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AMPK activation by long chain fatty acyl analogs

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ABSTRACT

The antidiabetic efficacy of first-line insulin sensitizers (e.g., metformin, glitazones) is accounted for by activation of AMP-activated protein kinase (AMPK). Long chain fatty acids (LCFA) activate AMPK, but their putative antidiabetic efficacy is masked by their β-oxidized or esterified lipid products. Substituted α,ω-dicarboxylic acids of 14–18 carbon atoms in length (MEDICA analogs) are not metabolized beyond their acyl-CoA thioesters, and may therefore simulate AMPK activation by LCFA while avoiding LCFA turnover into β-oxidized or esterified lipid products. MEDICA analogs are shown here to activate AMPK and some of its downstream targets in vivo, in cultured cells and in a cell-free system consisting of the ($\alpha_1\beta_1\gamma_1$)AMPK recombinant and LKB1-MO25-STRAD (AMPK-kinase) recombinant proteins. AMPK activation by MEDICA is accompanied by normalizing the hyperglycemia-hyperinsulinemia of diabetic db/db mice in vivo with suppression of hepatic glucose production in cultured liver cells. Activation of AMPK by MEDICA or LCFA is accounted for by (a) decreased intracellular ATP/AMP ratio and energy charge by the free acid, (b) activation of LKB1 phosphorylation of AMPK(Thr172) by the acyl-CoA thioester. The two activation modes are complementary since LKB1/AMPK activation by the CoA-thioester is fully evident under conditions of excess AMP. MEDICA analogs may expand the arsenal of AMPK activators used for treating diabetes type 2.

1. Introduction

AMP-activated serine/threonine protein kinase (AMPK) consists of alpha(α_1,α_2), beta(β_1,β_2) and gamma(γ_1,γ_2,γ_3)-heterotrimers of AMPK isoforms (reviewed in [1]). Most AMPK activity in liver is accounted for by the $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ heterotrimers [2]. Significant kinase activity requires phosphorylation of AMPK(Thr172) of the catalytic α-subunit by the upstream kinases LKB1-MO25-STRAD and/or Ca/calmodulin protein kinase (CaMKKβ) [1]. Binding of AMP to cystathionine β-synthase (CBS) motifs of the γ-subunit of AMPK results in allosteric activation of the phosphorylated AMPK as well as in suppressing dephosphorylation by protein phosphatase 2C (PP2C) [3–5]. Cellular AMPK may be similarly activated

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; CaMKKβ, Ca/calmodulin protein kinase; CBS, cystathionine β-synthase; Cl-DICA, tetrachloro-substituted hexadecanedioic acid; DN-AMPK, dominant negative AMPK; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; HGP, hepatic glucose production; LCFA, long chain fatty acids; MEDICA, tetramethyl-substituted α,ω-dicarboxylic acid; M14, tetramethyl-substituted tetradecanedioic acid; M16, tetramethyl-substituted hexadecanedioic acid; M18, tetramethyl-substituted octadecanedioic acid; PP, protein phosphatase.

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by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), being endogenously converted to ZMP [6]. Since ATP may compete for the AMP binding site and inhibit AMPK activity, the resultant activity of AMPK reflects the intracellular AMP/ATP ratio [1]. Changes in intracellular ADP/ATP may translate and be amplified into AMP/ATP ratio by the rapid equilibrium maintained by adenylate kinase (reviewed in [7]). AMPK sensing of the intracellular energy charge may account for its activation by stress conditions (e.g., hypoxia, heat shock, exercise, starvation). Activation of AMPK in response to intracellular energy depletion results in restoring cellular energy homeostasis by suppressing ATP-consuming pathways (e.g., lipogenesis, cholesterogenesis, gluconeogenesis, protein synthesis) while stimulating ATP production (e.g., glucose uptake and glycolysis, fatty acid oxidation, mitochondrial biogenesis) [1]. Some of the beneficial activities of leptin and adiponectin in the metabolic syndrome context as well as sensitization to insulin by metformin and thiazolidinediones are transduced, at least in part, by their activation of AMPK [8–11]. Hence, search for physiological and pharmacological activators of AMPK may help in characterizing the molecular etiology of the metabolic syndrome and lead to novel drugs for treating diabetes type 2.

The role played by long chain fatty acids (LCFA) in targeting AMPK has been extensively studied since the very beginning of searching for AMPK modulators [12]. AMPK activation by LCFA has been documented in perfused hearts, skeletal muscle and liver [13–15] as well as in cultured myotubes, hepatocytes and pancreatic islets [16–19]. However, the mode of action of LCFA in targeting AMPK still remains to be investigated in terms of (a) the immediate fatty acyl metabolites responsible for modulating AMPK, whether the free fatty acid, its respective acyl-CoA [12], or any of their short-chain fatty acid or esterified products [18,20–22]; (b) the putative primary role played by LCFA as direct allosteric activators of AMPK and/or its upstream kinases, as contrasted with their indirect role due to changes in energy charge mediated by their mitochondrial uncoupling activity [23]; (c) the role played by LCFA (or their metabolites) in modulating the activity of protein phosphatase PP2C [24] or PP2A [22].

In order to answer some of the above questions, AMPK activation by LCFA has recently been studied by Taylor et al. [25] and Watt et al. [17] in cell-free systems employing recombinant AMPK, LKB1-STRAD-MO25 and their respective model peptide substrates. However, the two concerned studies appear inconsistent. Thus, Taylor et al. [25] have reported inhibition of ($\alpha_2\beta_2\gamma_2$)AMPK(Thr172) phosphorylation by LCFA-CoA (but not by the respective free acid), thus leaving unanswered the mode of action of LCFA in activating AMPK in vivo and in cultured cells. On the other hand, Watt et al. [17] have reported activation of ($\alpha_1\beta_1\gamma_1$)AMPK(Thr172) phosphorylation by free fatty acids, thus conforming with previously reported cellular effects of LCFA in vivo and in cultured cells. However, AMPK activation by LCFA reported by Watt et al. [17], albeit significant, was marginal and essentially masked by added AMP.

To verify the immediate LCFA metabolites involved in activating AMPK in vivo, in cultured cells and in cell-free systems, the present study analyzes the mode of action of long chain α,α′-, β,β′- or γ,γ′-methyl-substituted α,ω-dicarboxylic acids (HOOC–C(α)R_2–C(β)R_2–C(γ)R_2–$(CH_2)_n$–C(γ)R_2–C(β)R_2–C(α)R_2–COOH, R = H or CH_3, n = 6–10) (MEDICA analogs [26]) in activating AMPK and some of its downstream targets. MEDICA analogs may be thioesterified endogenously into their respective monoacyl-CoA thioesters as verified both in vivo and in cultured cells [27]. However, the ω-carboxy-substitution of MEDICA analogs blocks MEDICA esterification into lipids, while the methyl-substitutions at the α,α′ or β,β′ positions block their β-oxidation. ATP-dependent CoA-thioesterification of MEDICA analogs to yield MEDICA-CoA does not result in sequestration of CoA and does not limit CoA-thioesterification of endogenous LCFA, as previously verified by profiling the content of liver acyl-CoAs in MEDICA-treated animals and cell cultures [27]. Analogs of the MEDICA series and their respective CoA thioesters may thus simulate the in vivo mode of action of natural LCFA in the absence of β-oxidized or esterified LCFA metabolites. Also, MEDICA analogs may highlight LCFA effects that could be masked by the rapid turnover of natural LCFA.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats: 170–250 g SD rats (Harlan, Indiana, USA) fed on Purina chow (Harlan) were dosed by gavage once daily with M16αα suspended in 1% CMC as indicated. Upon sacrifice, liver, epididymal fat and soleus muscle derived from non-fasting xylasine/katamine-anaesthetized rats were frozen immediately in liquid nitrogen and kept at −70 °C. Nonfasting blood was sampled in EDTA. 50 mg of frozen tissue were homogenized in 200 μl lysis buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM NaPPi, 10% glycerol, 1% Triton, 1 mM benzamidine, 1 mM PMSF, protease inhibitor cocktail (Sigma, Israel)), centrifuged at 14,000×*g* for 20 min, loaded on SDS-PAGE and analyzed by Western blot as indicated.

db/db mice: Twelve-week old C57BL/KsJ(db/db) mice (Jackson Laboratories, Bar Harbor) fed ad libitum with commercial rodent diet (Harlan Teklad) were dosed by gavage once daily with M16αα, troglitazone or vehicle (1% CMC) for 15 days as indicated. Mice were deprived of food 2 h before oral administration of the drugs until after blood sampling by tail vein puncture. Body weight and food consumption were measured once daily.

