Methotrexate (MTX) is a widely used anticancer and antirheumatic drug that has been postulated to protect against metabolic risk factors associated with type 2 diabetes, although the mechanism remains unknown. MTX inhibits 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (ATIC) and thereby slows the metabolism of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-5′-monophosphate (ZMP) and its precursor AICAR, which is a pharmacological AMPK activator. We explored whether MTX promotes AMPK activation in cultured myotubes and isolated skeletal muscle. We found MTX markedly reduced the threshold for AICAR-induced AMPK activation and potentiated glucose uptake and lipid oxidation. Gene silencing of the MTX target ATIC activated AMPK and stimulated lipid oxidation in cultured myotubes. Furthermore, MTX activated AMPK in wild-type HEK-293 cells. These effects were abolished in skeletal muscle lacking the muscle-specific, ZMP-sensitive AMPK-γ3 subunit and in HEK-293 cells expressing a ZMP-insensitive mutant AMPK-γ2 subunit. Collectively, our findings underscore a role for MTX as a direct molecular link between MTX and energy metabolism in skeletal muscle. Chronic therapy with methotrexate (MTX), a broadly used anticancer and antirheumatic drug, may protect rheumatic patients against metabolic risk factors associated with cardiovascular disease, obesity (1), and type 2 diabetes (2). This notion is supported by recent observations that MTX alleviates hyperglycemia and insulin resistance in diabetic (db/db) mice (3) and obese mice fed a high-fat diet (4). MTX is a folate antagonist and inhibits DNA replication (5), which explains its anticancer action but the not improvements in glucose homeostasis in type 2 diabetes or obesity. Suppression of chronic inflammation, thought to arise from MTX-stimulated adenosine release (6), may improve glucose homeostasis indirectly (7). Because anti-rheumatic drugs are not invariably associated with metabolic protection (1,8), other mechanisms are likely. Although MTX-induced adenosine release may have direct metabolic effects (4), its exact role is ambiguous, because adenosine receptor activation (9) and blockage (10) both improve insulin sensitivity. Clearly, the molecular underpinnings of MTX action in relation to metabolic disease remain undefined.

MTX has several pharmacological targets, including 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (ATIC) (11). ATIC is essential for the conversion of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-5′-monophosphate (ZMP) to inosine monophosphate in the final two steps of the de novo purine synthesis pathway. Thus, congenital ATIC deficiency or MTX therapy elevates intracellular ZMP content and excretion of its metabolites (6,12,13). The purine precursor ZMP is also an AMP mimetic and activates AMPK (14). AMPK is a heterotrimeric

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serine-threonine kinase composed of the catalytic α- and noncatalytic β- and γ-subunits (15). ZMP binds to the AMP-binding sites on the γ-subunits (16), and this is required for its ability to activate AMPK (17). AMPK plays a role in maintaining energy homeostasis and is currently a target for the treatment of type 2 diabetes (18–20). Thus, MTX might mitigate metabolic impairments by promoting ZMP-stimulated AMPK activation in key organs controlling glucose homeostasis.

AMPK activation increases glucose uptake, fatty acid oxidation, and mitochondrial biogenesis, which helps to ameliorate different aspects of metabolic dysregulation, including hyperglycemia and insulin resistance (19). MTX could promote ZMP accumulation and AMPK activation by suppressing ATIC, which is expressed and active in skeletal muscle (21,22). ZMP is also the active metabolite of the pharmacological AMPK activator AICAR (14). AICAR therapy promotes favorable metabolic reprogramming in skeletal muscle of sedentary (23) and diabetic (ob/ob) mice (24). However, a relatively high threshold for AMPK activation (25), in conjunction with poor bioavailability (26), limits the usefulness of AICAR in the treatment of type 2 diabetes (27). MTX may enhance AICAR-stimulated AMPK activation in skeletal muscle by suppressing ATIC-mediated ZMP clearance and overcome this problem.

Here, we show that MTX markedly reduces the threshold for AICAR-stimulated AMPK activation and potentiates glucose uptake and lipid oxidation in skeletal muscle. Thus, cotherapy with AICAR and MTX could represent a novel strategy to treat metabolic disorders and overcome current limitations of AICAR monotherapy.

