Activation of the AMP-activated Protein Kinase by the Anti-diabetic Drug Metformin in Vivo

ROLE OF MITOCHONDRIAL REACTIVE NITROGEN SPECIES*

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WITHDRAWN
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This article has been withdrawn by the authors. The Journal raised questions that the AMPK immunoblot in Fig. 3C was reused in Fig. 4A as ACC, lanes 1 and 4 of the AMPK-P immunoblot in Fig. 4A were duplicated, and lanes 3 and 4 of the AMPK-P immunoblot in Fig. 4F were reused in Fig. 5A as ACC-P.

Fifteen years after publication, the original data for these figures were not available for evaluation. The authors were able to provide to the Journal data from repeat experiments for Fig. 4A performed at the time of the original work, which they state confirm the results. The authors also provided the journal evidence that the duplications of Figs. 3C, 4F, and 5A occurred due to errors in the figure preparation. The authors offered to publish substitute figures based on the repeated experiments and corrected figures, alternatively, offered to repeat the experiments. However, the Journal declined both offers, a decision with which the authors respectfully disagree.

Further, the authors state that the results of this article are confirmed by the results of complementary experiments presented in the article, and the principal conclusion was further confirmed in publications from other laboratories (Quintero, M. et al. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 5379-5384; Guilherme, L. et al. (2006) Diabetes Care 29, 1083-1089).

The article, with confirmatory data supporting the results, can be obtained by contacting the authors. The authors stand by the experimental data and the conclusions of the article.

1 The abbreviations used are: AMPK, AMP-activated protein kinase; AMPK-CA, constitutively active AMPK kinase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide riboside; c-Src-DN, dominant-negative c-Src mutants; eNOS, endothelial nitric oxide synthase; GFP, green fluorescent protein; H, kinase-dead mutant; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; UCP, uncoupling protein; BAECs, bovine aortic endothelial cells; RNS, reactive nitrogen species; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PGIS, prostacyclin synthase.

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for the increase in glucose uptake in skeletal muscle (12). Phosphorylation of Thr\(^{172}\) of AMPK is necessary for its activity (16–17) and is regulated by the upstream enzyme LKB1, a recently identified AMPK kinase (18–19). Metformin does not activate AMPK in cell-free assays (20). Metformin does not influence the phosphorylation of the heterotrimeric AMPK complex by LKB1 in vitro (18, 19). Several studies demonstrate that metformin might activate AMPK by decreasing cellular energy charge, because it can act as an inhibitor of complex I of the respiratory chain (21, 22). However, two recent studies (20, 23) argue against this notion because, in these studies, metformin activates AMPK without affecting the AMP:ATP ratio. In addition, several studies suggest that there is a second AMPKK isoform that is not AMPK-dependent (18, 24–26). However, none of these studies have established the mechanism by which metformin activates AMPK.

Our recent studies indicate that ONOO\(^{-}\), a potent oxidant formed by nitric oxide (NO) and superoxide anions (O\(_2\)\(^{-}\)) at a diffusion-controlled rate, activates AMPK in cultured bovine aortic endothelial cells (BAECs) (27). We further characterize the mechanism by which NO/\(\mathrm{O}_2\) \& (ONOO\(^{-}\)) activates AMPK via a c-Src and PI3K-dependent mechanism without a change in cellular AMP or ATP content (28). This novel activation scheme may be implicated during hypoxia-reoxygenation, where we found that AMPK activation depends on ONOO\(^{-}\) formation, as well as activation of c-Src and PI3K (28). In the present study, we demonstrate that metformin inhibits the complex I of the respiratory chain to generate mitochondrial \(\mathrm{O}_2\)\(^{-}\) and then ONOO\(^{-}\), which by itself activates AMPK via a c-Src and PI3K-dependent mechanism (29). In addition, either depletion of mitochondria (28) or mitochondrial \(\mathrm{O}_2\)\(^{-}\) or adenosine deaminase (29) abrogates AMPK phosphorylation and activations (30), which might indicate that AMPK activation by metformin is mediated by mitochondrial RNs and PI3K pathway.

**EXPERIMENTAL PROCEDURES**

**Animals**

Female eNOS knockout (eNOS\(^{-/-}\)) and their littermates, C57BL6 mice, 10 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages with a 12-h light-dark cycle and given free access to water and normal chow. These mice were randomly divided into control and metformin-treated groups. The mice were abdominally injected with metformin (250 mg/kg) or AICAR (500 mg/kg) for 3 days, and the control mice received 0.9% physiological saline injection. The mice were euthanized with inhaled isoflurane. Mice hearts, kidneys, livers, and aorta were removed and immediately frozen in liquid nitrogen. The animal protocol was reviewed and approved by the University of Tennessee Institute Animal Care and Use Committee.

