The Role of Heparin Cofactor II in the Regulation of Insulin Sensitivity and Maintenance of Glucose Homeostasis in Humans and Mice

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Aim: Accelerated thrombin action is associated with insulin resistance. It is known that upon activation by binding to dermatan sulfate proteoglycans, heparin cofactor II (HCII) inactivates thrombin in tissues. Because HCII may be involved in glucose metabolism, we investigated the relationship between plasma HCII activity and insulin resistance.

Methods and Results: In a clinical study, statistical analysis was performed to examine the relationships between plasma HCII activity, glycosylated hemoglobin (HbA1c), fasting plasma glucose (FPG), and homeostasis model assessment-insulin resistance (HOMA-IR) in elderly Japanese individuals with lifestyle-related diseases. Multiple regression analysis showed significant inverse relationships between plasma HCII activity and HbA1c (p = 0.014), FPG (p = 0.007), and HOMA-IR (p = 0.041) in elderly Japanese subjects. In an animal study, HCII+/− mice and HCII−/− mice were fed with a normal diet or high-fat diet (HFD) until 25 weeks of age. HFD-fed HCII−/− mice exhibited larger adipocyte size, higher FPG level, hyperinsulinemia, compared to HFD-fed HCII+/− mice. In addition, HFD-fed HCII−/− mice exhibited augmented expression of monocyte chemoattractant protein-1 and tumor necrosis factor, and impaired phosphorylation of the serine/threonine kinase Akt and AMP-activated protein kinase in adipose tissue compared to HFD-fed HCII+/− mice. The expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase was also enhanced in the hepatic tissues of HFD-fed HCII−/− mice.

Conclusions: The present studies provide evidence to support the idea that HCII plays an important role in the maintenance of glucose homeostasis by regulating insulin sensitivity in both humans and mice. Stimulators of HCII production may serve as novel therapeutic tools for the treatment of type 2 diabetes.

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Key words: Glucose homeostasis, Heparin cofactor II, High-fat diet, Humans, Mice

Introduction

Previous clinical studies have provided considerable evidence for a relationship between obesity and thrombosis, citing the enhanced expression of prothrombotic molecules, including plasminogen activa-
tor inhibitor-1 and tissue factor, and increased platelet activation\textsuperscript{1). Additionally, it has been shown that the acceleration of thrombin action is associated with the development of insulin resistance, leading to metabolic syndrome and type 2 diabetes mellitus (T2DM)\textsuperscript{2). Thrombin activates platelets, vascular endothelial cells, vascular smooth muscle cells, macrophages, and fibroblasts via binding to their protease-activated receptors (PARs). PAR-1 activation by thrombin enhances procoagulation\textsuperscript{3}, chemotaxis\textsuperscript{4}, mitogenesis\textsuperscript{5}, and proliferation\textsuperscript{6} of these cells. Gene expression of PAR-2 has been observed in adipose tissues and macrophages of humans and rats, and PAR-2 has been shown to promote inflammation and metabolic dysfunction\textsuperscript{7). Taken together, the results indicated the possibility that both PAR-1 and PAR-2 have a significant role in the development of insulin resistance. We and another group of researchers demonstrated that thrombin promotes the release of inflammatory cytokines and growth factors from adipocytes and inhibits insulin-stimulated Akt phosphorylation\textsuperscript{8, 9}. We also showed that argatroban, a synthetic thrombin antagonist, can ameliorate insulin resistance in type 2 diabetic db/db mice\textsuperscript{8). Heparin cofactor II (HCII), like antithrombin, is a serine protease inhibitor (serpin) with a molecular weight of 65.6 kDa. HCII is synthesized by hepatocytes and secreted into the blood stream at a concentration of about 1.0 µmol/L\textsuperscript{10}. HCII, upon activation by binding to dermatan sulfate proteoglycans, specifically inhibits thrombin action in various tissue matrices. Thus, HCII can inhibit thrombin action in various tissues without affecting hemostasis. Although we previously reported that HCII protects against cardiovascular remodeling in mice and humans\textsuperscript{11-17}, the role of HCII in the regulation of glucose metabolism and insulin sensitivity has not yet been determined. Therefore, the present study aimed to clarify whether HCII is involved in glucose metabolism and insulin sensitivity in humans and mice.

Materials and Methods

Subjects for Cross-Sectional Study

We determined the relationship between plasma HCII activity and HbA1c levels using data sets from our previous clinical studies\textsuperscript{11, 13). Additionally, a total of 130 individuals seeking consultation regarding lifestyle-related disorders and who had never been treated with insulin were recruited from Tokushima University Hospital, Tokushima, Japan between January 2012 and May 2013. All subjects underwent a standardized interview and physical examination.

Diagnostic Criteria for Clinical Studies

Current smokers were defined as subjects who had smoked within 1 year prior to the study. Body mass index was calculated as an index of obesity, and blood pressure was measured twice and averaged. Hypertensive patients were defined as those with systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg, and individuals on antihypertensive medications. Hyperlipidemic patients were defined as those with low-density lipoprotein cholesterol (LDL-C) $\geq$ 140 mg/dl and/or triglyceride (TG) levels $\geq$ 150 mg/dl, and individuals on lipid-lowering medications. Diabetic patients were defined as individuals who were receiving oral hypoglycemic agents or individuals with glycosylated hemoglobin A1c (HbA1c) $\geq$ 6.5% or fasting plasma glucose (FPG) $\geq$ 126 mg/dl or 2 h plasma glucose $\geq$ 200 mg/dl during a 75 g oral glucose tolerance test. Exclusion criteria included overt cardiac failure, known malignancy, renal failure (serum creatinine $\geq$ 2.0 mg/dl), and malnutrition (serum albumin < 3.0 g/dl).

Biochemical Analysis

Before noon, overnight fasting blood samples were collected from the antecubital vein and assayed immediately for FPG, HbA1c, serum immunoreactive insulin (IRI), and serum lipid parameters including TG, HDL-C, and LDL-C. FPG and levels of TG, HDL-C, LDL-C, and creatinine were measured by enzymatic methods. HbA1c was assayed by latex agglutination. Serum IRI level was determined by the chemiluminescent enzyme immunoassay. An index of insulin resistance in the homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as FPG (mg/dl) $\times$ IRI (µU/ml)/405. For the measurements of plasma HCII activities, blood was drawn as described above, collected into a tube containing 1/10 volume of 3.8% sodium citrate, and centrifuged at 2,000 $\times$ g for 20 min. Plasma was stored at −80°C until use. Plasma HCII activity was measured on the basis of antithrombin activity in the presence of dermatan sulfate using the Stachrom® HCII assay kit (Diagnostica Stago, France). The intra-assay and inter-assay coefficients of variation of this kit were 3.9% and 4.3%, respectively. Our study followed the institutional guidelines of Tokushima University and was approved by the Institutional Review Board of Tokushima University Hospital. The ethics committee approved...
this study and the participants were required to sign an informed consent form prior to inclusion in the study, in accordance with the Declaration of Helsinki.