RESEARCH DESIGN AND METHODS

Antibodies and Reagents

Antibodies against phospho-AMPKα (Thr^172) and phospho-ACC (Ser^78) were from Cell Signaling Technology (Beverly, MA), the antibody against ATIC was from Sigma-Aldrich (Stockholm, Sweden), the GLUT4 antibody was from Millipore (Temecula, CA), and the antibody against GAPDH was from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL reagent and the protein molecular weight marker were from GE Healthcare (Uppsala, Sweden). The BCA Protein Assay kit was from Pierce (Rockford, IL), and the polyvinylidene fluoride Immobilon-P membrane was from Millipore (Bedford, MA). Cell culture materials were from Costar (Täby, Sweden), and [9–10(n)−3H]-palmitic acid and [1,2,3H]-2-deoxy-D-glucose were from Perkin-Elmer. All other reagents, unless otherwise specified, were of analytical grade and obtained from Sigma-Aldrich.

L6 Cells and Small Interfering RNA Transfection

L6 muscle cells were grown in α-minimum essential medium (α-MEM) supplemented with 10% FBS, 1% PenSt (100 units/mL penicillin and 100 μg/mL streptomycin), and 1% Fungigzone at 37°C in humidified air and 5% CO₂. They were differentiated into myotubes in α-MEM (2% FBS, 1% PenSt, and 1% Fungigzone). L6 cells were transfected with a pool of short interfering (si)RNAs (100 nmol/L) against ATIC mRNA or scrambled siRNA (Dharmacon, Chicago, IL) using the Eppendorf Transfection Kit on days 2 and 4 of differentiation. Experiments were performed 48–72 h after the last transfection.

Primary Human Skeletal Muscle Cells

Primary human skeletal muscle myoblasts were from Lonza Group Ltd (Basel, Switzerland) and maintained in DMEM/F12 supplemented with 20% FBS, 1% PenSt, and 1% Fungigzone at 37°C in humidified air and 7% CO₂. Myoblasts were differentiated into myotubes for 5 days in DMEM (2% FBS, 1% PenSt, and 1% Fungigzone). Serum-starved myotubes were incubated for 4 h and subsequently with or without 5 μmol/L MTX and/or 0.2 mmol/L AICAR for 5 h.

Metabolic Assays in L6 Myotubes

L6 myotubes were incubated in α-MEM (without nucleosides) with or without 5 μmol/L MTX for 16 h before the experiment. For fatty acid oxidation, myotubes were serum-starved for 4 h and then incubated with or without MTX (5 μmol/L) and/or AICAR (0.2 or 2 mmol/L) for 5 h in α-MEM, supplemented with 0.2% BSA, 20 μmol/L cold palmitate, and 0.5 μCi/mL [3H]-palmitic acid. Palmitate oxidation was determined by measuring the amount of [3H]2O in cell culture media. Nonmetabolized palmitate was adsorbed to charcoal and removed by centrifugation at 16,000 rpm for 15 min. For glucose uptake, L6 myotubes were incubated with or without MTX (5 μmol/L) and/or AICAR (0.2 mmol/L or 2 mmol/L) in serum-free α-MEM (without nucleosides) for 5 h. Myotubes were then washed with HEPES-buffered saline (140 mmol/L NaCl, 20 mmol/L HEPES, 5 mmol/L KCl, 2.5 mmol/L MgSO4, 1 mmol/L CaCl₂, pH 7.4) and subsequently incubated in HEPES-buffered saline supplemented with 10 μmol/L 2-deoxy-D-glucose and 0.75–1 μCi/mL [1,2,3H]-2-deoxy-D-glucose for 10 min. Nonspecific glucose transport was assessed in the presence of 10 μmol/L cytochalasin B. Myotubes were then washed with ice-cold stop solution (25 mmol/L glucose, 0.9% [w/v] NaCl) and lysed in 0.03% (w/v) SDS. Radioactivity was measured in cell lysates by liquid scintillation counting.

Experimental Animals

This study was approved by the Regional Animal Ethical Committee (Stockholm, Sweden). AMPK-γ3 knockout (AMPK-γ3/−/−) mice were bred onto a C57BL/6J genetic background and have been thoroughly characterized (28). Before all experiments, AMPK-γ3/−/− and wild-type (WT) mice were fasted for 4 h before being anesthetized with Avertin (0.02 mL/g i.p.).

