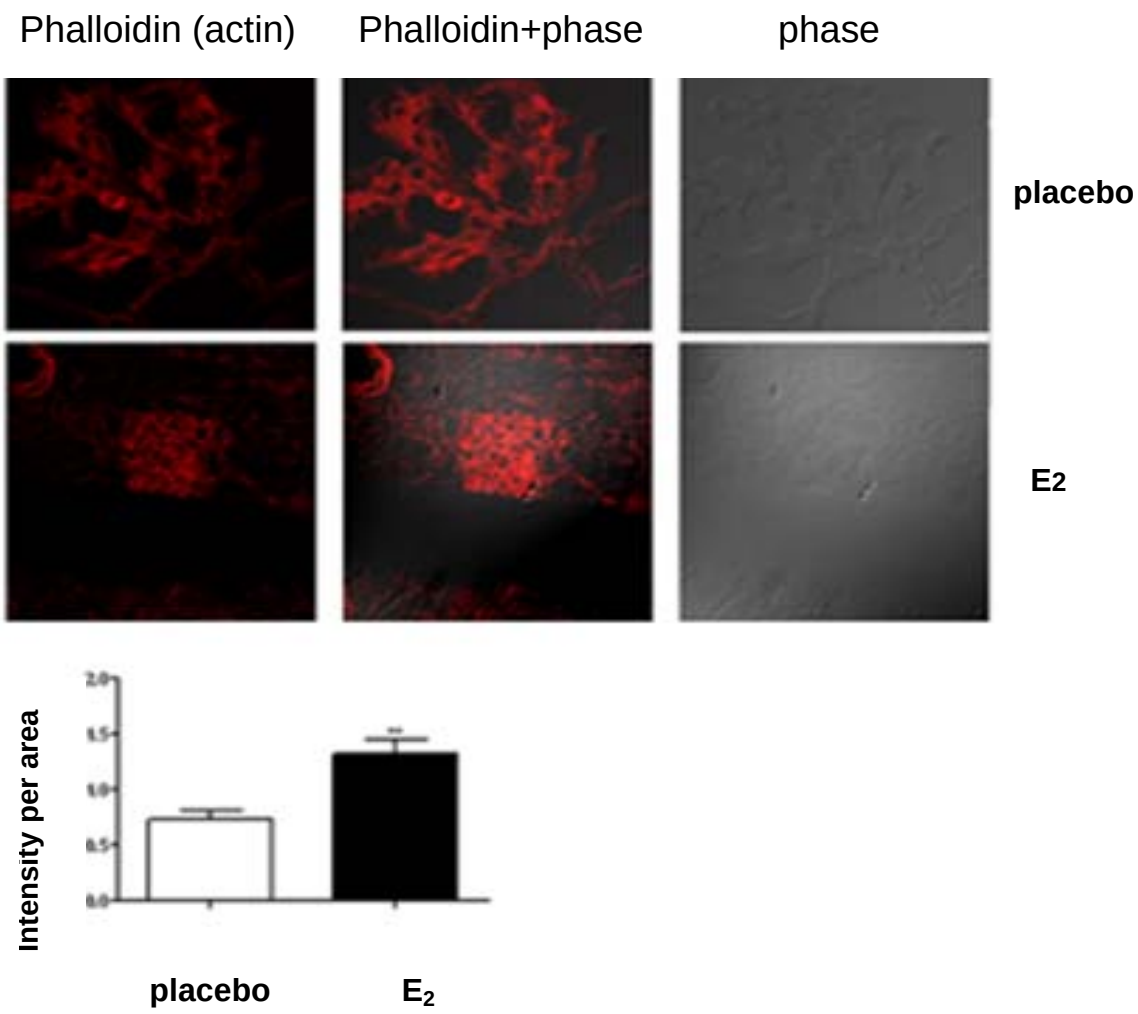


Fig.6

Podocytes isolated from female diabetic db/db mice treated *in vivo* with 17β-estradiol (E_2) have a higher percent of filamentous actin (F-actin) compared to podocytes isolated from placebo-treated mice.

