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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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[1] The abbreviations used are: AMPK, AMP-activated protein kinase; AMPK-CA, constitutively active AMPK kinase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidizole-4-carboxamide riboside; c-Src-DN, dominant-negative c-Src mutants; eNOS, endothelial nitric-oxide synthase; GFP, green fluorescent protein; L-NAME, L-nitroaginine methyl ester; 3-NT, 3-nitrotyrosine; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PDK1, phosphoinositide-dependent kinase 1; PDK-1-KD, PDK-1 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.

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 AMP-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 that $ONOO^-$ 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
generate mitochondrial O_2^- and then $ONOO^-$, which
AMPK activation via a c-Src and PI3K-dependent
addition, either depletion of mitochondria (ρ^0
mitochondrial O_2^-) or adenoviral overex
dismutases (SODs) as well as inhi
thase (NOS) abolished the m
phorylation and activities
of AMPK by metfor
dria-derived RNS. Fu
which increased 3-nitro
nitrogen species (RNS)
C57BL6, resulted in para
Ser^{79} phosphorylation in the
but not in those of eNOS kn
NO, which is required for RNS
tration 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.

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 chows. 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). [γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences. 1,1-Dimethylbiguanide (metformin), L-nitroarginine methyl ester (L-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) was from Molecular Probes (Eugene, OR). 5-Aminoimidazole-4-carboxamide riboside (AICAR) was from Toronto Research Chemicals (New York). The adenoviral constructs for SOD-1, SOD-2, uncoupling protein (UCP)-1, -2, and -3, and catalase were obtained from the University of Iowa Viral Vector Core facility. Antibody against 3-nitrotyrosine (3-NT), and the SAMS peptide were from Upstate Biotechnology (Lake Placid, NY). Antibodies against phospho-acetyl-CoA carboxygenase (ACC) (Ser^{79}), phospho-AMPK (Thr^{172}), and AMPK were obtained from Cell Signaling Inc. (Beverly, MA). Antibodies against ACC were obtained from Alpha Diagnostic International, Inc. (San Antonio, TX). Other chemicals and organic solvents of highest grade, if not indicated, were obtained from Fisher Scientific.

Methods

Cell Culture—Bovine aortic
EBM (endothelial cell bas
vine serum. Cells we
CO_2/95% air at 3
bromide
3 w
bunit II by both RT-PCR
grow in the absence of
cted with adenovirus ex-
P) as a control, or adenovirus
atalase, uncoupling protein (UCP)-1,
gative mutants for c-Src (c-Src-DN) (31),
DK1 (PDK1-KD, mutation of lysine 114 to
K-dominant negative mutants (AMPK-DN) (mu-
residue to arginine (27, 28)), or AMPK constitutively
nts (AMPK-CA (27)). Confluent BAECs were infected in
with 2% FCS overnight. The cells were then washed and
ubated in fresh EGM medium (endothelial cell growth medium with 2% fetal bovine serum) without FCS for an additional 18 h prior to experimentation. Using these conditions, infection efficiency was typically >80%, as determined by GFP expression.

Determination of Reactive Oxygen Species—Generation of reactive oxygen species (ROS) was assessed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) as described previously (35). ROS in cells cause oxidation of DCFH, yielding the fluorescent product 2′,7′-dichlorofluorescein (DCF). Cells were incubated with DCFH-DA (10 μM) under various experimental conditions. Cell monolayers were subsequently rinsed with PBS/BSA and fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. The monolayers were rinsed twice with PBS/BSA and scraped with rubber policemen. Cell suspensions were centrifuged at 1500 rpm for 5 min, the supernatants were discarded, and the pellets were resuspended in 0.5 ml of PBS/BSA. Cytofluorographic analysis was performed using a BD Biosciences FACScan (San Jose, CA). Acquisition was set at 5000 gated cells, and mean fluorescent intensities as well as the percentage of cells were measured in all samples using CellQuest software version 1.2 (BD Biosciences).

Assay of AMPK Activity—AMPK activity was assayed by using the SAMS peptide as previously described (28). Briefly, duplicate tubes with 200 μg of protein from each sample were prepared and mixed with 500 μl of IP buffer (lysis buffer plus 1 mM dithiothreitol). AMPK was then immunoprecipitated by adding 10 μg of polyclonal antibody against AMPK (Cell Signaling) and 25 μl of Protein A/G-agarose and incubated at 4 °C. After centrifugation (14,000 × *g*, 1 min), the beads were washed with IP buffer and then twice with 10× reaction buffer (400 mM HEPES, pH 7.4, 800 mM NaCl, 50 mM $MgCl_2$, 1 mM dithiothreitol). The AMPK activity was assayed by adding 50 μl of reaction mixtures, consisting of 5 μl of reaction buffer, 10 μl of SAMS peptide (1 mg/ml), 10 μl of ATP working stock consisting of 0.1 μl of 100 mM ATP, 1 μl of [^{32}P]ATP, and 8.9 μl of H_2O, 25 μl H_2O, or 25 μl of 400 μM AMP, and incubated at 37 °C for 10 min. The beads were quickly pelleted, and 25 μl 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

scintillation counter. The difference between the presence and absence of AMPK is calculated as the AMPK activity.

Assay of LKB1 Activity—LKB1 activity was assayed using recombinant AMPK$\alpha1\beta1\gamma1$, 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 AMPK$\alpha1\beta1\gamma1$ 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 tissues were 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-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were then sonicated twice for 10 s in an Ultrasonic Dismemberator with output 10% (Model 500, Fisher Scientific) and then centrifuged at 14,000 $\times$ *g* for 20 min at 4 °C. The pellets were discarded, and supernatants were assayed for protein concentration.

Proteins were subjected to Western blots, and immunoprecipitation was performed as described previously (36–38). The 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 antibod
prepared in 10 mM 3-NT in PBS. Antibody binding was then visu
by co-incubation of biotinylated anti-rabbit IgG for 30 mi
temperature with Fast Red chromogen in naphthol pho

Sections were washed and examined under a Leic
ative controls were performed by either eli
against 3-NT or by incubating sections
antibody. All picture were obtained
identical camera and print setting

Quantification of Western B
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Statistic Analysis—Data
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tistically significant.