Metabolic Assays in Isolated Skeletal Muscle

Extensor digitorum longus (EDL) and soleus muscle from WT or AMPK-γ3/−/− mice were preincubated for 2 h at 30°C in oxygenated (95% O₂ and 5% CO₂) Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 5 mmol/L glucose, 15 mmol/L mannitol, 5 mmol/L HEPES, and 0.1% (w/v) BSA with or without 10 μmol/L MTX. Thereafter, muscles
were incubated for 2 h with or without 10 μmol/L MTX and/or AICAR (0.2 mmol/L or 2 mmol/L). Glucose transport was assessed by measuring the uptake of [1,2,3H]-2-deoxy-D-glucose as described (28). Muscles were incubated for 20 min in KHB supplemented with 1 mmol/L 2-deoxy-D-glucose, 19 mmol/L mannitol, 2.5 μCi/mL [1,2,3H]-2-deoxy-D-glucose, and 0.7 μCi/mL [14C] mannitol. For fatty acid oxidation, KHB was supplemented with [H3]-palmitic acid (0.4 μCi/mL) and 0.3 mmol/L cold palmitate, and oxidation was determined by analyzing the [3H]-labeled water content using liquid scintillation counting as described above.

Lysate Preparation and Immunoblot Analysis

Myotubes were washed with ice-cold PBS and lysed in homogenization buffer (1% Protease Inhibitor Cocktail, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl2, 5 mmol/L Na3PO4, 0.5 mmol/L Na2VO4, 1% Triton X-100, 10% glycerol, 20 mmol/L Tris, 10 mmol/L NaF, 1 mmol/L EDTA, and 0.2 mmol/L phenylmethylsulfonyl fluoride, pH 7.8). Lysates were centrifuged for 15 min at 12,000 g (4°C). Muscle was homogenized in ice-cold buffer, followed by end-over-end rotation for 60 min (4°C) and centrifugation at 12,000 g for 10 min (4°C). Supernatants of cell or muscle lysates were collected, and protein content was measured (BCA Protein Assay). Proteins dissolved in Laemmli buffer (25 μg per well) were resolved on 4–12% Bis-Tris gel (Bio-Rad, Richmond, CA), transferred to polyvinylidene fluoride membrane, blocked with 7.5% nonfat milk, and probed overnight (4°C) with primary antibodies. All primary antibodies were diluted 1:1,000 in primary antibody buffer (Tris-buffered saline [TBS], 0.5% BSA, 0.5% NaNO3, pH 7.6). After the overnight incubation, membranes were washed with washing buffer (TBS, 0.02% Tween-20) and incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) for 1 h at room temperature. The secondary antibodies were diluted 1:25,000 in TBS supplemented with 5% nonfat milk. Immunoreactive proteins were visualized by enhanced chemiluminescence and quantified by the Quantity One 1-D Analysis Software (Bio-Rad). Data were normalized to GAPDH.

Plasmids and Generation of Stable WT and R531G AMPK-γ2 HEK-293 Cell Lines

Full-length human γ2 was amplified with primers designed to encode a 5′-BamHI site and a COOH-terminal FLAG tag, followed by an XhoI site. The resulting PCR product was cloned into the pcDNA5/FRT/TO plasmid (Invitrogen) to create pcDNA5/FRT/TO plasmid (Invitrogen). The resulting construct was performed using the QuickChange Site-Directed Mutagenesis system (Stratagene). The plasmid (POG44) expressing Flp recombinase was from Invitrogen. T-Rex HEK-293 cells containing a single FRT site (Invitrogen) were transfected with Fugene6 (Promega) using the plasmids POG44 and pcDNA5/FRT/TO/g2 at a ratio of 9:1. Fresh medium was added to the cells 24 h after transfection, and media containing 200 μg/mL hygromycin B and 15 μg/mL blasticidin was added 48 h after transfection. The medium was replaced every 3 days until foci could be identified, and individual foci were then selected and expanded. The expression of FLAG-tagged g2 was induced by adding 1 μg/mL tetracycline for 40 h, and expression was detected using immunofluorescence microscopy and Western blot using anti-FLAG antibodies.

