Enhanced activation of cellular AMPK by dual-small molecule treatment: AICAR and A769662

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[1]*Nestlé Institute of Health Sciences SA, EPFL Innovation Park, Lausanne, Switzerland;* [2]*Division of Endocrinology and Metabolism, Department of Medicine, McMaster University, Hamilton, Ontario, Canada;* [3]*Pôle de Recherche Cardiovasculaire, Université catholique de Louvain, Institut de Recherche Expérimentale et Clinique, Brussels, Belgium;* and [4]*Protein Chemistry and Metabolism, St. Vincent's Institute and Department of Medicine, University of Melbourne, Fitzroy, Victoria, Australia*

Ducommun S, Ford RJ, Bultot L, Deak M, Bertrand L, Kemp BE, Steinberg GR, Sakamoto K. Enhanced activation of cellular AMPK by dual-small molecule treatment: AICAR and A769662. *Am J Physiol Endocrinol Metab* 306: E688–E696, 2014. First published January 14, 2014; doi:10.1152/ajpendo.00672.2013.—AMP-activated protein kinase (AMPK) is a key cellular energy sensor and regulator of metabolic homeostasis. Activation of AMPK provides beneficial outcomes in fighting against metabolic disorders such as insulin resistance and type 2 diabetes. Currently, there is no allosteric AMPK activator available for the treatment of metabolic diseases, and limited compounds are available to robustly stimulate cellular/tissue AMPK in a specific manner. Here we investigated whether simultaneous administration of two different pharmacological AMPK activators, which bind and act on different sites, would result in an additive or synergistic effect on AMPK and its downstream signaling and physiological events in intact cells. We observed that cotreating primary hepatocytes with the AMP mimetic 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and a low dose (1 μM) of the allosteric activator A769662 produced a synergistic effect on AMPK Thr172 phosphorylation and catalytic activity, which was associated with a more profound increase/decrease in phosphorylation of downstream AMPK targets and inhibition of hepatic lipogenesis compared with single-compound treatment. Mechanistically, we found that cotreatment does not stimulate LKB1, upstream kinase for AMPK, but it protects against dephosphorylation of Thr172 phosphorylation by protein phosphatase PP2Cα in an additive manner in a cell-free assay. Collectively, we demonstrate that AICAR sensitizes the effect of A769662 and promotes AMPK activity and its downstream events. The study demonstrates the feasibility of promoting AMPK activity by using two activators with distinct modes of action in order to achieve a greater activation of AMPK and downstream signaling.

AMP-activated protein kinase; LKB1; A769662; 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; lipogenesis

AMP-ACTIVATED PROTEIN KINASE (AMPK) is a major regulator of cellular energy homeostasis that coordinates metabolic pathways in order to balance nutrient supply with energy demand (14, 15, 27, 34). AMPK is activated by various physiological and pathological energy stresses that increase intracellular ADP-to-ATP and AMP-to-ATP ratios, either by accelerating ATP consumption (e.g., muscle contraction) or by decreasing ATP generation (e.g., hypoxia, ischemia, mitochondrial poisoning). Once activated, AMPK acts to restore energy homeostasis by promoting ATP production pathways such as glucose uptake and fatty acid oxidation while simultaneously inhibiting ATP utilization pathways such as lipid and protein synthesis.

AMPK is a heterotrimeric complex containing a catalytic subunit (α) and two regulatory subunits (β, γ). Two isoforms of the α- and β-subunits exist (α1, α2, β1, β2), whereas there are three isoforms of the γ-subunit (γ1, γ2, γ3). Isoform expression varies among cells/tissues and also species (36, 41), with α1, β1, and γ1 appearing in general to be ubiquitously expressed isoforms. Some isoforms are known to be expressed in a cell/tissue-specific/restricted manner; for example, α2 is more predominantly expressed in skeletal muscle (22) and also γ3 is exclusively found in skeletal muscle (2, 44).

AMPK is activated by upstream kinases LKB1 and Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKKβ) via phosphorylation of Thr172 on the α catalytic subunit (1, 34). AMP binding to the γ-subunit allosterically activates AMPK, and ADP and/or AMP binding promotes Thr172 phosphorylation and protects it against dephosphorylation by protein phosphatases (e.g., PP2Cα), while ATP competes for binding at the same sites and antagonizes the effects of AMP (12, 25).

AMPK has emerged as a key drug target for metabolic disorders such as insulin resistance, cardiovascular disease, and type 2 diabetes (19). This is based on numerous reports demonstrating that activation of AMPK by physiological (e.g., exercise/muscle contraction) or pharmacological means brings about desirable effects that ameliorate metabolic dysfunctions by improving glycemic control and plasma lipid profiles (13, 28). Although a variety of compounds including several xenobiotics (which are known to elicit anti-obesity/diabetic effects) stimulate AMPK, it has been demonstrated that almost all of them [with some exceptions (3, 11, 16)] activate AMPK by an indirect mechanism (17). For instance, antidiabetic drugs like metformin (and its analog phenformin) as well as resveratrol, which display anti-obesity/diabetes effects [although controversial (38, 43)], activate AMPK indirectly by lowering ATP levels (i.e., increase in AMP-to-ATP ratio via suppression of mitochondrial respiration) (17, 26). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is a very widely used AMPK activator that is transported into the cell and phosphorylated to form ZMP, which mimics the effect of AMP by directly binding to the γ-subunit. There are a few direct AMPK activators reported in the literature, of which the small molecule A769662 is the only well-characterized/validated AMPK activator that stimulates AMPK via allosteric action and also protects active (phosphorylated) AMPKα at Thr172 from dephosphorylation through interacting with the β1-subunit.

Given that there are few compounds available to robustly stimulate cellular/tissue AMPK in a specific manner, we explored here whether simultaneous administration of two different pharmacological AMPK activators (AICAR and A769662), which are proposed to act on different sites/ regions (10, 11, 31, 33), would result in an additive or synergistic effect on AMPK and its downstream events compared with single-compound treatment (either AICAR or A769662) in intact cells.

MATERIALS AND METHODS

Materials. λ-Phosphatase was from New England Biolabs (Ipswich, MA). PP2Cα was from the Division of Signal Transduction Therapy (DSTT), University of Dundee (UK). AICAR was from Toronto Research Chemicals (Toronto, ON, Canada). A769662 was from Selleckchem (Houston, TX) or DSTT. Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Europe (Newmarket, UK). C_2C_{12} myoblasts were obtained from Sigma (Buchs, Switzerland). General and specific cell culture reagents, including Williams medium E (WME) and horse serum, were obtained from Life Technologies (Zug, Switzerland). [γ-^{32}P]ATP was obtained from PerkinElmer (Schwerzenbach, Switzerland).

