High-Throughput Assay for Modulators of Mitochondrial Membrane Potential Identifies a Novel Compound With Beneficial Effects on *db/db* Mice

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OBJECTIVE—Recently, several drugs have been shown to exert beneficial effects for metabolic syndrome through mild regulation of mitochondrial function. Hence, we explored a strategy of targeting mitochondrial function to improve glucose and lipid metabolism.

RESEARCH DESIGN AND METHODS—Mitochondrial membrane potential ($\Delta\psi m$) is a marker of mitochondrial function; therefore, we set up a high-throughput screening assay of $\Delta\psi m$ in L6 myotubes. The effects of a selected lead compound were investigated in vitro and in vivo in relation to metabolic syndrome.

RESULTS—A novel small-molecule compound, C1, was identified through this high-throughput screening. C1 depolarized $\Delta\psi m$ in L6 myotubes without cytotoxicity and led to increased cellular AMP-to-ATP ratio, activation of AMP-activated protein kinase (AMPK), and enhanced glucose uptake. It also stimulated the AMPK pathway in HepG2 cells, leading to decreased lipid content. Intriguingly, C1 inhibited respiration in L6 myotubes but did not affect respiration in isolated muscle mitochondria, suggesting that it may depolarize $\Delta\psi m$ indirectly by affecting the supply of electron donors. Acute administration of C1 in C57BL/6J mice markedly increased fat oxidation and the phosphorylation of AMPK and acetyl-CoA carboxylase in the liver. In diabetic *db/db* mice, chronic administration of C1 significantly reduced hyperglycemia, plasma fatty acids, glucose intolerance, and the mRNA levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver.

CONCLUSIONS—Our results demonstrate a novel small molecule that mildly depolarizes $\Delta\psi m$ and is able to improve glucose and lipid metabolism to exert beneficial effects for metabolic syndrome. These findings suggest that compounds regulating mitochondrial function may have therapeutic potential for type 2 diabetes. *Diabetes* 59:256–265, 2010

Current medications for type 2 diabetes are insufficient, and, as such, new antidiabetic agents that ameliorate insulin resistance and hyperlipidemia are needed to combat this disease. AMP-activated protein kinase (AMPK) serves as a "fuel gauge" in cells and represents an attractive target for treating metabolic disease (1,2). AMPK is activated under conditions that signify cellular stress, such as exercise, ischemia, and hypoxia, which induce an increment in the intracellular AMP-to-ATP ratio (3). Once activated, it orchestrates a variety of metabolic processes including stimulation of glucose uptake in muscle, increases in fatty acid oxidation, and inhibition of hepatic cholesterol and triglyceride synthesis and lipogenesis (4,5). These metabolic responses are primarily mediated through phosphorylation of enzymes or transcription factors and co-activators that regulate gene expression (3).

Interestingly, AMPK can also be activated by the antidiabetic drugs metformin and thiazolidinediones (TZDs) (6,7) and other natural products with antiobesity and antidiabetes properties, such as berberine (8) and resveratrol (9). The mechanism by which these agents exert their beneficial metabolic effects has been shown to be, at least in part, via modulating mitochondrial function (10–14). These findings raised the possibility of discovering novel therapeutics for metabolic disease from compounds that perturb mitochondrial function and thus activate AMPK.

In this study, we set up a high-throughput screening assay based on mitochondrial membrane potential ($\Delta\psi m$) and identified a small-molecule compound C1 that depolarizes $\Delta\psi m$ in a mild manner without causing cell toxicity. C1 increases cellular AMP-to-ATP ratio and activates AMPK, resulting in beneficial metabolic effects in L6 myotubes and HepG2 cells and in *db/db* mice. Interestingly, our studies indicate that C1 causes mild inhibition of mitochondrial respiration through a mechanism distinct from TZDs, metformin, or berberine (13). These findings suggest that targeted identification of mild mitochondrial modulators may represent an effective approach for the discovery of new therapeutics for type 2 diabetes.

RESEARCH DESIGN AND METHODS

Materials. Compound C, 5-aminoimidazole-4-carboxamide-1-D-ribofuranoside (AICAR), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), wortmannin, metformin, rosiglitazone, troglitazone, and insulin were obtained from Sigma Aldrich (St. Louis, MO). Radiochemical 2-deoxy-[³H]-D-glucose and Western blotting detection kits (enhanced chemiluminescence) and Hyperfilm were purchased from Amersham Biosciences (Uppsala, Sweden).

Cell culture. L6 myoblasts were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 4.5 g/l glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. For L6 myoblast differentiation, the concentration of FBS was decreased from 10 to 2%. Myotubes were used for experiments 5–7 days after differentiation. HepG2 cells were maintained in DMEM containing 10% FBS, 1 g/l glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Mitochondrial membrane potential assay. This assay was based on a previous report with modifications (15). Briefly, 7,000 L6 myoblasts per well were seeded into black 96-well optical-bottom plates (Greiner Bio-One, Dusseldorf, Germany) at 100 μl per well. On day 5 of differentiation, compounds were added and plates were incubated at 37°C for 10 min. After incubation, 100 μl fresh medium containing 0.2 μg JC-1 was added to each well. Plates were incubated for another 20 min at 37°C, and each well was washed three times with 100 μl Krebs-Ringer phosphate HEPES buffer. Fluorescence was measured in a Flexstation II plate reader (Molecular Device, Union City, CA) with first at lengths of excitation and emission (ex/em) 530 nm/580 nm ("red") and then at ex/em 485 nm/530 nm ("green"). The ratio of red to green reflects the Δψm. The coefficient of variation (CV%) and Z' factor for the quality control of high-throughput screening assay were estimated (16).

Adenine nucleotide extraction and measurement. L6 myotubes cultured in 60-mm dishes treated with C1 were washed with PBS (140 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4, 1.8 mmol/l KH_2PO_4) and trypsinized. The samples for cellular adenine nucleotides measurement were prepared and analyzed as previously described (17).

Measurement of 2-deoxy-[³H]-D-glucose uptake. 2-Deoxyglucose uptake was measured as described (17,18). Briefly, after stimulation with C1 or insulin, cells were washed three times with HEPES-buffered saline solution (20 mmol/l HEPES [pH 7.4], 136 mmol/l NaCl, 4.7 mmol/l KCl, 1.25 mmol/l $MgSO_4$, 1.2 mmol/l $CaCl_2$) followed by incubation with 2-deoxy-[³H]-D-glucose for 10 min. Cells were washed with ice-cold PBS, lysed using 0.1% Triton-X 100, and radioactivity counted.

Western blot analysis. Cell lysates were subjected to electrophoresis through 8% SDS-PAGE and blotted with antibodies for AMPK, phospho-AMPK, acetyl-CoA carboxylase (ACC), phospho-ACC, Akt, and phospho-Akt purchased from Cell Signaling Technology (Beverly, MA) and β-actin from Upstate (Billerica, MA). The immunoblots were visualized by chemiluminescence using the enhanced chemiluminescence Western Blotting System.

Determination of triglyceride and cholesterol contents. HepG2 cells cultured in 60-mm dishes were lysed in RIPA buffer (20 mmol/l Tris-HCl, pH 8.0, 1% Nonidet P-40, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonylfluoride, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 1 mmol/l EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). Triglyceride and total cholesterol contents were determined in cell lysates using a colorimetric assay and are expressed as milligram lipid per milligram of cellular protein as described (19,20).

