Insulin and SGK1 reduce the function of Na\(^+\)/monocarboxylate transporter 1 (SMCT1/SLC5A8)

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López-Barradas A, González-Cid T, Vázquez N, Gavi-Maza M, Reyes-Camacho A, Velázquez-Villegas LA, Ramírez V, Zandi-Nejad K, Mount DB, Torres N, Tovar AR, Romero MF, Gamba G, Plata C. Insulin and SGK1 reduce the function of Na\(^+\)/monocarboxylate transporter 1 (SMCT1/SLC5A8). Am J Physiol Cell Physiol 311: C720–C734, 2016. First published August 3, 2016; doi:10.1152/ajpcell.00104.2015.—SMCTs move several important fuel molecules that are involved in lipid, carbohydrate, and amino acid metabolism, but their regulation has been poorly studied. Insulin controls the translocation of several solutes that are involved in energetic cellular metabolism, including glucose. We studied the effect of insulin on the function of human SMCT1 expressed in Xenopus oocytes. The addition of insulin reduced α-keto-isocaproate (KIC)-dependent \(^{22}\)Na uptake by 29%. Consistent with this result, the coinjection of SMCT1 with SGK1 cRNA decreased the KIC-dependent \(^{22}\)Na uptake by 34%. The reduction of SMCT1 activity by SGK1 depends on its kinase activity, and it was observed that the coinjection of SMCT1 with S442D-SGK1 (a constitutively active mutant) decreased the KIC-dependent \(^{22}\)Na uptake by 50%. In contrast, an SMCT1 coinjection with K127M-SGK1 (an inactive mutant) had no effect on the KIC-dependent Na\(^+\) uptake. The decreasing SMCT1 function by insulin or SGK1 was corroborated by measuring \[^1\text{C}\]acetate uptake and the electric currents of SMCT1-injected oocytes. Previously, we found that SMCT2/Slc5a12-mRNA, but not SMCT1/Slc5a8-mRNA, is present in zebrafish pancreas (by in situ hybridization); however, Slc5a8 gene silencing was associated with the development of human pancreatic cancer. We confirmed that the mRNA and protein of both transporters were present in rat pancreas using RT-PCR with specific primers, Western blot analysis, and immunohistochemistry. Additionally, significant propionate-dependent \(^{22}\)Na uptake occurred in pancreatic islets and was reduced by insulin treatment. Our data indicate that human SMCT1 is regulated by insulin and SGK1 and that both SMCTs are present in the mammalian pancreas.

Na\(^+\)/monocarboxylate cotransporter with high affinity for MCs (SMCT1); solute carrier family 5 member 8 (SLC5A8); Na\(^+\)/monocarboxylate cotransporter with low affinity for MCs (SMCT2); solute carrier family 5, member 12 (SLC5A12); serum- and glucocorticoid-inducible kinase-1 (SGK1); α-keto-isocaproate; monocarboxylates
(64), suggesting that SLC5A8 is a tumor suppressor gene. It has been suggested that the protective effect of SLC5A8 against the development of malignancies is related to the inhibitory effect of butyrate, propionate, and pyruvate on the activity of the histone deacetylases HDAC1 and HDAC3 (60, 61). The inhibition of HDAC enzymes causes changes in the expression pattern of several apoptotic genes and cell cycle regulators (61). Interestingly, similar SLC5A12 gene alterations have not been linked to the protection or development of any malignancy.

Although the absence of SMCT1 is associated with the development of various malignancies, little is known regarding the regulation of SMCT1 function. It was shown that the SMCT mRNA expression in the kidney is downregulated by the deletion of the c/ebpδ transcription factor (59). SLC5A8 expression is also downregulated by TNF-α (4), whereas activin A upregulates SLC5A8 via Smad3 (66).

The activity of some Slc5 member proteins is controlled by kinases. Specifically, the activity of the Na+-glucose transporter 1 (SGLT1, encoded by Slc5a1 gene) and myo-inositol cotransporter (SMIT, encoded by Slc5a3 gene) are regulated by the serum- and glucocorticoid-inducible kinase-1 (SGK1) (9, 53). This kinase is ubiquitously expressed, and its expression is regulated by hormones, such as mineralocorticoids and glucocorticoids, and cellular stress (63). SGK1 is activated by insulin and growth factors via phosphatidylinositol 3-kinase (PI3K) and mTOR (30). In addition, SGK1 stimulates various ion channels and solute carriers and regulates the activity of several enzymes and transcription factors (30). Moreover, a gene variant of SGK1 is associated with an increase in blood pressure, obesity, and type 2 diabetes (28, 29).

The amino acid sequences of SMCTs contain several predicted phosphorylation sites, but there have been no reports that hormones or kinases control SMCT function. Interestingly, in the case of pancreatic tissue, there is species-specific SMCT1 localization because the Slc5a8 transcript is absent from the zebrafish pancreas (49) but could be found in the human pancreas and is silenced in pancreatic cancer (18, 43). This latter finding implies that the presence of SMCT1 is important in mammalian pancreatic cell metabolism. It is well known that insulin is one of the most important hormones in the regulation of cellular energy metabolism. Because several fuel metabolites (e.g., pyruvate, lactate, short-chain fatty acids) are transported by SMCT, we decided to study the regulation of SMCT1 activity by insulin and SGK1 and to analyze the presence of SMCT proteins in the mammalian pancreas.

METHODS

**In vitro cRNA translation.** cRNA for microinjection was synthesized using the T7 RNA polymerase mMESSAGE mMACHINE Kit (Ambion by Life Technologies) transcription system from cDNAs previously digested at their 3’ end as follows: Eco RI for SLC5A8-p77TS (8) and NotI for wild-type human SGK1-pGH19, the constitutively active and kinase-dead mutants SGK1-S422D and SGK1-K127M. The SGK1 clones were kindly provided by Dr. R. Lifton and were used previously in our laboratory (3, 27), cRNA integrity was assessed with an agarose gel, and the concentration was determined by absorbance reading at 260 nm (DU 640; Beckman, Fullerton, CA). cRNA was stored frozen in aliquots at −80°C until ready for use.

