RTEF-1 Attenuates Blood Glucose Levels by Regulating Insulin-Like Growth Factor Binding Protein-1 in the Endothelium

Angela F. Messmer-Blust, Melissa J. Philbrick, Shuzhen Guo, Jiaping Wu, Ping He, Shaodong Guo, Jian Li

Rationale: Related transcriptional enhancer factor-1 (RTEF-1) plays an important role in endothelial cell function by regulating angiogenesis; however, the mechanism underlying the role of RTEF-1 in the endothelium in vivo is not well defined.

Objective: We investigated the biological functions of RTEF-1 by disrupting the gene that encodes it in mice endothelium-specific RTEF-1-deficient transgenic mice (RTEF-1−/−).

Methods and Results: RTEF-1−/− mice showed significantly increased blood glucose levels and insulin resistance, accompanied by decreased levels of insulin-like growth factor binding protein-1 (IGFBP-1) mRNA in the endothelium and decreased serum IGFBP-1 levels. Additionally, the RTEF-1−/− phenotype was exacerbated when the mice were fed a high-fat diet, which correlated with decreased IGFBP-1 levels. In contrast, vascular endothelial cadherin/RTEF-1−/−overexpressing transgenic mice (VE-Cad/RTEF1) demonstrated improved glucose clearance and insulin sensitivity in response to a high-fat diet. Furthermore, we demonstrated that RTEF-1 upregulates IGFBP-1 through selective binding and promotion of transcription from the insulin response element site. Insulin prevented RTEF-1 expression and significantly inhibited IGFBP-1 transcription in endothelial cells in a dose-dependent fashion.

Conclusions: To the best of our knowledge, this is the first report demonstrating that RTEF-1 stimulates promoter activity through an insulin response element and also mediates the effects of insulin on gene expression. These results show that RTEF-1–stimulated IGFBP-1 expression may be central to the mechanism by which RTEF-1 attenuates blood glucose levels. These findings provide the basis for novel insights into the transcriptional regulation of IGFBP-1 and contribute to our understanding of the role of vascular endothelial cells in metabolism. (Circ Res. 2012;111:991-1001.)

Key Words: RTEF-1 ▪ IGFBP ▪ vascular endothelium ▪ metabolic syndrome

The endothelium is a complex organ with a multitude of properties essential for controlling vascular functions. Dysfunction of the vascular endothelium plays an important role in the pathogenesis of diabetic microangiopathy and macroangiopathy. Sustained hyperglycemia in metabolic syndrome causes alterations in a large number of transcription factors and mRNA transcripts, which ultimately leads to tissue damage. However, transcription factors involved in glucose homeostasis in response to insulin stimulation in the endothelium have not been fully studied. Previous studies from our laboratory have demonstrated that related transcriptional enhancer factor-1 (RTEF-1, also known as TEF-3 or TEAD4) plays an important role in endothelial cells via regulation of angiogenesis. More recently, studies have indicated that RTEF-1 drives communication between the endothelium and myocardium and enhances endothelium-dependent microvascular relaxation. RTEF-1 is a member of the transcriptional enhancer factor (TEF) family.

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Insulin-like growth factor binding proteins (IGFBPs) are key regulators of insulin-like growth factor (IGF) type 1 and 2 bioavailability at the cellular level and may exert IGF-independent effects on target cells. The IGFBP superfamily contains 6 high-affinity binding IGFBPs and 9 low-affinity binding IGFBP-related proteins. Growing evidence has shown an association of large- and small-vessel diseases with certain IGFBP-1 abnormalities, which suggests that they may be significant factors in the pathophysiology of cardiovascular disease. Furthermore, acute steady-state hyperinsulinemia decreases serum IGFBP-1 concentration to values that are 40% to 70% lower than baseline in normal individuals and in diabetic patients, which suggests that insulin is involved in the regulation of serum IGFBP-1 levels. Moreover, low levels of IGFBP-1 are associated with metabolic syndrome and cardiovascular diseases in cross-sectional studies and can predict ischemic heart disease mortality.

In this report, we examined RTEF-1 involvement in the endothelial-related effect of glucose regulation and its potential transcriptional target in vivo using diet-induced obese transgenic mice and in vitro using cultured endothelial cells to analyze the related mechanism. We demonstrate that RTEF-1 increases IGFBP-1 gene expression by interacting with its insulin response element (IRE). Evidence for a biological role of RTEF-1 in IGFBP-1 regulation includes decreased expression of IGFBP-1 in endothelium-specific RTEF-1 knockout mice (RTEF-1−/−) and in endothelial cells deficient in RTEF-1. Conversely, increased IGFBP-1 expression was observed in overexpressing RTEF-1 transgenic mice and in endothelial cells with forced RTEF-1 overexpression. Additionally, RTEF-1−/− mice exhibited increased blood glucose and insulin sensitivity, which was exacerbated in a high-fat diet (HFD), correlating with decreasing IGFBP-1 levels. Our findings suggest that RTEF-1 is integral in the endothelial regulation of IGFBP-1 and subsequent blood glucose homeostasis.

Methods

Generation of RTEF-1 Transgenic Mice

A conditional knockout line of RTEF-1 was generated by crossing homozygous TEAD4fl/fl ox mice (a gift from Dr Andres Buonanno, National Institute of Child Health and Human Development, National Institutes of Health) with transgenic mice expressing Cre recombinase under control of the endothelial cell–specific Tie2 promoter/enhancer (a gift from Dr Anthony Rosenzweig, Beth Israel Deaconess Medical Center). RTEF-1 transgenic mice were generated at the Beth Israel Deaconess Medical Center Transgenic Core Facility using the vascular endothelial cadherin (VE-Cad) promoter to drive endothelium-specific expression of human RTEF-1. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85–23, 1996) and was approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. For detailed protocols, please refer to the online-only Data Supplement.

Cell Culture and Transfection

Human microvascular endothelial cells (HMEC-1, Centers for Disease Control and Prevention) were cultured in MCDB-131, and human embryonic kidney cells 293 (HEK 293) were cultured in DMEM. Wild-type IGFBP-1 (IGFBP-1FL) or TK.IRS3 or VF-2 mutant promoter luciferase construct, control vector, PX40, and/or an increasing amount of RTEF-1 expression vector (generous gifts from Dr Alexandre Stewart, University of Ottawa, Canada) was transfected into HEK 293 cells, as shown in more detail in the online-only Data Supplement.