RES

Metformin Activates AMPK a ncreases the Phosphorylation of Its Downstream Enzymes, ACC-Ser^{79}, 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-Ser^{79} 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 Thr^{172} of AMPK, which is reported to be essential for the AMPK activity (16, 17). As shown in Fig. 1*a*, metformin concentrations dependently increased the phosphorylation of AMPK-Thr^{172}. Activation of AMPK was further confirmed by the enhanced phosphorylation of ACC-Ser^{79} (Fig. 1, *a* and *b*). Clinically relevant concentrations of metformin (~100 μM) significantly increased the phosphorylation of AMPK-Thr^{172} and ACC-Ser^{79}. However, increasing concentrations of metformin (up to 1 mM) caused a further increase in phosphorylation of AMPK-Thr^{172} and ACC-Ser^{79} (Fig. 1*a*). In addition, metformin concentration dependently enhanced AMPK activity, as assayed by phosphorylation of the SAMS peptide using [^{32}P]ATP assays (Fig. 1*b*). Furthermore, activation of AMPK by metformin was also time-dependent. Metformin rapidly increased the phosphorylations of AMPK-Thr^{172} and ACC-Ser^{79} 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-Thr^{172} 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-Thr^{172} 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-Thr^{172} phosphor and AMPK activity (Fig. 2, *a* and *b*). Inhibition of by wortmannin (100 nM) or LY294002 (50 tformin-enhanced AMPK-Thr^{172} ph AMPK activity (Fig. 2*b*), altho affected the basal level o 3*b*). Overexpression of (c-Src-DN) or PDK- ot of a GFP control, ased phosphorylation of r^{79} (Fig. 2*c*), thus indicating PK, at least in part, via c-Src and hibition of AMPK by both c-Src-DN and ther confirmed by decreasing phosphorylaMS peptide using [^{32}P]ATP assays (Fig. 2*d*). As Fig. 3*d*, both PDK-1-KD and c-Src-DN strongly ined 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 activated by AICAR, an AMPK agonist. As shown in Fig. 2*e*, 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 mimetic 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. 3*a*, 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 $O_2^{\cdot-}$, trigger tyrosine nitration of prostacyclin 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. 3*b*, exposure of BAECs to metformin (100 μM), a concentration in which AMPK was activated, significantly increased the detec-

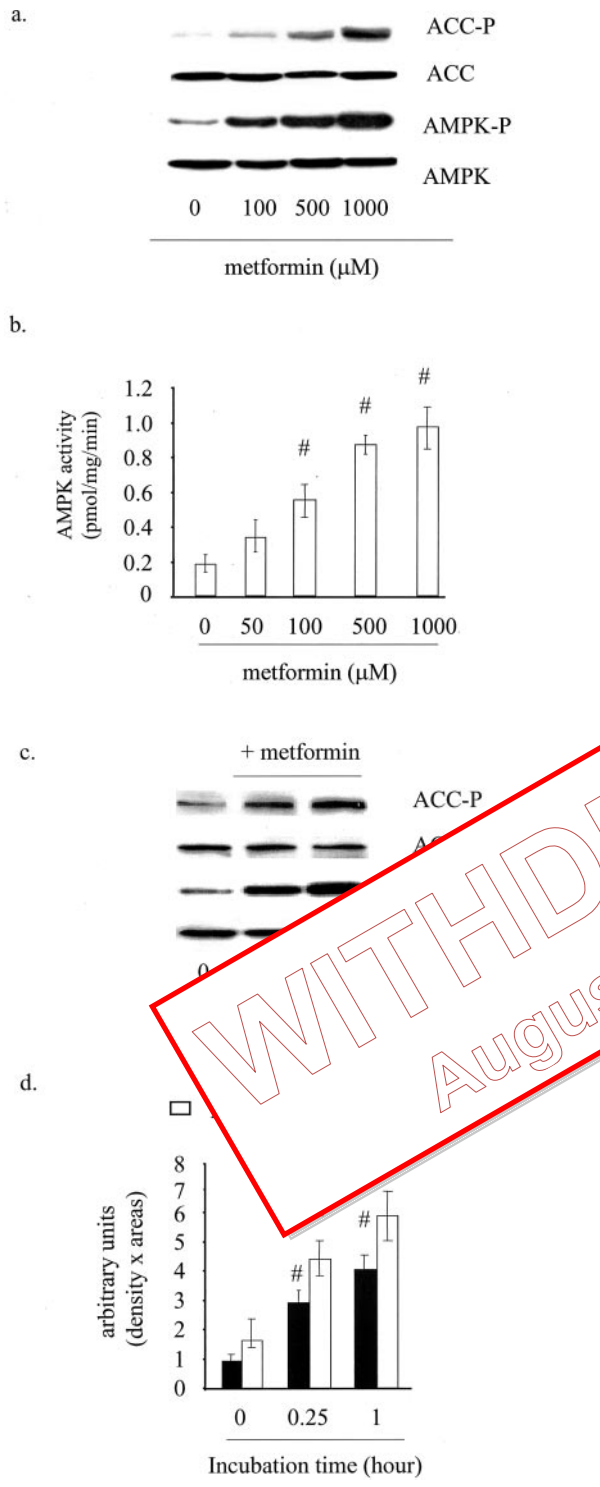

FIG. 1. **Metformin increases the phosphorylations of both AMPK-Thr^{172} and ACC-Ser^{79} in BAECs cells.** Confluent BAECs were treated with metformin as described under "Experimental Procedures." At the times indicated, cells were lysed and proteins were extracted, as described under "Methods." Proteins were separated in SDS-PAGE and detected by using the specific antibodies in Western blots. *a*, metformin (100 μM to 1 mM) caused a concentration-dependent increase in the phosphorylations of AMPK-Thr^{172} (*AMPK-P*) and ACC-Ser^{79} (*ACC-P*) in BAEC cells. The blot is representative of four individual experiments. *b*, metformin concentration dependently enhanced the phosphorylation of the SAMS peptide using [^{32}P]ATP assays ($n = 4$ or 5; #, $p < 0.05$, control *versus* metformin-treated). *c*, metformin increased in a time-dependent manner the phosphorylations of both AMPK and ACC in BAEC cells. The blot is representative of four blots obtained from four separate experiments. *d*, summary data for the time-dependent effects of metformin on both AMPK-P and ACC-P ($n = 4$; #, $p < 0.05$, control *versus* metformin-treated).

tion of tyrosine nitration of PGIS. Because PGIS nitration in metformin-treated BAECs was inhibited either by overexpressing SOD to scavenge O_2^-, 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-Thr^{172} 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-^{3}H]arginine into L-citrulline (not shown). As shown in Fig. 3*c*, either an overexpression of SOD-1 or SOD-2, or treatment of the cells with L-NAME, attenuated metformin-enhanced phosphorylation of AMPK-Th[illegible]g. 3*c*), ACC-Ser^{79} (not shown), and AMPK activity ([illegible]expression of catalase, which increased catal[illegible]-fold, as measured by the reduction [illegible]240 nm in BAECs overexpressi[illegible]rmin-enhanced AMPK-T[illegible]ivity, indicating that [illegible]metformin-enhanced [illegible]fection of cells with [illegible]no effect on metformin-[illegible]g a nonspecific effect of viral [illegible]usly showed that neither NO nor [illegible]K in BAECs (27), the attenuation of [illegible]sphorylation and AMPK activity by either [illegible]erexpression of SOD-1 and SOD-2 is likely ex-[illegible]y the formation of $ONOO^-$ during metformin expo-[illegible], which then activates both c-Src and AMPK kinase in the coordinated fashion observed.

