The Na⁺/Glucose Cotransporter Inhibitor Canagliflozin Activates AMPK by Inhibiting Mitochondrial Function and Increasing Cellular AMP Levels

Canagliflozin, dapagliflozin, and empagliflozin, all recently approved for treatment of type 2 diabetes, were derived from the natural product phlorizin. They reduce hyperglycemia by inhibiting glucose reuptake by sodium/glucose cotransporter (SGLT) 2 in the kidney, without affecting intestinal glucose uptake by SGLT1. We now report that canagliflozin also activates AMPK, an effect also seen with phloretin (the aglycone breakdown product of phlorizin), but not to any significant extent with dapagliflozin, empagliflozin, or phlorizin. AMPK activation occurred at canagliflozin concentrations measured in human plasma in clinical trials and was caused by inhibition of Complex I of the respiratory chain, leading to increases in cellular AMP or ADP. Although canagliflozin also inhibited cellular glucose uptake independently of SGLT2, this did not account for AMPK activation. Canagliflozin also inhibited lipid synthesis, an effect that was absent in AMPK knockout cells and that required phosphorylation of acetyl-CoA carboxylase (ACC) 1 and/or ACC2 at the AMPK sites. Oral administration of canagliflozin activated AMPK in mouse liver, although not in muscle, adipose tissue, or spleen. Because phosphorylation of ACC by AMPK is known to lower liver lipid content, these data suggest a potential additional benefit of canagliflozin therapy compared with other SGLT2 inhibitors.

A recently introduced approach to treatment of type 2 diabetes is selective inhibition of sodium/glucose cotransporter (SGLT) 2 (1). SGLT1 and SGLT2 are related transporters that carry glucose across apical membranes of polarized epithelial cells against concentration gradients, driven by Na⁺ gradients. SGLT1 is expressed in the small intestine and responsible for most glucose uptake across the brush border membrane of enterocytes, whereas SGLT2 is expressed in the kidney and responsible for most glucose reabsorption in the convoluted proximal tubules. The first identified SGLT inhibitor was a natural product, phlorizin, which is broken down in the small intestine to phloretin, the aglycone form (Fig. 1). Although phlorizin had beneficial effects in hyperglycemic animals (2), it inhibits SGLT1 and SGLT2, causing adverse gastrointestinal effects (3). This led to development of the synthetic analogs canagliflozin (4), dapagliflozin (5), and empagliflozin (6) (Fig. 1), which have 260-, 1,100-, and 2,700-fold selectivity for SGLT2 over SGLT1, respectively (6). In meta-analyses of clinical trials in type 2 diabetes, canagliflozin (7), dapagliflozin (8), or empagliflozin (9), as monotherapy or combined with existing therapies, all reduced fasting plasma glucose, HbA1c, and body weight. Canagliflozin also decreased plasma triglycerides (7).

The current front-line therapy for type 2 diabetes is metformin, a biguanide that lowers plasma glucose primarily by reducing hepatic glucose production (10). Metformin, and the related biguanide phenformin, inhibit Complex I of the respiratory chain (11,12) and activate the cellular energy sensor AMPK (13,14). Binding to the AMPK-γ subunit of AMP and/or ADP, which are elevated during cellular energy stress, causes conformational changes...
that activate the kinase via allosteric effects and promotion of net phosphorylation of Thr172 on the AMPK-α subunit (15–18). Metformin and phenformin increase ADP-to-ATP ratios and fail to activate AMPK containing a γ-subunit mutant that does not bind AMP/ADP (19), confirming that their AMPK-activating effects are mediated by increases in AMP/ADP. Once activated, AMPK acts to restore energy homeostasis by promoting catabolic pathways, including fatty acid oxidation, while inhibiting anabolic pathways, including fatty acid synthesis (15,16). Its opposing acute effects on fat synthesis and oxidation are due to phosphorylation of two acetyl-CoA carboxylase (ACC) isoforms, ACC1 and ACC2. Whether AMPK explains all therapeutic benefits of metformin has been controversial because its acute effects on hepatic glucose production in mice were reported to be AMPK independent (20,21). However, studies using knock-in mice, in which both ACC isoforms were replaced by mutants lacking the critical AMPK phosphorylation sites, suggested that the longer-term insulin-sensitizing effects of metformin are accounted for by phosphorylation and inactivation of ACC1/ACC2 by AMPK (22).