Retroviral Construction

The RTEF-1 (NM_014059.1) coding sequence was cloned into pBMN–green fluorescence protein (GFP) vector (Obigen, San Diego, CA) for retrovirus packaging, pBMN-GFP or pBMN-GFP–RTEF-1 was transfected to 293T with polyethylenimine with pSV-G, pPK3, and pCMV/α. The medium with retrovirus/RTEF-1 or retrovirus/GFP control was collected and filtered before being used to infect HMEC-1 cells.

siRNA Transfection

Small interfering RNAs (siRNAs) targeting human Ritef-1 were synthesized by GenePharma, Inc (Shanghai, China). Knockdown efficiency of 2 duplexes of RTEF-1 siRNAs or a nontarget control was determined by transfection into HMEC-1 cells at a final concentration of 50 nmol/L according to the manufacturer’s protocol. For details on this protocol, please refer to the online-only Data Supplement.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from the apex of mouse hearts from wild-type (WT) and transgenic mice, as well as the RTEF-1–overexpressing and GFP stably expressing HMEC-1 cell lines. A total of 2.0 μg of RNA from both endothelial cell lines stably transfected with RTEF-1 or isolated from the apex of WT, RTEF-1–overexpressing, and RTEF-1 endothelial cell–specific knockout transgenic mouse hearts was reverse-transcribed. Quantitative real-time polymerase chain reaction amplification was performed with SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol, as outlined in detail in the online-only Data Supplement.

ELISA and Blood Glucose Analysis

Blood glucose levels were obtained from feed-deprived (overnight), restrained, unanesthetized mice. Blood was obtained via submandibular bleed, and plasma IGFBP-1 concentration was measured by ELISA. Insulin resistance (IR) was assessed from fasting insulin and glucose levels by the previously validated homeostasis model assessment (HOMA-IR). Please refer to the online-only Data Supplement for details.

Metabolic Studies

Glucose tolerance tests and insulin tolerance tests were performed in 5-month-old conscious WT and transgenic mice. For glucose tolerance tests, glucose was injected intraperitoneally. Blood glucose was measured by tail bleeding at serial time points after glucose injection. For insulin tolerance tests, mice were injected with insulin and blood glucose was measured at the same time points as for glucose tolerance tests, as shown in detail in the online-only Data Supplement.
Promoter Activity and Chromatin Immunoprecipitation

HEK293 cells were transfected with constructs with or without RTEF-1, indicated in the Figure legends. Luciferase activity was determined using the dual luciferase assay system (Promega, Madison, WI). Chromatin immunoprecipitation was performed with the ChIP-IT Express Kit (Active Motif, Carlsbad, CA) in accordance with the manufacturer’s instructions, with primers shown in detail in the online-only Data Supplement.

Immunoblot Analysis

RTEF-1 and GFP stably transfected HMEC-1 cells were lysed and protein concentrations determined. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Whatman, Springfield Mill, United Kingdom), and subsequently blocked in TBS–Tween 20 containing 5% nonfat milk for 1 hour. The membranes were incubated with the indicated primary antibodies: Polyclonal anti-IGFBP-1 antibody; polyclonal anti-RTEF-1 antibody, monoclonal anti-vinculin followed by incubation with horseradish peroxidase–conjugated secondary antibodies anti-rabbit or anti-mouse IgG. Blots were developed with the chemiluminescence detection system according to the instructions of the manufacturer (Thermo Fisher Scientific, Rockford, IL). Densitometric analysis was performed with the National Institutes of Health software program Image J. The online-only Data Supplement provides more details.

Immunofluorescence

Hearts were removed from transgenic and littermate control mice, embedded in OCT compound, and frozen at −80°C. Tissues were sectioned, fixed in 4% paraformaldehyde, and stained with antibodies against RTEF, followed by incubation with goat anti-rabbit FITC and goat anti-mouse TRITC secondary antibodies (Santa Cruz, Santa Cruz, CA). Immunofluorescence stained sections were visualized with a DMS500B upright microscope, as shown in the online-only Data Supplement.

Blood Pressure Measurements

Blood pressure was measured noninvasively on conscious mice with a volume-pressure recording tail-cuff system (CODA system by Kent Scientific Corp, Torrington, CT). Additionally, blood pressure was confirmed by telemetry, as described in detail in the online-only Data Supplement.

Nuclear Magnetic Resonance Determination of Body Composition

Body composition was measured in both WT and RTEF-1−/− mice on both a normal chow diet (NCD) and a high fat diet (HFD) with quantitative nuclear magnetic resonance. Live, conscious, unrestrained mice were placed in small tubes and inserted into a Brucker model mq10 NMR analyzer (Brucker, Canada; Milton, Ontario, Canada). Total fat, lean, and water mass were recorded after 1 hour. The membranes were incubated with the indicated primary antibodies: Polyclonal anti-IGFBP-1 antibody; polyclonal anti-RTEF-1 antibody, monoclonal anti-vinculin followed by incubation with horseradish peroxidase–conjugated secondary antibodies anti-rabbit or anti-mouse IgG. Blots were developed with the chemiluminescence detection system according to the instructions of the manufacturer (Thermo Fisher Scientific, Rockford, IL). Densitometric analysis was performed with the National Institutes of Health software program Image J. The online-only Data Supplement provides more details.

Statistical Analysis

Data were obtained from at least 3 independent cell cultures or animals, as denoted in the Figure legends. Data are presented as mean±SEM. The trapezoidal rule was used to determine the area under the curve. HOMA-IR was calculated as (fasting glucose level×fasting insulin level)/22.4. The level of statistical significance was determined with Student 2-tailed t test when differences between the means of 2 populations were considered. Comparison of multiple time points between groups was made with a 1-way or 2-way repeated-measures ANOVA.

Results

IGFBP-1 Expression Is Attenuated in RTEF-1–Deficient Transgenic Mice

RTEF-1 is one of the earliest transcription factors expressed during mammalian development, and elimination of RTEF-1 expression in mice causes cardiac defects that result in embryonic lethality. To examine the importance of the RTEF-1 gene in endothelium, we generated a conditional endothelial cell–specific RTEF-1 knockout mouse (RTEF-1−/−). To conditionally delete the functional mouse RTEF-1 locus, 2 loxP sites were inserted into the introns flanking its second exon (Figure 1A). The targeting vector contained a pGK-neo cassette for positive selection and a pGK-tk cassette for negative selection against nonhomologous recombinants. Homozygous mice were bred as described previously and mated with Tie2-Cre mice that expressed Cre recombinase specifically in endothelial cells. RTEF-1−/− mice were genotyped by reverse transcription–polymerase chain reaction (Figure 1B). There were no gross morphological or developmental changes in the RTEF-1−/− transgenic mice and no evidence indicating that strong expression of Cre recombinase induced abnormalities in WT littermate mice. Additionally, to rule out the potential loss of RTEF-1 in hematopoietic cells using a Tie2-Cre promoter, bone marrow from WT and RTEF-1−/− mice were analyzed for RTEF-1 levels (Online Figure I). No significant differences were found.

