Pharmacological inhibition of Kv1.3 fails to modulate insulin sensitivity in diabetic mice or human insulin-sensitive tissues

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The effects of Kv1.3 ablation and pharmacological inhibition on insulin sensitivity suggest that this channel could be an attractive target for the treatment of type 2 diabetes. Therefore, the aims of the present study were to 1) determine whether Kv1.3 is functionally expressed in human and mouse peripheral insulin-sensitive tissues and whether inhibition of Kv1.3 alters glucose uptake in these tissues, 2) elucidate whether Kv1.3 is expressed in human skeletal muscle and adipose from healthy and/or type 2 diabetic individuals, 3) assess whether acute or chronic inhibition of Kv1.3 is sufficient to improve insulin sensitivity in hyperglycemic mice, and 4) determine whether acute central inhibition of Kv1.3 alters insulin sensitivity.

EXPERIMENTAL PROCEDURES

Whole cell patch clamp. Ionic currents were measured from human skeletal muscle cell (SkMC) and mouse 3T3-L1 adipocytes using the whole cell configuration of the patch clamp technique. Currents were recorded using an Axopatch 200B

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patch clamp amplifier, Digidata 1322A interface, and Clampex 9.0 software (Molecular Devices, Sunnyvale, CA). Electrodes were fabricated from borosilicate filament glass (1.5 OD \times 0.86 ID; Warner Instruments, Hamden, CT) using a Sutter Instruments P-97 Flaming/Brown micropipette puller. Cells were plated onto 25-mm poly-l-lysine coated glass coverslips and cultured \(\geq 24\) h prior to use. The coverslips were secured in an Attofluor chamber (Invitrogen), which was mounted to the stage of a Nikon TE2000S inverted microscope. The extracellular (bath) solution contained (in mM) 129 NaCl, 5 NaHCO\(_3\), 4.7 KCl, 1.2 KH\(_2\)PO\(_4\), 2 CaCl\(_2\), 1.2 MgSO\(_4\), 10 HEPES, and 10 glucose, pH 7.4, with NaOH. The intracellular pipette solution contained (in mM) 50 K\(_2\)SO\(_4\), 50 KCl, 5 MgCl\(_2\), 0.5 EGTA, and 10 HEPES, pH 7.2, with KOH. Only cells in which stable whole cell access was achieved with seal resistances \(>1\) Gohm were utilized for experiments. Cells were held at a holding potential of \(-70\) mV, and the small (\(-2\) mV) liquid junction potential was not corrected. Experiments were performed at room temperature.

**Human skeletal muscle cell culture and glucose uptake experiments.** For electrophysiological experiments, SkMCs (CC-2561; Lonza, Rockville, MD) were cultured to confluence in a 75-cm\(^2\) cell culture flask in skeletal muscle basal medium (Lonza). Once confluence was reached, cells were grown in skeletal muscle growth medium (Lonza) for 5–7 days until myotubes were visible. Cells were removed from flasks using 0.5% trypsin-EDTA solution, plated onto 25-mm poly-l-lysine-coated glass coverslips, and cultured \(\geq 24\) h prior to use. For glucose uptake experiments, SkMCs were plated in 96-well Cytostart plates (PerkinElmer, Waltham, MA) at 10,000 cells/well for a final concentration of 100 \(\mu\)M unlabeled 2-deoxy-glucose [1 \(\mu\)Ci/ml in a final concentration of 100 \(\mu\)M unlabeled 2-deoxy-glucose (PerkinElmer)] was added for 30 min. Solutions were removed and cells washed once with 0.15 ml of assay buffer and reapplied. Each well was counted for 2 min using a 1540 Trilux microbeta counter.

**Transient transfection of Kv1.3 into Chinese hamster ovary cells.** Chinese hamster ovary (CHO)-K1 cells (ATCC, Manassas, VA) were cultured in DMEM F-12 (Gibco) supplemented with 10% FBS, 50 U/ml penicillin, and 50 \(\mu\)g/ml streptomycin sulfate. CHO cells were transfected with cDNA encoding the human Kv1.3 (hKv1.3) channel, using the Mirus CHO transfection reagent kit (Mirus Bio, Madison, WI) according to the manufacturer’s instructions. Equal amounts of cDNA encoding hKv1.3 and green fluorescent protein were added to a 75-cm\(^2\) flask containing 60–80% confluent CHO cells 24 h prior to experimentation. Control CHO cells underwent the same transfection procedure without addition of hKv1.3 cDNA to the flask.