**Animal Preparations**

We used $HCII^{-/-}$ and $HCII^{+/+}$ 25-week-old male mice ($HCII^{-/-}$; Tokushima University, Graduate School of Biomedical Sciences, Tokushima, Japan) that we previously generated. In brief, we generated $HCII$-deficient mice by targeted disruption of the $HCII$ gene, mice were backcrossed for 10 generations with the C57BL/6J strain. Because the homozygous $HCII$-deficient mice were embryonic lethal, we used male heterozygote $HCII$-deficient ($HCII^{+/+}$) mice and male littermate WT ($HCII^{+/-}$) mice in all experiments of this study, as in our previous studies. These animals were housed in a specific pathogen-free facility under climate-controlled conditions with a 12-h light/dark cycle and were provided with either a normal diet (ND) or a high-fat diet (HFD: lard 58%, fish flour 30%, defatted soybean 10%, vitamins and minerals 2%) (Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum.

**Biochemical Analysis**

Plasma glucose levels were enzymatically measured (Wako Chemicals, Tokyo, Japan). Serum insulin levels, adiponectin levels, and leptin levels were determined using an insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Japan), an adiponectin ELISA kit (Ohtsuka Pharmaceutical, Co., Ltd., Japan) and a leptin ELISA kit (Morinaga Institute of Biological Science, Inc., Japan). HOMA-IR was calculated as fasting whole blood glucose (mg/dl) × fasting serum insulin (ng/ml)/405.

**Computed Tomography Scan**

The adiposity of the mice was determined using computed tomography (CT) scanning at 25 weeks of age. Mice were anesthetized with isoflurane gas, and images were acquired on a Latheta LCT-200 (Hitachi Aloka Medical, Ltd., Japan) at Tokushima Bioimaging Station, The University of Tokushima. Animals were scanned in a 48-mm-wide specimen holder with a resolution of 96 µm pixels. For all scans, the same number of views (796) was used, which represents the amount of data collected during a single 360° rotation around the object. In pilot experiments, optimal scanning conditions were evaluated for each tissue. Obtained CT images were analyzed with provided software. Density histograms of the volume within the selected range were generated, and the total volume of skeletal muscle and adipose tissue were calculated by the summation of the total density.

**Adipocyte Sizing**

Epididymal fat samples were fixed in osmium tetroxide (Sigma), suspended in isotonic saline, and passed through a 250-µm nylon filter to remove fibrous elements. Cells were filtered over a 25-µm nylon filter, collected in isotonic saline, and analyzed on a Coulter counter (Multisizer III, Coulter Electronics, Fullerton, CA), as previously described. The distribution of adipocyte size was analyzed for cells ranging 25–250 µm in diameter.

**Immunohistochemistry**

Mice were sacrificed by intraperitoneal injection of high-dose pentobarbital. White adipose tissues of epididymal fat were fixed overnight in 10% neutral buffered formalin, cut into 10-µm-thick cross sections, and stained with hematoxylin-eosin and F4/80 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antibody distribution was visualized with the avidin–biotin complex technique and Vector Red substrate (Vector Laboratories, Burlingame, CA). The summation of F4/80-positive cell numbers from five independent visual fields in each animal tissue was used to determine macrophage infiltration.

**Quantitative Real-Time PCR and PCR Array Analysis**

For real-time PCR analysis, epididymal fat tissues and hepatic tissues were excised. RNA extraction and reverse transcriptase-polymerase chain reactions (RT-PCR) were performed as previously described. In brief, epididymal fat tissues, hepatic tissues, and thigh skeletal muscle were homogenized in TRIzol (Invitrogen, Carlsbad, CA), and total RNA was extracted. One microgram of total RNA was used for cDNA synthesis with a QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA), according to the manufacturer’s instructions. The PCR mixture contained cDNA, synthesized from 2.5 ng of total RNA, 0.1 nmol/L forward and reverse primer mix, and SYBR Green (Platinum SYBR Green qPCR SuperMix-UDG, Invitrogen Carlsbad, CA). Assays were performed with a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification consisted of one stage of 2 min at 50°C and one stage of 2 min at 95°C followed by 40 cycles of a 2-step cycle: 15 s at 95°C and 30 s at 60°C. Quantitative RT-PCR analysis of 84 obesity-related genes was performed using Obesity RT² Profiler PCR Arrays PAMM-017Z (QIAGEN). Data analysis of the PCR array kit was performed using the manufacturer’s integrated web-based software for the PCR Array System (http://pcrdatalanalysis.sabiosciences.com/pcr/arrayanalysis.php) using ΔΔCt-based fold-change calculations. In addition, commercially avail-
able PCR primers were purchased from Perfect real-time primer (TAKARA BIO INC., Ohtsu, Japan) for F2r, Mep1, Tnf, Cntf, Cntfr, Pck1, G6pc, and Gapdh. The transcript levels of those genes were adjusted relative to Gapdh expression as an internal control.

**Western Blot Analysis**

For western blot analysis, murine epididymal fat tissues, hepatic tissues, and thigh skeletal muscle were excised. The phosphorylation of AMPKα and Akt with or without insulin stimulation (1 U/kg) was evaluated by western blot analysis. Protein extraction and western blot analysis were performed as previously described\(^{17, 19}\). In brief, 100-µg protein extracts from adipose tissue, hepatic tissues, and thigh skeletal muscle from HCCI\(^{−/−}\) and HCCI\(^{+/+}\) mice fed an ND or HFD were boiled for 5 min in Laemmli sample buffer and run on SDS-PAGE. The protein extracts were then transferred to a PVDF membrane (Millipore Corporation, Bedford, MA). The membrane was blocked for 20 min at room temperature with SuperBlock T20 TBS Blocking Buffer (Thermo Scientific, Rockford, IL). The blots were incubated overnight at 4°C with each primary antibody, followed by incubation for 1 h with anti-rabbit secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence with ECL-PLUS reagents (GE Healthcare, Buckinghamshire, UK) and exposure to a lumino image analyzer (LAS-3000 mini) (Fujifilm Corporation, Tokyo, Japan). The signals were quantified by densitometry using ImageJ version 1.47. About 8–12 independent samples were analyzed in each group, and the phosphospecific proteins were corrected by GAPDH as an internal control. We used primary antibodies against phosphorylated Akt (Ser473), phosphorylated AMPK (Thr172), and GAPDH (Cell Signaling Technology, Beverly, MA). All experimental procedures were performed in accordance with the guidelines from and approval of the Animal Research Committee of The University of Tokushima Graduate School.