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 O_2^- formation (39, 40), attenuated metformin-stimulated phosphorylation of both AMPK-Thr^{172} and ACC-Ser^{79} as well as AMPK activity. However, overexpression of p47^{phox}-dominant mutants, which inhibited angiotensin-triggered O_2^- 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-Thr^{172} phosphorylation. These data indicate that mitochondria, rather than NAD(P)H oxidase, was the source of O_2^- 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 O_2^- generation and apoptosis (42, 43), and inhibition of complex I might lead to O_2^- generation prior to the altered energy charge. Therefore, we further tested whether O_2^- 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 O_2^- prevents AMPK

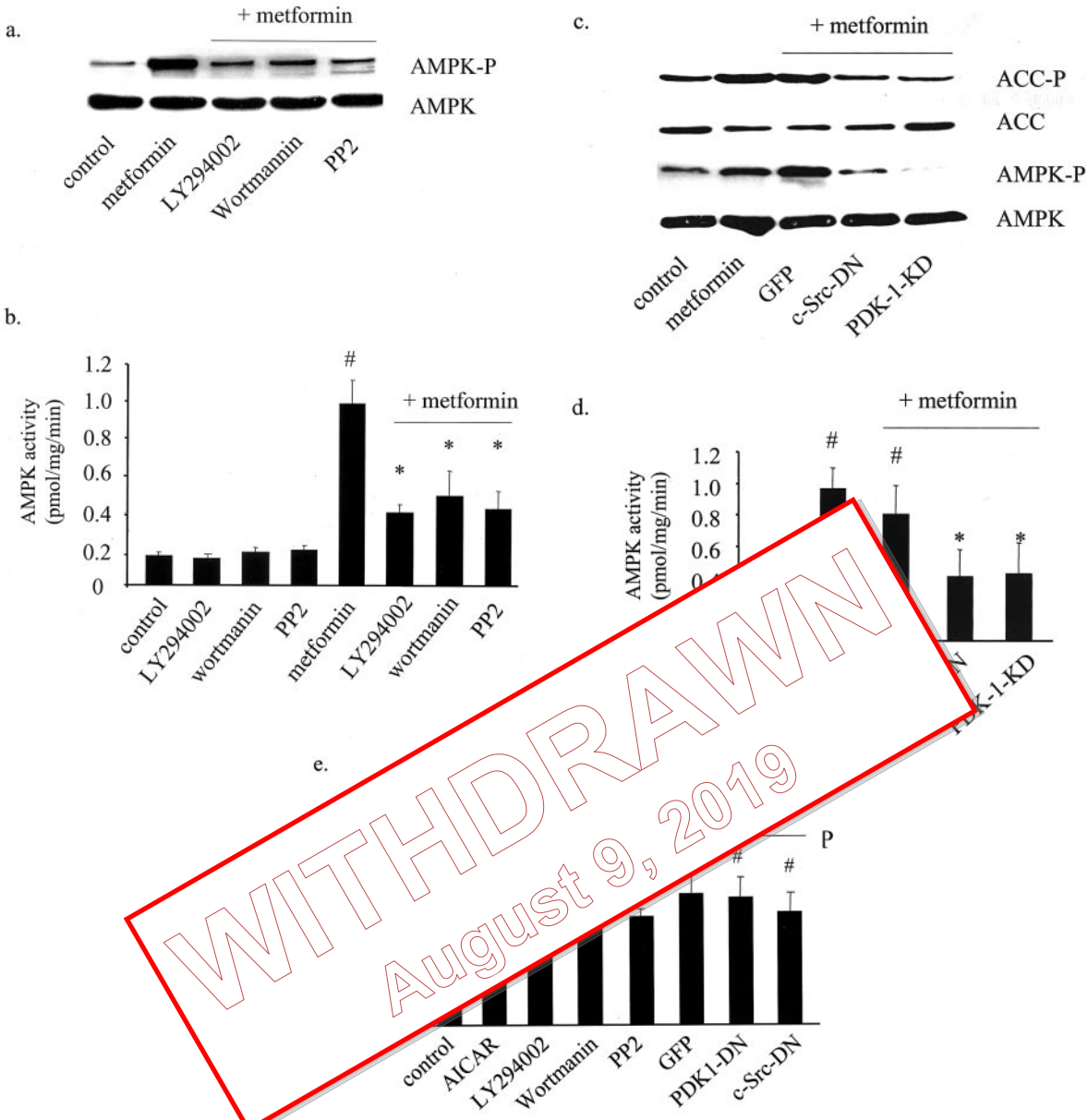

FIG. 2. **Activation of AMPK by metformin is c-Src-mediated and PI3K-dependent.** *a*, inhibition of c-Src with PP2 (10 μ[sca]m) or PI3K with wortmannin (100 nM) or LY294002 (50 μM) inhibited metformin (0.5 mM)-enhanced phosphorylations of AMPK and ACC. The blot is representative of five blots obtained from three independent experiments. *b*, inhibition of either c-Src or PI3K abolished metformin-enhanced AMPK activity ($n = 4$ or 5; #, $p < 0.05$, control *versus* metformin-treated; *, $p < 0.01$; metformin *versus* metformin plus either PP2, wortmannin, LY 294002, $n = 5$, *, $p < 0.01$). *c*, overexpression of either a kinase-inactive dominant negative mutant c-Src (c-Src-DN) or a kinase-dead PDK-1 (PDK1-KD), but not of a GFP control, blocked metformin-up-regulated phosphorylation of AMPK and ACC. This blot is representative of five blots obtained from five independent experiments (*d*). Overexpression of either PDK-1-KD or c-Src-DN, but not of a GFP control, also blocked metformin-up-regulated AMPK activity ($n = 6$, #, $p < 0.01$, control *versus* metformin; $n = 6$, *, $p < 0.01$, metformin *versus* metformin plus c-Src-DN or PDK-1-KD); *e*, inhibition of c-Src or PI3K did not alter AICAR-induced AMPK activation ($n = 6$, #, $p < 0.01$, control *versus* AICAR or AICAR with inhibitors).