Plasma glucose was determined by Glucometer Elite (Bayer Corporation, Germany). Plasma insulin was determined by RIA (Mercodia, Uppsala; Cat # 10-1150-10). Plasma triglycerides were determined by enzymatic colorimetric method (Roche, Basel).

Animal care and experimental procedures were in accordance with guidelines of the accredited animal ethics committee of the Hebrew University.

2.2. Cultured cells

2.2.1. Liver cells

HepG2 or H4IIE cells were grown in 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and F-12 medium, supple-

mented with 10% fetal calf serum. HepG2 cells were infected with Lentivirus consisting of dominant negative AMPK (rAMPKα1(D157A)) or constitutive active AMPK (rAMPKα1(aa1-312)) mutants in the presence of added 5 μg/ml polybrene for 2 cycles of 4 h each, followed by culturing in DMEM supplemented with 10% fetal calf serum for 4 d. MEDICA effects were determined in cells incubated in DMEM containing 10% fetal calf serum in the presence of added MEDICA analogs as indicated (MEDICA/albumin ratio of 0.3–3.3). Cell lysates were prepared and analyzed by Western blot as indicated.

2.2.2. HeLa cells

HeLa and HEK 293 cells were cultured in DMEM containing 10% fetal calf serum. HeLa cells were transfected with CMV-LKB1 and GFP plasmids using Lipofectamine 2000 reagent (Invitrogen, CA) according to manufacturer instructions (50% transfection yield). Following 3 h, cells were washed twice and further incubated in DMEM containing 10% fetal calf serum for 24 h. M16αα was added for the last 6 h of incubation and cell lysates were prepared and analyzed by Western blot as indicated.

2.2.3. 3T3-L1 cells

3T3-L1 cells were cultured in DMEM, 10% fetal calf serum, 25 mM L-glucose and 1% (v/v) Pen/Strep/Amphotericin (Biological Industries, Beit Haemek, Israel). Adipose conversion of confluent cultures was induced by added 11.5 μg/ml insulin, 4.0 μg/ml dexamethasone and 0.5 mM IBMX for 3 d, followed by further culturing in 11.5 μg/ml insulin for additional 2 d. Converted cells were maintained in DMEM supplemented with 10% fetal calf serum.

2.3. Cell lysis and Western blot

At the end of treatment, medium was removed and cells were immediately scraped with 1× sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 50 mM DTT, 1 mM Na-vanadate, 1 mM NaPPi, 1 mM PMSF and protease inhibitor cocktail (Sigma)), sonicated, boiled for 5 min and centrifuged for 5 min at 12,800×*g*. Lysate samples were subjected to SDS-PAGE, electrotransferred onto cellulose nitrate membranes (Schleicher & Schuell, Dassel, Germany) and probed with the indicated first antibody, followed by horseradish peroxidase-labeled second antibody. Bands were detected by ECL using LAS-3000. The intensity of individual bands in Western blots was determined by densitometry using TINA 2.10 software.

2.4. Hepatic glucose production (HGP)

H4IIE cells were treated with 500 nM dexamethasone and 0.1 mM 8-(4-chlorophenylthio)-cAMP in the presence or absence of added insulin (100 nM) or MEDICA analogs as indicated. Following overnight incubation, the culture medium was replaced by fresh glucose production buffer (glucose-free phenol red-depleted RPMI in the presence or absence of 20 mM sodium lactate and 2 mM sodium pyruvate) supplemented with dexamethasone, 8-(4-chlorophenylthio)-cAMP, insulin or MEDICA analogs as indicated. The cells were further incubated for additional 3 h at 37 °C and lactate/pyruvate-dependent glucose production in culture medium was measured by Amplex Red reagent (Invitrogen, CA).

2.5. Cell-free AMPK activation

Recombinant LKB1-MO25-STRAD (6 ng), SAMS peptide (133 μM) and MEDICA or LCFA as indicated were incubated in AMPK phosphorylation buffer (30 mM Hepes, pH 7.0, 57 mM NaCl, 6% glycerol, 0.57 mM EDTA, 3.6 mM MgCl2, 143 μM [gamma-^{32}P]ATP (s.a. 0.1 Ci/mmol), 140 μM AMP, 0.57 mM DTT), in a total volume of 30 μl. The reaction was started by adding recombinant ($\alpha_1\beta_1\gamma_1$)AMPK (180 ng) and was linear for 60 min at 30 °C. The reaction was quenched by spotting 15 μl of the reaction mixture on Whatman P81 filter paper while the rest of the reaction mixture was subjected to SDS-PAGE and analyzed by Western blot as indicated. The filter paper was washed four times with 1% phosphoric acid, rinsed in acetone, dried and counted in scintillation fluid.

2.6. Real time PCR

Total RNA was prepared from HepG2 cells, using the TRI reagent (Sigma) according to manufacturer instructions. First strand cDNA used as template was synthesized by reverse transcription using oligo(dT) as primer and the Reverse-iTMAX First Strand Kit (ABgene, Epson, UK). Glucose-6-phosphatase (G6Pase) expression normalized by GAPDH expression was quantified by real time PCR (Rotor Gene RG-3000A, Australia) using SYBER green Master Mix (Absolute Syber Green ROX Mix, ABgene, Epson, UK) using the following primers: G6Pase sense, 5′-TCATCTTGGTGTCCGTGATCG-3′; G6Pase antisense, 5′-TTTATCAGGGGCACGGAAGTG-3′; GAPDH sense, 5′-GTTGCTGTAGCCAAATTCGTTG-3′; GAPDH antisense, 5′-ACCCACTCCTCCACCTTTGA-3′.

2.7. Cellular adenine nucleotides

Following removal of culture medium, cells were scraped with cold 5% perchloric acid and the extract was centrifuged for 1 min at 14,000 rpm. Supernatant was immediately transferred to two pre-cooled tubes, one of which contained nucleotide mixture for calibration, and samples were stored at −70 °C. Before analysis, perchloric acid was extracted from the supernatants as described by Fryer et al. [11]. Nucleotides were separated and quantified by HPLC as previously described [4]. In spite of strict measures undertaken here in clamping intracellular nucleotide levels, and in line with previous reports in variable cell lines ([28,29], G. Za'tara, unpublished), quantified AMP amounted to 20–30% of total nucleotides. Hence, apparent AMP/ATP and Energy Charge [30] ratios were calculated from the quantified ATP and ADP values by applying rapid equilibrium of the adenylate kinase reaction with Keq of 1.05 [31].

2.8. Plasmids and viruses

rAMPKα1(D157A) mutant was prepared by mutating rAMPKα1 [32] using the QuickChange Site-Directed Mutagenesis kit

according to the manufacturer instructions (Stratagene, CA). cDNA encoding rAMPKα1-(1-312) was prepared by PCR using pcDNA3-rAMPKα1 as template, the sense primer 5′-GC-GGCCGCATGGCCGAGAAGCAGAAGCAC-3 and the antisense primer 5′-CCCGGGTTAGTACAGGCAGCTGAGGACC-3′.