**Materials**

Bovine aortic endothelial cells (BAECs) and cell culture media were purchased from Clonetics Inc. (Walkersville, MD). [\(^{32}\)P]ATP was obtained from PerkinElmer. Life Sciences. 1,1-Diethoxyiodoacetamide (metformin), \(t\)-nitroarginine methyl ester (\(t\)-NAME), FAD, NADPH, FMN, rotenone, ethidium bromide, and uridine were obtained from Sigma. Protein A/G-agarose and antibodies against LKB1 were from Santa Cruz Biotechnology (Santa Cruz, CA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) were from Molecular Probes (Eugene, OR). 5-Aminoimidazole-4-carboxyamide ribonucleotide (AICAR) was from Toronto Research Chemicals (New York). The adenoviral constructs for SOD-1, SOD-2, and PI3K (PDK1-KD, mutation of lysine 114 to glycine (32, 33)), AMPK-dominant negative mutants (AMPK-DN) (mutagenesis of lysine 45 residue to arginine (27, 28)), or AMPK constitutively active mutants (AMPK-CA (27)). Confuent BAECs were infected with 1\(\times\)10\(^{6}\) plaque-forming units/ml, as described previously (29). The cells were then washed and cultured in fresh EGM medium (endothelial cell growth medium with 2\% FCS overnight. The cells were then washed and cultured in fresh EGM medium (endothelial cell growth medium with 2\% Fetal bovine serum) without FCS for an additional 18-h period. By using these conditions, infection efficiency was typically \(\geq80\%\), as determined by GFP expression.

**Methods**

**Cell Culture—Bovine aortic endothelial cells (BAECs)** were grown in EBM (endothelial cell basal supplemented with 2\% fetal bovine serum. Cells were grown in humidified atmosphere of 5\% CO\(_2\)/95\% air at 37°C and 100\% humidity. Cells were transferred into mitochon-dria-depleted BAECs (p\(^{3}_{\mathrm{g}}\) cells), wild-type BAECs (p\(^{1}_{\mathrm{g}}\) cells), or endothelial cells, as described previously (29). The cells were stained with DCFH-DA (10\(\mu\)M). The cells were then incubated in fresh EGM medium (endothelial cell growth medium with 2\% fetal bovine serum) without FCS for an additional 18-h period. By using these conditions, infection efficiency was typically \(\geq80\%\), as determined by GFP expression.

**Determination of Reactive Oxygen Species—**Generation of reaction oxygen species (ROS) was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) as described previously (30). ROS was measured by oxidation of DCFH, yielding the fluorescent product 2',7'-dichlorofluorescein (DCF). Cells were incubated with DCFH-DA (10 \(\mu\)M) under various experimental conditions. Cell monolayers were subsequently rinsed with PBS/BSA and fixed with 1\% paraformalde-hyde in PBS for 10 min at room temperature. The monolayers were rinsed twice with PBS/BSA and then washed with 2\% FBS overnight. Cell layers were incubated with 2\% FBS for 18 h at 37°C. After centrifugation (14,000 \(\times\) g, 1 min), the cells were washed with PBS, and then twice with 10\(\times\) reaction buffer (400 mM HEPES, pH 7.4, 800 mM NaCl, 50 mM MgCl\(_2\), 1 mM dithiothreitol). The AMPK activity was assessed by using the SAMS peptide as previously described (28). Briefly, duplicate tubes with 200 \(\mu\)g of protein from each sample were prepared and mixed with 500 \(\mu\)l of IP buffer (lysate buffer plus 1 mmol dithiothreitol). AMPK was then immunoprecipitated by adding 10 \(\mu\)g of polyclonal antibody against AMPK (Cell Signaling) and 25 \(\mu\)l of Protein A/G-agarose and incubated at 4°C. After centrifugation (14,000 \(\times\) g, 1 min), the pellets were washed with IP buffer and then twice with 10\(\times\) reaction buffer (400 mM HEPES, pH 7.4, 800 mM NaCl, 50 mM MgCl\(_2\), 1 mM dithiothreitol). The AMPK activity was assessed by adding 50 \(\mu\)l of reaction mixtures, consisting of 5 \(\mu\)l of reaction buffer, 10 \(\mu\)l of SAMS peptide (1 mg/ml), 10 \(\mu\)l of ATP working stock consisting of 0.1 \(\mu\)l of 100 mM ATP, 1 \(\mu\)l of [\(^{32}\)P]ATP, and 0.9 \(\mu\)l of H\(_2\)O, 25 \(\mu\)l of 50 mM AMP, and incubated at 37°C for 10 min. The beads were then washed with 2\% FBS and incubated with 25 \(\mu\)l of supernatant was spotted onto P81 Whatman paper. The filter papers were then washed four or five times with 1\% phosphoric acid. After the final wash, the filters were quickly dried and counted in a