**Statistical Analysis**

In the clinical study, continuous variables were averaged; values were expressed as mean ± SD or as percentages for categorical parameters. Male gender, presence of hypertension, diabetes mellitus, hyperlipidemia, and current smoking were coded as dummy variables. The degree of association among independent variables for each glucose metabolism marker was assessed by multiple regression analysis. In the animal study, values for each parameter within a group are expressed as dot plots with mean bars. The Kruskal–Wallis test was used to assess the statistical significance.

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**Table 1. Clinical characteristics of subjects and multiple regression analysis for determinants of HbA1c, FPG and HOMA-IR**

| Variables       | mean ± SD | HbA1c coefficient | p-value | FPG coefficient | p-value | HOMA-IR coefficient | p-value |
|-----------------|-----------|--------------------|---------|-----------------|---------|--------------------|---------|
| Age (y.r.)      | 63.1 ± 9.9| 0.018              | 0.053   | 0.482           | 0.037   | 0.008              | 0.487   |
| Male/Female (n) | 60/70     | −0.029             | 0.891   | 5.389           | 0.312   | −0.052             | 0.844   |
| BMI (kg/m²)     | 24.8 ± 4.1| 0.027              | 0.238   | 0.826           | 0.150   | 0.130              | 0.001   |
| SBP (mmHg)      | 133.9 ± 20.3| 0.007              | 0.153   | 0.166           | 0.196   | 0.000              | 0.045   |
| LDL-C (mg/dl)   | 129.0 ± 30.5| 0.004              | 0.212   | 0.072           | 0.321   | 0.004              | 0.311   |
| HDL-C (mg/dl)   | 61.3 ± 16.1| −0.009             | 0.120   | −0.186          | 0.213   | −0.014             | 0.060   |
| TG (mg/dl)      | 120.3 ± 69.6| 0.000              | 0.731   | 0.018           | 0.581   | 0.000              | 0.790   |
| UA (mg/dl)      | 5.4 ± 1.4 | −0.064             | 0.388   | −1.398          | 0.461   | −0.091             | 0.361   |
| Cre (mg/dl)     | 0.73 ± 0.17| −0.496             | 0.493   | −19.329         | 0.293   | 0.202              | 0.830   |
| FPG (mg/dl)     | 108.6 ± 22.2|                   |        |                 |         |                    |         |
| HbA1c (%)       | 6.2 ± 0.9 |                    |        |                 |         |                    |         |
| HOMA-IR         | 1.71 ± 1.68|                   |        |                 |         |                    |         |
| HCCI activities (%) | 99.4 ± 18.4| −0.011             | 0.014   | −0.311          | 0.007   | −0.013             | 0.041   |
| Current Smoking (%) | 10.8% (14) | −0.105             | 0.662   | −3.999          | 0.541   | −0.058             | 0.864   |
| Hypertension (%) | 59.2% (77) | −0.090             | 0.673   | −2.772          | 0.608   | −0.165             | 0.547   |
| Hyperlipidemia (%) | 60.8% (79) | −0.182             | 0.345   | −2.598          | 0.593   | −0.000             | 0.486   |
| Diabetes Mellitus (%) | 30.0% (39) |                   |        |                 |         |                    |         |

BMI: body mass index, SBP: systolic blood pressure, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglyceride, UA: uric acid, Cre: creatinine, FPG: fasting plasma glucose, HbA1c: glycosylated hemoglobin A1c, HOMA: homeostatic model assessment, HCCI: heparin cofactor II.
Heparin Cofactor II and Hyperglycemia

HFD Prominently Increases Adipocyte Size in HCII⁺⁻ Mice

To examine whether the inhibition of thrombin action by HCII plays a role in the regulation of glucose metabolism, we used HCII⁺⁻ mice and compared their glucose metabolism with that of HCII⁺⁺ mice. Growth curve analysis revealed no difference between HCII⁺⁻ and HCII⁺⁺ mice fed ND (Fig. 2A). Both HCII⁺⁺ and HCII⁺⁻ mice who were fed HFD showed greater increase in body weight compared to mice who were fed ND (Fig. 2A). Body composition assessment by CT revealed no significant differences in the volume of skeletal muscles, subcutaneous fat, and visceral fat between HCII⁺⁻ and HCII⁺⁺ mice (Figs. 2B, 2C, & 2D). On the contrary, Coulter counter analysis revealed greater adipocyte diameters in HFD-fed HCII⁺⁻ mice than in HFD-fed HCII⁺⁺ mice (Figs. 2E & 2F).

HFD-Induced Obesity Causes Greater Hyperglycemia and Hyperinsulinemia in HCII⁺⁻ Mice

Although no obvious difference in FPG was observed between HCII⁺⁺ and HCII⁺⁻ mice fed ND, higher FPG levels were observed in HFD-fed HCII⁺⁻ mice than in HCII⁺⁺ mice in the HFD group (Fig. 2H). Fasting serum insulin levels more prominently increased in HCII⁺⁻ mice than in HCII⁺⁺ mice in the HFD group (Fig. 2I); this resulted in higher HOMA-IR values in HCII⁺⁻ mice than those in HCII⁺⁺ mice in the HFD group (Fig. 2I). There were no significant
differences in adiponectin or leptin serum levels between HCII⁺⁺ and HCII⁺⁻ mice in each diet group (Figs. 3A & 3B).  

**HFD Accelerates Inflammation and Macrophage Recruitment in the Adipose Tissue of HCII⁺⁻ Mice**

In the quantitative RT-PCR analysis of epididymal fat tissue, no significant difference in the gene expression levels of F2r, a murine homologue of thrombin receptor gene, was observed (Fig. 3C). Because chronic inflammation in adipose tissue plays pivotal roles in the development of insulin resistance, we evaluated the gene expression levels of monocyte chemo-tactic protein-1 (Mcp1) and tumor necrosis factor (Tnf) in epididymal fat tissue in mice. The expression levels of Mcp1 and Tnf genes significantly increased in HCII⁺⁻ mice compared to those in HCII⁺⁺ mice in the HFD group (Figs. 3D & 3E). In addition, immunohistochemical analysis revealed that F4/80 (cell-surface marker of macrophages)-positive cells were significantly increased in HCII⁺⁻ mice fed HFD compared to other groups (Figs. 3H & 3I). These results are consistent with the marked enhancement of Mcp1 and Tnf gene expression in HCII⁺⁻ mice with HFD-induced obesity (Figs. 3D & 3E).
in an in vitro study, we failed to prove that HCII and/or dermatan sulfate are able to increase both CNTF and CNTFR gene expression levels in adipocytes with statistical significance (Supplemental Figs. 2A & 2B). These results indicated that HCII seems to be indirectly associated with the CNTF–CNTF receptor axis in vivo.