**Western blotting.** Lysates of human adipose (control and type 2 diabetic donors) and skeletal muscle (control and type 2 diabetic donors; total protein or membrane only) were purchased from ProSci (Poway, CA). CHO cells transfected with Kv1.3 and control CHO cells were lysed and normalized to a concentration of 2 mg/ml in RIPA-SDS-PAGE sample buffer. Five microliters of Precision Plus Protein Dual Color Standards (no. 161-0374; Bio-Rad Laboratories) was loaded into the first lane of a Bio-Rad 4–15% Tris-HCl gel (cat. no. 161-1104), and 30 \(\mu\)g of the cell and tissue lysates were loaded into the rest of the lanes. The gel was run at 150 V for 1 h in Laemmli running buffer (250 mM Tris base, 1,920 mM glycine, 1% SDS, pH 8.3) and transferred overnight at 30 V onto a 6.5 \times 9 cm piece of polyvinylidene fluoride membrane (cat. no. 162-0174; Bio-Rad Laboratories). The transfer buffer contained 250 mM Tris base and 1,920 mM glycine. The membrane was rinsed once in Tris-buffered saline with Tween 20 (TBST; Cell Signaling Technology) and then blocked for 1 h in 20 ml of blocking solution (5% dry milk in TBST), followed by another rinse in TBST. The membrane was incubated in 10 ml of primary antibody (NeuroMab Anti-Kv1.3, no. 70-009) at 1 \(\mu\)g/ml in 3% BSA-TBST for 1 h. After three rinses in TBST, the membrane was incubated in ECL rabbit IgG and HRP-linked whole antibody (donkey, cat. no. NA934; Amersham) at 1:5,000 dilution for 1 h. The membrane was rinsed an additional three times in TBST, incubated in Pierce ECL Western Blotting Reagent, and imaged on a Fujifilm LAS-3000 imaging system. The same protocol was followed for the loading control reprobe using goat polyclonal anti-GAPDH primary antibody (sc-20357; Santa Cruz Biotechnology) at 1 \(\mu\)g/ml and donkey anti-goat secondary antibody (sc-2020; Santa Cruz Biotechnology) at 1:5,000 dilution.

**In vivo determination of insulin sensitivity.** All animals were housed in an Association for Assessment of Laboratory Animal Care-accredited facility, and all experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Pfizer. Animals were maintained on a 50,000 cells/well. One to two days later, cells were washed twice with assay buffer that contained (in mM) 130 NaCl, 5 KCl, 1.3 CaCl\(_2\), 1.3 MgSO\(_4\), 10 Na\(_2\)HPO\(_4\), 2 sodium pyruvate, and 0.1% BSA, pH 7.4, and incubated for 2–4 h in assay buffer prior to a 30-min drug incubation. One or 100 nM insulin was added to controls for 30 min. Trace 2-(14C)-1-deoxyglucose [1 \(\mu\)Ci/ml in a final concentration of 100 \(\mu\)M unlabeled 2-deoxy-glucose (PerkinElmer)] was added for 30 min. Solutions were removed and cells washed once with 0.15 ml of assay buffer and reapplied. Each well was counted for 2 min using a 1540 Trilux microbeta counter.
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For single-dose (acute) studies, male db/db mice (12–13 wk old) maintained on a chow diet were dosed with MgTX via ip injection or PAP-1 orally. PAP-1 was dissolved in peanut oil at a concentration of 6 mg/ml, whereas MgTX was dissolved in 0.1% BSA-0.9% NaCl. Two hours after dosing, baseline glucose was measured via tail snip, and mice were dosed with insulin (1.0 U/kg). Blood glucose measurements were taken from each animal at 0.25, 0.5, 1, 2, and 4 h post-insulin challenge. For multiple-dose (chronic) studies, male obese (ob/ob) mice with C57Bl/6J backgrounds (5–6 wk of age) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed four animals/cage. ob/ob Mice were dosed with peanut oil (Sigma), 0.5% methocellulose-water (MC), PAP-1, or thiazolidinedione (TZD; darglitazone) for 5 days. PAP-1 and TZD were dissolved in peanut oil and MC, respectively, at a volume of 5 mL/kg for oral (PO) dosing. PAP-1 and peanut oil were administered twice a day (bid). TZD and MC were administered in the morning, and those same animals were given MC in the evening. To determine plasma drug levels, animals were bled via retroorbital method 6 h post-morning dose on days 1, 3, and 4 and 3 h post-morning dose on days 2 and 5. Blood was collected in EDTA capillary tubes. Prior to the last dose on day 5, glucose measurements were obtained by tail snip to determine effect of repeated dosing on glucose levels. After the last dose, mice were fasted for 6 h. A blood sample via tail snip was obtained to establish baseline glucose levels. The mice were then dosed with either vehicle or insulin at 1.0 U/kg. Blood glucose measurements were taken from each animal at 0.25, 0.5, 1, 2, and 4 h post-insulin challenge. For all ITT experiments, glucose levels during the course of the experiment were analyzed using area under the curve (AUC) analysis. These data values were used to conduct statistical analysis between groups.