Lentivirus consisting of rAMPKα1 (D157A) or rAMPKα1 (aa1-312) mutants were prepared by subcloning the respective cDNA between Not1 and BamH1 sites of pHAGE (G.Mostoslavsky, Children's Hospital, Boston, MA), followed by cotransfecting HEK293 cells with the respective plasmids together with pCMVΔR8 [33] and pMD.G [34] plasmids by calcium phosphate precipitation. Transfected cells were cultured in DMEM containing 1% fetal calf serum for 48 h and the virus-containing media was collected.

2.9. Materials

Anti-AMPK, phosphor-AMPK(Thr172), acetyl CoA carboxylase(ACC) and phosphor-ACC(Ser79) antibodies were from Cell Signaling Technology (Boston, MA). Anti-Tubulin antibody was from Sigma. Recombinant LKB1-MO25-STRAD, SAMS and LKBtide were from Upstate Biotechnology (New York, NY). Recombinant ($\alpha_1\beta_1\gamma_1$)AMPK was prepared as previously described [4,35]. CMV-LKB1 plasmid was from OriGene Technologies (Rockville, MD). MEDICA analogs were synthesized as previously described [36]. Respective mono CoA-thioesters were synthesized and purified as previously described [37].

3. Results

3.1. AMPK activation by MEDICA analogs in vivo

AMPK activation by LCFA was simulated by incubating HepG2 cells in the presence of added MEDICA analogs of 14–18 carbon atoms in length (M14, M16, M18), tetramethyl-substituted at the αα′, ββ′ or γγ′ positions (e.g., α,α′-tetramethyltetradecanedioic acid, $(CH_2)_{10}$-$[C(CH_3)_2–COOH]_2$ (M14αα); β,β′-tetramethyltetradecanedioic, $(CH_2)_8$–$[C(CH_3)_2–CH_2–COOH]_2$ (M14ββ); γ,γ′-tetramethyltetradecanedioic acid, $(CH_2)_6$–$[C(CH_3)_2–CH_2–CH_2–COOH]_2$ (M14γγ); α,α′-tetramethylhexadecanedioic acid, $(CH_2)_{12}$-$[C(CH_3)_2–COOH]_2$ (M16αα); β,β′-tetramethylhexadecanedioic, $(CH_2)_{10}$–$[C(CH_3)_2–CH_2–COOH]_2$ (M16ββ); γ,γ′-tetramethylhexadecanedioic acid, $(CH_2)_8$–$[C(CH_3)_2–CH_2–CH_2–COOH]_2$ (M16γγ); α,α′-tetramethyloctadecanedioic acid, $(CH_2)_{14}$–$[C(CH_3)_2–COOH]_2$ (M18αα); β,β′-tetramethyloctadecanedioic, $(CH_2)_{12}$–$[C(CH_3)_2–CH_2–COOH]_2$ (M18ββ); γ,γ′-tetramethyloctadecanedioic acid, $(CH_2)_{10}$–$[C(CH_3)_2–CH_2–CH_2–COOH]_2$ (M18γγ)). Activation of AMPK(Thr172) phosphorylation by MEDICA analogs was dependent on their chain length and respective substitutions, with the higher efficacy being observed for αα′ analogs of increased chain length (e.g., M16αα, M18αα) (Fig. 1A and B). Total AMPK remained essentially unaffected by MEDICA. The specificity of AMPK activation by MEDICA analogs was further realized by lack of activation by αα-chloro α,ω-dicarboxylic acids (Cl-DICA analogs) of 16 carbon atoms in length ($(CH_2)_n$–$[CCl_2–COOH]_2$, $n = 12$) (Fig. 1B). In contrast to methyl-substituted MEDICA analogs, Cl-DICA analogs do not serve as substrates for endogenous ATP-dependent CoA-thioesterification [27], thus indicating a specific requirement for MEDICA/MEDICA-CoA in activating phosphorylation of AMPK(Thr172). Activation induced by 200 μM of M16αα surpassed that of 2 mM metformin (Fig. 1B). Activation of AMPK(Thr172) phosphorylation by M16αα was time dependent, being already observed within the first hour of exposure (Fig. 1C). M16αα concentrations required for activation of AMPK were significantly lower than those of myristic acid (Fig. 1D), reflecting the high turnover of the natural LCFA. The μM concentrations required for activation of AMPK by MEDICA analogs may reflect the high binding affinity of MEDICA analogs to medium albumin (estimated to be higher than 99%, independently of concentrations in the range of 0–0.9 mM (J. Bar-Tana, unpublished)), resulting in nM concentrations of the free acid in the culture medium.

Overexpression of a constitutive active AMPK(aa1-312) construct or activation of AMPK by MEDICA, LCFA or metformin in HepG2 cells resulted in increased ACC(Ser79) phosphorylation (Fig. 2). ACC(Ser79) phosphorylation in response to MEDICA analogs, LCFA or metformin reflected their specificity and characteristics in increasing AMPK(Thr172) phosphorylation (compare Figs. 1 and 2). Quantitative differences between AMPK(Thr172) (Fig. 1B) and ACC (Ser79) (Fig. 2B) phosphorylation by MEDICA analogs may reflect the variable stability of the respective phosphorylated motifs, or the interplay of additional targets of MEDICA in the ACC context [26]. ACC(Ser79) phosphorylation by M16αα was essentially eliminated by overexpressing a dominant-negative AMPK (DN-AMPK) (Fig. 2D), indicating that increased ACC(Ser79) phosphorylation by M16αα was transduced by AMPK activation.

Increased AMPK(Thr172) and ACC(Ser79) phosphorylation induced by MEDICA analogs was observed in other cell types (e.g., C2C12, 3T3-L1, HEK 293) (not shown) as well as in rat liver, muscle and adipose tissue of animals treated with M16αα in vivo (Fig. 3). AMPK activation by M16αα in vivo was accompanied by a significant decrease in nonfasting plasma glucose (18.6 ± 1.1 vs. $9.4 \pm 0.7^*$ mM) and triglycerides (1.13 ± 0.11 vs. $0.46 \pm 0.03^*$ mM) (mean ± S.E. ($n = 5$). $p < 0.05$ by t-test).

Activation of AMPK by M16 or M18 was accompanied by suppression of hepatic glucose-6-phosphatase (G6Pase) expression in HepG2 liver cells (Fig. 4A) and of lactate/pyruvate-dependent hepatic glucose production (HGP) induced by cAMP/dexamethasone in H4IIE hepatocytes (Fig. 4B) or HepG2 cells (not shown). Suppression of HGP by αα-MEDICA analogs of higher chain length surpassed that of shorter MEDICA analogs substituted in the γγ position, consistent with the higher efficacy of longer αα-MEDICA analogs in increasing AMPK(Thr172) and ACC(Ser79) phosphorylation (Figs. 1 and 2). Furthermore, in line with the higher efficacy of M16αα in activating AMPK as compared with metformin (Fig. 1B), suppression of HGP required 20-fold lower concentrations of M16αα as compared with metformin (not shown). The apparent lower concentrations of MEDICA analogs required for suppressing HGP as compared with those that activate AMPK(Thr172) and ACC(Ser79) phosphorylation may reflect the involvement of additional targets of MEDICA (e.g., Hepatocyte Nuclear Factor-4α (HNF-4α) [38]) in suppressing HGP.