**Xenopus laevis oocyte preparation.** All of the experiments involving oocytes were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at our Institutions. Oocytes were harvested surgically from adult female *Xenopus laevis* frogs (Nasco) under 0.17% tricaine anesthesia and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES/Tris pH 7.4) in the presence of collagenase B (2 mg/ml) for 1 h. After four washes in ND96, the oocytes were manually defolliculated and were incubated overnight at 18°C in ND96 supplemented with 5 mg/100 ml of gentamicin. The next day, stages V to VI oocytes were injected with 50 nl of water or 20 ng of cRNA per oocyte (49). The oocytes were then incubated for 2 or 3 days in ND96, which was changed every 24 h.

**22Na+ and [1-14C]acetate uptake.** SMCT1 functional activity was assessed by measuring 22Na+ and [1-14C]acetate uptake by groups of 10–12 oocytes 3 days after water (control) or cRNA injection. 22Na+ uptake assays were performed as described previously (49). Briefly, 22Na+ uptake was measured with the following protocol: an initial 30-min incubation period was performed in ND96 containing 1 mM NaCl, 200 μM amiloride, and 100 μM bumetanide to inhibit the activity of endogenous Na+/K+ pumps, amiloride-sensitive Na+ channels and Na+-K+2Cl^- cotransporter, respectively. When the effect of insulin was tested, bovine insulin (SIGMA) was added at a concentration of 0–15 U/ml or human insulin (Humulin 70/30, Eli Lilly) at 40 U/ml followed by a rapid wash in ND96 and 60 min uptake period in ND96 with 1.0 μCi/ml of 22Na+ (PerkinElmer Life Sciences) at 32°C in the presence or absence of propionate or KCl but with the inhibitors listed above. To evaluate the effect of insulin on SMCT1 over time, the [1-14C]acetate and 22Na+ uptake were measured with the following protocols. After the initial incubation in ND96 in the presence and absence of bovine insulin (12 U/ml), the oocytes were incubated in ND96 or zero Na+ isotonic solution (96 mM NDGCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES/Tris pH 7.4) containing 2 mM of potassium acetate and 1.0 μCi/ml of [1-14C]acetate (Amersham Biosciences) at 32°C to measure [1-14C]acetate uptake from 10 to 120 min. The 22Na+ uptake assays were also performed in the presence and absence of bovine insulin (12 U/ml) in ND96 as previously described, in the presence or absence of 2 mM Na+, 100 μM amiloride, and the inhibitors listed above, and 1.0 μCi/ml 22Na+ from 10 to 120 min. The effect of SGK1 on SMCT1 was also evaluated with the [1-14C]acetate uptake with a 60-min uptake period in ND96 or zero Na+ isotonic solution with 2 mM potassium acetate and 1.0 μCi/ml [1-14C]acetate. At the end of the uptake periods, oocytes were washed in ice-cold ND96 solution without the isotope to remove extracellular tracer. Next, individual oocytes were dissolved in 1% NaOH, and tracer activity was determined by β-scintillation counting. RNA-injected oocytes were compared with control oocytes from the same donor injected with water under identical conditions.

**Two-electrode voltage clamp.** Oocyte membrane currents were recorded using an OC-720C voltage clamp (Warner Instruments, Hamden, CT) filtered at 2–5 kHz, digitized at 10 kHz, and recorded with the PATCH MASTER software (HEKA, Germany); the data were analyzed as previously described (8, 49). For periods when the I-V protocols were not being run, oocytes were clamped at a holding potential (Vh) of −50 mV, and the current was monitored and recorded at 1 Hz. The I-V protocols consisted of 400-ms, 20-mV steps from Vh to −150 mV propionate, the inhibitors listed above, and 1.0 μCi/ml 22Na+ from 10 to 120 min. The effect of SGK1 on SMCT1 was also evaluated with the I-V protocol with a 60-min uptake period in ND96 or zero Na+ isotonic solution with 2 mM potassium acetate and 1.0 μCi/ml [1-14C]acetate. At the end of the uptake periods, oocytes were washed in ice-cold ND96 solution without the isotope to remove extracellular tracer. Next, individual oocytes were dissolved in 1% NaOH, and tracer activity was determined by β-scintillation counting. RNA-injected oocytes were compared with control oocytes from the same donor injected with water under identical conditions.

**Total RNA isolation from tissues.** RNA was extracted from the pancreas and kidney tissues of male Wistar rats weighing 200–250 g;
the tissues were homogenized in guanidinium buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0; 0.1 M 2-mercaptoethanol, and 0.5% N-laurylsarcosine using a polytron (PT2000; Kinematica, Lucerne, Switzerland) at its lowest setting. The homogenate was centrifuged at 12,000 g for 15 min at 18°C, and the resulting supernatant was layered onto a CsCl solution containing 5.7 M CsCl and 25 mM sodium acetate, pH 5.2. The CsCl gradient was formed by centrifugation at 113,000 g for 18 h at 18°C to yield total RNA. The RNA was precipitated with 100% ethanol and 3 M sodium acetate, pH 5.2, washed twice with 75% ethanol, and resuspended in water. The RNA was quantified by optical density at 260 nm and was stored at −80°C until use (35). Poly(A) RNA (mRNA) was purified from total RNA previously obtained by a single round of oligo(dT) cellulose chromatography (Amersham). Total RNA in pancreatic islets was extracted using TRIzol following the manufacturer’s recommendations (Invitrogen) (40).