HCII Insufficiency Reduces Phosphorylation of Akt and AMPK in the Adipose Tissue of Obese Mice

We investigated the phosphorylation of AMPK in adipose tissues (epididymal fat tissue), liver, and skeletal muscle of HCII+/+ and HCII−/− mice. Western blot analysis demonstrated that HFD-fed HCII+/−
were higher than those in HFD-fed HCII'''' mice (Figs. 5A and 5B).

**Discussion**

In the present study, we provide the first evidence that plasma HCII activity is inversely associated with FPG, HbA1c, and HOMA-IR in humans. Furthermore, fasting hyperglycemia and prominent insulin resistance were more frequently found in HFD-fed HCII'''' mice than that in HFD-fed HCII'''' mice.

Obesity-induced glucose metabolism disorder is recognized as chronic low-grade systemic inflammation. Recently, molecular bases of metabolic inflammation and their potential pathogenic roles in diabetes and cardiovascular disease have been investigated 21-23). Increasing evidence has revealed that the chemokine system is involved in chronic inflammation leading to obesity, insulin resistance, and T2DM. In this regard, MCP-1 is considered to play a pivotal role in obesity-induced insulin resistance24). In our previous study, we found that Mcp1 gene expression in 3T3-L1 adipocytes is markedly increased by thrombin treatment, suggesting that thrombin is a key molecule linking increased adiposity and an enhanced expression of MCP-18). In addition, we previously demonstrated that cuff injury of femoral arteries in HCII'''' mice prominently augmented neointimal hyperplasia with the enhancement of vascular Mcp1 gene expression12).
increase fatty acid oxidation and reduce insulin resistance via AMPK activation\(^27\)). Although we observed a reduced ratio of Cntf to Cntfr gene expression in HFD-treated HCII\(^{+/-}\) mice, the detailed mechanism underlying the influence of serine protease and/or its inhibitor on CNTF–CNTF receptor axis has been largely unknown. However, our in vivo and in vitro experimental results suggest that the aberrant balance between Cntf and Cntfr gene expression levels in HCII\(^{+/-}\) mice may be indirectly correlated with impaired AMPK activation. In addition, the finding of reduced phosphorylation of AMPK in HFD-treated HCII\(^{+/-}\) mice is consistent with the results of our previous study showing that HCII deficiency causes impaired AMPK phosphorylation in ischemic skeletal muscle, leading to insufficient angiogenic response\(^17\)). Thus, the present observations that reduced HCII activity leads to AMPK inactivation in adipose tissue may at least partly explain why HCII\(^{+/-}\) mice with HFD-induced obesity developed insulin resistance.

Therefore, HCII may be closely associated with the regulation of thrombin-MCP-1 axis.

TNF-\(\alpha\) has also been recognized as a key molecule linking obesity and insulin resistance. Because TNF-\(\alpha\) is overexpressed in the adipose tissue of obese animals and humans, and because obese mice lacking either TNF-\(\alpha\) or its receptor have attenuated insulin resistance\(^25\), it is plausible that the prominent gene expression of inf was observed in HFD-treated HCII\(^{+/-}\) mice with insulin resistance. Moreover, Kalle et al. reported that HCII-deficient mice exhibit increased susceptibility to Pseudomonas aeruginosa infection with elevated inflammatory plasma cytokines such as interleukin 6, TNF-\(\alpha\), MCP-1, and interferon-\(\gamma\)\(^26\). Their study suggested that HCII is an immunological regulator having broad anti-inflammatory effects. Taken together, these results are consistent with the notion that HCII deficiency promotes macrophage infiltration, leading to low-grade, chronic inflammation in adipose tissue.

In the present study, we found that Cntfr was significantly downregulated in HCII\(^{+/-}\) mice. CNTF is known to prevent obesity and ameliorate glucose tolerance. Watt et al. demonstrated that CNTF signals

![Fig. 5. Enhanced gene expression of gluconeogenesis-related factors in HFD-treated HCII\(^{+/-}\) mice](image)

Gene expression levels of Pck1 (A) and G6pc (B) in hepatic tissues of HCII\(^{+/-}\) and HCII\(^{++/-}\) mice fed ND or HFD (\(n=12\) or 13 in each group). Bars represent the mean values in each group. *\(p<0.05\), **\(p<0.01\)
glucose uptake in muscle and adipocytes, and the dysregulation of hepatic glucose production\textsuperscript{29}. As our previous study provided evidence that thrombin stimulation blunts Akt phosphorylation in 3T3L-1 adipocytes\textsuperscript{30}, we evaluated Akt phosphorylation in the adipose tissue of mice and found that HC\textsuperscript{II} deficiency causes insufficient activation of Akt in HFD-induced obesity. It has been demonstrated that macrophage infiltration and secreted factors from the macrophages in adipocytes inhibit Akt phosphorylation, subsequently impairing insulin action\textsuperscript{30}. The enhancement of macrophage infiltration along with increased gene expression of \textit{Mcp1} induced by HC\textsuperscript{II} deficiency may partially explain Akt inactivation. From these results, it is plausible to assume that Akt inactivation, along with AMPK inactivation, in the adipose tissue of HC\textsuperscript{II\textsuperscript{+/−}} mice impair glucose transport and insulin action.

Gluconeogenesis is one of the major mechanisms to maintain blood glucose levels and prevent hypoglycemia. The acceleration of gluconeogenesis in the liver is largely responsible for fasting hyperglycemia in patients with T2DM\textsuperscript{31}. Gluconeogenesis is regulated by the activity of two rate-limiting enzymes, PEPCK and G6 Pase\textsuperscript{20}. Therefore, we evaluated the expression levels of these genes in the liver, and found that the mRNA levels of both \textit{Pck1} (murine PEPCK homologue) and \textit{G6pc} (murine G6 Pase homologue) in HFD-fed HC\textsuperscript{II\textsuperscript{+/−}} mice were higher than those in HFD-fed HC\textsuperscript{II\textsuperscript{+/+}} mice. Previous studies showed that thrombin activates NAD(P)H oxidase; oxidative stress is known to be associated with increased expression of PEPCK and G6 Pase. Thus, it is possible that HC\textsuperscript{II} deficiency augments oxidative stress by activating the tissue thrombin-PAR-1 axis and enhances PEPCK and G6 Pase expression, leading to fasting hyperglycemia.