Originally, DNA microarray data from RTEF-1–overexpressing endothelial cells illustrated that a handful of insulin-regulated genes had significant changes, including IGFBP family members (data not shown), which was verified by quantitative polymerase chain reaction (Figure 1C) immunoblot (Figure 1D). IGFBP-1 levels were decreased significantly in the apex of the RTEF-1−/− hearts compared with littermate controls. We further confirmed that the absence of RTEF-1 was localized in the endothelium by double immunostaining with antibodies to endothelial marker CD31 and RTEF-1 in hearts from RTEF-1−/− and littermate controls (Figure 1E).

Effect of RTEF-1 Ablation on Diet-Induced Obesity

We next examined the metabolic effect of RTEF-1 deletion on mice fed a NCD versus a HFD. Five-week-old male RTEF-1−/− and WT mice were fed either NCD or HFD (42% kcal from fat) for 15 weeks. The HFD is a well-established model for obesity-induced insulin resistance. Although the HFD increased growth rate and final body weight compared with mice fed the NCD, RTEF-1−/− did not significantly alter body weight with either diet (Table), nor did it change the percentage of skeletal muscle or adipose tissue in WT and RTEF-1−/− mice (Online Figure IIA and IIB). Obesity is typically associated with increased insulin sensitivity and elevated circulating concentrations of glucose and insulin. Initially, blood glucose levels were screened in RTEF-1−/− mice and WT mice, and as shown in Figure 2A, fasting blood glucose levels were modestly increased in RTEF-1−/− compared with WT mice on the NCD. Despite having a similar body weight and fat-pad mass, overnight fasting blood glucose (Figure 2A) and 6-hour fasting plasma insulin concentrations (Figure 2B) were increased in obese RTEF-1−/− mice compared with littermate controls fed a HFD, which suggests an impaired insulin sensitivity. Serum insulin levels were increased 2-fold (Figure 2B) in RTEF-1−/− versus littermate controls fed either...
Immunoblot analysis of hearts from both wild-type (WT) and RTEF-1 −/− (column with DAPI staining, showing that RTEF-1 is not present in the RTEF-1−/− endothelium (representative picture from n=4). L/W mice immunostained for DAPI and CD31, together with RTEF-1. Merged images of the left and middle columns are shown in the right.

Quantitative densitometry data (n=3; *≤0.05). However, RTEF-1 −/− mice fed a HFD were significant compared with WT mice maintained on the HFD (Figure 2C). Additionally, circulating insulin to regulate fasting blood glucose levels was impaired (Online Figure IIIA and IIIB).

**Effect of RTEF-1 Ablation on Cardiac Function**

To assess the effect of endothelium-specific removal of RTEF-1 and obesity on the heart, cardiac parameters of RTEF-1−/− mice were assessed (Table). No significant difference in heart rate or systolic blood pressure was detected between the groups. Additionally, telemetry was performed to confirm the blood pressure measurements taken in the RTEF-1−/− and control mice by a volume-pressure recording tail-cuff system (Online Figure IV). Although no difference in blood pressure was detected, echocardiography revealed a significant decline of heart function in both control and RTEF-1−/− mice fed a HFD (Table 1; 45.8±2.9% versus 37.5±6%).

**RTEF-1 Induced IGFBP-1 Expression in Endothelial Cells**

To confirm that IGFBP-1 might be a potential target gene of RTEF-1 and investigate the mechanism involved, RTEF-1 was overexpressed in HMEC-1 by retroviral infection. RTEF-1 and IGFBP mRNA were examined by quantitative real-time polymerase chain reaction (Figure 4A). IGFBP-1 mRNA expression and secreted IGFBP-1 (Figure 4B) were increased markedly in RTEF-1–overexpressing cells compared with control cells. To verify that RTEF-1 was
Table. Systolic BP and Cardiac Function of R TEF−/− Compared With Control Littermates

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R TEF−/−</th>
<th>WT P Value</th>
<th>R TEF−/− P Value, NCD vs HFD</th>
<th>R TEF−/− P Value, NCD vs HFD</th>
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</thead>
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<tr>
<td>Body weight, g</td>
<td>28.4±3.1</td>
<td>30±1.1</td>
<td>0.23</td>
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<td>0.006</td>
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<tr>
<td>NCD</td>
<td>37.2±3.8</td>
<td>38.45±2.6</td>
<td>0.43</td>
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<td></td>
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<tr>
<td>Body composition (% of body weight)</td>
<td></td>
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<tr>
<td>Lean mass</td>
<td>68.2±5.2</td>
<td>69.72±6</td>
<td>0.62</td>
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<td>0.002</td>
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<tr>
<td>NCD</td>
<td>55.7±5.4</td>
<td>49.81±5.8</td>
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<tr>
<td>Fat mass (% of body weight)</td>
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<tr>
<td>NCD</td>
<td>12.2±5.7</td>
<td>11.72±7.1</td>
<td>0.41</td>
<td>0.0007</td>
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<td>HFD</td>
<td>29.9±8.9</td>
<td>36.87±6.6</td>
<td>0.52</td>
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<td>Heart rate, bpm</td>
<td>674.9±55.7</td>
<td>704.8±38.2</td>
<td>0.08</td>
<td>0.12</td>
<td>0.0007</td>
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<tr>
<td>NCD</td>
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<td>736.7±25.8*</td>
<td>0.03</td>
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<tr>
<td>HFD</td>
<td>711.9±32.6</td>
<td>736.7±25.8*</td>
<td>0.03</td>
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<td>Systolic BP, mm Hg</td>
<td>99.7±3</td>
<td>109.1±7</td>
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<tr>
<td>NCD</td>
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<tr>
<td>HFD</td>
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<td>111±6</td>
<td>0.32</td>
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<tr>
<td>% FS</td>
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<tr>
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<td>37.5±6.2†</td>
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<td>LVDd, mm</td>
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<td>3.21±0.2†</td>
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<tr>
<td>NCD</td>
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<td>1.64±0.1†</td>
<td>0.0008</td>
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<tr>
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<td>1.64±0.1†</td>
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<td>0.6</td>
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<tr>
<td>HFD</td>
<td>1.0±0.05</td>
<td>0.83±0.09†</td>
<td>0.01</td>
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</table>

BP indicates blood pressure; RTEF, related transcriptional enhancer factor; WT, wild type; NCD, normal chow diet; HFD, high-fat diet; FS, fractional shortening; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; IVSd, interventricular septal thickness at diastole; LVPWd, left ventricular posterior wall dimension; IVSs, interventricular septal thickness at systole; LVPWS, left ventricular posterior wall dimension at systole.