**Adenoviral Infection**

Bovine aortic endothelial cells (BAECs) were infected with adenovirus expressing p53 (p53) as a control, or adenovirus expressing p53 dominant-negative mutants (p53-DN) (31), or adenovirus expressing p53-DN but not in those of eNOS knockout mice (eNOS\(^{-/-}\)) and ACC-Ser\(^{197}\) phosphorylation in the hearts of C57BL6 mice but not in those of eNOS knockout mice (eNOS\(^{-/-}\)) lacking NO, which is required for NOS function. Because administration of AICAR, an AMPK agonist, caused an AMPK activation in both C57BL6 and eNOS knockout mice and the eNOS\(^{-/-}\) mice expressed normal levels of AMPK, these results strongly indicate that metformin, unlike AICAR, activated AMPK via RNs such as ONOO\(^{-}\). Taken together, our data indicate that ONOO\(^{-}\) is required for metformin to activate AMPK both in vitro and in vivo. We conclude that activation of AMPK by metformin is mediated by mitochondrial RNs and PI3K pathway.
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Assay of LKB1 Activity—LKB1 activity was assayed using recombinant LKB1 (28), as described previously (18). Briefly, LKB1 was immunoprecipitated from BAECs (1 mg of protein) by incubation with a polyclonal antibody against LKB1 (Santa Cruz Biotechnology) overnight at 4 °C. LKB1 activity present in the immune complex was determined by its ability to activate recombinant AMPKα1β1γ1 in the kinase buffer for 30 min at 37 °C. AMPK activity in the supernatant was measured using SAMS peptide assay described above. We define 1 milliunit of LKB1 activity as the amount required to increase the activity of recombinant AMPK by 1 nmol/min/mg.

Immunoprecipitation and Western Blotting—Cells or mouse aortic tissue was homogenated in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were then sonicated twice for 10 s in an Ultrasonic Dismembrator with output 10% (Model 500, Fisher Scientific) and then centrifuged at 14,000 g for 20 min at 4 °C. The pellets were discarded, and supernatants were assayed for protein concentration.

Antibodies—The following antibodies were used: (1) rabbit polyclonal antibody against phosphorylated Thr172 of AMPK, (2) rabbit polyclonal antibody against ACC-Ser79. All antibody bindings were detected by using ECL-Plus.

Immunohistochemical Staining of 3-Nitrotyrosine—Immunohistochemical staining of 3-nitrotyrosine was performed as described previously (37). Briefly, isolated mouse hearts were fixed in 4% paraformaldehyde overnight. Sections, each of 9 μm, were mounted on slides and blocked with protein blocking agent (Biogenex) at room temperature for 30 min. After having removed the blocking solution, tissues were incubated with a rabbit polyclonal antibody against 3-NT (15 μg/ml) overnight at 4 °C. Control sections were stained with the 3-NT antibody prepared in 10 mM 3-NT in PBS. Antibody binding was then visualized by co-incubation of biotinylated anti-rabbit IgG for 30 min at room temperature with Fast Red chromogen in naphthol phosphate buffer. Sections were washed and examined under a Leica microscope. Negative control sections were incubated with a non-specific primary antibody. All pictures were obtained under 10-fold magnification with a Leica microscope.

Statistical Analysis—Data are presented as mean +/− S.E. Differences were analyzed by one-way ANOVA followed by appropriate comparison tests. A P value was considered as statistically significant.

Results

Metformin Activates AMPK and Increases the Phosphorylation of Its Downstream Enzymes, ACC-Ser79, in Cultured BAEC Cells—Previous studies have demonstrated that metformin activates AMPK in both hepatocytes and skeletal muscle (11, 12). Activation of AMPK phosphorylates ACC-Ser79 in hypoxic BAECs (27, 28) and in ischemic cardiac myocytes (7). To investigate whether or not metformin activated AMPK to phosphorylate ACC, confluent BAECs were treated with different concentrations of metformin for 0.25 to 1 h. AMPK activation was monitored in Western blots by staining with a specific antibody against phosphorylated Thr172 of AMPK, which is reported to be essential for the AMPK activity (16, 17). As shown in Fig. 1a, metformin concentrations dependently increased the phosphorylation of AMPK-Thr172. Activation of AMPK was further confirmed by the enhanced phosphorylation of ACC-Ser79 (Fig. 1, a and b). Clinically relevant concentrations of metformin (~100 μM) significantly increased the phosphorylation of AMPK-Thr172 and ACC-Ser79. However, increasing concentrations of metformin (up to 1 mM) caused a further increase in phosphorylation of AMPK-Thr172 and ACC-Ser79 (Fig. 1a). In addition, metformin concentration dependently enhanced AMPK activity, as assayed by phosphorylation of the SAMS peptide using [32P]ATP assays (Fig. 1b). Furthermore, activation of AMPK by metformin was also time-dependent. Metformin rapidly increased the phosphorylations of AMPK-Thr172 and ACC-Ser79 as early as 15 min, reaching maximal at 1 h without affecting the total content of AMPK, as assayed by the AMPK-α subunit, indicating that increased AMPK-Thr172 phosphorylation by metformin was not from an increased AMPK expression (Fig. 1, c and d). Because 0.5 mM metformin caused an optimal activation of AMPK at 1 h, we used this concentration of metformin and 1-h incubation for the following studies.