Oxidative stress and chronic inflammation are known to be associated with the development of metabolic diseases, including obesity and diabetes\textsuperscript{32}. Because we previously reported that HC\textsuperscript{II\textsuperscript{−/−}} mice manifest excessive superoxide production at the sites of vascular injury, the observed glucose metabolism disorder in the present study may be associated with an abnormal oxidative stress state in HFD-treated HC\textsuperscript{II\textsuperscript{+/−}} mice.

As thrombin inactivation is effectively caused by complex formation with HCII and dermatan sulfate (DS), DS has an important role in the modulation of thrombin action. Because glycosaminoglycans (GAGs) undergo structural and functional remodeling in various pathological states including hyperglycemia and the levels of GAGs such as chondroitin/DS are significantly decreased in diabetic condition\textsuperscript{33, 34}, the amount and functional changes of DS may be associated with thrombin inhibition by HCII.

In conclusion, the present study provides evidence supporting the assumption that HCII, a serine protease inhibitor, plays an important role in maintaining glucose homeostasis by regulating insulin sensitivity in both humans and mice. Our findings suggest that the measurement of plasma HCII activity may be useful for identifying patients at high risk for T2DM and cardiovascular complications. In addition, the stimulation of HCII production may be a novel therapeutic approach to ameliorate cardiovascular diseases and diabetic conditions in patients with T2DM.

Conflicts of Interest/Disclosures

The authors report no conflicts of interest or disclosures.

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References

1) Samad F and Ruf W. Inflammation, obesity, and thrombosis. Blood. 2013; 122: 3415-3422
2) Sanchez C, Poggi M, Morange PE, Defoort C, Martin JC, Tanguy S, Dutour A, Grino M and Alessi MC. Diet modulates endogenous thrombin generation, a biological estimate of thrombosis risk, independently of the metabolic status. Arterioscler Thromb Vasc Biol. 2012; 32: 2394-2404
3) McNamara CA, Sarembock IJ, Gimple LW, Fenton JW, 2nd, Couglin SR and Owens GK. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. J Clin Invest. 1993; 91: 94-98
4) Derian CK, Damiano BP, D’Andrea MR and Andrade-Gordon P. Thrombin regulation of cell function through protease-activated receptors: implications for therapeutic intervention. Biochemistry (Mosc). 2002; 67: 56-64
5) Griffin CT, Srinivasan Y, Zheng YW, Huang W and Couglin SR. A role for thrombin receptor signaling in endothelial cells during embryonic development. Science. 2001; 293: 1666-1670
6) Cheung WM, D’Andrea MR, Andrade-Gordon P and Damiano BP. Altered vascular injury responses in mice deficient in protease-activated receptor-1. Arterioscler Thromb Vasc Biol. 1999; 19: 3014-3024
7) Lim J, Iyer A, Liu L, Suen JY, Lohman RJ, Seow V, Yau MK, Brown L and Fairlie DP. Diet-induced obesity, adipose inflammation, and metabolic dysfunction correlating with PAR2 expression are attenuated by PAR2 antagonism. FASEB J. 2013; 27: 4757-4767
8) Mihara M, Aihara K, Ikeda Y, Yoshida S, Kinouchi M,
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Kurahashi K, Fujinaka Y, Akaike M and Matsumoto T. Inhibition of thrombin action ameliorates insulin resistance in type 2 diabetic db/db mice. Endocrinology. 2010; 151: 513-519

9) Strande JL and Phillips SA. Thrombin increases inflammatory cytokine and angiogenic growth factor secretion in human adipose cells in vitro. J Inflamm. 2009; 6: 4

10) Tollefsen DM, Pestka CA and Monafo WJ. Activation of heparin cofactor II by dermatan sulfate. J Biol Chem. 1983; 258: 6713-6716

11) Aihara K, Azuma H, Takamori N, Kanagawa Y, Akaike M, Fujimura M, Yoshida T, Hashizume S, Kato M, Yamaguchi H, Kato S, Ikeda Y, Arase T, Kondo A and Matsumoto T. Heparin cofactor II is a novel protective factor against carotid atherosclerosis in elderly individuals. Circulation. 2004; 109: 2761-2765

12) Aihara K, Azuma H, Akaike M, Ikeda Y, Sata M, Takamori N, Yagi S, Iwase T, Sumitomo Y, Kawanoh H, Yamada T, Fukuda T, Matsumoto T, Sekine K, Sato T, Nakamichi Y, Yamamoto Y, Yoshimura K, Watanabe T, Nakamura T, Oomizu A, Tsukada M, Hayashi H, Sudo T, Kato S and Matsumoto T. Strain-dependent embryonic lethality and exaggerated vascular remodeling in heparin cofactor II-deficient mice. J Clin Invest. 2007; 117: 1514-1526

13) Aihara K, Azuma H, Akaike M, Kurobe H, Takamori N, Ikeda Y, Sumitomo Y, Yoshida S, Yagi S, Iwase T, Ishikawa K, Sata M, Kitagawa T and Matsumoto T. Heparin cofactor II is an independent protective factor against peripheral arterial disease in elderly subjects with cardiovascular risk factors. J Atheroscler Thromb. 2009; 16: 127-134

14) Sumitomo-Ueda Y, Aihara K, Ise T, Yoshida S, Ikeda Y, Uemoto R, Yagi S, Iwase T, Ishikawa K, Hirata Y, Akaike M, Sata M, Kato S and Matsumoto T. Heparin cofactor II protects against angiotensin II-induced cardiac remodeling via attenuation of oxidative stress in mice. Hypertension. 2010; 56: 430-436

15) Takamori N, Azuma H, Kato M, Hashizume S, Aihara K, Akaike M, Tamura K and Matsumoto T. High plasma heparin cofactor II activity is associated with reduced incidence of in-stent restenosis after percutaneous coronary intervention. Circulation. 2004; 109: 481-486

16) Ise T, Aihara K, Sumitomo-Ueda Y, Yoshida S, Ikeda Y, Yagi S, Iwase T, Yamada H, Akaike M, Sata M and Matsumoto T. Plasma heparin cofactor II activity is inversely associated with left atrial volume and diastolic dysfunction in humans with cardiovascular risk factors. Hypertens Res. 2011; 34: 225-231

17) Ikeda Y, Aihara K, Yoshida S, Iwase T, Tajima S, Izawa-Ishizawa Y, Kihira Y, Ishizaka K, Tomita S, Tsuchiya K, Sata M, Akaike M, Kato S, Matsumoto T and Tamaki T. Heparin cofactor II, a serine protease inhibitor, promotes angiogenesis via activation of the AMP-activated protein kinase-endothelial nitric-oxide synthase signaling pathway. J Biol Chem. 2012; 287: 34256-34263