*P≤0.05.
†P≤0.001.

necessary to increase IGFBP-1 levels, siRNAs were used to knockdown RTEF-1 expression. RTEF-1 levels were decreased significantly in human umbilical vein endothelial cells, which resulted in an almost 70% reduction in IGFBP-1 levels (Figure 4C). When HMEC-1 cells were treated with insulin, IGFBP-1 mRNA levels decreased (Figure 4D). Increasing insulin concentrations also dose-dependently decreased RTEF-1 mRNA levels (Figure 4E). Furthermore, when cells were treated with RTEF-1 siRNA, insulin was unable to significantly regulate IGFBP-1 levels compared with control (Figure 4F).

IGFBP-1 Is a Potential Target Gene of RTEF-1

To determine whether IGFBP-1 was regulated by RTEF-1 on a transcriptional level via the IGFBP-1 promoter, we performed luciferase reporter assays. The IGFBP-1 promoter exhibited an RTEF-1 dose-dependent increase in activity, exhibiting a maximum of a 3.7±0.4-fold increase (Figure 5B). Many of the important cis-regulatory elements are located within 500 bp of the transcription start site in the IGFBP-1 promoter (Figure 5A). The hepatocyte nuclear factor 1 (HNF1) binding region, cAMP response element (CRE), IRE, glucocorticoid response element (GRE), and TATA element are highly conserved among the human, rat,
IGFBP-1 has been reported to be regulated by other transcription factors, such as FOXO1, through its IRE. 27,28 The IGFBP-1 promoter contains an IRE that includes 2 insulin response sites (IRSs) located 100 bp 5′ to the RNA cap site, IRSA (CAAAACAA) and IRSB (TTATTTTG), and each is sufficient to mediate the negative effects of insulin on promoter activity. To determine whether RTEF-1 possibly regulates IGFBP-1 via its IRS, an array of IRSs (TK.IRS3) were introduced immediately upstream from the thymidine kinase promoter. 27 The TK.IRS3 was sufficient to confer effects of RTEF-1 on promoter activity (Figure 5C and 5D), as evidenced by the 4.9±0.03-fold increase in the TK.IRS3 luciferase reporter construct. This was confirmed by the inability of RTEF-1 to increase promoter activity in the IGFBP-1 late vascular endothelial growth factor through its Sp1 site. 2

Figure 2. RTEF-1−/− (related transcriptional enhancer factor-1–deficient) transgenic mice have lower insulin-like growth factor binding protein (IGFBP-1) levels and increased insulin resistance according to the homeostasis model assessment. A, Blood glucose levels were obtained from food-deprived (overnight), restrained, unanesthetized mice. Blood was obtained via submandibular bleed, and glucose was quantified with a CVS TrueTrack glucose monitor at 8 weeks of age on wild-type (WT) and RTEF-1−/− mice fed a normal chow (NCD) or high-fat diet (HFD). Blood glucose was significantly higher in the transgenic mice that lacked RTEF-1 in the endothelium in both the NCD and HFD (P<0.022; n=18 mice). B, Serum insulin concentration was measured by ELISA with a maximum absorbance of 450 nm. The limit of sensitivity is 0.121 ng/mL. C, Serum IGFBP-1 concentration was measured by ELISA. IGFBP-1 concentration was measured with a maximum absorbance of 450 nm. The limit of sensitivity is 0.121 ng/mL. n=3; *P<0.035. IGFBP-1 levels correlated inversely with blood glucose levels in these mice.

Figure 3. Obese RTEF-1−/− (related transcriptional enhancer factor-1–deficient) transgenic mice have decreased glucose tolerance and increased insulin resistance. A, Glucose tolerance in wild-type (WT) and RTEF-1−/− mice. Glucose was measured before and 30, 60, and 120 minutes after intraperitoneal glucose injection at 15 weeks of age. B, Insulin tolerance in WT and RTEF-1−/− was analyzed; glucose was measured before and 30, 60, and 120 minutes after intraperitoneal insulin injection. Data are presented as mean±SD (n=7–9 animals per group). *P<0.05 and **P<0.01 for RTEF-1−/− vs WT. C, Glucose tolerance in WT and RTEF-1−/− mice was measured before and 30, 60, and 120 minutes after intraperitoneal insulin injection. Data are presented as mean±SD (n=7–9 animals per group). *P<0.05 and **P<0.01 for RTEF-1−/− vs WT.
VF2 mutant, which lacked the IRE sequence (Figure 5B and 5E). Additionally, as shown in Figure 5D, the IRE mutation disrupted the ability of RTEF-1 to stimulate IGFBP-1 promoter activity in a dose-dependent manner, whereas the IRS sequence was sufficient to confer effects of RTEF-1 on IGFBP-1 promoter activity, as shown in Figure 5C.

To further investigate the IRE as the key element in RTEF-1 regulation of IGFBP-1 transcription, a chromatin immunoprecipitation assay was performed (Figure 5F). Primers were designed to flank the IRE sequence of IGFBP-1, and the chromatin immunoprecipitation assay demonstrated that RTEF-1 specifically bound to the IGFBP-1 IRE promoter region. Taken together, these results imply that RTEF-1, much like the forkhead transcription factors, exerts effects on IGFBP-1 promoter activity through its IRE.

Overexpression of RTEF-1 in the Endothelium

Improved Glucose Tolerance in Obese Mice

Some of the physiological consequences of diet-induced obesity are insulin resistance, glucose intolerance, and the eventual development of overt diabetes. We evaluated whether mice overexpressing RTEF-1 showed any changes in glucose tolerance after prolonged exposure to a HFD. Interestingly, Figure 6A demonstrates that blood glucose levels were increased in WT mice fed a HFD compared with the VE-Cad/RTEF-1 mice. In correlation with the previous data in the RTEF-1−/− mice, serum IGFBP-1 levels in both WT and VE-Cad/RTEF-1 mice were decreased in response to the HFD; however, VE-Cad/RTEF-1 mice had significantly higher levels than WT (Figure 6B).

