TRPM2 $\text{Ca}^{2+}$ channel regulates energy balance and glucose metabolism

Zhiyou Zhang,1 Wenyi Zhang,2 Dae Young Jung,1,3 Hwi Jin Ko,1,3 Yongjin Lee,3 Eunjung Lee,3 John Jun,1 Zhexi Ma,1 Francis Kim,1 Nicholas Tsvitsianos,3 Kathryn Chapman,4 Alastair Morrison,4 Marcus P. Cooper,5 Barbara A. Miller,2 and Jason K. Kim1,5
1Department of Cellular and Molecular Physiology and 2Department of Pediatrics, Division of Pediatric Hematology and Oncology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania; 3Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts; 4Molecular Discovery Research, GlaxoSmithKline, Essex, United Kingdom; and 5Department of Medicine and 6Division of Endocrinology, Metabolism, and Diabetes, University of Massachusetts Medical School, Worcester, Massachusetts

Submitted 11 May 2011; accepted in final form 8 January 2012

INJECTION RESISTANCE IS A MAJOR CHARACTERISTIC of type 2 diabetes and is causally associated with obesity (11). Recent studies have shown an important role of adipose tissue inflammation in obesity-mediated insulin resistance, and multiple factors in stress kinase signaling are involved in underlying mechanisms (33, 34). Identifying new proteins that regulate obesity, inflammation, and insulin resistance remains a key therapeutic approach to treat type 2 diabetes.

Transient receptor potential melastatin 2 (TRPM2) is a member of the transient receptor potential (TRP) channel superfamily, a diverse group of cation-permeable channels expressed in many tissues (20, 26). Substantial evidence demonstrates that TRPM2 acts as a sensor for reactive oxygen species (ROS) in oxidative stress, and it plays a critical role in oxidant-induced cell injury and death (9, 39). In oxidative stress, increased intracellular ROS results in ADP-ribose production, which binds to the TRPM2 COOH-terminal NUDT9-H domain to further enhance channel opening (14, 24, 32). TRPM2 currents also have a strong requirement for calcium at the intracellular surface of the plasma membrane (6, 19). Interaction between calmodulin and an IQ-like motif in the NH2 terminus of TRPM2 is strengthened when calmodulin is calcium bound, providing positive feedback for TRPM2 activation and leading to an increase in the intracellular calcium concentration [Ca$^{2+}$]i (30). TRPM2 has been shown to be involved in oxidant injury to striatal cells (8), cardiomyocytes (37), endothelial cells (10), and pancreatic β-cells (15). A role for TRPM2 in inflammation has also been demonstrated. TRPM2 is involved in oxidant-induced cell death in monocytes (9, 40). In TRPM2-deficient mice, hydrogen peroxide-induced calcium influx and production of macrophage inflammatory protein-2 were impaired in monocytes, leading to reduced inflammation (36).

In this study, we examined the metabolic role of TRPM2-Ca$^{2+}$ channel by performing a series of metabolic studies following chronic high-fat feeding in TRPM2-deficient mice. Importantly, we demonstrate a major role of TRPM2-Ca$^{2+}$ channel in regulating energy expenditure, inflammation, and insulin resistance.