Antibodies. Total acetyl-CoA carboxylase (ACC; no. 3676), phospho-ACC1 (Ser79; no. 3661), total Raptor (no. 2280), phospho-Raptor (Ser792; no. 2083), total AMPKα (no. 2532), phospho-AMPKα (Thr172 and Ser485/Ser491; no. 2535 and no. 4185), total AMPKβ1 (no. 4182), phospho-AMPKβ1 (Ser108 and Ser182; no. 4181 and no. 4186), total LKB1 (no. 3047), total salt-inducible kinase (SIK)2 (no. 6919), total p70 S6 kinase (p70S6K; no. 2708), and phospho-p70S6K (Thr389; no. 9234) antibodies were from Cell Signaling Technology (Danvers, MA). The antibody against α-tubulin (no. T6074) was from Sigma. Total AMPKα1 and AMPKα2 isoform-specific antibodies were raised against the previously validated peptide sequences (30) TSPPDSFLDDHHLTR (α1) and MDDSAMHIP-PGLKPH (α2) custom-generated by DSTT, MRC Protein Phosphorylation and Ubiquitylation Unit, University of Dundee and affinity purified in house. Total SIK2 antibody for immunoprecipitation was from DSTT (S227B, 3rd bleed).

Animals. Wild-type C57BL/6 mice were obtained from Jackson Laboratories. Generation and breeding of AMPKβ1$^{-/-}$ and littermate AMPKβ1$^{+/+}$ mice were described previously (6). All protocols for animal use and euthanasia were approved by the McMaster University Animal Research Ethics Board.

C_2C_{12} cell culture. C_2C_{12} myoblasts were cultured in DMEM supplemented with 20% FBS. Myoblasts were differentiated to myotubes during 7 days in DMEM supplemented with 1% horse serum and treated for 30 min with compounds as indicated in Fig. 1, *D* and *E.*

Primary mouse hepatocytes. Primary hepatocytes were isolated by collagenase perfusion and prepared as previously described (9). Briefly, cells were plated on collagen-coated six-well plates in WME containing 10% FBS and 1% antibiotic-antimycotic and allowed to adhere for 4 h before being washed with PBS and provided with fresh medium. For all protocols, hepatocytes were incubated overnight at 37°C and experiments were performed the following morning. [^{3}H]acetate lipogenesis was executed as described previously (8) with minor modifications. Cells were washed with PBS and incubated in serum-free WME for 2–3 h. Serum-free medium containing [^{3}H]acetate (10 μCi/ml; PerkinElmer) and 0.5 mM unlabeled sodium acetate (Sigma) was then applied, and cells were incubated in the presence or absence of AICAR and/or A769662 for 45 min. Cells were washed twice with PBS, and lipids were extracted in chloroform-methanol 1:2 for determination of radiolabel incorporation into total lipids as previously described (6). For protein experiments, hepatocytes were washed and incubated in serum-free WME for 2–3 h and then exposed to no drug, AICAR, and/or A769662 for 45 min. Medium was quickly

removed, and cells were lysed on ice in 4°C lysis buffer [in mM: 50 HEPES, 150 NaCl, 100 NaF, 10 Na-pyrophosphate, 5 EDTA, 250 sucrose, 1 DTT, and 1 Na-orthovanadate, with 1% Triton X and 1 tablet/50 ml Complete protease inhibitor cocktail (Roche)]. Plates were scraped, and lysates were stored at −80°C for later analyses.

Immunoblotting. Lysates were denatured in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane. Membranes were blocked for 1 h in 10 mM Tris (pH 7.6), 137 mM NaCl, 0.1% (vol/vol) Tween 20 (TBST) containing 5% (wt/vol) skimmed milk. Membranes were incubated in primary antibody prepared in TBST containing 1% (wt/vol) BSA overnight at 4°C. Detection was performed with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagent.

Cloning and recombinant AMPK expression, purification, and activation. The coding regions of AMPKα2 (NM_006252), AMPKβ1 (NM_006253), and AMPKγ1 (NM_002733) were amplified from brain RNA (Agilent) with a SuperScript III RT-PCR kit (Invitrogen). The resulting PCR products were ligated into intermediate vectors with a Strataclone PCR cloning kit (Agilent). Site-directed mutagenesis was carried out according to the QuikChange method (Stratagene) using KOD polymerase (Novagen). The sequences of all constructs were verified with the BigDyeR Terminator 3.1 kit and 3500XL Genetic analyzer (Applied Biosystems) in house.

Active heterotrimeric AMPK complexes were prepared by a modification of the method described previously (23). Briefly, BL21(DE3) were transformed with the polycistronic vector pET15B-6HIS-α2β1γ1 or pET15B-6HIS-α2β1(S108A)γ1 and cultured in 4l auto-induction medium (overnight, 18°C) (37). AMPK complexes were isolated from clarified lysates with His60 Ni Superflow Resin (Clontech). Imidazole was removed by buffer exchange over Sephadex G-25, and AMPK complexes were activated by in vitro phosphorylation with GST-CaMKKβ. GST-CaMKKβ was removed with glutathione Sepharose 4B (GE Healthcare), and AMPK complexes were further purified over Superdex200 10/300 (GE Healthcare). Preparations were stored in 12.5 mM HEPES pH 7.4, 75 mM NaCl, 0.5 mM DTT, 50% (vol/vol) glycerol at −20°C.

AMPK and SIK2 activity assay. AMPK was immunoprecipitated from 30 μg of lysate with antibodies against α1- or α2-subunit with protein G Sepharose. The immune complex (endogenous AMPK), or recombinant AMPK was assayed for phosphotransferase activity toward *AMARA* peptide (AMARAASAAALARRR) with [γ-^{32}P]ATP, as previously described (21). SIK2 was immunoprecipitated from 250 μg of lysate with 2 μg of anti-SIK2 antibody and protein G Sepharose. The assay was performed as previously described (18) with Sakamototide peptide (ALNRTSSDSALHRRR) as substrate.