To determine whether IGFBP-1 levels were affected in VE-Cad/RTEF-1 mice, we analyzed IGFBP-1 levels in these mice. Figure 6C demonstrates that VE-Cad/RTEF-1 mice had significantly higher IGFBP-1 serum levels than littermate controls, despite the decrease that resulted from the HFD. In addition, both the AUC and HOMA-IR were significantly increased in WT mice compared with VE-Cad/RTEF-1 transgenic mice, which indicates that the ability of circulating insulin to regulate fasting blood glucose levels was not significantly impaired in VE-Cad/RTEF-1 mice (Online Figure III). When intraperitoneal glucose tolerance test experiments were performed after 24 weeks of a HFD, VE-Cad/RTEF-1 mice displayed a markedly improved glucose tolerance and insulin sensitivity compared with the WT mice fed a similar HFD, which suggests protection from the HFD-induced insulin resistance.

Discussion

The present studies revealed an important role of RTEF-1 in the regulation of IGFBP-1 and potential glucose homeostasis. First, we demonstrated that IGFBP-1 is a target gene of RTEF-1 and that RTEF-1 can increase IGFBP-1 levels in the endothelium both in vitro and in vivo. Second, we identified an IRE element in the IGFBP-1 promoter to which RTEF-1 binds directly. Third, ablation of RTEF-1 from the endothelium significantly decreased circulating IGFBP-1 serum levels,
Figure 5. RTEF-1 (related transcriptional enhancer factor-1) upregulates transcription of insulin-like growth factor binding protein (IGFBP-1) through its insulin response element site. A, IGFBP-1 full-length (FL) promoter sequences designating binding elements. B, Schematic of IGFBP-1 promoter. IGFBP-1 FL promoter was transiently cotransfected with increasing concentrations of RTEF-1, and luciferase activity was examined. C, Transient transfections using a set of IGFBP-1 promoter luciferase constructs (300 ng) and control vector (600 ng; black bar) or equal amount of RTEF-1 cDNA (gray bar). D, The truncated IRS-3 repeat promoter was transiently cotransfected with increasing concentrations of RTEF-1, and luciferase activity was examined. E, The mutant VF-2 promoter was transiently cotransfected with increasing concentrations of RTEF-1, and luciferase activity was examined. F, Chromatin immunoprecipitation assays were performed by immunoprecipitating chromatin from human microvascular endothelial cells (HMEC-1) with control IgG or an anti-RTEF-1 antibody and performing reverse transcription–polymerase chain reaction with primers to the insulin response element site on the IGFBP-1 promoter or another control to the actin promoter. The results are from 3 independent experiments (mean±SD). *P≤0.05; **P≤0.01.
vascular endothelial growth factor 3 in endothelial cells, and insulin sensitivity. However, the RTEF-1−/− mice showed significantly impaired glucose tolerance, hyperinsulinemia, and impaired insulin sensitivity. In contrast, when these mice were fed a HFD, they were glucocompetent when assessed by an intraperitoneal glucose tolerance test, and showed only slightly impaired insulin sensitivity. Blood glucose was significantly lower in the transgenic mice that overexpressed RTEF-1 in the endothelium (*P<0.05; n=18 mice). Serum IGFBP-1 concentration was measured with an ELISA. IGFBP-1 concentration was measured with a maximum absorbance of 450 nm. The limit of sensitivity is ≥31.2 pg/mL (n=3; *P<0.05). IGFBP-1 levels inversely correlated with increased glucose intolerance and insulin resistance, and decreased fractional shortening. Finally, IGFBP-1 expression regulated by RTEF-1 overexpression resulted in improved glucose tolerance and insulin sensitivity.

As a member of the transcriptional enhancer factor family, RTEF-1 binds to the muscle-CAT regulatory element in promoters of muscular genes to direct gene expression. However, the abilities of RTEF-1 in nonmuscle cells have not been fully investigated. We have previously reported that RTEF-1 is involved in hypoxia-induced angiogenesis through its target genes, including hypoxia-inducible factor-1α and vascular endothelial growth factor in endothelial cells, and that RTEF-1 increases endothelium-specific coronary microvascular relaxation, which indicates an importance in further understanding the role of RTEF-1 in endothelium. Endothelium-specific RTEF-1-deficient transgenic mice provide an efficient model to dissect the function of RTEF-1 in endothelium. Interestingly, RTEF-1−/− transgenic mice fed a HFD were normoglycemic in the fasting state, appeared to be glucocompetent when assessed by an intraperitoneal glucose tolerance test, and showed only slightly impaired insulin sensitivity. In contrast, when these mice were fed a HFD, both the transgenic mice and littermate controls demonstrated impaired glucose tolerance, hyperinsulinemia, and impaired insulin sensitivity. However, the RTEF-1−/− mice showed significantly increased hyperglycemia, impaired glucose regulation, and increased HOMA-IR scores, the main features of insulin-resistant states. These findings revealed a novel ability of RTEF-1, as a transcription factor, to be involved in glucose regulation via IGFBP-1.

The present study indicates that IGFBP-1 is a potential target gene of RTEF-1 and plays a key role in RTEF-1 regulation of glucose homeostasis. Previous reports indicated that lower concentrations of IGFBP-1 were found in patients with diabetes and macrovascular disease. The present findings substantiate the conclusion that IGFBP-1 is negatively associated with insulin resistance. This indicates IGFBP-1 may simply be a marker of metabolic improvements and reduced insulinemia, yet more current research suggests that IGFBP-1 exerts direct metabolic effects.

In human longitudinal studies, IGFBP-1 concentrations were lower in obese children with metabolic syndrome than in obese children without. Additionally, mean IGFBP-1 levels were significantly lower in human subjects with abdominal obesity (P<0.001), elevated fasting glucose (P<0.001), hypertension (P<0.001), low high-density lipoprotein cholesterol (P<0.001), and metabolic syndrome (P<0.001) than in control subjects without these metabolic abnormalities. In rodent models, overexpression of IGFBP-1 resulted in dysregulation of the insulin/IGFBP axis and is a model of reduced sensitivity of IGFBP-1 to insulin regulation. However, more recent studies in mice that backcrossed human-IGFBP-1 to a C57BL/6 background demonstrated that IGFBP-1 had a protective effect on susceptibility to glucose intolerance and insulin resistance when the mice were challenged with nutritional obesity, which correlates with other recent data pooled from rodent studies on IGFBP-1 in glucose regulation.

