TRB3 is stimulated in diabetic kidneys, regulated by the ER stress marker CHOP, and is a suppressor of podocyte MCP-1

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The prevalence of diabetes is reaching epidemic levels and projections suggest that there will be 44 million diabetics in the United States by 2034 (17). Approximately 30–40% of these patients will develop diabetic nephropathy; therefore, it will be imperative to develop new strategies and novel molecular approaches to slow the progression of this devastating disease. Podocytes are highly specialized glomerular epithelial cells that play a vital role in glomerular filtration (37), and diabetic kidney disease is associated with podocyte loss and defective podocyte function (26). In diabetes, the mediators of podocyte injury include reactive oxygen species (ROS), angiotensin II (ANG II), matrix metalloproteinases, prosta...

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lines under Institutional Animal Care and Use Committee-approved protocols. Type 1 diabetes was induced in 10-wk-old male C57BL/6 mice by low-dose streptozotocin (STZ) injection (50 mg/kg × 5 days). Hyperglycemia was confirmed in the diabetic mice (STZ treatment: blood glucoses > 350 mg/dl) and mice were euthanized 8 wk after induction of type 1 diabetes.

For the model of type 2 diabetes, we used five db/db: BKS.Cg-Dock7m+/+ Leptob J (stock number 642) obtained from Jackson Labs (Bar Harbor, ME) and five db/m nondiabetic, lean heterozygote littermates.

**Immunofluorescence studies.** Frozen sections (10 μm) of control and STZ-treated mouse kidneys were stained with antibodies to TRB3 (1:200; Marc Montminy), podocin (1:200), anti-rabbit Alexa fluor 488 (1:400; Molecular Probes, Invitrogen) and mounted with Prolong Gold. The sections were visualized with an Olympus IX81 inverted spinning disk confocal microscope with Slidebook 4.1. Frozen sections (5 μm) of db/db and db/db mice were stained with TRB3 (1:200), podocin (1:200), anti-rabbit Alexa fluor 488 (1:400), and anti-goat Alexa fluor 594 (1:400) and visualized with a Zeiss LSM 510 laser-scanning confocal microscope.

**Podocytes.** Conditionally immortalized podocytes were kindly provided by Dr. P. Mundel and Dr. S. Shankland and propagated at 33°C (permissive conditions) on type I collagen-coated plastic plates with IFN-γ as previously described (51). For differentiation, cells were transferred to 37°C for 14 days and semiquantitative PCR studies were used to verify expression of synaptopodin (Table 1).

**Real-time PCR.** RNA, cDNA, and real-time PCR studies were performed as previously described (1). For quantification of TRB3, we used TaqMan Gene Expression Assays (TRB3: Mm00454875_m1; TRB1: Mm00454876_m1). Amplification efficiencies were normalized against RPL19 (Table 1) and relative fold increases were calculated using the Pfaffl technique of relative quantification (38). Each experiment was performed in triplicate and at least three times.

**Western blotting.** Cellular lysates were prepared with Cell Lysis Buffer (Cell Signaling, Beverly, MA) with protease inhibitors (31). Samples were run on NuPAGE Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membranes (Invitrogen). The following antibodies were used: TRB3 (1:400; Calbiochem, La Jolla, CA), Chop/GADD153 (1:400; SC-793, SCBT), actin (1:400; SC-1616, SCBT) and detection was performed with ECL Plus Detection Reagents (GE Healthcare, Piscataway, NJ).

**Palmitate-BSA preparation.** Fatty acid and endotoxin-free BSA (5.5 g; Sigma, no. A8806) were added to 50 ml of Krebs Ringer bicarbonate buffer. Palmitate (10 mM final; Sigma) was prepared in 1 ml of ethanol and then added to the BSA under nitrogen. Samples were filter sterilized and concentrations were assayed using the NEFA-HR kit (Wako Diagnostics, Richmond, VA). In the same manner, the BSA control was prepared with ethanol.

