

Corrections

BIOCHEMISTRY

Correction for "Endothelial nitric oxide synthase negatively regulates hydrogen peroxide-stimulated AMP-activated protein kinase in endothelial cells," by Benjamin Y. Jin, Juliano L. Sartoretto, Vadim N. Gladyshev, and Thomas Michel, which appeared in issue 41, October 13, 2009, of *Proc Natl Acad Sci USA* (106:17343–17348; first published September 25, 2009; 10.1073/pnas.0907409106).

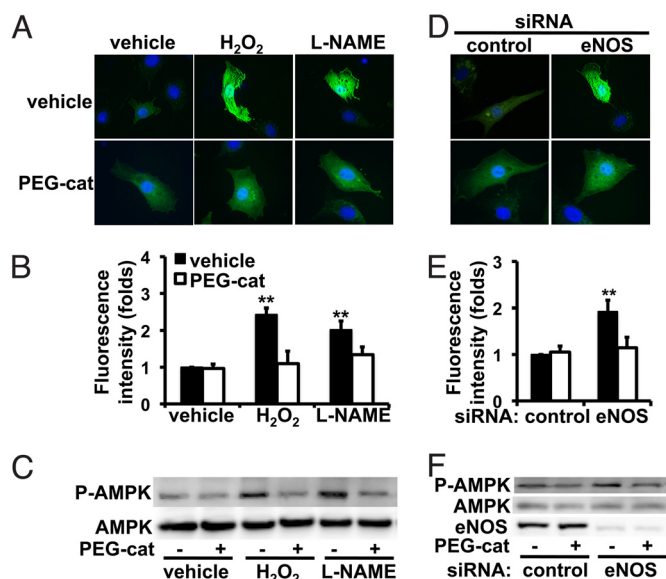


Fig. 5. Intracellular H₂O₂ detection by HyPer: Effects of eNOS inhibition and reversal by PEG-catalase. Endothelial cells were transfected with the HyPer plasmid (28), and single cell images were obtained 48 h later; cells were incubated with PEG-catalase or vehicle, as noted, and processed either for imaging or immunoblot analyses. (A–C) Results of treatments with H₂O₂ (200 μ M) or L-NAME (100 μ M) following incubation of cells with PEG-catalase or vehicle. The results in D–F are from endothelial cells transfected with control or eNOS siRNA constructs and then treated with PEG-catalase or vehicle, as shown. (A and D) Representative images of HyPer-transfected cells that were treated as shown, then fixed and stained with Hoechst 33342, and analyzed for HyPer fluorescence. (B and E) Pooled data from 15 individual HyPer transfected cells from three experiments, quantitated for the determination of fluorescence intensity using MetaMorph software. (C and F) Representative immunoblot experiments in which endothelial cells were treated with PEG-catalase or vehicle, and either treated with L-NAME or H₂O₂ (C) or transfected with control or eNOS siRNA (F) as described in the text, and then analyzed in immunoblots probed with antibodies as shown. **, $P < 0.01$.

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The authors note that, due to a printer's error, Figs. 5 and 6 appeared incorrectly. Fig. 5 appeared with incorrect labels and without error bars or graph lines. Fig. 6 appeared with incorrect labels and with a panel C that was included in error. In addition, the legend did not accurately define the P values indicated by asterisks. These errors do not affect the conclusions of the article. The corrected figures and legends appear below.

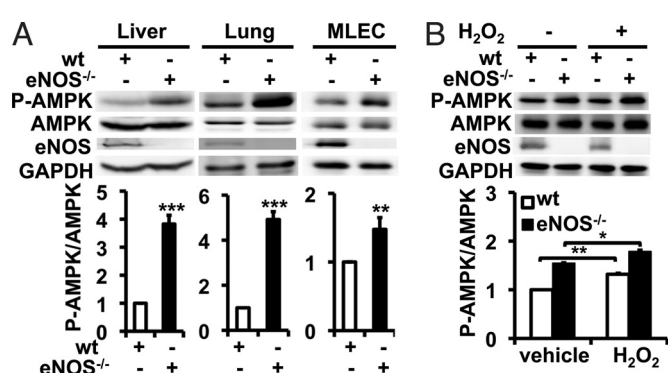


Fig. 6. AMPK phosphorylation in tissues and cells from eNOS^{-/-} mice. This figure shows immunoblot analyses of liver, lung, or endothelial cells from wild-type (wt) or eNOS^{-/-} mice. (A) Liver, lung tissues, and isolated lung endothelial cells (MLECs) from wild-type and eNOS^{-/-} mice were analyzed in immunoblots probed with antibodies as indicated. Shown below are pooled data from at least three experiments quantitating AMPK phosphorylation. (B) MLECs from wild-type or eNOS^{-/-} mice were treated with H₂O₂ (100 μ M for 30 min). The experiment shown is a representative of five similar experiments showing that MLECs from eNOS^{-/-} mice have increased basal as well as H₂O₂-stimulated AMPK phosphorylation relative to MLECs from wild-type mice; pooled data are also shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Endothelial nitric oxide synthase negatively regulates hydrogen peroxide-stimulated AMP-activated protein kinase in endothelial cells

