

Inhibition of Inducible Nitric-oxide Synthase by Activators of AMP-activated Protein Kinase

A NEW MECHANISM OF ACTION OF INSULIN-SENSITIZING DRUGS*[§]

Received for publication, February 8, 2004

Published, JBC Papers in Press, February 25, 2004, DOI 10.1074/jbc.M401390200

Geneviève Pilon[‡], Patrice Dallaire[§], and André Marette[¶]

From the Department of Anatomy and Physiology and Lipid Research Unit, Laval University Hospital Research Center, Sainte-Foy, Québec G1V 4G2, Canada

AMP-activated protein kinase (AMPK), an energy-sensing enzyme that is activated in response to cellular stress, is a critical signaling molecule for the regulation of multiple metabolic processes. AMPK has recently emerged as an attractive novel target for the treatment of obesity and type 2 diabetes because its activation increases fatty acid oxidation and improves glucose homeostasis. Here we show that pharmacological activation of AMPK by insulin-sensitizing drugs markedly inhibits inducible nitric-oxide synthase (iNOS), a proinflammatory mediator in endotoxic shock and in chronic inflammatory states including obesity-linked diabetes. AMPK-mediated iNOS inhibition was observed in several cell types (myocytes, adipocytes, macrophages) and primarily resulted from post-transcriptional regulation of the iNOS protein. AMPK activation *in vivo* also blunted iNOS induction in muscle and adipose tissues of endotoxin-challenged rats. Reduction of AMPK expression by small interfering RNA reversed the inhibitory effects of AMPK activators on iNOS expression and nitric oxide production in myocytes. These results indicate that AMPK is a novel anti-inflammatory signaling pathway and thus represents a promising therapeutic target for immune-inflammatory disorders.

acts to switch off ATP-consuming pathways and switch on alternative pathways for ATP regeneration. AMPK is a heterotrimer consisting of a catalytic α -subunit and two regulatory subunits, β and γ . In response to cellular energy depletion, as reflected by an increase in the AMP/ATP ratio, AMPK is phosphorylated and activated by a still uncharacterized upstream AMPK kinase (2). It also can be activated allosterically by increases in the AMP/ATP and creatine/creatine-P ratios. The metabolic function of AMPK perhaps has been documented best in exercising skeletal muscle, where its activation seems to contribute to increased glucose transport and fatty acid oxidation (3, 4). AMPK can be activated chemically with 5-aminoimidazole-4-carboxamide riboside (AICAR), which is taken up by cells and phosphorylated by adenosine kinase to form 5-aminoimidazole-4-carboxamide ribonucleoside, a nucleotide that mimics the effect of AMP (5). More recent studies also have identified AMPK as the mediator of the metabolic effects of the adipose-derived peptidic hormones leptin and adiponectin in skeletal muscle and liver (6–8).

Chronic treatment of animal models of type 2 diabetes with the AMPK activator AICAR improves glucose homeostasis and insulin sensitivity (9–11). These beneficial effects are thought to be explained mainly by the well known actions of AMPK on glucose metabolism and lipid oxidation in muscle and liver. Accordingly, the anti-diabetic drugs metformin and rosiglitazone also activate AMPK, and this is believed to contribute to their insulin-sensitizing actions in diabetic subjects (12–15). However, the potential effects of AMPK activation on proinflammatory mediators of insulin resistance have not been investigated yet. Indeed, an inflammatory component also is present in obesity-linked diabetes (16, 17) as reflected by increased systemic and tissue concentrations of the proinflammatory cytokines TNF- α and interleukin-6 in obese human subjects (18, 19) and several animal models of obesity (20–22). Furthermore, we recently have reported that inducible nitric-oxide synthase (iNOS), a cytokine-inducible proinflammatory mediator in several pathological conditions, is overexpressed in muscle and fat of genetic and dietary models of obesity and type 2 diabetes (22). Targeted disruption of the iNOS gene was found to protect high fat-fed obese mice from developing insulin resistance and to significantly improve glucose tolerance (22). A role for iNOS in mediating insulin resistance also is supported by the finding that pharmacological inhibition of iNOS reverses impaired insulin action in cultured myocytes exposed to cytokines and the endotoxin LPS (23). Because an inflammatory component that includes iNOS is involved in the pathogenesis of muscle insulin resistance in obesity-linked type 2 diabetes, we asked whether AMPK activation might improve

AMP-activated protein kinase (AMPK)¹ is emerging as an important energy-sensing/signaling system in mammalian tissues. It is a member of a metabolite-sensing protein kinase family that acts as a fuel gauge by monitoring cellular energy levels (1). When AMPK “senses” decreased energy storage, it

* This work was supported by grants from the Canadian Institutes for Health Research (to A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at www.jbc.org) contains a supplemental figure.

[‡] Supported by a Canadian Institutes for Health Research doctoral award.

[§] Supported by a Canadian Diabetes Association doctoral award.

[¶] A Canadian Institutes for Health Research scientist. To whom correspondence should be addressed: Dept. of Physiology & Lipid Research Unit, Laval University Hospital Research Center, 2705 Laurier Blvd., Ste-Foy, Québec G1V 4G2, Canada. Tel.: 418-656-4141 (ext. 7908); Fax: 418-654-2176; E-mail: andre.marette@crchul.ulaval.ca.

¹ The abbreviations used are: AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide-riboside; TNF- α , tumor necrosis factor- α ; NO, nitric oxide; iNOS, inducible nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; LPS, lipopolysaccharide; ACC, acetyl-CoA carboxylase; BW, body weight; IRS-1, insulin receptor substrate-1; PI, phosphatidylinositol; IFN, interferon; RT, reverse transcription; siRNA, small interfering RNA; PPAR, peroxisome proliferator-activated receptor; PG, prostaglandin; eNOS, endothelial nitric oxide synthase; 15d PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂.

