Renin–Angiotensin System

Novel Mechanism of Blood Pressure Regulation By Forkhead Box Class O1–Mediated Transcriptional Control of Hepatic Angiotensinogen

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Abstract—The renin–angiotensin system is a major determinant of blood pressure regulation. It consists of a cascade of enzymatic reactions involving 3 components: angiotensinogen, renin, and angiotensin-converting enzyme, which generate angiotensin II as a biologically active product. Angiotensinogen is largely produced in the liver, acting as a major determinant of the circulating renin–angiotensin system, which exerts acute hemodynamic effects on blood pressure regulation. How the expression of angiotensinogen is regulated is not completely understood. Here, we hypothesize that angiotensinogen is regulated by forkhead transcription factor forkhead box class O1 (Foxo1), an insulin-suppressed transcription factor, and thereby controls blood pressure in mice. We generated liver-specific Foxo1 knockout mice, which exhibited a reduction in plasma angiotensinogen and angiotensin II levels and a significant decrease in blood pressure. Using hepatocyte cultures, we demonstrated that overexpression of Foxo1 increased angiotensinogen expression, whereas hepatocytes lacking Foxo1 demonstrated a reduction of angiotensinogen gene expression and partially impaired insulin inhibition on angiotensinogen gene expression. Furthermore, mouse angiotensinogen promoter analysis demonstrated that the angiotensinogen promoter region contains a functional Foxo1-binding site, which is responsible for both Foxo1 stimulation and insulin suppression on the promoter activity. Together, these data demonstrate that Foxo1 regulates hepatic angiotensinogen gene expression and controls plasma angiotensinogen and angiotensin II levels, modulating blood pressure control in mice. (Hypertension. 2014;64:1131-1140.)

Key Words: angiotensinogen ■ hypertension ■ liver

Angiotensinogen is a precursor of angiotensin II (Ang II), an octapeptide pressor hormone that has a key role in mediating vascular constriction and regulating salt and fluid homeostasis.1 Elevation of Ang II contributes to hypertension, atherosclerosis, cardiac hypertrophy, and heart failure.2,3 Angiotensinogen is expressed and synthesized largely in the liver and secreted into the blood circulation where it is processed by the kidney-derived aspartyl protease renin to produce angiotensin I, which is in turn hydrolyzed by angiotensin-converting enzyme to generate Ang II. Overexpression of the angiotensinogen gene in mice increases blood pressure,4 whereas mice lacking the angiotensinogen gene systemically exhibited hypotension.5,6 Mice lacking angiotensinogen in liver have a significant reduction in plasma angiotensinogen and Ang II levels and have reduced blood pressure. Thus, liver is a major source for plasma angiotensinogen and Ang II in control of blood pressure.7 Determining the mechanisms by which angiotensinogen gene expression is regulated is important for understanding the molecular basis for blood pressure control.

Insulin is known to decrease angiotensinogen gene expression in hepatocytes,8 although other hormones and nutrients, such as high glucose, increase angiotensinogen gene expression.9,10 Over the past decade, we have identified an insulin-regulated forkhead transcription factor, Foxo1, which stimulates expression of target genes encoding gluconeogenic enzymes and hence promotes hepatic glucose production.11–13 The forkhead transcription factor family has >80 members and each contains a conserved DNA-binding domain that forms a forkhead winged/helix structure interacting with DNA. They are grouped into 19 classes from FoxA to FoxS based on DNA sequence homology.14 The O class of forkhead transcription factor family (FoxO) consists of Foxo1, Foxo3, Foxo4, and Foxo6 and each has 3 consensus Akt phosphorylation motifs (RxRxxT/S, R-arginine, x-any amino acid, and T/S- threonine/serine- target of Akt).11,12 Foxo1 is involved in a wide range of events, including embryonic development, cell metabolism, survival, and diseases.13,15

Insulin suppresses the gene transcription via a conserved insulin response element (IRE) in the promoter region of