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 Thr^{172} and ACC-Ser^{79} (Fig. 4*a*). 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. 4*a*). 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*). In contrast, protein level of mitochondrial F1Fo-ATPase α (encoded by the genomic DNA) was not affected (Fig. 4*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

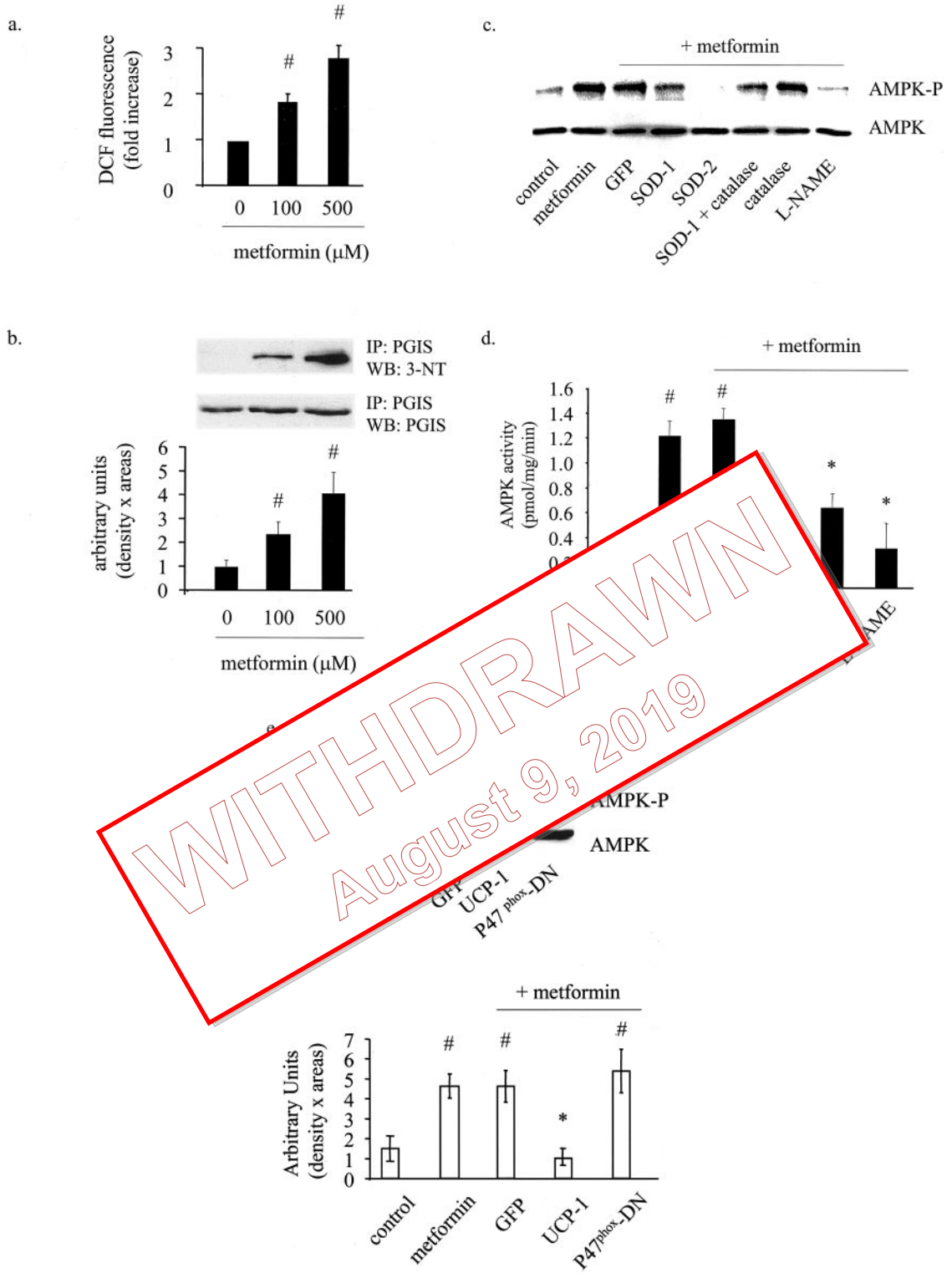

FIG. 3. **Activation of AMPK by metformin is both $ONOO^-$ and PI3K-dependent.** *a*, metformin enhances intracellular ROS generation. Intracellular ROS was detected by the DCF fluorescence as described under "Experimental Procedures." Exposure of BAECs to either 0.1 or 0.5 mM metformin significantly 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 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 (Thr^{172}) 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 $p47^{phox}$-DN, attenuated metformin-enhanced phosphorylations of AMPK-Thr^{172}. The *lower panel* gives the summary data ($n = 3$ or 4, #, $p < 0.05$ control *versus* metformin; $n = 3$ or 4, *, $p < 0.05$, metformin *versus* metformin plus UCP-1).