We now report that canagliflozin activates AMPK, in intact cells and in vivo, by a mechanism involving inhibition of respiratory chain Complex I. Our results raise the possibility that some therapeutic benefits of canagliflozin might occur via AMPK activation rather than SGLT2 inhibition.

RESEARCH DESIGN AND METHODS

Materials and Antibodies
Canagliflozin, dapagliflozin, and empagliflozin were from Selleck Chemicals, and phlorizin, phloretin, metformin, phenformin, AICAR, and 2,4-dinitrophenol (DNP) were from Sigma-Aldrich. A769662 was synthesized as described (23). Antibodies against phosphorylated (p)Thr172 on AMPK-α (pThr172, #2531) were from Cell Signaling Technology. In Fig. 7, antibodies against pACC (#3661) and total ACC (#3676) were from Cell Signaling Technology. In other figures, total ACC was detected using streptavidin directly conjugated to 800 nm fluorescein (Rockland Immunochemicals), and pACC (14) and total AMPK-α (24) antibodies were as previously described. Anti-GLUT1 (#325510) was from Abcam, and anti-SGLT2 (sc-47402) was from Santa Cruz Biotechnology.

Cell Culture and Lysis
HEK-293 cells and wild-type (WT) and AMPK knockout mouse embryonic fibroblasts (MEFs) (25) were grown in DMEM with 25 mmol/L glucose and 10% FBS. Cell lysates were prepared as described previously (19). For Western blots shown in Fig. 6C, tissues were homogenized in 5 vols of HES buffer (20 mmol/L Na HEPES [pH 7.4], 1 mmol/L EDTA, 250 mmol/L sucrose; Roche complete protease inhibitor cocktail) with a Dounce homogenizer and centrifuged (7,050 × g, 20 min, 4°C). The pellet was resuspended in HES buffer and layered on top of buffer containing 1.12 mol/L sucrose before centrifugation in a swing-out rotor (41,500 × g, 60 min, 4°C). Membranes were collected from the interface of the sucrose layers, diluted in HES buffer, and centrifuged (150,000 × g, 60 min, 4°C). The resultant plasma membrane–rich pellets were resuspended in HES buffer (0.2–0.4 mL).

Immunoprecipitate Kinase Assays and Other Analyses
Methods for AMPK assay in immunoprecipitates, SDS-PAGE, Western blotting, and determination of cellular ADP-to-ATP ratios and oxygen consumption in HEK-293 cells were described previously (19). Lipid synthesis in MEFs was analyzed by starving cells of serum for 3 h and then treating with drug or vehicle in the presence of [14C]acetate (1 mCi/mL)/0.4 mmol/L Na acetate for 3 h. Cells were washed with PBS before extraction to determine incorporation of label into total lipid (26). Fatty acid oxidation was measured as etomoxir-sensitive 3H2O production from [3H]palmitate. MEFs were preincubated with AMPK activators for 30 min before incubation in [3H]palmitic acid (8 μCi/mL, 110 μmol/L), carnitine (50 μmol/L), fatty acid–free BSA (0.5 mg/mL) in Earle’s HEPES (116 mmol/L NaCl, 5.3 mmol/L KCl, 0.8 mmol/L MgSO4, 1.8 mmol/L CaCl2, 1 mmol/L NaH2PO4, 20 mmol/L HEPES-NaOH, pH 7.4) in the presence or absence of etomoxir (50 μmol/L) for 90 min at 37°C. The 3H2O generated was separated and quantified as previously described (27).

Animal Experiments
All animal procedures were approved by the McMaster University Animal Ethics Research Board. Male and female mice (16–20 weeks), WT or ACC1/ACC2 (S79A/S212A) double knock-in (DKI), were housed in a pathogen-free facility under a 12-h light/dark cycle at 23°C, with ad libitum access to standard chow and water. Primary hepatocytes were generated from WT and DKI mice and the following day were treated for 4 h with canagliflozin or vehicle before assessing AMPK and ACC phosphorylation and lipid synthesis, as previously described (22,28). For experiments to examine AMPK and ACC phosphorylation in vivo, canagliflozin or vehicle (saline solution containing 0.5% carboxymethyl cellulose, 0.025% Tween-20) was administered by oral gavage (100 mg/kg, 10 μL/g). Mice were anesthetized
and tissues snap frozen in situ as previously described (22). Measurements of the respiratory exchange ratio (RER) were performed in metabolic cages using a protocol (29) in which mice were fasted overnight and refed with chow for 2 h before being gavaged with canagliflozin or vehicle at the time of food withdrawal. In separate experiments, mice were treated as above, blood was collected from a nick in the tail, and glucose was measured using a Roche glucometer.