**RT-PCR.** Reverse transcription reactions were prepared with 2.5 μg of total RNA from the pancreas, kidney, or pancreatic islets using an oligo(dT) primer and MMLV reverse transcriptase (Invitrogen). PCR amplification was performed using Taq polymerase (Invitrogen) in a GeneAmp PCR System 9600 programmable thermal controller (Perkin Elmer), using the following PCR amplification conditions with 35 cycles: denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 1 min 30 s at 72°C; the initial and final extension cycles were 1 min and 7 min, respectively, at 72°C. Each PCR amplification was performed in a total volume of 25 μl containing 2 μl of RT product (cDNA), 800 nM of each primer, 1.5 mM MgCl2, 400 μM of dNTPs and 0.5 μl of Taq polymerase (Invitrogen). The amplification product was separated by electrophoresis on a 1% agarose-Tris-acetate-EDTA gel containing ethidium bromide and was viewed under UV light. Alternatively, the product was separated by electrophoresis on a 5% acrylamide gel for higher resolution. The amplified product was sequenced to establish its molecular identity. The specific primer sequences were designed to amplify rat Slc5a12/SMCT2 and Slc5a8/SMCT1 at their carboxy-termini. The primer pairs used were: Slc5a8/SMCT1 sense 5′-CTTCTCTgggCTTg-TTTTCTTG-3′, antisense 5′-ATCGgggTCTAATTTTggT-3′, length 390 bp; Slc5a12/SMCT2 sense 5′-ATTACCTTgACAgTg-CgAtg-3′, antisense 5′-TCTTTCAAAGAACATCTTTg-3′, length 321 bp. The amplified products were excised and were eluted from the gel. The fragments were cloned into the vector pCR4-TOPO (Invitrogen) sequenced, and sequenced. RT-PCR amplification was performed using specific rabbit antibodies against mouse SMCT1 and SMCT2 that were produced by the laboratory of Dr. Mount. The antibodies were used at concentrations of 1:3,000, respectively. The primary antibody was incubated overnight for 18 h at 4°C. The secondary antibody (anti-rabbit IgG, HRP) was used at a concentration of 1:5,000, respectively. The membranes were incubated overnight at 4°C, washed and then incubated at room temperature with alkaline phosphatase-conjugated secondary anti-rabbit IgG antibody (Bio-Rad) diluted to 1:5,000 to detect anti SMCT1 and anti-SMCT2, and donkey anti-goat IgG-HRP:sc-2020 (Santa Cruz Biotechnology) (1:5,000) was used to detect SLCS5A8 (H-18) and SLCS5A12 (S-17) antibodies. The respective blocking peptides (Santa Cruz Biotechnology) of SLCS5A8 (H-18):sc-34194P and SLCS5A12 (S-17):sc-162221P antibodies were used for the preabsorption of antibodies (negative controls). The antibodies were combined with twofold excess of blocking peptides in 500 μl of TBS, incubated for 2 h at room temperature. Following blocking, the membrane was incubated with the diluted antibody/peptide mixture with TBS (1:100/1:50) at 4°C overnight. In addition, for the WB analysis with the SLCS5A8 (H-18) antibody, extracts from oocytes injected with mSlc5a8-cRNA (25 ng by oocyte) and coinjected with mSlc5a8-cRNA/Slc5a8 siRNA (m:sc-45290 (Santa Cruz Biotechnology) (25/25 ng by oocyte) were used as positive and negative controls, respectively. The bands were detected using Immun-Star Chemiluminescent Protein (Bio-Rad).

**Immunohistochemistry.** For immunohistochemistry, the examination was performed using male rat pancreas. Tissue sections were deparaffinized at 60°C for 30 min and were immersed for 3 min in xylene, then in different percentages of ethanol (100, 95, and 70% twice) and finally in distilled water. The sections were blocked with peroxidase/methanol solution for 10 min and were washed with phosphate-buffered saline (PBS) and 0.05% Tween 20. The slides were blocked for 30 min with a Background Sniper solution (Biocare Medical). The primary antibodies used were the rabbit antibodies against mouse SMCT1 (1:50), SMCT2 (1:50) or goat antibodies SLCS5A8 (H-18) (1:50) or SLCS5A12 (H-17) (1:50). Background sniper solution instead of primary antibody was used as the negative control. The primary antibody was incubated overnight for 18 h at 25°C. After incubation, the sections were washed with PBS-Tween 0.05% and were incubated for 30 min with rabbit or goat Immunoblotting. Biotin Link (Biocare Medical), followed by a PBS-Tween 0.05% wash and a 30-min incubation with Rabbit or Goat Immunoblotting. HRP Label (Biocare Medical). After washing the sections using PBS-Tween 0.05%, the reaction was developed with diaminobenzidine (DAKO) for ~1 min. The slides were washed with distilled water and counterstained with hematoxylin solution.

**Rat pancreatic islet extract.** Male Wistar rats weighing 200–250 g were used to obtain pancreatic islets, following standard methodology (20, 40, 55). Briefly, the pancreas was perfused with 10 ml of Hank’s balanced salt solution (HBSS) supplemented with 25 ml of 4% serum albumin and Spinner salts. Each pancreas was removed, finely chopped in ice-cold HBSS containing 3 mg of collagenase-P (Roche) for 5 min on ice, and then was incubated with 3 ml of HBSS containing 1.0 U/ml bovine insulin were incubated in a similar solution containing 5 U/ml bovine insulin, followed by another 60 min in HBSS containing 1.0 μCi/ml of [22Na] (PerkinElmer Life Sciences) in the presence or absence of 5 mM amiloride; [1-14C]acetate uptake by the islets treated with insulin was measured in cold HBSS solution and were lysed in 500 μl of 10% SDS. [22Na] uptake by the islets was determined in a beta radiation counter (Beckman).

**Data analysis.** All of the results presented are based on a minimum of three experiments, or for [22Na] and [1-14C]acetate uptake experiments used at least 10–12 oocytes per group for each experiment. The results are presented as the means of uptake values within groups ± SE unless otherwise stated. Prism 5.0 was used to fit the data.
insulin curve. The significance of the differences between groups was evaluated by Student’s *t*-test and one-way analysis of variance.