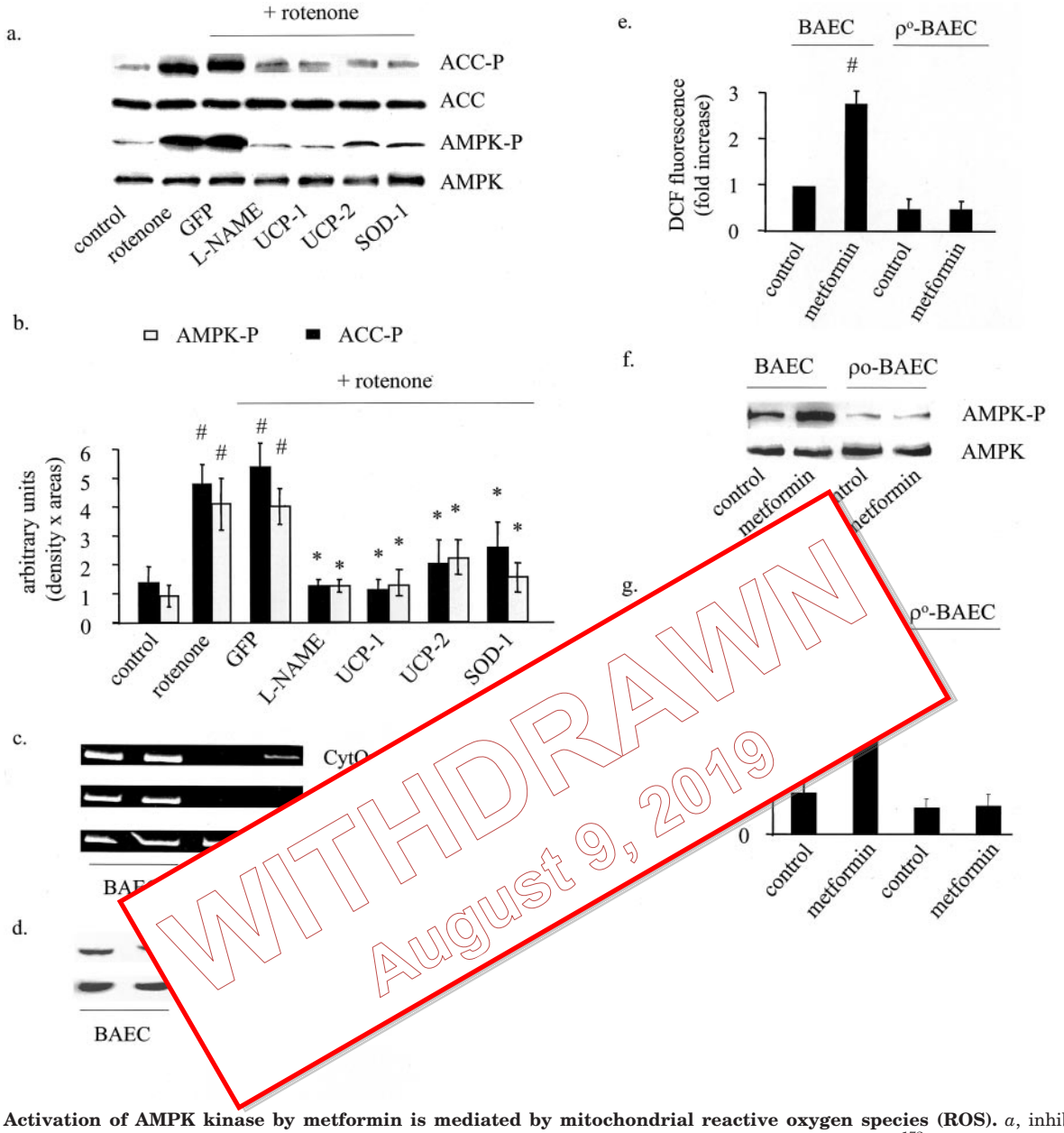

FIG. 4. **Activation of AMPK kinase by metformin is mediated by mitochondrial reactive oxygen species (ROS).** *a*, inhibition of complex I activates AMPK in BAECs. BAECs were exposed to rotenone (100 nM) for 30 min and AMPK, and AMPK-Thr^{172}-P were detected by using the specific antibodies. The blot is representative of three blots from independent experiments. *b*, the *lower panel* gives the summary data ($n = 3$, #, $p < 0.05$ control *versus* rotenone; *, $p < 0.05$, rotenone *versus* rotenone plus treatments. *c*, confluent monolayers of BAECs were incubated in EGM/2% fetal bovine serum alone or in media containing ethidium bromide (50 ng/ml), pyruvate (110 μg/ml), and uridine (50 μg/ml) for 3 weeks. After incubation, cells were harvested, total RNA were extracted, and mRNA for cytochrome oxidase (*CytOx*) were estimated by reverse transcription-PCR (30). The message for glyceraldehyde-3-phosphate dehydrogenase was used to ensure equal loading. Data are representative of four independent experiments. *d*, BAEC cells were treated as described above. After treatment, cells were lysed and proteins were separated by SDS-PAGE and Western blotted using the antibodies specific for the cytochrome oxidase II and F1F0ATPase α. The blot is representative of four independent experiments. *e*, metformin (0.5 mM) increased DCF fluorescence in wild type of BAECs but not in ρ^o-BAECs. *f*, metformin (0.5 mM) increased phosphorylation in BAECs but not in ρ^o-BAECs. The blot is representative of at least three blots obtained from independent experiments. *g*, metformin activates AMPK in BAECs but not in ρ^o-BAECs ($n = 5$, #, $p < 0.05$, control *versus* metformin).

ROS in ρ^o-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-Thr^{172} and AMPK activity in wild type of BAECs, affected neither the phosphorylation of AMPK-Thr^{172} nor AMPK activity in ρ^o-BAECs (Fig. 4, *f* and *g*). These observations further corroborated that metformin requires functional mitochondria to activate AMPK in endothelial cells. Because ρ^o-BAECs also failed to produce oxidants in response to metformin (Fig. 4*e*), 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-Thr^{172} phosphorylation and AMPK activity. As shown in Fig. 5 (*a* and *b*), administration of metformin significantly increased the phosphorylations of AMPK-Thr^{172} and ACC-Ser^{79} in C57BL6 mice but not in eNOS$^{-/-}$ mice, whereas AICAR activated AMPK-Thr^{172} 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*.