MATERIALS AND METHODS

TRPM2-deficient mice and high-fat diet study. Frozen TRPM2 embryos were obtained from GlaxoSmithKline, and TRPM2-deficient (KO) mice were bred to colonies in B. A. Miller’s laboratory at the Pennsylvania State College of Medicine. TRPM2-KO mice were generated at GSK using homologous recombination in mouse embryonic stem cells and subsequent blastocyst injection of appropriately targeted ES cells. Exons 19 and 20 of TRPM2, which encode transmembrane domains three and four, were deleted (13). A TRPM2 cDNA with the same deletion was tested in vitro; after hydrogen peroxide treatment, no significant rise in [Ca$^{2+}$]i was detected by digital video analysis of Fura-2-loaded human embryonic kidney-293T cells expressing this construct compared with wild-type (WT) TRPM2. TRPM2-KO mice were back-crossed to the C57BL/6 genetic background, and WT littermates were used in this study. For diet-induced obesity, mice were fed a high-fat diet (HFD) (55% fat by calories; Harlan Teklad TD93075) ad libitum for 4–10 mo. The animal studies were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State College of Medicine. All studies at GSK were conducted after review by the GSK Institutional Animal Care and Use Committee and in accordance with the GSK Policy on the Care, Welfare, and Treatment of Laboratory Animals.
**Body composition and energy balance measurement.** Mice were housed under controlled temperature and lighting, with free access to food and water. Whole body fat and lean mass were noninvasively measured using 1H-MRS (Echo Medical Systems, Houston, TX). The food/water intake, energy expenditure, respiratory exchange ratio, and physical activity were assessed for 3 days using metabolic cages (TSE Systems, Chesterfield, MO). We used the TSE Systems LabMaster platform with easy-to-use calorimetry featuring fully automated monitoring for food and water and XYZ activity. LabMaster cages that are most similar to facility home cages were used, thereby allowing the use of bedding in the cage and minimizing any animal anxiety during the experimental period. The system provides intuitive software with flexibility for experimental setup and data utilization.

**Glucose and insulin tolerance tests.** Following an overnight fast, a glucose tolerance test was performed using an intraperitoneal injection of 1 g/kg body wt of 20% glucose in mice, and serum glucose levels were measured using glucometer. Insulin tolerance test was performed in the fed state using an intraperitoneal injection of 0.75 U/kg body wt of human insulin.

**Hyperinsulinemic euglycemic clamp.** Following chow or HFD, a survival surgery was performed at 4–5 days before clamp experiments to establish an indwelling catheter in jugular vein. On the day of experiment, mice were fasted overnight (~15 h), and a 2-h hyperinsulinemic euglycemic clamp was conducted in conscious mice with a primed and continuous infusion of human insulin (150 mU/kg body wt priming followed by 2.5 mU·kg⁻¹·min⁻¹; Humulin; Eli Lilly) (12). To maintain euglycemia, 20% glucose was infused at variable rates during clamps. Whole body glucose turnover was assessed with a continuous infusion of [3-¹⁴C]glucose, and 2-deoxy-[¹⁴C]glucose uptake in individual organs. At the end of the clamps, mice were anesthetized, and tissues were taken for biochemical analysis (12).

**Immunoblot analysis.** Immunoblotting was performed using powdered heart samples that were dissolved in lysis buffer (50 mM HEPES, pH 7.3, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM NaVO₄, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 2 mM PMSF, 10 mM benzamidine, 10% glycerol, 1% Triton X-100, 1 mM microcinyl LR, 100 mM okadaic acid and cocktail protease inhibitor) and sonicated for 10 s. The samples were incubated on ice for 30 min and centrifuged at 15,000 g for 15 min at 4°C, and the supernatants were harvested. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad). Fifty micrograms of total protein per well for PKCα/IRS-1 (IRS-1) was loaded. The proteins were dissolved in SDS gel sample buffer and resolved by electrophoresis in 10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad), blocked in 5% nonfat milk in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20, and incubated with IRS-1 antibodies (1:1,000 dilution; Upstate), anti-Akt and anti-phospho-Akt (Ser⁴⁷³) antibodies (1:1,000 dilution; Cell Signaling Technology), anti-phospho-Akt (Thr³⁸²) antibodies (1:1,000 dilution; Cell Signaling Technology), anti-GSK-3β (Ser⁹⁵) antibodies (1:1,000 dilution; Cell Signaling Technology), and anti-calmodulin antibodies (1:1,000 dilution; Calbiochem). The membranes were then incubated in the appropriate goat anti-rabbit IgG or goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Pierce). Blots were developed in SuperSignal West Pico (Pierce) and exposed to X-ray film. The film was scanned by a GS-800 scanner (Bio-Rad), and the density was quantified by Quantity One software (Bio-Rad).