RESULTS

Negative effect of insulin and SGK on SMCT1 function. The effect of insulin on the function of human SMCT1 was studied by measuring $^{22}$Na$^+$ uptake in Xenopus laevis oocytes injected with human (h) SMCT1-cRNA as previously described in METHODS. Figure 1 shows a summary of four experiments in oocytes from different frogs. SMCT1 cRNA injection induced a significant increase in KIC-dependent $^{22}$Na$^+$ uptake [SMCT1ND96 0.64 ± 0.06 (n = 51 oocytes) vs. SMCT1KIC 11.92 ± 0.42 nmol-oocyte$^{-1}$·h$^{-1}$ (n = 52 oocytes); P < 0.001]. The increased $^{22}$Na$^+$ uptake induced by SMCT1 cRNA was reduced by pretreatment with insulin (SMCT1KIC/insulin = 9.29 ± 0.39 nmol-oocyte$^{-1}$·h$^{-1}$, n = 50; P < 0.001). Interestingly, insulin activated an endogenous sodium transport KIC independently in the oocytes; this was appreciated in the absence of KIC [H$_2$O ND96 0.00 ± 0.06 (n = 50)] vs. SMCT1KIC 0.06 (n = 51)]. The increased $^{22}$Na$^+$ uptake of oocytes incubated with insulin was not different. A similar reduction of KIC-dependent $^{22}$Na$^+$ uptake was observed in the absence of the KIC of the respective $^{22}$Na$^+$ uptake of oocytes in the presence of KIC. One hundred percent of the function was assigned to KIC-dependent $^{22}$Na$^+$ uptake of SMCT1-oocytes without insulin.

Effect of insulin on SMCT1 function is dose dependent. We assessed the effect of bovine insulin and SGK1 on SMCT1-oocytes in the transport of monocarboxylates using $^{1-14}$Cacetate. Figure 3A is a summary of three experiments showing that the Na$^+$-dependent $^{1-14}$Cacetate uptake in the SMCT1-oocytes was significantly reduced by 12 U/ml bovine insulin [SMCT1 3.72 ± 0.24 (n = 33) vs. SMCT1insulin 2.24 ± 0.26 nmol-oocyte$^{-1}$·h$^{-1}$ (n = 34); P < 0.001]. In the same figure, we observed endogenous Na$^+$-dependent $^{1-14}$Cacetate uptake activated by insulin in the control oocytes [H$_2$O ND96 0.09 ± 0.30 (n = 34) vs. H$_2$O insulin 0.82 ± 0.55 nmol-oocyte$^{-1}$·h$^{-1}$ (n = 34); P < 0.001]. The transport of endogenous acetate did not mask the negative effect of insulin on the function of SMCT1 at 2 h of $^{1-14}$Cacetate uptake, it certainly provided us with a guide to consider that the effect of insulin on SMCT1 was greater than the observed effect.

The insulin effect on Na$^+$-dependent $^{1-14}$Cacetate uptake and propionate-dependent $^{22}$Na$^+$ uptake of SMCT1-oocytes was evaluated over time. As observed in Fig. 3B, the Na$^+$-dependent $^{1-14}$Cacetate uptake of SMCT1-oocytes pretreated with or without insulin was linear during the first minute. After 30 min, the slopes of the curves changed and tended to reach a constant $^{1-14}$Cacetate uptake. As shown in Fig. 3B, during the first minutes, there was no significant difference between the $^{1-14}$Cacetate uptake of oocytes pretreated with insulin compared with untreated oocytes; however, after 60 min, there was a significant reduction in the group preincubated with insulin.
Fig. 2. The effect of insulin on SMCT1 function is dose dependent. A: raw data for the curve with (+) and without (−) 10 U/ml insulin in the presence (gray bar) or absence (white bar) of 2 mM KIC. Oocytes injected with water ± KIC were the control groups for the hSMCT1-oocyte groups. *Statistical significance at P < 0.001 (hSMCT1KIC vs. hSMCT1+insulin): B: the insulin concentration was varied (0 to 15 U/ml) to determine the percentage of KIC-dependent \( \text{[1-14C]} \)acetate uptake by SMCT1. The \( \text{[22Na]}^{+} \) uptake data were normalized to 100% of the KIC-dependent function with a previous subtraction of background (hSMCT1-oocytes without 2 mM KIC).

Fig. 3. Time curves of \( \text{[1-14C]} \)acetate and \( \text{[22Na]}^{+} \) uptake of SMCT1. A: raw data of the 2-h \( \text{[1-14C]} \)acetate uptake used in time curve of the Na\(^{+}\)-dependent \( \text{[1-14C]} \)acetate uptake in oocytes. Each bar represents the \( \text{[1-14C]} \)acetate uptake average ± SE of −30 oocytes injected with hSMCT1/SLC5A8-cRNA or water (control) from 3 separate experiments with cells from different donor frogs. The oocyte groups were incubated in the presence of 2 mM potassium acetate preincubated with (+) and without (−) 12 U/ml insulin in the presence (gray bar) or absence (white bar) of Na\(^{+}\): B: Na\(^{+}\)-dependent \( \text{[1-14C]} \)acetate uptake time curve in the absence (black line) and presence (dash line) of 12 U/ml insulin. C: propionate-dependent \( \text{[22Na]}^{+} \) uptake time curve in the absence (black line) and presence (dash line) of 12 U/ml insulin. *Statistical significance at \( P < 0.01 \) between hSMCT1 vs. hSMCT1 + insulin at the respective times.
inhibitors of sodium transport. Considering that SMCT1 is a secondary transport and its function depends on the gradient of its solutes (Na⁺, MC⁻) produced mainly by primary sodium transporters, we expected that the presence of ouabain, amiloride, and bumetanide may induce a significant reduction not only in the function of endogenous sodium acetate transport activated by insulin but also in the acetate transporter induced by SMCT1.

Therefore, to confirm our argument, experiments of [1-14C]acetate uptake with SMCT1-oocytes were performed in the presence and absence of sodium transport inhibitors. As observed in Fig. 4A, the presence of sodium transport inhibitors reduced significantly the [1-14C]acetate uptake of the SMCT1-oocytes group in the presence of sodium [1.50 ± 0.02 (n = 21) vs. 0.69 ± 0.04 nmol-oocyte⁻¹·h⁻¹ (n = 24); P < 0.001]. The residual SMCT1 function was sufficient to assess the effect of insulin on SMCT1, as was observed in the [22Na⁺] uptake experiments with SMCT1-oocytes because, in the presence of insulin, [1-14C]acetate uptake was also reduced with respect to SMCT1-oocytes without insulin [0.69 ± 0.04 (n = 24) vs. 0.35 ± 0.03 nmol-oocyte⁻¹·h⁻¹ (n = 23); P < 0.001].