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Hydrogen peroxide and other reactive oxygen species are intimately involved in endothelial cell signaling. In many cell types, the AMP-activated protein kinase (AMPK) has been implicated in the control of metabolic responses, but the role of endothelial cell redox signaling in the modulation of AMPK remains to be completely defined. We used RNA interference and pharmacological methods to establish that H₂O₂ is a critical activator of AMPK in cultured bovine aortic endothelial cells (BAECs). H₂O₂ treatment of BAECs rapidly and significantly increases the phosphorylation of AMPK. The EC₅₀ for H₂O₂-promoted phosphorylation of AMPK is 65 ± 15 μM, within the physiological range of cellular H₂O₂ concentrations. The Ca²⁺/calmodulin-dependent protein kinase kinase-β (CaMKKβ) inhibitor STO-609 abolishes H₂O₂-dependent AMPK activation, whereas eNOS inhibitors enhance AMPK activation. Similarly, siRNA-mediated knockdown of CaMKKβ abrogates AMPK activation, whereas siRNA-mediated knockdown of eNOS leads to a striking increase in AMPK phosphorylation. Cellular imaging studies using the H₂O₂ biosensor HyPer show that siRNA-mediated eNOS knockdown leads to a marked increase in intracellular H₂O₂ generation, which is blocked by PEG-catalase. eNOS^{-/-} mice show a marked increase in AMPK phosphorylation in liver and lung compared to wild-type mice. Lung endothelial cells from eNOS^{-/-} mice also show a significant increase in AMPK phosphorylation. Taken together, these results establish that CaMKKβ is critically involved in mediating the phosphorylation of AMPK promoted by H₂O₂ in endothelial cells, and document that eNOS is an important negative regulator of AMPK phosphorylation and intracellular H₂O₂ generation in endothelial cells.

eNOS | signal transduction

The term “reactive oxygen species” (ROS) is used to describe a class of molecules capable of oxidizing molecular targets in cells and tissues. ROS modulate both physiological and pathophysiological responses, and increased production of ROS is implicated in cardiovascular disease states (1, 2). Hydrogen peroxide (H₂O₂) is a stable cell-permeant ROS that modulates diverse endothelial cell processes, including vascular remodeling and endothelium-regulated vasorelaxation (1, 3, 4). The molecular mechanisms of H₂O₂-dependent modulation of these endothelial cell responses are incompletely understood. Basal intracellular levels of H₂O₂, typically in the range of 25–75 μM (5), are involved in normal processes of endothelial cell function (6). However, under pathological conditions, excessive ROS production has detrimental consequences in the vascular wall (7).

In many cells, H₂O₂-induced cellular responses involve the modulation of protein kinase signaling pathways (8, 9). AMPK is a heterotrimeric serine/threonine kinase (10) that can be activated by a rise in the AMP/ATP ratio; activated AMPK may phosphorylate metabolic enzymes that switch on ATP-generating catabolic pathways and switch off anabolic pathways. However, AMP-dependent activation of AMPK is only part of the regulatory pathway: full AMPK activation requires the phosphorylation of the enzyme at threonine-172 in the α subunit (11). At least two AMPK-activating kinases have been identified: the tumor suppressor kinase LKB1 and the Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKKβ) (12, 13).

The regulation of AMPK has been most thoroughly studied in “metabolic” tissues such as adipose tissue, muscle, and liver. More recently, AMPK has been characterized in vascular endothelial cells. AMPK in endothelial cells plays a role in cell energy flux (14), apoptosis (15), and regulation of inflammation, angiogenesis, and tissue perfusion (16). Endothelial AMPK is activated by a broad range of stimuli, including hypoxia (17); metformin, peroxynitrite (18), and adiponectin (19); and vasoactive mediators such as S1P, VEGF (20, 21), bradykinin (29), or thrombin (22). The link between AMPK and eNOS activation has been extensively studied. Kemp and colleagues first showed that AMPK phosphorylates eNOS at serine-1177, leading to enzyme activation (23). NO formation has been implicated in the angiogenic effects of AMPK (24). Several studies reported that H₂O₂ promotes eNOS activation and phosphorylation in endothelial cells—but without implicating AMPK—and yet other studies reported that H₂O₂ treatment promotes AMPK phosphorylation, but without implicating eNOS (13, 25, 26). Thus, multiple reports have suggested intriguing correlations and complex interactions between H₂O₂, AMPK, and eNOS activation pathways, yet the relationship between AMPK, H₂O₂, and eNOS remains incompletely understood.