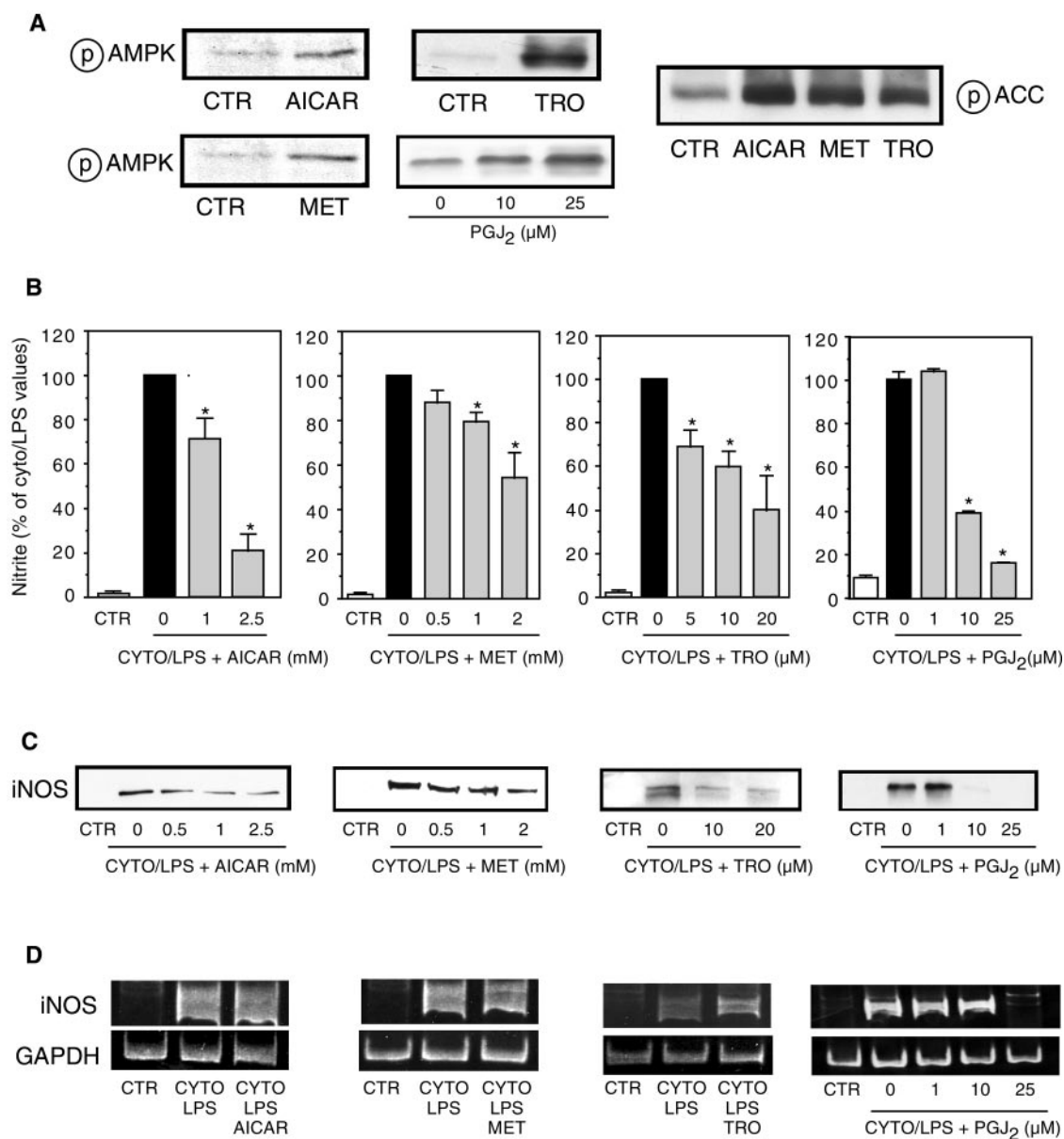


FIG. 1. Effects of AMPK activators on NO production and iNOS expression in L6 myocytes. Cells were treated for 24 h with or without cytokines (TNF- α , 10 ng/ml, and IFN- γ , 200 units/ml) and LPS (10 μ g/ml) (CYTO/LPS) in the presence of AICAR (2.5 mM), metformin (MET) (2 mM), troglitazone (TRO) (10 μ M), or 15-deoxy-PGJ₂ (10 and 25 μ M). **A**, representative immunoblots of ACC (Ser-79) and AMPK (Thr-172) phosphorylation following treatment with AMPK activators. **B**, NO production was measured by nitrite accumulation in the incubation medium. Results are expressed as mean \pm S.E. for 3–6 individual experiments. **C**, representative immunoblots of iNOS protein expression. iNOS migrated as an \sim 130-kDa band. **D**, representative RT-PCR for iNOS and glyceraldehyde-3-phosphate dehydrogenase (internal control) mRNAs. Immunoblots and RT-PCR are representative of 3–6 individual experiments. *, $p < 0.05$ as compared with cytokine/LPS values.

insulin action in muscle cells through inhibition of iNOS, and if so, whether AMPK is a key iNOS inhibitory pathway in other cell types where increased iNOS expression is pathogenic.

EXPERIMENTAL PROCEDURES

Materials—AICAR was purchased from Toronto Research Chemicals (Toronto, Canada). Troglitazone and 15-deoxy-PGJ₂ were from Cayman Chemical Co. (Ann Arbor, MI). Interferon- γ and TNF- α were from Research Diagnostics (Flanders, NJ) and RD Systems (Minneapolis, MN), respectively. 1400W was from Biomol Research Laboratories (Plymouth, PA), and Oligofectamine was from Invitrogen. ADP-Sepharose 4B beads were from Amersham Biosciences, and the Renaissance enhanced chemiluminescence kit was from PerkinElmer Life Sciences (Boston, MA). All other chemicals were from Sigma. Monoclonal antibodies against iNOS and eNOS and a polyclonal antibody against nNOS were obtained from Transduction Laboratories (Mississauga, Canada). Polyclonal antibodies against AMPK (which recognizes both α 1- and α 2-AMPK), phospho-AMPK, and phospho-ACC (acetyl-CoA carboxylase) were purchased from New England Biolabs (Beverly, MA). IRS-1

antibody (C-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture—L6 myoblasts and 3T3-L1 fibroblasts were grown in α -minimum Eagle's medium (10% fetal bovine serum) or α -Dulbecco's modified Eagle's medium (20% calf serum) containing 1% antibiotic/antimycotic solution and differentiated into myotubes and adipocytes as described previously (23, 24). Bone marrow-derived macrophages were a kind gift of Dr. M. Olivier (CHUL Research Center, Quebec, Canada). Generation and immortalization of the macrophages from mouse femurs was performed as described previously (25). Induction of iNOS by cytokines and/or LPS in L6 myotubes, 3T3-L1 adipocytes, and macrophages and the effects of AMPK-activating agents were carried out as described in the figure legends. The accumulation of nitrite in the incubation medium was used as an index of NO production. Nitrite was determined spectrophotometrically as described previously (23).

RNA Interference—Small interfering RNAs (siRNAs) were synthesized by Dharmacon Research (Lafayette, CO) and annealed according to the manufacturer's instructions. The sequence of the 19-nucleotide (plus 2 amino acids overhanging at 5') siRNAs were as follows: α 1-

AMPK, GCAUAUGCUGCAGGUAGAU; α 2-AMPK, CGUCAUUGAUGAUGAGGCU; unrelated siRNA, AUUGUAUGCGAUCGAGAC. L6 myotubes in 12-well plates were transfected 24 h prior to cytokine and LPS treatment with 0.27 μ M α 1- and α 2-AMPK siRNAs by using the Oligofectamine reagent. Mock controls were transfected with unrelated siRNA.

Animals—This study was approved by the Animal Care and Handling Committee of Laval University. Male Sprague-Dawley rats (200–250 g) purchased from Charles River (Montréal, Canada) were used in these studies. Rats were pretreated with a single intraperitoneal injection of AICAR (1 g/kg, BW), metformin (100 mg/kg, BW) or saline (vehicle) 30 min prior to LPS (20 mg/kg, BW, intraperitoneally) or saline treatment. Animals were sacrificed 4 h later, and hind limb muscles and white adipose tissues were removed and stored at -80°C until further processing.