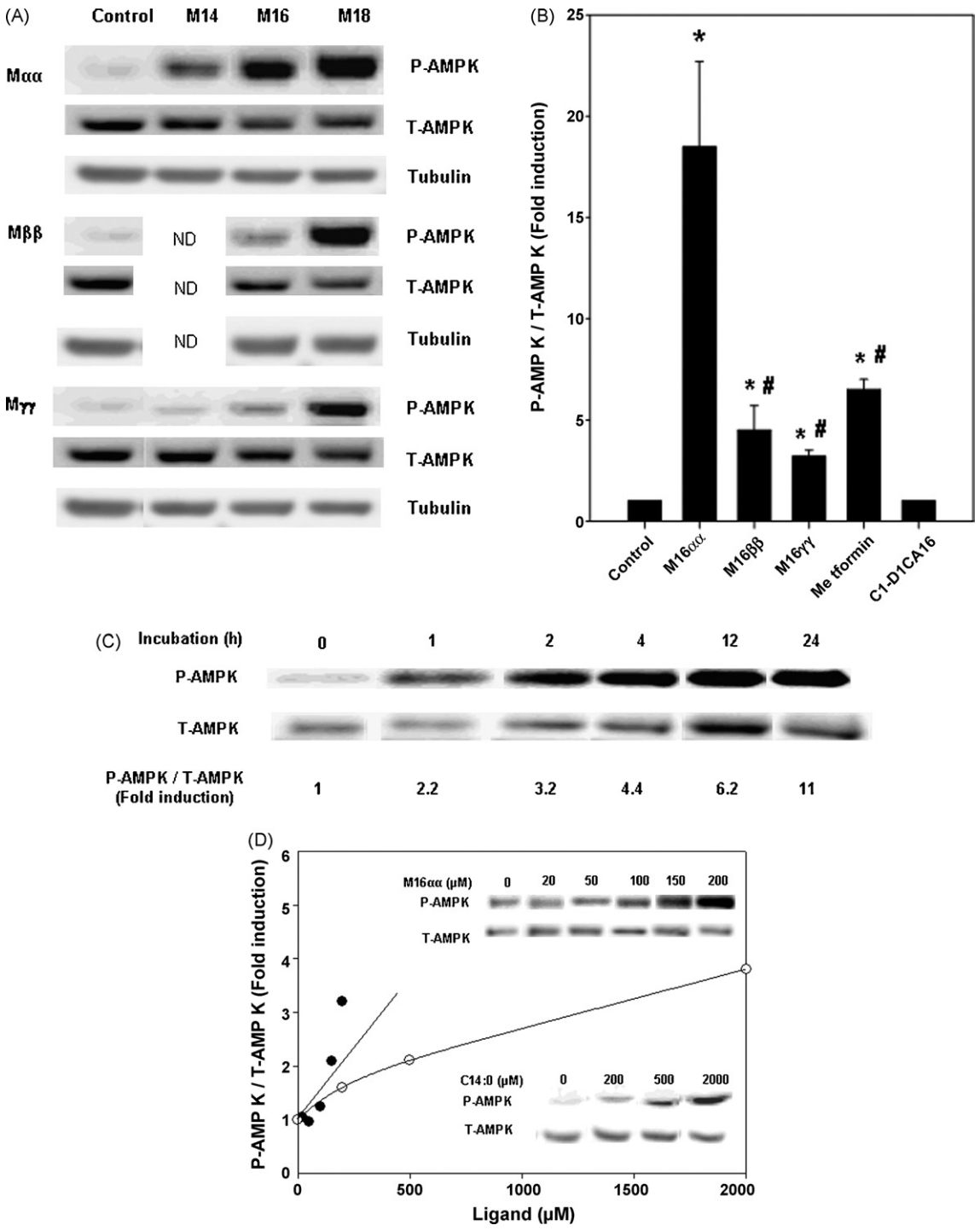

Fig. 1 – AMPK activation by MEDICA in HepG2 cells. (A) Phospho-AMPK(Thr172) and total AMPK in cell lysates of HepG2 cells incubated for 24 h with 200 μM of respective MEDICA analogs. Representative experiment out of three independent experiments. ND—not determined. (B) Phospho-AMPK(Thr172)/total AMPK ratio in cell lysates of HepG2 cells incubated for 24 h with 200 μM M16αα, M16ββ or M16γγ, 200 μM Cl-DICA or 2 mM metformin. Mean ± S.E. of three independent experiments in duplicates. *Significant as compared with nontreated cells. #Significant as compared with M16αα ($p < 0.05$ by one-way ANOVA). (C) Time curve of phosphor-AMPK(Thr172)/total AMPK ratio in cell lysates of HepG2 cells incubated with 200 μM M16αα. Representative experiment out of three independent experiments in duplicates. (D) Phospho-AMPK(Thr172)/total AMPK ratio in cell lysates of HepG2 cells incubated with increasing M16αα (●) or C14:0 (○) for 2 h as indicated. Representative experiments out of three independent experiments for each ligand in duplicates. Inset—Representative blots.