Activation of AMPK by Metformin Is c-Src-mediated and PI3K-dependent—Our previous studies demonstrate that ONOO− activates AMPK via both c-Src and PI3K pathways (28). Thus, it was interesting to investigate if c-Src/PI3K pathways contributed to the metformin-induced AMPK activation. As shown in Fig. 2 (a and b), metformin significantly increased AMPK-Thr172 phosphorylation and AMPK activity but without altering AMP sensitivity or dependence. In addition, we found that PP2 (10 μM), which selectively inhibits both c-Src activity and c-Src phosphorylation, attenuated metformin-induced AMPK-Thr172 phosphorylation and AMPK activity (Fig. 2, a and b). Inhibition of AMPK by both wortmannin (100 nM) or LY294002 (50 μM) abolished metformin-enhanced AMPK-Thr172 phosphorylation and AMPK activity (Fig. 2b), although wortmannin, which affected the basal level of AMPK, was ineffective (Fig. 2a). Overexpression of C-Src or PI3K-ΔN, both c-Src/PI3K pathways, increased phosphorylation of AMPK-Thr172 (Fig. 2c), thus indicating that c-Src and PI3K pathways contribute to AMPK activation, at least in part, via c-Src and PI3K pathways. Inhibition of AMPK by both c-Src-DN and PI3K-ΔN strongly inhibited AMPK activity that was enhanced by metformin. Thus, these data indicate that metformin activates a signaling pathway involving c-Src, PI3K, and PDK1 that leads to activation of AMPK.

It was interesting to investigate if c-Src-PI3K affected the AMPK activity by AICAR, an AMPK agonist. As shown in Fig. 2e, inhibition of c-Src or PI3K did not alter AICAR-enhanced AMPK activity. These data suggest that AICAR, which is converted by adenosine kinase to AICAR monophosphate, a cellular mimic of AMPK, did not require c-Src/PI3K to cause AMPK activation.

Activation of AMPK by Metformin is ONOO−-dependent—Because our studies indicate that metformin, like authentic ONOO−, activates AMPK in a c-Src/PI3K-dependent manner, we next determined if endogenous ONOO− was involved in AMPK activation caused by metformin. We first investigated if exposure to metformin increased reactive oxygen species (ROS) in BAECs. Generation of intracellular ROS was assessed using DCFH-DA, which is oxidized by intracellular ROS to yield the fluorescent product DCF. As shown in Fig. 3a, exposure of BAECs to low concentrations of metformin (100 μM), a concentration in which AMPK was activated, significantly increased the DCF fluorescence, indicating that metformin increased intracellular ROS generation.

Our previous studies demonstrate that ONOO−, but not its two precursors, NO or O2−, trigger tyrosine nitration of prosta-cyclin synthase (PGIS) (36–38). Thus, tyrosine nitration of PGIS can be used as an index for the formation of ONOO− in cultured BAECs exposed to metformin. As shown in Fig. 3b, exposure of BAECs to metformin (100 μM), a concentration in which AMPK was activated, significantly increased the detec-
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Fig. 1. Metformin increases the phosphorylations of both AMPK-Thr172 and ACC-Ser79 in BAECs cells. Confluent BAECs were treated with metformin as described under “Experimental Procedures.” At the times indicated, cells were lysed and proteins were extracted, as described under “Methods.” Proteins were separated in SDS-PAGE and detected by using the specific antibodies in Western blots.

Identification of Mitochondria as the Source of Oxidants—We next investigated the source of oxidants by which metformin activated AMPK in BAECs. As shown in Fig. 3 (e and f), adenoviral overexpression of UCP-1, which blocks electron transfer of the respiratory chain and prevents O2 formation (39, 40), attenuated metformin-stimulated phosphorylation of both AMPK-Thr172 and ACC-Ser79 as well as AMPK activity. However, overexpression of p47phox-dominant mutants, which inhibited angiotensin-triggered O2 production in BAECs by 70–7% and abolishes the activity of NAD(P)H oxidases (41), had little effect (Fig. 3, d and e) in metformin-enhanced AMPK-Thr172 phosphorylation. These data indicate that mitochondrion, rather than NAD(P)H oxidase, was the source of O2 and ONOO- in cells exposed to metformin.