Culture of WT and R531G AMPK-γ2 HEK-293 Cells and Cell Treatments

HEK-293 cells expressing WT or R531G AMPK-γ2 were generated as described (17). T-Rex-Flp-In HEK-293 cells (Invitrogen) containing a single FRT site were cultured in DMEM containing 4.5 g/L glucose, 10% (v/v) FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 15 μg/mL blasticidin, and 100 μg/mL zeocin. After transfection, T-Rex-Flp-In HEK-293 cells stably expressing WT or R531G AMPK-γ2 were cultured as above except that zeocin was replaced by 200 μg/mL hygromycin B. Forty hours after the induction of protein, 3 μmol/L MTX/DMSO was added to cells for 16 h, followed by 1 h of AICAR in different concentrations.

AMPK Activity Assays

WT HEK-293 cells or mutant HEK-293 (RG) cells were assayed for AMPK activity as described (17). Activity of the purified rat liver AMPK, which is approximately an equal mixture of the α1β1γ1 and α2β1γ1 heterotrimers (29), was measured in the presence or absence of AMP or protein phosphatase 2C (PP2C) as described (30).

Statistics

Results are presented as means ± SEM or means ± SD. Comparisons among multiple groups were performed using ANOVA, followed by the Bonferroni or Dunnett post hoc test. The Student t test was used when two groups were compared. Statistical significance was established at P < 0.05.

RESULTS

MTX Promotes AMPK Activation in Cultured Myotubes

To determine whether MTX modulates AMPK activation, L6 myotubes and primary human myotubes were exposed to 5 μmol/L MTX and subthreshold (0.2 mmol/L) and/or maximally effective (2 mmol/L) concentrations of AICAR (14,25). AMPK activation was determined by measuring AMPK Thr172 and acetyl-CoA carboxylase Ser79 (ACC) phosphorylation. Exposure of cultured myotubes to 0.2 mmol/L AICAR alone did not elevate AMPK or ACC phosphorylation (Fig. 1A–D), demonstrating that 0.2 mmol/L AICAR is below the threshold for AMPK activation. Conversely, coincubation with MTX and 0.2 mmol/L AICAR robustly increased phosphorylation of AMPK (Fig. 1A and B) and ACC (Fig. 1C and D), demonstrating that MTX markedly reduces the threshold for AMPK activation.

MTX Promotes Metabolic Effects in Skeletal Muscle via AMPK Activation

To investigate whether the augmented AICAR-stimulated ACC phosphorylation in MTX-treated myotubes translates
into increased lipid utilization, palmitate oxidation was measured in L6 myotubes incubated with MTX (5 μmol/L) and/or AICAR (0.2 or 2 mmol/L). Although 0.2 mmol/L AICAR alone failed to increase palmitate oxidation, treatment with 0.2 mmol/L AICAR and MTX together stimulated palmitate oxidation in L6 myotubes (Fig. 2A).

We next examined whether AICAR-stimulated glucose uptake was increased in L6 myotubes incubated with MTX (5 μmol/L) and/or AICAR (0.2 or 2 mmol/L). Although 0.2 mmol/L AICAR alone failed to increase palmitate oxidation, treatment with 0.2 mmol/L AICAR and MTX together stimulated palmitate oxidation in L6 myotubes (Fig. 2A).

MTX Promotes AMPK Activation Through Indirect Mechanisms
To confirm that MTX treatment inhibited ATIC, ZMP content was measured in L6 cells exposed to 0.2 mmol/L AICAR and/or 5 μmol/L MTX. AICAR alone did not elevate ZMP content, indicative of the high ZMP turnover (Fig. 3A). However, a combined treatment with MTX and 0.2 mmol/L AICAR markedly elevated glucose uptake. MTX enhanced glucose uptake in myotubes treated with a saturating concentration of AICAR (2 mmol/L), indicating AICAR responsiveness was improved. Glucose transport was also determined in EDL and soleus muscle treated with 0.2 mmol/L AICAR and/or 10 μmol/L MTX (Fig. 2C). MTX robustly augmented AICAR-stimulated glucose uptake in EDL muscle, underscoring the effect of MTX to potentiate AICAR responsiveness. Although soleus muscle was less responsive to AICAR, glucose uptake was elevated upon MTX and AICAR exposure. MTX treatment did not affect GLUT4 protein abundance or GLUT4 mRNA expression (data not shown).

