Tonicity-Responsive Enhancer-Binding Protein Mediates Hyperglycemia-Induced Inflammation and Vascular and Renal Injury

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ABSTRACT

Diabetic nephropathy (DN) has become the single leading cause of ESRD in developed nations. Bearing in mind the paucity of effective treatment for DN and progressive CKD, novel targets for treatment are sorely needed. We previously reported that increased activity of tonicity-responsive enhancer-binding protein (TonEBP) in monocytes was associated with early DN in humans. We now extend these findings by testing the hypotheses that TonEBP in macrophages promotes hyperglycemia-mediated proinflammatory activation and chronic renal inflammation leading to DN and CKD, and TonEBP genetic variability in humans is associated with inflammatory, renal, and vascular function–related phenotypes. In a mouse model of DN, compared with the wild-type phenotype, TonEBP haplodeficiency associated with reduced activation of macrophages by hyperglycemia, fewer macrophages in the kidney, lower renal expression of proinflammatory genes, and attenuated DN. Furthermore, in a cohort of healthy humans, genetic variants within TonEBP associated with renal function, BP, and systemic inflammation. One of the genetic variants associated with renal function was replicated in a large population-based cohort. These findings suggest that TonEBP is a promising target for minimizing diabetes- and stress-induced inflammation and renovascular injury.


Diabetic nephropathy (DN) is a complex disease with progressive decline in renal function that involves multiple pathways including inflammation, endothelial injury, and tubular injury. Only 30% of patients with type 1 diabetes and 40% of those with type 2 diabetes develop DN, indicating considerable individual variations in susceptibility.1 Genome-wide studies have uncovered many genetic variants associated with CKD; nevertheless, they only account for a small proportion of the total genetic contribution.2–4 As such, complementary approaches, such as animal models and translational candidate gene approaches, are needed to better help delineate genetic contributors and novel targets for treatment.

In macrophages, TonEBP (tonicity-responsive enhancer-binding protein) is the rate-limiting component of “NFκB enhanceosome” in which TonEBP...
binds activated NFκB, histone acetyltransferase p300, and RNA polymerase II on the promoters of the TNF-α and other inflammatory genes.3,4 Haplo-deficiency of TonEBP results in a reduced NFκB enhancesome activity and blunted macrophage activation in response to inflammatory signals. Thus, TonEBP haplo-deficient animals display dramatically reduced inflammatory injury in animal models of rheumatoid arthritis7,8 and atherosclerosis.9 We previously reported that among patients with approximately 30 years of type 1 diabetes, proteinuria was associated with approximately 50% higher TonEBP activity in monocytes.10 Here, we extend these results by showing in a mouse model that TonEBP mediates the hyperglycemia-induced proinflammatory activation of macrophages leading to renal infiltration of macrophages and DN. In humans, we find TonEBP-associated single nucleotide polymorphisms (SNPs) to be associated with systemic inflammatory markers, BP, and renal function. Our animal-based findings along with consistent SNP-based association in related phenotypes in humans suggest that genetic variability in TonEBP leads to differential susceptibility to inflammatory responses, vascular injury, and CKD, in response to stressors such as hyperglycemia.

RESULTS

Hyperglycemia Stimulates M1 Polarization and Migration of Macrophages via Upregulation of TonEBP

We sought to investigate the underlying molecular mechanism for our previously noted association between TonEBP activity in monocytes and DN in patients with type 1 diabetes.10 Because previous studies demonstrated the role of macrophage-mediated inflammation in the development of DN,11,12 we decided to examine macrophages in a mouse model of type 1 diabetes. In order to mimic the differences in the level of TonEBP activity, we used the TonEBP heterozygous (TonEBP+/Δ) mice because they display TonEBP haplo-deficiency.7,9,13 We made the TonEBP+/Δ mice and their TonEBP wild-type (TonEBP+/+) littermates hyperglycemic by injecting streptozotocin (STZ) as shown in Figure 1A. When peritoneal macrophages (PMs) from the TonEBP+/+ animals were examined, higher mRNA expression for TonEBP and M1 polarization, as indicated by increased proinflammatory gene expression, in diabetic animals compared with nondiabetic animals was observed (Figure 1B). These changes were reproduced in Raw264.7 cells cultured in high glucose (Figure 1C): raising glucose concentration to 25 mM resulted in higher TonEBP expression in a manner synergistic with LPS, whereas addition of mannitol to the same osmolality did not. Furthermore, the high glucose–enhanced TonEBP expression was associated with elevated NFκB activity in the presence of LPS (Figure 1D). Because most of the M1 genes were NFκB target genes, expression of M1 genes was elevated as expected (Figure 1E). These observations indicate that the enhanced TonEBP expression and M1 polarization of macrophages from diabetic animals (Figure 1B) are due, at least in part, to hyperglycemia.