The percentage of sodium-dependent [1-14C]acetate uptake of SMCT1 in the presence and absence of sodium transport inhibitors and insulin was calculated with the subtraction of the [1-14C]acetate uptake of the control group (H₂O-oocytes and the SMCT1-oocytes incubated without Na⁺) with the respective SMCT1 groups. The values obtained in the SMCT1 groups in the presence or absence of sodium transport inhibitors were considered as 100% function with respect to the SMCT1 groups in the presence of insulin. These percentages were used to evaluate whether the negative effect of insulin on SMCT1 function was different in the presence of sodium transport inhibitors. As shown in Fig. 4B, insulin reduced in a similar percentage the sodium-dependent [1-14C]acetate uptake of SMCT1 in the absence or presence of ouabain, amiloride, and bumetanide. Interestingly, as predicted, the endogenous sodium acetate transport activated by insulin was dependent on the function of sodium transporters as observed in Fig. 4A because the increment of sodium-dependent [1-14C]acetate uptake in the H₂O-oocytes was not observed in the presence of ouabain, amiloride, and bumetanide. These results indicated that the negative effect of insulin on SMCT1 function was not dependent on modifications of the solute gradient, and it may be an effect associated with the activation of the signal transduction pathway induced by the hormone.

In the presence of sodium transport inhibitors, the negative effect of insulin on [1-14C]acetate uptake in SMCT1-oocytes incubated with 2 mM acetate was observed at 30 min; although [1-14C]acetate uptake was low, a significant difference between the SMCT1-oocytes and H₂O-oocytes groups without insulin was found [0.46 ± 0.02 vs. 0.30 ± 0.01 nmol-oocyte⁻¹·30 min⁻¹ (n = 12); P < 0.001]. This difference was also observed in SMCT1-oocytes in the absence and presence of insulin [0.46 ± 0.02 vs. 0.38 ± 0.03 nmol-oocyte⁻¹·30 min⁻¹ (n = 12); P < 0.01]. These data indicated that the effect of insulin on SMCT1 function occurs rapidly after the incubation of oocytes with the hormone.

The negative effect of SGK1 was also observed in the [1-14C]acetate uptake of SMCT1. On the other hand, as shown in Fig. 5, the [1-14C]acetate uptake in oocytes co injected with SMCT1/SGK1 was significantly lower than those injected only with SMCT1 [SMCT1: 2.34 ± 0.27 (n = 30) vs. SMCT1/SGK1: 0.79 ± 0.11 nmol-oocyte⁻¹·h⁻¹ (n = 34); P < 0.001 uptake]. Unlike control-oocytes preincubated with insulin, the presence of SGK1 did not activate any endogenous acetate transport in the oocytes. Therefore, if we calculated the percentages of sodium-dependent [1-14C]acetate uptake reduction of SMCT1 subtracting the [1-14C]acetate uptake of the groups in the absence of sodium in the respective oocytes group under similar experimental conditions but with sodium, we observed that the sodium-dependent [1-14C]acetate uptake of SMCT1 was reduced by 67% in the presence of SGK1 and by 62% when exposed to insulin.

**Effect of mutant SGK on SMCT1 function.** To determine whether the reduction of SMCT1 activity was associated with the kinase activity of SGK1, we used two different mutants of this enzyme, namely SGK1-S442D, which mimics a constitutively active kinase (9, 42, 53), and SGK1-K127M, an inactive kinase (5, 19). As shown in Fig. 6, KIC-dependent [22Na⁺] uptake was reduced in the group of oocytes co injected with the constitutively active SGK1-S442D mutant and SMCT1 [7.50 ± 0.27 nmol-oocyte⁻¹·h⁻¹ (n = 58 oocytes), P < 0.001]
compared with the group injected with SMCT1 alone (13.35 ± 0.25 nmol-oocyte−1·h−1, n = 58 oocytes) or coinjected with wild-type SGK1 [9.21 ± 0.41 nmol-oocyte−1·h−1 (n = 58 oocytes), P < 0.001]. The catalytically inactive SGK1-K127M coinjected with SMCT1 did not inhibit 22Na+ uptake (12.02 ± 0.37 nmol-oocyte−1·h−1, n = 53 oocytes). The KIC-dependent 22Na+ uptake in oocytes coinjected with SMCT1/SGK1 or SMCT1/SGK1-S442D was reduced by approximately 40% and 50%, respectively. These results indicated that the reduction in KIC-dependent 22Na+ uptake is directly related to the catalytic activity of SGK1.

The ionic current induced by SMCT1 was reduced by the presence of SGK1 and insulin. The reduction in SMCT1 function in oocytes coinjected with SGK1 or exposed to 10 U/ml insulin was associated with membrane potential changes (∆Vm) when the oocytes were perfused for 1 min with 1 mM propionate. As shown in Fig. 7C, SMCT1-injected oocytes exposed to insulin (∆Vm = +32.3 ± 3.3 mV, n = 6, P < 0.001) before perfusion with propionate or coinjected with SGK1 (∆Vm = +18 ± 4.3 mV, n = 8, P < 0.001) showed a smaller depolarization than those injected only with SMCT1 in the absence of insulin (∆Vm = +84 ± 5.4 mV, n = 17). These data correlated with the decrease in the intensity of the propionate-dependent current in the same oocytes, as demonstrated by the current-voltage curve (Fig. 7B). As observed by the voltage-clamp experiments in Xenopus oocytes in the presence of propionate (Fig. 7A), SMCT1-injected oocytes induced a significant propionate-dependent current (@ −150 mV = −1.82 ± 0.15 µA, n = 17, P < 0.01) compared with H2O-injected oocytes (@ −150 mV = −0.12 ± 0.03 µA, n = 10). The propionate-dependent current was reduced by 52% when oocytes were exposed to insulin (@ −150 mV = −0.87 ± 0.12 µA, n = 6, P < 0.01) and by 85% in the presence of SGK1 (@ −150 mV = −0.27 ± 0.15 µA, n = 8, P < 0.01).