To clarify more precise information regarding the role of RTEF-1 in the regulation of IGFBP-1 and its physiological
effect, we used an endothelial cell–specific conditional knock-out (Tie2-Cre/loxP) and an overexpressing (VE-Cad) transgenic model. Tie2 is expressed not only in the endothelium but also in early hematopoietic progenitors, which differentiate into hematopoietic, lymphoid, and endothelial cell lineages. Although no significant differences were found in RTEF-1−/− mice in hematopoietic cells (Online Figure I), it is possible that this could be caused by the low expression of RTEF-1 in the bone marrow, and deletion of RTEF-1 in hematopoietic and lymphoid cells may contribute to the phenotype observed in the present study.

Differential distributions of IGFBP-1 have been found in endothelial cells; IGFBP-1 expression in the endothelium is influenced by various conditions, growth factors, and molecules. Recent data demonstrate novel actions of IGFBP-1 with rescue of endothelial function in a diabetic model of insulin resistance via increased endothelial nitric oxide synthase production and preserved insulin sensitivity. IGFBP-1 levels were attenuated in human retinal endothelial cells of diabetic origin compared with nondiabetic origin. In the present study, we have demonstrated a novel pathway in which RTEF-1 regulates IGFBP-1 expression in endothelial cells. Endothelium-specific RTEF-1 transgenic mice provide an efficient model to dissect the function of RTEF-1 in endothelium. The present study has demonstrated that RTEF-1 regulates IGFBP-1 promoter activity through its IRS in endothelial cells. Insulin influences gene expression in multiple tissues and can suppress expression of a number of genes that contain a conserved IRS [CAAAA(C/T)AA], including IGFBP-1. One of the main branching pathways activated by insulin is the IRS pathway, termed the metabolic signal, which leads to activation of kinases dependent on phosphoinositide 3-kinase 3-kinase. On the basis of growing evidence that impaired signaling through phosphoinositide 3-kinase contributes to obesity-induced insulin resistance in peripheral tissues, the hypothesis that obesity similarly impairs IGFBP-1 signaling through phosphoinositide 3-kinase provides a plausible mechanism to explain this phenomenon. Regulation of IGFBP-1 and modulation of the IRS/phosphoinositide 3-kinase pathway are highly dynamic and remain to be further examined.

These findings represent a novel mechanism by which RTEF-1 regulates IGFBP-1 in the endothelium and suggest a protective role of RTEF-1 in the pathogenesis of diabetes and subsequent vasculopathy.

Acknowledgments
We thank Dr Andres Buonanno (National Institutes of Health, NCIH) for TEAD4 loxP mice; Dr Alexandre Stewart (University of Ottawa, Canada) for RTEF-1 constructs; Dr Anthony Rosenzweig (Beth Israel Deaconess Medical Center, Harvard) for the Tie2-Cre mice and Drs Glenn Rowe and Pablo Quintero for helpful scientific discussions.

Sources of Funding
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Disclosures
None.

References
RTEF-1 Regulates IGFBP-1 in Endothelial Cells

Novelty and Significance

What Is Known?

- Related transcriptional enhancer factor-1 (RTEF-1) plays an important role in cardiac and endothelial cell function.
- Insulin-like growth factor binding proteins (IGFBPs) are key regulators of insulin-like growth factor at the cellular level.
- Low levels of IGFBP-1 are associated with metabolic syndrome and cardiovascular diseases.

What New Information Does This Article Contribute?

- RTEF-1 increases IGFBP-1 gene expression by interacting with its insulin response element.
- RTEF-1 deficiency in endothelial cells exhibited increased blood glucose and insulin sensitivity in vivo.

- The increased blood glucose and insulin sensitivity shown in RTEF-1 deficiency in vivo was exacerbated in a high-fat diet, correlating with decreasing IGFBP-1 levels.

The transcriptional enhancer factor family regulates multiple genes expressed in both cardiac myocytes and endothelial cells. In the present study, we identified that RTEF-1 directly regulates IGFBP-1, and we determined a novel interaction site between RTEF-1 and the IGFBP-1 promoter. By regulating IGFBP-1, the signaling cascade of RTEF-1 impacts glucose levels and insulin resistance both in vitro and in vivo. These findings represent a novel mechanism by which RTEF-1 regulates IGFBP-1 in the endothelium and suggest a protective role of RTEF-1 in the pathogenesis of diabetes and subsequent vasculopathy.
RTEF-1 Attenuates Blood Glucose Levels by Regulating Insulin-Like Growth Factor Binding Protein-1 in the Endothelium

Angela F. Messmer-Blust, Melissa J. Philbrick, Shuzhen Guo, Jiaping Wu, Ping He, Shaodong Guo and Jian Li

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Materials and Methods

Generation of RTEF-1 transgenic mice

A conditional knockout (KO) line of RTEF-1 was generated by crossing homozygous TEAD4lox/lox mice (a gift from Dr. Andres Buonanno, NICHHD, NIH) with transgenic mice expressing Cre recombinase under control of the endothelial cell-specific Tie2 promoter/enhancer (a gift from Dr. Anthony Rosenzweig, BIDMC)\(^1\). Mice were screened by PCR to verify germline transmission, using the following primers as described previously\(^2\):

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>P1</td>
<td>CTAGCATTAAGGAATGTCCCGA</td>
</tr>
<tr>
<td>P2</td>
<td>CTCAACATACAGTTTGAAGCAC</td>
</tr>
<tr>
<td>P3</td>
<td>CGTATAGCATACTTATACGAAG</td>
</tr>
<tr>
<td>P4</td>
<td>GTGTTCTTAGAGGTACGTCA</td>
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</table>

RTEF-1 transgenic mice were generated at the BIDMC Transgenic Core Facility using the vascular endothelial (VE)-cadherin promoter to drive endothelial-specific expression of human RTEF-1\(^3\). RTEF-1 transgenic mice were genotyped by PCR as described previously\(^4\), and floxed littermates were used as control mice in all of the mouse studies. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, 1996) and was approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

Cell culture and transfection

Human microvascular endothelial cells (HMEC-1, CDC) were cultured in MCDB-131, and human embryonic kidney cells 293 (HEK 293) were cultured in DMEM, both supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin, at 37 °C in 95% air and 5% CO2 atmosphere. HEK 293 cells were transfected with DNA (1 µg/10⁵ cells) using polyethylenimine (PEI) according to previously optimized transfections at a ratio of 1 (DNA) to 3 (PEI). Wild-type IGFBP-1 (IGFBP-1FL) or TK.IRS3 or VF-2 mutant promoter luciferase construct, control vector, PXJ40 and/or an
increasing amount of RTEF-1 expression vector (generous gifts from Dr. Alexandre Stewart, University of Ottawa, Canada) were transfected into HEK 293 cells. PXJ40/lacZ (0.01 μg) was co-transfected to determine transfection efficiency of approximately 70–80%. After transfection, cells were incubated for 48 h before analysis.