Significance Statement

TonEBP (tonicity-responsive enhancer-binding protein), also known as Nfat5, is a transcription factor with a physiologic role in the response to osmotic stress in epithelial cells of the renal medulla. Recent evidence points to additional functions in macrophages. This study provides evidence that TonEBP constitutes a causal link between hyperglycemia and induction of proinflammatory gene expression in macrophages, renal infiltration by macrophages, and macrophage-mediated renal injury. Beyond this, the investigators find in a cohort of healthy humans that genetic variations in the TonEBP gene are associated with systemic inflammation and renal function. TonEBP is a novel target for therapy development for diabetic complications including diabetic nephropathy.

We asked whether the elevated expression of TonEBP in response to hyperglycemia was responsible for the elevated NFκB activity and M1 polarization. When TonEBP was knocked down, the LPS-induced activation of NFκB (Figure 1F) and M1 gene expression was reduced without changes in TLR4 mRNA expression (Figure 1G) in high-glucose conditions. Likewise, the induction of M1 genes by LPS was reduced in PMs and bone marrow–derived macrophages (BMDMs) obtained from the TonEBP+/Δ mice compared with those obtained from their TonEBP+/+ littermates (Figure 1H). These data demonstrate that in macrophages TonEBP is induced by hyperglycemia leading to activation of NFκB and elevation of M1 gene expression. In the TonEBP haplo-deficient animals, the M1 gene induction in response to diabetes was blunted (Figure 1B), as expected.

We showed previously that haplo-deficiency of TonEBP in bone marrow cells resulted in approximately 80% reduction in the size of atherosclerotic plaques in a mouse model of atherosclerosis.9 The reduced atherosclerotic lesion was associated with reduced cell migration of BMDMs. We asked whether hyperglycemia affected migration of macrophages. We found that BMDMs from wild-type animals were stimulated by an increase in glucose concentration to 25 mM, but not by addition of mannitol to the same osmolality (Figure 2A). This cell migration was dramatically reduced in BMDMs obtained from the TonEBP haplo-deficient animals (Figure 2B). Taken together, the data in Figures 1 and 2 demonstrate that hyperglycemia enhances TonEBP expression in macrophages, leading to M1 polarization and increased migration of macrophages. A modest, approximately 50% reduction in TonEBP expression resulted in a dramatic decrease in the M1 polarization and migration.

TonEBP Haplo-Deficiency Displays Reduced Number of Renal Macrophages and Renal Inflammation in Mouse Model of DN

Given the reduced activation and migration of macrophages in response to hyperglycemia in the TonEBP haplo-deficient animals (see above), we asked whether reduced renal macrophages were observed in these animals in a mouse model of DN: deficiency of endothelial nitric oxide synthase (eNOS),
i.e., TonEBP+/Δ, eNOS−/− versus TonEBP+/+, eNOS−/− (Figure 3, A and B; see also Supplemental Material for details). Renal macrophage numbers assessed by F4/80 mRNA expression (Figure 3C) and immunohistochemical analyses of F4/80 (Figure 3D) were higher in the TonEBP+/+ animals on the eNOS−/− background compared with those on the eNOS+/+ background. In those animals on the eNOS−/− background, but not those on the eNOS+/+ background, both the mRNA
have been implicated in DN both in patients and animals.14–16 IL-6, MCP-1, IP-10, IL-8, TNF-α, IL-1β, RANTES, IL-18, and INF-γ (Figure 4, A–I). All of these genes have been implicated in DN both in patients and animals.15,16 In correlation with the reduced IL-6 mRNA expression, IL-6 signaling measured by phosphorylation of STAT3 was reduced (Figure 4J), suggesting that the lower gene expression in the TonEBP haplo-deficient (Figure 6C) mirrored the changes in fibrosis, suggesting that changes in TGF-β expression were responsible for the variations in fibrosis. Thus, diabetic renal injuries were dramatically tempered in the TonEBP haplo-deficient animals in association with reduced macrophage infiltration and renal inflammation.