Measurement of respiration in L6 myotubes and isolated mitochondria. Mitochondria were isolated from mouse quadriceps muscle using a method previously described (13). Respiration measurements in L6 myotubes and isolated mitochondria were conducted at 37°C in a Clark type oxygen electrode (Strathkelvin Instruments, Motherwell, Scotland). For L6 myotubes, respiration measurements were conducted using cell culture medium, while for mitochondria, a standard respiration medium was used (13). Cells or mitochondria were transferred to the electrode chamber, and after attaining a steady rate of oxygen consumption, C1 was added dose-dependently, and its effect on oxygen consumption was recorded.

Determination of lactate content. The cells were cultured in a 24-well plate and treated with C1 and positive control in serum-free cell culture medium for the indicated time. Lactate in the medium was measured with a lactate assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animal experiments. All animal experiments were approved by the Animal Ethics Committee of either the Shanghai Institute of Materia Medica or Garvan Institute of Medical Research/St. Vincent's Hospital, where the experiments were conducted. Male C57BL/6J *db/db* and lean C57BL/6J mice were housed in a temperature-controlled room (22 ± 2°C), with a light/dark cycle of 12 h. At 8–14 weeks of age, mice were randomly assigned to acute or chronic treatment. The acute effects of C1 (50 mg/kg) on oxygen consumption rate (VO_2) and respiratory exchange ratio were measured using an eight-chamber indirect calorimeter (Oxymax series; Columbus Instruments, Columbus, OH) as described previously (21). To examine the acute effects of C1 on activation of AMPK, lean mice were given intraperitoneal injections of vehicle (0.9% NaCl), C1 (20 mg/kg), or AICAR (400 mg/kg) as a positive control. Animals were killed after 2 h with the liver freeze clamped for the determination of phosphorylation of AMPK and ACC. For chronic treatment, lean and *db/db* mice received oral administration of either vehicle (0.5% methylcellulose) or C1 (50 mg · kg⁻¹ · day⁻¹) or metformin (250 mg · kg⁻¹ · day⁻¹) for 4 weeks. Body weight and food intake were recorded daily. Fasting and random-fed blood glucose levels were measured every other week. Glucose tolerance test (2 g/kg glucose i.p.) was performed in overnight-fasted mice as described before (13). Plasma concentrations of nonesterified fatty acids (NEFAs) and triglyceride were measured using kits from Wako Diagnostics (Richmond, VA) and Nanjing Jiancheng Bioengineering Institute (Nanjing, China), respectively. Plasma insulin content was measured by using a rodent insulin enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, St. Charles, MO). After 4 weeks of treatment, animals were killed with the liver freeze clamped for the determination of gene expression.

RNA isolation and real-time PCR. Gene expression was analyzed by real-time PCR (RT-PCR). Total RNA prepared from mouse livers was extracted with Trizol (Invitrogen). Complementary DNA generated by M-MLV Reverse Transcriptase (Promega, Madison, WI) was analyzed by quantitative PCR using an SYBR Premix Ex *Taq* (TaKaRa, Dalian, China). All samples were run in duplex with and normalized to tubulin expression. The sequences of oligonucleotide primers used are described elsewhere (22).

Glucose output assay. Primary hepatocytes were prepared from Sprague-Dawley rats as previously described (23,24) and were plated with DMEM containing 1 g/l glucose (Invitrogen) in 12-well plates (5×10^5 cells in each well). After 4-h attachment, cells were changed to serum-free medium and incubated with 5, 10, and 20 μmol/l C1 for 16 h. The medium was then replaced with 500 μl glucose production buffer consisting of glucose-free DMEM, without phenol red, supplemented with 20 mmol/l sodium lactate and 2 mmol/l sodium pyruvate. After a 4.5-h incubation, 50 μl medium was collected and the glucose concentration was measured with a colorimetric glucose assay kit (Fudan-Zhangjiang, Shanghai, China).

Statistical analysis. Results are presented as means ± SE. Differences between groups were analyzed by Student's *t* test. For respiration measurements, the effect of C1 treatment is given as a percentage of intraindividual control values (100%), with *P* values calculated by paired *t* tests. $P < 0.05$ was regarded as statistically significant.

RESULTS

Identification of C1 by Δψm and AMPK assays in L6 myotubes. Δψm provides an index of mitochondrial function, and therefore to screen for novel compounds that can affect mitochondrial function, we developed a high-throughput screening assay in a 96-well format for Δψm in L6 myotubes using JC-1, a positively charged fluorescent compound. Initially, we characterized this assay using the mitochondrial uncoupler CCCP as a control and observed a dose-dependent decrease in Δψm with CCCP (Fig. 1A). The CV% and Z' factor determined with CCCP were 8.5 and 0.6, respectively, which meet the criteria for quality control of a high-throughput screening assay.

Next, the effect of berberine, TZDs, and metformin on Δψm was examined, since we have previously reported that these agents exert their beneficial effects, in part, by perturbing mitochondrial function (13). A decrease in Δψm of ~20% was observed when L6 myotubes were treated with the above compounds for 30 min (Fig. 1B). We also tested the AMPK activator AICAR (25,26) and observed no effect on Δψm. A recent study reported that AICAR (0.5 mmol/l) inhibits oxidative phosphorylation in primary mouse hepatocytes (27), and thus it would be expected to also decrease Δψm. However, there is clearly a difference in sensitivity between cell types, as we have found that even at a concentration of 2 mmol/l, AICAR does not inhibit respiration in L6 myotubes (data not shown).