**siRNA transfection**

siRNAs targeting human RTEF-1 were synthesized by Genepharma, Inc. (Shanghai, China). Knockdown efficiency of the two duplexes of RTEF-1 siRNAs (siRNA-1: 5’-GGG CAG ACC UCA ACA CCA ATT-3’, 5’-UUG GUG UUG AGG UCU GCC CAG-3’ and siRNA-2: 5’-ACC CAA GAU GCU GUG UAU UTT-3’, 5’-AAU ACA CAG CAU CUU GGG UTT-3’) or a nontarget control (5’-UUC UCC GAA CGU GUC ACG UTT-3’, 5’-ACG UGA CAC GUU CGG AGA ATT-3’) was determined by transfection into HMEC-1 cells at a final concentration of 50 nM according to the manufacturer’s protocol. Briefly, a master mix of Lipofectamine 2000 (Invitrogen, CA) was diluted with 1ml of OPTI-MEM (Invitrogen, CA) and incubated for 5 min. The Lipofectamine 2000 dilution was added to the DNA/siRNA dilution, incubated for 20 min, and added dropwise to the cells. Five hours after transfection, media was changed and the cells were allowed to recover overnight.

**Quantitative real-time PCR analysis**

Total RNA was extracted from the apex of mouse hearts from wild type and transgenic mice, as well as the RTEF-1 o/e and GFP stably expressing HMEC-1 cell lines using Trizol (Invitrogen, CA) according to the manufacturer’s instructions. A total of 2.0μg of RNA from both endothelial cell lines stably transfected with RTEF-1 or isolated from the apex of wildtype (WT), RTEF-1 o/e and RTEF-1 EC-specific KO transgenic mouse hearts were reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA) with random primers according to the manufacturer’s
protocol. Quantitative real-time PCR (QPCR) amplification was done using SYBR Green Master Mix (Applied Biosystems, CA) according to the manufacturer’s protocol with the following primers:

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTEF-1</td>
<td>5'-CCACGAAGGTCTGCTCTTTC-3'</td>
<td>5'-AAGTTTCTCCAGCAGCTGT-3'</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>5'-AAATAGGAAGGAGCCCTGCC-3'</td>
<td>5'-GGGTAGACACACCAGCAGT-3'</td>
</tr>
<tr>
<td>IGF-1</td>
<td>5'-TGGTGGATGCTCTTCAGTTC-3'</td>
<td>5'-GACAGAGCGAGCTGACTTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGGTGAACGAGGATTGAG-3'</td>
<td>5'-CTCCTGCGACTTCAACAGCA-3'</td>
</tr>
</tbody>
</table>

Real time quantitative PCR was performed in an under SDS 7000 System (Applied Biosystems, CA). For all individual cDNAs, amplification of each specific mRNA sequence was performed in at least 3 independently performed PCR experiments. For each reaction, expression was calculated as $2^{-\Delta C_t}$, where $\Delta C_t$ is the difference between the $C_t$ for the gene of interest and the $C_t$ for the housekeeping gene, GAPDH.

**ELISA and Blood Glucose analysis**

Mice ages 8-10 weeks were maintained on a standard 12:12-h light-dark cycle and received a standard diet. Blood glucose levels were obtained from feed-deprived (overnight), restrained un-anesthetized mice. Blood was obtained via submandibular bleed, and glucose was quantified using a CVS TRUEtrack glucose monitor (Home Diagnostics, FL). Plasma IGFBP-1 concentration was measured by ELISA following the instructions of the manufacturer (Boster Biotechnology, Wuhan, China). IGFBP-1 concentration was measured with a maximum absorbance of 450 nm. The limit of sensitivity is at or above 31.2 pg/ml. After submandibular bleeds, feed was returned to the cages and mice were allowed to recover. Insulin resistance (IR) was assessed from fasting insulin and glucose levels by the previously validated homeostasis model assessment (HOMA-IR)$^5$.

**Metabolic Studies**

GTT and ITT assays were performed in 5-month-old conscious WT and transgenic mice ($n = 6-8$ for each genotype and diet). For GTT, mice were fasted overnight and given free access to water. Glucose (1 g/kg
body weight) was injected intraperitoneally. Blood glucose was measured by tail bleeding using CVS TRUEtrack glucometer (Home Diagnostics, FL) at 0, 30, 60, and 120 minutes after glucose injection. For ITT, mice were injected with insulin (0.75 U/kg body weight; Eli Lilly and Co. MI) intraperitoneally, and blood glucose was measured at the same timepoints for GTT.

Promoter Activity and Chromatin Immunoprecipitation (ChIP)

HEK293 cells were transfected with following constructs:

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL IGFBP-1.Luc</td>
<td>-116 GCTCACAAGCAAAACAAACTTATTTTGAACACGGGG -81</td>
</tr>
<tr>
<td>Mutant IGFBP-1.Luc</td>
<td>-116 GCTAGCAAGCACCGACGACCAGGCCTGAACACGGGG -81</td>
</tr>
<tr>
<td>TK81.IRS3</td>
<td>IRSA-IRSA-IRSA-Thym-Kin -81</td>
</tr>
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</table>

with or without RTEF-1, indicated in the figure legends, using PEI (Polysciences), and incubated for 24 hours prior to measurement of luciferase activity. Luciferase activity was determined using the dual luciferase assay system (Promega). Chromatin immunoprecipitation (ChIP) was performed with the ChIP-IT Express Kit (Active Motif, Carlsbad, CA) in accordance with the manufacturer’s instructions. The IGFBP-1 and actin promoters were amplified with the primer pairs: 5’-CCCTAAACAACGGGACAAACA-3’ and 5’-TTG CAC CAG GAG GTT AAT GA-3’, and 5’-TGCACTGTGCGCGGAACG-3’ and 5’-TCGAGCCATAAAAAGGCAA-3’, respectively. The IGFBP-1 primers were designed to the insulin response sequence.