**Immunoprecipitation.** Powdered heart samples of TRPM2-KO and WT mice were dissolved in lysis buffer as described above and sonicated for 10 s. The samples were incubated on ice for 30 min and centrifuged at 15,000 g for 15 min at 4°C, and the supernatants were harvested. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad). Immunoprecipitation was performed with 3 mg of total protein and 4 µg of anti-IRS-1 antibodies (Upstate) rotating overnight at 4°C. IRS-1 and anti-IRS-1 antibody complexes were pulled down by protein A sepharose CL-4B beads (Amersham Biosciences). The beads were rinsed three times by PBS. Gel sample buffer was added, boiled for 10 min, and loaded to SDS gel. Western blot was performed, and the membrane was blocked by 5% BSA in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20 and incubated by anti-phosphotyrosine antibodies (1:1,000 dilution; Upstate) overnight at 4°C and stripped; the membrane was to be reprobed by anti-IRS-1 antibodies.

**Superoxide dismutase activity.** The OxiSelect superoxide dismutase (SOD) assay kit (Cell Biolabs, San Diego, CA) was used for the assay. Briefly, 50 µg of heart samples was homogenized in 500 µl of cold 1× lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Triton-100) and centrifuged at 12,000 g for 10 min to collect the tissue lysate supernatant. Then, 150 µg of heart lysates was prepared in 96-well microtiter plate, including a blank. A mixture containing 5 µl of xanthine solution, 5 µl of chromagen solution, and 10 µl of 10× SOD assay buffer was added to each well, and finally, 10 µl of prediluted xanthine oxidase solution was added to each well. Mixtures were incubated for 1 h at 37°C, and absorbance was measured at 490 nm on a microplate reader.

**RNA analysis.** The adipose mRNA expression was examined with quantitative real-time PCR using a 7500 Fast Real Time PCR machine. Taqman assays were used for quantitation. The relative mRNA expression was normalized by measurement of the amount of Gapdh mRNA (no. 4352339E) in each sample using Taqman assays (Applied Biosystems).

**Statistical analysis.** Differences between groups were examined for statistical significance using Student’s t-test or analysis of variance with Fisher’s test.

**RESULTS**

TRPM2 deficiency increases insulin sensitivity and glucose metabolism in heart. Using ¹H-MRS, whole body fat mass and lean mass were measured and found to be comparable between male TRPM2-deficient mice and WT littermates (Fig. 1A). Intraperitoneal glucose tolerance tests showed improved glucose clearance (tolerance) in TRPM2-deficient mice, resulting in significantly lower area under the curve of glucose tolerance tests in TRPM2-deficient mice (1,202 ± 120 vs. 1,780 ± 97 mM·min⁻¹·l⁻¹ in WT mice, P < 0.01; Fig. 1B). To directly measure insulin sensitivity, we performed a 2-h hyperinsulinemic euglycemic clamp combined with [³⁵H]glucose infusion in conscious mice. Basal glucose and insulin levels were measured following an overnight fast, and they were similar in both groups of mice. During the clamp, plasma glucose levels were maintained at 7 mM, and plasma insulin levels were raised to 250 pM in both groups of mice (Fig. 1, C and D). Steady-state glucose infusion rates required to maintain euglycemia during clamps were significantly elevated in TRPM2-deficient mice (P = 0.016; Fig. 1E). Insulin-stimulated whole body glucose turnover and glycogen synthesis rates were increased by 15–30% in TRPM2-deficient mice (P < 0.03; Fig. 1, F and G). During the clamp, 2-[¹⁴C]deoxyglucose was injected as a bolus to measure glucose uptake in individual organs. Insulin-stimulated glucose uptake in heart was significantly elevated in TRPM2-deficient mice (P < 0.02; Fig. 1H). Insulin-stimulated glucose uptake in white adipose tissue tended to increase in TRPM2-deficient mice (P = 0.08; Fig. 1I). In contrast, muscle glucose uptake (376 ± 63 vs. 425 ± 60 mmol·l⁻¹·min⁻¹ in TRPM2-deficient mice) and hepatic insulin action (95 ± 2 vs. 93 ± 5% suppression of hepatic glucose production in...
TRPM2-deficient mice) were not significantly affected by TRPM2 deletion.