For central Kv1.3 inhibition, conscious male ob/ob mice (6–7 wk old) were given a single 5-μl intracerebroventricular (icv) injection of either sterile aprotic 0.9% saline or MgTX. Solutions were administered using a 20-μl gas-tight Hamilton syringe and 27-gauge needle with a fixed stopper. MgTX. Solutions were administered using a 20-μl gas-tight Hamilton syringe and 27-gauge needle with a fixed stopper.

Measurement of PAP-1 concentration in plasma and MgTX concentration in cerebral spinal fluid. PAP-1-containing plasma samples were stored at −20°C before analysis. Plasma samples (10 μl) were extracted using protein precipitation extraction with acetonitrile (100 μl) containing 250 ng/ml internal standard (a Pfizer propriety compound). The mixture was vortexed for 1 min and centrifuged at 3,000 rpm. The supernatant was injected onto a high-performance liquid chromatography (HPLC) column for analysis. For detection of PAP-1 levels in whole brain, brains were weighed and added to PBS buffer at a ratio of 1:4 (brain weight-PBS buffer) and homogenized. For MgTX analysis, standard samples were prepared in artificial cerebral spinal fluid (CSF) with the concentration range of 1.71 to 875 nM. An aliquot of 100 μl of MgTX in artificial CSF (147 mM NaCl, 1.3 mM CaCl2, 2.7 mM KCl, 1 mM MgCl2) was mixed with 100 μl of buffer solution [5 mM ammonium acetate with 0.1% formic acid-methanol (9:1)] before analysis. Replicates of CSF samples from the same time point were combined, aliquoted, and mixed with buffer before analysis.

HPLC gradient separations were performed on a Shimadzu 10ADvp Binary HPLC system (Shimadzu Scientific Instruments, Columbia, MD). Sample injection was performed with a CTC PAL autosampler (LEAP Technology). A gradient separation was performed using mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile). The gradient range was from 5% mobile phase B to 95% mobile phase B. Chromatographic separations were performed on a Phenomenex (Torance, CA) C18 2.1 × 30 mm, 5-μm HPLC column for PAP-1 analysis and on a Halo Amide-RP 3 × 30 mm, 2.7-μm column (Mac_Mod Analytical, Chadds Ford, PA). The mobile phase flow rate was 0.3 ml/min for PAP-1 analysis and 0.6 ml/min for MgTX analysis. The injection volume was 5 μl.

PAP-1 and MgTX were detected and quantified by tandem mass spectrometry, which was performed on a Sciex API 4000 system equipped with a turbo-ion spray source (Applied Biosystems, Foster City, CA) and operated in positive ion mode. Monitoring-Reaction-Monitoring was conducted using the ion transition 351.1 → 149.1 for PAP-1 and 836.6 → 187.2 for MgTX. The instrument operation and data analysis were performed with Analyst version 1.4 (Applied Biosystems).

Data analysis. Data are presented as means ± SE. Data were analyzed using GraphPad Prism software or Excel and the appropriate statistical analysis (Student’s t-test or 1-way ANOVA). In all figures, P < 0.05.

RESULTS

Kv1.3-specific blockers do not alter K+ currents or glucose uptake in human SkMC or mouse 3T3-L1 adipocytes. Electrophysiological recordings of voltage-gated K+ channel currents were performed on human SkMC and mouse 3T3-L1 adipocytes to determine the contribution of the Kv1.3 channel to the generation of voltage-gated K+ currents in these cells. Depolarization of SkMC from a holding potential of −70 to +50 mV for 250 s generated robust delayed rectifier currents (13.1 ± 4 pA/pF peak end-pulse current at +50 mV; n = 6). To investigate the contribution of Kv1.3 to the generation of these currents, cells were treated with the small-molecule Kv1.3 inhibitor PAP-1 (22). Since PAP-1 is a use-dependent blocker of Kv1.3, binding only to the inactivated state of the channel (22), a pulse protocol designed to drive the channel into the inactivated state was utilized for all blockers tested. Cells were held at a holding potential of −70 mV and depolarized to +40 mV for 2 s every minute for 10 min, with currents evoked during the last test pulse used for analysis. Treatment of SkMC with 10 nM PAP-1, a concentration fivefold greater than the IC50 for Kv1.3 channels, had no effect on the peak amplitude of the voltage-gated currents (peak end-pulse current = 104.7 ± 3.0% of control, n = 4; Fig. 1, A and B). Voltage-gated
K^+ currents from SkMCs were also unaltered by treatment with 10 nM MgTX (106.5 ± 11.2% of control, n = 5; Fig. 1 A and B). Unlike the Kv1.3-specific blockers, the nonselective voltage-gated K^+ channel blocker tetraethylammonium (TEA; 10 mM) significantly decreased these currents (29.5 ± 1.4% of control, P < 0.05, n = 3; Fig. 1 A and B), suggesting that the measured currents were generated by activation of voltage-gated K^+ channels.

