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ENDOTHELIAL NITRIC OXIDE SYNTHASE NEGATIVELY REGULATES HYDROGEN PEROXIDE-STIMULATED AMP-ACTIVATED PROTEIN KINASE IN ENDOTHELIAL CELLS

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Abbreviations: AMPK, 5'-AMP-activated protein kinase; siRNA, small interfering RNA; FBS, fetal bovine serum; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; CaMKKβ, calcium/calmodulin-dependent protein kinase kinase β; ACC, acetyl-CoA carboxylase; ANOVA, analysis of variance.

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Abstract

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 H_2O_2 is a critical activator of AMPK in cultured bovine aortic endothelial cells (BAEC). H_2O_2 treatment of BAEC rapidly and significantly increases the phosphorylation of AMPK at Thr172, as analyzed in immunoblots probed with phosphorylation state-specific antibodies. The EC_{50} for H_2O_2–promoted phosphorylation of AMPK is 65±15 μM, within the physiological range of cellular H_2O_2 concentrations. The Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CaMKKβ) inhibitor STO-609 abolishes H_2O_2-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 H_2O_2 biosensor HyPer show that siRNA-mediated eNOS knockdown leads to a marked increase in intracellular H_2O_2 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 H_2O_2 in endothelial cells, and document that eNOS is an important negative regulator of AMPK phosphorylation and intracellular H_2O_2 generation in endothelial cells.

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The term "reactive oxygen species" (ROS) is used to describe a class of molecules capable of oxidizing a broad array of molecular targets in cells and tissues. ROS modulate both physiological and pathophysiological cellular responses, and increased production of ROS has been implicated in the pathogenesis of cardiovascular diseases (1, 2). Hydrogen peroxide (H_2O_2) is a cell-permeant stable ROS that modulates diverse endothelial cell processes, including cell proliferation, cytoskeletal structure, vascular remodeling, and endothelium-regulated vasorelaxation (1, 3, 4). However, the molecular mechanisms of H_2O_2–dependent modulation of these endothelial cell responses are incompletely understood. A low basal intracellular level of H_2O_2, typically in the range of 25-75 μM (5), appears to be involved in normal processes of endothelial cell growth and proliferation (6). However, under pathological conditions, excessive ROS production leads to a detrimental consequences in the vascular wall (7).

In many cells, H_2O_2–induced cellular responses appear to involve the modulation of protein kinase signaling pathways (8, 9). The AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase composed of a catalytic α subunit and regulatory β and γ subunits (10). AMPK can be activated by a rise in the AMP/ATP ratio, and the activated AMPK may phosphorylate metabolic enzymes that switch on ATP-generating catabolic pathways and switch off ATP-requiring 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 activation loop of the α subunit (11). At least two AMPK-activating kinases have been identified: the tumor suppressor kinase LKB1 and the Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) (12, 13).

The cellular 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 may play a role in cell energy flux (14),

protection from apoptosis (15), as well as in the regulation of inflammation, angiogenesis, and tissue perfusion (16). Endothelial AMPK may be activated by a broad range of cellular and chemical stimuli, including tissue hypoxia (17); hypoglycemic drugs such as metformin; peroxynitrite (18); adiponectin (19); and vasoactive mediators such as S1P, VEGF (20, 21), bradykinin (29), or thrombin (22). The link between AMPK and the key vascular signaling protein endothelial nitric oxide synthase activation has been extensively studied. Kemp and colleagues were the first to show that AMPK phosphorylates eNOS at serine residue 1177, leading to enzyme activation (23). NO formation has been implicated in the angiogenic effects of AMPK (24). Several studies have reported that H_2O_2 promotes eNOS activation and phosphorylation in endothelial cells- but without implicating AMPK- and yet other studies have reported that H_2O_2 treatment promotes AMPK phosphorylation, but without implicating eNOS or endothelial responses (13, 25, 26). Thus, multiple reports have suggested intriguing correlations and complex interactions between H_2O_2, AMPK, and eNOS activation pathways, but the relationship between AMPK, H_2O_2, and eNOS remains incompletely understood.