Insulin signaling was examined using Western blot in heart samples obtained at the end of insulin clamps. Insulin-stimulated IRS-1 tyrosine phosphorylation was elevated >10-fold in TRPM2-deficient mice, but total IRS-1 protein levels were not altered significantly in these mice (0.29 ± 0.08 vs. 0.21 ± 0.03 in TRPM2-deficient mice for IRS-1/β-actin levels). As a result, the ratio of IRS-1 tyrosine phosphorylation to IRS-1 total protein levels in heart was elevated significantly in TRPM2-deficient mice (P < 0.02; Fig. 2A). Since Ca²⁺ homeostasis and calmodulin were shown to affect insulin signaling, we measured calmodulin expression in the TRPM2-deficient heart (21). Heart calmodulin levels were reduced significantly in TRPM2-deficient mice, which is consistent with increased insulin signaling and glucose metabolism in these mice (Fig. 2B). These data indicate that TRPM2 deficiency increases insulin sensitivity and glucose metabolism in heart possibly by reducing myocardial expression of calmodulin.

TRPM2 deficiency enhances energy expenditure and attenuates diet-induced obesity. Since TRPM2 deficiency increased insulin sensitivity, we next determined whether TRPM2 affects diet-induced obesity and insulin resistance. To this end, a HFD (55% fat by calories) was fed ad libitum to male TRPM2-deficient mice and WT littermates, and body composition was measured before and after 2, 3, and 4 mo of HFD. Whereas WT mice became obese in response to HFD, TRPM2-deficient mice were partly resistant to diet-induced obesity (Fig. 3A). Whole body fat mass was significantly lower in TRPM2-deficient mice compared with WT mice after 2, 3, and 4 mo of HFD (Fig. 3B). Whole body lean mass was unaffected by TRPM2 deletion (Fig. 3C). In a separate cohort of mice, high-fat feeding was maintained for 10 mo, and TRPM2-deficient mice remained less obese than WT mice after 10 mo of HFD (Fig. 3D).

To determine why TRPM2-deficient mice became less obese during high-fat feeding, we measured energy balance using metabolic cages in TRPM2-deficient and WT mice fed a chow diet and after 4 mo of high-fat feeding. Rates of VO₂ consump-
tion and V\textsubscript{CO\textsubscript{2}} production normalized to whole body lean mass (measured using \textsuperscript{1}H-MRS) were significantly elevated in chow-fed TRPM2-deficient mice compared with chow-fed WT mice (Fig. 4, A–C). After 4 mo of high-fat feeding, V\textsubscript{CO\textsubscript{2}} production and energy expenditure rates normalized to whole body lean mass remained significantly higher in HFD-fed TRPM2-deficient mice (Fig. 4, D and E). TRPM2 deficiency caused a selective change in energy expenditure since daily food intake (1.8 ± 0.2 vs. 1.6 ± 0.1 g·HFD intake\textsuperscript{-1}·day\textsuperscript{-1} in TRPM2-deficient mice) and physical activity (150,297 ± 22,333 vs. 138,672 ± 17,045 movements/day in TRPM2-deficient mice) were not altered significantly in HFD-fed TRPM2-deficient mice. Altogether, these data demonstrate that TRPM2 deficiency enhances energy expenditure in mice.

TRPM2-deficient mice are more insulin sensitive following HFD. The effects of TRPM2 deletion on diet-induced insulin resistance were examined in mice after 4 mo of high-fat feeding. Glucose tolerance tests showed that WT mice developed severe insulin resistance after HFD since glucose levels failed to return to basal levels following intraperitoneal glucose injection (Fig. 5A). In contrast, HFD-fed TRPM2-deficient mice were able to clear glucose that returned near-basal levels at 120 min following glucose injection. Basal plasma insulin levels were significantly lower in HFD-fed TRPM2-deficient mice compared with HFD-fed WT mice, suggesting increased insulin sensitivity in TRPM2-deficient mice (Fig. 5B). Since adipose-derived hormones are known to affect insulin sensitivity (33), we measured circulating levels of leptin and resistin, which were not affected significantly by TRPM2 deletion (leptin: 16.3 ± 5.9 vs. 13.8 ± 0.9 ng/ml in TRPM2-deficient mice; adiponectin: 18.2 ± 3.5 vs. 15.2 ± 1.0 ng/ml in TRPM2-deficient mice).