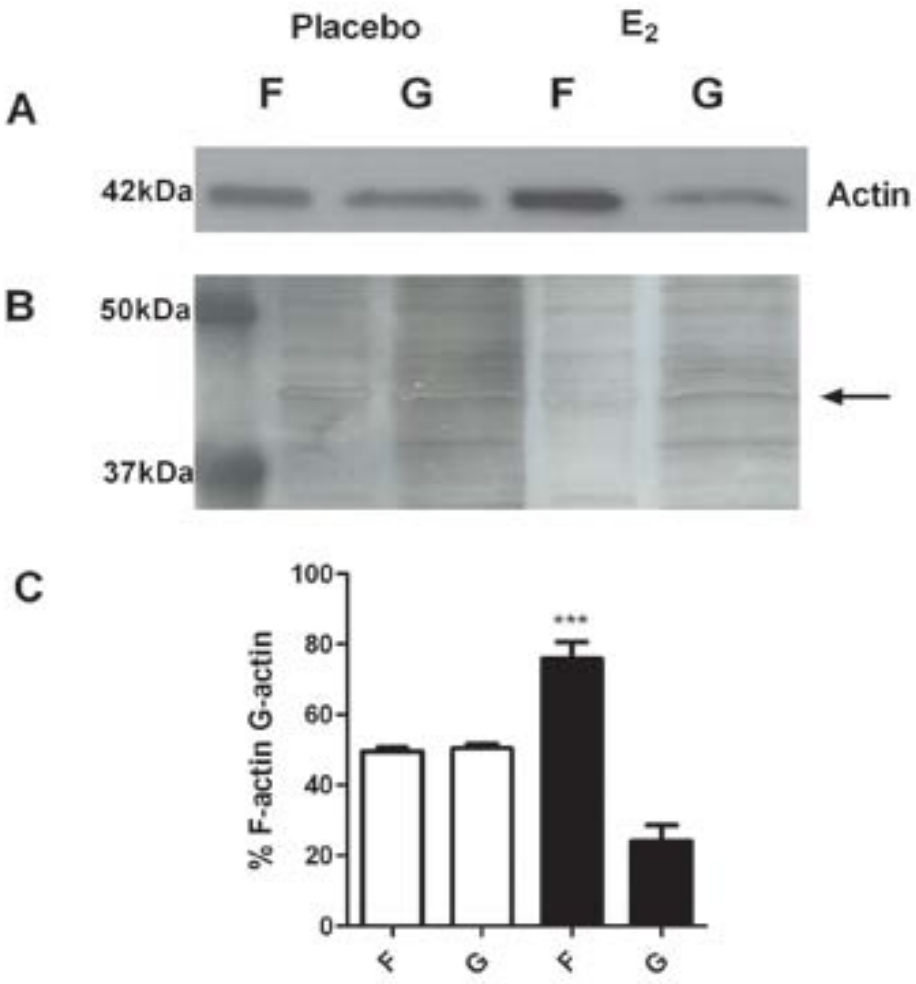


Fig.7

Podocytes isolated from female diabetic db/db mice treated *in vivo* with 17β-estradiol (E_2) have decreased expression of Hsp25, decreased mRNA expression, and repressed transcriptional activation compared to podocytes isolated from placebo treated mice.