We then proceeded to randomly screen our compound library, which contains pure synthetic compounds and natural products collected from different sources, and from this screen, we identified a small molecule (449.14 Da), named C1 (Fig. 1C), as a compound able to depolarize Δψm in a dose-dependent manner within a 30-min treatment. Compared with CCCP (Fig. 1D), which depolarized Δψm within a narrow dose range, C1 depolarized Δψm in a relatively mild manner, reaching <50% even at the
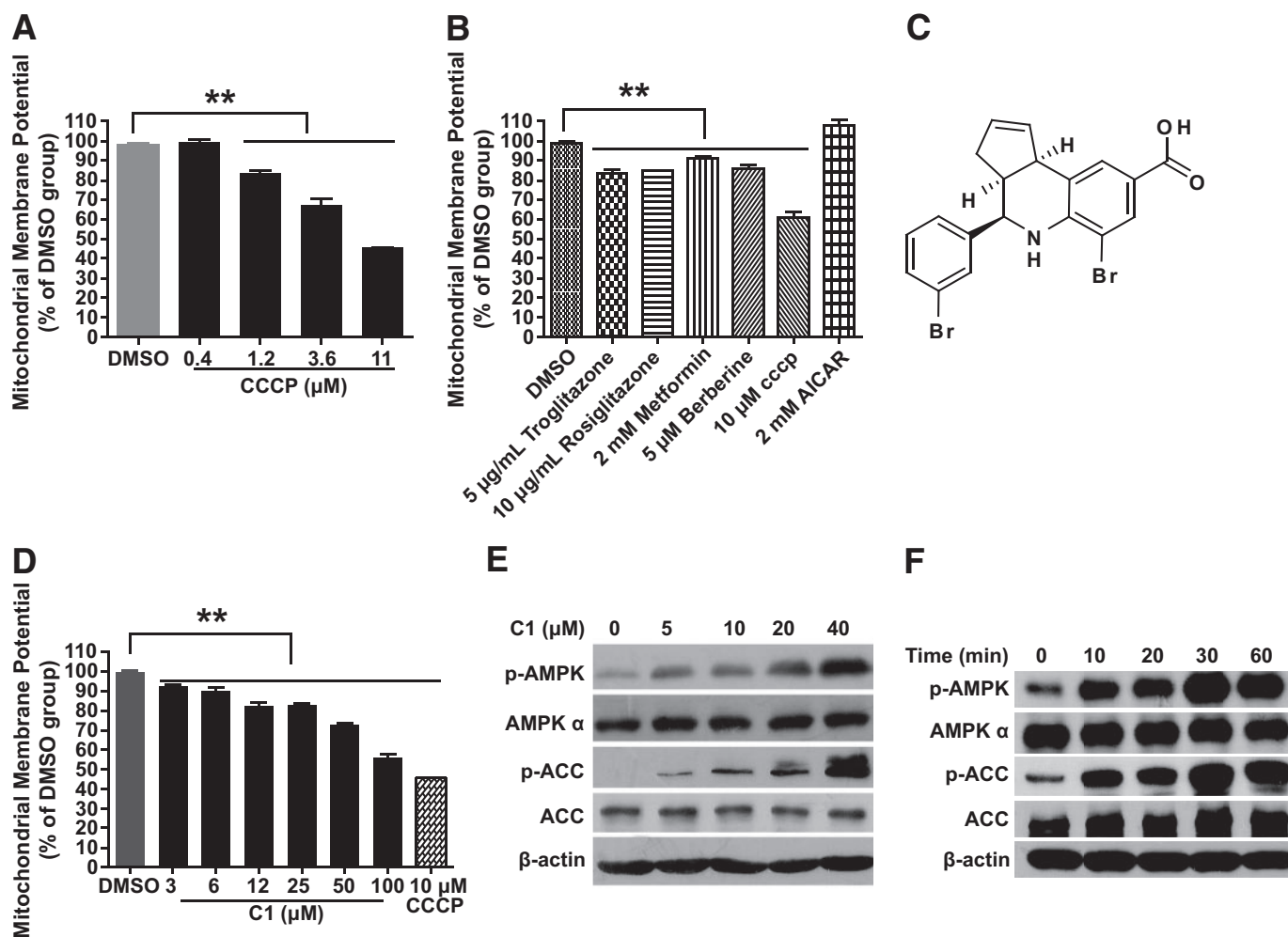
FIG. 1. High-throughput screening for mild inhibitors of Δψm and AMPK assays in L6 myotubes. For the Δψm assay, L6 myotubes in a 96-well plate were treated with indicated agents for 10 min and then incubated with 1 μg/ml JC-1 for another 20 min. Cells were washed with Krebs-Ringer phosphate HEPES buffer three times and then used for fluorescence intensity measurement. *A*: Detection of CCCP induced Δψm depolarization in a JC-1–based fluorescent high-throughput assay in L6 myotubes. CCCP depolarizes Δψm in a dose-dependent manner. *B*: Effect of known antidiabetic agents on Δψm in L6 myotubes. Berberine (5 μmol/l), troglitazone (5 μg/ml), rosiglitazone (10 μg/ml), metformin (2 mmol/l), and AICAR (2 mmol/l) on Δψm. *C*: Chemical structure of compound C1. *D*: Dose-dependent depolarization of Δψm by C1. *E* and *F*: Effects of C1 on the AMPK signaling pathway by Western blots. For the dose-dependent assays, C1 was incubated with L6 myotubes for 60 min and for the time course measurement 20 μmol/l C1 was used. Total cell lysates were prepared from L6 myotubes, resolved by 8% SDS-PAGE and immunoblotted with specific antibodies as indicated. Results are presented as percentage of vehicle (DMSO) control group. **$P < 0.01$ compared with DMSO group ($n = 3$ independent experiments).

highest test concentration (100 μmol/l). For subsequent experiments, a concentration of 20 μmol/l was used, since this dose gave a decrease in the Δψm (~20%) similar to the other antidiabetic agents tested (Fig. 1*B*).

Because AMPK acts as an energy sensor, we examined if treatment of L6 myotubes with C1 would lead to activation of AMPK. We observed a dose- and time-dependent activation of AMPK by C1 as evidenced by the increase in Thr-172 phosphorylation of the α-catalytic subunit of AMPK and the increase in Ser-79 phosphorylation of its downstream target ACC (Fig. 1*E* and *F*).

C1 stimulates glucose uptake via the AMPK pathway in L6 myotubes. Several studies have linked AMPK activation with the stimulation of glucose uptake and utilization by skeletal muscle (28,29). As shown in Fig. 2*A*, C1 stimulated glucose uptake dose-dependently, with 20 μmol/l C1 resulting in a nearly twofold increase compared with controls. To further elucidate the mechanism of C1-stimulated glucose uptake, we measured the effects of 100 nmol/l insulin and 20 μmol/l C1, respectively, or together on glucose uptake. As shown in Fig. 2*B*, when L6

myotubes were treated with insulin and C1 together, glucose uptake was stimulated to a higher extent compared with single treatment with insulin or C1. This additive effect indicated that C1 stimulated glucose uptake in L6 myotubes through a pathway distinct from the insulin-signaling pathway. Indeed, while the phosphatidylinositol 3′ kinase inhibitor wortmannin was able to block glucose uptake and Ser-473 phosphorylation of Akt by insulin, C1 did not activate Akt and wortmannin did not inhibit the stimulation of glucose uptake by C1 (Fig. 2*C* and *D*). We next examined whether the effects of C1 were dependent on AMPK by using the AMPK inhibitor compound C. Compound C pretreatment abolished the ability of C1 to increase glucose uptake (Fig. 2*C*) and blocked the phosphorylation of AMPK and ACC by C1 (Fig. 2*D*).