Figure 1—MTX promotes AMPK activation in cultured myotubes. Phosphorylation (p) of AMPK (pAMPK) at Thr172 (A and B) and its target pACC at Ser79 (C and D) were determined in L6 myotubes and in myotubes cultured from primary human skeletal muscle cells (HMSC) upon exposure to 5 μmol/L MTX and/or 0.2 mmol/L or 2 mmol/L AICAR (5 h). Myotubes were preincubated with 5 μmol/L MTX or vehicle for 16 h before the experiment. Results are means ± SEM (n = 6). #P < 0.05 vs. basal, *P < 0.05 vs. AICAR (0.2 mmol/L).

Figure 2—MTX promotes metabolic effects in skeletal muscle via AMPK activation. Palmitate oxidation (n = 7–8; except basal and MTX, where n = 15) (A) and glucose uptake (n = 24) (B) in L6 myotubes upon exposure to 5 μmol/L MTX and/or 0.2 mmol/L or 2 mmol/L AICAR (5 h). L6 myotubes were preincubated with 5 μmol/L MTX or vehicle for 16 h before the palmitate oxidation or glucose uptake assay. C: Glucose uptake in isolated EDL and soleus muscle after exposure to 10 μmol/L MTX, 120 nmol/L insulin, and/or 2 mmol/L AICAR (n = 6–8). EDL and soleus muscle were preincubated with 10 μmol/L MTX or vehicle for 2 h before the glucose uptake assay. Results are means ± SEM. #P < 0.05 vs. basal, *P < 0.05 vs. AICAR (0.2 mmol/L) or as indicated.
that MTX promotes AMPK activation in cultured myotubes primarily by decreasing ZMP clearance, although ATP depletion may play a secondary role.

To establish a causal link between MTX-induced alterations in cellular nucleotide content and AMPK activation, WT HEK-293 and RG cell lines were exposed to MTX (3 μmol/L) and/or different concentrations of AICAR (Fig. 3C–F). RG cells harbor a single substitution (R531G) within the AMPK-γ2 subunit that renders AMPK insensitive to stimulation by AMP and ZMP (17). ZMP content and the ADP-to-ATP ratio were both increased in WT cells treated with MTX and AICAR (Fig. 3C and D). The elevated ZMP content during MTX and AICAR treatment was paralleled by an increase in AMPK activity (Fig. 3E). Notably, MTX alone was sufficient to activate AMPK in WT cells. However, the increase in AMPK activity was almost entirely abrogated in RG cells during coincubation with MTX and AICAR (Fig. 3F), and MTX alone did not stimulate AMPK activation in RG cells. Because AMPK complexes in RG cells remain responsive to direct AMPK activators other than AMP and ZMP, such as A-769662 (17), this suggests that MTX is not a direct AMPK activator.

**MTX Is Not a Direct AMPK Activator**

To evaluate whether MTX activates AMPK allosterically, the activity of AMPK, purified from rat liver, was measured during incubation with AMP and/or increasing concentrations of MTX (Fig. 4A). AMPK activity was increased in the presence of 200 μmol/L AMP, but MTX alone or combined with AMP did not alter AMPK activity. We also determined whether MTX protects AMPK from dephosphorylation by PP2C, which dephosphorylates and thereby inactivates AMPK (35). Purified AMPK was incubated with PP2C and 200 μmol/L AMP or different concentrations of MTX (Fig. 4B). AMP almost completely protected AMPK from dephosphorylation by PP2C, but MTX did not, showing that MTX does not inhibit PP2C-mediated dephosphorylation of AMPK.