Measurement of Plasma and Tissue Nitrite and Nitrate—Nitrite and nitrate levels in plasma, skeletal muscles, and adipose tissue were measured by fluorometry (26). Briefly, blood was collected in tubes containing EDTA and centrifuged for 10 min at $3200 \times g$ to obtain plasma. Tissues were grounded in liquid nitrogen using a mortar. The tissue powder was resuspended in 5 volumes of Tris-EDTA buffer (20 mM Tris, pH 7.5, 10 mM EDTA) containing a protease inhibitor mixture. Tissue lysates were centrifuged at $2800 \times g$ for 20 min, and protein concentration of the supernatant was measured by the BCA protein assay. Plasma and tissue lysates then were centrifuged at $5000 \times g$ (4°C) overnight in an Ultrafree M -Microcentrifuge 10,000 NMWL filter unit. Nitrate was reduced to nitrite using nitrate reductase and the NADPH regenerating system as described previously (26). The fluorescence was measured at λ_{ex} of 360 nm and λ_{em} of 450 nm.

Determination of Nitric-oxide Synthase Protein Expression and Western Blot Analysis—Muscle and adipose tissue lysates (1 mg of protein) were used to purify nitric-oxide synthase enzymes using 2',5'-ADP-Sepharose resin as described previously (22). Samples were subjected to SDS-PAGE, and immunoblotting was performed as described previously (27). Immunoreactive bands were detected by the enhanced chemiluminescence method.

RNA Analysis—Total cellular RNA was isolated using guanidium thiocyanate-phenol-chloroform extraction, and iNOS mRNA was measured by semiquantitative reverse transcriptase-polymerase chain reaction as described previously (23, 24).

PI 3-Kinase Activity—Cell lysates (500 μ g) were immunoprecipitated with 2 μ g of anti-IRS-1 coupled to protein A-Sepharose overnight at 4°C . PI 3-kinase activity was determined as described previously (22).

Data Analysis—All data are presented as means \pm S.E. The effects of the treatments were compared by an analysis of variance followed by Fisher's post hoc test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

AMPK Activators Inhibit iNOS and Reverse Insulin Resistance in Skeletal Muscle Cells—We first tested the effect of the AMPK activator AICAR on iNOS induction in L6 myocytes chronically exposed to cytokines and LPS, which is a model of chronic inflammation associated with insulin resistance (23, 27). As shown in Fig. 1A, treatment of muscle cells with stimulated AMPK activity as reflected by increased phosphorylation of AMPK on Thr-172 (a site known to activate the enzyme (28)) as well as by its ability to enhance phosphorylation of ACC, a downstream target of AMPK and a good correlate of its activation (29). The anti-diabetic drug metformin and the PPAR γ agonists troglitazone and 15dPGJ2 also activated AMPK in these cells (Fig. 1A). Chronic exposure of L6 myocytes to cytokines (TNF- α and IFN- γ) and LPS markedly induced NO production as detected by the accumulation of the stable bio-product nitrite in the culture medium (Fig. 1B). This effect was explained by a robust induction of iNOS mRNA and protein levels (Fig. 1, C and D). Neither eNOS nor nNOS is expressed in control or cytokine/LPS-treated L6 myocytes, and NO production was completely inhibited by co-incubation with the specific iNOS inhibitor 1400W (30) (data not shown).

Addition of AICAR caused a dose-dependent reduction of cytokine/LPS-induced NO production, reaching a maximal 80% inhibition at 2.5 mM (Fig. 1B). Treatment with either metformin or PPAR γ agonists also decreased (by 50–90%) the

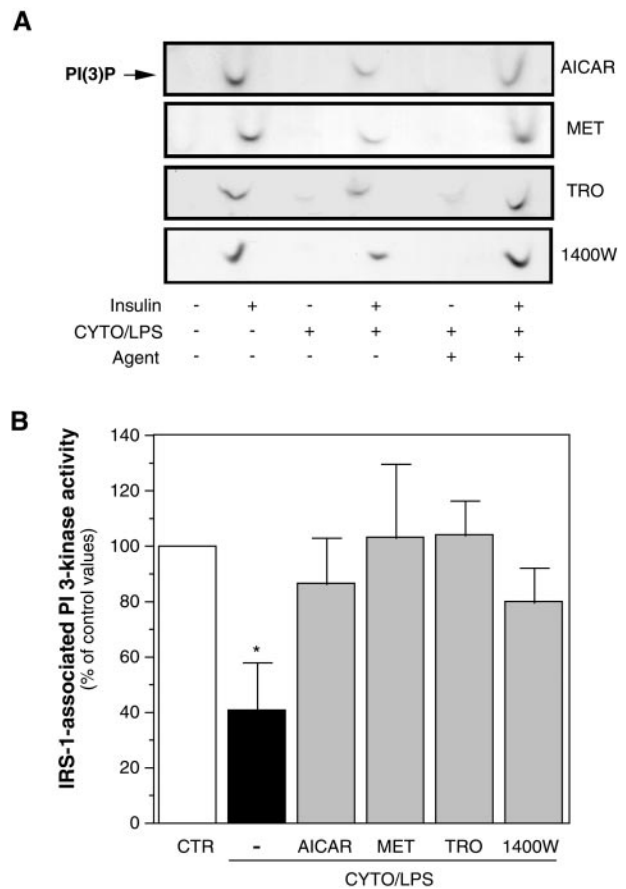


FIG. 2. Effects of cytokine/LPS and AMPK activators on IRS-1-associated PI 3-kinase activity in L6 myocytes. PI 3-kinase was measured in anti-IRS-1 immunoprecipitates from lysates of L6 myocytes treated for 24 h with or without cytokines (TNF- α , 10 ng/ml, and IFN- γ , 200 units/ml) and LPS (10 μ g/ml) (CYTO/LPS) in the presence of AICAR (2.5 mM), metformin (MET) (2 mM), troglitazone (TRO) (10 μ M), or the specific iNOS inhibitor 1400W (0.1 mM). Quantification of ^{32}P incorporated into PI 3-phosphate (PI(3)P) was expressed relative to insulin-stimulated values in control (CTR) cells. Representative autoradiographs are shown in A. Results in B are expressed as mean \pm S.E. from 3–6 individual experiments. *, $p < 0.05$ as compared with control values.

effect of cytokine/LPS on NO production. All AMPK activators inhibited NO production through the reduction of cellular iNOS protein levels (Fig. 1C). AMPK activators failed to change the cellular expression of desmin, the main intermediate filament protein in myocytes, here used as an internal control (data not shown). Reduction of iNOS protein expression was not linked to suppression of iNOS transcription because iNOS mRNA levels were not affected by AICAR or the anti-diabetic drugs metformin and troglitazone (Fig. 1D). However, high concentrations of 15dPGJ2 did reduce iNOS mRNA levels (see 25 μ M 15dPGJ2 in Fig. 1D). In additional studies, we also tested whether AICAR could inhibit iNOS-mediated NO production either through reduction in substrate (L-arginine) uptake or by direct inhibition of iNOS enzymatic activity. L-Arginine uptake by muscle cells was not found to be affected by AICAR (2.5 mM) (1297 ± 209 versus 1369 ± 90 pmol/min/mg for control and AICAR-treated cells, respectively). Furthermore, AICAR did not affect iNOS subunit dimerization and failed to reduce iNOS catalytic activity in cytokine/LPS-treated myocytes, as assessed by the formation of L-[^3H]citrulline from L-[^3H]arginine (see Figs. A and B in supplemental material). It has been reported that AICAR increases adenosine release by ischemic heart tissue (31), and this could contribute to iNOS inhibition. However, adenosine *per se* failed to affect iNOS