C1 decreases lipid contents via the AMPK pathway in HepG2 cells. To investigate whether C1 also influences lipid metabolism, we tested its effects in human hepatoma HepG2 cells. As shown in Fig. 3*A*, C1 decreased the contents of triglyceride and cholesterol dose-dependently,
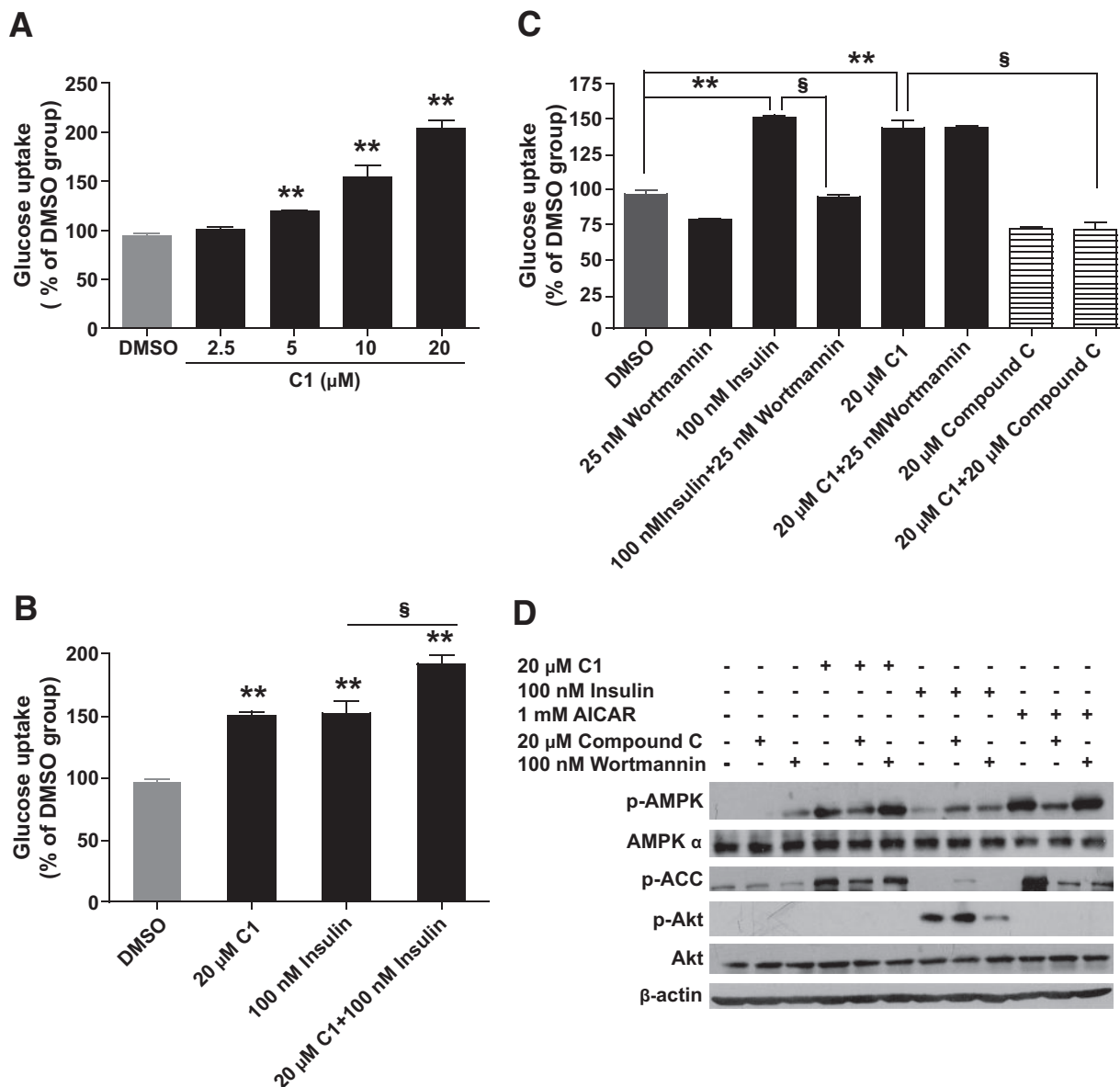
FIG. 2. Effect of C1 on glucose uptake in relation to the AMPK and phosphatidylinositol 3-kinase/Akt signaling pathways. *A*: Dose-dependent effects of C1 on glucose uptake. L6 myotubes were treated for 1 h with a different dose of C1 as described in RESEARCH DESIGN AND METHODS. *B*: Additive effect of C1 on insulin-stimulated glucose uptake. Cells were treated with 100 nmol/l insulin and 20 μmol/l C1 for 30 min and 1 h, respectively. *C*: Influences of wortmannin and compound C on C1-induced glucose uptake. After serum deprivation for 2 h, 25 nmol/l wortmannin or 20 μmol/l compound C was added to the cells for 30 min before and during the incubation with 20 μmol/l C1 for 1 h. A total of 100 nmol/l insulin was added in the last 30 min. *D*: The effects of C1 on AMPK, ACC, and Akt phosphorylation. **$P < 0.01$ compared with DMSO group; §$P < 0.05$. $n = 3$ independent experiments.

with 5 μmol/l C1 able to lower lipid contents with a similar potency to that of 2 mmol/l metformin. Similar to the L6 myotubes, C1 dose-dependently activated the AMPK pathway in HepG2 cells, as evidenced by the increase in AMPK and ACC phosphorylation (Fig. 3*B*). The effects of C1 and metformin on ACC phosphorylation and decreasing triglyceride content could be partially blocked by compound C pretreatment (Fig. 3*C* and *D*), suggesting that, like metformin (19), the lipid-lowering effect of C1 is largely mediated through AMPK.

C1 influences mitochondrial metabolism via a mechanism different from TZDs and metformin. ATP generation occurs primarily in mitochondria, and a decrease in Δψm will lead to a reduced ATP production, an increase in the AMP-to-ATP ratio, and activation of AMPK (30,31). Because the activation of AMPK by TZDs and metformin

involves inhibition of respiratory complex I, we wondered if C1 shares a similar mechanism. C1 treatment for 1 h increased the AMP-to-ATP ratio in a dose-dependent manner, up to fourfold greater than basal levels at a concentration of 40 μmol/l (Fig. 4*A*). We also observed a time-dependent increase in the AMP-to-ATP ratio with 20 μmol/l C1 (Fig. 4*B*). To determine if the change in nucleotide ratio was due to an effect on cellular respiration, we examined oxygen consumption in L6 myotubes and observed a dose-dependent inhibition of respiration at similar concentrations of C1 (Fig. 4*C*). To determine if this effect was due to a specific inhibition of mitochondrial function, we isolated muscle mitochondria and examined the effect of C1 on ADP-stimulated respiration in the presence of complex I (glutamate) or complex II (succinate) substrates. Surprisingly, C1 did not inhibit mitochon-
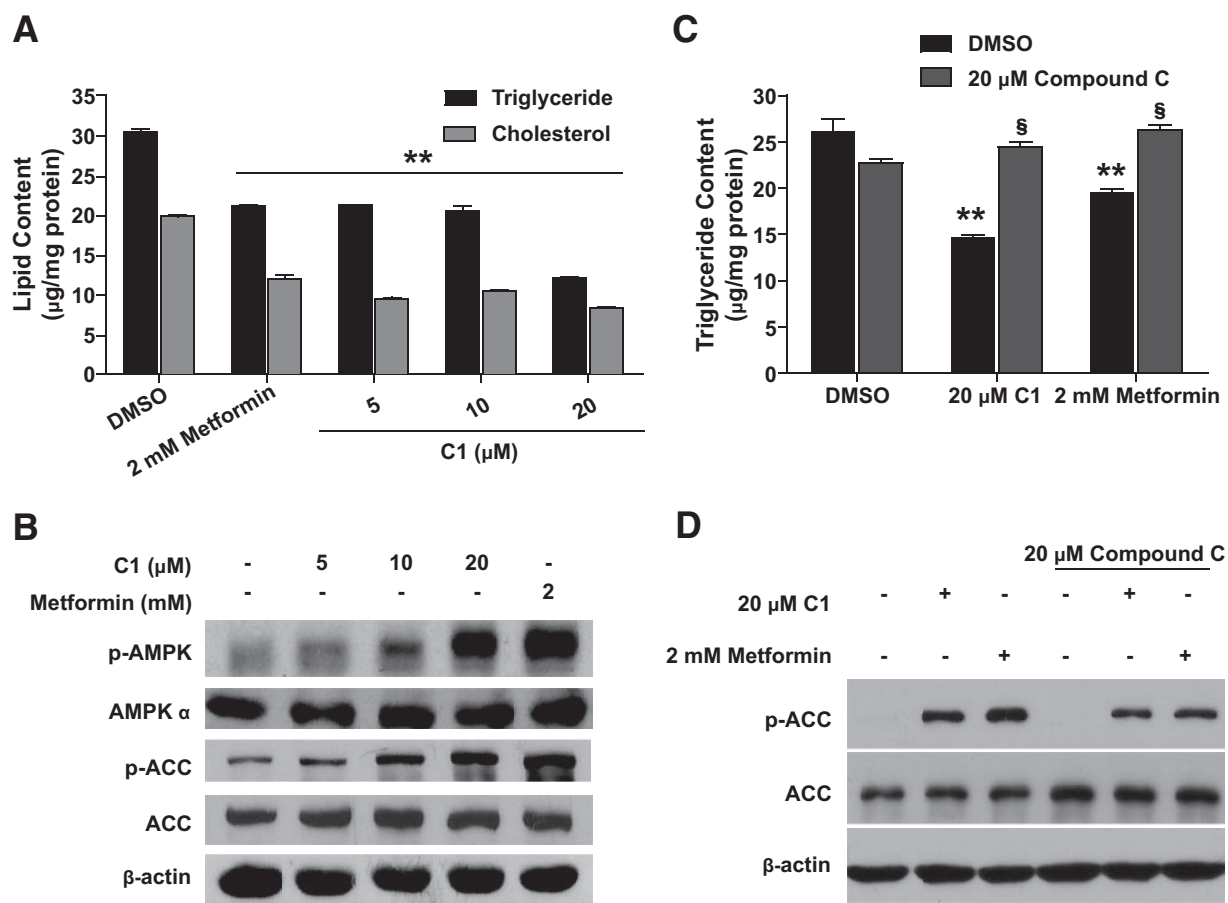
FIG. 3. Effects of C1 on intracellular lipid contents and AMPK signaling pathway. Experiments were performed in HepG2 cells. Cells were starved in serum-free medium overnight and treated with different doses of C1 or 2 mmol/l metformin as a positive control for 24 h before the experiments. *A*: C1 decreases lipid content dose-dependently. *B*: Dose-dependent effects of C1 on AMPK and ACC phosphorylation. *C*: Inhibition by compound C (20 μmol/l) of ACC phosphorylation induced by C1 or metformin. *D*: Reversal of C1 or metformin induced reduction of cell lipid content by compound C (20 μmol/l). **$P < 0.01$ compared with DMSO group; §$P < 0.01$ compared with the sample without inhibitor treatment ($n = 3$ independent experiments).