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target genes.\textsuperscript{11,12} The IRE sequence that we have identified is 5′-CAAAACAACG3′, which Foxo1 binds to in the promoter region and activates target gene transcription.\textsuperscript{11,16} In response to insulin stimulation, Akt is activated and then inhibits Foxo1 by phosphorylating threonine/serine residues at T\textsuperscript{24}, S\textsuperscript{256}, and S\textsuperscript{319}. These phosphorylation events control Foxo1 protein turnover and promote Foxo1 cytoplasmic localization and ubiquitination, impairing Foxo1 interactions with the IRE on the target gene’s promoter region and suppressing gene transcription.\textsuperscript{11,12}

At first, we performed an online analysis of mouse angiotensinogen promoter region with a transcription element search system (http://www.cbil.upenn.edu) and found that there are 2 potential IREs or Foxo1-binding sites, prompting us to test the hypothesis that Foxo1 may regulate angiotensinogen gene expression in the liver; thereby controlling plasma angiotensinogen and Ang II and further modulating blood pressure in animals. This study provides in vitro and genetic evidence that demonstrates the role of liver Foxo1 in regulating angiotensinogen gene expression and blood pressure in mice.

Materials and Methods

Mice

All animal experiments were performed according to procedures approved by the Texas A&M Health Science Center Institutional Animal Care and Use Committee. The floxed Foxo1 mice (Foxo1\textsuperscript{-floxed}) and albumin-Cre mice, in which cre recombinase is specifically expressed in the liver, were previously described.\textsuperscript{13} All of the mice were on a C57BL/6 and 129 Sv mixed background and were maintained on regular chow (Prolabo Isopro 5P76).

DNA Cloning, Mutagenesis, and Reporter Gene Assay

Mouse angiotensinogen promoter regions were amplified by polymerase chain reaction (PCR), using mouse tail DNA and cloned into a luciferase reporter gene. The angiotensinogen promoter region-1 spanning 1.5 kb upstream from the transcriptional initiation site (−1.5kb) was amplified with PCR primers: 5′-tttcttctctggctgcagcaagcttcgtcaaagaccctct-3′ and 5′-tatatacagccag-3′, an 0.8 kb region upstream from the 1.5 kb promoter was designated as the angiotensinogen promoter region-2, and amplified by PCR, with the primers: 5′-agttgttacctgcgtctgctgcttcagcaagacagt-3′ and 5′-agagtaagcttacagaacagcgcgttgtgtgttg-3′, and an 0.66 kb region upstream from the 0.8 kb promoter region was designated as the angiotensinogen promoter region-3, and amplified by PCR, with primers: 5′-acagctgacagcagcaagctgcgtcgttggaag-3′ and 5′-tttattaattctgtctgctgcttcagcaagacagt-3′. The 3 PCR promoter fragments were cloned into the pGL3-luciferase reporter gene (Promega), generating Agtp-1.5 kb, Agtp-800 bp, and Agtp-660 bp luciferase reporter constructs. The mutation of the Foxo1-binding site on the Agtp-800 bp promoter was achieved by in vitro mutagenesis, with PCR primers: 5′-ctctttcttggctgcagcaagcttcgtcaaagaccctct-3′ and 5′-gaagctgctgtctgctgcttcagcaagacagt-3′, using a site-specific mutagenesis kit (Stratagen). In the Agtp-800 bp promoter region, 3 reporter constructs containing 5′ deletion of 200 bp, 400 bp, and 600 bp were generated and designated as Agtp-600, Agtp-400, and Agtp-200 bp, respectively. All cloned DNA fragments and mutations were confirmed by DNA sequencing. HepG2 cells were cultured in DMEM/10% fetal bovine serum and transfected by pAlter-Max plasmid DNA, with or without expression of Foxo1, using TransIT-293 transfection reagent (Mirus, Madison, WI), as previously described.\textsuperscript{13}

Chemicals and Antibodies

Foxo1, pFoxo1-S\textsuperscript{213}, Akt, pAkt-T\textsuperscript{308}, extracellular signal-regulated kinase 1 and 2 (ERK1/2), pERK1/2-T\textsuperscript{202}/Y\textsuperscript{204}, GAPDH, and α-actin antibodies were from Cell Signaling Technology (Billerica, MA), and angiotensinogen antibody was purchased from Immuno-Biological laboratories, Inc (Japan). Insulin and collagenase were purchased from Sigma and Percoll from Amersham.