**Measurements of Oxygen Uptake/Respiration in Primary Mouse Hepatocytes**
Mitochondrial respiration was measured by high-resolution respirometry (Oxygraph-2 k; Oroboros Instruments, Innsbruck, Austria) at 37°C and room air-saturated O₂ tension in respiration buffer (MIRS5) containing EGTA (0.5 mmol/L), MgCl₂ (3 mmol/L), K-lactobionate (60 mmol/L), KH₂PO₄ (10 mmol/L), HEPES (20 mmol/L), sucrose (110 mmol/L), and fatty acid–free BSA (1 g/L). Primary hepatocytes from WT mice were generated as described above. The following day they were suspended in 2 mL of respiration buffer, and 800 μL of the suspension was added to the respiration chambers. Digitonin (8.1 μmol/L) was added to permeabilize the cells, and the assay was initiated 5 min later. To measure Complex I–supported respiration, glutamate (5 mmol/L), malate (2 mmol/L), and ADP (2.5 mmol/L) were added, and respiration was allowed to reach steady state. To measure Complex II–supported respiration, rotenone (1.25 μmol/L), succinate (10 mmol/L), and ADP (2.5 mmol/L) were added, and respiration was allowed to reach steady state. Drugs were then added to respiring cells at the indicated concentrations.

**Measurements of Glucose Uptake**
Cells were incubated in glucose-free Krebs-Ringer phosphate buffer for 2 h, with AICAR present for the last 1 h or canagliflozin/dapagliflozin for the last 15 min. 2-Deoxyglucose (2DG; 50 μmol/L, 1 μCi/mL) was added, and cytochalasin B-sensitive 2DG uptake was measured over 10 min (30).

**Statistical Analysis**
Significance of differences was assessed using the Student t test, one-way or two-way ANOVA as appropriate, using the Sidak test after ANOVA. Values of P < 0.05 were considered statistically significant.

**RESULTS**

**Canagliflozin and Phloretin, but not Dapagliflozin, Empagliflozin, or Phlorizin, Significantly Activate AMPK in HEK-293 Cells**
To assess the effect of canagliflozin on AMPK, we initially used HEK-293 cells. A daily 300-mg dose of canagliflozin produces a peak plasma concentration in humans of ~10 μmol/L (31), so we tested concentrations from 1 to 30 μmol/L. After incubation for 1 h (Fig. 2A), canagliflozin increased AMPK activity at concentrations from 1 to 30 μmol/L, with 10 μmol/L giving a similar activation to that observed with 300 μmol/L A769662, which activates AMPK by direct binding between the α- and β-subunits (32), or berberine, a mitochondrial inhibitor that activates AMPK by increasing the cellular AMP-to-ATP ratio (19). AMPK activation by canagliflozin, A769662, and berberine was associated with increased phosphorylation of Thr172 on AMPK and of the primary AMPK site on ACC (pACC) (Fig. 2A; quantified results shown in Supplementary Fig. 1A and B). Figure 2B shows that the effect of 30 μmol/L canagliflozin was rapid, reaching a maximum by 20 min.

Figure 2C and D compares the effects of canagliflozin, dapagliflozin, and empagliflozin. Although dapagliflozin and empagliflozin both activated AMPK, this required concentrations >30 μmol/L, and even at 100 μmol/L, their effects were small compared with canagliflozin. Effects on phosphorylation of AMPK and ACC (Fig. 2F–H, quantified results in Supplementary Fig. 1C–F) were consistent with this. Because single doses of dapagliflozin (20 mg) or empagliflozin (50 mg) produce peak plasma concentrations of only 1–2 μmol/L (33,34), that these inhibitors would produce significant AMPK activation at normal therapeutic doses seems unlikely.

We also examined the effects of the natural product phlorizin and its aglycone form, phloretin. Interestingly, phloretin activated AMPK and promoted phosphorylation of AMPK and ACC at concentrations slightly higher than canagliflozin. However, phlorizin only affected these parameters marginally at much higher concentrations (Fig. 2E, I, and J; quantification of blots in Supplementary Fig. 1G and H).