drial respiration with either substrate (Fig. 4*D*). C1 also had no effect on mitochondrial respiration in the absence of ADP (data not shown).

Because a decrease in aerobic respiration may lead to an elevation in anaerobic respiration to compensate, we tested whether C1 increased lactate release, a marker of anaerobic respiration. C1 (20 μmol/l) acutely increased lactate production from L6 myotubes by 20 and 65% at 1 and 4 h, respectively (Fig. 4*E*). A similar effect was observed for cells treated with 50 μmol/l troglitazone. In HepG2 cells, both C1 and metformin also increased lactate release within 1–4 h (Fig. 4*F*).

Acute effects of C1 in mice. Based on the in vitro data, we examined whether C1 could acutely affect whole-body metabolism in C57BL/6J lean mice. Animals were dosed with 50 mg/kg C1 and in the 6 h following, we observed no change in VO_2 (data not shown), but a significant decrease in respiratory exchange ratio, indicating a shift to fatty acid utilization (Fig. 5*A* and *B*). To investigate if AMPK might be involved, we measured the phosphorylation of AMPK and ACC in the liver, 2 h after C1 treatment, and observed a clear activation of this pathway (Fig. 5*C*), to a degree similar to that of the positive control AICAR (Fig. 5*D* and *E*) (21).

Chronic effects of C1 in mice. To assess the antidiabetic potential of C1 in vivo, *db/db* mice and lean mice were administered 50 mg · kg^{-1} · day^{-1} for 4 weeks. We compared the efficacy of C1 with the well-known antidiabetic agent metformin (250 mg · kg^{-1} · day^{-1}). Food intake and body weight gain were checked daily, and no significant differences were observed between the three groups (Table 1). In lean mice, plasma parameters were generally unchanged by C1 and metformin treatment, apart from a small increase (12%) in random-fed glucose levels in C1-treated animals. In *db/db* mice, random-fed and fasting blood glucose levels were decreased 42 and 21%, respectively, by C1 treatment, while metformin decreased blood glucose levels by ~20% (Table 1). Plasma levels of insulin and triglyceride were unchanged by C1 or metformin treatment, but NEFA levels were decreased by ~20% in C1-treated *db/db* mice. C1 and metformin did not alter glucose tolerance in lean mice (Fig. 6*A* and *B*); however, in *db/db* mice, both agents improved glucose clearance, as evidence by the ~25% decrease in the AUC (Fig. 6*C* and *D*). Liver gluconeogenesis plays an important role in regulating glucose levels; therefore, we assessed the expression of two key gluconeogenic enzymes, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), in response to C1 treatment. The expression of G6Pase and PEPCK was approximately threefold higher in *db/db* mice compared with lean controls (Fig. 6*E* and *F*). C1 treatment reduced the expression of G6Pase and PEPCK by 45 and 62%, respectively, in *db/db* mice. Interestingly, in lean mice, C1 treatment did not affect PEPCK
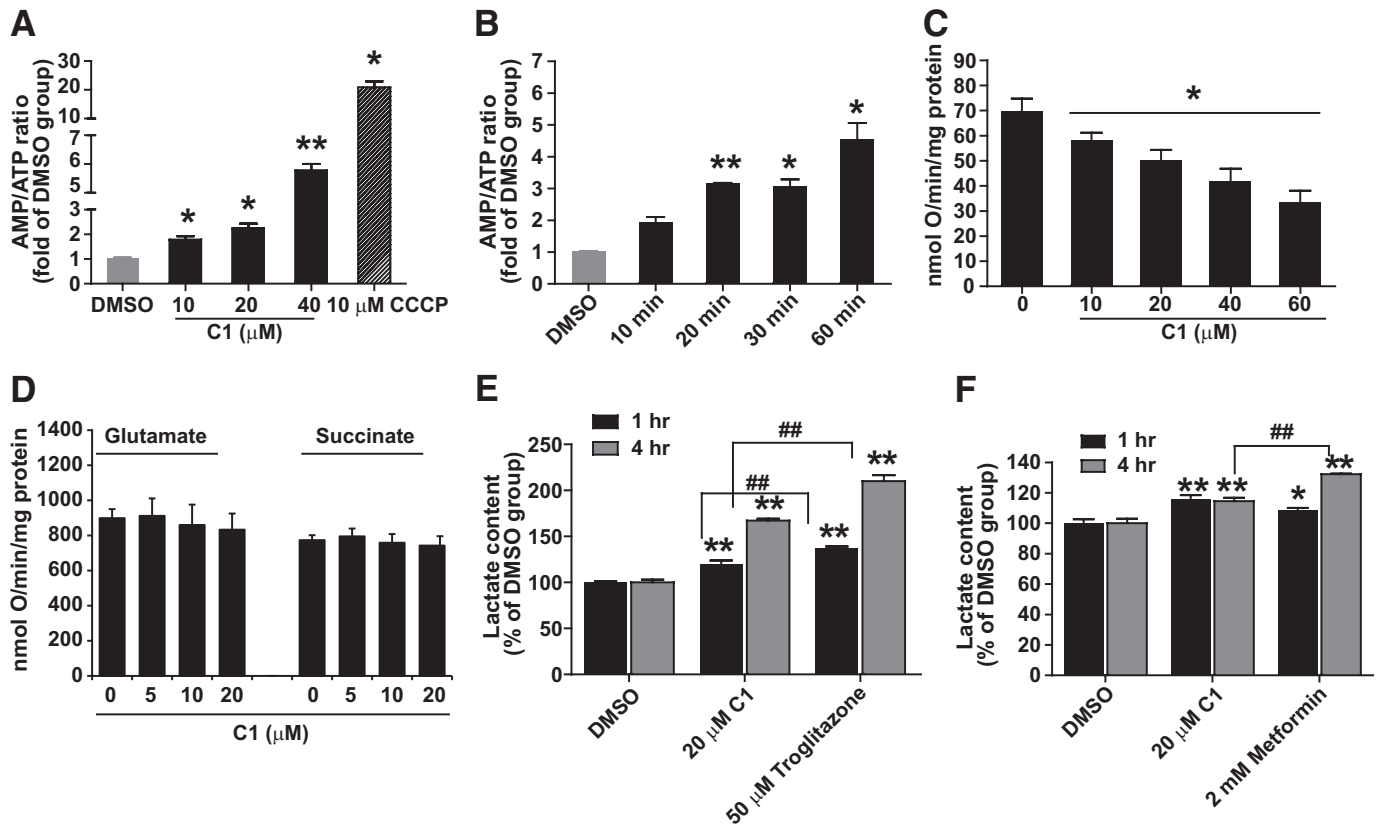
FIG. 4. Effects of C1 on AMP-to-ATP ratio and respiration. Cellular ATP and AMP content were measured by HPLC as mentioned in RESEARCH DESIGN AND METHODS. Results are presented as fold over DMSO group. *A*: Dose-dependent effects of C1 on AMP-to-ATP ratio after incubation for 1 h in L6 myotubes ($n = 3$). *B*: Time course of C1 (20 μmol/l) induced changes in AMP-to-ATP ratio ($n = 3$). *C*: Effect of C1 to inhibit the respiration of intact L6 myotubes ($n = 6$). *D*: Effect of C1 on the respiration of isolated mitochondria from mouse quadriceps muscle ($n = 4$). *E* and *F*: Time-dependent effect of C1 (20 μmol/l) on lactate release from L6 myotubes and HepG2 cells, respectively ($n = 3$). *$P < 0.05$; **$P < 0.01$ compared with DMSO group; ##$P < 0.01$ between C1 and troglitazone or metformin. Results are presented as percentage of DMSO group.