**Chromatin immunoprecipitation.** Fully differentiated podocytes were treated with 50 μM H₂O₂ or control for 4 h and then harvested with Accutase (Innovative Cell Technologies, San Diego, CA). Chromatin immunoprecipitation (ChIP) studies were performed per the manufacturer’s instructions (EZ ChIP, Millipore, Billerica, MA) and

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**Table 1. PCR primers used**

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*Fig. 1. TRB3 expression increases in diabetic mouse kidneys. A: C57BL/6 mice were treated with low-dose streptozotocin (STZ) and kidneys were harvested 8 wk later. Real-time PCR studies revealed a 5-fold increase in TRB3 expression in the STZ-treated mice (n = 5 mice per group). B: TRB3 expression was also increased in the kidneys of 24-wk-old db/db mice (n = 5 mice per group). *P < 0.05 vs. control mice, **P < 0.05 vs. db/db mice. Student’s t-test and bars represent SE. Frozen sections of control and STZ-treated (×600) (C) and db/m and db/db (×1000) mice (D) were stained with anti-TRB3 and anti-podocin. There is an upregulation of TRB3 staining in the glomeruli of the diabetic mice (STZ, db/db) when compared with the controls (control, db/m).
as previously described (48). Immunoprecipitation was performed with 4 μg of the following antibodies: CHOP/GADD153 (SC-793), C/EBPβ (SC-150X), and anti-rabbit IgG (SCBT) at 4°C. DNA was purified and PCR was performed with primers corresponding to the proximal CHOP/C/EBPβ binding site 61 bp from the TRB3 transcription start site (ChIP prox; Table 1).

Plasmids and transient transfections. A TRB3 expression plasmid was generated as previously described, pcDNA3-HA-TRB3 (31). A CHO plasmid was amplified from mCHOP10;pcDNA1 (David Ron Lab) and cloned into pcDNA3. Fully differentiated podocytes (14 days at 37°C) were removed from 10-cm plates with Accutase (Innovative Cell Technologies, San Diego, CA) and replated and transfected in 96-well plates (~9 × 10^4 cells/well) with 100 μl Opti-MEM (Invitrogen) and 1.5 μg/well Lipofectamine 2000 (Invitrogen) using 750 ng/well of pcDNA3-HA-TRB3 and its control pcDNA3. For the MCP-1 studies, cells were transfected in 96-well plates (Innovative Cell Technologies, San Diego, CA) and replated and incubated for 24 h and Western blotting was performed.

Statistics. Differences were analyzed using the Student’s t-test, ANOVAs with post hoc Tukey tests for pair-wise comparisons. Analysis was accomplished with SPSS 11 (Chicago, IL).

RESULTS

TRB3 expression increases in diabetic kidneys. Diabetes was induced in 10-wk-old male C57BL/6 mice by low-dose STZ injection. Control mice received vehicle (citrate buffer). Hyperglycemia was confirmed in the diabetic mice and mice were euthanized 8 wk after induction of type 1 diabetes. Figure 1A demonstrates that there was a fivefold increase in TRB3 mRNA expression in the diabetic kidneys compared with the controls. TRB1 and TRB2 mRNA expression were not increased in the diabetic kidneys (data not shown). Expression of TRB3 mRNA was also significantly increased in 24-wk-old db/db mice compared with db/m controls (Fig. 1B). Immunofluorescence studies confirmed that there was upregulation of TRB3 protein expression in the glomeruli of the STZ-treated and db/db mice when compared with the control and db/m mice (Fig. 1, C and D).

Podocytes express TRB3, and high glucose does not augment expression of TRB3. Conditionally immortalized murine podocytes express TRB3 (Fig. 2). During their differentiation, murine podocytes were incubated for 10 days in normal (5.6 mM glucose)- and high-glucose (30.6 mM glucose) conditions. Cells were also incubated in mannitol to control for the osmolarity. There were no significant changes in TRB3 expression in any of the conditions (Fig. 2).