Hypotension and Hyperreninemia in TonEBP Haplo-Deficiency

A recent report shows that the SNP rs33063 in the TonEBP gene is associated with pulse pressure. Because the functional significance of the associated variant was not established, we tested whether reduced TonEBP levels were associated with BP using the TonEBP haplo-deficient mice. Consistent with the human association studies, these animals displayed reduced systolic BP (SBP). We also noted elevated renal renin expression and circulating renin levels (Figure 7), presumably as a compensatory reaction to the reduced BP.

Association of TonEBP Polymorphisms with Inflammatory, Vascular, and Renal Function Markers in Humans

Data in the previous sections demonstrate the relevance of TonEBP to glycemic stress–induced inflammation, renal function, and BP. Moreover, previously published associations of TonEBP expression with inflammation, rheumatoid arthritis, atherosclerosis, and DN in humans raised the possibility that variations in TonEBP might affect similar phenotypes in humans. Accordingly, we performed a look-up of TonEBP variant association in a highly homogeneous cohort of healthy individuals with minimal confounders (see Methods and Supplemental Material for details) with measures of inflammation, renal function, and BP.

We identified a total of 320 SNPs on the basis of full sequencing of the TonEBP gene region, from which we identified 16 haplotype blocks and selected 16 single haplotype
and found a significant association between rs118095741 and absolute monocyte count \((P=0.002)\). We also found rs74956396 to be suggestively associated with serum IL-1\(\beta\) \((P<0.01)\) and homocysteine \((P<0.01)\), whereas rs244416 was independently also suggestively associated with IL-1\(\beta\) \((P<0.01)\). For our BP phenotypes, we found rs2287970 to be significantly associated with diastolic BP (DBP) \((\beta=1.4, P=0.003)\) and suggestively with SBP \((\beta=1.65, P=0.04)\). Lastly, for our renal phenotypes, we found a significant association between rs17297179 and eGFR \((\beta=6.3, P=0.003)\) and suggestive association between rs17232663 and albuminuria \((\beta=0.36, P<0.01)\). Further details are provided in the Supplemental Material. Secondary adjustments for eGFR and BP, as appropriate, did not modify our findings (see Supplemental Material). Functional annotations for our top identified SNPs are also summarized in Supplemental Table 3.

We also attempted to replicate our renal function associated SNP within the open source CKDGen consortium meta-GWAS results for eGFR \((n=67,093)\) and albuminuria.\(^{25}\) Neither our albuminuria sequence based variant rs17232663 nor any SNPs in strong linkage disequilibrium (LD) with it were identified in the more limited HapMap based CKDGen database and hence could not be tested for replication. Our eGFR variant rs17297179 or other variants in strong LD were similarly not available. However, we were able to identify rs1064825, our second-best association with eGFR, which is in moderate LD with our top SNP (rs17297179), to be associated with eGFR in CKDGen \((\beta=0.006, P<0.003)\), hence validating our finding.

In summary, in a highly homogeneous cohort of healthy individuals with minimal confounders, gene variants in TonEBP are associated with inflammation, renal function, and BP, all in accordance with our mouse TonEBP haplo-deficiency findings described here and previously. The association with renal function is replicated in the CKDGen cohort, suggesting its relevance to renal function.