18) Sakai T, Sakaue H, Nakamura T, Okada M, Matsuki Y, Watanabe E, Hiramatsu R, Nakayama K, Nakayama KI and Kasuga M. Skp2 controls adipocyte proliferation during the development of obesity. J Biol Chem. 2007; 282: 2038-2046
### Supplemental Table 1.

| Characteristics            | Total (n = 306) | Men (n = 154) | Women (n = 152) | p-value     |
|----------------------------|-----------------|---------------|-----------------|-------------|
| Age (yr)                   | 68.9 ± 11.1     | 67.2 ± 10.8   | 70.6 ± 11.1     | <0.01       |
| Body mass index (kg/m²)    | 23.2 ± 3.7      | 23.6 ± 3.8    | 22.8 ± 3.5      | NS          |
| SBP (mmHg)                 | 138.3 ± 20.2    | 138.2 ± 20.2  | 138.5 ± 20.3    | NS          |
| Total cholesterol (mg/dL)  | 198.1 ± 39.6    | 187.3 ± 40.9  | 209.1 ± 35.1    | <0.0001     |
| LDL-cholesterol (mg/dL)    | 124.5 ± 34.5    | 114.4 ± 35.5  | 134.8 ± 30.3    | <0.0001     |
| HDL-cholesterol (mg/dL)    | 47.0 ± 13.2     | 44.2 ± 12.4   | 49.9 ± 13.4     | <0.0005     |
| Triglyceride (mg/dL)       | 131.6 ± 85.4    | 143.5 ± 104.1 | 119.5 ± 58.8    | <0.05       |
| Lipid peroxide (nmol/mL)   | 0.45 ± 0.23     | 0.48 ± 0.26   | 0.41 ± 0.18     | <0.005      |
| Lipoprotein(a) (mg/dL)     | 23.0 ± 17.1     | 22.9 ± 15.7   | 23.1 ± 18.5     | NS          |
| HbA1c (%)                  | 5.9 ± 1.7       | 6.1 ± 1.8     | 5.8 ± 1.6       | NS          |
| Antithrombin activity (%)  | 93.1 ± 14.8     | 89.5 ± 14.3   | 96.6 ± 14.5     | <0.0005     |
| Heparin cofactor II activity (%) | 94.0 ± 18.3  | 92.1 ± 18.2   | 95.9 ± 18.2     | NS          |
| Maximum plaque thickness (mm) | 2.04 ± 1.13    | 2.15 ± 1.18   | 1.93 ± 1.07     | NS          |
| Current smoking (%)        | 28.4            | 48.7          | 7.9             | <0.0001     |
| Hypertension (%)           | 57.8            | 55.8          | 59.9            | NS          |
| Hyperlipidemia (%)         | 42.5            | 35.7          | 49.3            | <0.05       |
| Diabetes mellitus (%)      | 30.7            | 36.4          | 25              | <0.05       |

Values are means ± SD or percentages.
Baseline characteristics were compared between men and women by unpaired t test or χ² test for independence.
(Referred from the article by Aihara et al. Circulation. 2004; 109: 2761-2765)

### Supplemental Table 2.

| Characteristics        | ABI ≥ 0.9 (n = 432) | ABI < 0.9 (n = 62) | p-value (ABI ≥ 0.9 vs < 0.9) |
|------------------------|---------------------|--------------------|-----------------------------|
| Male/Female            | 233/199             | 41/21              | 0.065                       |
| Age (yr)               | 66.4 ± 11.0         | 71.0 ± 9.5         | 0.001                       |
| BMI (kg/m²)            | 23.7 ± 3.5          | 22.4 ± 2.8         | 0.001                       |
| SBP (mmHg)             | 136.2 ± 20.5        | 143.6 ± 20.4       | 0.001                       |
| LDL-cholesterol (mg/dL)| 121.0 ± 35.0        | 129.2 ± 40.8       | 0.134                       |
| HDL-cholesterol (mg/dL)| 50.9 ± 16.9         | 50.7 ± 24.0        | 0.938                       |
| Triglyceride (mg/dL)   | 139.4 ± 86.2        | 136.2 ± 78.2       | 0.762                       |
| Lipoprotein(a) (mg/dL) | 20.7 ± 15.8         | 27.0 ± 27.4        | 0.081                       |
| HbA1c (%)              | 6.0 ± 1.7           | 7.3 ± 6.7          | 0.148                       |
| Antithrombin activity  | 97.8 ± 15.8         | 94.7 ± 17.0        | 0.173                       |
| Heparin cofactor II activity (%) | 94.6 ± 17.8 | 87.5 ± 19.7 | 0.009                       |
| Current smoking (%)    | 127 (29.4)          | 23 (41.1)          | 0.003                       |
| Hypertension (%)       | 266 (61.6)          | 46 (74.2)          | 0.041                       |
| Hyperlipidemia (%)     | 189 (43.4)          | 29 (46.8)          | 0.658                       |
| Diabetes mellitus (%)  | 121 (28.0)          | 33 (53.2)          | 0.001                       |

Values are means ± SD or percentages.
Baseline characteristics were compared between ABI ≥ 0.9 and ABI < 0.9 by unpaired t test or χ² test for independence.
(Referred from the article by Aihara et al. J Atheroscler Thromb. 2009; 16: 127-134.)
Supplemental Table 3.