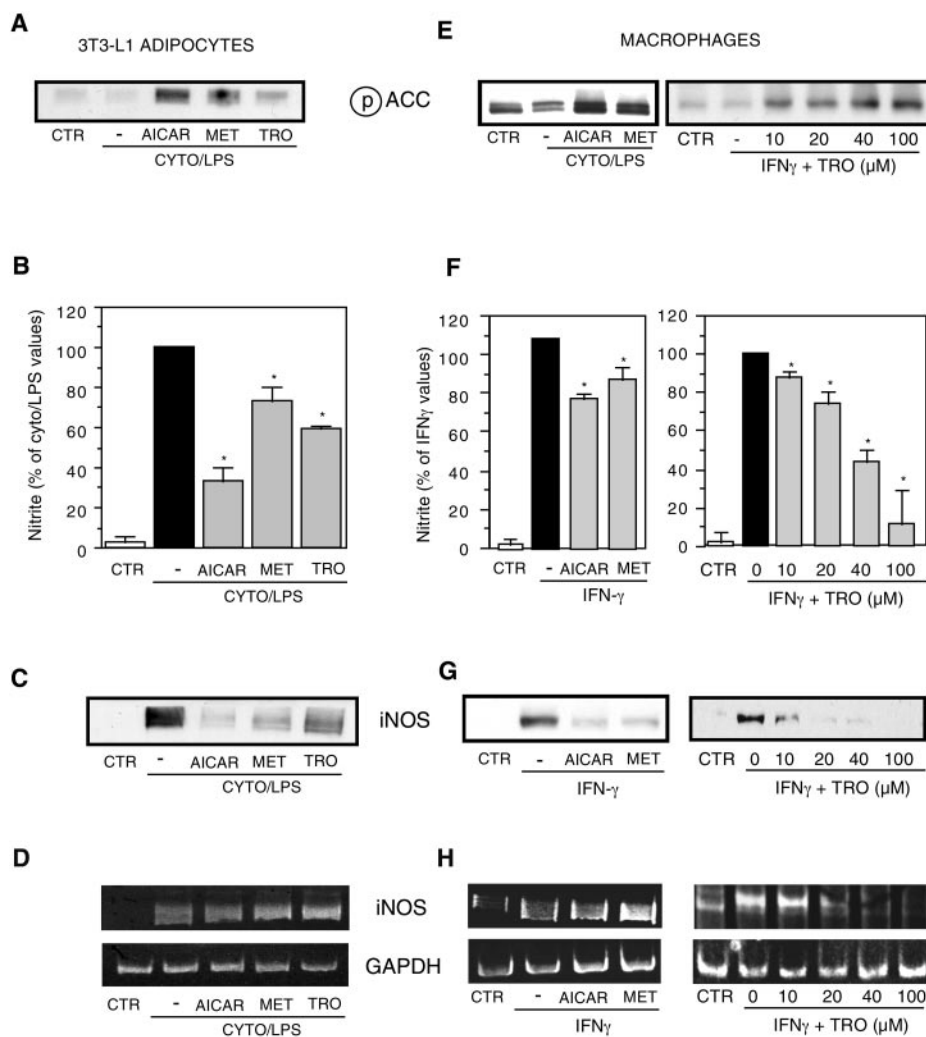


FIG. 3. Effects of AMPK activators on NO production and iNOS expression in 3T3-L1 adipocytes and macrophages. 3T3-L1 adipocytes (A–D) were treated for 24 h with or without cytokines (TNF- α , 10 ng/ml, and IFN- γ , 200 units/ml) and LPS (10 μ g/ml) (CYTO/LPS) in the presence of AICAR (2.5 mM), metformin (MET) (2 mM), or troglitazone (TRO) (10 μ M). Macrophages (E–H) were treated for 10 h with IFN- γ (200 units/ml) and the same AMPK activators. A and E, representative immunoblots of ACC Ser-79 phosphorylation following treatment with AMPK activators. B and F, measurement of nitrite production in the incubation medium. Data are from 2–4 independent experiments and are expressed relative to nitrite production in cytokine/LPS cells in the absence of AMPK activators. C and G, representative immunoblots of iNOS protein expression. D and H, representative RT-PCR of iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. Immunoblots and RT-PCR are representative of 2–4 independent experiments *, $p < 0.05$ as compared with cytokine/LPS values.

activity in L6 myocytes, and the inhibitory effect of AICAR on NO production was not decreased by blocking adenosine receptors with 8-(*p*-sulfophenyl) theophylline (see Figs. C and D in supplemental material), ruling out any role for adenosine release in AMPK-dependent iNOS inhibition.

To test whether AMPK-induced iNOS inhibition reverses the insulin resistance-promoting effects of cytokine/LPS, next we measured the effects of AMPK activators on insulin action in L6 myocytes. We chose to assess insulin-induced activation of PI 3-kinase, an essential mediator of the metabolic actions of insulin, rather than glucose uptake because the latter is increased by AMPK through insulin-independent pathways. As shown in Fig. 2, A and B, cytokines and LPS treatment caused insulin resistance as revealed by a 60% reduction in the ability of insulin to stimulate IRS-1-associated PI 3-kinase activity as compared with control cells. However, addition of either AICAR, metformin, or troglitazone to cytokine/LPS-treated cells fully restored insulin action on PI 3-kinase. AMPK activating agents failed to increase PI 3-kinase in the absence of insulin (Fig. 2A). The restoration of insulin action by AMPK-activating agents was similar to that obtained with the specific iNOS inhibitor 1400W (Fig. 2B).

AMPK Activators Inhibit iNOS in Adipocytes and Macrophages—Next we tested whether AMPK activation inhibits iNOS in other cell types. We have reported that adipose tissue is a major site of iNOS expression in endotoxemia and that chronic treatment of adipocytes with cytokines and endotoxins reproduces this inflammatory response *in vitro* (24). As shown in Fig. 3A, addition of AICAR or anti-diabetic drugs to cultured

3T3-L1 adipocytes activated AMPK as reflected by enhanced ACC phosphorylation. Activation of AMPK reduced cytokine/LPS-mediated NO production (Fig. 3B) and cellular iNOS protein content (Fig. 3C) but not iNOS mRNA levels (Fig. 3D). Monocytes and macrophages are major sites of iNOS expression, particularly in infectious or inflammatory diseases. Sustained production of NO endows macrophages with cytostatic or cytotoxic activity against several pathogens and tumor cells, but it also can exert deleterious proinflammatory effects on host cells (32). Activation of AMPK by AICAR and metformin inhibited iNOS induction upon exposure of macrophages to interferon- γ (Fig. 3, E and F). The PPAR γ ligand troglitazone also increased AMPK and caused a dose-dependent inhibition of NO production in these cells (Fig. 3, E and F). The inhibitory effects of AMPK activators also were linked to reduction in iNOS protein induction through a post-transcriptional mechanism (Fig. 3, G and H). However, at higher concentrations (> 10 μ M) of troglitazone, a reduction in iNOS mRNA levels was also observed.