Phosphatase protection assay. The assay was performed as previously described (5) with some modifications. Briefly, fully phosphorylated recombinant AMPK α2β1γ1 complex (200 ng) (as described above) was incubated for 15 min at 30°C in the presence of PP2Cα (20 ng) and the indicated concentrations of AICAR and/or A769662 in 50 mM HEPES pH 7.4, 10 mM MgCl$_2$, 100 μM EGTA. The dephosphorylation reaction was stopped by 20-fold dilution in 50 mM HEPES pH 7.4, 100 mM NaCl, 0.03% (vol/vol) Brij 35, 1 mM DTT. AMPK activity was then measured in the presence of 200 μM AMP as described above.

Statistical analysis. Data are expressed as means ± SE or SD as indicated. Statistical analysis was performed by unpaired, two-tailed Student's *t*-test or two-way ANOVA with Bonferroni post hoc test. Differences between groups were considered statistically significant when $P < 0.05$.

RESULTS

Cotreatment of mouse primary hepatocytes with AICAR and A769662 exhibits synergistic effect on AMPK activity. We first assessed whether coincubation of two different pharmacological AMPK activators (AICAR and A769662), which are

proposed to act on different sites/regions (10, 11, 31, 33), would result in an additive or synergistic effect on AMPK activity compared with single-compound treatment (either AICAR or A769662) in intact cells. Mouse primary hepatocytes were treated with an increasing dose of AICAR (0, 0.01, 0.03, 0.1, 0.3, and 1 mM) in the absence or presence of a fixed concentration of A769662 (10 μM), and AMPK phosphorylation (Thr172) was assessed by immunoblotting using a phospho-specific antibody. As illustrated in Fig. 1*A*, A769662 (10 μM) alone showed no detectable increase in AMPK phosphorylation whereas AICAR stimulated Thr172 phosphorylation in a dose-dependent manner. Strikingly, when hepatocytes were coincubated with AICAR and A769662, AMPK phosphoryla-

tion, as judged by phospho-Thr172 signal and also upper shifting of band mobility of total AMPKα, was robustly enhanced compared with the respective single AICAR dose (Fig. 1*A*). As anticipated, the AMPK phosphorylation was tightly associated with AMPK (α1) phosphotransferase activity measured in vitro, demonstrating that the cotreatment results in a significant increase in AMPK activity at AICAR doses ranging from 0.1 to 1 mM compared with the respective single AICAR dose (Fig. 1*B*). We next assessed cellular AMPK activity by measuring phosphorylation of bona fide AMPK substrates such as ACC and Raptor in mouse primary hepatocytes. ACC phosphorylation (Ser79 on ACC1) was readily saturated at relatively low concentrations of AICAR (0.03

Fig. 1. Cotreatment of mouse primary hepatocytes with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and A769662 exhibits a synergistic effect on AMP-activated protein kinase (AMPK) activity. Primary hepatocytes were isolated from C57BL/6 (12–15 wk old) mice and cultured overnight. Cells were serum-starved for 2–3 h and then treated with vehicle, AICAR, and/or A769662 for 45 min before cell lysis. *A*: immunoblotting was performed with 20 μg per lane and with the indicated antibodies. *B*: AMPKα1 complexes were immunoprecipitated from 30 μg of extract with 1 μg of anti-AMPKα1 and protein G Sepharose. Immune complexes were assayed for AMPK activity (in duplicate) as described in MATERIALS AND METHODS. Results are expressed as mean ± SE pmol P_i incorporated·min^{-1}·mg^{-1}. *Significance (AICAR vs. respective AICAR + A769662), $P < 0.05$; $n = 3$/condition. *C*: AMPKα1 complexes were immunoprecipitated (IP) from lysate (30 μg) of hepatocytes treated with 1 mM AICAR and 10 μM A769662 with 1 μg of anti-AMPKα1 and protein G Sepharose. Immune complexes were incubated in the absence or presence of 400 U of λ-phosphatase for 15 min at 30°C. Reactions were terminated with Laemmli buffer and analyzed by immunoblotting with anti-AMPKα1. *D* and *E*: differentiated C₂C₁₂ cells were treated with vehicle, AICAR, and/or A769662 for 30 min before cell lysis. *D*: immunoblots were performed with 30 μg of protein per lane and with the indicated antibodies. *E*: AMPKα1 complexes were immunoprecipitated from 30 μg of extract with 1 μg of anti-AMPKα1 and protein G Sepharose. Immune complexes were assayed for AMPK activity (in duplicate). Results are expressed as mean ± SE pmol P_i incorporated·min^{-1}·mg^{-1}. *Significance (AICAR vs. respective AICAR + A769662), $P < 0.05$; $n = 3$/condition.
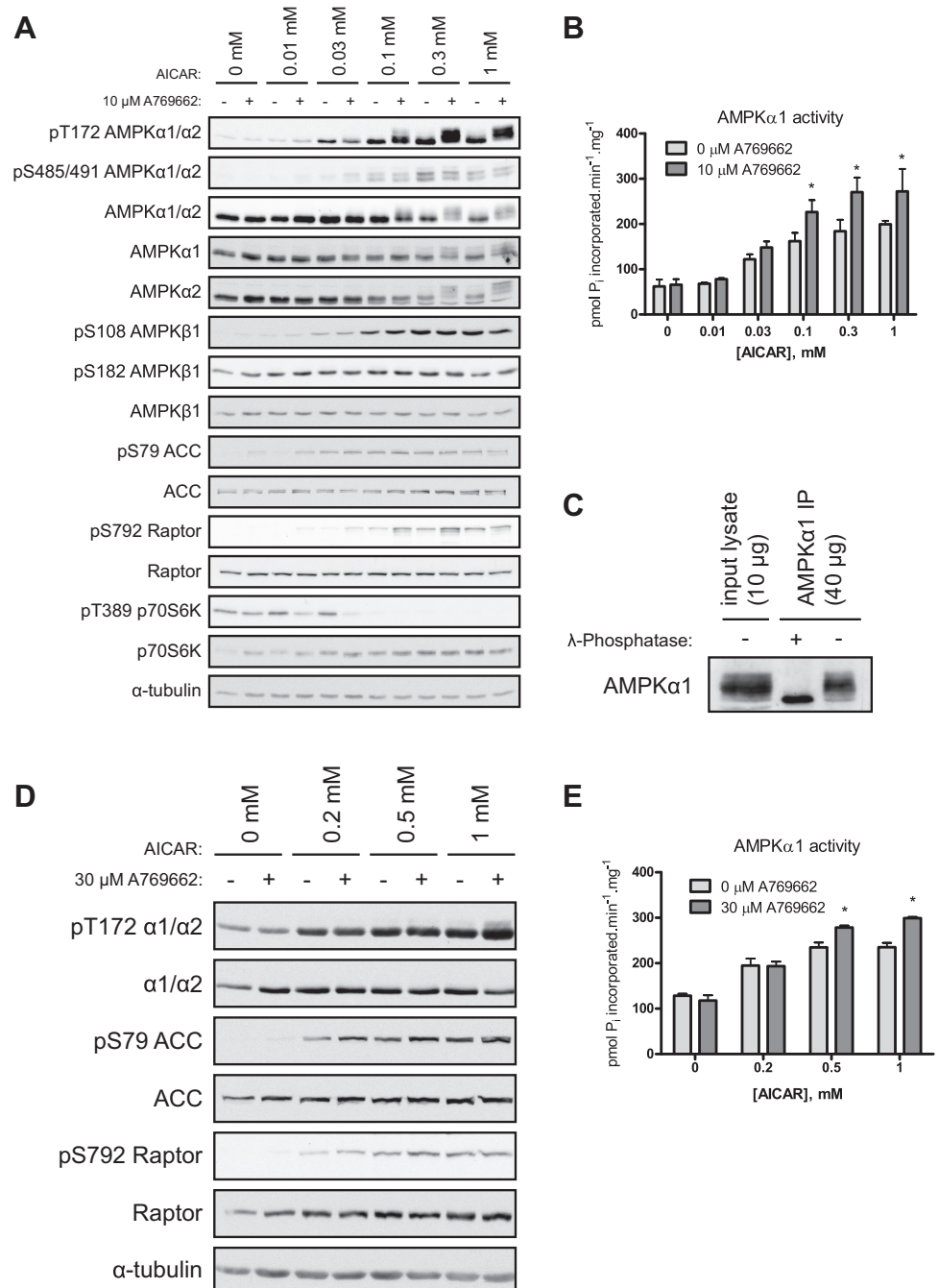
mM), whereas Raptor phosphorylation (Ser792) exhibited a pattern (dose-dependent increase) similar to AMPK phosphorylation (Thr172) and activity (Fig. 1A). This phosphorylation on Raptor, which has been proposed to inhibit mammalian target of rapamycin complex 1 (mTORC1), was associated with a decrease in phosphorylation (Thr389) of p70S6K, an established mTORC1 substrate.