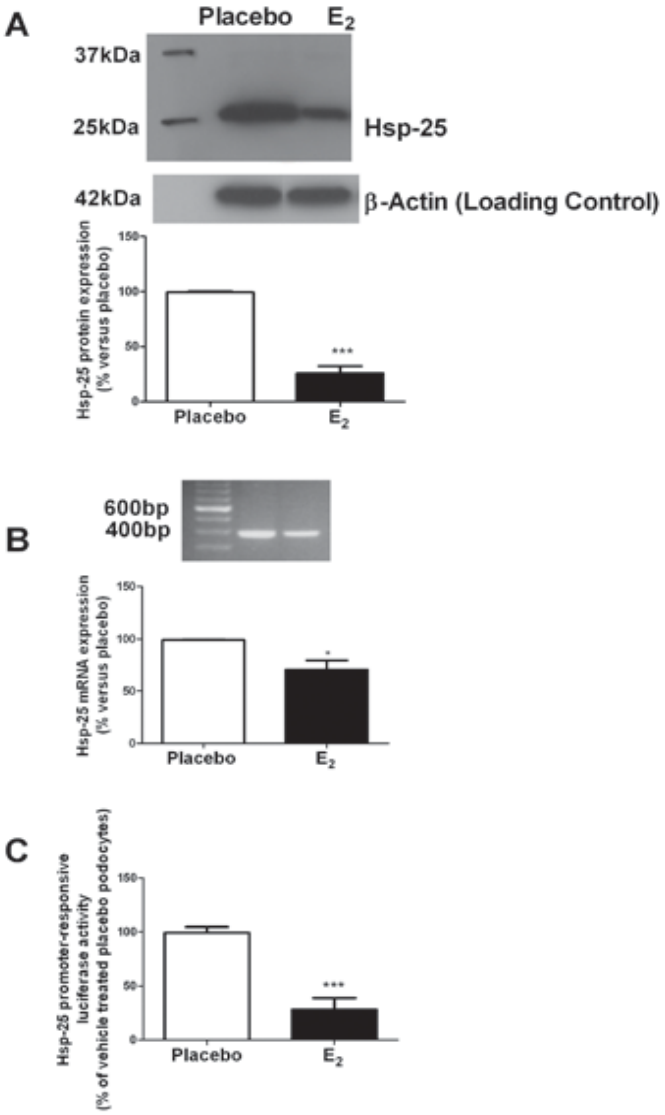


Fig.8

Inhibition of increased Rac1 expression in podocytes isolated from female diabetic db/db mice treated *in vivo* with 17β-estradiol (E_2) increases the percentage of G-actin and Hsp25 protein expression.