Measurement of Plasma Ang II Concentration

Ang II from plasma was extracted in 1 mol/L acetic acid, passed over a Discovery Supelco C-18 column (Supelco, Bellefonte, PA), and eluted with methanol, as previously described.\textsuperscript{17} A standard curve was generated from binding of a constant amount of biotinylated angiotensin peptide with increasing concentrations of nonbiotinylated peptide.

Measurement of Blood Pressure

Mice were anesthetized with isoflurane and placed on a heating pad, and rectal temperature maintained between 36.0°C and 37.5°C. The left carotid artery was cannulated with a catheter (FTH-1212B-4518, 1.2F P-V catheter with 4.5 mm electrode spacing, Scisense Inc, Canada) and connected to a transducer and data acquisition systems (iWorx IX/228S Data Acquisition System with the Scisense Advantage pV control unit version 5.0). Alternatively, measurement of systolic blood pressure was also performed on conscious, restrained mice via the Visitech BP-2000 tail-cuff system. Briefly, systolic blood pressure was quantified for 3 days and each cycle consisted of 10 preliminary tail-cuff inflation/deflations for daily acclimation, followed by 10 additional cycles that were recorded. Criteria for inclusion of measurements from individual mice were 24 of 10 successful measurements, with a SD ≤50 required for inclusion, as previously described.\textsuperscript{18}

Protein Analysis and Western Blot

Immunoblotting analysis using specific antibodies was previously described.\textsuperscript{19} Signal intensity was measured and analyzed by NIH Image J software. For angiotensinogen measurements, plasma samples were analyzed by Western blot using angiotensinogen antibody and total plasma protein by Ponceau Red (Bio-Red).

Chromatin Immunoprecipitation assay

Chromatin immunoprecipitation assay was performed as described previously.\textsuperscript{20} Immunoprecipitated DNA was analyzed by PCR with following primers: 5′-gactgaaagtgcagctgctgctgc-3′ and 5′-ctcaagagttacatcgtgc-3′.

Primary Hepatocyte Isolation and Cell Culture

Mouse hepatocytes were isolated from 8- to 12-week-old mice with a protocol previously described.\textsuperscript{13} After cell attachment, hepatocytes were cultured in serum-free medium overnight and then treated with or without 100 nmol/L insulin for 18 hours before further analysis.

Adenovirus Infection of Hepatocytes

Mouse primary hepatocytes were cultured in DMEM/10% FBS medium for 8 hours then transfected by adding adenovirus expressing green fluorescent protein, Foxo1-wild type (wt), constitutively active Foxo1-T/S/S-A in which 3 Akt phosphorylation sites on T\textsuperscript{24}, S\textsuperscript{256}, and S\textsuperscript{319} are mutated to alanine (A), and Foxo1-DNA-binding domain (DBD) that expresses DBD, which was previously described.\textsuperscript{21} Cells were infected with adenovirus with 50 multiplicity of infection for 8 hours and then changed to fresh DMEM medium with serum for another 8 hours of culturing. Cells were serum starved for 8 hours before insulin intervention. For protein kinase inhibitor treatment, the inhibitor PD98059 or wortmannin was added to cells for 0.5 hours before addition of insulin and before cellular protein lysates were prepared for immunoblotting.