**Canagliflozin Activates AMPK by Inhibiting Complex I of the Respiratory Chain**
To test whether canagliflozin activated AMPK by increasing cellular AMP or ADP, we tested its effects in HEK-293 cells expressing the WT AMPK-γ2 subunit (WT cells) or the AMP/ADP-insensitive R531G mutant (RG cells) (19). Canagliflozin activated AMPK and promoted its phosphorylation in WT cells but not in RG cells; similar results were obtained with phloretin (Fig. 3; quantification of blots in Supplementary Fig. 2). These results suggest that canagliflozin and phloretin activate AMPK by increasing cellular AMP or ADP.

Cellular AMP levels are low and difficult to measure in cultured cells, so we routinely measure ADP-to-ATP ratios as a surrogate for AMP-to-ATP ratios (17). Increasing concentrations of canagliflozin caused increases in the ADP-to-ATP ratio that were significant at 10 and 30 μmol/L, with 30 μmol/L canagliflozin producing an effect similar to 10 mmol/L phenformin (Fig. 4A). Similar results were obtained with phloretin (Fig. 4B). The increase in the cellular ADP-to-ATP ratio due to canagliflozin was accompanied by a reduction of cellular oxygen consumption (Fig. 4C). The effect of 30 μmol/L canagliflozin was smaller, although more rapid, than that of 10 mmol/L phenformin. When the uncoupler 2,4-DNP was added 60 min later, there was a large increase in oxygen consumption (representing the maximal respiratory rate when mitochondria were not limited by the supply of ADP) in cells treated with DMSO. However, as canagliflozin concentrations increased, the effects of 2,4-DNP were eliminated, suggesting that,
like phenformin, canagliflozin inhibited the respiratory chain rather than the F1 ATP synthase.

We next used primary mouse hepatocytes permeabilized with digitonin and measured the function of respiratory chain Complexes I and II. Figure 4D and E shows that canagliflozin and dapagliflozin inhibited Complex I–supported respiration in a concentration-dependent manner, whereas any effect on Complex II was marginal. The inhibitory effect of canagliflozin on Complex I was significantly greater than that of dapagliflozin, with estimated half-maximal effects at 18 ± 1 μmol/L for canagliflozin and 40 ± 3 μmol/L for dapagliflozin.
AMPK-Dependent and -Independent Effects of Canagliflozin on Cellular Metabolism

To examine effects of canagliflozin on metabolic effects mediated by AMPK, we initially used immortalized MEFs with a double knockout (DKO) of AMPK-α1 and -α2 or WT controls. Figure 5A shows that AMPK was activated by concentrations of canagliflozin above 1 μmol/L in WT cells, associated with phosphorylation of AMPK and ACC (blots quantified in Supplementary Fig. 3A and B). As expected, AMPK activity was undetectable in DKO cells, and although ACC expression was normal, there was no basal or canagliflozin-stimulated ACC phosphorylation. We next measured lipid synthesis using [14C]acetate and fatty acid oxidation using [3H]palmitate (Fig. 5B and C). A769662 and phenformin, which activate AMPK by different mechanisms, caused inhibition of lipid synthesis in the WT cells, whereas canagliflozin caused increasing inhibition at 10 and 30 μmol/L. As expected, the inhibition by A769662 was abolished in DKO cells, but surprisingly, lipid synthesis was stimulated by both phenformin and canagliflozin in DKO cells. Fatty acid oxidation (Fig. 5C) was stimulated by AICAR, and this was abolished in the DKO cells, showing that it was AMPK-dependent. However, phenformin and canagliflozin inhibited fatty acid oxidation in the WT and DKO cells. The likely explanation for this is that these drugs, although activating AMPK, inhibit the respiratory chain and, therefore, prevent reoxidation of NADH and FADH₂ generated by fat oxidation, an effect that would be AMPK-independent and that has been previously observed in primary hepatocytes treated with metformin (22).