An interesting observation of the AMPK immunoblot signal (both phospho and total) mentioned above is the band shift upon cotreatment, which is attributable to both AMPKα1 and AMPKα2 isoforms (as shown by immunoblots using isoform-specific antibodies) (Fig. 1A). Band shifts on SDS-PAGE are typically observed when proteins are phosphorylated at multiple residues. To test whether this was the case for AMPKα, we immunoprecipitated endogenous AMPKα1 from extracts of hepatocytes (which had been stimulated with 1 mM AICAR and 10 μM A769662), and the resulting immunoprecipitants were incubated with a recombinant protein phosphatase (λ-phosphatase) for 15 min in vitro. We observed that the upper band shift induced by the cotreatment was abolished after the phosphatase treatment (Fig. 1C). To determine whether the cotreatment promoted phosphorylation of sites on AMPKα apart from the Thr172 in the activation T-loop residue, we checked another known phosphorylation site (Ser485 on α1 and Ser491 on α2), which is outside of the kinase domain and is proposed to be regulated by Akt (also known as PKB) (20) and p70S6K (4). AICAR alone stimulated Ser485/Ser491 phosphorylation in a dose-dependent manner, while there was no further increase in phosphorylation of this site with coincubation with A769662 (10 μM) (Fig. 1A). This suggests that the Ser485/Ser491 site is unlikely to be responsible for the band shift observed in the total and phospho-Thr172 AMPKα signals. It would be interesting to perform phosphopeptide mapping of adenovirally expressed AMPKα in primary hepatocytes treated with AICAR and A769662 and then identify/determine whether there are novel phosphorylation sites that are uniquely regulated by the cotreatment. Given that the effect of A769662 in activating AMPK requires the β1 isoform in the trimeric complex and the Ser108 residue is implicated to play a key role (16, 31, 33), we examined whether two known autophosphorylation sites on AMPKβ1 (Ser108 and Ser182) were regulated by a single or dual treatment with AMPK-activating compound(s) in primary hepatocytes. Ser108 phosphorylation was increased in a dose-dependent manner upon AICAR stimulation alone but was not further increased when cotreated with A769662 (10 μM). Ser182 phosphorylation was detectable in unstimulated cells, which was not altered by either AICAR or A769662 treatment (Fig. 1A).

To determine whether the synergistic effect of the cotreatment on AMPK phosphorylation/activation can be observed in other cell types apart from primary hepatocytes, C_2C_{12} (mouse skeletal muscle cell line) myotubes were treated with an increasing dose of AICAR (0, 0.2, 0.5, and 1 mM) with or without a fixed concentration of A769662 (30 μM). We chose to use 30 μM, as opposed to 10 μM used in hepatoyctes, because our previous work (10) and pilot experiments (data not shown) revealed that the dose required to activate de novo AMPK (by monitoring ACC phosphorylation) is different among cell types. While 1–10 μM is sufficient to activate AMPK in hepatocytes, 10–30 μM is required to stimulate AMPK in C_2C_{12} (and also mouse embryonic

fibroblasts) (unpublished data). Similar to the results observed in primary hepatocytes, AICAR increased AMPK T-loop phosphorylation (Thr172) in a dose-dependent fashion, whereas A769662 (30 μM) alone did not, and the cotreatment promoted further phosphorylation and activity of AMPK as well as phosphorylation of AMPK substrates (Fig. 1D) compared with AICAR treatment alone (at 0.5 and 1 mM) (Fig. 1, D and E). We also observed that the cotreatment (AICAR + A769662) promotes phosphorylation of AMPK and its downstream targets (i.e., ACC and Raptor) in other cell types such as NIH-3T3 fibroblasts (data not shown).