Quantitative Real-Time PCR Analysis

Mouse liver RNA isolation was extracted with Trizol reagent (Invitrogen) and PCR performed as previously described.\textsuperscript{13} PCR primers used for angiotensinogen were 5′-gggagggcagagggcttcagc-3′ and 5′-gaaggggctgctacaggtgc-3′.
Statistical Analysis
All results are presented as the mean±SEM and analyzed by ANOVA to determine P values. P<0.05 was considered statistically significant, as previously described.22,23

Results
Generation of Liver-Specific Foxo1 Gene Knockout (L-F1KO) Mice
We intercrossed Foxo1<sup>1<sup>LoxP</sup></sup> with albumin-Cre transgenic mice (Cre<sup>Alb</sup>) to produce mice without liver Foxo1 (L-F1KO mice) and control littermates that include Foxo1<sup>1<sup>LoxP</sup></sup> mice or albumin-Cre mice (Figure 1A). All of the mice were born in Mendelian ratios, and F1KO mice were indistinguishable in appearance from control littermates. At the age of 10 weeks, F1KO mice exhibited a 15% to 20% reduction in blood glucose concentrations and no change in serum insulin compared with control littermates. The metabolic phenotype of F1KO mice was extensively characterized in our previous study.13

L-F1KO Mice Exhibit Lower Blood Pressure
We examined the blood pressure of mice under anesthesia with the pressure–volume loop system. At 12 weeks of age, L-F1KO mice had reduced blood pressure: a 14 mm Hg reduction of systolic blood pressure compared with control mice (P<0.05, n=6); a 11 mm Hg reduction in diastolic blood pressure (P<0.05, n=6); and a 19 mm Hg reduction in the mean arterial pressure (P<0.05, n=6), although the L-F1KO mice exhibited no difference in heart rate (Figure 1B–1E). To confirm further the reduction in blood pressure in L-F1KO mice, we measured blood pressure of mice under conscious conditions with a tail-cuff. The L-F1KO mice exhibited a reduction of systolic blood pressure by 15 mm Hg (P<0.05, n=6; Figure 1F). Clearly, the liver-specific Foxo1 deletion resulted in lower blood pressure in mice.

Hepatic Deficiency of Foxo1 Decreases Plasma Angiotensinogen and Ang II Concentration
We next determined the plasma Ang II concentration in these mice. The plasma Ang II concentration was reduced by 55% in L-F1KO mice, compared with control (P<0.05, n=6; Figure 2A). Angiotensinogen in the plasma was reduced by ≈50% in L-F1KO mice, as determined by Western blot (Figure 2B). The angiotensinogen, which is largely produced by the liver, demonstrated a 52% reduction in protein expression in L-F1KO liver, compared with control (Figure 2C). In addition, loss of Foxo1 in F1KO liver had no changes in intracellular signaling, including phosphorylation of Akt and ERK1/2, as determined by Western blot (Figure 2D). Together,

Figure 1. Generation of liver-specific forkhead box class O1 knockout (L-F1KO) mice and measurement of blood pressure. A, Location of loxP sites on the Foxo1 gene in Foxo1-<sup>loxP</sup> mice and deletion of exon 2 of Foxo1 containing DNA-binding domain in L-F1KO mice. B–D, The blood pressure of 12-week-old male F1KO and control littermates under anesthesia was measured by pressure–volume loop experiments and tail-cuff experiments when mice are under conscious conditions (E and F). Data are expressed as the mean±SEM; *P<0.05 vs control (n=8–12 mice/group). CNTR indicates control; DBP, diastolic blood pressure; MAP, mean arterial pressure; and SBP, systolic blood pressure.
these results indicate that Foxo1 regulates angiotensinogen gene expression in the liver and significantly controls plasma angiotensinogen and Ang II levels.

Overexpression of Foxo1 Stimulates Angiotensinogen Expression in Hepatocytes

We next examined whether Foxo1 stimulates angiotensinogen gene expression in hepatocytes. Forced expression of Foxo1-wt in hepatocytes, by 2.5-fold at the protein level, was achieved by adenovirus infection, resulting in a 1.3-fold increase in angiotensinogen protein expression (Figure 3A and 3B). A similar effect was observed with forced expression of the Foxo1-T/S/S-A mutant that prevents Akt-mediated phosphorylation. However, expression of Foxo1-DBD that contains the DBD without transcriptional activation domain did not increase angiotensinogen protein expression (Figure 3A and 3B). Consistent with the previous study, overexpression of Foxo1-wt, Foxo1-T/S/S-A, or Foxo1-DBD enhanced Akt phosphorylation with little effect on ERK1/2 phosphorylation, likely owing to Foxo1 interaction with tribbles homolog-3, an endogenous Akt inhibitor. These results indicate that overexpression of Foxo1 enhances angiotensinogen protein expression in hepatocytes.