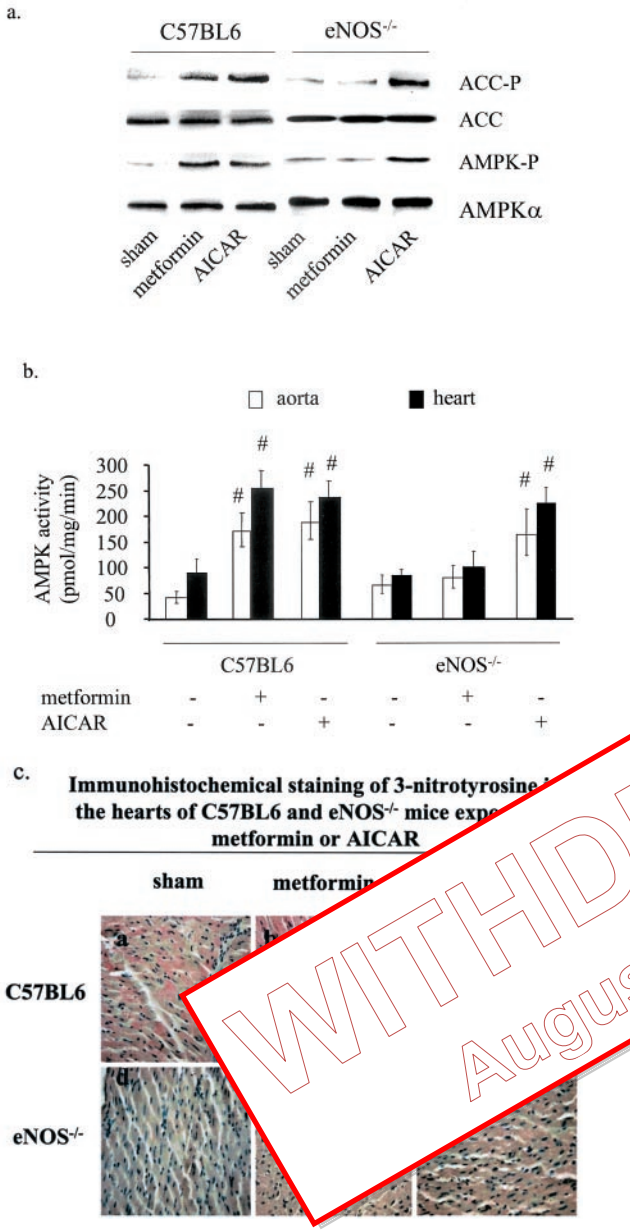

FIG. 5. **Activation of AMPK by metformin is ONOO$^-$-dependent *in vivo*.** Mice were treated with metformin or AICAR as described under "Experimental Procedures." Mice aorta and hearts were isolated and assayed for AMPK activity as described under "Experimental Procedures." *a*, administration of metformin or AICAR in mice increased phosphorylation of AMPK-Thr^{172} and ACC-Ser^{79}. Isolated aorta or hearts were homogenized as described under "Methods," and proteins were separated by SDS-PAGE and Western blotted with the antibodies against AMPK-P and ACC-P. Of note is that meformin increased AMPK-P and ACC-P in C57BL6 mice but not in eNOS$^{-/-}$ mice. The blot is representative of four independent experiments. *b*, metformin activates AMPK in C57BL6 but not in eNOS$^{-/-}$ *in vivo*. AMPK activity was assayed using ^{32}P-SAMS peptide, as described under "Experimental Procedures." Metformin significantly increased AMPK activity in aorta and hearts from C57BL6, but not in those from eNOS$^{-/-}$ mice. In contrast, AICAR activated AMPK in both C57BL6 and eNOS$^{-/-}$ mice ($n = 5$ or 6, #, $p < 0.01$, sham *versus* metformin). *c*, metformin increases 3-nitrotyrosine staining in C57BL6 mice but not in eNOS$^{-/-}$ mice. 3-Nitrotyrosine was detected by immunohistochemical staining with polyclonal antibody against 3-nitrotyrosine in the hearts from C57BL6 mice or eNOS$^{-/-}$ mice with or without metformin for 3 days. *a*, sham-treated C57BL6 mice; *b*, metformin-treated C57BL6 mice; *c*, C57BL6 mice treated with AICAR; *d*, sham-treated eNOS$^{-/-}$ mice; *e*, eNOS$^{-/-}$ mice exposed to metformin; *f*, eNOS$^{-/-}$ mice treated with AICAR. Of note is that metformin but not AICAR increased 3-nitrotyrosine in C57BL6. 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.

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. 5*a*). Metformin but not AICAR significantly increased 3-NT staining in C57BL6 mice (Fig. 5, *b* and *c*). In contrast, there was no 3-NT staining in the hearts isolated from sham-treated eNOS$^{-/-}$, 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 [illegible] studies (18, 19) have identified that LKB1 acts as an [illegible]PK upstream kinase, AMPK kinase. Exposure [illegible]549 cells (which lack LKB1) to either ON[illegible] not activate AMPK, although [illegible]hese cells.[2] These data ind[illegible]NOO$^-$-induced AMPK [illegible]ect upstream enzyme

[illegible]ased LKB1 activity *in* [illegible]pitated using a specific an- [illegible]Cruz Biotechnology) from heart [illegible]protein) isolated from C57BL6 and [illegible]f without metformin or AICAR and then [illegible]was assayed by using recombinant AMPK [illegible]substrate. As shown in Fig. 6*a*, neither metformin [illegible]AR altered LKB1 activity in mice treated with met- [illegible]min 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 Western blotted for AMPK, or *vice versa*. As shown in Fig. 6*b*, metformin significantly increased the co-immunoprecipitation of LKB1 and AMPK-α1 in C57BL6 mice but not in eNOS$^{-/-}$ mice. Metformin did not increase the co-immunoprecipitation of LKB1 with AMPK-α2 (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 ^{32}P-SAMS peptide phosphorylation without adding exogenous

[2] M.-H. Zou, B. J. Davis, S. S. Kirkpatrick, and J. S. Nelson, unpublished data.