**DISCUSSION**

Numerous underlying molecular mechanisms involved in glucose toxicity have been defined, including oxidative stress and advanced glycan end products (for a review, see\(^ {26}\)). Here, we have demonstrated that hyperglycemia is a proinflammatory
signal for macrophages (Figure 8). The effects of hyperglycemia are similar to those of LPS: like LPS, hyperglycemia enhances TonEBP expression which in turn drives the expression of proinflammatory genes via stimulation of NFκB. In addition, hyperglycemia stimulates the migration of macrophages, another key proinflammatory phenotype, which is also TonEBP-dependent. Even more interesting is the finding that TonEBP haplo-deficiency is associated with dramatically reduced expression of the proinflammatory genes as well as migration in macrophages. The reduced renal injury in response to STZ-induced diabetes in the TonEBP haplo-deficient animals can be readily explained by the reduced macrophage numbers in the kidney in combination with reduced expression of proinflammatory genes by the macrophages. These data provide a mechanistic basis for the association of TonEBP activity in monocytes and DN in patients with type 1 diabetes. In line with this, we previously reported that TonEBP haplo-deficiency in bone marrow cells led to an 80% reduction in the size of atherosclerotic lesions. The same pathway in monocytes can also explain the dramatically reduced rheumatoid arthritis in the TonEBP haplo-deficient animals.

Other investigators have reported that monocytes isolated from humans with both type 1 and type 2 diabetes display an inflammatory phenotype because they secrete higher levels of proinflammatory cytokines. The data presented here reveal that hyperglycemia stimulates NFκB via induction of TonEBP: TonEBP is a transcriptional cofactor of NFκB by way of recruiting histone acetyl transferase p300 leading to increased expression of proinflammatory cytokines and COX-2. Thus, TonEBP is a critical mediator of diabetes-induced inflammation in macrophages.

Given our animal-based findings, we examined whether TonEBP gene variants in humans were associated with phenotypes that are affected by TonEBP expression levels in our animal model and noted multiple consistent findings. This included significant associations with serum MMP-1 and absolute monocyte counts. This corroborates our previous finding of increases in TonEBP expression in monocytes of individuals with DN. Because IL-1β is a direct transcriptional target of TonEBP, the suggestive association of TonEBP SNPs with circulating levels of IL-1β and homocysteine observed in this study supports the notion that TonEBP genetic variability may possibly affect IL-1β expression in humans. The previously noted association between TonEBP variants and pulse pressure also confirms our association with BP, whereas the associations with eGFR and albuminuria are novel. Interestingly, in a cohort which included >700 individuals with DN, Kavanagh et al. found rs17297207 in TonEBP to be weakly associated with DN (OR, 0.66; P=0.04). The rs17297207 SNP from the Kavanagh et al. study

Figure 4. TonEBP haplo-deficiency reduces renal expression of proinflammatory genes and STAT3 activation in a mouse model of DN. mRNA for IL-6 (A), MCP-1 (B), IP-10 (C), IL-8 (D), TNF-α (E), IL-1β (F), RANTES (G), IL-18 (H), and IFN-γ (I) was measured from the kidney samples described in Figure 3 using qRT-PCR. Animals injected with VH are shown in open bars, and those injected with STZ in filled bars. Mean±SEM, n=8. *P<0.05 compared with corresponding VH. #P<0.05 compared with corresponding TonEBP+/+. (J) Representative images of immunohistochemical staining for phosphorylated STAT3. Arrowheads denote intense signals in tubulointerstitial areas. VH, vehicle; +/+, wild-type; +/-, heterozygous; --/--, knock out.
is in perfect LD with rs118095741, which we found to be significantly associated with serum monocyte count (P=0.002) and MMP-1 measures in our population. This is also in agreement with our previously noted association of a near 50% increase in TonEBP expression in monocytes of type 1 diabetic individuals with proteinuria.10

Although several variants were each associated with multiple related phenotypes, as in our animal model, we also noted a significant amount of distinct associations between several variants and phenotypes. A plausible explanation for this is that these variants are associated with differential expression of distinct TonEBP isoforms. Indeed, given that this gene can regulate hundreds of genes,5,7 both as enhancer and suppressor, a complex regulatory process is arguably required. For example, it has been shown to stimulate genes as a DNA binding transcription factor31 or as a transcriptional cofactor for NFκB and other DNA binding proteins,6 while suppressing other genes by recruiting histone methylase32 or by recruiting DNA methylase to promoter regions (unpublished observations by H.H.L.). It has also been noted to have seven splice isoforms (Ensembl, GRCh38.p10), which in addition to tissue-specific enhancer and repressor can provide additional mechanisms for differential regulation of various downstream pathways. Consistent with our findings, three distinct TonEBP signals have been associated with various phenotypes, such as: (1) rs7193778 with uric acid and CRP,33,34 rs33063 with pulse pressure,21,35 and rs9980 with plasma osmolality,35 which are all in strong LD with each other and rs244416 from our analysis (associated with IL-1β and DBP); (2) rs12599391 with age at menarche36 and rs6499244 with allogeneic hematopoietic stem cell transplantation outcomes,37 which are in LD with each other but not with any of our identified SNP’s; and (3) rs17297207 with DN,30 which is in LD with our monocyte count-associated SNP (rs118095741). Lastly, in an interesting human case of TonEBP haplo-insufficiency, disturbances in both innate and adaptive immunity leading to intestinal autoimmunity38 were noted, again supporting our findings.