Inhibition of the Complex I of Mitochondria Activates AMPK in BAECs—Previous studies demonstrate that metformin activates AMPK by a depleted energy charge resulting from the inhibition of the mitochondrial respiratory chain complex I (21, 22). Recent studies argue against this notion, because activation of AMPK by metformin occurs prior to depletion of the cellular energy charge (20, 23). Inhibition of complex I by rotenone is reported to lead to O2 generation and apoptosis (42, 43), and inhibition of complex I might lead to O2 generation prior to the altered energy charge. Therefore, we further tested whether O2 instead of energy depletion, both of which are likely caused by the complex I inhibition (42, 43), was involved in the activation of AMPK by metformin. To test this hypothesis, we first investigated whether inhibition of the complex I with rotenone activates AMPK and if scavenging O2 prevents AMPK activation of tyrosine nitration of PGIS. Because PGIS nitration in metformin-treated BAECs was inhibited either by overexpressing SOD to scavenge O2 or by treating the cells with L-NAME (1 mM) to prevent formation of NO, these results indicate that metformin triggers an NO-derived oxidant, likely ONOO-, in BAECs.

To investigate whether metformin activates AMPK via ONOO-, we monitored AMPK-Thr172 phosphorylation and AMPK activity under conditions where ONOO- was inhibited. The overexpression of SOD-1 and SOD-2 was confirmed by a 2.7- and 3.3-fold increase in SOD-1 and SOD-2 proteins, respectively, as detected in Western blots using the specific antibodies (Research Diagnostic Inc.). Further, compared with GFP-infected cells, SOD activities, as measured by the reduction in pyrogallol autoxidation, were increased 2.1- and 2.5-fold in the cells overexpressing SOD-1 and SOD-2 (not shown). 1 mM L-NAME inhibited 100% eNOS activity, as seen by conversion of L-[3H]arginine into L-citrulline (not shown). As shown in Fig. 3c, either an overexpression of SOD-1 or SOD-2, or treatment of the cells with L-NAME, attenuated metformin-enhanced phosphorylation of AMPK-Thr172 (Fig. 3c), ACC-Ser79 (not shown), and AMPK activity (Fig. 3d). Overexpression of catalase, which increased catalase activity by 2.8-fold, as measured by the reduction of 1% H2O2 absorption at 240 nm in BAECs overexpressing catalase, did not alter metformin-enhanced AMPK-Thr172 phosphorylation and AMPK activity, indicating that overexpression of catalase, either an overexpression of SOD-1 or SOD-2, or treatment of the cells with L-NAME, attenuated metformin-enhanced phosphorylation of AMPK-Thr172 and ACC-Ser79, either by overexpressing SOD-1 or SOD-2, or treatment of the cells with L-NAME, attenuated metformin-enhanced phosphorylation of AMPK-Thr172 and ACC-Ser79, excluding a nonspecific effect of viral infection. Because we previously showed that neither NO nor NOS inhibition of the complex I with rotenone is reported to lead to O2 generation and apoptosis (42, 43), was involved in the activation of AMPK by metformin exposure, which then activates both c-Src and AMPK kinase in the coordinated fashion observed.

Inhibition of the Complex I of Mitochondria Activates AMPK in BAECs—Previous studies demonstrate that metformin activates AMPK by a depleted energy charge resulting from the inhibition of the mitochondrial respiratory chain complex I (21, 22). Recent studies argue against this notion, because activation of AMPK by metformin occurs prior to depletion of the cellular energy charge (20, 23). Inhibition of complex I by rotenone is reported to lead to O2 generation and apoptosis (42, 43), and inhibition of complex I might lead to O2 generation prior to the altered energy charge. Therefore, we further tested whether O2 instead of energy depletion, both of which are likely caused by the complex I inhibition (42, 43), was involved in the activation of AMPK by metformin. To test this hypothesis, we first investigated whether inhibition of the complex I with rotenone activates AMPK and if scavenging O2 prevents AMPK...
activation by rotenone in BAECs. Inhibition of the complex I with rotenone (100 nM) increased the DCF fluorescence by 1.9-fold in BAECs after 2-h exposure. In parallel, rotenone significantly increased the phosphorylation of both AMPK Thr172 and ACC-Ser79 (Fig. 4a). Importantly, similar to metformin, overexpression of SOD or uncoupling proteins or inhibition with L-NAME blunted rotenone-activated phosphorylation of AMPK-Thr 172 and ACC-Ser 79 (Fig. 4a). These results support the notion that inhibition of complex I is capable of generating ROS, which in return leads to activation of AMPK via ONOO⁻.