To confirm improved insulin sensitivity, we performed a 2-h hyperinsulinemic euglycemic clamp in conscious mice after 4 mo of HFD. Basal plasma glucose levels tended to be lower in HFD-fed TRPM2-deficient mice (173 ± 6 mg/dl vs. 206 ± 20

Fig. 2. Increased insulin signaling in TRPM2-deficient heart. A: heart samples were obtained at the end of insulin clamps, and insulin signaling was measured as insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation normalized to total IRS-1 protein levels. B: calmodulin protein levels were determined from heart samples using Western blot. *P < 0.05 vs. WT mice.

Fig. 3. TRPM2-deficient mice are resistant to diet-induced obesity. A: body weight was measured during 4 mo of high-fat diet (HFD; n = 7–10, *P < 0.05 vs. 0 mo). B and C: whole body fat and lean mass were measured using \textsuperscript{1}H-MRS before HFD (0 mo) and at 2, 3, and 4 mo of HFD (n = 7–10, *P < 0.05 vs. 0 mo). D: another group of mice were fed a HFD for 10 mo, and body weights were measured at the end (n = 8; *P < 0.05 vs. WT-HFD). ◇ and □ WT; ■, TRPM2-KO.
mg/dl in HFD-fed WT mice, \( P < 0.01 \). Plasma insulin levels were similarly raised and maintained at \( \sim \)350 pM in both groups of mice (Fig. 5B). Steady-state glucose infusion rates during clamps were elevated significantly in HFD-fed TRPM2-deficient mice compared with HFD-fed WT mice (Fig. 5C). Insulin-stimulated whole body glucose turnover was increased by \( \sim \)40% in HFD-fed TRPM2-deficient mice (Fig. 5D), confirming enhanced insulin sensitivity in these mice. Using 2-[\( ^{14} \)C]deoxyglucose injection during clamps, glucose uptake was measured in individual organs. Insulin-stimulated glucose uptake was increased significantly in skeletal muscle and heart of HFD-fed TRPM2-deficient mice (Fig. 5, E and F). Glucose uptake into white adipose tissue was also elevated in TRPM2-deficient mice (6.5 \( \pm \) 1.2 vs. 3.4 \( \pm \) 0.5 nmol·g\(^{-1}\)·min\(^{-1}\) in WT mice, \( P = 0.02 \)). Hepatic insulin action also increased by \( \sim \)60% in HFD-fed TRPM2-deficient mice, although this difference did not reach statistical significance.

TRPM2 deletion enhances insulin signaling and reduces calmodulin levels in heart. To determine the mechanism of increased glucose metabolism in HFD-fed TRPM2-deficient mice, insulin signaling was measured in heart samples obtained at the end of the clamps. Insulin-stimulated Ser\(^{373}\) phosphorylation of Akt, an important downstream signaling that mediates glucose transport, was increased more than sevenfold in HFD-fed TRPM2-deficient mice (Fig. 5G). Insulin-stimulated IRS-1 tyrosine phosphorylation was also significantly elevated in HFD-fed TRPM2-deficient mice (Fig. 5H). Insulin-mediated phosphorylation of a downstream insulin-signaling protein, GSK-3\( \beta \), was further increased more than threefold in HFD-fed TRPM2-deficient mice (Fig. 5I). Thus, these data demonstrate that TRPM2 deficiency improved insulin signaling and glucose metabolism following high-fat feeding in mice.