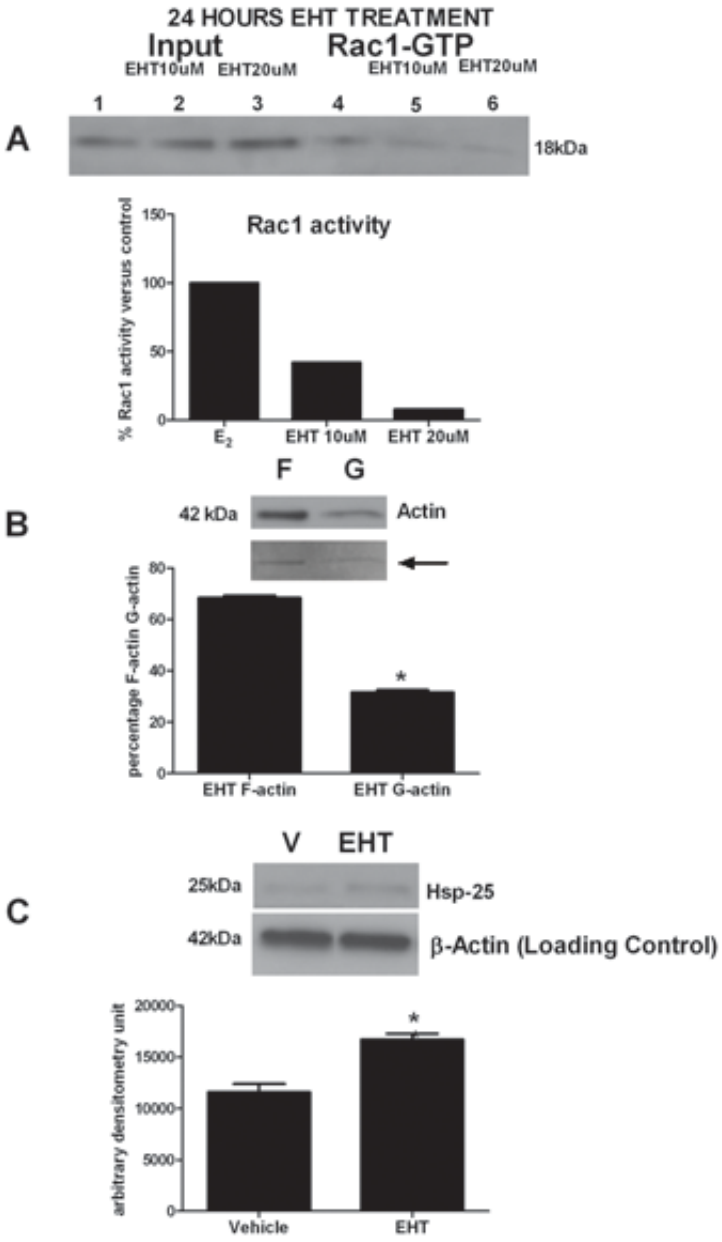


Fig.9

Immunofluorescence staining of Cleaved Caspase-9 and expression of Caspase-9 decreased in podocytes isolated from female diabetic db/db mice *in vivo* treated with 17β-estradiol (E_2).

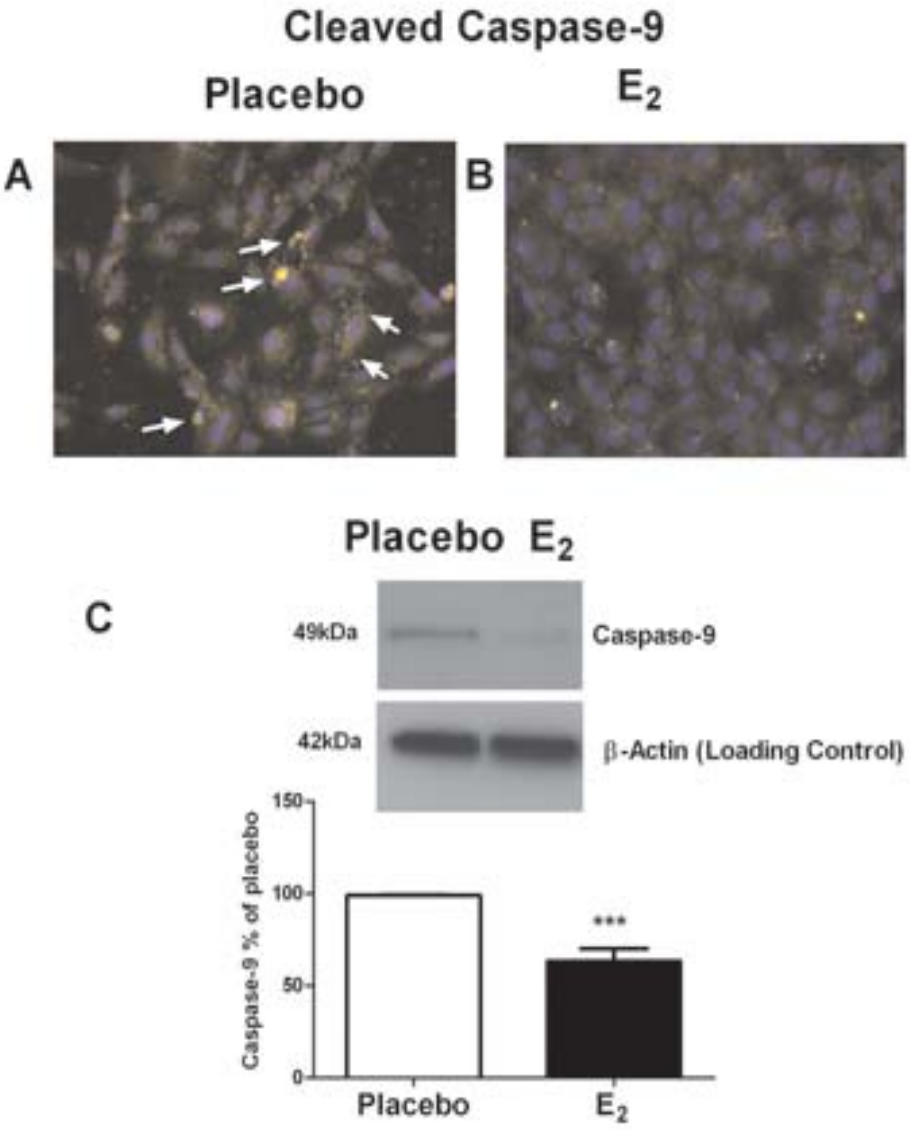
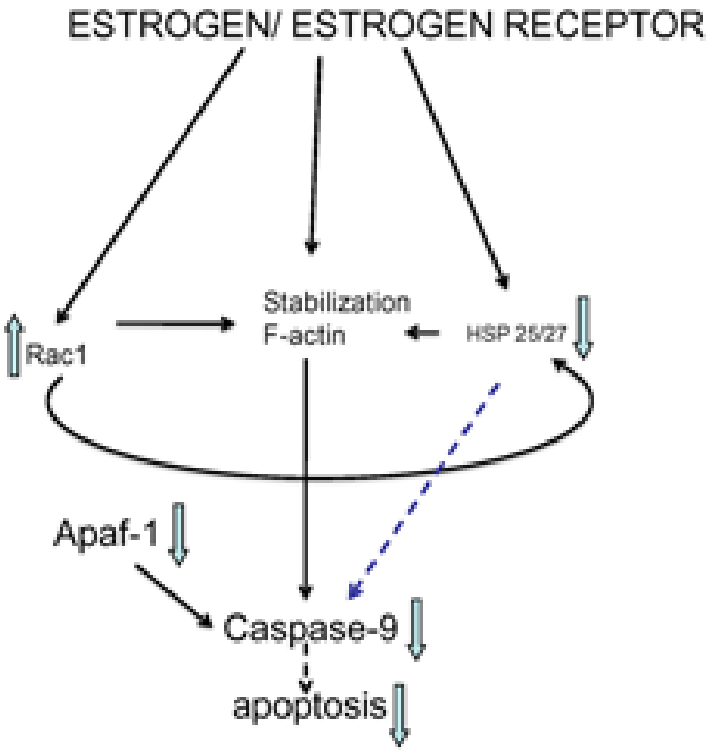