In the present studies, we investigate the signaling mechanisms involved in AMPK activation in endothelial cells stimulated with H₂O₂. We demonstrate that H₂O₂ activates AMPK in endothelial cells, and establish that CaMKKβ is the key upstream kinase responsible for H₂O₂-dependent AMPK phosphorylation and endothelial tube formation. Importantly, we identify a role of eNOS in regulating AMPK phosphorylation and activation: eNOS negatively modulates AMPK phosphorylation both in cultured endothelial cells and in tissues from eNOS^{-/-} mice. These studies establish stimulatory and inhibitory links between H₂O₂, CaMKKβ, AMPK, and eNOS signaling pathways in the vascular endothelium.

H₂O₂-Mediated AMPK Activation. The effects of H₂O₂ on phosphorylation of AMPK and its substrate acetyl CoA carboxylase (ACC) in BAECs are shown in Fig. 1A. BAECs were treated for 30 min with varying concentrations of H₂O₂, and cell lysates were analyzed in immunoblots probed with antibodies against phospho-AMPK, total AMPK, phospho-ACC, or total ACC. H₂O₂ treatment increases AMPK and ACC phosphorylation in a dose-dependent manner; total AMPK or ACC abundance does not change, and there is no change in cell viability (Fig. S1). Fig. 1B and Fig. S2A shows pooled data from five similar dose-response experiments quantitating AMPK and ACC phosphorylation, respectively; the EC₅₀ for H₂O₂-promoted AMPK phosphorylation is 65 ± 15 μM, a value close to the physiological H₂O₂

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The authors declare no conflict of interest.

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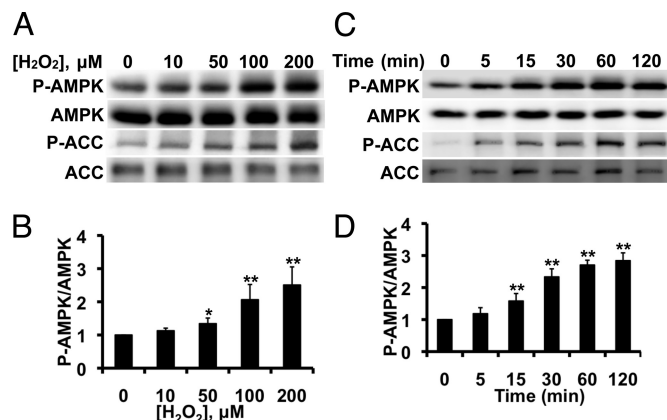


Fig. 1. H_2O_2 -mediated AMPK phosphorylation in endothelial cells. Shown in this figure are the results of immunoblots analyzed in endothelial cells treated with H_2O_2 . (A) Representative immunoblot from a dose-response experiment analyzed in cells stimulated with the indicated concentrations of H_2O_2 for 30 min and probed with antibodies as shown; (B) pooled data from five independent experiments, analyzing the intensities corresponding to phospho-AMPK and total AMPK by quantitative chemiluminescence. (C) Representative time course experiment in BAECs treated with 200 μM H_2O_2 for the indicated times and analyzed in immunoblots probed with antibodies as shown; (D) pooled data from five independent experiments. *, $P < 0.05$, and **, $P < 0.01$ by ANOVA.

concentration in these cells (6). Fig. 1C shows a time course of H_2O_2 -induced phosphorylation of AMPK and ACC, and Fig. 1D and Fig. S2B shows the quantitative analysis of pooled data from five similar experiments. Within 5 min after addition of H_2O_2 (200 μM) to BAECs, AMPK and ACC phosphorylation increase significantly, and this signal is sustained for at least 120 min. Furthermore, as previously reported (25), we found that H_2O_2 promoted phosphorylation of eNOS at serine-1179 (Fig. S2C–F).

Effects of Protein Kinase or NOS Inhibitors on H_2O_2 -Mediated Phosphorylation Responses. Fig. 2 shows experiments analyzing H_2O_2 -induced