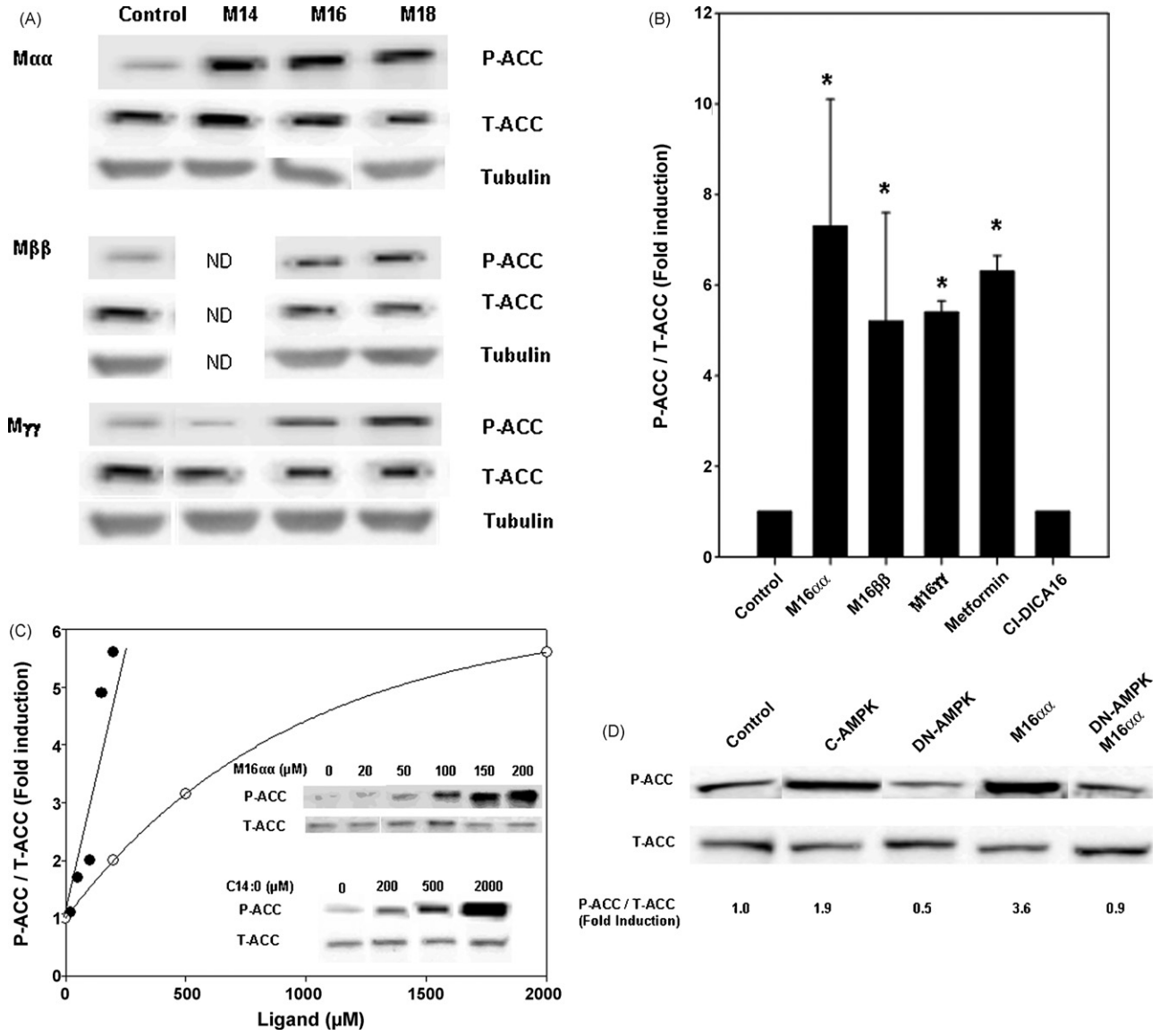

Fig. 2 – ACC(Ser79) phosphorylation by MEDICA in HepG2 cells. (A) Phospho-ACC(Ser79) and total ACC in cell lysates of HepG2 cells incubated for 24 h with 200 μM of respective MEDICA analogs. Representative experiment out of three independent experiments in duplicates. ND—not determined. (B) Phospho-ACC(Ser79) to total ACC ratio in cell lysates of HepG2 cells incubated for 24 h with 200 μM M16αα, M16ββ or M16γγ, 200 μM Cl-DICA or 2 mM metformin. Mean ± S.E. of three independent experiments in duplicates. *Significant as compared with nontreated cells ($p < 0.05$ by one-way ANOVA). (C) Phospho-ACC(Ser79) to total ACC ratio in cell lysates of HepG2 cells incubated with increasing M16αα or C14:0 for 2 h as indicated. Representative experiment out of three independent experiments for each ligand in duplicates. Inset—Representative blots. (D) Phospho-ACC(Ser79) to total ACC ratio in cell lysates of HepG2 cells infected with empty virus, constitutive AMPK (C-AMPK), dominant negative AMPK (DN-AMPK) and treated for 8 h with 200 μM M16αα as indicated. Representative experiment out of two independent experiments in duplicates.

Suppression of HGP by M16αα in liver cells was accompanied by amelioration of hyperglycemia and insulin resistance in db/db mice. Thus, treating db/db mice with M16αα for 15 d resulted in normalizing fasting blood glucose levels (Fig. 5A) and plasma triglycerides (1.68 ± 0.20 and 1.10 ± 0.15 mM for nontreated and M16αα-treated mice, respectively (mean ± S.E. $p < 0.05$ by *t*-test)), with a concomitant decrease in plasma insulin (Fig. 5B). The efficacy of M16αα surpassed that of troglitazone. Furthermore, in contrast to troglitazone where sensitization to insulin was accompanied by 7% increase in average food consumption and considerable increase in weight gain, sensitization to insulin by M16αα was accompanied by 14% decrease in average food consumption and in the absence of increased weight gain (0.99 ± 0.41, 0.93 ± 0.31 and 6.00 ± 0.44* g weight gain for nontreated, M16αα- and troglitazone-treated mice, respectively (Mean ± S.E. $p < 0.05$ by *t*-test)).

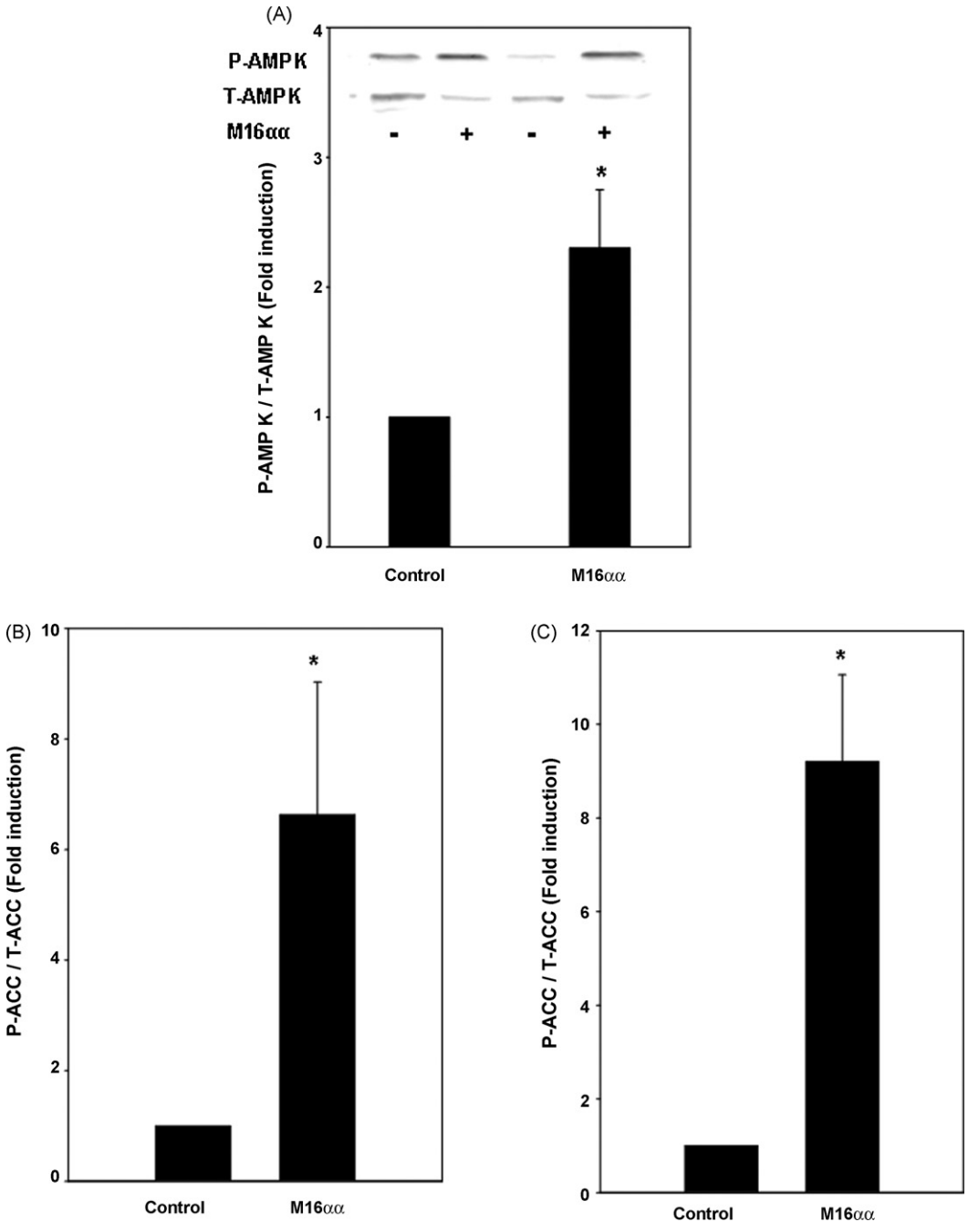

Fig. 3 – AMPK activation by MEDICA analogs in vivo. SD rats were treated with 80 mg M16αα/kg body weight/d for 14 consecutive days. (A) Liver phospho-AMPK(Thr172), total AMPK and phospho-AMPK(Thr172)/total AMPK ratio. Inset—Representative blots. (B) Soleus phosphor-ACC(Ser79) to total ACC ratio. (C) Epididymal fat phosphor-ACC(Ser79) to total ACC ratio. Mean ± S.E. (n = 5). *Significant as compared with vehicle-treated rats ($p < 0.05$ by Mann–Whitney).

3.2. *Mode of action of MEDICA*

The role played by upstream AMPK kinases in activating AMPK phosphorylation by MEDICA was verified in HeLa cells that lack expression of LKB1 but do express CaMKKβ [39]. M16αα failed to activate AMPK(Thr172) phosphorylation in the absence of LKB1 while overexpression of LKB1 resulted in AMPK activation (Fig. 6), pointing to the obligatory role played by LKB1 in AMPK activation by MEDICA analogs.