Metformin Did Not Activate AMPK in Mitochondria-lacking ρ-o-BAECs—To further establish if mitochondria is the target of metformin, we created BAECs lacking functional mitochondria (so-called ρ-o cells). To this purpose, BAECs were incubated with ethidium bromide (which depletes mitochondrial DNA) and supplemented with an alternative source of energy and nucleotides (pyruvate and uridine) (29). Incubation of BAECs with ethidium bromide (50 ng/ml), pyruvate (110 μg/ml), and uridine (50 μg/ml) for 3 weeks dramatically reduced the expression of both cytochrome oxidase II and III mRNA and depleted their protein levels completely (Fig. 4, c and d). Further, these ρ-o cells did not survive if uridine was omitted from the media (data not shown). Taken together, these observations indicated that ρ-o-BAECs lacked functional mitochondria.

We next investigated if metformin increased intracellular
FIG. 3. **Activation of AMPK by metformin is both ONOO⁻ and PI3K-dependent.** 

a, metformin enhances intracellular ROS generation. Intracellular ROS was detected by the DCF fluorescence as described under “Experimental Procedures.” Exposure of BAECs to either 0.1 or 0.5 mM metformin significantly increased DCF fluorescence (n = 6, #, p < 0.01, control versus metformin); b, increased tyrosine nitration of prostacyclin synthase (PGIS) in BAECs exposed to metformin. Confluent BAECs were exposed to metformin at concentrations indicated for 24 h. PGIS proteins were first immunoprecipitated with a monoclonal antibody against PGIS and then Western blotted for either PGIS or 3-NT using a monoclonal antibody against PGIS or 3-NT (n = 4, #, p < 0.05 control versus metformin-treated); c, ONOO⁻-dependent activation of AMPK in BAECs exposed to metformin. Phosphorylation of AMPK (Thr172) was attenuated by the NOS inhibitor, l-NAME, or overexpression of SOD-1 or SOD-2. The blot is representative of three to four blots obtained from independent experiments; d, decreased formation of ONOO⁻ by overexpression of SOD-1, SOD-2 or adding the NOS inhibitor, l-NAME, blocked metformin-enhanced AMPK activity in BAECs (n = 5, #, p < 0.01, control versus metformin; n = 5, *, p < 0.01 metformin versus metformin plus SOD or l-NAME). e, overexpression of UCP-1, but not p47phox-DN, attenuated metformin-enhanced phosphorylations of AMPK-Thr172. The **lower panel** gives the summary data (n = 3 or 4, *, p < 0.05 control versus metformin; n = 3 or 4, #, p < 0.01, metformin versus metformin plus UCP-1).
ROS in \( \rho^- \)-BAECs. As expected, metformin failed to increase ROS, as assayed by the formation of DCF fluorescence. In addition, metformin, which significantly increased the phosphorylation of AMPK-Thr172 and AMPK activity in wild type of BAECs but not in \( \rho^- \)-BAECs (Fig. 4, f and g). These observations further corroborated that metformin requires functional mitochondria to activate AMPK in endothelial cells. Because \( \rho^- \)-BAECs also failed to produce oxidants in response to metformin (Fig. 4e), the results strongly support that metformin-activated AMPK is via mitochondria-derived ROS.

**Activation of AMPK by Metformin in Vivo**—To further establish if ONOO\(^-\) was involved in AMPK activation by metformin in vivo, metformin or AICAR were given to eNOS\(^{-/-}\) mice (attenuate ONOO\(^-\) by lacking eNOS-derived NO) or to the wild type C57BL6 mice. Mouse aorta and hearts were isolated for assaying AMPK-Thr172 phosphorylation and AMPK activity. As shown in Fig. 5 (a and b), administration of metformin significantly increased the phosphorylations of AMPK-Thr172 and ACC-Ser79 in C57BL6 mice but not in eNOS\(^{-/-}\) mice, whereas AICAR activated AMPK-Thr172 phosphorylation and increased AMPK activity in both C57BL6 and eNOS\(^{-/-}\) mice, suggesting a role of ONOO\(^-\) in the activation of AMPK by metformin in vivo.
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Activation of AMPK by Metformin is ONOO−-dependent in Vivo—To elucidate if metformin increases RNS such as ONOO− in vivo, we immunohistochemically stained 3-NT, a footprint of RNS, in the hearts isolated from mice. The staining with the 3-NT antibody was only weakly visible in the heart isolated from sham-treated C57BL6 mice (Fig. 5a). Metformin but not AICAR significantly increased 3-NT staining in C57BL6 mice (Fig. 5b and c). In contrast, there was no 3-NT staining in the hearts isolated from sham-treated eNOS−/− mice, and neither metformin nor AICAR increased 3-NT antibody staining in the hearts of eNOS−/− mice (Fig. 5, d and e), suggesting that NO from eNOS was needed for 3-nitrotyrosine formation by metformin. The specificity of 3-NT staining was confirmed by the absence of staining when the antibody was omitted or was diluted in 10 mM 3-NT (data not shown). Because metformin activated AMPK in C57BL6 but not in eNOS−/−, these results suggest that ONOO− is likely to be involved in the activation of AMPK by metformin in vivo.