Foxo1 Deficiency Impairs Insulin Inhibition on Angiotensinogen mRNA Expression in Hepatocytes

Because insulin decreases angiotensinogen mRNA expression in liver, we assessed the role of Foxo1 in insulin inhibition.
on angiotensinogen gene expression. We treated control and L-F1KO hepatocytes with 100 nmol/L insulin for 24 hours, and angiotensinogen mRNA expression was analyzed. Angiotensinogen mRNA expression levels were reduced by 40% in insulin-treated control hepatocytes (Figure 4A). Although angiotensinogen mRNA was reduced by 30% in F1KO hepatocytes compared with control, insulin further reduced angiotensinogen mRNA levels by 15% in F1KO hepatocytes \( (P<0.05); \text{Figure 4B}. \) Although the ability of insulin to suppress angiotensinogen mRNA expression was not completely disrupted in hepatocytes lacking Foxo1, Foxo1 deletion impaired insulin inhibition on the angiotensinogen mRNA expression. These results suggest that insulin inhibits angiotensinogen gene expression in both a Foxo1-dependent and Foxo1-independent manner.

Insulin Suppresses Angiotensinogen mRNA Expression Through Phosphatidylinositol 3-Kinase and Mitogen-Activated Protein Kinase Pathways

Insulin activates both phosphatidylinositol 3-kinases (PI3K) and mitogen-activated protein (MAP) kinases, regulating expression of many genes in control of metabolism and growth. We next determined whether activation of PI3K and mitogen-activated protein kinases (MAPK) is required for insulin to inhibit angiotensinogen mRNA expression. One hundred nanomole per liter insulin treatment for 18 hours in control hepatocytes reduced angiotensinogen mRNA and protein levels by \( \approx 40\% \), whereas treatment with a kinase inhibitor for PI3K (wortmannin) or ERK1/2 MAPK (PD98059) partially blunted insulin inhibition on the angiotensinogen mRNA level (Figure 5A and 5B). After 18 hours of insulin treatment in cells, Akt and Foxo1 phosphorylation, rather than ERK1/2 phosphorylation, was evident (Figure 5B), although treatment of insulin for 0.5 hours robustly stimulated Akt, Foxo1, and ERK1/2 phosphorylation (data not shown), which was shown in cardiomyocytes. \( ^{23} \)

To assess further whether insulin regulates Foxo1-stimulated angiotensinogen mRNA expression, we measured angiotensinogen mRNA expression in hepatocytes infected by adenovirus expressing control green fluorescent protein, Foxo1-wt, Foxo1-T/S-A, and Foxo1-DBD. Overexpression of Foxo1-wt and Foxo1-T/S-A significantly increased endogenous angiotensinogen mRNA by 2.0-fold and 2.5-fold, respectively, compared with expression of green fluorescent protein control (Figure 5C). However, expression of Foxo1-DBD slightly decreased the basal level of angiotensinogen mRNA \( (P<0.09); \) Thus, Foxo1 stimulates endogenous angiotensinogen gene expression at the gene transcriptional level.

Insulin treatment inhibited Foxo1-wt-stimulated angiotensinogen expression, by 40% \( (P<0.05), \) whereas insulin inhibition on the angiotensinogen mRNA level was slightly affected in cells with expression of constitutively active Foxo1-T/S/S-A or Foxo1-DBD (Figure 5C). The results further support the concept that Foxo1 stimulates angiotensinogen gene transcription while blocking Akt-mediated phosphorylation of Foxo1 or inhibition of PI3K partially impaired insulin inhibition on angiotensinogen gene transcription.