| Position | Symbol | Description | Fold Change | 95% CI      | p-Value   |
|----------|--------|-------------|-------------|-------------|-----------|
| A01      | Adcyap1| Adenylate cyclase activating polypeptide 1 | 1.3553      | (0.00001, 2.95) | 0.517354 |
| A02      | Adcyap1r1| Adenylate cyclase activating polypeptide 1 receptor 1 | 0.6598 | (0.30, 1.02) | 0.248703 |
| A03      | Adipoq | Adiponectin, C1Q and collagen domain containing | 0.7423 | (0.48, 1.01) | 0.101473 |
| A04      | Adipor1| Adiponectin receptor 1 | 0.8187 | (0.67, 0.96) | 0.046135 |
| A05      | Adipor2| Adiponectin receptor 2 | 0.7263 | (0.37, 1.08) | 0.237507 |
| A06      | Adra2b| Adrenergic receptor, alpha 2b | 1.005 | (0.42, 1.59) | 0.926584 |
| A07      | Adrb1 | Adrenergic receptor, beta 1 | 1.0728 | (0.23, 1.91) | 0.752198 |
| A08      | Agrp  | Agouti related protein | 0.7386 | (0.53, 0.95) | 0.071847 |
| A09      | Apoa4 | Apolipoprotein A-I | 0.8082 | (0.55, 1.06) | 0.207946 |
| A10      | Arrn  | Attractin | 0.7263 | (0.55, 0.90) | 0.036849 |
| A11      | Bdnf  | Brain derived neurotrophic factor | 1.3117 | (0.75, 1.87) | 0.178754 |
| A12      | Brx3  | Bombesin-like receptor 3 | 0.8082 | (0.55, 1.06) | 0.207946 |
| B01      | C3    | Complement component 3 | 1.2533 | (0.86, 1.65) | 0.160931 |
| B02      | Calca| Calcitonin/calcitonin-related polypeptide, alpha | 0.7306 | (0.36, 1.10) | 0.234034 |
| B03      | Calcr| Calcitonin receptor | 1.3796 | (0.00001, 3.05) | 0.518477 |
| B04      | Campt| CART prepropeptide | 0.6637 | (0.41, 0.92) | 0.07998 |
| B05      | Cck   | Cholecystokinin | 3.1821 | (0.00001, 9.22) | 0.275644 |
| B06      | Cckar | Cholecystokinin A receptor | 1.9166 | (0.00001, 6.61) | 0.35273 |
| B07      | Clps  | Colipase, pancreatic | 1.417 | (0.03, 2.80) | 0.895452 |
| B08      | Cnr1  | Cannabinoid receptor 1 (brain) | 0.9184 | (0.56, 1.27) | 0.77961 |
| B09      | Cnfr  | Ciliary neurotrophic factor receptor | 0.4749 | (0.29, 0.66) | 0.005242 |
| B10      | Cpd   | Carboxypeptidase D | 1.0539 | (0.76, 1.35) | 0.666465 |
| B11      | Cpe   | Carboxypeptidase E | 1.0792 | (0.56, 1.60) | 0.6268 |
| B12      | Cshr1 | Corticotropin releasing hormone receptor 1 | 1.2511 | (0.00, 2.46) | 0.508164 |
| C01      | Drd1a | Dopamine receptor D1A | 0.4642 | (0.12, 0.81) | 0.1923 |
| C02      | Drd2  | Dopamine receptor D2 | 0.6443 | (0.42, 0.87) | 0.046368 |
| C03      | Gal   | Galanin | 0.8442 | (0.23, 1.46) | 0.930742 |
| C04      | Galr1 | Galanin receptor 1 | 1.434 | (0.35, 2.52) | 0.323728 |
| C05      | Gcg   | Glucagon | 0.7156 | (0.50, 0.94) | 0.063372 |
| C06      | Gcgr  | Glucagon receptor | 0.6864 | (0.00001, 1.88) | 0.15625 |
| C07      | Gh    | Growth hormone | 1.0612 | (0.39, 1.73) | 0.578551 |
| C08      | Ghr   | Growth hormone receptor | 0.8019 | (0.52, 1.08) | 0.199447 |
| C09      | Ghrl  | Ghrelin | 0.5401 | (0.21, 0.87) | 0.135176 |
| C10      | Ghrtr| Growth hormone secretagogue receptor | 1.3499 | (0.00001, 3.00) | 0.455813 |
| C11      | Glp1r | Glucagon-like peptide 1 receptor | 0.7071 | (0.39, 1.03) | 0.157053 |
| C12      | Mchr1 | Melanin-concentrating hormone receptor 1 | 1.3553 | (0.00001, 2.95) | 0.705335 |
| D01      | Gep   | Gastrin releasing peptide | 1.3526 | (0.00001, 2.74) | 0.919938 |
| D02      | Gspr  | Gastrin releasing peptide receptor | 0.903 | (0.38, 1.43) | 0.278168 |
| D03      | Hcrt  | Hypocretin | 1.2483 | (0.00001, 2.53) | 0.509901 |
| D04      | Hcrr1| Hypocretin (orexin) receptor 1 | 0.5946 | (0.34, 0.85) | 0.051138 |
| D05      | Hrh1  | Histamine receptor H1 | 1.6213 | (0.56, 2.68) | 0.239564 |
| D06      | Htr2c | 5-hydroxytryptamine (serotonin) receptor 2C | 1.005 | (0.37, 1.64) | 0.449317 |
| D07      | Iapp  | Islet amyloid polypeptide | 0.9238 | (0.47, 1.37) | 0.388305 |
| D08      | Il1a  | Interleukin 1 alpha | 0.6837 | (0.06, 1.31) | 0.257391 |
| D09      | Il1b  | Interleukin 1 beta | 1.879 | (0.96, 2.80) | 0.065358 |
| D10      | Il1r1 | Interleukin 1 receptor, type 1 | 0.8671 | (0.61, 1.13) | 0.298529 |
| D11      | Il6   | Interleukin 6 | 1.3906 | (0.22, 2.56) | 0.235615 |
| D12      | Il6ra | Interleukin 6 receptor, alpha | 1.022 | (0.68, 1.37) | 0.948864 |
(Cont Supplemental Table 3) Fold Change (comparing to HCII-WT mice fed a HFD)