Limitations of our findings include the lack of information on the putative functional SNPs driving our associations, as well as their effect on TonEBP expression. Accordingly, we cannot prove that the noted associations between the TonEBP SNPs in our and previous studies are due to differential expression of TonEBP. Moreover, our set of statistically suggestive findings should not be over-interpreted without further validation with more data in independent cohorts. However, there are factors that suggest these suggestive findings, in addition to our significantly associated findings, are worth further study. Most notably, we have already established, via our animal-based experiments, mechanistic pathways by which TonEBP deficiency can affect our selected outcomes. Second, many of our findings are at least partially corroborated by related previous publications. Last, our findings are highly consistent across our mouse model and identified human phenotypes. These findings provide further impetus for the targeting of TonEBP as a potential novel treatment for inhibiting inflammation-based renal injury. Lastly, we previously showed that cerulenin could suppress the TonEBP-mediated proinflammatory activation of NFκB and downstream inflammation,16 demonstrating that the targeting of TonEBP may be a viable therapeutic option.

**CONCISE METHODS**

**Animals**

All animal procedures were approved by the Institutional Animal Care and Use Committee at Ulsan National Institute of Science and Tech-
The TnEBP+/Δ mice on C57BL/6 background were obtained from Dr. S.N. Ho. They were crossed back to the C57BL/6 line (The Jackson Laboratory, Bar Harbor, ME) to produce TnEBP+/Δ animals and their TnEBP+/+ littermates. Where indicated, the animals were bred to the eNOS-decient (eNOS2/2) line on C75BL/6 (The Jackson Laboratory) to produce TnEBP+/Δ, eNOS2/2 mice and their littermates—TonEBP+/+, eNOS2/2. Mice were kept on a 12-hour light/dark cycle with free access to standard chow and water. Males were selected and made diabetic by daily intraperitoneal injections of freshly prepared STZ (50 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) in 0.1 M citrate buffer (pH 4.5) for 4 days. Animals displaying fasting blood glucose levels >250 mg/dl after 2 weeks of STZ injections were considered diabetic. Control, nondiabetic animals were injected with the buffer. Six or 7 weeks post the STZ injections the animals were analyzed for macrophages (see below) and nephropathy. Body weight and blood glucose levels were monitored weekly. After spot urine was collected, animals were euthanized with intraperitoneal injection of Zoletil 50 (10 mg/kg; Virbac Laboratories, Carros, France) and Rompun (15 mg/kg; Bayer, Leverkusen, Germany) to collect blood samples and tissue specimens. Fractional excretion of sodium (FeNa), urine osmolality, urine creatinine, urine albumin, and BUN were measured as described previously with slight modification.39,40 Tissue sections were stained with periodic acid–Schiff and Trichrome to assess glomerular injury and renal interstitial fibrosis, respectively. Randomly selected fields were analyzed to quantify injuries and fibrosis. For BP and renin analyses, 5-week-old animals were used.

**Figure 6.** TnEBP haplo-deficiency reduces tubulointerstitial fibrosis and TGF-β expression. (A) Representative images of Masson’s trichrome staining of kidney sections. (B) Blue areas representing collagen deposition were measured and expressed as percentage. Mean±SEM, n=8. *P<0.05 compared with corresponding VH. #P<0.05 compared with corresponding TnEBP+/+. (C) Representative images of immunohistochemical staining for TGF-β. Asterisks denote intense signals in tubulointerstitial areas. VH, vehicle; +/+ , wild-type; +/Δ, heterozygous; −/−, knock out.