Low concentration (1 μM) of A769662 is sufficient to significantly increase effect of AICAR on AMPK activity. To determine whether an A769662 dose lower than 10 μM would elicit the synergistic effect of the AICAR-A769662 cotreatment on AMPK catalytic activity, primary mouse hepatocytes were treated with increasing concentrations of A769662 (0, 1, 3, 10, 30, and 100 μM) combined with a fixed AICAR dose (100 μM). While 1 μM A769662 had no effect on AMPK phosphorylation, this dose was readily sufficient to robustly increase the effects of AICAR on phosphorylation of AMPK and its substrates (ACC and Raptor) as well as the inhibition of p70S6K phosphorylation (Fig. 2A). Given that Thr172 and its surrounding residues are conserved between AMPKα1 and AMPKα2 isoforms and thus the phospho-specific antibody detects both isoforms, we examined whether the cotreatment selectively activates one isoform or both. Individual AMPKα isoforms were immunoprecipitated from the hepatocyte lysates (with isoform-specific antibodies) followed by an in vitro kinase assay. We observed that the cotreatment resulted in an increase in AMPK activity at A769662 doses ranging from 1 to 30 μM for AMPKα1-containing complexes and 1 and 10 μM for AMPKα2-containing complexes (Fig. 2B). Cotreatment with higher doses of A769662 (100 μM for AMPKα1, 30 and 100 μM for AMPKα2) failed to increase AMPK activity above AICAR treatment alone, most likely because of a toxic effect of the compound on hepatocytes.

Synergistic effect of A769662 and AICAR cotreatment is ablated in AMPKβ1-deficient hepatocytes. A769662 has been reported to be selective to act on β1-containing complexes of AMPK (16, 33). Therefore we investigated whether the synergistic effect of the cotreatment (AICAR and A769662) would be lost in primary hepatocytes lacking AMPKβ1. As previously described (33), in AMPKβ1$^{-/-}$ hepatocytes there is a profound loss of AMPKα catalytic subunits due to instability in the absence of the predominant scaffold β1 regulatory subunit in those mouse hepatocytes (Fig. 3A). β1-Null hepatocytes were treated with an increasing dose of AICAR (0, 0.01, 0.03, 0.1, 0.3, and 1 mM) and with a fixed dose of A769662 (10 μM). AMPK phosphorylation was still dose-dependently increased by AICAR treatment alone (Fig. 3B). In contrast, the elevation of AMPK phosphorylation upon cotreatment with A769662 observed in wild-type hepatocytes (Fig. 1A) was ablated in the β1-null hepatocytes (Fig. 3B), and therefore the activity of AMPK was not affected by the cotreatment either (Fig. 3C). This confirms that the β1 specificity of A769662 is retained/confirmed and that synergistic effect of the cotreatment (AICAR + A769662) can only take place in intact cells/tissues expressing β1-containing complexes.
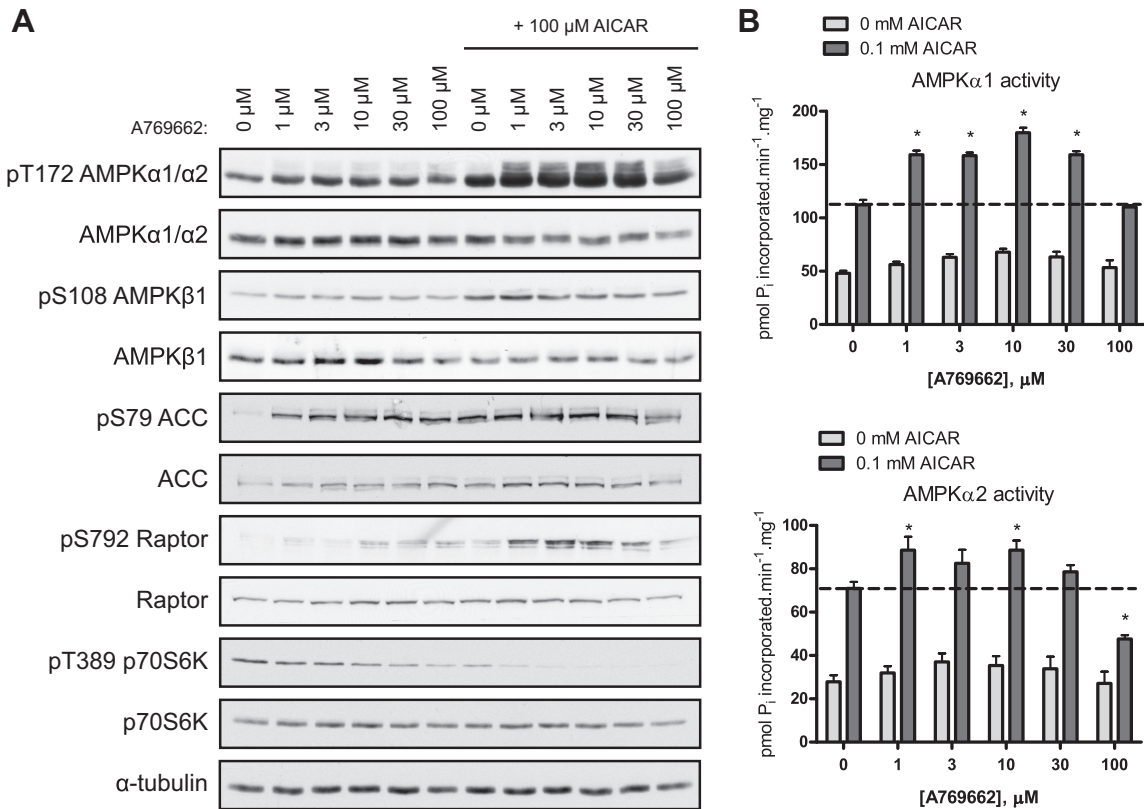
Fig. 2. A low concentration of A769662 is sufficient to increase the effect of AICAR on AMPK activity. Primary hepatocytes were isolated from C57BL/6 mice (12–15 wk old) and cultured overnight. Cells were serum starved for 2–3 h and then treated with vehicle, AICAR, and/or A769662 for 45 min before cell lysis. A: immunoblotting was performed with 30 μg of protein and the indicated antibodies. B: AMPKα1 or α2 complexes were immunoprecipitated from 30 μg of lysate with anti-AMPKα1 or anti-AMPKα2. Immune complexes were assayed for AMPK activity (in duplicate). Results are expressed as mean ± SE pmol P_i incorporated·min^{-1}·mg^{-1}. *Significance (AICAR vs. respective AICAR + A769662), $P < 0.05$; $n = 3$/condition.