Podocyte TRB3 expression is induced by ROS. It is generally accepted that the pathogenesis underlying diabetic kidney disease is due in part to the generation of ROS (24). Thus, we tested the hypothesis that ROS enhance expression of TRB3. Indeed, TRB3 mRNA expression was increased in conditionally immortalized, fully differentiated podocytes treated for 4 h with H2O2 (Fig. 3A). Additionally, we investigated whether other ROS generators such as the xanthine/xanthine oxidase (XO) reaction augment TRB3 expression. Conditionally immortalized and differentiated podocytes were treated for 3 h with 100 μM xanthine and 5 μM/XO and similar to H2O2, the xanthine/XO reaction augmented TRB3 expression (Fig. 3B).

FFA augment TRB3 expression. Many research groups showed that oxidative stress associated with diabetes is due to hyperglycemia (11, 24, 42). However, in our podocyte studies, high-glucose conditions did not augment TRB3 expression. Early in diabetes, before the development of hyperglycemia, FFA can induce the production of ROS (12, 30). Since palmitate is the most abundant FFA in the plasma, we tested whether palmitate induces TRB3 expression. Palmitate potently increased TRB3 mRNA levels in podocytes (Fig. 4A). Palmitate circulates in the blood bound to albumin, therefore BSA was used to control for the amounts of BSA bound to palmitate (1.3% BSA is the average of 3 studies performed. *P < 0.05 vs. control; 1-way ANOVA (A) and Student’s t-test (B).

Fig. 2. High-glucose conditions do not regulate TRB3 expression. Conditionally immortalized podocytes were differentiated and incubated for 14 days in high-glucose conditions. Mannitol was used as an osmotic control. The results are the average of 3 studies performed.

Fig. 3. Reactive oxygen species (ROS) augment TRB3 expression. Fully differentiated, conditionally immortalized podocytes were treated for 4 h with H2O2 (A) and 3 h with xanthine and xanthine oxidase (B). Real-time PCR demonstrated that ROS augment TRB3 mRNA expression. Each study was performed at least 3 times and the averages of the results are shown. *P < 0.05 vs. control mice; 1-way ANOVA (A) and Student’s t-test (B).
control for 650 μM palmitate). BSA did not appear to regulate TRB3 expression. Importantly, Fig. 4B confirms that FFA and ROS also increase podocyte TRB3 protein expression. However, ROS and palmitate do not appear to have additive effects on TRB3 expression (Fig. 4C).

**DISCUSSION**

In the current studies, we demonstrate for the first time that TRB3 expression is enhanced in kidneys derived from diabetic mice and further show that it is expressed in the glomeruli and in podocytes. Interestingly, hyperglycemia does not induce TRB3 expression. Instead, ROS and FFA augment podocyte TRB3 expression. TRB3 inhibits MCP-1 expression in podocytes. To further investigate the role that TRB3 may play in the diabetic kidney, podocytes were transfected with either a TRB3 expression plasmid or with an empty plasmid control (pcDNA3). Expression of TRB3 was confirmed by Western blotting (Fig. 7B). Cells were then left unstimulated or stimulated with phorbol 12-myristate 13-acetate (PMA/TPA). Twenty-four hours after stimulation, we assessed MCP-1 protein expression by ELISA. PMA significantly augmented expression of MCP-1 and overexpression of TRB3 inhibited basal and stimulated protein expression of MCP-1.
of phosphatidylinositol-3-kinase and ethanol (5, 10, 16a, 29, 32, 34, 35, 46, 61). Upregulation of TRB3 expression has previously been described in livers (10) and hearts (56) of diabetic mice and we extend these findings to diabetic kidneys. In our studies, high glucose did not augment TRB3 expression. This is not surprising, as TRB3 expression increases in mouse livers under fasting conditions (10). The diabetic milieu is associated with oxidative stress (11) and in our studies ROS potently augmented TRB3 expression. Additionally, Tang and colleagues (56) showed that advanced glycation endproducts induce TRB3 expression and this is associated with overexpression of type 1 collagen in rat cardiac fibroblasts.