**Figure 7.** TnEBP haplo-deficiency is associated with reduced SBP, hyperreninemia, and elevated renal expression of renin. Male TnEBP+/Δ mice or their TnEBP+/+ littermates were analyzed for body weight (A), pulse rate (B), SBP (C), plasma renin activity (D), and renal renin immunoblot (E). Mean±SEM, n=8. *P<0.05 compared with corresponding TnEBP+/+. VH, vehicle; +/+ , wild-type; +/Δ, heterozygous; −/−, knock out.
Table 1. Top SNP associations with selected traits

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For IL-1β, WBC, c-IMT, and MMP-1, log transformations were used to calculate effect size—IL-1β in pg/ml, MMP-1 in ng/ml. SNP, the SNP nucleotide associated with the outcome; allele, reference allele; MAF, minor allele frequency; n, sample size; effect size for SNP for selected traits; adjusted for age, sex, and family structure; MMP-1, MMP-1 (interstitial collagenease); WBC, white blood cell count; monocyte, absolute monocyte count; DBP, DBP (mmHg); albuminuria, log-transformed urinary albumin-to-creatinine ratio (mg/gm); eGFR, eGFR (ml/min per 1.73 m²). *Statistical significance on the basis of P<0.003 (0.05/16 haplotypes=0.003).

**Transfection and Luciferase Reporter Assay**

Dicer-substrate small interfering RNA (siRNA) targeting TonEBP (5'-CCAGUUCCACAUAGUAACACCUg-3') and nontargeting negative control (scrambled) siRNA (5'-CGUUAACUCGUUGAUAACCCGUA-3') were purchased from Integrated DNA Technologies (Coralville, IA). Raw264.7 cells were transfected for 1 day with 2 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected cells were cultured for another day in fresh culture medium before LPS treatment. For NF-κB luciferase assays, siRNA-transfected cells were transfected with the NF-κB–dependent luciferase reporter plasmid (pGL4.32[luc2P/NF-κB-RE/Hygro]; Promega, Madison, WI) using Lipofectamine 2000 reagent (Invitrogen). The pRL-TK vector was co-transfected to normalize for transfection efficiency. After 24 hours, the cells were treated with LPS for 8 hours. The cell lysates were prepared with a passive lysis buffer and used to measure the luciferase activity according to the manufacturer’s instructions for the luciferase reporter assay system (Promega). The luciferase assays were carried out using a GloMax 96 Microplate Luminometer (Promega).

**RNA Isolation and Real-Time PCR**

Total RNA was isolated using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen). After reverse transcription, quantitative PCR was performed using SYBR Green I Master and LightCycler 480 II (Roche Applied Sciences, Indianapolis, IN) and primers described in Supplemental Table 4. Resulting cycle threshold (Ct) values were normalized with cyclophilin A and the ΔΔCt method was then used to express values as fold over control samples.

**Western Blotting**

Protein extraction from tissues was performed as previously described.39 Equal amounts of protein from samples were separated by SDS-PAGE and immunoblotted using specific antibodies. The antigen-antibody binding was detected by enhanced chemiluminescence western blotting detection reagents (GE Healthcare, Little Chalfont, UK). Primary antibodies used were anti-TonEBP antibody,31 anti-iNOS antibody (BD Biosciences, Franklin Lakes, NJ), and anti-Hsc70 (Rockland, Gilbertsville, PA).

**Macrophage Migration Assay**

Macrophage migration was measured using a modified Boyden chamber (NeuroProbe, Gaithersburg, MD). BMDMs were plated in the upper chamber on a 5-μm porous membrane and cultured in DMEM containing normal glucose (5.5 mM), high glucose (25 mM), or 5.5 mM glucose +19.5 mM mannitol. MCP-1 (10 ng/ml) was added to the lower chamber as a chemoattractant. After 16 hours, cells were removed from the upper side of membranes and nuclei of migratory cells on the lower side of the membrane were stained with DAPI. The number of migratory cells was visualized by fluorescence microscopy and quantified using ImageJ software.