**MTX Depends on AMPK To Promote Lipid Oxidation**

Our results have established that MTX enhances AICAR action in skeletal muscle. To determine whether MTX depends on AMPK to promote metabolic alterations in skeletal muscle, we studied EDL and soleus muscles from WT and AMPK-γ3−/− mice. Isolated EDL and soleus muscles were incubated in the absence or presence of 10 μmol/L MTX and/or a subthreshold concentration of AICAR (0.2 mmol/L). Treatment with 0.2 mmol/L AICAR and MTX was sufficient to markedly stimulate AMPK activation and palmitate oxidation in WT EDL and soleus muscle. MTX also failed to enhance AICAR-stimulated

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<th>Table 1 — Effect of MTX on ZMP content and adenylate energy charge in AICAR-treated murine skeletal muscle</th>
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ZMP content, expressed as the ZMP-to-ATP ratio, and adenylate energy charge, calculated as ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]), were determined in WT EDL and soleus muscle after exposure to 0.2 mmol/L AICAR and 10 μmol/L MTX (2 h). EDL and soleus muscle were preincubated with 10 μmol/L MTX or vehicle for 2 h before the experiment. ZMP was below detection limit under basal conditions (data not shown). Results are means ± SEM (n = 3–4).
Methotrexate Enhances AMPK Activation

Suppression of ATIC Directly Promotes AMPK Activation and Increases Lipid Oxidation

L6 myotubes were used to validate ATIC as an MTX target (Fig. 6A). Expression of ATIC was suppressed in L6 myotubes using siRNA (Fig. 6B). Exposure of myotubes transfected with scrambled siRNA to 0.2 mmol/L AICAR did not increase ZMP content (Fig. 6C). Conversely, stimulation with 0.2 mmol/L AICAR robustly elevated ZMP content in ATIC-depleted myotubes (Fig. 6C), indicating diminished ZMP clearance. The ADP-to-ATP ratio was also elevated 12–15% in ATIC-depleted cells (Fig. 6D), possibly reflecting purine depletion due to decreased de novo synthesis. The increased ZMP content in ATIC-depleted myotubes treated with AICAR increased AMPK and ACC phosphorylation and palmitate oxidation (Fig. 6E–G), consistent with the hypothesis that decreased ZMP clearance promotes AMPK activation. Notably, ATIC depletion alone increased AMPK phosphorylation and palmitate oxidation (Fig. 6E and G), underscoring the link between ATIC suppression and AMPK activation in skeletal muscle.

Gene silencing suppressed ATIC expression 70% (Fig. 6B). To test whether inhibition of residual ATIC activity provides an additional stimulus for AMPK activation, L6 myotubes were transfected with siRNA, subsequently exposed to MTX, and incubated in the presence of 0.2 mmol/L AICAR. In ATIC-depleted myotubes, MTX robustly stimulated AMPK and ACC phosphorylation as well as palmitate oxidation (Fig. 6H–J). ATIC depletion in conjunction with MTX exposure achieved maximal AMPK activation, because incubation with 0.2 mmol/L AICAR failed to exert an additional effect. However, a combined treatment with 0.2 mmol/L AICAR and MTX triggered a similar response in control myotubes transfected with scrambled siRNA.

DISCUSSION

Epidemiological (1,2) and experimental (3,4) evidence suggests that MTX may protect against type 2 diabetes, but the underlying mechanisms have been unclear. We provide evidence that MTX potentiates AICAR-stimulated AMPK activation and glucose uptake as well as lipid oxidation in skeletal muscle. We also demonstrate that suppression of ATIC, a well-characterized MTX target (11), directly promotes AMPK activation in cultured myotubes. Finally, MTX alone is sufficient to stimulate AMPK activation in HEK-293 cells. These results highlight a role for AMPK as a link between MTX and skeletal muscle energy metabolism.

Pharmacological activation of AMPK in skeletal muscle represents a promising strategy to bypass insulin-resistant pathways to stimulate glucose uptake and improve insulin sensitivity in type 2 diabetes (19). However, the search for peripheral AMPK activators has been challenging. AMPK activators, such as A-769662, target AMPK-β1 complexes that are poorly expressed in skeletal muscle (37,38), whereas AICAR has clinical limitations due to its poor bioavailability (18,26). Even with intravenous infusion, the plasma concentration of AICAR (0.16–0.18 mmol/L) remains below the threshold for AMPK activation in skeletal muscle (27,39). Here we report coincubation with 0.2 mmol/L AICAR and MTX activates AMPK...
and stimulates glucose uptake and lipid oxidation in skeletal muscle. These results are consistent with effects of MTX on AICAR in cancer cells (32). Thus, combined therapy with MTX and AICAR might provide a novel pharmacological strategy to overcome current limitations of AICAR monotherapy.