Our group and others showed that PPAR-α ligands upregulate TRB3 expression in lymphocytes and hepatocytes (23, 48).

Fig. 5. ROS and FFA induce C/EBP homologous protein (CHOP) expression and augment recruitment of CHOP and C/EBPβ to the proximal TRB3 promoter. Fully differentiated podocytes were treated for 4 h with H$_2$O$_2$ (A) and 24 h with palmitate (Palm) or BSA control (B). CHOP expression was assessed by real-time PCR. *P < 0.05 vs. control (1-way ANOVA). C: as above, podocytes were treated for 24 h with Palm, BSA, H$_2$O$_2$, and tunicamycin. Western blotting was performed and CHOP protein expression was assessed. This blot is representative of studies performed at least 3 times. D: Western blotting for GRP78/Bip expression was also performed. E: differentiated podocytes were treated for 4 h with 50 μM H$_2$O$_2$ or control and chromatin immunoprecipitation (ChIP) studies were performed. Immunoprecipitation (IP) was performed with antibodies directed to CHOP, C/EBPβ, and the control IgG. E: represents real-time PCR amplification. *P < 0.05 vs. control, 1-way ANOVA. F: represents the 5’ region of the murine TRB3 promoter with the potential CHOP/C/EBPβ binding site at −61. Arrows represent the site of primers used for the ChIP studies. G: represents semiquantitative PCR ChIP results (34 cycles). H: fully differentiated podocytes were transfected with pcDNA3-CHOP or control vector and 24 h later, TRB3 expression was assessed by Western blotting.

Fig. 6. CHOP increases in diabetic mouse kidneys. Kidneys from STZ-treated (A) and db/db mice (B) and controls were harvested and mRNA expression of CHOP was evaluated by real-time PCR. There was a significant increase in CHOP expression in the db/db mice compared with the db/m controls and a trend for an increase in the STZ-treated mice (n = 5 mice per group, Student’s t-test).

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reducing NF-κB and AP-1 activation of inflammatory genes such as MCP-1, transforming growth factor (TGF)-β, and plasminogen activator-1 (PAI)-1.

endoplasmic reticulum (ER) stress and activation of MAPK pathways. We propose that enhanced TRB3 expression could inhibit MAPK signaling thereby reducing the activity of ACC and ultimately promoting the oxidation of FFA. These findings are likely to be of physiological relevance in other cell types and current studies are underway to test these hypotheses.

Little work has been published focusing on the effects of FFA on podocyte function. Using similar physiologic concentrations of palmitate, Saleem and Welsh’s group (25) recently demonstrated that palmitate blocks insulin-stimulated glucose uptake in human podocytes. Palmitate treatment was also associated with reduced phosphorylation of insulin receptor substrate 1 and AKT (25). It is possible that in these studies, TRB3 is augmented by palmitate and this in turn inhibits phosphorylation of AKT.

Tribbles, the Drosophila homolog of TRB3, inhibits mitosis and causes G2 cell cycle arrest (15, 28, 47). Our previous work in lymphocytes showed that TRB3 induces G2 cell cycle arrest (48), and this is likely related to its ability to reduce transcription of the cyclin B1 promoter (31). Recent work in brain tumor cells suggests that TRB3 inhibits AKT and that this in turn activates autophagy and caspase-mediated apoptotic pathways (43). TRB3 also mediates human monocyte-derived macrophage apoptosis (49), and knockdown of TRB3 reduces ER stress-induced apoptosis (32). Diabetic kidney disease is associated with podocyte loss and defective podocyte function (26). However, in podocytes overexpressing TRB3, we have not yet observed increased apoptosis. In fact, TRB3 does not universally cause cell death. TRB3 was induced fivefold by erythropoietin and was associated with erythroblast survival (45). In postmitotic neuronally differentiated PC12 cells, coexpression of TRB3 with ATF4 prevented ATF4-induced apoptosis (33). Thus, TRB3’s effects on survival or apoptosis are likely cell type and context dependent, and studies are underway to evaluate their survival effect on podocytes.