Lipogenesis is robustly inhibited in hepatocytes treated with AICAR and A769662. Next we investigated whether the AICAR-A769662 cotreatment would also have an effect on a downstream physiological outcome of AMPK activation, of which one established example is inhibition of lipogenesis in

hepatocytes. AMPK inhibits lipogenesis through phosphorylation of its targets ACC1 and ACC2 (8). Primary mouse hepatocytes were left untreated or treated with AICAR (0.03 or 0.1 mM) in the presence or absence of A769662 (10 μM), and incorporation of [³H]acetate (a widely used lipogenic sub-

Fig. 3. The synergistic effect of A769662 and AICAR cotreatment is ablated in AMPKβ1-deficient hepatocytes. Primary hepatocytes were isolated from AMPKβ1$^{-/-}$ mice and wild-type (WT) littermates (12–15 wk old) and cultured overnight. Cells were serum starved for 2–3 h and then treated with vehicle, AICAR, and/or A769662 for 45 min before cell lysis. A: vehicle-treated hepatocyte lysates were analyzed by immunoblotting using 30 μg of protein and the indicated antibodies. B: hepatocyte lysates from AMPKβ1$^{-/-}$ mice were analyzed by immunoblotting using 30 μg of protein extracts and the indicated antibodies. C: AMPKα1 complexes were immunoprecipitated from 30 μg of extract with 1 μg of anti-AMPKα1 and protein G Sepharose. Immune complexes were assayed for AMPK activity (in duplicate). Results are expressed as mean ± SE pmol P_i incorporated·min^{-1}·mg^{-1}; $n = 3$.
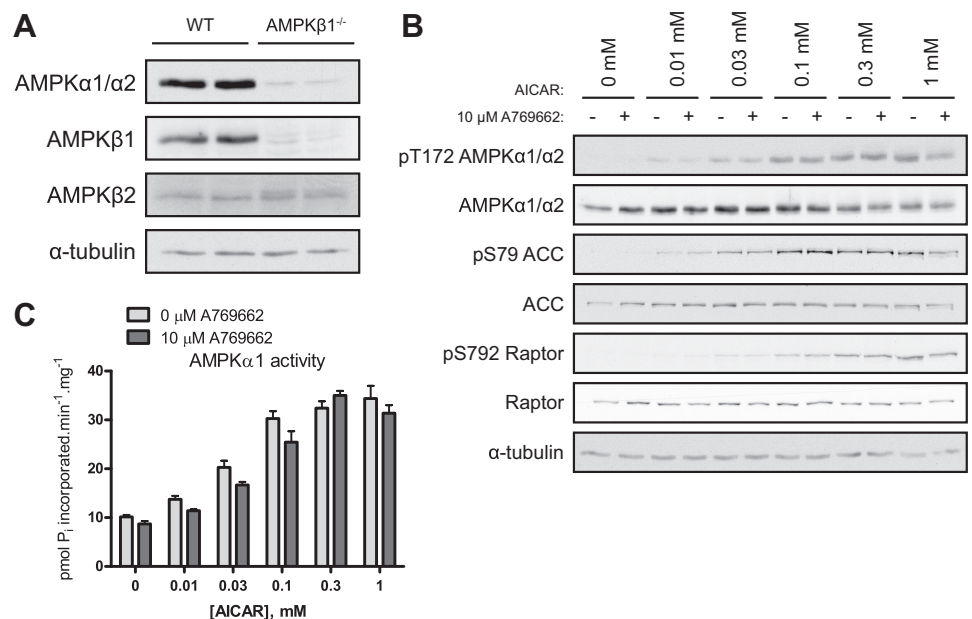
strate) into the lipid pool was measured as a readout of de novo lipogenesis. AICAR treatment decreased lipogenesis to ~60% or ~40% (for 0.03 mM and 0.1 mM, respectively) of the basal rate (100%), while 10 μM A769662 decreased lipogenesis to ~40%. Cotreatment of AICAR (0.03 and 0.1 mM) with A769662 (10 μM) further reduced lipogenesis to ~30% and ~25% of the basal rate, demonstrating that both compounds could inhibit lipogenesis in hepatocytes in an additive manner (Fig. 4).

Coincubation with AMP and A769662 displays additive protection effect on AMPK phosphorylation against dephosphorylation by protein phosphatase. We and others previously reported that not only does A769662 directly activate AMPK but its binding (independently of canonical AMP/ZMP-binding sites) also prevents Thr172 dephosphorylation in a cell-free assay (10, 33). To test whether the mechanism by which cotreatment with AMP and A769662 promotes AMPK phosphorylation in intact cells is due to protection against dephosphorylation by protein phosphatase, a recombinant AMPKα2β1γ1 (which had been purified from *E. coli* and phosphorylated/activated by recombinant CaMKKβ in vitro as described in MATERIALS AND METHODS) was incubated with recombinant PP2Cα in the presence of AICAR, A769662, or both compounds together in vitro. As previously shown, both AMP (saturated ~3–10 μM) and A769662 (0.1 μM) robustly protected against dephosphorylation by PP2Cα. As anticipated in the control experiment, there was no dephosphorylation/deactivation of AMPK in the absence of Mg^{2+}, as it is an essential cofactor in the activation of PP2Cα. Interestingly, cotreatment with various doses of AMP and a fixed dose of A769662 (0.1 μM) showed an additive protective effect against dephosphorylation by PP2Cα (Fig. 5A). To more directly demonstrate that the observed additive effect is indeed due to A769662 interaction with β1-subunit, we used a recombinant AMPK complex containing a A769662-resistant mutant (β1-S108A). In the AMPKα2β1(S108A)γ1 complex, AMP was able to protect against dephosphorylation by PP2Cα, while the effect of A769662 was abolished (Fig. 5B). We examined whether the enhanced Thr172 phosphorylation by the cotreatment is due to activation of the upstream kinase of AMPK,

LKB1, in mouse primary hepatoytes and measured activity of a known LKB1-dependent substrate, SIK2, expressed in the liver (Fig. 5C). We observed that both SIK activity and LKB1 expression were not altered with or without cotreatment. These results may explain the effect of the cotreatment in vivo, where AICAR/ZMP would promote AMPK phosphorylation (Thr172) by LKB1 and in turn allow A769662 to exert its effect by protecting AMPK from dephosphorylation by protein phosphatases present in the cells.