Activation of AMPK by *in Vivo* Injection of AICAR and Metformin Inhibits iNOS in Muscle and Adipose Tissues of LPS-treated Rats—To test whether AMPK also blunts iNOS induction *in vivo*, we assessed the ability of AICAR and metformin to inhibit iNOS in skeletal muscle and adipose tissue of rats injected with the bacterial endotoxin LPS, a model of septic shock associated with insulin resistance (27). Pretreatment of LPS-challenged rats with AICAR or metformin increased ACC phosphorylation (Fig. 4A) and significantly reduced NO production in the plasma as well as in muscle (gastrocnemius and tibialis) and adipose tissues (Fig. 4B). AICAR and metformin

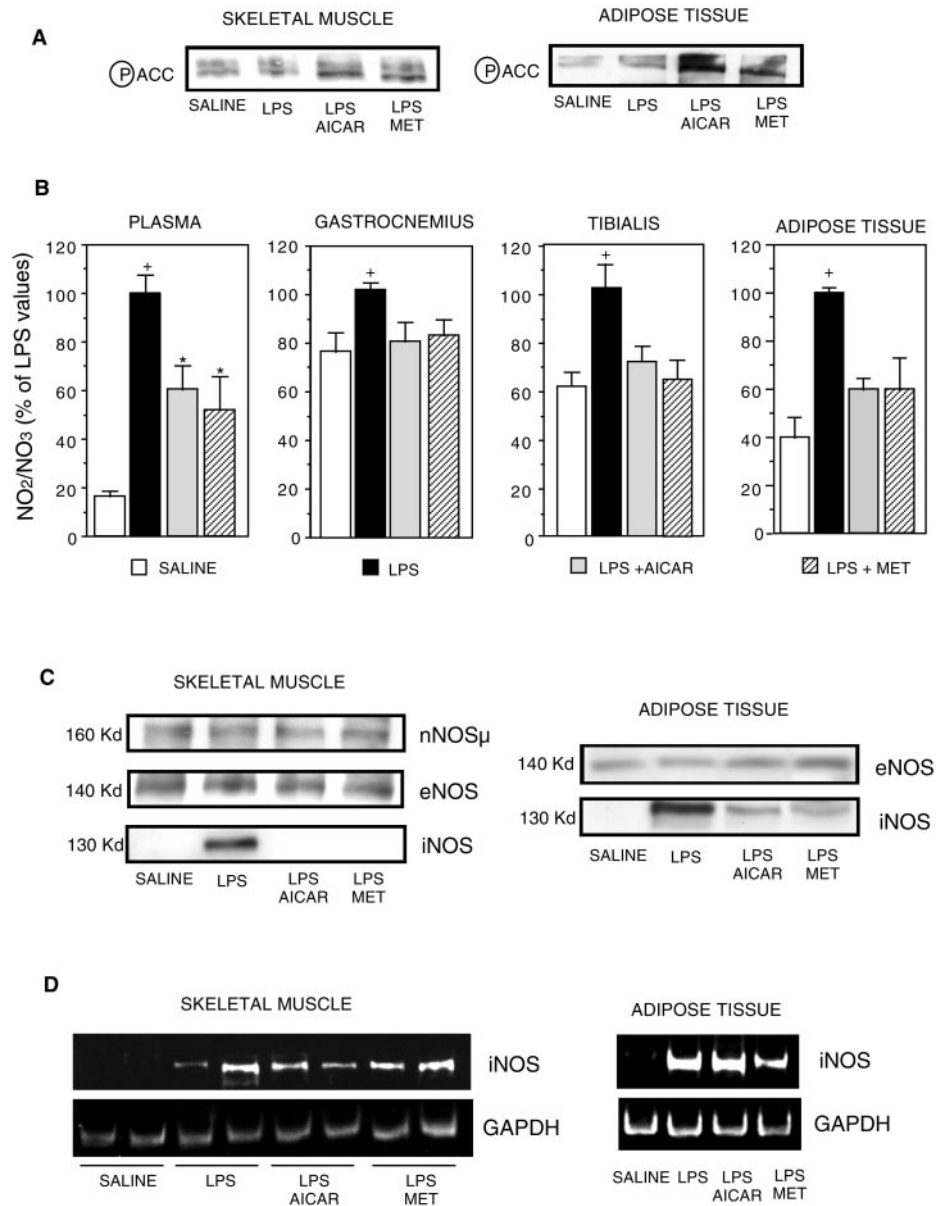


FIG. 4. Effect of AICAR and metformin on iNOS in skeletal muscle and adipose tissue *in vivo*. Rats were pretreated with a single intraperitoneal injection of AICAR (1 g/kg, BW), metformin (MET) (100 mg/kg, BW), or saline (vehicle) 30 min prior to injection of LPS (20 mg/kg, BW, intraperitoneally). Tissues and plasma were sampled 4 h after LPS injection. **A**, immunoblots of ACC (Ser-79) phosphorylation in muscle and fat tissues. **B**, measurement of nitrite and nitrate in plasma, gastrocnemius, tibialis anterior, and adipose tissue. Results are expressed as mean \pm S.E. from 4 animals. **C**, representative immunoblots of eNOS, nNOS μ , and iNOS protein expression in muscle and fat tissues. **D**, representative RT-PCR of iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs in muscle and adipose tissue. Immunoblots and RT-PCR data are representative of 4 independent experiments with different rats. +, $p < 0.05$ as compared with saline values.

treatments prevented induction of iNOS protein expression in muscle and fat tissues of septic rats (Fig. 4C). In marked contrast, neither eNOS nor nNOS μ enzymes were affected by LPS or AMPK activators in muscle and fat (nNOS μ was not detectable in fat), highlighting the isoform-specific effect of AMPK on iNOS protein. As observed for cell lines, the inhibitory effects of AMPK activators on iNOS-mediated NO production was explained by post-transcriptional mechanisms because LPS-induced iNOS mRNA expression was not affected by AICAR or metformin treatments (Fig. 4D). However, one notable exception is metformin, which caused a small but reproducible reduction in iNOS mRNA only in adipose tissue.

RNA Interference-mediated Depletion of α -AMPK Reverses the Inhibitory Effects of AMPK Activators on iNOS Protein and NO Production—To further establish whether AICAR and anti-diabetic drugs inhibit iNOS through AMPK activation, we next used RNA interference technology to “knock down” endogenous AMPK α -subunits. Immunoblotting experiments confirmed that transfection of siRNA specific for the $\alpha 1$ and $\alpha 2$ catalytic subunits reduced α -AMPK expression to $53 \pm 5\%$ of that of cells mock transfected with unrelated siRNA (Fig. 5A). Moreover, combined siRNA treatment resulted in a comparable inhibition

of AMPK and ACC phosphorylation by AMPK activators (Fig. 5A). In AMPK-depleted myocytes, the ability of AICAR, metformin, and troglitazone to inhibit cytokine/LPS-induced NO production and iNOS protein expression was reduced significantly as compared with cells treated with unrelated siRNA (Fig. 5B). These results confirm that these agents blunt iNOS expression and NO production at least in part through activation of AMPK.