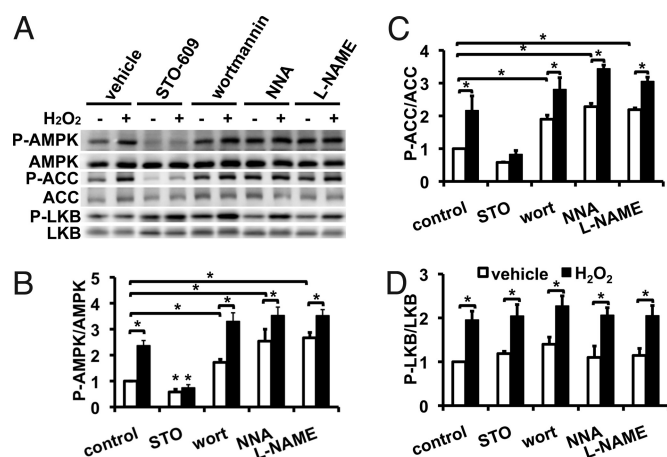


Fig. 2. Effects of protein kinase inhibitors and NOS inhibitors on H_2O_2 -induced AMPK phosphorylation. (A) Representative immunoblot analyzed in endothelial cells treated with H_2O_2 (200 μM , 30 min) after first being incubated for 30 min with inhibitors as shown: STO-609 (CaMKK β inhibitor, 10 $\mu\text{g}/\text{mL}$); wortmannin (PI3-kinase inhibitor, 10 μM); N-nitro-L-arginine (NNA, NOS inhibitor 10 μM); or L-arginine methyl ester (L-NAME, NOS inhibitor, 100 μM). Cell lysates were subjected to immunoblotting using antibodies as shown. (B–D) Quantitative analyses of pooled data from three to five independent experiments, analyzing the phosphorylation responses for AMPK, ACC, and LKB1, respectively.

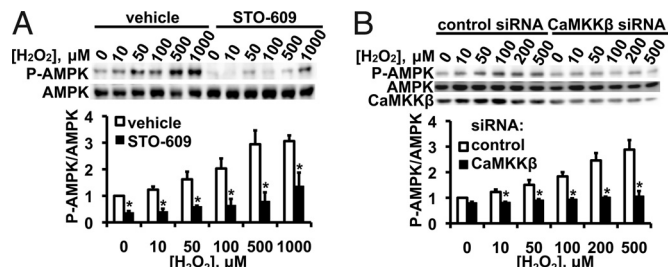


Fig. 3. CaMKK β inhibitor STO-609 and siRNA-mediated CaMKK β knockdown: Effects on H_2O_2 -stimulated AMPK phosphorylation. This figure shows the results of dose-response experiments exploring AMPK phosphorylation in BAECs treated with the CaMKK β inhibitor STO-609 (A), or transfected with CaMKK β or control siRNA (B). The cells were then incubated for 30 min with the indicated concentrations of H_2O_2 . For each panel, an immunoblot from a representative experiment is shown above; the results from pooled data analyzed by quantitative chemiluminescence are shown below; *, $P < 0.05$.

AMPK phosphorylation in BAECs treated with the CaMKK β inhibitor STO-609, with the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin, or with the NOS inhibitors N-nitro-L-arginine (NNA) or L-arginine methyl ester (L-NAME). STO-609 suppresses basal phosphorylation of AMPK and ACC and effectively abrogates H_2O_2 -stimulated phosphorylation of AMPK and ACC ($n = 5$, $P < 0.05$); however, STO-609 does not block H_2O_2 -promoted Akt phosphorylation (Fig. S3). The PI3K inhibitor wortmannin induces a small but statistically significant increase in basal and H_2O_2 -mediated AMPK and ACC phosphorylation ($n = 3$, $P < 0.05$). While wortmannin does not inhibit H_2O_2 -promoted AMPK phosphorylation, wortmannin blocks H_2O_2 -promoted Akt phosphorylation, as previously reported (25; Fig. S3). As shown in Fig. 2, NOS inhibitors significantly increase basal AMPK and ACC phosphorylation (2.1 ± 0.2 -fold increase, $n = 6$, $P < 0.05$), to the point that there was only nominal additional phosphorylation when H_2O_2 was added (Fig. 2). We explored the effects of STO-609, wortmannin, NNA, and L-NAME on phosphorylation of another AMPK kinase LKB1. As shown in Fig. 2A, STO-609 treatment had no effect on LKB1 phosphorylation, while suppressing AMPK phosphorylation; LKB1 phosphorylation was unaffected by the NOS inhibitors and by wortmannin.

Effects of siRNA-Mediated Knockdown of CaMKK β on H_2O_2 -Mediated Phosphorylation Responses. Fig. 3A shows the H_2O_2 dose response for AMPK phosphorylation in the presence and absence of the CaMKK β inhibitor STO-609. At H_2O_2 concentrations up to 500 μM , AMPK phosphorylation is abrogated by STO-609. At a H_2O_2 concentration of 1 mM, some AMPK phosphorylation was seen despite the presence of STO-609, suggesting that other mechanisms for AMPK phosphorylation may come into play at high H_2O_2 concentrations. We next performed experiments using CaMKK β siRNA, and found that siRNA-mediated knockdown of CaMKK β blocked AMPK phosphorylation in response to H_2O_2 (Fig. 3B).

Roles of eNOS in AMPK Activation. siRNA-mediated knockdown of eNOS led to a marked increase in AMPK phosphorylation (Fig. 4): there was a 3.3 ± 0.3 -fold increase in basal AMPK phosphorylation ($n = 4$, $P < 0.01$), to the point that only a nominal (albeit statistically significant) increase in AMPK phosphorylation was seen with the addition of H_2O_2 . Cells treated with eNOS inhibitors show robust AMPK phosphorylation, to the point that there is only a small increase in phosphorylation following addition of H_2O_2 (Fig. 4B). However, siRNA-mediated CaMKK β knockdown attenuates the NOS inhibitor-mediated increase in AMPK phosphorylation (Fig. 4B), suggesting that eNOS-dependent AMPK activation requires CaMKK β .