DISCUSSION

We here show that cotreatment of cells with two distinct AMPK activators (AICAR and A769662), which act on different sites/regions, exhibits a synergistic effect on AMPK and its downstream targets/physiological consequences compared with single-compound treatment in intact cells, including primary hepatocytes (7) and muscle cells (C$_2$C$_{12}$). We have also confirmed that the cotreatment displays similar results in other cell types such as NIH-3T3 fibroblasts (data not shown). A notable observation was that in the presence of AICAR a low concentration of A769662 (as low as 1 μM) produces a robust synergistic effect on Thr172 phosphorylation and activation of AMPK, which indicates that AICAR/ZMP binding profoundly sensitizes the effect of A769662. This was particularly striking because we previously observed that, although A769662 significantly increased ACC phosphorylation (presumably through direct/allosteric activation of AMPK) at 1–10 μM, it failed to display detectable increase in Thr172 phosphorylation up to ~100–200 μM in mouse hepatocytes (7). We initially hypothesized that the conformational change of AMPK complex induced by ZMP binding to AMPK γ-subunit facilitates A769662 interaction not only to β1-containing complex but also to β2-containing complex (which is not normally activated by the compound when treated alone); therefore cotreatment would enhance global (β1/β2-complex) AMPK activation in cells. However, experiments performed employing AMPKβ1-deficient hepatocytes ruled out this hypothesis and involvement of the β2 isoform in the observed effects (Fig. 3).

It has been shown that coincubation of the fully phosphorylated (Thr172 residue by CaMKKβ in vitro) AMPKαβγ complex (prepared from bacteria) with AMP and A769662 does not display an additive allosteric effect on kinase activity in a cell-free assay (31), while in intact mammalian cells we observed that the coincubation promoted AMPK activity, which was associated with an increase in Thr172 phosphorylation in the activation T loop of AMPKα catalytic subunit. Therefore, it is reasonable to assume that the effect of cotreatment on AMPK Thr172 phosphorylation is due to an increase in the activity of the upstream kinase of AMPK (i.e., LKB1), protection against dephosphorylation via protein phosphatase(s), or the combination of both. We initially measured de novo activity of LKB1 by assaying its substrate, SIK2 (also known as QIK) (1, 18), and observed that cotreatment had no effect (Fig. 5C). This observation is consistent with our previous finding that LKB1 is a constitutively active kinase (29) and AMPK activity is uniquely regulated among other related kinases by its ability to be controlled through adenine nucleotide (i.e., ADP and AMP) binding to the γ-subunit (24, 32). Consequently, we showed that the cotreatment displayed a significant additive effect on protection from dephosphoryla-
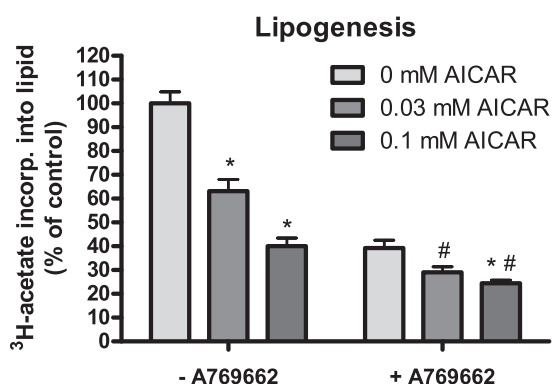