Metformin Increased the Association of LKB1 (AMPK Kinase) and AMPK in Vivo—Two recent studies (18, 19) have identified that LKB1 acts as an upstream kinase, AMPK kinase. Exposure of HeLa S3 and A549 cells (which lack LKB1) to either ONOO− or metformin did not activate AMPK, although metformin activated AMPK in these cells.2 These data indicate that ONOO−-induced AMPK activation in vivo does not occur via an upstream enzyme such as LKB1. In vivo, metformin increased LKB1 activity in AMPK upstream kinase, AMPK, and proteins were separated by SDS-PAGE and Western blotted for AMPK, or vice versa. As shown in Fig. 6a, neither metformin nor AICAR altered LKB1 activity in mice treated with metformin or AICAR. In addition, LKB1 activity in eNOS−/− mice was similar to those in C57BL6 mice.

It was interesting to investigate the mechanism by which metformin activated AMPK without altering LKB1 activity. Because metformin did not increase AMPK in vitro assays excluding a direct interaction of metformin with AMPK (20), we investigated if metformin activated AMPK by increasing the interaction of AMPK and LKB1. To determine the interaction of LKB1 and AMPK, LKB1 was first immunoprecipitated and then assayed for AMPK activity. Metformin significantly increased the co-immunoprecipitation of LKB1 and AMPK in C57BL6 mice but not in eNOS−/− mice. Metformin did not increase the co-immunoprecipitation of LKB1 with AMPK in mice (data not shown), suggesting that AMPK-α2 in heart might be activated by an AMPK kinase other than LKB1. In contrast, AICAR did not increase the co-immunoprecipitation of LKB1 with either AMPK-α1 or AMPK-α2 in either C57BL6 or eNOS−/−. These results further support that the mechanism by which metformin activates AMPK differs from AICAR, the latter being converted by adenosine kinase to AICAR monophosphate, a cellular mimetic of AMPK.

To further confirm if metformin increased the co-immunoprecipitation of LKB1 with AMPK, we measured AMPK activity in the immunoprecipitate using an antibody against LKB1. LKB1 was first immunoprecipitated and then assayed for AMPK activity. Metformin and AICAR activated AMPK in both C57BL6 and eNOS−/− mice. The specificity of antibody against 3-nitrotyrosine is seen by loss of the staining if 3-NT antibody is omitted or if the antibody is diluted in 10 mM 3-nitrotyrosine.

2 M.-H. Zou, B. J. Davis, S. S. Kirkpatrick, and J. S. Nelson, unpublished data.
AMPK. Because the SAMS peptide is a specific substrate for AMPK but not LKB1, the increased 32P-SAMS phosphorylation (an index for AMPK activity) in metformin-treated C57BL6 mice can only be explained by the increased co-immunoprecipitation of AMPK with LKB1 in vivo.

Because inhibition of PI3K significantly attenuated AMPK activation that was enhanced by metformin, we further investigated if PI3K contributed to the increased association of AMPK and LKB1. Interestingly, metformin did not alter LKB1 activity (Fig. 6d) but increased co-immunoprecipitation of LKB1 and AMPK (Fig. 6e). Furthermore, inhibition of PI3K with wortmannin significantly attenuated the co-immunoprecipitation of AMPK and LKB1 that was enhanced by metformin. These results suggest that activation of PI3K by met-
Previous studies have established that metformin exerts its therapeutic effects in diabetes partly via AMPK activation (11, 12). Although activation of AMPK by metformin has been demonstrated in various tissues (11, 12), and a mechanism other than AMP/ATP has been suggested (18–19, 23–26), none has yet been identified. The present study has, for the first time, demonstrated that metformin via mitochondrial oxidants is PI3K-dependent pathway (Fig. 7).