Canagliflozin Inhibits Lipogenesis in Hepatocytes Through a Mechanism Involving Phosphorylation of ACC by AMPK

The inhibition of lipid synthesis by canagliflozin in Fig. 5B was associated with phosphorylation of ACC at the AMPK sites (Fig. 5D). Figure 5E shows that increasing concentrations of canagliflozin caused progressive inhibition of lipid synthesis in WT hepatocytes, whereas canagliflozin caused increasing inhibition at 10 and 30 μmol/L. As expected, the inhibition by A769662 was abolished in DKO cells, but surprisingly, lipid synthesis was stimulated by both phenformin and canagliflozin in DKO cells. Fatty acid oxidation (Fig. 5C) was stimulated by AICAR, and this was abolished in the DKO cells, showing that it was AMPK-dependent. However, phenformin and canagliflozin inhibited fatty acid oxidation in the WT and DKO cells. The likely explanation for this is that these drugs, although activating AMPK, inhibit the respiratory chain and, therefore, prevent reoxidation of NADH and FADH₂ generated by fat oxidation, an effect that would be AMPK-independent and that has been previously observed in primary hepatocytes treated with metformin (22).

Canagliflozin Inhibits Glucose Uptake in HEK-293 Cells and MEFs

We next assessed the effects of the drugs on glucose transport by measuring 2DG uptake. Canagliflozin inhibited...
uptake by 50–60% in HEK-293 cells and MEFs, whereas the AMPK activator AICAR had no effect. The results were identical in WT MEFs and AMPK DKO MEFs, showing that this effect of canagliflozin was AMPK-independent.

We also assessed the expression of SGLT2 in these cell types using Western blotting. HEK-293 cells and mouse liver, but not MEFs, expressed a polypeptide that comigrated with SGLT2 in mouse kidney. HEK-293 cells, MEFs, and mouse liver also appeared to express GLUT1, although the band did not always comigrate with the bands in control tissue, possibly due to variable glycosylation (Fig. 6C). Attempts to measure expression of other glucose transporters (GLUT3, SGLT1) by Western blotting were inconclusive. The effect of canagliflozin to inhibit glucose uptake in HEK-293 cells and MEFs appears to be yet another off-target effect, because dapagliflozin had no effect on 2DG uptake in either cell type (Fig. 6A and B). To further confirm that the activation of AMPK in HEK-293
cells was not secondary to its effects on glucose uptake, we compared the effects of canaglifl ozin with complete glucose removal from the medium (Fig. 6D; quantification of blots in Supplementary Fig. 3C and D). Although removal of all glucose caused a modest activation and Thr172 phosphorylation of AMPK, activation by 30 μmol/L canaglifl ozin was more than threefold larger. Because inhibition of glucose uptake by canaglifl ozin was only partial (Fig. 6A), the

Figure 5—Effects of canaglifl ozin on AMPK, lipid synthesis, and fatty acid oxidation in intact cells. A: WT and AMPK-α1−/− and AMPK-α2−/− MEFs (AMPK-α1/α2 DKO) were incubated with the indicated concentrations of canaglifl ozin for 1 h, and AMPK activity was determined (upper panel) in anti-AMPK-α immunoprecipitates (mean ± SEM, n = 4). Assays were performed in the DKO cells, although the activity, as expected, was negligible. *P < 0.05 and ****P < 0.0001 indicate significant differences from the vehicle control. The lower panel shows phosphorylation of AMPK and ACC analyzed in duplicate dishes of cells from the same experiment (see Supplementary Fig. 3 for quantification of these blots). B: Lipid synthesis (incorporation of radioactivity from [14C]acetate into total lipid, i.e., fatty acids and sterols) in WT and AMPK-α1/α2 DKO MEFs incubated in the indicated concentrations of A769662, phenformin, or canaglifl ozin for 1 h (mean ± SEM, n = 4). **P < 0.01 and ****P < 0.0001 indicate significant differences from vehicle controls. C: Fatty acid oxidation (incorporation of radioactivity from [3H]palmitate into 3H2O) in WT and AMPK-α1/α2 DKO MEFs incubated in the indicated concentrations of phenformin, AICAR, or canaglifl ozin for 1 h. Results are mean ± SEM, n = 4. ****P < 0.0001 indicates significant differences from vehicle controls. D: Lipid synthesis and protein phosphorylation in primary mouse hepatocytes from WT and ACC1/ACC2 DKI mice. The upper panel shows incorporation of radioactivity from [3H]acetate into total lipid measured after 4 h (mean ± SEM; cells from three mice, each performed in triplicate), ****P < 0.0001 indicates significant differences from control within each genotype. †P < 0.05, ††P < 0.01, and ††††P < 0.0001 indicate significant differences between genotypes at the same drug concentration. The lower panel shows analysis of protein phosphorylation by Western blotting of a representative example. E: Quantification of the increases in phosphorylation in WT hepatocytes of Thr172 on AMPK (left) or Ser79 plus Ser212 on ACC1/ACC2 (right), normalized for the expression of total AMPK or ACC1/ACC2 and expressed relative to control (mean ± SEM; n = 4 for pT172/AMPK-α, and n = 5 for pACC/ACC). *P < 0.05, **P < 0.01, and ****P < 0.0001 indicate significant differences from vehicle controls.
effect of canagliflozin on AMPK is unlikely to be due to a reduced supply of glucose for catabolism.