Lipogenesis

Fig. 4. Lipogenesis is robustly inhibited in hepatocytes treated with AICAR and A769662. Primary hepatocytes were isolated from C57BL/6 mice (12–15 wk old) and cultured overnight. Cells were serum starved for 2–3 h and labeled with [^{3}H]acetate for 45 min in the presence of vehicle, AICAR, and/or 10 μM A769662. De novo lipogenesis was measured as described in MATERIALS AND METHODS. Results are expressed as mean ± SE [^{3}H]acetate incorporated into lipids (% of control). *P < 0.05 (comparing the effect of AICAR doses within [−A769662] group or [+A769662] group); #P < 0.05 (comparing the effect of A769662 to the respective AICAR doses); n = 12/condition.
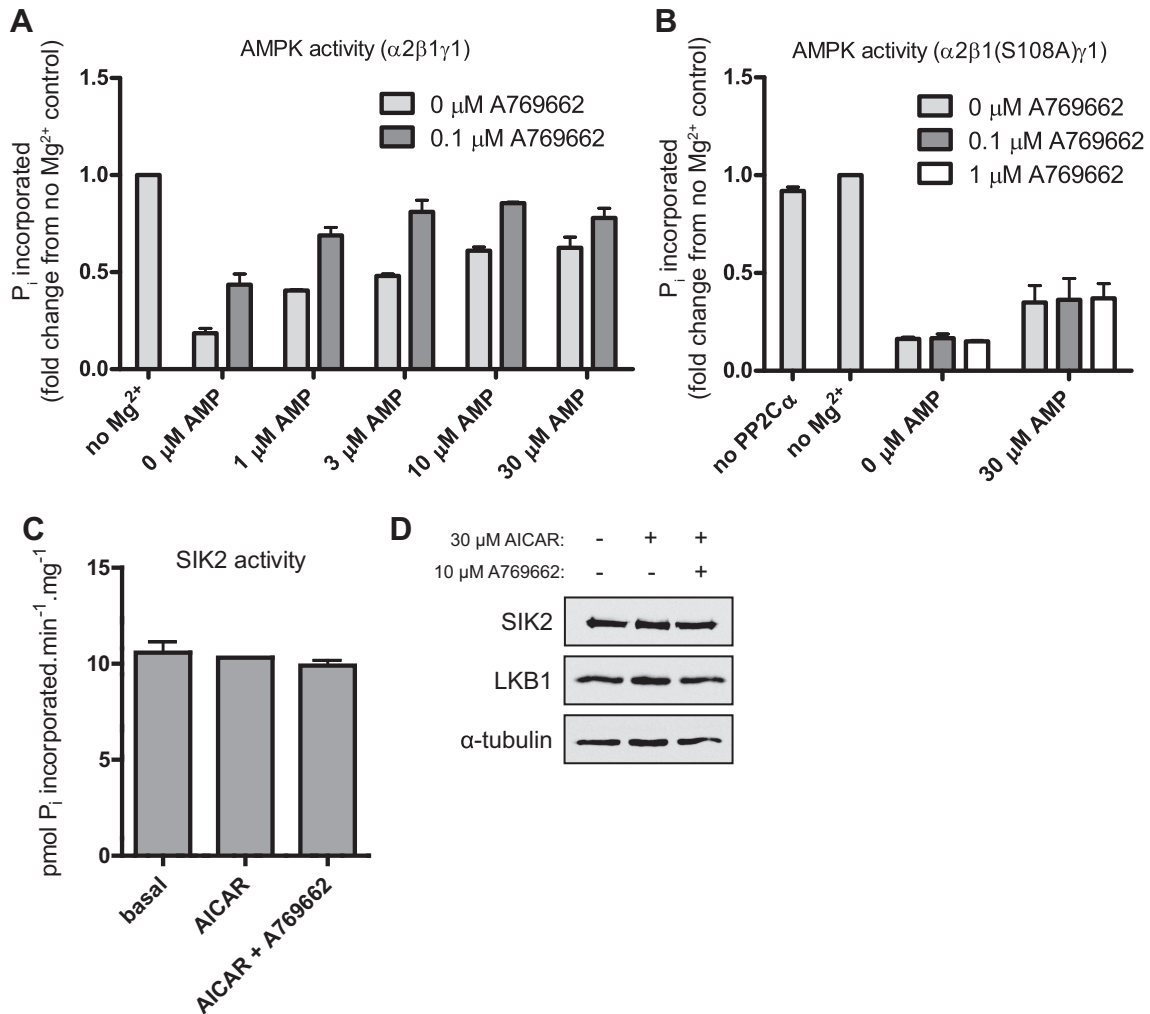
Fig. 5. Coincubation with AICAR and A769662 displays an additive protection effect on AMPK phosphorylation against dephosphorylation by a protein phosphatase. Phosphatase protection assays were performed with recombinant AMPK complex and PP2Cα (20 ng) as described in MATERIALS AND METHODS. *A*: prephosphorylated/activated recombinant AMPKα2β1γ1 was dephosphorylated by a recombinant PP2Cα in the presence or absence of its cofactor Mg^{2+} and assayed for AMPK phosphotransferase activity in vitro. Results are expressed as Pi incorporated (fold change from no Mg^{2+} control ± SE); *n* = 2. *B*: prephosphorylated/activated recombinant AMPKα2β1(Ser108Ala)γ1 was dephosphorylated by a recombinant PP2Cα in the presence or absence of its cofactor Mg^{2+} and assayed for AMPK phosphotransferase activity in vitro. Results are expressed as Pi incorporated (fold change from no Mg^{2+} control ± SE); *n* = 3. *C* and *D*: primary hepatocytes were isolated from C57BL/6 mice (12–15 wk old) and cultured overnight. Cells were serum starved for 2–3 h and then treated with vehicle, 30 μM AICAR, and/or 10 μM A769662 for 45 min. *C*: salt-inducible kinase (SIK)2 was immunoprecipitated from extracts and assayed as described in MATERIALS AND METHODS. Results are expressed as mean ± SE pmol Pi incorporated·min^{-1}·mg^{-1}; *n* = 2/condition (assay in duplicate). *D*: immunoblotting was performed with 25 μg extract per lane and the indicated antibodies.

tion via protein phosphatase in a cell-free assay (Fig. 5*A*). However, currently we are not able to demonstrate this in a cellular context/assay, as the identity of AMPK phosphatase(s) is still unknown/elusive [although there are some reported candidates (35, 40)].

Another key observation was that the enhanced AMPK activation by cotreatment resulted in a more profound inhibition of lipogenesis compared with single-compound treatment in primary hepatocytes (Fig. 4). It would be interesting to see whether the cotreatment strategy also works efficiently in the whole animal and elicits more pronounced physiological effects downstream of AMPK, for example, in reversing steatosis/fatty liver caused by high-fat diet feeding or leptin/leptin receptor deficiency (i.e., *ob/ob* or *db/db* mice). This cotreatment strategy might be attractive from the pharmaceutical point of view, as it would potentially be feasible to reduce the

concentration of the compounds by promoting sensitivity by dual treatment. However, one caveat is that it only works in cells/tissues expressing the AMPKβ1 isoform. It has been reported that the β2 isoform is predominantly expressed in skeletal muscle and that there is very little β1 isoform, resulting in insufficient AMPK activation to elicit glucose uptake in isolated rodent skeletal muscles in response to A769662 (33, 39). However, this cotreatment strategy is still a useful tool to study the function of AMPK in various cell systems given that the majority of cultured cell lines express the β1 isoform and may be resistant to AICAR. Interestingly, a recent paper has demonstrated that a patented AMPK activator termed "991" possesses structural similarity and shares a common binding site with A769662 in the carbohydrate-binding domain of the AMPKβ subunit, as evidenced by a cocrystallization study (42). However, 991 is more effective in stimulating AMPK in

both cell-based and cell-free assays that include AMPK complexes containing β2. It would be interesting to test whether 991 also elicits synergistic activation of AMPK at very low concentration when cotreated with AICAR.

In summary, we demonstrated that coincubation of cells with AICAR and a low concentration of A769662 resulted in a synergistic effect on AMPK Thr172 phosphorylation, which appears to be at least partially due to the compound binding to distinct sites causing conformation changes favorable to protection against dephosphorylation by phosphatase(s). Interestingly, we have recently observed in a parallel study that A769662 treatment of cardiac myocytes enhanced the activation of AMPK and the subsequent stimulation of glucose uptake induced by various indirect AMPK activators that raise cellular AMP-to-ATP ratio, including phenformin, oligomycin, and hypoxia (Timmermans A and Bertrand L, unpublished observations). All in all, these results suggest that it is feasible to promote AMPK activity by using two activators with distinct modes of action in order to achieve a greater resulting physiological effect of AMPK in intact cells.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: S.D., R.J.F., L. Bultot, and M.D. performed experiments; S.D., R.J.F., L. Bultot, G.R.S., and K.S. analyzed data; S.D., R.J.F., L. Bultot, G.R.S., and K.S. interpreted results of experiments; S.D. prepared figures; S.D. and K.S. drafted manuscript; S.D., R.J.F., L. Bultot, M.D., L. Bertrand, B.E.K., G.R.S., and K.S. approved final version of manuscript; R.J.F., L. Bultot, M.D., L. Bertrand, B.E.K., and G.R.S. edited and revised manuscript; L. Bertrand and K.S. conception and design of research.

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