DISCUSSION

Elucidation of the multiple roles of NO in health and disease states has uncovered many areas where manipulation of NO production would be of therapeutic benefit. Thus, whereas focal iNOS induction and NO production is an important anti-infectious and anti-tumor mechanism of innate immunity (33), high amounts of iNOS-derived NO also can lead to self-tissue destruction in autoimmune diseases, allograft rejection, sepsis, rheumatoid arthritis, chronic heart failure, and other disorders accompanied by excessive activation of the immune system (see Refs. 34–37). iNOS induction also is implicated in chronic metabolic disorders such as atherosclerosis (38, 39) and obesity-linked diabetes (22, 40) in which an inflammatory condition

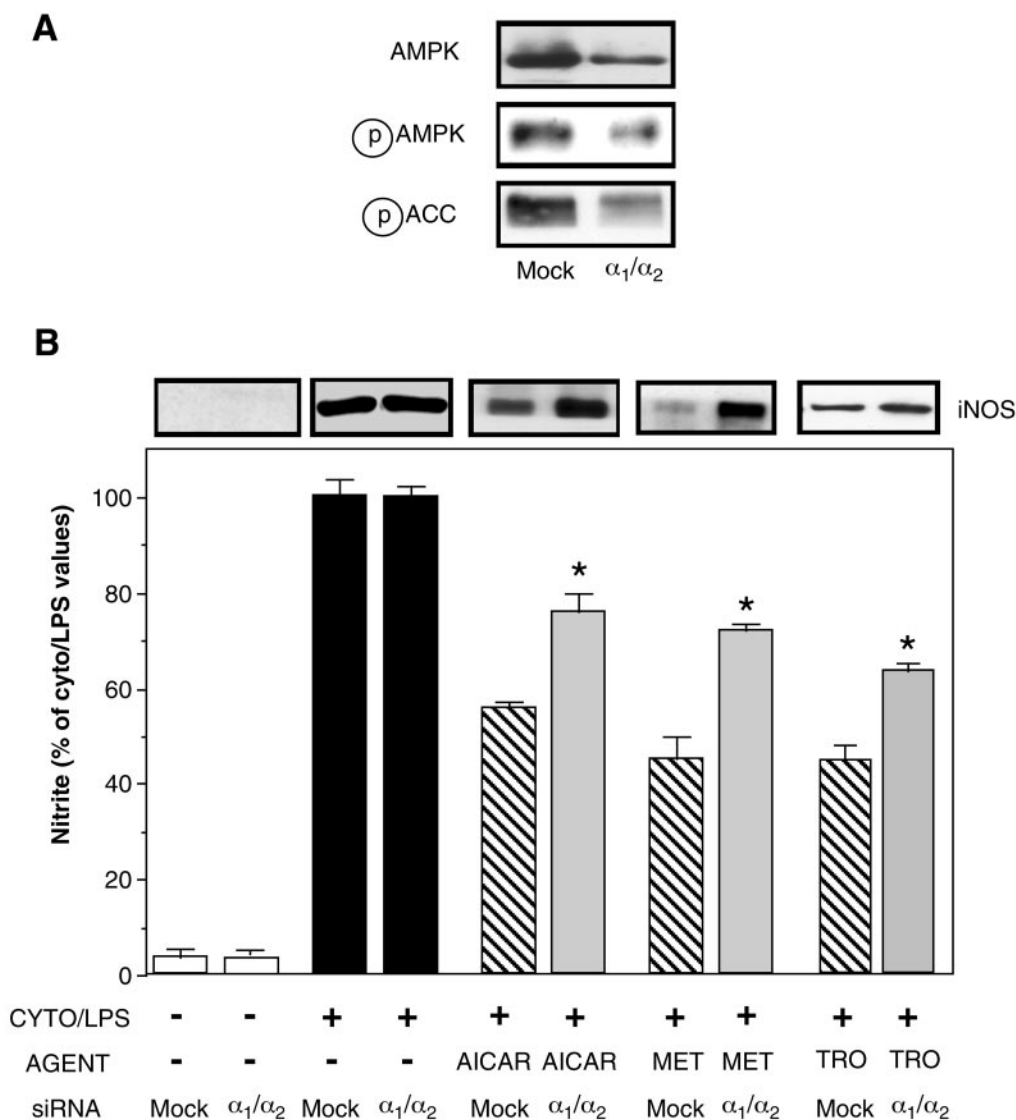


FIG. 5. siRNA-mediated reduction in AMPK expression reverses the inhibitory effects of AMPK activators on iNOS expression and activity. L6 myocytes were transfected with 0.27 μM α_1 - and α_2 -AMPK siRNAs by using Oligofectamine reagent. Mock controls were transfected with unrelated siRNA. Twenty-four h after transfection, cells were treated with cytokines, LPS, and AMPK activators as described in Fig. 1. *A*, representative immunoblots of AMPK protein expression, AMPK (Thr-172) phosphorylation, and ACC (Ser-79) phosphorylation in siRNA (α_1/α_2) and mock transfected cells. *B*, cellular iNOS protein expression and nitrite accumulation in the medium of siRNA (α_1/α_2) and mock transfected cells. Nitrite data are mean \pm S.E. of 3–7 individual experiments. Immunoblots in *A* and *B* are representative of at least 3 experiments. *, $p < 0.05$ as compared with mock controls. *MET*, metformin; *TRO*, troglitazone.

also is believed to play a pathogenic role. Such wide implication has produced an intense interest in understanding the modulation of iNOS expression and activity, with the goal of finding novel mechanisms of control of this high output NO pathway. For these reasons, the design of selective iNOS inhibitors and the identification of iNOS inhibitory pathways have received much attention in recent years. In the present study, we show for the first time that pharmacological activation of AMPK by AICAR and two classes of anti-diabetic drugs inhibit iNOS induction in cells and tissues exposed to inflammatory mediators. Gene silencing experiments confirmed that AMPK-activating agents blunt iNOS-mediated NO production at least in part via activation of AMPK. Indeed, depletion of α -AMPK in L6 myocytes by using the combination of α_1 - and α_2 -AMPK siRNA species led to a significant loss in the ability of AICAR, metformin, and troglitazone to reduce cytokine/LPS-induced iNOS protein expression and NO production.

Metformin and the PPAR γ agonist rosiglitazone activate AMPK, and this is believed to contribute to their insulin-sensitizing actions in diabetic subjects (12–15, 41). These anti-

diabetic effects mainly have been attributed to the lipid- and glucose-related metabolic effects of AMPK activation. However, our data strongly suggest that inhibition of iNOS induction and reduced NO formation represent a novel mechanism by which AMPK improves insulin action in muscle. Indeed, we show here that AMPK activation by several drugs reduces iNOS induction in L6 myocytes chronically exposed to cytokines and LPS, an *in vitro* model of insulin resistance for glucose transport (23). Accordingly, we also found that iNOS-mediated impairment in insulin-induced PI 3-kinase activation can be reversed by AMPK activators. Furthermore, the inhibitory effect of AMPK activation on iNOS was confirmed *in vivo* in a model of septic shock associated with insulin resistance (27). Further studies will be needed to test whether long term AMPK activation also can prevent or reduce iNOS induction in lower grade but chronic inflammatory disorders associated with insulin resistance such as atherosclerosis and obesity-linked diabetes.