expression, but caused an almost twofold increase in G6Pase. To more directly examine the effect of C1 on gluconeogenesis, we measured glucose output in primary hepatocytes. As shown in Fig. 6*G*, overnight treatment of hepatocytes with C1 dose-dependently inhibited glucose output by 25–35%.

DISCUSSION

Interest in mitochondrial biology has undergone a resurgence in recent years, with the discovery that mitochondrial dysfunction is linked with several diseases, including obesity and type 2 diabetes (32). Intriguingly, mild perturbation of mitochondrial function has been associated with beneficial effects for obesity and type 2 diabetes (10–14,28,33). Metformin and TZDs, drugs widely prescribed for the treatment of type 2 diabetes, exert their antidiabetic effects partly through inhibition of respiratory complex I (10–12). We have recently shown that berberine, a natural product with antidiabetic effects, also inhibits complex I of the respiratory chain (13). A common finding of these reports is that inhibition of mitochondrial function leads to activation of AMPK in tissues such as muscle and liver, and the pleiotropic actions of AMPK likely mediate most of the beneficial effects of these compounds. Therefore, we hypothesized that small molecules, which depolarize $\Delta\psi$m, would activate AMPK and have beneficial effects on these metabolic diseases. Herein, we set up a high-throughput screening assay based on $\Delta\psi$m and dis-

covered a small molecule C1 as a novel modulator of mitochondrial function.

C1 treatment caused depolarization of $\Delta\psi$m in L6 myotubes in a relatively mild fashion, and a consequence of this was activation of AMPK and improvements in metabolic parameters both in vitro and in vivo. In principle, several factors may lead to a drop in $\Delta\psi$m, including inhibition of mitochondrial respiration, induction of proton leakage (uncoupling), or opening of the mitochondrial permeability transition pore. Mitochondrial permeability transition pore opening is tightly correlated with initiation of apoptosis (34), and we excluded this pathway, since we observed no induction of markers of apoptosis after C1 treatment (data not shown). We also ruled out C1 acting directly as an uncoupling agent, as it did not accelerate respiration rate in isolated mitochondria, as typical uncoupling agents do (35). Similarly, uncoupling agents have been shown to induce cell death (36), and we observed no obvious toxicity on viability of a range of different cell types (L6, Hela, A549, and PC12), even at high doses of C1 (data not shown). Collectively, our results suggested that the likely mode of action of C1 to decrease $\Delta\psi$m was through inhibition of mitochondrial respiration.

Several antidiabetic agents, such as troglitazone, rosiglitazone, metformin, and berberine, have been shown to inhibit mitochondrial respiratory complex I (10–14,37). With our assay system, we confirmed that these agents, as expected, caused a decrease in $\Delta\psi$m. Given that the effect
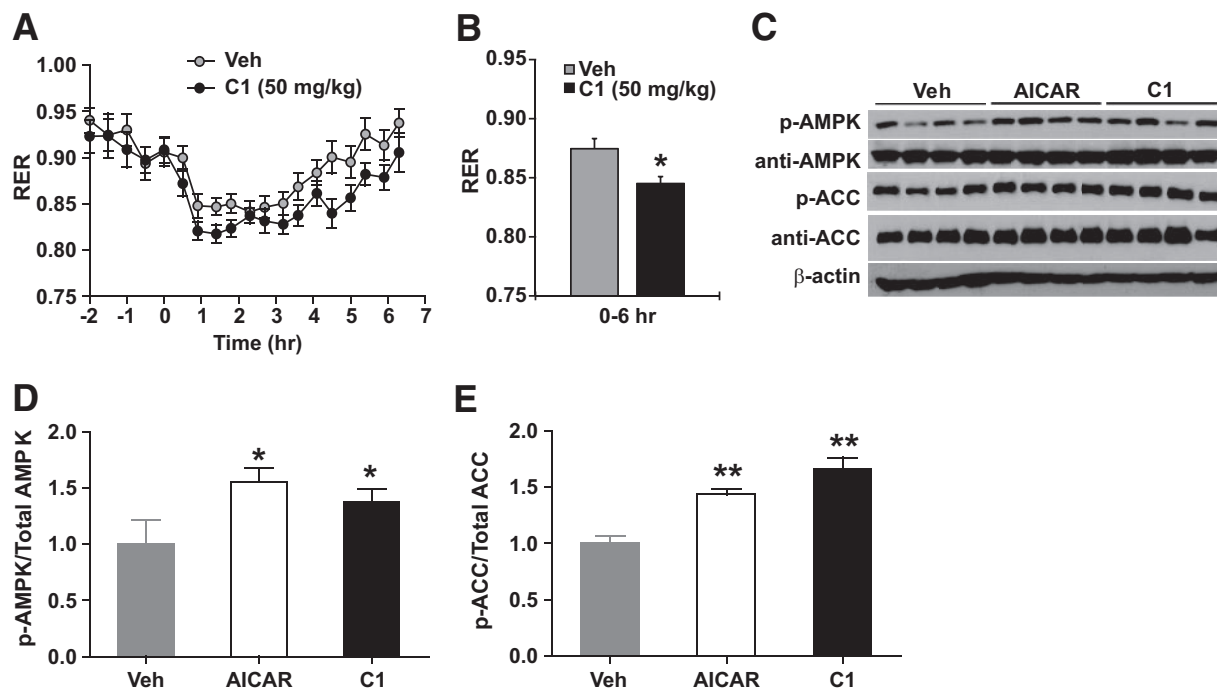
FIG. 5. Acute effect of C1 on respiratory exchange ratio (RER) and the AMPK pathway. For RER measurement, lean mice were placed in a metabolic chamber at 9:00 A.M., and after 2 h of rest, C1 (50 mg/kg) or saline (vehicle) was administered by oral gavage. For acute experiment, C57BL/6J lean mice were given either vehicle (0.9% NaCl) or C1 (20 mg/kg) or AICAR (400 mg/kg) by intraperitoneal injection. After 2-h treatment, animals were killed, with liver freeze-clamped for the determination of phosphorylation of AMPK and ACC. *A*: Changes of RER throughout the period of monitoring. *B*: Comparison of the average RER results before and after drug administration. *C*: The phosphorylation state of liver AMPK and ACC. *D* and *E*: The ratio of phosphorylation level versus protein level of AMPK and ACC. **$P < 0.01$; *$P < 0.05$ compared with vehicle ($n = 7$–12).