To identify a potential mechanism of increased insulin signaling, calmodulin levels were measured in heart and skeletal muscle of age-matched (\( \sim \)6 mo of age) male TRPM2-KO and WT littermates fed chow diet (\( n = 5 \) for each group) and after \( \sim \)4 mo of HFD (\( n = 4 \) for each group). All data are expressed per kg of whole body lean mass measured using \(^{1}H\)-MRS. A: 24-h \( VO_2 \) consumption rates in chow-fed mice. B: 24-h \( VCO_2 \) production rates in chow-fed mice. C: 24-h average \( VO_2 \) consumption and \( VCO_2 \) production rates in chow-fed mice. D: 24-h \( VCO_2 \) production rates in HFD-fed mice. E: 24-h average energy expenditure rates in HFD-fed mice. *\( P < 0.05 \) vs. WT or HFD-fed WT mice.
that SOD activity was not affected by high-fat feeding or TRPM2 deficiency (Fig. 6C).

Next, we examined insulin secretion in vivo by conducting a 2-h hyperglycemic clamp in a separate cohort of TRPM2-deficient and WT mice after 4 mo of high-fat feeding. Plasma glucose levels were quickly raised and maintained at ~300 mg/dl by intravenous infusion of 20% glucose in both groups of mice (Fig. 6D). HFD-fed TRPM2-deficient mice showed markedly lower plasma insulin levels compared with HFD-fed WT mice during hyperglycemic clamps, indicating alterations in glucose-induced insulin secretion in TRPM2-deficient mice (Fig. 6E) (31).

TRPM2 deficiency upregulates metabolic gene expression and attenuates diet-induced inflammation in adipose tissue. To determine the underlying mechanism responsible for dramatic effects of TRPM2 deletion on obesity, energy expenditure, and insulin resistance, metabolic gene expression was measured using quantitative real-time PCR in white adipose tissues obtained from HFD-fed TRPM2-deficient mice and WT littermates. TRPM2 deletion caused a significant upregulation of major lipid metabolic genes, including peroxisome proliferator-activated receptor (PPAR)γ coactivator (PGC)-1α, PGC-1β, and PPARα (Fig. 7A). Adipose protein levels of PGC-1α were also increased in HFD-fed TRPM2-deficient mice (Fig. 7B). Furthermore, TRPM2-deficient mice showed a more than twofold increase in estrogen-related receptor (ERR)α and an ~40% increase in medium-chain acyl-CoA dehydrogenase (MCAD) gene expressions in adipose tissue (Fig. 7A). Adipose expression of the transcription factor regulating mitochondrial biogenesis, mitochondrial transcriptional factor A (TFAM), was increased by ~40% in TRPM2-deficient mice (Fig. 7A). Since TRPM2 is expressed in other organs, including skeletal muscle, our findings do not rule out that partial resistance to diet-induced obesity in TRPM2-deficient mice may be due to alterations in mitochondrial oxidation in other organs, such as skeletal muscle.

Since TRPM2-deficient mice were resistant to diet-induced obesity, we examined the effects of TRPM2 deletion on systemic and local inflammation. Following HFD, circulating monocyte chemoattractant protein-1 levels were markedly re-
duced in TRPM2-deficient mice compared with WT mice (Fig. 7C). In contrast, serum levels of IL-1α, IL-1β, IL-10, keratinocyte-derived chemokine (KC), and TNFα were not significantly altered in TRPM2-deficient mice (data not shown). TRPM2-deficient mice also showed less adipose tissue inflammation, as reflected by significant reductions in macrophage-selective CD68 levels compared with HFD-fed WT mice (Fig. 7D). Adipose expression of F4/80 was increased almost three-fold following HFD in WT mice, but this increase was attenuated in HFD-fed TRPM2-deficient mice (Fig. 7E). Local levels of IL-1β, IL-6, and KC in white adipose tissue were reduced significantly in HFD-fed TRPM2-deficient mice (Fig. 8, A–C). Last, diet-induced hepatic inflammation was blunted in TRPM2-deficient mice, as shown by significant reductions in CD68 and Toll-like receptor 4 (TLR4) expression in liver (Fig. 8, D and E).