AMPK-γ3 is the muscle-specific isoform of the AMP- and ZMP-sensitive AMPK-γ subunit, which exists in three isoforms (γ1–γ3) (40), and is a major isoform involved in exercise-induced AMPK activation (41). Although AMPK-γ3 is expressed primarily in glycolytic muscles (28,36), a complete failure of MTX to enhance AICAR action in AMPK-γ3−/− EDL muscle was unexpected because the AMPK-γ1 and -γ2 isoforms are also expressed (36). Furthermore, MTX enhanced AICAR-stimulated AMPK phosphorylation in WT and AMPK-γ3−/− soleus muscle, despite low endogenous expression in WT soleus muscle (28,36). Thus, MTX preferentially promotes activation of AMPK-γ3–containing complexes in glycolytic skeletal muscle. The γ3 subunit offers an entry point to tissue-specific regulation of AMPK function and a possible therapeutic target for treatment of insulin resistance in type 2 diabetes (28). Activating mutations in the γ3 isoform are associated with skeletal muscle remodeling analogous to exercise training, including augmented mitochondrial biogenesis and fat oxidation as well as protection against insulin resistance (28,42). Cotherapy with MTX and AICAR may therefore promote a plethora of beneficial metabolic alterations, including mimicking the effects of exercise training (23).

MTX promotes AMPK activation in cultured myotubes and WT cells primarily via inhibition of ATIC, whereas ATP depletion may also have a secondary role. However, MTX did not inhibit ATIC or evoke energy depletion in marine EDL and soleus muscles, although AICAR action was markedly enhanced. This failure to suppress ATIC activity may be explained by the fact that MTX is an

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**Figure 5**—MTX is dependent on AMPK to promote lipid oxidation in isolated skeletal muscle. Phosphorylation (p) of AMPK (pAMPK) (A and B) and its target pACC (C and D) and palmitate oxidation (E and F) were measured in WT or AMPK-γ3−/− EDL and soleus muscle after exposure to 10 μmol/L MTX and/or 0.2 mmol/L AICAR (2 h). EDL and soleus muscle were preincubated with 10 μmol/L MTX or vehicle for 2 h before the experiment. Results are means ± SEM (n = 12). *P < 0.05 vs. AICAR.
efficient ATIC inhibitor only after MTX polyglutamates are formed by the action of folypolyglutamate synthetase, which is a slow process (11). Thus, the exposure time during the isolated skeletal muscle incubation may have been insufficient for MTX to depress ATIC activity. Although our data do not exclude a role for ATIC inhibition of glycinamide ribonucleotide transformylase (GART), an enzyme upstream of ATIC in the de novo pathway of purine synthesis (Fig. 7), may critically determine the level of endogenous ZMP. Concurrent inhibition of ATIC and GART by MTX suppresses the entire de novo synthesis pathway and decreases endogenous ZMP formation (31). This provides a possible explanation why MTX alone did not increase the content of endogenous ZMP in cultured myotubes, although the clearance of ZMP derived from AICAR was decreased. Similarly, MTX increases ZMP content and enhances AMPK activation in AICAR-treated cancer cells, but not when used alone (32). Conversely, ATIC depletion elevated basal AMPK phosphorylation and palmitate oxidation in cultured myotubes, suggesting selective inhibition of ATIC in skeletal muscle may introduce a metabolic block leading to ZMP accumulation and AMPK activation.

MTX has a greater propensity to inhibit ATIC than GART (11,31). Patients receiving low-dose MTX exhibit increased urinary excretion of 5-aminoimidazole-4-carboxamide (13), a ZMP degradation product, which is indicative of ZMP accumulation due to ATIC inhibition. Conversely, high-dose MTX fails to increase urinary excretion of endogenous AICAR (45). Scaling down the MTX dosage may create a therapeutic window for more selective ATIC inhibition with subsequent ZMP accumulation and AMPK activation in skeletal muscle and other metabolic tissues. Consistent with this hypothesis, low-dose MTX therapy increased intracellular ZMP content in mice (6). Furthermore, low-dose MTX monotherapy increases skeletal muscle GLUT4 abundance in diabetic (db/db) mice (3) and suppresses adipose tissue lipolysis in obese mice (4), consistent with AMPK activation in skeletal muscle (24,46) and adipose tissue (14), respectively. Finally, we report MTX alone is sufficient to induce AMPK activation in HEK-293 cells, thus providing proof-of-concept that MTX acts as AMPK activator rather than just as an enhancer of effects of AICAR.