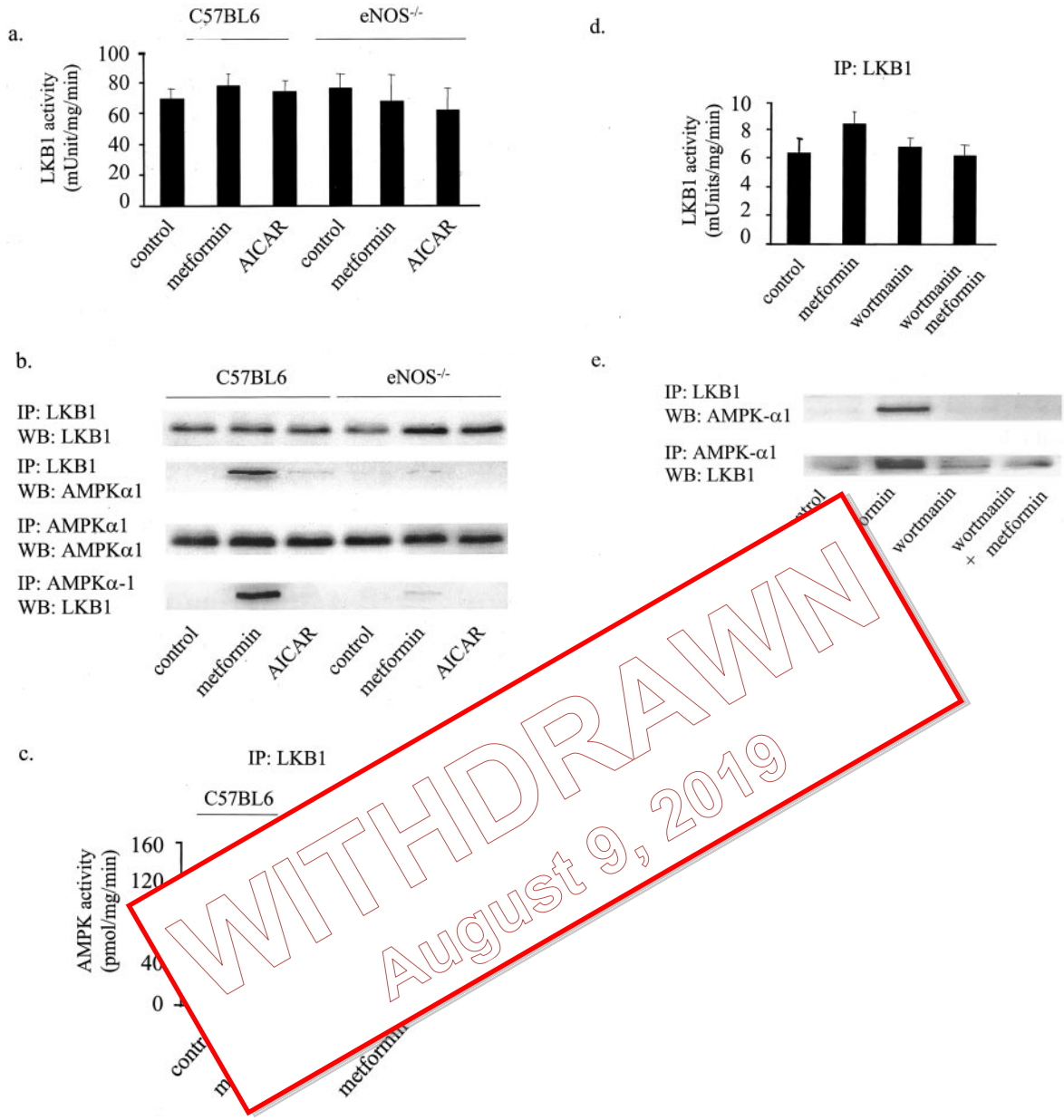

FIG. 6. **Metformin increases the interaction of LKB1 and AMPK.** *a*, metformin or AICAR did not alter LKB1 activity *in vivo* ($n = 7$). LKB1 was first immunoprecipitated with a specific antibody against LKB1 (Santa Cruz Biotechnology) from sham- or metformin-treated mice, and LKB1 activity was assayed by adding recombinant AMPK$\alpha 1\beta 1\gamma 1$, as described under "Methods." *b*, metformin but not AICAR increased co-immunoprecipitation of LKB1 and AMPK-α. To determine the interaction of LKB1 and AMPK, LKB1 was immunoprecipitated and stained for AMPK, or *vice versa*. The blots are representative of three obtained from three independent experiments. *c*, increased AMPK activity in the LKB1 immunoprecipitates in mice treated with metformin. LKB1 was first immunoprecipitated from the heart homogenates from sham-treated or metformin-treated mice. AMPK activity was assayed by the phosphorylation of the SAMS peptides without adding exogenous recombinant AMPK, as described under "Experimental Procedures." Compared with sham-treated mice, metformin significantly increased LKB1 co-immunoprecipitated AMPK activity in C57BL6 mice but not in eNOS$^{-/-}$ mice ($n = 5$, #, $p < 0.05$). *d*, metformin did not alter LKB1 activity in BAECs ($n = 5$). Confluent BAECs were exposed to metformin (0.5 mM) at 37 °C for 1 h. The cells were scraped and lysed as described under "Methods." LKB1 activity was assayed using recombinant AMPK, as described above. *e*, inhibition of PI3K with wortmannin attenuated metformin-enhanced co-immunoprecipitation of LKB1 and AMPK. Confluent BAECs were exposed to metformin (0.5 mM) at 37 °C for 1 h. The cells were scraped and lysed as described under "Methods." To study the interaction of LKB1 and AMPK, LKB1 was immunoprecipitated and Western blotted for AMPK or *vice versa*. Of that, inhibition of PI3K with wortmannin abolished metformin-enhanced co-immunoprecipitation of AMPK and LKB1. The blots are representative of three blots obtained from three individual experiments.

AMPK. Because the SAMS peptide is a specific substrate for AMPK but not LKB1, the increased ^{32}P-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. 6*d*) but increased co-immunoprecipitation of LKB1 and AMPK (Fig. 6*e*). 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-

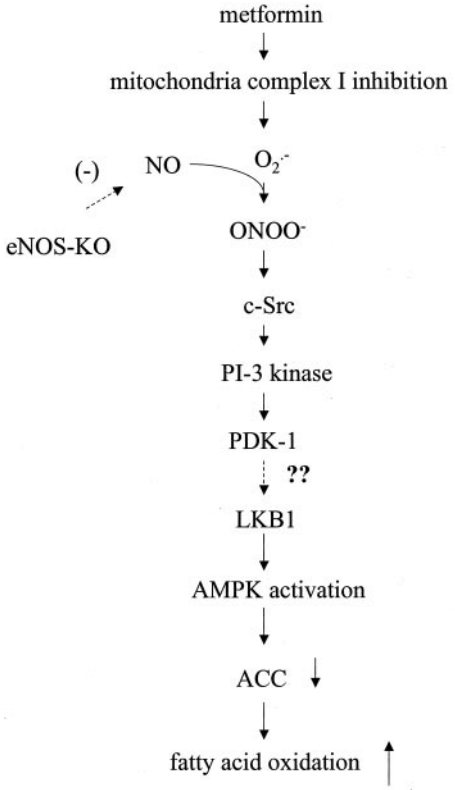

FIG. 7. **Activation of AMPK by metformin is RNS m… PI3K-dependent.** We hypothesize that inhibition… respiratory chain complex I by metformin will gen… with NO generated from mitochondrial or fr… form RNS such as ONOO⁻. ONOO⁻ activ… unknown molecule or a second mes… LKB1 to form an active complex…