AMPK activation also suppressed iNOS protein expression and NO production in cytokine/LPS-treated adipocytes and in adipose tissue of LPS-challenged rats. Whereas the precise role

of iNOS-derived NO in adipose cells still is not fully understood, it is thought to be involved in the modulation of lipid metabolism in endotoxemia (24) and may be a paracrine mediator of skeletal muscle insulin resistance in obesity-linked diabetes (22). Activation of AMPK in macrophages, an important site of iNOS expression, also caused inhibition of interferon- γ -induced NO production. The latter finding is of important clinical relevance because macrophage/monocyte iNOS induction has been implicated in the pathogenesis or promotion of several inflammatory/immune disorders such as septic and hemorrhagic shock (42, 43), multiple sclerosis (44), myocardial infarction (45, 46), inflammatory bowel disease (47, 48), and pancreatic β -cell dysfunction (49).

It will be important to dissect out the molecular mechanisms by which AMPK activators cause iNOS inhibition. It is well established that inflammatory cytokines and LPS trigger iNOS transcription through a complex network of intracellular pathways including NF- κ B, Janus kinase/signal transducers and activators of transcription, and mitogen-activated protein kinase (50, 51). Moreover, PPAR γ ligands rosiglitazone and 15dPGJ₂ were previously shown to reduce iNOS expression in several cell types (49, 52) through transcriptional repression (52). Therefore, we were expecting that PPAR γ -dependent activation of AMPK would inhibit iNOS by reducing the transcription of iNOS. Surprisingly, we found that all AMPK activating agents, including the PPAR γ agonists troglitazone and 15dPGJ₂, decreased iNOS protein but barely affected mRNA expression. Only when higher concentrations of troglitazone and 15dPGJ₂ were used did we observe a reduction in iNOS transcript levels. It should be noted that recent studies have shown that inhibition of iNOS transcription by rosiglitazone may result from activation of both PPAR γ and PPAR δ (53). Thus, high concentrations of rosiglitazone inhibit iNOS in PPAR γ ^{-/-} macrophages at least in part by activating PPAR δ . Troglitazone is 100-fold less potent than rosiglitazone for PPAR γ , but its affinity for PPAR δ has not been established. Therefore, it cannot be excluded that its inhibitory action on iNOS may be mediated in part through PPAR δ . Studies of cells lacking both PPAR γ and PPAR δ will be needed to answer this important issue.

The inhibitory effects of AMPK activators on NO production were highly correlated with the reduction in iNOS protein content, suggesting that AMPK modulates iNOS protein turnover. In this regard, recent reports suggest that AMPK switches off protein synthesis either through suppression of the mTOR-p70S6 kinase pathway (54) or by direct activation of eukaryotic elongation factor 2 kinase, resulting in the phosphorylation and inactivation of eukaryotic elongation factor 2 (55). It also is possible that AMPK reduces iNOS protein content by promoting its ubiquitination, a process required for targeting iNOS through the proteasome proteolysis pathway (56). However, ubiquitination also has been reported to be involved in the proteolytic degradation of nNOS (57) whereas the inhibitory effects of AMPK on NO production appear to be specific for the iNOS isoform. Indeed, neither nNOS nor eNOS proteins were affected by AICAR or metformin treatments in muscle and adipose tissue of LPS-challenged rats, and AMPK activators blunted LPS-induced NO production but did not reduce basal NO formation by constitutive nitric-oxide synthase enzymes. This argues for inhibition of protein synthesis rather than ubiquitin-mediated proteasomal degradation as the principal mechanism by which AMPK regulates iNOS protein turnover.

The identification of AMPK as a potent inhibitory pathway for iNOS may provide a molecular basis for the recent observations that exercise training, which increases AMPK expression (9), improved exercise capacity in association with reduced

skeletal muscle iNOS expression in patients with chronic heart failure (37). Our data further suggest that AMPK represents a promising therapeutic target for immune-inflammatory disorders in which iNOS induction is pathogenic but not critical for health (*i.e.* non-infectious diseases). These include metabolic disorders such as atherosclerosis and obesity-linked diabetes. However, even for those inflammatory conditions, caution must be exerted because iNOS also can play more subtle yet important physiological roles with potential adverse consequences attendant on its inhibition in diseased states. For example, iNOS plays a protective role in the development of transplant arteriosclerosis (58) and also is critical for optimal wound healing (59), which calls into question the use of iNOS inhibitory agents to treat diseases such as atherosclerosis and diabetes. Bearing these cautions in mind, it is crucial to elucidate the molecular mechanisms by which AMPK inhibits iNOS because this may help design novel strategies for an effective and tissue-specific inhibition of iNOS in inflammatory settings while limiting adverse physiological consequences.

Acknowledgments—We thank Bruno Marcotte and Sandra Favret for expert technical assistance. We also thank Dr. Daniel Konrad for helpful advice on the use of siRNA and Drs. Yves Deshaies and Claude H. Côté for critical reading of the manuscript.