LCFA [23] as well as MEDICA analogs [40,41] (but not Cl-DICA analogs) have been reported to uncouple mitochondrial oxidative phosphorylation due to their protonophoric activity and gating the mitochondrial transition pore. Mitochondrial uncoupling by M16ββ has been previously reported to result in increase in freeze clamped hepatic ADP/ATP ratio in vivo as

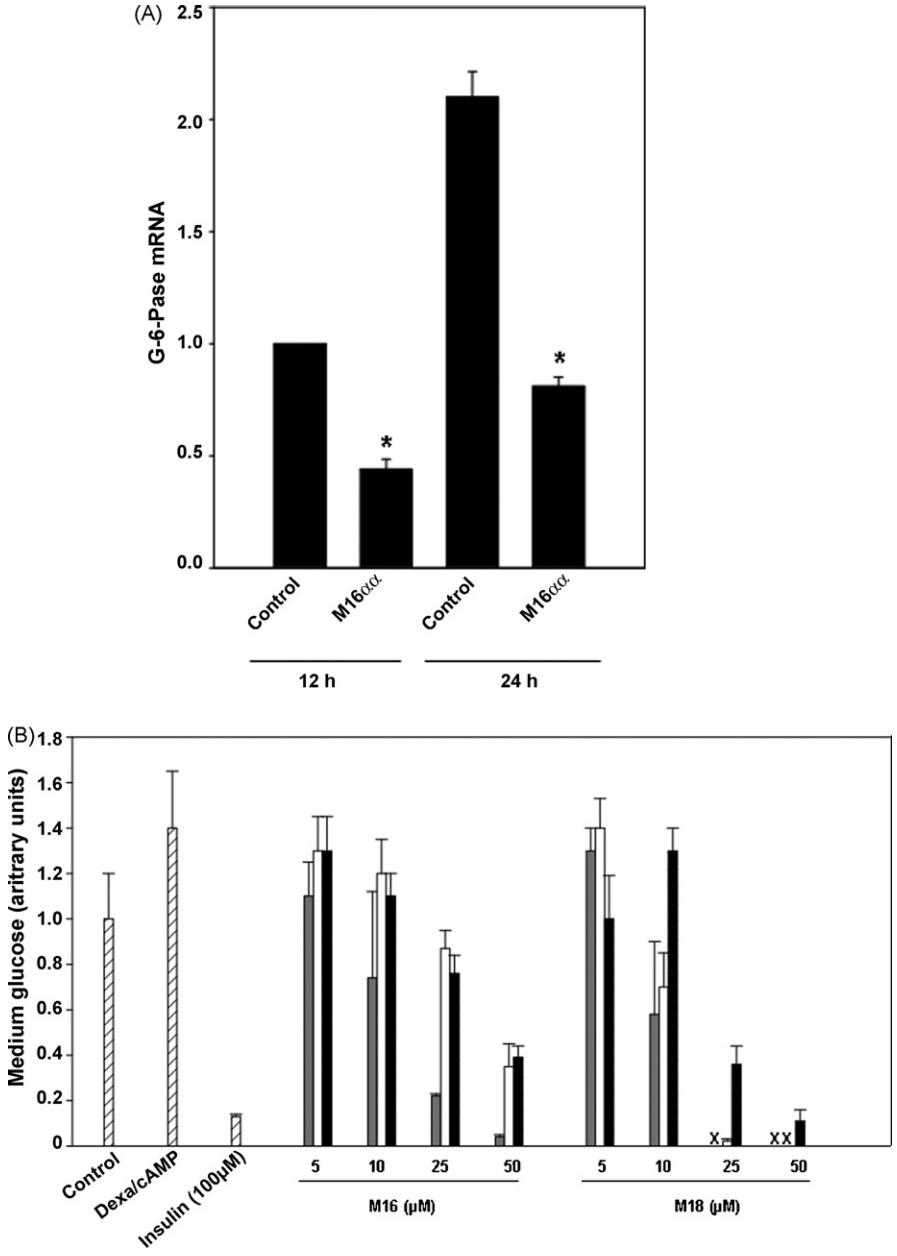

Fig. 4 – Inhibition of hepatic glucose production by MEDICA analogs. (A) HepG2 cells were incubated with 200 μM M16αα for 12 or 24 h as indicated. G6Pase mRNA normalized by GAPDH was quantified by real time PCR as described in Section 2. Mean ± S.E. of three independent experiments in triplicates. *Significant as compared with respective nontreated cultures ($p < 0.05$ by t-test). (B) H4IIE cells were incubated with dexamethasone and 8-(4-chlorophenylthio)-cAMP in the presence of added insulin, M16 or M18 analogs as indicated. Glucose production in the absence of added MEDICA (hatched bars), in the presence of respective Mαα analogs (grey bars), respective Mββ analogs (blank bars) and respective Mγγ analogs (black bars) was determined as described in Section 2. Mean ± S.E. Representative experiment out of two independent experiments in triplicates. X—below detection.

verified by [^{31}P]NMR spectroscopy [41]. Perturbation of adenine nucleotide levels by M16αα and M16ββ was further studied here in cell lines. In line with our previous findings in vivo, treatment with MEDICA analogs resulted in pronounced increase in ADP/ATP and AMP/ATP ratio with concomitant decrease in energy charge in HepG2, HEK293 and 3T3-L1 cell lines (Table 1). Hence, AMPK activation by MEDICA may partially be accounted for by perturbation of the intracellular energy charge.

The role played by MEDICA in activating AMPK independently of changes in intracellular energy charge was evaluated in a cell-free system consisting of LKB1-MO25-STRAD recombinant, ($\alpha_1\beta_1\gamma_1$)AMPK recombinant [4], SAMS peptide serving as AMPK substrate, [^{32}P]ATP and saturating AMP. The overall

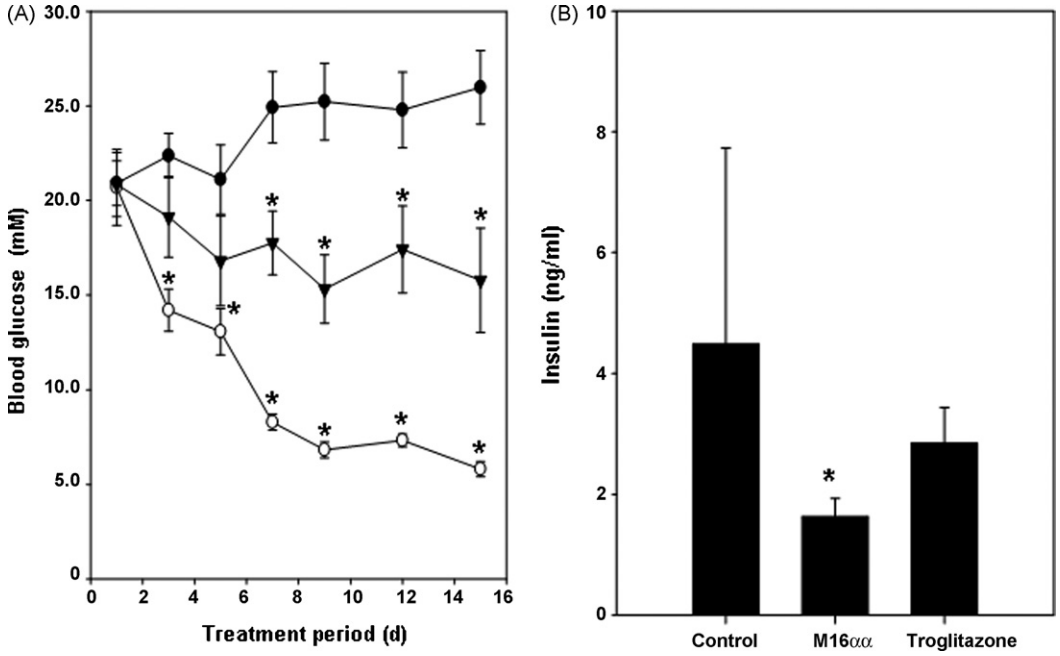

Fig. 5 – Antidiabetic efficacy of M16αα in db/db mice. db/db mice were treated for 15 consecutive days with 30 mg M16αα/kg body weight/d (○), 80 mg troglitazone/kg body weight/d (▼) or vehicle (●). Blood glucose (A) and plasma insulin (B) were determined as described in Section 2. Mean ± S.E. (n = 8). *Significant as compared with vehicle-treated animals ($p < 0.05$ by one-way nested ANOVA). Representative experiment out of two independent experiments.