To confirm that Kv1.3 currents could be inhibited by PAP-1 under the recording conditions used for these studies, the effect of PAP-1 on voltage-gated K^+ currents elicited from Kv1.3-overexpressing CHO cells was measured. CHO cells were transiently transfected with a human Kv1.3-encoding plasmid 24 h prior to electrophysiological recording. Kv1.3-CHO cells were depolarized from −70 mV to +40 mV for 2 s every minute for 10 min in the presence of 10 and 100 nM PAP-1. Treatment with 10 nM PAP-1 for 10 min inhibited the Kv1.3 current by ∼50% at the end of the depolarizing pulse, whereas 100 nM PAP-1 caused an almost complete inhibition of the current (Fig. 1 C).

In contrast to the robust outward currents observed in SkMCs, currents elicited from 3T3-L1 adipocytes were largely inward currents, with little outward current generated at physiologically relevant membrane potentials (Fig. 2 A and B). Specifically, the peak inward current generated by 3T3-L1 cells at −100 mV was −8.1 ± 1.2 pA/pF, whereas the peak outward current generated at +40 mV measured 4.4 ± 1.9 pA/pF (n = 6). These currents were nearly linear and resembled a leak current rather than a voltage-gated K^+-selective current. On average, outward currents were generated only at membrane potentials positive to −5 mV (Fig. 2 B).

To determine whether Kv1.3 channels serve to regulate glucose utilization, the effects of Kv1.3 blockers on glucose uptake into SkMC and 3T3-L1 adipocytes was assessed. In SkMCs, insulin (30 nM) induced a 64.6 ± 8.8% increase in glucose uptake compared with the uptake in the absence of insulin (n = 3, P < 0.05; Fig. 3 A). In contrast, treatment of cells with TEA, PAP-1, or MgTX was unable to significantly increase glucose uptake above basal levels. Specifically, the change in glucose uptake relative to basal was 26.9 ± 7.7% in the presence of 10 nM TEA, 25.8 ± 14.8% in 10 mM PAP-1, and 11.9 ± 8.4% following treatment with 10 mM MgTX (P > 0.05; Fig. 3 A). TEA, PAP-1, and MgTX also failed to alter glucose uptake in the presence of insulin (% uptake = 103.2 ± 11.1, 94.6 ± 8.1, and 81.3 ± 18.1%, respectively, of the response to 30 nM insulin, P > 0.05; Fig. 3 C). Similarly, treatment of 3T3-L1 adipocytes with insulin generated robust glucose uptake (Fig. 3 B). Glucose uptake increased 91.7 ± 41.8 and 259.4 ± 99.5% above basal in the presence of 1 and 100 nM insulin, respectively (n = 3–4, P < 0.05; Fig. 3 B). However, treatment of 3T3-L1 cells with TEA, PAP-1, or MgTX did not modulate glucose uptake. Glucose uptake was −0.15 ± 0.01% of basal in the presence of 10 nM TEA, 11.8 ± 1.0% of basal when exposed to 10 nM PAP-1, and 2.4 ± 0.2% of basal in the presence of 10 nM MgTX (Fig. 3 B). TEA, PAP-1, and MgTX also failed to alter glucose uptake in the presence of insulin (% uptake = 94.9 ± 13.9, 98.4 ± 15.0, and
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To investigate the expression of Kv1.3 in native human tissue involved in peripheral insulin sensitivity, Kv1.3 expression was measured in lysates of human skeletal muscle and adipose from nondiabetic and diabetic individuals using a Kv1.3-specific antibody and Western blotting. CHO cells transiently transfected with Kv1.3 were utilized as a positive control for Kv1.3 expression, whereas untransfected CHO cells served as a negative control. As shown in Fig. 4, Kv1.3 expression was readily detected in Kv1.3-transfected CHO cells, whereas expression was not observed in untransfected cells, suggesting specificity of this antibody for Kv1.3. To determine Kv1.3 expression in peripheral insulin-sensitive tissues, a total of three Western blots were performed on lysates from two different normal and type 2 diabetic donors for each tissue tested. In contrast to the Kv1.3 expression observed in the positive control, expression of Kv1.3 was not observed in any sample of human skeletal muscle or adipose tested, suggesting the absence of Kv1.3 expression in these peripheral insulin-sensitive tissues in humans.