Fig.10

Schematic design depicting possible estrogen contribution to actin stabilization.



Ongoing studies:

Based on our studies in persuit of regulation of ER without hormons we have focused our attention on a natural compound, resveratrol (RSV). Resveratrol is a phytoestrogen found in grapes that is present in red wine and may have similar properties to endogenous estrogens. As mentionated above endogenous estrogens are steroid hormones synthesized by humans and other mammals; these hormones bind to estrogen receptors within cells. The estrogen-receptor complex interacts with unique sequences in DNA (estrogen response elements; EREs) to modulate the expression of estrogen-responsive gene. A compound that binds to estrogen receptors and elicits similar responses to endogenous estrogens is considered an estrogen agonist, while a compound that binds estrogen receptors but prevents or inhibits the response elicited by endogenous estrogens is considered an estrogen antagonist. The chemical structure of resveratrol is very similar to that of the synthetic estrogen agonist, diethylstilbestrol, suggesting that resveratrol might also function as an estrogen agonist. However, in cell culture experiments resveratrol acts as an estrogen agonist under some conditions and an estrogen antagonist under other conditions (72;73).

In estrogen receptor-positive breast cancer cells, resveratrol acts as an estrogen agonist in the absence of the endogenous estrogen, 17β-estradiol, but acts as an estrogen antagonist in the presence of 17β-estradiol (74;75).

At present, it appears that resveratrol has the potential to act as an estrogen agonist or antagonist depending on cell type, estrogen receptor isoform (ER alpha or ER beta), and the presence of endogenous estrogens.

It has 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 (76). 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 (77). RSV has been shown to attenuate glomerulosclerosis by an antioxidative mechanism and by reducing the expression of Sir-2 and p38 in diabetic kidneys (78). Finally, Ding et al. demonstrated that Resveratrol attenuates renal hypertrophy in STZ-DM rats by AMPK activation (79).

Our future studies focus on Resveratrol protection against diabetic glomerulosclerosis in a diabetic mice model (db/db). Through ER-mediated effects, we found regulation of estrogen receptor α and β after treatment with resveratrol, decrease in extracellular signal-regulated kinase (ERK) activation, and increase in matrix metalloproteinases (mmp-2) leading to decrease in accumulation of collagen in the glomerulous. Previous reports of research, both in humans and in animals, have revealed that activation of the ERK pathway in glomeruli may play an important role in progression during the early stages of diabetic nephropathy (80;81).

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