formin or ONOO⁻ n… molecule that enhance… resulting a consequent a…

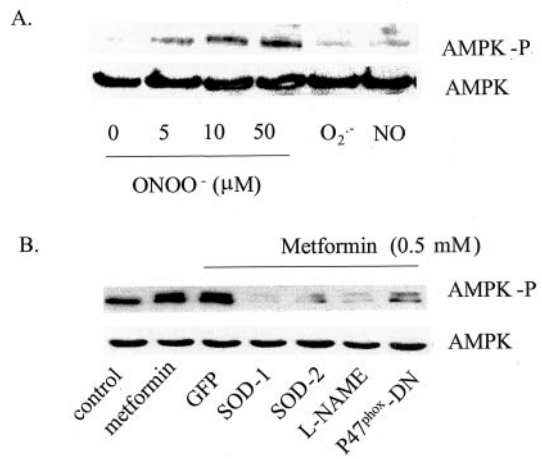

FIG. 8. **ONOO⁻-dependent AMPK activation in isolated rat cardiomyocytes, as described previously (41).** The sub-cultured myocytes were treated with authentic ONOO⁻ at concentrations indicated or with metformin. Phosphorylated AMPK (Thr^{172}) and total AMPK were assayed by using the sp… antibodies as described under "Experimental Procedures." … dose-dependently increased the phosphorylation of A… ontrast, $O_2^{\cdot-}$ (generated from 10 milliunits/ml xa… sence of 0.1 mM hypoxanthine) or NO (50 … two precursors of ONOO⁻, caused … ylation. *B*, metformin activa… h after isolation, myocytes … 1, SOD-2, $p47^{phox}$ domi… ^{phox}-DN), and L-NAME … ection, myocytes were … MPK-Thr^{172} and AMPK … ne specific antibodies. Overex… S by L-NAME blocked metformin… results suggest that metformin is able … ac myocyte, possibly mediated by ONOO⁻. … of those obtained from three separate …

…on (27) and addition of authentic ONOO⁻ did not affect … AMP/ATP ratio (28), the attenuation of both AMPK-Thr^{172} and ACC-Ser^{79} 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 $O_2^{\cdot-}$ from the respiratory chain (39–43). In the present study, inhibition of mitochondrial complex I with rotenone, like metformin, activated AMPK in BAECs. Overexpression of UCP proteins, which inhibit the electron transport and produce $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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^o-BAECs lacking functional mitochondria. Exposure of ρ^o-BAECs to metformin did not enhance intracellular ROS release. Because metformin did not activate AMPK in mitochondria-depleted ρ^o-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-Thr^{172} and ACC-Ser^{79}. Because metformin increased ROS in BAECs and ONOO⁻-activated AMPK via c-Src/PI3K-dependent pathways (28), these data

Previous studies have esta… formin exerts its therapeutic effects in diabetes, … partly via AMPK activation (11, 12). Although activati… 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, likely ONOO⁻, activates AMPK via a c-Src-mediated, PI3K-dependent pathway (Fig. 7).

The evidence supporting activation of AMPK by the increased formation of mitochondria-derived oxidants is severalfold. 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-Thr^{172}. Further, metformin increased RNS in C57B6 mice but not eNOS$^{-/-}$ mice, indicating that metformin increased RNS both *in vitro* and *in vivo*.

Second, inhibition of ONOO⁻ formation by overexpression of SOD (to scavenge $O_2^{\cdot-}$) 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 $O_2^{\cdot-}$ alone have no effect on AMPK

also suggest that metformin, like authentic $ONOO^-$, leads to AMPK activation via c-Src/PI3K-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. 8*A*), and further overexpression of SOD or inhibition of NOS by L-NA blocked metformin-induced AMPK activation (Fig. 8 results suggest that metformin is able to acti non-vascular cells such as cardiac myocytes pendent fashion. Finally, there is grow chondria constitutively express mi duces NO to regulate mitoc metabolism (44–46). Th from mitochondrial with mitochondria-de chondria, which diffuses to activate AMPK. There might delete both components of sulting in a loss of AMPK activation by and *g*). Other possibilities include that metfo also activate AMPK by hydrogen peroxide, because absence of NO O_2^- generated by the complex I inhibition can be converted into hydrogen peroxide by mitochondrial SOD, which is reported to activate AMPK by increasing AMP (47) and might help explain activation of AMPK by metformin in hepatocytes (11), cultured vascular smooth muscle cells,[2] or HeLa-S3 cells overexpressing LKB1 (18, 19). However, because the reaction of NO with O_2^- is at least three times faster than O_2^- dismutation by SOD (36–38), $ONOO^-$ will be predominantly factor formed in the presence of NO.

Another important finding of the present study is that we, for the first time, provide evidence that activation of PI3K by metformin or by $ONOO^-$ might enhance AMPK activity by increasing association of LKB1 with AMPK, although metformin did not alter LKB1 activity. Our finding that metformin increased AMPK without altering LKB1 activity was consistent with previous work in COS-7 (18), where LKB1 was not activated by phenformin or AICAR. How PI3K leads to the AMPK·LKB1 complex remains unknown. Our previous studies (27, 28) demonstrate that activation of PI3K is not sufficient to activate AMPK, and insulin or growth factors that stimulate the PI3K/PDK1 pathway either do not affect AMPK in most cell types, or in one or two cases have been reported to cause AMPK inhibition (48). In addition, AMPK activity is normal in PDK-1 (−/−) ES cells compared with PDK1 (+/+) control cells where the activity of several kinases of the AGC family is severely affected (49). Therefore, it is very likely that PI3K/PDK1 will not serve as the direct upstream enzyme for LKB1. However, it is possible that activation of PI3K by metformin or $ONOO^-$ might generate a metabolite or other molecule within the cell that binds to AMPK, which converts it into a better substrate for LKB1 to promote its activation by the LKB1 complex, similar to the regulation of Akt/PKB by PI3K-PDK1 (50). Our results, demonstrating that the inhibition of PI3K attenuated the association of LKB1 and AMPK, strongly support this hypothesis. Further experiments will be necessary to elucidate how PI3K increases the interaction of LKB1 and AMPK.

Our previous work has also demonstrated that high concentrations of $ONOO^-$ (>100 μM) uncouple eNOS (36) and inactivate prostacyclin synthase (37, 38) in diabetes. In addition, we have found that low concentrations of $ONOO^-$ (1–10 μM) activate AMPK and increase AMPK-dependent eNOS-Ser^{1177} phosphorylation (27, 28). Thus, the concentrations of $ONOO^-$ required for AMPK activation are at least 10-fold lower than those needed for eNOS u ing and PGIS inhibition, indicating that $ONOO^-$ tially activate AMPK. In addition, we have reatment with the AMPK activators, b n, prevents oxidant stress such as cells exposed to 30 mM gl that sub-toxic concen- relevant concentra- condition" the cells, ve oxidant stress trig- e plasma concentrations of with the drug are estimated to be ncentrations of metformin are clearly s of $ONOO^-$ formation, which will im- function by AMPK activation instead of in- elial cells.

ummary, we demonstrate for the first time that met- min increases mitochondria-derived $ONOO^-$ to activate AMPK in c-Src/PI3K-dependent manners. Activation of AMPK by metformin might contribute to the beneficial effects of metformin in treating diabetes.

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