Effect of Kv1.3 inhibition on insulin sensitivity of diabetic mouse models measured in vivo. The effect of Kv1.3 inhibition on insulin sensitivity in vivo was measured using multiple inhibitors, dosing paradigms, and diabetic models. To confirm the findings of Xu et al. (31), which demonstrated a significant increase in insulin sensitivity in multiple mouse strains in response to a single dose of MgTX, db/db mice were given a single ip injection of MgTX (0.5 μg/g), followed 2 h later by an ITT. No effect of MgTX on insulin sensitivity was observed despite the 50-fold higher dose of MgTX used in the present study (glucose AUC = 882 ± 86 mg/dl for vehicle vs. 1,050 ± 47 mg/dl for MgTX, n = 8, P > 0.05; Fig. 5, A and B). In addition to MgTX, the small-molecule Kv1.3 inhibitor PAP-1 was utilized to investigate the effects of acute Kv1.3 inhibition by a nonpeptide inhibitor, which binds to a different region of Kv1.3 than MgTX. A single dose pharmacokinetic study revealed that orally dosed PAP-1 achieves near-maximal plasma concentration at 2 h, which is sustained between 2 and 4 h postdose (Fig. 5C) but shows significant brain impairment. The 50 mg/kg single oral dose of PAP-1 used in these studies yielded a peak free plasma concentration of 111 nM, which is 55-fold greater than the IC_{50} for inhibition of Kv1.3. Similar to MgTX, no effect on insulin sensitivity was observed following acute treatment with PAP-1 (glucose AUC = 1,106 ± 76 mg/dl for vehicle vs. 1,087 ± 77 for PAP-1, respectively, n = 8, P > 0.05; Fig. 5, D and E).

To determine whether chronic inhibition of Kv1.3 is required for modulation of insulin sensitivity, ob/ob mice were dosed orally for 5 days with PAP-1, and plasma glucose levels were measured on day 6 in response to an ITT. Given that PAP-1 is ~25-fold selective for Kv1.3 over Kv1.5, the dose of PAP-1 used in these studies (10 mg/kg bid) was chosen such that the free drug concentration in the plasma would never exceed a level that could block other Kv channels. To verify this, the free PAP-1 concentration was measured on each day of the study. As a positive control, a separate group of animals were orally dosed with darglitazone (3 mg/kg qd) for 5 days.

Following a 6-h fast, both drug and control groups were treated with insulin (1 U/kg), and plasma glucose was measured at multiple time points over 4 h. As expected, treatment of ob/ob mice for 5 days with darglitazone lowered basal glucose levels. Specifically, darglitazone treatment decreased glucose by 58.5 ± 3.2% (P < 0.05, n = 10) compared with vehicle (Fig. 6, A and B). In contrast, neither the fasting plasma glucose nor the glucose response to insulin of the group treated with 10 mg/kg PAP-1 was altered significantly from control (13.1 ± 1.4% decrease compared with vehicle, n = 10; Fig. 6, A and B) despite achieving free PAP-1 concentrations that would be expected to block Kv1.3 channels in the periphery (Fig. 6C). ITT experiments were also performed in ob/ob mice treated with higher doses of PAP-1 (50 mg/kg every 12 h for 5 days), which generated free plasma drug concentrations no longer specific for Kv1.3 (Fig. 6F). In contrast to mice given 10 mg/kg PAP-1, the plasma glucose levels of animals in the 50 mg/kg group decreased by 22.3 ± 1.8% (P < 0.05, n = 12; Fig. 6, D and E).

Acute central Kv1.3 inhibition is not sufficient to improve insulin sensitivity in ob/ob mice. To determine whether acute inhibition of Kv1.3 in the brain is capable of modulating insulin sensitivity in vivo, ob/ob mice were administered MgTX centrally via icv injection 10 min prior to an ITT. MgTX was utilized for these studies since it is soluble in the aqueous solution used for injection into the brain. The dose of MgTX injected (5 μl of a 473 nM MgTX solution) yielded a maximum concentration of 30 nM MgTX and sustained concentrations in excess of 400 pM in the CSF for >120 min (Fig. 7C). These concentrations are 8–600 times greater...
than the IC_{50} for Kv1.3 (8). Intracerebroventricular injection of MgTX caused a 24.5 ± 0.1% decrease in insulin sensitivity, which was observed as an increase in the glucose AUC measurement (P < 0.05, n = 9–10; Fig. 7, A and B).