Identification of a Foxo1-Binding Site on the Angiotensinogen Promoter Region

We next tested whether Foxo1 directly regulates angiotensinogen gene expression through interactions with the promoter region. Promoter analysis of the mouse angiotensinogen gene suggests that 2 loci are potential Foxo1-binding sites or IREs-CAAAAACA (Figure 6A). To determine whether these promoter regions are responsive to Foxo1 stimulation or insulin suppression, we cloned 3 promoter regions into the pGL3-luciferase reporter gene construct. Promoter region-1 has 1.5 kb upstream of the transcriptional initiation site \( (~1.5 \text{ kb}), \) containing an IRE distinct from the Foxo1-binding site, mediating insulin suppression through ERK1/2 MAP kinase activation. \( ^{23} \)

Transfection of the reporter gene in HepG2 cells indicated that \(-1.5 \text{ kb of the promoter region-1} \) (Agtp-1.5 kb) was responsive to both Foxo1 stimulation and insulin suppression, we cloned 3 promoter regions into the pGL3-promoter regions are responsive to Foxo1 stimulation or insulin suppression. Hence, we made 2 constructs, each with a potential Foxo1-binding site, which can be responsive to both Foxo1 stimulation and insulin suppression, whereas Agtp-600 bp and Agtp-400 bp were responsible for both Foxo1 stimulation and insulin suppression, similar to the Agtp-800 bp (Figure 6E).

Overexpression of Foxo1 stimulated the Agtp-800 bp in a dose-dependent manner (Figure 7A). To confirm further the
functional Foxo1-binding site in Agt-p800bp, we mutated the Foxo1-binding site to a non-IRE sequence, as we previously reported.11 These mutations in the IRE in the Agtp-800 bp promoter completely abrogated Foxo1 stimulation on promoter activity (Figure 7A). The flanking sequence of the Foxo1-binding site in the promoter region-2 is shown in Figure 7B. We further performed chromatin immunoprecipitation to examine whether Foxo1 binds to the functional Foxo1-binding site in mouse liver. The chromatin immunoprecipitation results indicate that the immunoprecipitate using antibody against Foxo1, rather than IgG, contains the DNA fragment from region-2 (Figure 7C) and that the DNA fragment from promoter region-3 was not observed (data not shown). Together, these results indicate that Foxo1 interacts with a Foxo1-binding site in angiotensinogen promoter region-2 and mediates insulin inhibition on angiotensinogen gene expression, which is summarized in Figure 7D.

**Discussion**

In this study, we demonstrate 3 important and novel findings: (1) L-F1KO mice lacking liver Foxo1 gene exhibit a reduction in plasma angiotensinogen and Ang II and a 19 mm Hg reduction in the mean arterial pressure; (2) Foxo1 significantly stimulates angiotensinogen gene expression in hepatocytes; and (3) a functional Foxo1-binding site in the mouse angiotensinogen promoter region is identified, which is responsible for both Foxo1 stimulation and insulin inhibition. Angiotensinogen is the most upstream and essential component of the renin–angiotensin system (RAS) to generate bioactive peptide Ang II, a hormone that exerts potent influence on vascular constriction and cardiovascular remodeling.26 Chemical inhibitors for angiotensin-converting enzyme and Ang II receptor that target angiotensinogen downstream actions are effective agents in treating hypertension and cardiac failure.26 In addition, chemical suppression of hepatic angiotensinogen using small interference RNA is sufficient for achieving a sustained lower blood pressure in animal models.27 Our study demonstrated that hepatic Foxo1 regulates angiotensinogen gene expression and controls blood pressure, providing a novel regulatory mechanism for the RAS. Of note is that the reduction of blood pressure in F1KO mice may not solely be mediated by decreases in angiotensinogen expression, and other factors secondary to the loss of liver Foxo1, such as hypoglycemia, may indirectly influence blood pressure.