We show that MTX enhances AICAR-stimulated AMPK activation in skeletal muscle as well as in cells of kidney origin (HEK-293 cells). Taken together with studies in different types of cancer cells (31,32), these data suggest that MTX potentiates effects of AICAR not only in skeletal muscle but also in other organs. For instance, ATIC is expressed and active in the heart (21,47), indicating MTX could enhance AICAR action in cardiomyocytes. Also, liver expresses ATIC (47), and MTX-treated hepatic carcinoma cells release adenosine (48), which results from

activate AMPK in RG cells, although they are normally responsive to AMPK activators other than ZMP and AMP (17). However, MTX decreases the concentration of S-adenosylmethionine (43), a biological methyl group donor that promotes AMPK inactivation (44), which may provide one possible ATIC-independent pathway.

Figure 6—Suppression of ATIC promotes AMPK activation and increases lipid oxidation in L6 myotubes by a direct mechanism. A: ATIC expression was determined by immunoblot in L6 myotubes, differentiated primary human skeletal muscle cells (HSMC), HEK-293 cells, and in murine EDL and soleus muscle. B: ATIC protein expression in L6 myotubes after transfection with scrambled siRNA (Scr) or siRNA against ATIC (siATIC). ZMP content (C), the ADP-to-ATP ratio (D), AMPK phosphorylation (pAMPK) (E), ACC phosphorylation (pACC) (F), and palmitate oxidation (G) measured in control or ATIC-depleted L6 myotubes upon exposure to 0.2 mmol/L AICAR (5 h). Results for nucleotide measurements are means for basal (n = 2) and means ± SEM (n = 4) for AICAR. Results for all others are means ± SEM (n = 8). *P < 0.05 vs. basal (Scr). #P < 0.05 vs. AICAR (Scr). ATIC-depleted or control L6 myotubes were preincubated with 5 μmol/L MTX for 16 h. AMPK phosphorylation (h), ACC phosphorylation (l), and palmitate oxidation (j) were determined upon exposure to 0.2 mmol/L AICAR (5 h). Results are means ± SEM (n = 6). **P < 0.05 vs. MTX (Scr).
MTX-induced ATIC inhibition (6). Consistent with the involvement of ATIC, we determined that MTX enhances ZMP accumulation and AMPK activation in AICAR-treated H4IIE cells of liver origin (data not shown). Aside from ATIC expression, differences in MTX uptake might also determine tissue responsiveness to cotherapy with AICAR and MTX. Indeed, cells lacking MTX transporters are resistant to MTX despite ATIC expression (32,49). Collectively, these studies indicate that MTX is likely to enhance AICAR action in all tissues that express ATIC and MTX uptake transporters.

In conclusion, MTX markedly reduces the threshold for AICAR-induced AMPK activation and increases glucose uptake and lipid oxidation in skeletal muscle. The underlying mechanisms include reduced ZMP clearance due to ATIC inhibition and ATP depletion as well as ATIC-independent effects. Collectively, our results highlight a role for AMPK as a direct link between MTX and energy metabolism. Cotherapy with AICAR and MTX or another MTX-based agent represents a novel strategy to treat metabolic disorders and overcome current limitations of AICAR monotherapy.

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References

Figure 7—MTX promotes AMPK activation in skeletal muscle through different mechanisms. Clearance of AICAR through the de novo purine synthesis pathway limits effectiveness of AICAR as an AMPK activator in skeletal muscle. Cotherapy with AICAR and MTX may overcome these limitations by reducing the threshold for AMPK activation in skeletal muscle. Underlying mechanisms include diminished ZMP clearance and energy deprivation. However, other ATIC-independent mechanisms are likely involved. IMP, inosine monophosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate.
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