DISCUSSION

TRPM2 Ca2+-permeable cation channel was shown recently to regulate insulin secretion and glucose homeostasis in mice (31). Using isolated β-cells, TRPM2-KO mice showed blunted glucose-induced insulin secretion when β-cells were incubated in a high-glucose environment (≥11 mM) (31). With recent evidence implicating a causal relationship between Ca2+ homeostasis, endoplasmic reticulum (ER) stress, and insulin resistance (4, 16, 38), the present study investigated a potential role of TRPM2 in diet-induced insulin resistance. The hyperinsulinemic euglycemic clamp involves an intravenous infusion of exogenous insulin to raise plasma insulin level and maintain a physiological insulin level during the measurement of glucose metabolism. Thus, this experiment directly measures insulin sensitivity and is not affected by alterations in endogenous insulin secretion. This is reflected by a comparable level of plasma insulin that was achieved during the euglycemic clamp in TRPM2-deficient and WT mice. In this setting, our data indicate that TRPM2 deficiency enhances insulin sensitivity in diet-induced obese mice, and this is attributed partly to a significant increase in skeletal muscle and cardiac glucose metabolism.

Previous studies have shown that Ca2+ regulates calmodulin binding to IRS-1 and suppresses downstream phosphatidylinositol 3-kinase-dependent insulin signaling (21). Indeed, calmodulin overexpression reduced insulin-stimulated IRS-1 tyrosine phosphorylation and caused insulin resistance (17). Our present findings indicate that chronic high-fat feeding increases calmodulin levels in heart and skeletal muscle of WT mice, and TRPM2 deficiency attenuates diet-induced increase in calmodulin levels. These effects of TRPM2 on calmodulin were associated with enhanced insulin signaling in TRPM2-deficient mice, which is consistent with a possible role of Ca2+/calmodulin signaling in increased glucose metabolism in these mice. This is demonstrated clearly with significant increases in insulin-stimulated glucose uptake in skeletal muscle, heart, and adipose tissue of TRPM2-deficient mice compared with WT mice following high-fat feeding. However, in chow-fed mice, elevated glucose uptake in heart and adipose tissue does not fully account for increased whole body glucose disposal in TRPM2-deficient mice. This likely suggests that either other insulin-sensitive organs such as different muscle fiber types may contribute to increased whole body glucose turnover or

![Calmodulin levels in Heart](A)

![Calmodulin levels in Muscle](B)

![SOD Activity in Heart](C)

![Hyperglycemic Clamp](D)

![Hyperglycemic Clamp](E)

Fig. 6. A–C: calmodulin levels and superoxide dismutase (SOD) activity in heart and skeletal muscle (gastrocnemius) in TRPM2-KO and WT mice fed chow diet and after 4 mo of HFD. Data represent means ± SE; n = 5 for each group. *P < 0.05 vs. WT-chow mice. D and E: hyperglycemic clamps were performed to assess in vivo insulin secretion in awake TRPM2-KO and WT mice after 4 mo of high-fat feeding. Data represent means ± SE; n = 6 for each group.
The in vivo glucose uptake measurement is not sensitive enough to detect small but significant changes in tissue glucose metabolism in TRPM2-deficient mice.

The TRPM2 calcium-permeable channel, by regulating intracellular calcium concentration \([\text{Ca}^{2+}]_i\), plays an important role in oxidative stress, which is involved in obesity-mediated insulin resistance (7, 9). Therefore, we examined the effects of chronic high-fat feeding in TRPM2-deficient mice. Interestingly, TRPM2-deficient mice became significantly less obese compared with WT mice following 2–4 mo of HFD. After 10 mo of HFD, TRPM2-deficient mice weighted 25% less than WT mice. The metabolic cage study indicates that TRPM2-deficient mice are resistant to diet-induced obesity because of a selective increase in energy expenditure without changes in food intake or physical activity. Furthermore, quantitative real-time PCR analysis found that major adipose genes involved in lipid metabolism and mitochondrial function/biogenesis, such as PGC-1\(\alpha\), PGC-1\(\beta\), PPAR\(\alpha\), ERR\(\alpha\), MCAD, and TFAM, were significantly upregulated in TRPM2-deficient mice. In that regard, PGC-1\(\alpha\), PGC-1\(\beta\), and PPAR\(\alpha\) are known to regulate mitochondrial lipid oxidation in adipose tissue (18). ERR\(\alpha\) was shown recently to be activated by PGC-1\(\alpha\) and mediate transcriptional effects of PGC-1\(\alpha\) on mitochondrial lipid oxidative genes such as MCAD and mitochondrial biogenesis (27, 29). These results collectively suggest that TRPM2 deficiency enhances energy expenditure by increasing mitochondrial function and biogenesis in adipose tissue.