of C1 on $\Delta\psi m$ was of a comparable magnitude to the above compounds, it seemed possible that C1 might act via a similar mechanism. C1 dose-dependently inhibited respiration in L6 myotubes, and this lead to an increase in the AMP-to-ATP ratio. However, to our surprise, when we examined isolated mitochondria, C1 did not affect ADP-stimulated respiration with either glutamate or succinate as the substrate. Therefore, C1 does not appear to inhibit respiration via a direct effect on mitochondrial respiratory complexes or the phosphorylation system (i.e., ATP synthase and adenine nucleotide exchanger) (10). Another important factor regulating mitochondrial respiration is a constant supply of electron donors (e.g., NADH) (30). There are several shuttle systems (e.g., malate-aspartate shuttle, α-glycerophosphate shuttle) that oxidize cytosolic NADH and transfer the reducing equivalents to the mitochondrial respiratory chain (38). Inhibition of one of these shuttles could lead to impairment in respiration in intact cells, which would not be apparent using glutamate or

succinate as substrates in isolated mitochondria. For example, the anti-inflammatory drug indomethacin, which inhibits the α-glycerophosphate shuttle, inhibits glucose oxidation in intact kidney tubules, without affecting pyruvate oxidation in rat kidney mitochondria (39,40). Our similar results for C1 suggest that it may also inhibit one of these shuttle systems, and our finding of increased lactate release support this, as a rise in cytosolic NADH/NAD$^+$ would favor the lactate dehydrogenase reaction (41).

A consequence of reduced mitochondrial respiration and an elevated AMP-to-ATP ratio is activation of the AMPK pathway, which induces a rapid shutdown of anabolic processes and stimulation of catabolic processes to try and overcome metabolic stress (26,28). Given its effect on $\Delta\psi m$ it was not unexpected that we observed robust stimulation of the AMPK pathway in L6 myotubes and HepG2 cells with C1. The effects of C1 to stimulate glucose uptake in L6 myotubes and to inhibit lipid synthesis in HepG2 cells were also shown to be largely dependent on

TABLE 1
Chronic effects of C1 and metformin on metabolic parameters in mice

Plasma parameters	*db/db*-Veh	*db/db*-C1	*db/db*-Met	Ln-Veh	Ln-C1	Ln-Met
Food intake (g · day^{-1} · mouse^{-1})	4.41 ± 0.09	4.39 ± 0.07	4.04 ± 0.09	2.94 ± 0.04	2.88 ± 0.15	2.89 ± 0.05
Body weight gain (g)	4.5 ± 0.51	5.05 ± 0.40	4.32 ± 0.27	3.00 ± 0.77	2.51 ± 0.47	1.8 ± 0.21
Fasting blood glucose (mmol/l)	21.9 ± 1.9	17.3 ± 1.5*	17.0 ± 2.9*	10.7 ± 0.5	11.2 ± 0.5	10.0 ± 0.6
Random-fed blood glucose (mmol/l)	20.3 ± 1.8	11.8 ± 0.7*	16.3 ± 1.8*	11.0 ± 0.3	12.3 ± 0.6*	10.8 ± 0.6
Plasma insulin (ng/ml)	8.06 ± 1.37	10.99 ± 2.09	8.29 ± 1.13	0.48 ± 0.03	0.43 ± 0.03	0.48 ± 0.03
Plasma NEFA (mEq/l)	1.18 ± 0.09	0.96 ± 0.05*	1.12 ± 0.16	0.72 ± 0.05	0.80 ± 0.07	0.83 ± 0.07
Plasma triglyceride (mmol/l)	0.85 ± 0.03	0.82 ± 0.04	0.91 ± 0.18	0.66 ± 0.06	0.63 ± 0.05	0.67 ± 0.03

Data are means ± SE ($n = 7$–10). Daily food consumption and body weight gain were measured during the study. Blood samples from mice were collected after oral administration of vehicle (Veh) (0.5% methylcellulose) or C1 (50 mg · kg^{-1} · day^{-1}) or metformin (Met) (250 mg · kg^{-1} · day^{-1}) for 4 weeks. *$P < 0.05$ vs. vehicle.
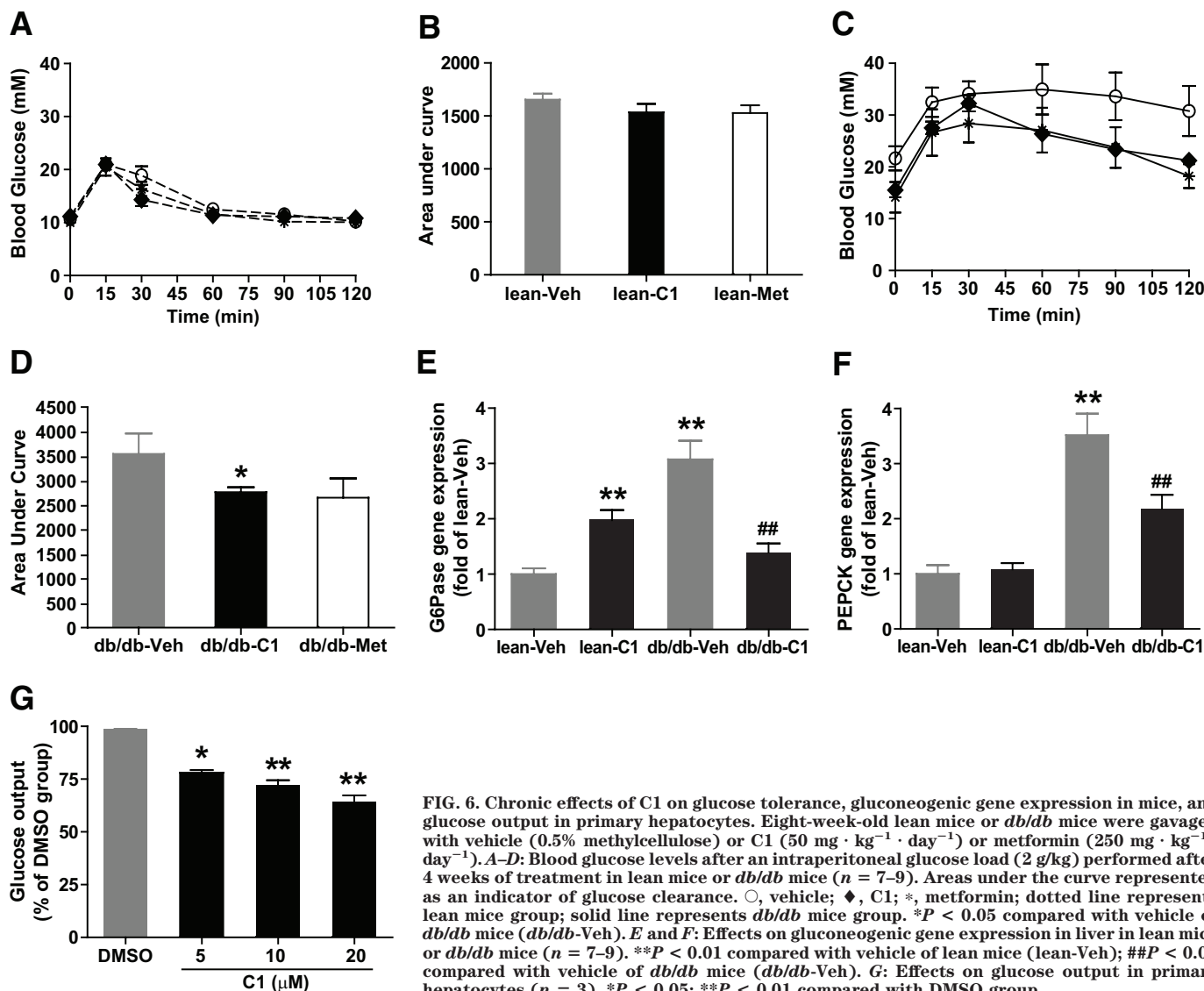
FIG. 6. Chronic effects of C1 on glucose tolerance, gluconeogenic gene expression in mice, and glucose output in primary hepatocytes. Eight-week-old lean mice or *db/db* mice were gavaged with vehicle (0.5% methylcellulose) or C1 (50 mg · kg^{-1} · day^{-1}) or metformin (250 mg · kg^{-1} · day^{-1}). *A–D*: Blood glucose levels after an intraperitoneal glucose load (2 g/kg) performed after 4 weeks of treatment in lean mice or *db/db* mice ($n = 7–9$). Areas under the curve represented as an indicator of glucose clearance. ○, vehicle; ♦, C1; *, metformin; dotted line represents lean mice group; solid line represents *db/db* mice group. *$P < 0.05$ compared with vehicle of *db/db* mice (*db/db*-Veh). *E* and *F*: Effects on gluconeogenic gene expression in liver in lean mice or *db/db* mice ($n = 7–9$). **$P < 0.01$ compared with vehicle of lean mice (lean-Veh); ##$P < 0.01$ compared with vehicle of *db/db* mice (*db/db*-Veh). *G*: Effects on glucose output in primary hepatocytes ($n = 3$). *$P < 0.05$; **$P < 0.01$ compared with DMSO group.