| Position | Symbol | Description | Fold Change | 95% CI | p-Value |
|----------|--------|-------------|-------------|--------|---------|
| E01      | Ins1   | Insulin I   | 1.3486      | (0.00001, 2.93) | 0.517016 |
| E02      | Ins2   | Insulin II  | 0.7571      | (0.54, 0.98)    | 0.109651 |
| E03      | Insr   | Insulin receptor | 0.6552      | (0.36, 0.95)    | 0.119759 |
| E04      | Lep    | Leptin      | 1.2422      | (0.50, 1.99)    | 0.223664 |
| E05      | Lepr   | Leptin receptor | 0.9284      | (0.45, 1.41)    | 0.979422 |
| E06      | Mc3r   | Melanocortin 3 receptor | 0.9257      | (0.47, 1.38)    | 0.390157 |
| E07      | Nmb    | Neuromedin B | 0.6029      | (0.26, 0.95)    | 0.263443 |
| E08      | Nmbr   | Neuromedin B receptor | 0.5223      | (0.07, 0.97)    | 0.293177 |
| E09      | Nmu    | Neuromedin U | 0.5917      | (0.30, 0.88)    | 0.126651 |
| E10      | Nmur1  | Neuromedin U receptor 1 | 0.6235      | (0.00001, 1.34) | 0.297705 |
| E11      | Npy    | Neuropeptide Y | 1.6439      | (0.00001, 3.61) | 0.820424 |
| E12      | Npy1r  | Neuropeptide Y receptor Y1 | 0.8459      | (0.47, 1.22)    | 0.369206 |
| F01      | Nr3c1  | Nuclear receptor subfamily 3, group C, member 1 | 0.7198      | (0.57, 0.87)    | 0.008339 |
| F02      | Nrk2   | Neurotrophic tyrosine kinase, receptor, type 2 | 0.9904      | (0.62, 1.19)    | 0.576791 |
| F03      | Nts    | Neurotensin | 1.4875      | (0.00001, 3.75) | 0.900008 |
| F04      | Nsr1   | Neurotensin receptor 1 | 1.1476      | (0.49, 1.80)    | 0.709402 |
| F05      | Oprk1  | Opioid receptor, kappa 1 | 0.8476      | (0.54, 1.16)    | 0.285696 |
| F06      | Oprm1  | Opioid receptor, mu 1 | 0.7586      | (0.47, 1.05)    | 0.140887 |
| F07      | Sigma1 | Sigma non-opioid intracellular receptor 1 | 0.7738      | (0.61, 0.94)    | 0.034028 |
| F08      | Pomc   | Pro-opiomelanocortin-alpha | 0.6988      | (0.51, 0.89)    | 0.025299 |
| F09      | Ppara  | Peroxisome proliferator activated receptor alpha | 0.5285      | (0.16, 0.90)    | 0.042418 |
| F10      | Pparg  | Peroxisome proliferator activated receptor gamma | 0.6199      | (0.35, 0.89)    | 0.078419 |
| F11      | Pparg1a| Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha | 0.3884      | (0.19, 0.59)    | 0.014167 |
| F12      | Pthr   | Prolactin releasing hormone receptor | 0.9367      | (0.46, 1.41)    | 0.600798 |
| G01      | Ptnp1  | Protein tyrosine phosphatase, non-receptor type 1 | 0.7908      | (0.65, 0.93)    | 0.026759 |
| G02      | Ppy    | Peptide YY | 0.7489      | (0.34, 1.16)    | 0.173387 |
| G03      | Ramp3  | Receptor (calcitonin) activity modifying protein 3 | 1.188       | (0.00001, 2.47) | 0.620546 |
| G04      | Sort1  | Sortilin 1 | 0.6481      | (0.43, 0.87)    | 0.025804 |
| G05      | Sst    | Somatostatin | 0.7177      | (0.50, 0.94)    | 0.066392 |
| G06      | Sstr2  | Somatostatin receptor 2 | 0.732       | (0.02, 1.44)    | 0.364054 |
| G07      | Thrb   | Thyroid hormone receptor beta | 0.9442      | (0.67, 1.22)    | 0.856099 |
| G08      | Tnf    | Tumor necrosis factor | 1.2758      | (0.07, 2.49)    | 0.671972 |
| G09      | Tih    | Thyrotropin releasing hormone | 0.77        | (0.36, 1.18)    | 0.495715 |
| G10      | Ucn    | Urocortin | 0.6571      | (0.41, 0.91)    | 0.080877 |
| G11      | Ucp1   | Uncoupling protein 1 (mitochondrial, proton carrier) | 0.9574      | (0.57, 1.35)    | 0.920895 |
| G12      | Zfp91  | Zinc finger protein 91 | 0.856       | (0.68, 1.03)    | 0.174067 |
| H01      | Gusb   | Glucuronidase, beta | 1.001       | (0.64, 1.36)    | 0.781711 |
| H02      | Hprt   | Hypoxanthine guanine phosphoribosyl transferase | 0.8501      | (0.70, 1.00)    | 0.110994 |
| H03      | Hsp90ab1| Heat shock protein 90 alpha (cytosolic), class B member 1 | 1.1052      | (0.84, 1.37)    | 0.398062 |
| H04      | Gapdh  | Glyceraldehyde-3-phosphate dehydrogenase | 0.8409      | (0.65, 1.03)    | 0.147452 |
| H05      | Acrb   | Actin, beta | 1.2645      | (0.90, 1.63)    | 0.138857 |
| H06      | MGDC   | Mouse Genomic DNA Contamination | 1.1151      | (0.21, 2.02)    | 0.489386 |
| H07      | RTC    | Reverse Transcription Control | 1.2311      | (0.00, 2.46)    | 0.508164 |
| H08      | RTC    | Reverse Transcription Control | 1.1544      | (0.14, 2.17)    | 0.497475 |
| H09      | RTC    | Reverse Transcription Control | 0.8228      | (0.55, 1.10)    | 0.237255 |
| H10      | PPC    | Positive PCR Control | 0.9423      | (0.46, 1.43)    | 0.405995 |
| H11      | PPC    | Positive PCR Control | 0.9564      | (0.48, 1.43)    | 0.469732 |
| H12      | PPC    | Positive PCR Control | 0.9564      | (0.44, 1.47)    | 0.4568   |
Supplemental Fig. 1. Scatter plots of plasma HCl activity and HbA1c values in our previous studies (A: \(n=130\) and B: \(n=494\)), the present study (C: \(n=130\)), and all studies combined (D: \(n=930\)).

**A**
\[ \begin{align*}
Y &= -0.013X + 7.183 \\
R^2 &= 0.025, p<0.01
\end{align*} \]

Data set from Aihara et al. Circulation. 2004;109: 2761

**B**
\[ \begin{align*}
Y &= -0.013X + 7.366 \\
R^2 &= 0.016, p<0.01
\end{align*} \]

Data set from Aihara et al. J Atheroscler Thromb. 2009;16:127

**C**
\[ \begin{align*}
Y &= -0.011X + 6.854 \\
R^2 &= 0.060, p<0.01
\end{align*} \]

The present study

**D**
\[ \begin{align*}
Y &= -0.013X + 7.2831 \\
R^2 &= 0.022, p<0.001
\end{align*} \]

Combined data from A to C

Supplemental Fig. 2. The 3T3-L1 preadipocytes were cultured as previously described (Mihara M et al. Endocrinology. 2010; 151: 513-519.) and the cells plated onto six-well dishes were treated with thrombin (1 U/ml) or human purified heparin cofactor II (HClII) (100 nM) or dermatan sulfate (DS) (5 µg/ml) for 48 h. \(n=6\) in each group. NS means not significant.
Supplemental Fig. 3. Quantitative results of phosphorylated AMPK-to-GAPDH protein ratios and phosphorylated Akt-to-GAPDH protein ratios in hepatic tissues and skeletal muscle (thigh) of $\text{HCII}^{+/+}$ mice and $\text{HCII}^{+/-}$ mice with ND or HFD feeding, $n = 8$ to 12 in each group. Bars represent mean values in each group. *$p < 0.05$, NS means not significant.