**Immunohistochemistry**

Immunohistochemistry was performed as described previously.39 The F4/80, STAT3, and TGF-β1 were detected in 4-μm tissue sections by incubating tissue slides for 12 hours with specific antibodies against F4/80 (Serotec, Oxford, UK), STAT3 (Cell signaling, Boston, MA), and TGF-β1 (Proteintech, Chicago, IL) at 4°C.

**Histologic Analyses**

Kidneys were fixed with 2% paraformaldehyde-lysin-peridate and embedded in paraffin. Sections were stained with periodic acid–Schiff reaction plus hematoxylin counterstain. For quantitative assessment of glomerular injury, >50 glomeruli were examined from each animal. Percentage of glomeruli displaying mesangioysis, mesangial expansion, microaneurysm, nodular lesions, and sclerosis was assessed in a blinded manner.

**Blood Collection, Renin Activity, and Renin Immunoblot**

Blood was taken from conscious mice by tail vein puncture and collected into a 75-μl hematocrit tube that contained 1 μl of 125 mM EDTA at its tip. Plasma was collected by centrifugation and stored frozen. Renin concentration was measured by Gammacoat plasma renin activity immununoassay kit (DiaSorin, Stillwater, MN). Renal renin expression was evaluated using immunoblot analysis. Renin was detected by incubating for 12 hours with specific antibody, goat polyclonal renin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Relative OD of the band in each lane was normalized relative to the density of the Hsc70 band from the same gel.

**BP and Heart Rate**

SBP and heart rate of TonEBP heterozygotes and their wild-type littermates were determined by noninvasive tail-cuff BP system (Hatteras Instruments SC-1000, Cary, NC). Animals were conditioned by placing them into the holding device on three consecutive days before the first measurement. BP was determined for 3 days in a row, and values were calculated as averages of these three measurements for each individual mouse.
Human Population, Genotyping, and Phenotype Analyses

Our primary population consisted of 868 participants from the HAPI Heart study. These participants were healthy white individuals from the Old order closed Amish founder population in Lancaster, PA. Subjects recruited for this study were ≥20 years old and were excluded if they had severe hypertension (BP >180/105 mmHg); malignancy; or kidney, liver, or untreated thyroid disease. Additionally, all participants were not on any active medications at the time of the study. This unique genetically and environmentally homogenous population was selected to minimize potential confounders and enhance the ability to detect genetic contributions to a variety of cardiovascular phenotypes, including selected markers of systemic inflammation. Resting protocol–based SBP and DBP measures were also obtained while participants were off any antihypertensive medications. Baseline measures of inflammatory markers: serum IL-1β an inflammatory cytokine, homocysteine, MMP-1, matrix metalloprotease-9 (MMP-9), C-reactive protein (CRP), monocyte count, and white blood cell count as markers of systemic inflammation. Albuminuria was on the basis of spot morning collected urine albumin-to-creatinine ratio. GFR was estimated on the basis of serum creatinine using the Modification of Diet in Renal Disease formula. Details regarding methods for selection of individuals and phenotype measures have been previously published.

Genotyping by whole-genome sequencing was done under the National Heart, Lung, and Blood Institute (NHLBI) Trans-Omics for Precision Medicine program (http://www.nhlbi.nih.gov/research/resources/nhlbi-precision-medicine-initiative/topmed). We selected all SNPs with MAF ≥3% within a 2-kb window of the TonEBP gene. We then used Haploview to identify the number of haplotype blocks and tagging SNPs. We then looked at the association between the identified tagging SNPs and our selected phenotypes. Once we identified our top candidate SNPs for each phenotype, we used the HaploReg software package V4.1 to extract the functional annotation for each SNP of interest. For each variant, we looked for its predicted chromatin segmentation, including histone markers, focusing on enhancers as well as DNase I hypersensitive sites across a variety of tissue and cell types. Expression quantitative trait loci annotation was on the basis of the Genotype-Tissue Expression Project, the Geuvadis RNA-sequencing project, and the latest NHLBI-supported GRASP databases.

All SNP to phenotype associations were adjusted for age, sex, and family relatedness using the Mixed Models Analysis for Populations and Pedigrees (MMAP) program (http://edn.som.umaryland.edu/mmap/index.php). All baseline inflammatory marker and albuminuria values were natural logarithm transformed to remove skewness.

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DISCLOSURES
None.

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