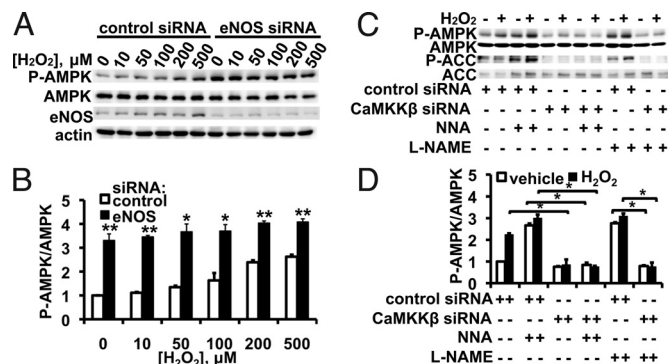


Fig. 4. siRNA-mediated eNOS knockdown and H_2O_2 -mediated AMPK phosphorylation. In the experiment shown in (A) endothelial cells were transfected with control or eNOS siRNA; 48 h after transfection, cells were treated with indicated concentrations of H_2O_2 for 30 min. The blot shown is a representative of five similarly designed experiments that yielded equivalent results. (B) Endothelial cells were transfected with control siRNA or with siRNA targeting CaMKK β ; 48 h after transfection, cells were first treated with vehicle or with the NOS inhibitors NNA (10 μ M) or L-NAME (100 μ M) for 30 min, and incubated with H_2O_2 (200 μ M for 30 min) or vehicle as indicated. The blot shown is a representative of five similar experiments.

Intracellular H_2O_2 in AMPK Activation. Cellular imaging approaches to detect intracellular H_2O_2 used the H_2O_2 biosensor, HyPer (28), transfected into BAECs (Fig. 5). Exogenous H_2O_2 causes an

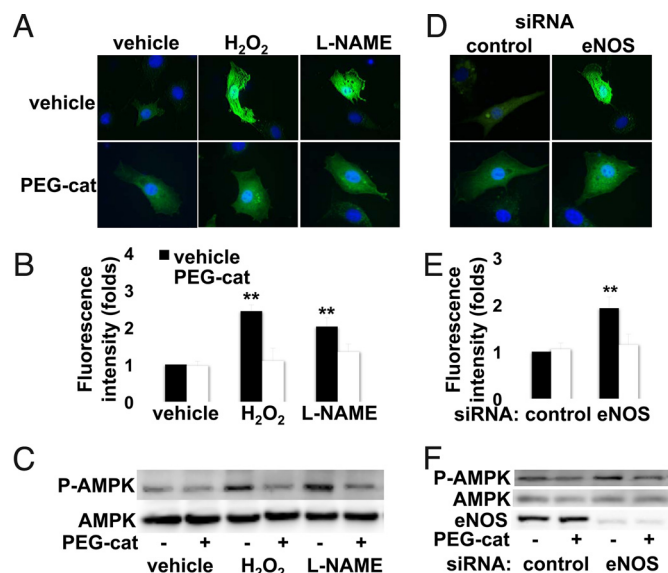


Fig. 5. Intracellular H_2O_2 detection by HyPer: Effects of eNOS inhibition and reversal by PEG-catalase. Endothelial cells were transfected with the HyPer plasmid (28), and single cell images were obtained 48 h later; cells were incubated with PEG-catalase or vehicle, as noted, and processed either for imaging or immunoblot analyses. (A–C) Results of treatments with H_2O_2 (200 μ M) or L-NAME (100 μ M) following incubation of cells with PEG-catalase or vehicle. The results in (D–F) are from endothelial cells transfected with control or eNOS siRNA constructs and then treated with PEG-catalase or vehicle, as shown. (A and D) Representative images of HyPer-transfected cells that were treated as shown, then fixed and stained with Hoechst 33342, and analyzed for HyPer fluorescence. (B and E) Pooled data from 15 individual HyPer transfected cells from three experiments, quantitated for the determination of fluorescence intensity using MetaMorph software. (C and F) Representative immunoblot experiments in which endothelial cells were treated with PEG-catalase or vehicle, and either treated with L-NAME or H_2O_2 (C) or transfected with control or eNOS siRNA (F) as described in the text, and then analyzed in immunoblots probed with antibodies as shown. **, $P < 0.01$.