AMPK, as these effects could be mostly abrogated by treatment with the AMPK inhibitor compound C. In fact, the ability of AMPK to overcome a mild metabolic stress represents an important protective mechanism, and it has been shown that other agents that mildly inhibit or uncouple mitochondria also increase glucose transport and inhibit lipid synthesis (8,28,42,43).

Based on our in vitro findings, we sought to test the effect of C1 in vivo. Pharmacokinetic studies in rats showed favorable bioavailability of C1 (43%) and plasma concentrations in the range that depolarized $\Delta\psi$m and activated AMPK in vitro (supplementary Fig. 1 in the online appendix, available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0223/DC1). Initially, we administered a single dose of C1 to mice, and while this did not significantly affect whole-body energy expenditure, we did observe a reduction in the respiratory exchange ratio, suggesting a switch to whole-body fatty acid oxidation. These findings of elevated fat utilization are consistent with activation of the AMPK pathway (26), which we observed in the liver of lean animals dosed with C1. We next assessed the chronic effects of 4 weeks of treatment of C1 in *db/db* mice and lean mice. Neither C1 nor the antidiabetic drug metformin had any major effect in lean

mice. In *db/db* mice, C1 improved glucose homeostasis to a degree similar to that of metformin, as evidenced by the reduction in blood glucose levels and improved glucose tolerance. C1 also decreased plasma NEFA levels, which is potentially a consequence of the enhanced whole-body fat utilization we observed. Liver gluconeogenesis is a critical component of glucose homeostasis, and we observed a dose-dependent reduction in glucose output from primary hepatocytes and a downregulation of hepatic PEPCK and G6Pase expression by C1 in *db/db* mice. Interestingly, the expression of G6Pase, but not PEPCK, was significantly upregulated by C1 treatment in lean animals. A number of studies have reported that AMPK activators decrease gluconeogenic gene expression (44,45); however, similar to our findings, a recent study showed that AICAR treatment of *ob/ob* mice potently upregulated the expression of G6Pase in liver, while at the same time reducing the expression of PEPCK (44). The reason for the disparity in findings regarding AMPK activators and G6Pase expression is unclear at present.

Overall, we propose a scheme whereby C1 causes mild inhibition of mitochondrial respiration, through a mechanism distinct from TZDs, metformin, or berberine, ultimately leading to activation of AMPK, with beneficial
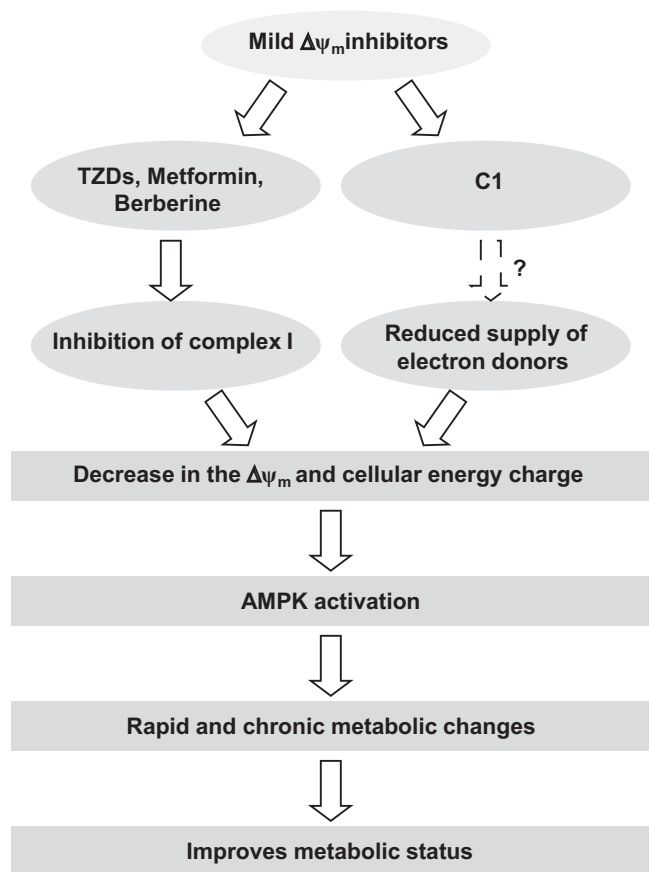
FIG. 7. Proposed mechanisms of C1 and other mitochondrial function modulators on energy metabolism. C1, TZDs, metformin, and berberine all activate AMPK to produce beneficial metabolic changes to ameliorate metabolic syndrome via depolarization of $\Delta\psi m$ and the resultant increase in the AMP-to-ATP ratio. However, upstream mechanisms leading to the depolarization of $\Delta\psi m$ are different. Whereas TZDs, metformin, and berberine inhibit complex I, C1 depolarizes $\Delta\psi m$ likely through reducing the supply of electron donors, rather than directly acting on complex I.

metabolic outcomes (Fig. 7). Despite the encouraging results with C1 and the fact that many prominent antidiabetic compounds are known to inhibit mitochondrial function, there are several potential concerns regarding the use of compounds that target mitochondria. For example, in addition to their role in energy production, mitochondria play an important role in apoptotic signaling and calcium regulation (46,47), and disruption of these pathways may compromise cellular homeostasis. Furthermore, inhibiting ATP production in certain cells (e.g., pancreatic β-cells) will have deleterious functional consequences, as these cells rely on ATP as a key signaling intermediate (30). We did not observe any obvious cytotoxic effects of C1 in our cell and animal studies, and the only potentially negative finding was an increase in lactate production in our cell studies. Lactic acidosis is an undesirable side effect of metformin, and further studies are required to determine if increased lactate levels occur in vivo in response to all compounds that inhibit mitochondrial function, and whether this outweighs their beneficial effects on metabolism through AMPK activation.

In summary, we have used a screen for $\Delta\psi m$ to identify a novel small molecule C1 that activates the AMPK pathway and exerts beneficial metabolic effects in vitro and in vivo related to metabolic syndrome. Unlike other antidiabetic agents that also work through the AMPK pathway,

C1 does not appear to directly affect mitochondrial complex I, but indirectly inhibits mitochondrial function, potentially through impairing the supply of electron donors. Our findings therefore suggest that high-throughput screening for compounds that mildly perturb mitochondrial function is a promising approach to discover novel therapeutics for the treatment of type 2 diabetes and other related metabolic diseases.

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