Immunoblot Analysis

RTEF-1 and GFP-stably transfected HMEC-1 cells were lysed in cold RIPA buffer (Boston Bio-Products, MA). Conditioned medium was removed and concentrated using centrifugal ultracel-10k filters (Millipore, MA). Protein concentrations were determined with the DC Protein Standard Assay (Bio-Rad, Munich, Germany). Samples were subjected to SDS–PAGE, transferred to nitrocellulose membranes (Whatman, Springfield Mill, UK) and subsequently blocked in TBS–Tween 20 containing 5% non-fat milk for 1h. The membranes were incubated with the indicated primary antibodies: polyclonal anti-
IGFBP-1 antibody with 1:500 dilution, (Millipore, MA); polyclonal anti-RTEF-1 antibody with 1:10000 dilution, (Genemed Synthesis, CA), monoclonal anti-vinculin with 1:65000 dilution followed by incubation with horseradish peroxidase-conjugated secondary antibodies anti-rabbit IgG with 1:3000 dilution, (Calbiochem, CA) or anti-mouse IgG with 1:2000 dilution (Vector Labs, CA). Blots were developed using the chemiluminescence detection system according to the instructions of the manufacturer (Thermo Fisher, PA). Densitometric analysis was done using the NIH software program, Image J.

**Immunofluorescence**

Hearts were removed from transgenic and littermate control mice, embedded in O.C.T. compound (Sakura Finetek USA Inc.) and frozen at -80°C. Tissues were sectioned with a Cryostat CM Model 3050S-3-1-1 (Leica, Wetzlar, Germany), fixed in 4% paraformaldehyde, and stained with antibodies against RTEF-1 (Genemed, CA) with 1:750 dilution and CD31 (Sigma) with a 1:500 dilution followed by incubation with goat anti-rabbit FITC and goat anti-mouse TRITC (Santa Cruz Inc, CA) secondary antibodies, respectively, at a dilution of 1:500. Immunofluorescence stained sections were visualized with a DM5000B upright microscope (Leica, Germany).

**Blood Pressure Measurements**

Blood pressure was measured non-invasively on conscious mice using a volume pressure recording tail-cuff system (CODA™ system by Kent Scientific Corporation). Two measurements were taken per mouse and were averaged after 20 cycles subsequent to 8 acclimation cycles; all false readings were excluded. The CODA system analyzes six measurements: systolic, diastolic pressure, mean pressure, rate, blood flow and blood volume6-8 (BIDMC, Metabolic Core Facility). Additionally, blood pressure was confirmed using telemetry. Briefly, the catheter was secured in place with a 6-0 silk suture and arterial blood pressure was measured by inserting a 1.4 Fr high-fidelity pressure catheter (SPR-671, Millar catheters) in a carotid artery. The catheter was calibrated before each experiment. Heart rate and aortic pressure were
recorded at 2 kHz and analyzed using a built-in analytic program in PowerLab software Chart 5 (AD Instruments, CO; BIDMC, Cardiophysiology Core Facility).

**Statistical analysis**

Data was obtained from at least three independent cell cultures or animals, as denoted in the figure legends. Data are presented as means ± SEM. The trapezoidal rule was used to determine the area under the curve (AUC). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as (fasting glucose level × fasting insulin level)/22.4. The level of statistical significance was determined using Student's two-tailed t-test when differences between the means of two populations were considered. Comparison of multiple time points between groups was made using a 1-way or 2-way repeated measures ANOVA.

**References:**

Bone marrow was extracted from both WT and RTEF-/- mice to determine if RTEF-1 levels were decreased in hematopoietic cells.

**Online Figure I.**

Bone marrow was extracted from both WT and RTEF-/- mice to determine if RTEF-1 levels were decreased in hematopoietic cells.
Online Figure II.

A. Body composition was determined by NMR in WT vs. RTEF-1/- mice on both NCD and HFD diets.

B. White and brown mouse adipose tissues were harvested and measured as a percentage of total body weight.
**Online Figure III A & B.**

A. Area under the curve (AUC) for GTT and ITTs was calculated for NCD WT and RTEF-1-/- mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and RTEF-1-/- mice on a NCD.

B. Area under the curve (AUC) for GTT and ITTs was calculated for NCD WT and RTEF-1-/- mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and RTEF-1-/- mice on a HFD. **C.** Area under the curve (AUC) for GTT and ITTs was calculated for WT and VE-CAD/RTEF-1 mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and VE-CAD/RTEF-1 mice on a HFD.
Online Figure III C.

Area under the curve (AUC) for GTT and ITTs was calculated for WT and VE-CAD/RTEF-1 mice. HOMA-IR calculated from fasting glucose and insulin levels of WT and VE-CAD/RTEF-1 mice on a HFD.
Online Figure IV.

Mice were anesthetized and blood pressure and LV function measured in both control and RTEF-1-/- mice using a Millar pressure system. The parameters included 1) Systolic blood pressure (SBP), 2) Diasolic blood pressure (DBP), 3) LV systolic pressure (LVSP), 4) LV ending diasolic pressure (LV EDP), 5) maximum dP/dt (dP/dt_{MAX}) and 6) minimum dP/dt_{MIN} are shown. Data were analyzed using SPSS analysis software.* p< 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RTEF-1 -/-</th>
</tr>
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<tbody>
<tr>
<td>SBP</td>
<td>104.05 ± 1.7</td>
<td>99.3 ± 2.6</td>
</tr>
<tr>
<td>DBP</td>
<td>69.4 ± 0.2</td>
<td>67.6 ± 2.1</td>
</tr>
<tr>
<td>LVSP</td>
<td>105.45 ± 6.5</td>
<td>99.5 ± 6.6</td>
</tr>
<tr>
<td>LV EDP</td>
<td>6.3 ± 0.28</td>
<td>7.8 ± 0.49 *</td>
</tr>
<tr>
<td>dP/dt_{MAX}</td>
<td>6993 ± 1033</td>
<td>7291 ± 562</td>
</tr>
<tr>
<td>dP/dt_{MIN}</td>
<td>-6331.5 ± 47.37</td>
<td>-7022 ± 185</td>
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</table>