**DISCUSSION**

The voltage-gated K\(^+\) channel Kv1.3 has been proposed as a novel target for the treatment of diabetes (5). In humans, a single nucleotide polymorphism in the promoter of the Kv1.3 gene has been linked to impaired glucose tolerance and decreased insulin sensitivity (23), although a follow-up study by this same group failed to replicate these findings (10). Previous studies in rodents have suggested that Kv1.3 is expressed in several insulin-sensitive tissues, including adipose, skeletal muscle (31), and the olfactory bulb (7); however, direct electrophysiological recording of Kv1.3 currents was never demonstrated, and data from human tissues is lacking. In addition, Kv1.3-knockout mice are resistant to diet-induced obesity and show enhanced insulin sensitivity (30, 31), although a clear mechanistic basis for this phenotype has not been identified. In the present study, a multidisciplinary strategy utilizing distinct pharmacological inhibitors, human insulin-sensitive tissues, multiple diabetic mouse models, in vivo and in vitro assays of insulin sensitivity, and direct electrophysiological recording of voltage-gated K\(^+\) channel activity was utilized to evaluate the role of Kv1.3 in regulating insulin sensitivity of peripheral insulin-sensitive tissues. The results suggest that 1) Kv1.3 is not functionally expressed in human SkMC or mouse 3T3-L1 adipocytes, 2) specific blocking of Kv1.3 with PAP-1 or MgTX does not modulate basal or insulin-induced glucose uptake in human SkMC or mouse 3T3-L1 adipocytes, 3) Kv1.3 is not expressed in human adipose or skeletal muscle from healthy or type 2 diabetic donors, 4) insulin sensitivity of diabetic mouse models is not altered by acute or chronic treatment with pharmacologically relevant doses of PAP-1 or MgTX, and 5) insulin sensitivity of ob/ob mice is improved with chronic, suprapharmacological, nonselective doses of PAP-1 and dampened with acute central administration of MgTX.

Kv1.3 has been shown to regulate glucose uptake in mouse peripheral insulin-sensitive tissues (31). Plasma membrane expression of Kv1.3 has been demonstrated in mouse 3T3-L1 adipocytes using immunofluorescence, and inhibition of Kv1.3...
with MgTX has been shown to increase GLUT4 translocation to the plasma membrane of mouse skeletal muscle, adipose, and 3T3-L1 adipocytes and glucose uptake into mouse adipose tissue (14, 30, 31). Although Xu et al. (31) have reported expression of Kv1.3 protein in 3T3-L1 adipocytes and shown that treatment with Psora-4 (a PAP-1 like compound with lower selectivity than PAP-1) increases GLUT4 translocation in these cells, electrophysiological characterization of the channel and measurement of glucose uptake into 3T3-L1 adipocytes was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells. Although the present study did not investigate the effect of Kv1.3 inhibition on glucose uptake into mouse adipose tissue, the expression of the channel in human adipose tissue was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells. Although the present study did not investigate the effect of Kv1.3 inhibition on glucose uptake into mouse adipose tissue, the expression of the channel in human adipose tissue was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells. Although the present study did not investigate the effect of Kv1.3 inhibition on glucose uptake into mouse adipose tissue, the expression of the channel in human adipose tissue was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells. Although the present study did not investigate the effect of Kv1.3 inhibition on glucose uptake into mouse adipose tissue, the expression of the channel in human adipose tissue was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells. Although the present study did not investigate the effect of Kv1.3 inhibition on glucose uptake into mouse adipose tissue, the expression of the channel in human adipose tissue was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells. Although the present study did not investigate the effect of Kv1.3 inhibition on glucose uptake into mouse adipose tissue, the expression of the channel in human adipose tissue was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells. Although the present study did not investigate the effect of Kv1.3 inhibition on glucose uptake into mouse adipose tissue, the expression of the channel in human adipose tissue was never reported by this group. In the present study, there was no effect of Kv1.3 inhibition on glucose uptake into these cells. Consistent with this observation, voltage-gated K⁺ currents are not generated at physiologically relevant membrane potentials in these cells.