Transcripts of SMCT1 and SMCT2 are expressed in the rat pancreas. Using the mouse cDNA sequences of SMCT2 and SMCT1, we performed a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) alignment to identify homologous sequences in the rat genomic database. Two sequences were identified: XM_576209.2 for SMCT1 and XM_001080126.1 for SMCT2. The rat sequences were 40% identical to each other and ~85% and ~87% identical to their respective human orthologs.

The sequences of the rat (r) SMCTs were used to design specific primers for each SMCT. The sequences were amplified using specific primers with no identity overlapped. The predicted amplified sequences were an amplicon of 390 bp long (1672–2062) for rSMCT1 and 321 bp long (1992–2313) for rSMCT2.

As shown in Fig. 8, we found rSMCT transcripts in the kidney, pancreas, and pancreatic islets. The products amplified by RT-PCR were run on 1% agarose (Fig. 8A) or 5% acrylamide (Fig. 8B) gels. The expected band sizes of ~390 bp for SMCT1 and ~321 bp for SMCT2 were observed using both kidney and pancreas mRNA (cDNA+), but not in the negative controls where water or pancreas mRNA was added. The bands with other sizes not expected for us probably corresponded to immature mRNA or mRNA of SMCT isoforms, but not to genomic DNA. To confirm that pancreas total RNA was free of genomic DNA, the PCR was performed with the total RNA and both primer pairs of each SMCT. The expected band sizes were not observed (Fig. 8A, first two wells). The cDNA products amplified from the pancreas and kidney were sequenced (Perkin Elmer/Applied Biosystems Model 3730), and the sequences obtained were analyzed with BLAST to identify homologous sequences. The BLAST showed that the products amplified of the ~390- and ~321-bp sequences were identical to the rSMCT1 and rSMCT2 sequences, respectively.
Proteins of SMCT1 and SMCT2 are present in the rat pancreas. SMCT protein expression was confirmed by Western blot analysis (WB) of the pancreas and other rat tissues using specific antibodies against mouse (m) SMCT1 (Fig. 9A) and mSMCT2 (Fig. 9B). WB analysis of rSMCT1 revealed one band that migrated at a level corresponding to a molecular weight of 75 kDa in the pancreas, kidney, liver, and colon tissues but not in the protein extracts from oocytes previously injected with human SMCT1-cRNA, zebrafish (z) SMCT2-cRNA, or water. Considering only the amino acid sequence of SMCT (610 – 620 aa), the molecular weight expected by SMCT1 and SMCT2 proteins was approximately 66 – 68 kDa, and we assume that the bands observed corresponded to the size previously reported for the N-glycosylated form of hSMCT1/SLC5A8, which contains two N-glycosylated sites (N480 and N485) (46) that are also present in rSlc5a8 (N481 and N485). Interestingly, the mSMCT1 antibody did not detect hSMCT1 in our experimental conditions, possibly because of the differences between the human and mouse protein sequences that detected the SMCT1 antibody. The human peptide region homologous to the mSMCT1 antigen has five different amino acids, two of which change a nonpolar amino acid to a polar amino acid (F to C and I to S). By contrast, the homologous rSMCT region has four different amino acids, but they did not change the polarity of the peptide region. This difference is likely the reason why the mSMCT1 antibody is specific to murine species.

We also observed two rSMCT2 bands that migrate at a level corresponding to a molecular weight less than 75 kDa in the pancreas and liver tissues and also in zSMCT2 oocyte protein extract (Fig. 9B). Bands were observed in the kidney and pancreas but were not present in the colon, as previously reported (24) and were absent from water and SMCT1-injected oocyte protein extracts, demonstrating that anti-mSMCT2 did not cross-react with SMCT1 and is specific for the SMCT2 protein (Fig. 9B). We considered that the higher molecular weight band corresponding to the glycosylated form of this protein, which also has two putative N-glycosylated sites (N219 and N480), and the bands with smaller molecular weights most likely correspond to non-glycosylated or otherwise posttranslationally modified forms of this transporter. Certainly, a weight difference between the bands with a higher molecular weight in the pancreas and kidney was observed. Previously, a similar observation was reported between the bands detected with SMCT1 antibodies in the colon and thyroid with respect to the bands observed in the kidney in WB analysis (46). These data suggest that posttrans-
To demonstrate that oocytes injected with mSMCT1-cRNA produced the SMCT1 protein observed in the WB analysis with the SLC5a8 (H-18) antibody and the translation of mSMCT1-cRNA was blocked by Slc5a8 siRNA (m), we assessed the movement of acetate following [1-14C]acetate uptake in the presence and absence of Na+. Figure 11C shows that mSMCT1-cRNA-injected oocytes displayed increased [1-14C]acetate uptake (1.76 ± 0.30 nmol-oocyte⁻¹·h⁻¹, n = 10) compared with the controls injected with H2O (0.36 ± 0.02 nmol-oocyte⁻¹·h⁻¹, n = 10) or in the absence of Na⁺ (0.22 ± 0.02 nmol-oocyte⁻¹·h⁻¹, n = 10). However, when mSMCT1-cRNA was co-injected with Slc5a8 siRNA (m) (25 ng/25 ng), the [1-14C]acetate uptake was significantly reduced (0.97 ± 0.1 nmol-oocyte⁻¹·h⁻¹, n = 10), likely because of the decreased amount of mSMCT1 protein synthesized by oocytes co-injected with Slc5a8 siRNA (m), as demonstrated in the WB analysis, in which the SMCT1 protein was not detected in the protein extracts of oocytes (100 µg) co-injected with mSMCT1cRNA/Slc5a8 siRNA (m) compared with similar amounts of protein extracts of oocytes injected with mSMCT1-cRNA. Additionally, this observation indicates that the SLC5A8 (H-18) antibody requires a certain concentration of SMCT1 in the protein extract to observe the specific band of mSMCT1 because, even though there was lower [1-14C]acetate uptake in co-injected oocytes with mSMCT1cRNA/Slc5a8 siRNA (m), indicating the presence of SMCT1, the concentration of SMCT1 in the protein extract was not sufficient to see a band in Fig. 11A. The sensitivity of the SLC5A8 (H-18)
antibody to the SMCT1 concentration in the protein extract was also observed in samples of the pancreas, as shown in Fig. 10, and the intensity of the band corresponding to SMCT1 increases when the amount of protein extract of the pancreas also increases.