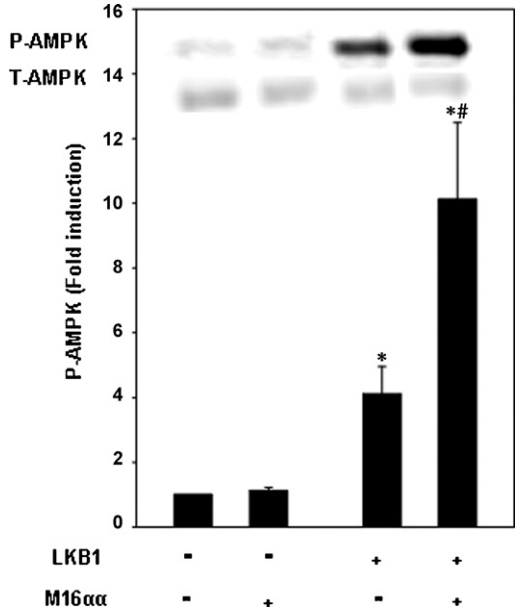

Fig. 6 – LKB1/AMPK activation by MEDICA in HeLa cells. HeLa cells were transfected in duplicates with 0.01 μg CMV-LKB1 as described in Section 2 and were further incubated for 6 h with 200 μM M16αα as indicated. Phospho-AMPK(Thr172)/total AMPK ratio was determined in cell lysates as described in Section 2. Mean ± S.E. of four independent experiments in duplicates. *Significant as compared with cells transfected with empty plasmid ($p < 0.05$ by Mann–Whitney). #Significant as compared with cells transfected with LKB1 in the absence of M16αα ($p < 0.05$ by Mann–Whitney). Inset—Representative blot.

reaction leading from LKB1 to SAMS phosphorylation was measured by quantifying [^{32}P]SAMS, while the partial reaction consisting of AMPK(Thr172) phosphorylation by LKB1 was verified by Western blot using antiphosphor-AMPK(Thr172) antibodies. The mode of action of M16αα has been verified in the presence of M16αα-COOH free acid or the M16αα-CoA thioester. [^{32}P]SAMS production was activated 6-fold by M16αα-CoA at saturation (Fig. 7A), thus pointing to direct activation of the overall reaction by M16αα-CoA, while indicating that activation by AMP and M16αα-CoA were not mutually exclusive. Similarly, phosphorylation of AMPK(Thr172) by LKB1 was activated dose-dependently by M16αα-CoA (Fig. 7A), indicating that M16αα-CoA activation of the overall reaction may be accounted for by its activation of the first partial reaction yielding phospho-AMPK(Thr172). These studies have been complemented by verifying the effect of MEDICA on the phosphorylation of SAMS catalyzed by phospho-AMPK(Thr172). Two phospho-AMPK(Thr172) preparations have been studied, namely, phospho-AMPK(Thr172) recombinant generated by phosphorylation by CaMKKβ [4] or the partially purified rat liver phospho-AMPK(Thr172) (Upstate, Cat # 14-305). Phosphorylation of SAMS by recombinant phospho-AMPK(Thr172) or by the rat liver phospho-AMPK(Thr172) preparation remained unaffected by M16αα-CoA (not shown), indicating that M16αα-CoA activation of the overall reaction leading from LKB1 to SAMS phosphorylation via phospho-AMPK(Thr172) (Fig. 7A) was essentially accounted for by the activation of LKB1 phosphorylation of AMPK(Thr172). In contrast to M16αα-CoA, SAMS phosphorylation or AMPK(Thr172) phosphorylation by LKB1 were not activated by the M16αα-COOH free acid (Fig. 7A), nor by free CoA (not shown), pointing to the specificity of the CoA-

Table 1 – Adenine nucleotides in MEDICA-treated cells

	ATP	ADP	AMP	ADP/ATP	AMP/ATP	Energy Charge
HepG2	10.2 ± 0.3	1.83 ± 0.05	0.32 ± 0.01	0.188 ± 0.003	0.033 ± 0.001	0.900 ± 0.002
HepG2 + M16αα	8.6 ± 0.3*	1.90 ± 0.05	0.43 ± 0.01*	0.230 ± 0.003*	0.050 ± 0.001*	0.871 ± 0.002*
HEK293	8.9 ± 1.1	1.40 ± 0.15	0.21 ± 0.02	0.160 ± 0.004	0.026 ± 0.001	0.912 ± 0.003
HEK293 + M16αα	7.8 ± 0.9	1.40 ± 0.13	0.26 ± 0.02	0.190 ± 0.005*	0.037 ± 0.002*	0.895 ± 0.003*
3T3L1	8.1 ± 0.6	0.77 ± 0.00	0.07 ± 0.00	0.095 ± 0.002	0.009 ± 0.000	0.949 ± 0.001
3T3L1 + M16ββ	6.2 ± 0.6	1.10 ± 0.09*	0.19 ± 0.02*	0.181 ± 0.003*	0.03 ± 0.01*	0.902 ± 0.001*

HepG2 and HEK293 cells were incubated with 200 μM M16αα for 4 h and 1h, respectively, as described in Materials and Methods. 3T3-L1 cells were incubated with 350 μM M16ββ for 24 h as described in Materials and Methods. ATP and ADP (nmol/sample extract) were determined and quantified as described in Methods. Apparent AMP (e.g., ADP^2/ATP), AMP/ATP (e.g., ADP^2/ATP^2) and Energy Charge (e.g., (ATP + 0.5ADP)/(ATP + ADP + AMP) were calculated by applying rapid equilibrium of the adenylate kinase reaction with Keq of 1.05 [31]. Mean ± S.E. of three independent experiments in triplicates.

* Significant as compared with nontreated cells ($p < 0.05$ by Mann–Whitney test).

thioester in activating the overall LKB1/AMPK/SAMS reaction in the cell-free system. In contrast to activation of AMPK(Thr172) phosphorylation, LKB1-tide phosphorylation by LKB1 was marginally (1.3-fold), albeit significantly, activated by M16αα-CoA (Fig. 7C), indicating that activation of AMPK(Thr172) phosphorylation by M16αα-CoA specifically required the LKB1/AMPK context, rather than reflecting activation of LKB1 proper.

Activation of AMPK by LCFA was further verified in the cell-free system in the presence of added myristic (C14:0) free acid or myristoyl-CoA. Similarly to MEDICA-CoA, myristoyl-CoA, but not the respective free acid, activated AMPK(Thr172) phosphorylation by LKB1 with concomitant activation of SAMS phosphorylation (Fig. 7B). In line with a previous report [42], metformin was ineffective in activating AMPK or SAMS phosphorylation in the cell-free system (not shown).