Canagliflozin Activates AMPK in Mice In Vivo

To test whether canagliflozin activated AMPK in vivo, it was administered to mice (100 mg/kg) by oral gavage, and tissues were collected 3 h later by freeze clamping in situ, which preserves the activation state of AMPK (35). In liver, Thr172 phosphorylation of AMPK was significantly increased by this treatment, as was the phosphorylation of ACC and Raptor at AMPK sites (Fig. 7A and Supplementary Fig. 4). By contrast, significant increases in phosphorylation of AMPK, ACC, and Raptor were not observed in muscle (tibialis interior), gonadal white adipose tissue, or spleen (Supplementary Fig. 5A–C). We also measured the effects on the RER of oral administration of canagliflozin at the time of withdrawing food from previously fasted mice that had been reed for 2 h. Canagliflozin caused a more rapid drop in RER than vehicle in WT mice, indicating a more rapid shift back toward fat rather than carbohydrate oxidation (Fig. 7B). However, this was also observed in ACC1/ACC2 DKI mice, showing that the effect was independent of ACC phosphorylation and, therefore, presumably of AMPK (Fig. 7C). This reduction in RER by canagliflozin was likely secondary to the reduction of blood glucose, which was similar in WT and ACC DKI mice (Fig. 7D).

DISCUSSION

Recent clinical trials suggest that the SGLT2 inhibitors canagliflozin, dapagliflozin, and empagliflozin show promise for reversal of hyperglycemia, either as monotherapy or as adjuncts to existing therapy. Compared with dapagliflozin, canagliflozin also has consistently favorable effects on plasma lipid profiles (7,8,36,37). These differential effects on plasma lipids prompted us to investigate whether canagliflozin might have SGLT2-independent effects. Our results show that canagliflozin causes a substantial activation of AMPK in human and mouse cells at concentrations corresponding to the peak plasma concentrations achieved after therapeutic doses in humans. By contrast, dapagliflozin and empagliflozin only caused a modest AMPK activation at concentrations well above their peak plasma concentrations. Thus, activation of AMPK by dapagliflozin or empagliflozin is less likely to be significant in vivo.

Our results demonstrate that AMPK activation is primarily due to inhibition of Complex I of the respiratory chain, leading to increases in cellular AMP/ADP that bind to the γ-subunit and promote Thr172 phosphorylation.
Thus, canaglifl ozin:

1. increased cellular ADP-to-ATP ratios;
2. increased AMPK activation and Thr172 phosphorylation in cells expressing the WT but not the AMP/ADP-insensitive R531G mutant of AMPK-γ2;
3. inhibited oxygen uptake in HEK-293 cells; and
4. inhibited oxygen uptake in permeabilized mouse hepatocytes provided with substrates that feed into Complex I.

Dapaglifl ozin also caused a less potent effect on Complex I, although only at concentrations (10–30 μmol/L) higher than those observed in human plasma with normal doses. We also found that canaglifl ozin, but not dapaglifl ozin, inhibited 2DG uptake in HEK-293 cells and MEFs in an AMPK-independent manner, indicating that it had additional off-target effects on glucose transport, presumably due to inhibition of another glucose transporter such as GLUT1. Indeed, previous studies in L6 myotubes have indicated that 10 μmol/L canaglifl ozin can inhibit glucose uptake by ~50%, an effect that was attributed to GLUT1 inhibition (38). However, this is unlikely to account for the AMPK activation observed in our experiments, because even complete removal of glucose from the medium had only a modest effect on AMPK activity compared with canaglifl ozin.