To determine whether a role for Kv1.3 exists in human skeletal muscle, the sensitivity of voltage-gated K⁺ currents and glucose uptake to Kv1.3 inhibitors in human skeletal muscle cells was measured. Voltage-gated K⁺ currents were found to be insensitive to Kv1.3-specific concentrations of PAP-1, whereas currents generated from Kv1.3-expressing CHO cells under identical recording conditions were found to be sensitive to PAP-1. It should be noted that the experiments measuring inhibition of Kv1.3 current by PAP-1 in CHO cells likely overestimated the concentration of PAP-1 required to inhibit the channel. In these experiments PAP-1 was present for only 10 min, and the experiments utilized short (2 s) depolarizing pulses to inactivate Kv1.3, thus allowing the use-dependent PAP-1 to bind the inactivated channel. Longer duration depolarization of cells in 10 nM PAP-1 results in a greater inhibition of the channel than shorter pulses (22), since a greater fraction of the channels is driven to inactivation. Therefore, although these experiments were not designed to determine the pharmacology of PAP-1 against Kv1.3, which has already been well established in the literature (22), these experiments are sufficient to show that PAP-1 is capable of inhibiting Kv1.3 under the conditions employed in these studies. Furthermore, whereas ~50% inhibition of Kv1.3 current by 10 nM PAP-1 was observed in overexpressing CHO cells, no inhibition of current was observed in human skeletal muscle. Treatment with suprapharmacological concentrations of MgTX (10 nM) also failed to inhibit these currents, although treatment with the nonspecific K⁺ channel blocker TEA was able to decrease the currents in these cells.
uptake into human SkMC was not altered by Kv1.3 inhibition in either the presence or absence of insulin. Consistent with these observations, Kv1.3 expression was not observed in human skeletal muscle lysates from healthy or type 2 diabetic donors. These findings suggest that Kv1.3 is not expressed in human skeletal muscle and does not have a role in modulating the sensitivity of human skeletal muscle to insulin.

The Kv1 family of potassium channels consists of eight distinct subunits (Kv1.1–1.8) that can form homotetrameric or heterotetrameric assemblies (11). The formation of heterotetrameric channels can significantly alter the kinetic and pharmacological properties of the channel. For example, Kv1.3 has been shown to form functional heterotetrameric channels with Kv1.5 in mouse macrophages (26). When measured in a heterologous system expressing either homotetrameric Kv1.3 or heterotetrameric Kv1.3/Kv1.5 channels, the heterotetrameric channels were found to be ~100-fold less sensitive to blocking by MgTX than the homotetrameric channels (26). Thus, if functional Kv1.3 channels exist as heterotetrameric assemblies in the systems examined in our study, it is possible that the channels would not have been inhibited by PAP-1 or MgTX. However, this appears unlikely for two reasons. First, 10 nM MgTX was employed in all ex vivo studies, which is 200-fold greater than the IC50 against homotetrameric Kv1.3 [50 pM (8)]. Thus, based on the study by Vicente et al. (26), the concentration of MgTX employed in ex vivo studies would still be 100-fold greater than the IC50 for heterotetrameric channels composed of Kv1.3 and Kv1.5 and should therefore have revealed a MgTX-sensitive component if one was present. Second, even if Kv1.3 heterotetramers were expressed in human adipose and skeletal muscle, the Kv1.3 component of those channels would have been identified in Western blotting.

Fig. 6. Measurement of plasma glucose concentration in response to an ITT in ob/ob mice treated for 5 days with low- and high-dose PAP-1. A and D: plasma glucose concentration of 2 control and 2 drug-treated groups of mice measured 0–4 h after ip injection with 1.0 U/kg insulin. The PAP-1-treated group in A received 10 mg/kg PAP-1 bid orally, whereas the PAP-1-treated group in D received 50 mg/kg PAP-1 bid orally. Both darglitazone-treated groups received 3 mg/kg qd orally. B and E: mean plasma glucose levels of control and drug-treated groups of mice measured in response to an ITT, as illustrated in A and D. Mean glucose levels were calculated as the AUC of data generated in the ITT experiments illustrated in A and D. *P < 0.05. C and F: free plasma and brain concentration of PAP-1 measured on the indicated day of dosing. The numbers above each data point represent the mean free concentration in nM. On days 1, 3, and 4, animals were bled 6 h post-morning dose. On days 2 and 5, animals were bled 3 h post-morning dose.
experiments. However, Kv1.3 expression was not observed in the skeletal muscle or adipose samples from healthy or type 2 diabetic donors profiled in the present study. In contrast, in the present studies, the dosing paradigm used (5 days) may not have been long enough to generate changes in the activity of inflammatory pathways. However, in Kv1.3-knockout animals, chronic inhibition of inflammatory pathways may prevent an inflammation-mediated decrease in insulin sensitivity (5), thus accounting for the enhanced insulin sensitivity observed in the knockout animals (31).