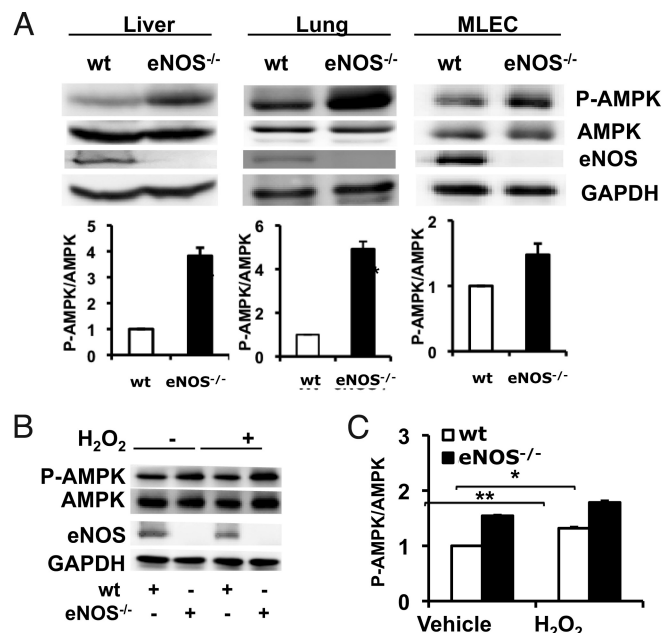


Fig. 6. AMPK phosphorylation in tissues and cells from eNOS $^{-/-}$ mice. This figure shows immunoblot analyses of liver, lung, or endothelial cells from wild-type (wt) or eNOS $^{-/-}$ mice. (A) Liver, lung tissues and isolated lung endothelial cells (MLECs) from wild-type and eNOS $^{-/-}$ mice were analyzed in immunoblots probed with antibodies as indicated. Shown below are pooled data from at least three experiments quantitating AMPK phosphorylation; ***, $P < 0.001$. (B) MLECs from wild-type or eNOS $^{-/-}$ mice were treated with H_2O_2 (100 μ M for 30 min). The experiment shown is a representative of five similar experiments showing that MLECs from eNOS $^{-/-}$ mice have increased basal as well as H_2O_2 -stimulated AMPK phosphorylation relative to MLECs from wild-type mice; pooled data are also shown, *, $P < 0.05$.

approximate 2.5-fold increase in fluorescence in HyPer-transfected endothelial cells relative to vehicle-treated HyPer-transfected cells ($P < 0.01$). We next used HyPer to detect endogenous production of H_2O_2 . Treatment of BAECs with the NOS inhibitor L-NAME leads to a striking 2.0-fold increase in intracellular H_2O_2 production (Fig. 5). Importantly, siRNA-mediated eNOS knockdown increases the production of H_2O_2 to a similar magnitude (Fig. 5). These same interventions (L-NAME treatment and siRNA-mediated eNOS knockdown) also similarly increase AMPK phosphorylation (Fig. 4). Conversely, treatment of endothelial cells with PEG-catalase to degrade intracellular H_2O_2 (27) suppresses both the increase in H_2O_2 generation (Fig. 5 A, B, D, and E) as well as the increase in AMPK phosphorylation (Fig. 5 C and F) that are seen after eNOS inhibition with L-NAME (Fig. 5 A–C) or following siRNA-mediated eNOS knockdown (Fig. 5 D–F). Cells cultured in high glucose media are known to show an increase in AMPK phosphorylation as well as an increase in reactive oxygen species, including H_2O_2 , relative to cells cultured in physiological levels of glucose (1, 2). Using the HyPer biosensor to detect intracellular H_2O_2 , we found that BAECs cultured in high glucose (30 mM) had elevated levels of H_2O_2 compared to cells cultured in 5 mM glucose, associated with an increase in AMPK phosphorylation (Fig. S4).

AMPK Activation in Tissues and Cells from eNOS $^{-/-}$ Mice. We next studied AMPK phosphorylation and expression in tissues and cells isolated from wild type and eNOS $^{-/-}$ mice. In the liver and lung of eNOS $^{-/-}$ mice, AMPK phosphorylation is strikingly increased compared to the level of AMPK phosphorylation seen in corresponding tissues of wild-type mice, with no change in overall levels of AMPK expression (Fig. 6). In lung tissue of eNOS $^{-/-}$ mice, AMPK phosphorylation is increased by 4.9 ± 0.3 -fold compared to wild-type littermates

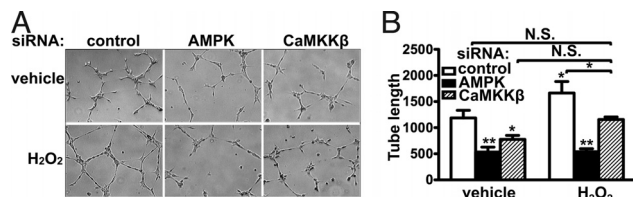


Fig. 7. siRNA-mediated knockdown of AMPK and CaMKK β impairs H₂O₂-induced endothelial cell tube formation. Endothelial cells were transfected with siRNA constructs targeting AMPK or CaMKK β , and analyzed in the Matrigel tube formation assay. (A) Representative images of endothelial Matrigel tube formation in H₂O₂-treated endothelial cells transfected with siRNA constructs as shown. (B) Pooled data from three independent tube formation experiments, plotting the total normalized tube length relative to the total tube length as measured for untreated endothelial cells transfected with control siRNA. *, $P < 0.05$, **, $P < 0.01$.