**Transcriptional Regulation of Angiotensinogen Gene Expression by Foxo1**

Multiple hormones and nutrients stimulate expression of angiotensinogen at the gene transcriptional level, whereas insulin is a potent inhibitor for angiotensinogen gene transcription.5,25 Foxo1 is a well-studied transcription factor that is suppressed by insulin/insulin-like growth factor-1 (IGF-1) signaling through activation of PI3K and Akt.11 Foxo1 stimulates angiotensinogen gene expression, an effect suppressed by insulin, providing a molecular link via insulin inhibition of angiotensinogen gene transcription. Conversely, glucagon, glucocorticoid, isoproterenol, Ang II, estrogen, thyroid hormone, cytokine interleukin-6, and high glucose all stimulate angiotensinogen gene expression in a variety of cells involving distinct receptors and intracellular protein kinase activation.28 Receptors of the glucocorticoid receptor, estrogen receptor-α, and transcription factors CCAAT-enhancer–binding proteins, GATA-binding protein 1, and nuclear factor kappa-B interact with the five prime untranslated region or −1.5kb promoter region of angiotensinogen, in which some single nucleotide polymorphisms have been implicated in hypertension.129 In
response to hormone stimulation, such as catecholamines and glucocorticoids, elevation of intracellular cAMP and cAMP-dependent protein kinase A activate cAMP-responsive element binding protein that recruits global transcriptional cofactor p300/CBP enhancing angiotensinogen gene expression.\(^{28}\) Foxo1 has been shown to interact with cAMP-responsive element binding protein and C/EBP-enhancing expression of genes in control of glucose homeostasis.\(^{30-32}\) Thus, we predict that Foxo1 may not only serve as a mediator in insulin/IGF-1 → PI3K → Akt signaling but also mediate effects of other signaling cascades, such as protein kinase A promoting angiotensinogen gene expression. Moreover, high glucose promotes glycosylation of Foxo1, thereby enhancing the target gene expression of G6Pase.\(^{33,34}\) Thus, Foxo1 may also mediate the effect of glucose on inducing angiotensinogen, a new target gene expression in cells. Alternatively, high glucose activates p38α MAPK\(^{35-37}\) and inactivates Akt, resulting in Foxo1 activation, by promoting degradation of insulin receptor substrate-1, 2 (IRS1, -2).\(^{23}\) Taken together, Foxo1 may promote angiotensinogen gene transcription through integrating different intracellular signaling cascades.

### Role of Foxo1 in Suppression of Angiotensinogen Gene Expression by Insulin

Foxo1 serves as a key component in the insulin/IGF-1 signaling pathway downstream from PI3K,\(^ {12}\) and our studies also indicate that insulin inhibits angiotensinogen gene expression, through both PI3K and MAPK pathways. Early reports demonstrated that activation of ERK1/2 MAPK, by insulin, mediates the effect of insulin inhibition on angiotensinogen gene transcription in kidney cells.\(^ {38}\) Moreover, rat angiotensinogen promoter region analysis suggested a distinct IRE, such as 5′CCTTTCCGCCCTTCA3′, was located within the rat −1.5 kb promoter region, mediating insulin suppression on angiotensinogen gene expression, through interacting with heterogeneous nuclear ribonucleoprotein K, downstream from ERK1/2 MAPK.\(^ {39,40}\) In this study, we showed that inhibitors of either ERK1/2 or PI3K partially blocked insulin inhibition on endogenous angiotensinogen expression in hepatocytes and established that the mouse angiotensinogen promoter region-2, contains a functional Foxo1-binding site (CAAAACAA) responsible for insulin suppression on angiotensinogen gene expression.