With respect to WB analysis using the SLC5a12 (S-17) antibody, a band that migrated at a level corresponding to a molecular weight between 75 kDa and 50 kDa in both protein extracts of the kidney and pancreas was observed (Fig. 10, C and G). The bands disappeared when the antibody was absorbed with the blocking peptide Slc5a12 (S-17)P (Fig. 10, D and H).

Therefore, our data revealed the presence of mRNA transcripts and proteins for both SMCT transporters in pancreatic tissue.

Propionate-dependent $^{22}$Na$^+$ uptake and SMCT proteins are present in pancreatic islet tissue. We determined the activity of the SMCT cotransporter in the pancreas by assessing $^{22}$Na$^+$ uptake in the presence or absence of propionate. Figure 12A shows a significant difference in the uptake of $^{22}$Na$^+$ between islets incubated in the presence (89 ± 18 nmol·20 islets$^{-1}$·h$^{-1}$) or absence (36 ± 8 nmol·20 islets$^{-1}$·h$^{-1}$) of 5 mM propionate. These data indicated that sodium-dependent monocarboxylate transporters are active in pancreatic islets. Interestingly, pro-

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**Fig. 10.** SMCT1 and SMCT2 proteins are present in the rat pancreas and kidney. A Western blot analysis was performed with different kidney protein extracts (K1–K4) from rats (80 µg, 8% SDS-PAGE) using Slc5a8 (H-18) or Slc5a12 (S-17) antibodies to detected SMCT1 (A) and SMCT2 proteins (C), respectively. WB analysis was also performed with different amounts (100, 200, 400 µg) of pancreas protein extracts (P100–P400) and with 50 µg of kidney protein extracts from rats using SMCT1 (E) and SMCT2 antibodies (G). As negative controls, the WB analysis was performed using Slc5a8 (H-18) (B, F) and Slc5a12 (S-17) antibodies (D, H) with their respective blocking peptides. Actin (commercial antibody) was used as a loading control for each protein sample (blot at bottom of each panel).

**Fig. 11.** The Slc5a8 (H-18) antibody specifically detects the mSMCT1 protein synthesized by oocytes injected with mSMCT1-cRNA in WB analysis. A: as controls in the WB analysis with the Slc5a8 (H-18) antibody, protein extracts were used from oocytes injected with H$_2$O (negative control), mouse SMCT1-RNAc (positive control), and coinjected SMCT1-RNAc/siRNA Slc5a8 (m) (negative control) to detect SMCT1 in the kidney protein extract. B: WB analysis with anti-V5 to detect the SMCT1 protein in oocytes injected with V5-hSMCT1-cRNA. Actin (commercial antibody) was used as a loading control for each protein sample (blot below A and B). C: $[^1]$H$_2$O acetate uptake (average ± SEs) in the bar graph of different oocyte groups injected with water, mSMCT1-cRNA, and mSMCT1-cRNA/siRNAslc5a8 (m) in the presence of 2 mM potassium acetate with Na$^+$ (gray bar) and the absence of Na$^+$ (white bar). *Statistical significance at $P < 0.01$. 

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pionate-dependent $^{22}\text{Na}^{+}$ uptake was almost completely blocked in the presence of insulin and ibuprofen, as shown in Fig. 12B.

To confirm the location in the islets of both SMCTs, we performed immunohistochemistry analysis in pancreatic tissue with anti-SMCT1 (Fig. 13C), anti-SMCT2 (Fig. 13E), SLC5a8 (H-18) (Fig. 13D), and SLC5a12 (S-17) antibodies. As shown in Fig. 12, both proteins were located in the pancreatic islets. Immunohistochemistry analysis with SLC5a8 (H-18) and SLC5a12 (S-17) antibodies showed a stronger signal in the islets. Interestingly, the proteins were not only present in the cytoplasmic membrane but also in the cytosol. This location suggests that the signal transduction involved in the migration of vesicles to the membrane as well as the mechanisms of internalization of proteins present in cytoplasmic membrane regulate the cell functions of these proteins. Although it has been reported that SMCT1 is also located in the cell nucleus (18), the location of these proteins in the nucleus of pancreatic islets was not clear in our results.

**DISCUSSION**

Insulin regulates the function of SGK1, one of the proteins involved in insulin signal transduction. This study shows that SGK1 kinase affects SMCT1 function in a manner similar to insulin, in that both insulin and SGK1 reduce SMCT1 function. Moreover, the functional reduction caused by insulin and SGK1 are not synergistic, suggesting that SGK1 and insulin share a transduction pathway.

The regulatory effect of SGK1 on SMCT1 function is different from its role in other transport systems. In most systems, SGK1 kinase increases transport activity. For example, SGK1 increases ENaC activity by inhibiting the ubiquitin-ligase Nedd4-2. By phosphorylating Nedd4-2, SGK1 prevents the ubiquitination of the carrier protein. This event prevents the removal of ENaC from the membrane and its subsequent destruction, resulting in an increased number of ENaC channels in the plasma membrane and thereby increasing the overall cellular ENaC activity (30).