($n = 3$, $P < 0.001$). There is also a 3.8 ± 0.3 -fold increase in AMPK phosphorylation in the liver of eNOS $^{-/-}$ mice compared to wild-type mice ($n = 3$, $P < 0.001$). In contrast to the increase in AMPK phosphorylation in liver and lung, in several other tissues we examined, including arterial preparations from aorta and carotid artery, heart, brown fat, and white fat, we found no difference in AMPK phosphorylation or expression in eNOS $^{-/-}$ mice compared to wild-type animals. As shown in Fig. 6, AMPK phosphorylation in lung endothelial cells cultured from eNOS $^{-/-}$ mice is significantly increased compared to wild-type mice (1.5 ± 0.1 -fold increase, $n = 3$, $P < 0.01$). H₂O₂-induced AMPK phosphorylation also is enhanced in lung endothelial cells isolated from eNOS $^{-/-}$ mice compared to cells from wild-type mice ($n = 3$, $P < 0.05$; Fig. 6B and C).

The Role of AMPK and CaMKK β in Endothelial Cell Tube Formation. H₂O₂ has been reported to play a role in angiogenesis (29). We investigated the effects of siRNA-mediated knockdown of AMPK and CaMKK β on H₂O₂ responses in the Matrigel tube formation assay, which is commonly used as an index of angiogenesis (30). H₂O₂ treatment enhances endothelial tube formation. siRNA-mediated knockdown of AMPK or CaMKK β suppresses basal endothelial tube formation and blocks the response to H₂O₂; control siRNA is without effect (Fig. 7A). Quantitative analyses of tube formation (20) confirm that siRNA-mediated knockdown of AMPK or CaMKK β reduces both basal and H₂O₂-stimulated endothelial tube formation (Fig. 7B).

Discussion

These studies have explored the endothelial cell signaling pathways modulated by H₂O₂ in the context of AMPK regulation. Reactive oxygen species are produced by diverse vascular cells (31–33). The production of superoxide anion (O₂ $^{\cdot-}$) in the vasculature has been extensively analyzed, yet the short half-life and small radius of diffusion of O₂ $^{\cdot-}$ limit its role as an important paracrine agent in vascular biology (1, 3). The O₂ $^{\cdot-}$ metabolite H₂O₂ is an important cellular signaling agent that modulates diverse aspects of endothelial cell physiology and pathophysiology (1, 3, 34). H₂O₂ has been reported to activate AMPK by inducing oxidative stress (13, 27), and H₂O₂ is involved in some receptor-mediated pathways of AMPK activation (35). We assessed AMPK activation in endothelial cells by measuring AMPK phosphorylation and by quantitating phosphorylation of the AMPK substrate ACC (Fig. 1). Under all conditions, the level of AMPK phosphorylation paralleled the phosphorylation of its substrate ACC, indicating that phosphorylation of AMPK can serve as an effective marker for enzyme activation. We found that H₂O₂ induces AMPK phosphorylation in a time-dependent and dose-dependent manner, with an EC₅₀ value of 65 ± 15 μ M, a concentration in the range of physiological concentrations of H₂O₂ (6). Following siRNA-mediated knockdown of AMPK or CaMKK β , H₂O₂ treatment no longer increases

endothelial tube formation (Fig. 7), suggesting that H₂O₂-modulated angiogenic responses can be modulated by the CaMKK β /AMPK pathway.

Two separate lines of evidence establish a key role for CaMKK β in AMPK activation by H₂O₂. Pharmacological inhibition of CaMKK β by STO-609 completely abolishes H₂O₂-induced AMPK activation (Fig. 2). In addition, siRNA-mediated downregulation of CaMKK β suppresses H₂O₂-induced AMPK activation (Fig. 3). These findings suggest that CaMKK β is critically involved in modulating H₂O₂-induced AMPK activation. We also found that phosphorylation of the AMPK kinase LKB1 is increased by H₂O₂ treatment (Fig. 2). However, the H₂O₂-promoted increase in LKB1 phosphorylation is not blocked by the CaMKK β inhibitor STO-609 under conditions where H₂O₂-promoted AMPK phosphorylation is completely blocked. Taken together, these findings argue against a central role for LKB1 in H₂O₂-induced AMPK phosphorylation.