Angiotensinogen gene expression is suppressed by insulin, whereas insulin has a minimal effect on reducing blood pressure. Insulin not only promotes vascular relaxation by activating PI3K and Akt and stimulating endothelial nitric oxide synthase activity in vascular cells but also stimulates vasoconstrictor action by inducing secretion of endothelin-1, largely via ERK1/2 MAPK activation.\(^ {41,42}\) In a healthy lean individual,
physiological concentrations of insulin also increase venous catecholamine levels and sympathetic nervous activity for vasoconstriction,\textsuperscript{42,43} which may mask the effect of insulin on reducing blood pressure by decreasing hepatic Foxo1 and associated angiotensinogen and plasma Ang II. Thus, insulin has both vasodilator and vasoconstrictor actions, such that the net hemodynamic effect of insulin on blood pressure is minimal in healthy humans.\textsuperscript{43,44}

Of note is that IGF-1, an insulin-like growth factor produced by the liver, which shares IRS1 and IRS2, the intracellular signaling molecules of insulin, also activates Akt and inhibits Foxo1.\textsuperscript{23} An inverse relationship between IGF-1 and blood pressure has recently been reported in humans.\textsuperscript{45} Thus, a reduction of angiotensinogen by suppression of Foxo1 after an increase of IGF-1 and associated Akt activation in liver or other tissues may also provide a link to reduced blood pressure in humans.

**Role of Foxo1 in Promoting the RAS in Type 2 Diabetes Mellitus and Metabolic Syndrome**

Foxo1 nuclear localization and activation is widely present in tissues of animals with insulin resistance and type 2 diabetes mellitus,\textsuperscript{46} in which elevation of angiotensinogen and other RAS components is concurrent.\textsuperscript{47} Loss of IRS1 and IRS2, the 2 major mediators of insulin/IGF-1 signaling, after metabolic stress, chronic hyperinsulinemia, over nutrition, or inflammation provides a molecular basis of insulin resistance and type 2 diabetes mellitus.\textsuperscript{23,34,48} Loss of IRS1 and IRS2 results in inactivation of PI3K/Akt and Foxo1 activation,\textsuperscript{22,23,34} which may not only disrupt glucose homeostasis via excessive hepatic glucose production but also contribute to elevation of the circulating RAS components via angiotensinogen gene expression. Although tissues of animals with insulin resistance or deficiency often maintain ERK1/2 MAPK activity\textsuperscript{34} that reduces angiotensinogen gene expression making angiotensinogen gene expression complex in diabetes mellitus, Foxo1 activation may play a dominant role increasing angiotensinogen gene expression in liver and plasma angiotensinogen and Ang II, linking to high incidence of hypertension during insulin resistance and diabetes mellitus. Hypertension is closely associated with the metabolic syndrome and has become increasingly prevalent during the past several decades together with obesity and type 2 diabetes mellitus.\textsuperscript{34} It is well documented that hypertension occurs in several mouse models with insulin resistance, including high-fat diet and db/db mice.\textsuperscript{18,49}

**Perspectives**

On the basis of the results that hepatic Foxo1 ablation reduces angiotensinogen and Ang II in the blood circulation, we think that the Foxo1→angiotensinogen→Ang II axis likely provides a unifying mechanism in many other tissues, such as fat, brain, and kidney where Foxo1 is ubiquitously expressed. A variety of tissues have intracellular or local RAS components and Ang II receptors.\textsuperscript{47,50} We expect that Foxo1 activation, after insulin resistance, may contribute to an excess of local RAS synthesis by stimulating angiotensinogen gene expression. The Foxo1→angiotensinogen→Ang II pathway may contribute to the incidence and progression of hypertension, cardiomyopathy, neuropathy, and nephropathy in patients with type 2 diabetes mellitus.
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Disclosures

None.

References

What Is New?

- We have identified Foxo1, a known insulin-suppressed transcription factor through PI3K/Akt activation, involving control of liver angiotensinogen gene expression and plasma angiotensinogen and angiotensin II levels and modulating blood pressure in mice.

What Is Relevant?

- Hypertension is closely associated with insulin resistance, a major mechanism of metabolic syndrome; however, the molecular link between blood pressure control and insulin signaling is lacking.

Summary

This study provides a novel regulatory mechanism for the renin–angiotensin system and blood pressure control by the Foxo1→angiotensinogen→angiotensin II pathway.
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