Recent studies have shown that alterations in intracellular \([\text{Ca}^{2+}]_i\) levels and calcium/calmodulin-dependent protein kinase activity modulate mitochondrial biogenesis and glucose metabolism in L6 muscle cells (35). This may involve activation of p38 mitogen-activated protein kinase and glucose transporter 4 biogenesis via the PGC-1\(\alpha\)-dependent pathway (35). Moreover, intracellular release of ER \([\text{Ca}^{2+}]_i\) was shown to stimulate calpain-dependent degradation of PGC-1\(\alpha\), and low intracellular \([\text{Ca}^{2+}]_i\) levels mediated by TRPM2 deficiency may inhibit PGC-1\(\alpha\) degradation, resulting in elevated protein levels (25). Further studies are needed to determine the underlying mechanism by which intracellular \([\text{Ca}^{2+}]_i\) regulates mitochondrial biogenesis and function.

Obesity is a major cause of insulin resistance, and obesity-mediated insulin resistance involves defects in insulin signaling and glucose metabolism in peripheral organs (1, 22). Improved insulin sensitivity in HFD-fed TRPM2-deficient mice was associated with increases in serine phosphorylation of Akt and GSK-3\(\beta\), and insulin stimulates glycogen metabolism by phosphorylating and inactivating GSK-3\(\beta\) through Akt-mediated signaling (3). Recent studies have shown that adipose tissue inflammation plays a major role in obesity-mediated insulin resistance in humans and animal models (5,
33). Since TRPM2-deficient mice were resistant to diet-induced obesity, we examined the effects of TRPM2 deletion on inflammation. Our findings indicate that local inflammation in adipose tissue and liver following HFD was greatly attenuated in TRPM2-deficient mice. TLR4 was shown to be activated by fatty acids to mediate obesity-induced inflammation (28), and reduced TLR4 expression was associated with lower CD68 levels in liver of HFD-fed TRPM2 deficient mice. Although attenuated inflammation may be due to less obesity in HFD-fed TRPM2-deficient mice, we cannot rule out a direct role of adipose genes such as ERRα/H9251, which was shown to be dysregulated in inflammatory arthritis (2). Overall, our findings indicate that resistance to diet-induced obesity is at least partly responsible for reduced inflammation and increased glucose metabolism in TRPM2-deficient mice.

Our observation that TRPM2 deficiency enhances energy expenditure and protects mice from diet-induced obesity, inflammation, and insulin resistance identifies TRPM2 as a novel therapeutic target to treat type 2 diabetes. Although TRPM2 deletion may affect β-cell secretion of insulin, chronic hyperinsulinemia is not necessarily beneficial because it has been shown to exacerbate obesity and cause insulin resistance (23). In that regard, reduced insulin secretion in the face of enhanced insulin sensitivity in selective organs, as with TRPM2 deficiency, may provide a better overall metabolic state.

GRANTS

This study was supported by grants from the US Public Health Service (R01-DK-80756 and U24-DK-093000 to J.K. Kim), American Diabetes Association (7-07-RA-80 to J.K. Kim), and American Heart Association (0855492D to J.K. Kim), a grant from the Pennsylvania Department of Health using Tobacco Settlement Funds (J.K. Kim), a Children’s Miracle Network Grant (to W. Zhang), and an endowment from the Four Diamonds Fund of the Pennsylvania State University (to B.A. Miller). Part of this study was conducted by the Pennsylvania State-Hershey Diabetes and Obesity Institute Core Facility.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

TRPM2 REGULATES GLUCOSE METABOLISM

10. E816