The mechanisms whereby H₂O₂ modulates CaMKK β remain to be completely defined. H₂O₂-induced oxidative stress may lead to an increased level of intracellular AMP, leading to AMPK phosphorylation. However, AMP has no effect on the activity of CaMKK β (13), so our finding that CaMKK β inhibition abolishes H₂O₂-stimulated AMPK activation suggests that H₂O₂-induced AMPK phosphorylation does not importantly involve changes in AMP levels, at least at physiological levels of H₂O₂ (Fig. 3). The signaling pathways leading to activation of CaMKK β are incompletely understood, although a role for calcium-calmodulin has been established. A growing literature on protein kinase regulation has identified redox-active cysteine thiols as critical determinants of the activity of some kinases (4, 8). For example, the cGMP-dependent protein kinase undergoes oxidation at key thiol residues, leading to kinase activation independent of cGMP (4, 8). An intriguing if speculative hypothesis in the context of these studies is that redox-active cysteine thiols in CaMKK β might undergo reversible oxidation as well as S-nitrosylation, each modification with opposing effects on CaMKK β activity.

eNOS is a Ca²⁺/calmodulin-dependent enzyme that is regulated by phosphorylation at multiple residues (23, 36). AMPK is one of several kinases that stimulate eNOS phosphorylation (17, 20, 37). In contrast to the inhibitory effects of the CaMKK β inhibitor STO-609 on AMPK activation, the PI3K inhibitor wortmannin failed to suppress AMPK phosphorylation (Fig. 2). Treatment of endothelial cells with NOS inhibitors leads to a significant increase in basal AMPK and ACC phosphorylation (Fig. 2), suggesting that blockade of NO synthesis leads to an increase in AMPK phosphorylation. Indeed, following eNOS inhibition with NNA or L-NAME, AMPK appears to become fully activated, with only a nominal additional response after subsequent treatment with H₂O₂ (Figs. 2 and 4). siRNA-mediated knockdown of CaMKK β abolishes the effect of eNOS inhibitors on AMPK activation (Fig. 4), suggesting that eNOS-dependent activation of AMPK involves CaMKK β . Further evidence for an inhibitory role for eNOS in modulating AMPK signaling pathways comes from our studies using eNOS siRNA (Fig. 4), in which we found that siRNA-mediated eNOS knockdown enhances AMPK phosphorylation. Finally, our observations in eNOS $^{-/-}$ mice are consistent with our findings in cultured endothelial cells: AMPK phosphorylation is increased in liver and lung tissues and in lung endothelial cells in eNOS $^{-/-}$ mice compared to wild-type mice (Fig. 6).

Our studies document an important relationship between H₂O₂ and eNOS in the reciprocal regulation of the CaMKK β /AMPK pathway. Our experiments using the HyPer H₂O₂ biosensor have been particularly informative in the analysis of intracellular H₂O₂ generation in endothelial cells. Exogenous H₂O₂ promotes phosphorylation of AMPK (Fig. 1) and eNOS (Fig. S2), associated with a strong intracellular HyPer signal (Fig. 5). Importantly, the suppression of the eNOS pathway in endothelial cells—either by enzyme inhibition with L-NAME or by siRNA-mediated eNOS knockdown—leads both to AMPK phosphorylation (Fig. 4) as well as marked increases in intracellular H₂O₂ (Fig. 5). The effects of siRNA-mediated eNOS knock-

immunoblotted with specific antibodies using protocols provided by the manufacturers. Immunoblots were analyzed by quantitative chemiluminescence using a ChemImager 4000 (Alpha-Innotech) and reported in arbitrary units.

Intracellular H₂O₂ Detection by HyPer. BAECs were transfected with the cytosol-targeted Hyper plasmid and imaged 48 h after transfection following the protocol described (28). After cell treatments, single-cell imaging was performed using a Nikon TE2000 microscope with a Perkin-Elmer spinning disk confocal system. Image intensities were quantified using MetaMorph software.

Tube Formation Assay. One hundred microliters of growth-factor reduced Matrigel (BD Biosciences) were added to wells in a 48-well plate, and 10⁴ cells were added to each Matrigel-coated well. Quantitative assays of tube formation were performed as reported (20).

Analyses of Tissues and Cells from Wild-Type and eNOS^{null} Mice. C57BL/6J wild type and eNOS^{-/-} mice from Jackson Laboratory were euthanized, and liver

and lung were harvested and homogenized using a Polytron homogenizer in a 50 mM Tris-HCl pH 7.4; 5 mM EGTA; 2 mM EDTA; 100 mM NaF; 2 mM Na₃VO₃; and Sigma protease inhibitor mixture. Following determination of protein concentrations, equal quantities of liver or lung lysates were analyzed in immunoblots. Mouse lung endothelial cells were isolated (44) and maintained in DMEM supplemented with endothelial cell growth factor and 20% (vol/vol) FBS. Cells between passages 3 and 5 were studied.

Statistical Analysis. All experiments were performed at least three times. Mean values for individual experiments were expressed as means ± SE. Statistical differences were assessed by ANOVA. A *P* value less than 0.05 was considered significant.

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