Genetic ablation of Kv1.3 significantly increases basal metabolic rate in mice (30). In addition, deletion of the gene encoding Kv1.3 prevents weight gain associated with genetic ablation of the melanocortin-4 receptor, a critical part of the hypothalamic pathway involved in the control of metabolism and food intake (24). These observations obtained from genetic mouse models suggest that Kv1.3 may play a role in the CNS to regulate overall energy metabolism. To determine whether acute inhibition of Kv1.3 in the brain is capable of increasing insulin sensitivity, MgTX was administered directly into the hypothalamic pathway involved in the control of metabolism and food intake (24). These observations obtained from genetic mouse models suggest that Kv1.3 may play a role in the CNS to regulate overall energy metabolism. To determine whether acute inhibition of Kv1.3 in the brain is capable of increasing insulin sensitivity, MgTX was administered directly into the hypothalamic pathway involved in the control of metabolism and food intake (24). These observations obtained from genetic mouse models suggest that Kv1.3 may play a role in the CNS to regulate overall energy metabolism. To determine whether acute inhibition of Kv1.3 in the brain is capable of increasing insulin sensitivity, MgTX was administered directly into the hypothalamic pathway involved in the control of metabolism and food intake (24). These observations obtained from genetic mouse models suggest that Kv1.3 may play a role in the CNS to regulate overall energy metabolism. To determine whether acute inhibition of Kv1.3 in the brain is capable of increasing insulin sensitivity, MgTX was administered directly into the hypothalamic pathway involved in the control of metabolism and food intake (24).
Kv1.3 DOES NOT MODULATE PERIPHERAL INSULIN SENSITIVITY

CSF of ob/ob mice via icv injection. MgTX was chosen for these studies since the concentration of PAP-1 required for injection exceeded the solubility in vehicles compatible with in vivo experiments. Surprisingly, acute central MgTX exposure led to a decrease in insulin sensitivity in our studies. It is possible that longer-term central exposure to Kv1.3 blockers is required to increase insulin sensitivity. Alternatively, Kv1.3 activity within the olfactory bulb may be responsible for the effects of Kv1.3 on insulin sensitivity. Specifically, the olfactory bulb exhibits high insulin receptor kinase expression, the activation of which results in tyrosine phosphorylation of Kv1.3 and a reduction in channel activity (7). Although it has been shown that intranasal delivery of insulin does not alter peripheral glucose homeostasis despite causing Kv1.3 phosphorylation in the olfactory bulb (16), it is possible that direct pharmacological inhibition of Kv1.3 in this region would be capable of altering peripheral glucose homeostasis. Such investigation would need to utilize intranasal delivery of a Kv1.3 inhibitor, since icv injections such as those employed in the present study are unlikely to generate sufficient drug exposure in the olfactory bulb (18). In support of a role for the olfactory bulb as the locus for the effects of Kv1.3 on energy homeostasis, Tucker et al. (25) have demonstrated that Kv1.3-knockout animals are no longer resistant to diet-induced weight gain and display a decreased metabolic rate following bilateral olfactory bulbectomy.

In addition to a potential role for Kv1.3 in the olfactory bulb, Kv1.3 may function within the peripheral nervous system to regulate metabolism. Kv1.3 has been demonstrated to be functionally expressed in postganglionic sympathetic neurons, where it contributes to maintenance of the resting membrane potential (6). Inhibition of Kv1.3 with MgTX was found to increase membrane depolarization, resulting in increased nor-adrenergine release from these neurons. Thus, genetic ablation of Kv1.3 would be expected to result in enhanced sympathetic tone, potentially accounting for the increased metabolic rate observed in Kv1.3-knockout animals (30).

The present findings contrast with previously published work that demonstrated an increase in peripheral insulin sensitivity in response to Kv1.3 inhibition. There are several potential explanations for these discrepancies. The present studies were focused largely on elucidating the role of Kv1.3 in human insulin-sensitive tissues, as opposed to the studies of Li et al. (14) and Xu et al. (30, 31), which utilized mouse tissues exclusively. In addition, Kv1.3 activity has never been measured directly in any of the previous studies that suggested a role for Kv1.3 in the modulation of insulin sensitivity (14, 30, 31). Rather, these studies have assumed that Kv1.3 was selectively inhibited in response to treatment with a pharmacological agent, an assumption often combined with indirect readouts of insulin sensitivity (i.e., GLUT4 translocation) (14, 31) to draw conclusions about the role of Kv1.3. In contrast, the present study is the first to directly measure voltage-gated K + currents in peripheral insulin-sensitive tissues and show that these currents are insensitive to blocking by Kv1.3 inhibitors. In addition, the present study utilized pharmacokinetic measurements of PAP-1 to ensure that Kv1.3-selective concentrations of the compound were achieved in in vivo studies, whereas previous studies utilized only MgTX (31) and did not measure exposure levels, thus leaving open the possibility that nonspecific inhibitor concentrations were generated in these experiments.

Overall, the present findings are not supportive of a role for Kv1.3 in the regulation of glucose homeostasis or peripheral insulin sensitivity in mice or humans. These findings do not preclude a role for chronic central modulation of Kv1.3 in the regulation of food intake, energy expenditure, or effects on glucose homeostasis that are secondary to weight loss, which may account for the phenotype of Kv1.3-knockout mice.

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DISCLOSURES

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

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