REFERENCES

- Hardie, D. G., Scott, J. W., Pan, D. A. & Hudson, E. R. (2003) *FEBS Lett.* **546**, 113–120
- Hong, S. P., Leiper, F. C., Woods, A., Carling, D. & Carlson, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 8839–8843
- Winder, W. W. (2001) *J. Appl. Physiol.* **91**, 1017–1028
- Sakamoto, K. & Goodyear, L. J. (2002) *J. Appl. Physiol.* **93**, 369–383
- Corton, J. M., Gillespie, J. G., Hawley, S. A. & Hardie, D. G. (1995) *Eur. J. Biochem.* **229**, 558–565
- Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D. & Kahn, B. B. (2002) *Nature* **415**, 339–343
- Tomas, E., Tsao, T. S., Saha, A. K., Murrey, H. E., Zhang C. C., Itani, S. I., Lodish, H. F. & Ruderman, N. B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 16309–16313
- Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B. & Kadowaki, T. (2002) *Nat. Med.* **8**, 1288–1295
- Winder, W. W. (2000) *Diabetes Technol. Ther.* **2**, 441–448
- Halseth, A. E., Ensor, N. J., White, T. A., Ross, S. A. & Gulve, E. A. (2002) *Biochem. Biophys. Res. Commun.* **294**, 798–805
- Fiedler, M., Zierath, J. R., Selen, G., Wallberg-Henriksson, H., Liang, Y. & Sakariassen, K. S. (2001) *Diabetologia* **44**, 2180–2186
- Musi, N., Hirshman, M. F., Nygren, J., Svanfeldt, M., Bavenholm, P., Rooyackers, O., Zhou, G., Williamson, J. M., Ljunqvist, O., Efendic, S., Moller, D. E., Thorell, A. & Goodyear, L. J. (2002) *Diabetes* **51**, 2074–2081
- Hawley, S. A., Gadalla, A. E., Olsen, G. S. & Hardie, D. G. (2002) *Diabetes* **51**, 2420–2425
- Fryer, L. G., Parbu-Patel, A. & Carling, D. (2002) *J. Biol. Chem.* **277**, 25226–25232
- Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J. & Moller, D. E. (2001) *J. Clin. Invest.* **108**, 1167–1174
- Pickup, J. C. & Crook, M. A. (1998) *Diabetologia* **41**, 1241–1248
- Marette, A. (2002) *Curr. Opin. Clin. Nutr. Metab. Care* **5**, 377–383
- Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L. & Spiegelman, B. M. (1995) *J. Clin. Investig.* **95**, 2409–2415
- Yudkin, J. S., Stehouwer, C. D., Emeis, J. J. & Coppack, S. W. (1999) *Arterioscler. Thromb. Vasc. Biol.* **19**, 972–978
- Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. (1993) *Science* **259**, 87–91
- Hotamisligil, G. S. & Spiegelman, B. M. (1994) *Diabetes* **43**, 1271–1278
- Perreault, M. & Marette, A. (2001) *Nat. Med.* **7**, 1138–1143
- Bedard, S., Marcotte, B. & Marette, A. (1997) *Biochem. J.* **325**, 487–493
- Kapur, S., Marcotte, B. & Marette, A. (1999) *Am. J. Physiol.* **276**, E635–E641
- Forget, G., Siminovitch, K. A., Brochu, S., Rivest, S., Radzioch, D. & Olivier, M. (2001) *Eur. J. Immunol.* **31**, 3185–3196
- Rao, A. M., Dogan, A., Hatcher, J. F. & Dempsey, R. J. (1998) *Brain Res.* **793**, 265–270
- Kapur, S., Bedard, S., Marcotte, B., Cote, C. H. & Marette, A. (1997) *Diabetes* **46**, 1691–1700
- Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D. & Hardie, D. G. (1996) *J. Biol. Chem.* **271**, 27879–27887
- Winder, W. W. & Hardie, D. G. (1999) *Am. J. Physiol.* **277**, E1–E10
- Garvey, E. P., Oplinger, J. A., Furfine, E. S., Kiff, R. J., Laszlo, F., Whittle, B. J. & Knowles, R. G. (1997) *J. Biol. Chem.* **272**, 4959–4963
- Gruber, H. E., Hoffer, M. E., McAllister, D. R., Laikind, P. K., Lane, T. A., Schmid-Schoenbein, G. W. & Engler, R. L. (1989) *Circulation* **80**, 1400–1411
- MacMicking, J., Xie, Q. W. & Nathan, C. (1997) *Annu. Rev. Immunol.* **15**,

- 323–350
33. Bogdan, C., Rollinghoff, M. & Diefenbach, A. (2000) *Immunol. Rev.* **173**, 17–26
34. Nathan, C. (1997) *J. Clin. Investig.* **100**, 2417–2423
35. Parkinson, J. F., Mitrovic, B. & Merrill, J. E. (1997) *J. Mol. Med.* **75**, 174–186
36. Stichtenoth, D. O. & Frolich, J. C. (1998) *Br. J. Rheumatol.* **37**, 246–257
37. Gielen, S., Adams, V., Mobius-Winkler, S., Linke, A., Erbs, S., Yu, J., Kempf, W., Schubert, A., Schuler, G. & Hambrecht, R. (2003) *J. Am. Coll. Cardiol.* **42**, 861–868
38. Cromheeke, K. M., Kockx, M. M., De Meyer, G. R., Bosmans, J. M., Bult, H., Beelaerts, W. J., Vrints, C. J. & Herman, A. G. (1999) *Cardiovasc. Res.* **43**, 744–754
39. Behr-Roussel, D., Rupin, A., Simonet, S., Bonhomme, E., Coumailleau, S., Cordi, A., Serkiz, B., Fabiani, J. N. & Verbeuren, T. J. (2000) *Circulation* **102**, 1033–1038
40. Shimabukuro, M., Ohneda, M., Lee, Y. & Unger, R. H. (1997) *J. Clin. Investig.* **100**, 290–295
41. Musi, N. & Goodyear, L. J. (2002) *Curr. Drug Targets Immune Endocr. Metabol. Disord.* **2**, 119–127
42. Angele, M. K., Schwacha, M. G., Smail, N., Catania, R. A., Ayala, A., Cioffi, W. G. & Chaudry, I. H. (1999) *Am. J. Physiol.* **276**, C285–C290
43. Le Roy, D., Heumann, D., Glauser, M. P., Mauel, J., Smith, J. & Betz Corradin, S. (1998) *Shock* **10**, 37–42
44. Smith, K. J. & Lassmann, H. (2002) *Lancet Neurol.* **1**, 232–241
45. Bachmaier, K., Neu, N., Pummerer, C., Duncan, G. S., Mak, T. W., Matsuyama, T. & Penninger, J. M. (1997) *Circulation* **96**, 585–591
46. Wildhirt, S. M., Dudek, R. R., Suzuki, H. & Bing, R. J. (1995) *Int. J. Cardiol.* **50**, 253–261
47. Bauer, A. J., Schwarz, N. T., Moore, B. A., Turler, A. & Kalff, J. C. (2002) *Curr. Opin. Crit. Care* **8**, 152–157
48. Southey, A., Tanaka, S., Murakami, T., Miyoshi, H., Ishizuka, T., Sugiura, M., Kawashima, K. & Sugita, T. (1997) *Int. J. Immunopharmacol.* **19**, 669–676
49. Kwon, G., Xu, G., Marshall, C. A. & McDaniel, M. L. (1999) *J. Biol. Chem.* **274**, 18702–18708
50. Blanchette, J., Jaramillo, M. & Olivier, M. (2003) *Immunology* **108**, 513–522
51. Adams, V., Nehrhoff, B., Spate, U., Linke, A., Schulze, P. C., Baur, A., Gielen, S., Hambrecht, R. & Schuler, G. (2002) *Cardiovasc. Res.* **54**, 95–104
52. Li, M., Pascual, G. & Glass, C. K. (2000) *Mol. Cell. Biol.* **20**, 4699–4707
53. Welch, J. S., Ricote, M., Akiyama, T. E., Gonzalez, F. J. & Glass, C. K. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6712–6717
54. Bolster, D. R., Crozier, S. J., Kimball, S. R. & Jefferson, L. S. (2002) *J. Biol. Chem.* **277**, 23977–23980
55. Horman, S., Browne, G., Krause, U., Patel, J., Vertommen, D., Bertrand, L., Lavoigne, A., Hue, L., Proud, C. & Rider, M. (2002) *Curr. Biol.* **12**, 1419–1423
56. Kolodziejewski, P. J., Musial, A., Koo, J. S. & Eissa, N. T. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12315–12320
57. Bender, A. T., Demady, D. R. & Osawa, Y. (2000) *J. Biol. Chem.* **275**, 17407–17411
58. Qian, Z., Gelzer-Bell, R., Yang, S. X., Cao, W., Ohnishi, T., Wasowska, B. A., Hruban, R. H., Rodriguez, E. R., Baldwin, W. M., III & Lowenstein, C. J. (2001) *Circulation* **104**, 2369–2375
59. Yamasaki, K., Edington, H. D., McClosky, C., Tzeng, E., Lizonova, A., Kovetski, I., Steed, D. L. & Billiar, T. R. (1998) *J. Clin. Investig.* **101**, 967–971