Our results indicate that the effect of SGK1 on SMCT1 does not occur through this ubiquitin pathway, as SGK1 reduces SMCT1 function. These data suggest an alternative route, possibly by direct phosphorylation of SMCTs by SGK1, rather than through Nedd4-2-mediated ubiquitination. Alternatively, SGK1 might modify or regulate other proteins that then reduce SMCT1 function or membrane permanence. Interestingly, the classic SGK1 consensus site, RXRXX(S/T)-F (X = any amino acid, R = arginine, F = hydrophobic amino acid), is not present in SMCT1. However, the SMCT1 sequence contains some potential phosphorylation sites for PKA and p38 MAPK that match the consensus phosphorylation site for SGK1 (17, 28). As SGK1 likely acts on these phosphorylation sites to reduce SMCT1 function, future investigations should focus on the details of this signaling pathway. Our data indicate that the inhibitory effect of SGK1 on SMCT1 function depends on its kinase activity. Future experiments will need to determine whether the inhibitory effect is by the direct phosphorylation of SMCT1 or by the phosphorylation of other proteins that interact with SMCT1, thereby modifying its function.

SGK1 increases glucose movement by glucose transporters (SGLT1 or GLUT1, GLUT4) but reduces monocarboxylate (pyruvate, lactate) movement by SMCT1. These distinct effects are interesting because glucose and monocarboxylate are involved in antagonistic metabolic pathways: glycolysis and gluconeogenesis, respectively. Additionally, it is interesting that both SMCTs are located in gluconeogenic tissues, such as the kidney and liver. These observations indicate that SMCTs likely play an important role in regulating cell metabolism by controlling the entry of fuel substrates, such as lactate and pyruvate. Importantly, because the SMCTs are Na$^{+}$ coupled, they will always bring these fuels into the cell at physiological voltages and gradients, while the MCT transporters (which are coupled to the H$^{+}$ gradient) do not always have a thermodynamically favorable and inwardly directed driving force.

Our results show that the monocarboxylate α-keto-isocaprate acid (KIC), derived from leucine catabolism, is carried by SMCT1 (Figs. 1 and 2). Interestingly, KIC is an insulin secretagogue that is much more potent than leucine and is as potent as glucose (36). KIC is involved in the synthesis of intermediates of the citric acid (Krebs) cycle (anaplerosis). It has been proposed that insulin secretion is dependent not only on the mitochondrial ATP concentration but also on anaplerotic processes that increase the levels of citric acid cycle intermediates.
thereby possibly altering the synthesis of second messengers involved in pancreatic insulin secretion (7, 26, 36). It is believed that KIC most likely stimulates insulin secretion by its transamination with endogenous glutamate to form leucine and $\alpha$-ketoglutarate, thereby increasing $\alpha$-ketoglutarate metabolism, and by the conversion of KIC to acetyl-CoA.

In previous studies, we demonstrated the presence of Slc5a12, but not Slc5a8 mRNA, in the pancreas of zebrafish embryos (49). Nonetheless, the absence of the SMCT1 in humans (due to methylation of the Slc5a8 gene) is associated with pancreatic cancer, suggesting that the carrier protein is necessary for human pancreatic cells. In the present study, we demonstrate the presence of Na$^+$-dependent propionate transport in mammalian pancreatic islets. These same cells also express the proteins and transcripts of both SMCTs (SMCT1/Slc5a8, SMCT2/Slc5a12). These data suggest a physiological role for pancreatic islet SMCTs in regulating the entry of KIC and other monocarboxylates that may be necessary mediators in pancreatic insulin secretion. Moreover, because SMCT1 is an electrogenic transporter, the resulting depolarization during SMCT1 activity may further contribute to the depolarization processes required for the release of insulin in the pancreas.

These findings are further supported by reports demonstrating that the MCT transporters (Slc16a1-4) are also expressed in the pancreas at very low levels (67). MCT1 expression is involved in insulin secretion because Slc16a1/MCT1 overexpression causes increased insulin secretion in response to greater entry of pyruvate or lactate into $\beta$-cells (22). Likewise, a dominantly inherited hypoglycemic disorder, physical exercise-induced hyperinsulinism disease (EIHI), results from the overexpression of SLCD1A1 (MCT1) due to an alteration in gene silencing (41). Recently, MCT1 (Slc16a1) overexpression in mouse pancreatic $\beta$-cells was also reported to result in hyperinsulinemia during exercise (52).
It is not difficult to imagine that the presence and function of the three types of monocarboxylate transporters, all of which move the same monocarboxylates into the pancreas, are regulated by the metabolic changes that generate their substrates. Thus, based on our work, we speculate that the secretion of pancreatic insulin regulates itself by controlling Na\(^+\)–H\(^+\)-coupled monocarboxylate transporters to regulate the entry of monocarboxylates, such as KIC, pyruvate, and lactate, thereby preventing increases in the ATP levels. These coordinated processes, which are involved in insulin secretion, would avoid hyperinsulinism and hypoglycemic events during exercise and in normal or pathological metabolic situations by increasing blood monocarboxylate concentration. Moreover, if the elevated entrance of monocarboxylates (e.g., lactate or KIC) into pancreatic \(\beta\)-cells causes insulin hypersecretion (22), it is not difficult to imagine an insulin-regulated feedback mechanism in the pancreatic islet. This process likely not only causes a reduction in the MC transporter numbers but also reduces MC transporter function, as our data with SMCT1 indicate.

The chromosomal localization of Ske5a8 and Ske5a12 make them positional candidates for proteins involved in diseases, such as non-insulin-dependent type 2 diabetes (NIDDM2 on 12q22-24, MIM 601407) (11, 34, 47) and familial combined hyperlipidemia (11p14) (39, 54), respectively. These SMCTs are found in various tissues (kidney, brain, eyes, muscle, pancreas) that are seriously affected by metabolic diseases, such as diabetes (e.g., diabetic nephropathy), supporting the pancreas) that are seriously affected by metabolic diseases, such as diabetes (e.g., diabetic nephropathy), supporting the idea that alterations in the SMCT function may be involved in the progression of these chronic degenerative diseases. These disease and metabolic implications will require future study to discover how SMCT transporters are associated with metabolic abnormalities that involve insulin secretion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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