CHOP (C/EBPδ, DDIT, GADD153) (39) is a transcription factor induced by and used as a marker of ER stress (36). CHOP can function as a transcriptional activator or suppressor (59) and in vivo C/EBPβ and CHOP form dimers in cellular stress (13). In lymphocytes and podocytes, we showed that CHOP and C/EBPβ bind to and activate the murine TRB3 promoter (48). Additionally, in this study, we demonstrate that transfection of CHOP into podocytes enhances TRB3 expression. Pavenstadt’s group (4) first demonstrated that in podocytes, ROS induce CHOP expression. In their study, ROS did not increase podocyte proliferation, cell death, or growth arrest. Podocytes that retrovirally overexpressed CHOP had reduced expression of β1 and α3-integrin; unexpectedly, CHOP overexpression was associated with enhanced cell adhesion to collagen IV-coated plates (4). Although CHOP is considered to induce apoptosis in certain cell types, its function in podocytes remains incompletely understood.

Kidney injury in diabetic nephropathy is associated with the recruitment and activation of monocytes and macrophages in the kidney (14, 58). MCP-1 attracts and activates monocytes and macrophages and MCP-1 levels correlate with kidney injury in humans and diabetic mouse models (58). Mice deficient in MCP-1 have less manifestations of diabetic kidney disease (7, 57) and in general reduced MCP-1 levels attenuate expression of inflammatory renal diseases (21, 58). Evidence

PPAR-α ligands are used clinically to reduce hypertriglyceridemia and lower the risks of cardiovascular disease (9). Montminy’s group (40) elegantly demonstrated that TRB3 stimulates liver lipolysis by promoting the degradation of acetyl-coenzyme A carboxylase, the rate-limiting enzyme of fatty acid synthesis. We show for the first time that FFA induce TRB3 expression. This observation is currently limited to podocytes; however, we propose that TRB3 could function in a negative feedback loop that is designed to regulate the turnover of FFA. High levels of FFA could induce TRB3 expression, thereby reducing the activity of ACC and ultimately promoting the oxidation of FFA. These findings are likely to be of physiological relevance in other cell types and current studies are underway to test these hypotheses.

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suggests that podocytes are an important source of MCP-1 (6, 7, 16) and our studies support these observations. Podocytes in culture express CCR2 (the MCP-1 receptor) (6) and podocyte CCR2 expression is greatly increased in patients with diabetic nephropathy (57). Recombinant human MCP-1 induces podocyte proliferation, migration (6), and reduces nephrin expression (57).

In our studies, TRB3 inhibits expression of MCP-1. Previously, we showed that TRB3 functions as a transcriptional inhibitor of cyclin B1 (31). TRB3 also inhibits the transcriptional activity of NF-κB (61), AP-1 (22), ATF-4 (33, 34), and PPAR-γ (53). The MCP-1 promoter has NF-κB, AP-1, and PPAR-γ response elements, and it is likely that TRB3 inhibits expression of MCP-1 at the transcriptional level. Several studies supported the concept that TRB3 may function as a scaffold protein to inhibit MAPK signaling cascades (19, 22, 56). MAPKs phosphorylate and activate transcription factors in diabetic kidney injury driven by NF-κB and AP-1, including TGF-β and plasminogen activator-1 (Fig. 8) (44).

Because MCP-1 plays a central role in inflammation associated with diabetic kidney disease, it is possible that further augmentation of TRB3 expression during the course of diabetes could be a therapeutic target in diabetes and other chronic inflammatory renal diseases.

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