Interestingly, we found that phloretin, the aglycone derivative of phlorizin, also activated AMPK, although phlorizin itself was much less effective. Like canaglifl ozin,
ylation. Surprisingly, phenformin and canagliflozin, correlating with a complete loss of ACC phosphorylation. These inhibitory effects were abolished in MEFs lacking AMPK, consistent with a complete loss of ACC phosphorylation. Surprisingly, phenformin and canagliflozin (but not A769662) stimulated lipid synthesis in DKO cells. When we measured fatty acid oxidation in the same cells, phenformin and canagliflozin inhibited the pathway in an AMPK-independent manner, which is expected because both compounds inhibit Complex I. The major fates of cellular acetyl-CoA are oxidation by the tricarboxylic acid cycle or incorporation into lipids. Thus, in the absence of AMPK to inhibit lipid synthesis, acetate flux might be diverted into lipid synthesis as oxidation was inhibited. This provides an explanation for the paradoxical activation of lipid synthesis by phenformin and canagliflozin observed in AMPK knockout cells.

Our results in isolated mouse hepatocytes demonstrate that the effects of canagliflozin on lipid synthesis are mediated by phosphorylation of ACC. Thus, a moderate and therapeutically relevant concentration of canagliflozin (10 μmol/L) inhibited lipid synthesis in WT hepatocytes but failed to do so in DKI cells from mice where both ACC isoforms lacked the critical AMPK site. At higher canagliflozin (30 μmol/L), there was some inhibition even in DKI hepatocytes, although significantly less than in WT cells; this is most likely because both ACC isoforms use ATP as a direct substrate. Once the increase in the cellular ADP-to-ATP ratio becomes substantial, as with 30 μmol/L canagliflozin (Fig. 4A), decreases in ATP may limit flux through ACC independently of AMPK. This is a revealing demonstration of the physiological role of AMPK: as ATP falls under situations of energetic stress, the kinase limits the function of energy-consuming pathways before the ATP concentration has dropped to levels where it becomes limiting for the pathway itself. This occurs because AMPK is more sensitive to ATP depletion than the ATP-consuming enzymes in the pathway.

Our results show that oral administration of canagliflozin increased Thr172 phosphorylation of AMPK in liver in vivo and phosphorylation of ACC and Raptor, two of its well-recognized downstream targets. Although we only tested effects of the drug in normal mice, there is no reason to believe the results would be any different in diabetic models; for example, berberine, which activates AMPK by the same mechanism as canagliflozin (19), activates AMPK normally in db/db mice (41). Canagliflozin also caused a more rapid drop in the RER when administered to fed mice, indicating a more rapid switch to fat versus carbohydrate oxidation. However, this was still observed in the DKI mice and was, therefore, presumably independent of AMPK. It is possible that a reduction in blood glucose caused by canagliflozin causes increased fat oxidation due to competition between glucose and fat for substrate oxidation (42) and that this obscures any effect due to ACC phosphorylation by AMPK. This might be addressed in future studies using SGLT2 knockout mice.

Our findings raise the interesting question of whether dual therapy with canagliflozin and metformin would be more effective than canagliflozin alone. In a recently reported clinical trial of newly diagnosed subjects with type 2 diabetes (43), monotherapy with canagliflozin was more effective in lowering HbA1c than metformin. Although dual therapy was more efficacious than canagliflozin alone, the effects of the two drugs were not additive, as might be expected if they had distinct mechanisms of action. By contrast, in a similar trial using metformin and dapagliflozin, monotherapy with dapagliflozin was not more effective than metformin, and the effects of dual therapy were closer to being additive (44).

Finally, although the long-term effects of metformin to inhibit hepatic glucose production in mice are AMPK-dependent (22), studies have suggested that its rapid effects are AMPK-independent (20,21). However, the latter authors agree that the primary effect of metformin is to inhibit the respiratory chain and increase cellular AMP in the liver, which is then proposed to affect other AMP-sensitive targets such as fructose-1,6-bisphosphatase (20) or adenylate cyclase (21). Because canagliflozin inhibits the respiratory chain and activates AMPK in the liver, it is conceivable that these AMP-dependent but AMPK-independent effects of metformin might also be mimicked by canagliflozin.

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References
1. Rosenwasser RF, Sutton S, Sutton D, Choksi R, Epstein BJ. SGLT-2 inhibitors and their potential in the treatment of diabetes. Diabetes Metab Syndr Obes 2013;6:453–467