The evidence supporting activation of AMPK by the increased formation of mitochondria-derived oxidants is several-fold. First, exposure to metformin significantly increased intracellular RNS, as evidenced by an increased detection of tyrosine nitration of PGIS, an established marker for the NO-derived oxidant, ONOO⁻. In addition, the concentrations of metformin (100–500 μM) triggering RNS formation and PGIS nitration were similar to those required for the minimally effective concentrations required for phosphorylation and activation of AMPK-Thr172. Further, metformin increased RNS in C57B6 mice but not eNOS-KO mice, indicating that metformin increased RNS both in vitro and in vivo.

Second, inhibition of ONOO⁻ formation by overexpression of SOD (to scavenge O₂⁻) or NOS inhibition with l-NAME (to prevent the formation of NO) attenuated metformin-enhanced phosphorylation of both AMPK and ACC as well as AMPK activity. Because NO or O₂⁻ alone have no effect on AMPK activation (27) and addition of authentic ONOO⁻ did not affect the AMP/ATP ratio (28), the attenuation of both AMPK-Thr172 and ACC-Ser79 by both l-NAME and SOD in cells exposed to metformin suggests the involvement of ONOO⁻.

Third, metformin has been previously demonstrated as a weak inhibitor for mitochondrial respiratory chain complex I. Inhibition of the mitochondrial respiratory chain complex I not only inhibits ATP synthesis but also leads to release of O₂⁻ from the respiratory chain (39–43). In the present study, inhibition of mitochondrial complex I with retorone, like metformin, activated AMPK in BAECs. Overexpression of UCP proteins, which inhibit the electron transport and produce O₂⁻ by the respiratory chain, abolished metformin-enhanced phosphorylation of both ACC and AMPK. These results indicate that inhibition of complex I could produce oxidants to activate AMPK. In contrast, overexpression of adenovirus encoding p47(phox)-DN, which blocked angiotensin-II-stimulated NADPH oxidases and then O₂⁻ release (data not shown), did not alter AMPK activated by metformin. These results suggest that mitochondria instead of NAD(P)H oxidases were the major source of oxidants in cells exposed to metformin.

Fourth, these results were further corroborated by the findings in ρ⁻-BAECs lacking functional mitochondria. Exposure of ρ⁻-BAECs to metformin did not enhance intracellular ROS release. Because metformin did not activate AMPK in mitochondria-depleted ρ⁻-BAECs, these results indicate that activation of AMPK by metformin is mitochondrial ROS-dependent.

Fifth, inhibition of c-Src or PI3K activity by pharmacological inhibition or dominant negative mutants also blocked the phosphorylation of both AMPK-Thr172 and ACC-Ser79. Because metformin increased ROS in BAECs and ONOO⁻-activated AMPK via c-Src/PI3K-dependent pathways (28), these data
also suggest that metformin, like authentic ONOO−, leads to AMPK activation via c-Src/P13K-dependent pathway.

Finally, metformin significantly increased AMPK activity in the aortae and hearts of C57BL6 mice but not those of eNOS−/−, although eNOS−/− mice expressed AMPK. Because eNOS−/− mice did not generate ONOO− in response to metformin, the data strongly suggest that ONOO− is required for AMPK activation by metformin. In addition, administration of AICAR, a cell-permeable AMPK agonist, increased AMPK activity in both aorta and hearts in mice lacking eNOS, indicating that ONOO− is not required for AMPK activation by AICAR. Because metformin increased ROS both in vitro and in vivo, and because both inhibition of NOS with l-NAME or deficiency of NOS blocked metformin-induced AMPK activation, these results strongly suggest that NO-derived oxidants such as ONOO− might be required for AMPK activation by metformin.

Our data show an essential role of endogenous NO in the activation of AMPK in vascular tissues. This concept might also be applicable to non-vascular tissues, and metformin might be able to activate AMPK by increasing mitochondria-derived ONOO− even in non-endothelial cells. First, NO generated from vascular cells can migrate and diffuse into parenchymal cells such as myocytes, skeletal muscle, etc. Second, NO can be generated from other type of NOS, such as neuronal NOS or inducible NOS localized in non-vascular tissues. Indeed, we have obtained data showing that exogenous ONOO− activated AMPK in cultured myocytes (Fig. 8A), and further overexpression of SOD or inhibition of NOS by l-NAME blocked metformin-induced AMPK activation (Fig. 8B). These results suggest that metformin is able to activate non-vascular cells such as cardiac myocytes in a dependent fashion. Finally, there is growing evidence that mitochondria constitutively express mito- 

chondria, which diffuses into other cellular compartments (44–46). The reaction of NO with O2 generates NO to regulate mitochondrial metabolism (44–46).

In summary, we demonstrate for the first time that metformin increases mitochondria-derived ONOO− to activate AMPK in c-Src/P13K-dependent manners. Activation of AMPK by metformin might contribute to the beneficial effects of metformin in treating diabetes.

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