4. Discussion

MEDICA analogs and LCFA were shown here to increase phosphorylation of AMPK(Thr172) and some of its downstream targets in cultured cells, in vivo and in cell-free system, being accounted for by (a) decreased energy charge in cultured cells (Table 1) or in vivo [41], as a result of gating the mitochondrial transition pore by the free MEDICA acid or LCFA [23,40], (b) direct activation of LKB1-dependent phosphorylation of AMPK(Thr172) by the MEDICA-CoA or LCFA-CoA thioester. Lack of activation of AMPK phosphorylation by Cl-DICA (Fig. 1) may thus be accounted for by its inefficacy in gating the mitochondrial transition pore and in serving as substrate for endogenous CoA-thioesterification [27]. Since activation by MEDICA-CoA or LCFA-CoA thioesters was evident in the cell-free system in the presence of AMP in excess, the AMP-dependent and independent modes of AMPK activation by MEDICA/LCFA may synergize in vivo, via activation of LKB1-dependent phosphorylation of AMPK(Thr172), complemented by activation of the phospho-AMPK(Thr172) by the increase in AMP/ATP ratio. The contribution made by each mode of action may reflect the actual intracellular concentrations of the free acid and the respective acyl-CoA in a specific cell type, and their efficacies in targeting their respective targets. Since the liver acyl-CoA profile reflects the composition of dietary MEDICA/LCFA [27], the two modes of AMPK activation may be affected by dietary composition and drug dosage. AMPK activation by MEDICA analogs may indicate that activation by natural LCFA may not require their β-oxidized or esterified lipid products, but mediated by LCFA/LCFA-CoA per se. These findings are in line with previous reports that have documented activation of AMPK by LCFA in vivo [13–19]. Also, our findings are essentially in line with those of Watt et al. [17], reporting marginal activation of AMPK by the free acid in a cell-free system. It is noteworthy, however, that activation of AMPK phosphorylation by the acyl-CoA is far more significant (Fig. 7). Inhibition of LKB1/AMPK recombinants by acyl-CoA reported by Taylor et al. [25] does not concur with the in vivo data and was perhaps specific for the ($\alpha_2\beta_2\gamma_2$)AMPK isoform. Activation of AMPK by MEDICA analogs is noteworthy in light of the reported inefficacy of the more polar related 8-hydroxy-α,α′-tetramethyl-α,ω-pentadecanedioic acid (ESP 55016) [43]. The efficacy of MEDICA analogs may reflect their higher affinity for a putative hydrophobic binding domain in LKB1/AMPK.

AMPK activation by MEDICA analogs may complement and amplify the hypolipidemic [44] and antidiabetic [45] effects of MEDICA previously ascribed to suppression of HNF-4α transcriptional activity due to inhibition of its intrinsic thioesterase activity by high affinity binding of MEDICA-CoA ([38] and references therein). Thus, suppression of HNF-4α transcriptional activity may further be amplified by MEDICA-induced AMPK phosphorylation of HNF-4α resulting in its ubiquitination and proteolytic degradation ([46], G. Za'tara, unpublished). Moreover, AMPK activation by MEDICA may allow for antidiabetic effects of MEDICA in the context of tissues that do not express HNF-4α (e.g., muscle, adipose fat). Furthermore, AMPK activation by MEDICA resulting in ACC phosphorylation, may account for the robust decrease in malonyl-CoA with concomitant increase in fatty acid oxidation, previously reported in MEDICA-treated animals and cultured cells, and ascribed to inhibition of ATP-citrate lyase activity [26]. The effect exerted by MEDICA analogs in db/db mice (Fig. 5) and fa/fa rats [45] may indeed reflect their antidiabetic activity in terms of the LKB1/AMPK target [47,48] combined with their HNF-4α [38] and ATP-citrate lyase [26] targets, in the context of liver, muscle and adipose fat. Hence, MEDICA analogs may expand the arsenal of AMPK activators used for treating diabetes type 2. Furthermore, MEDICA mode of action may offer a molecular basis for the surprisingly beneficial effects of high-fat low-carbohydrate diets in treating aspects of the metabolic syndrome [49].

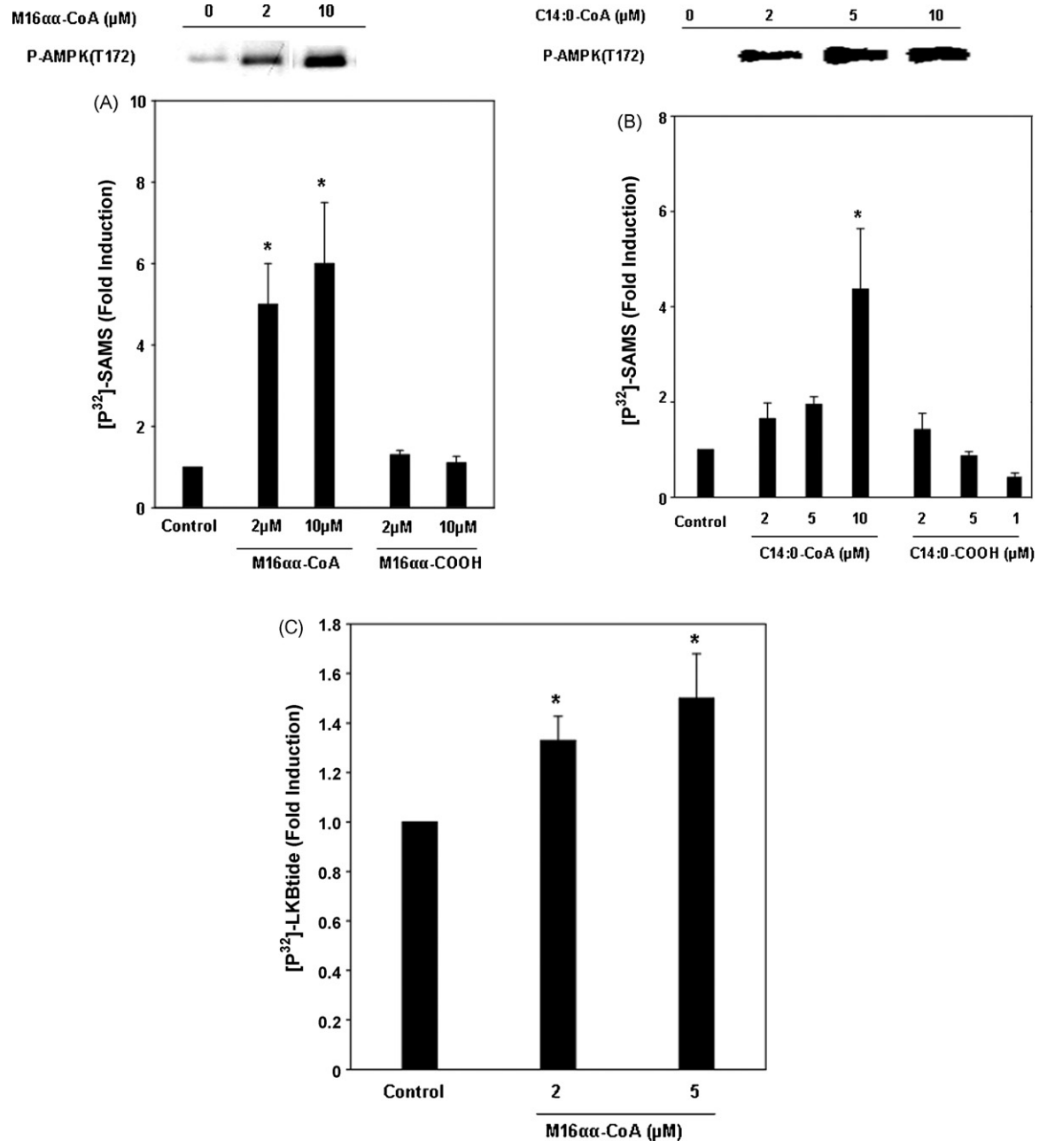

Fig. 7 – LKB1/AMPK activation by M16αα and C14:0 in cell-free system, recombinant LKB1-MO25-STRAD, recombinant ($\alpha_1\beta_1\gamma_1$)AMPK and SAMS peptide were incubated for 30 min with M16αα-COOH free acid or M16αα-CoA (A), or with C14:0-COOH free acid or C14:0-CoA (B) as indicated, followed by measuring [^{32}P]SAMS and phosphor-AMPK(Thr172) as described in Section 2. Base line phosphorylation of SAMS amounted to 10 pmol. Mean ± S.E. of three independent experiments in duplicates. *Significant as compared with activity in the absence of added ligand ($p < 0.05$ by *t*-test). Inset—Representative blot. (C) Recombinant LKB1-MO25-STRAD and LKBtide (340 μM) were incubated for 20 min as described by Taylor et al. [25] with M16αα-CoA as indicated, followed by measuring [^{32}P]LKBtide. Mean ± S.E. of three independent experiments in duplicates. *Significant as compared with activity in the absence of added ligand ($p < 0.05$ by Mann–Whitney).

Acknowledgements

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