In the present studies, we investigate the signaling mechanisms involved in the activation of AMPK in endothelial cells stimulated with H_2O_2, and explore the functional consequences of H_2O_2 –mediated AMPK activation. We demonstrate that H_2O_2 activates AMPK in endothelial cells, and establish that CaMKKβ is the key upstream kinase responsible for H_2O_2–dependent AMPK phosphorylation and endothelial tube formation. Importantly, we identify a previously unrecognized 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 H_2O_2, CaMKKβ, AMPK, and eNOS signaling pathways in the vascular endothelium.

Results

H_2O_2-mediated AMPK activation. We first studied the effects of H_2O_2 on the phosphorylation of AMPK and its substrate acetyl CoA carboxylase (ACC) in bovine aortic endothelial cells (BAEC). Figure 1A shows the results of a dose response to H_2O_2 for phosphorylation of AMPK and ACC in BAEC. In this experiment, BAEC were treated for 30 minutes with varying concentrations of H_2O_2, and cell lysates were analyzed in immunoblots probed with antibodies against phospho-AMPK, total AMPK, phospho-ACC or total ACC. As can be seen in Figure 1, H_2O_2 treatment increases AMPK and ACC phosphorylation in a dose-dependent manner; total AMPK or ACC abundance does not change. There is no change in endothelial cell viability under any of the H_2O_2 incubation conditions used in these studies, as determined by trypan blue exclusion assay (Supplemental Figure S6). Figure 1B and S1A shows pooled data from five similar dose response experiments quantitating AMPK and ACC phosphorylation, respectively; the EC_{50} for H_2O_2-promoted AMPK phosphorylation is 65±15 μM, a value close to the physiological H_2O_2 concentration in these cells (6). Figure 1C shows a typical time course of H_2O_2 induced phosphorylation of AMPK and ACC, and Figure 1D and S1B shows the corresponding quantitative analysis of pooled data from 5 similar experiments. Within 5 min after the addition of H_2O_2 (200 μM) to BAEC, AMPK and ACC phosphorylation increase significantly, and this signal is sustained for at least 120 minutes. Furthermore, as previously reported (25), we found that H_2O_2 treatment promoted phosphorylation of eNOS at serine 1179 (Supplemental Figure S1 C-F).

Effects of protein kinase or NOS inhibitors on H_2O_2-mediated phosphorylation responses. We next used pharmacological inhibitors to probe key endothelial signaling pathways involved in H_2O_2-mediated AMPK phosphorylation. Figure 2 shows the results of experiments analyzing H_2O_2-induced AMPK phosphorylation in endothelial cells treated with the CaMKKβ 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). We found that 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 (Figure S2). The PI3K inhibitor wortmannin induces a small but statistically significant *increase* in basal and H_2O_2-mediated AMPK and ACC phosphorylation (n=3, $p<0.01$). While wortmannin does not inhibit H_2O_2-promoted AMPK phosphorylation, wortmannin blocks H_2O_2-promoted Akt phosphorylation, as has been previously reported (25; Figure S2). As shown in Figure 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 (Figure 2). We then explored the effects of STO-609, wortmannin, NNA, and L-NAME on phosphorylation of another AMPK kinase LKB1. As shown in Figure 2A, STO-609 treatment had no effect on phosphorylation of LKB1, while completely suppressing AMPK phosphorylation; LKB1 phosphorylation was unaffected by the NOS inhibitors and the PI3K inhibitor wortmannin.

Effects of siRNA-mediated knockdown of CaMKKβ on H_2O_2-mediated phosphorylation responses. Figure 3A shows the H_2O_2 dose response for AMPK phosphorylation in the presence and absence of the CaMKKβ 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 CaMKKβ siRNA, and found that siRNA-mediated knockdown of CaMKKβ blocked AMPK phosphorylation in response to H_2O_2 (Figure 3B).

Roles of eNOS in AMPK activation. We used RNA interference methods to further explore the role of eNOS in AMPK regulation. siRNA-mediated knockdown of eNOS led to a marked increase in AMPK phosphorylation (Figure 4): there was a 2.4±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. We next explored AMPK phosphorylation in endothelial cells treated with the eNOS enzyme inhibitors in the context of knockdown of CaMKKβ. 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 (Figure 4B). However, siRNA-mediated CaMKKβ knockdown attenuates the NOS inhibitor-mediated increase in AMPK phosphorylation (Figure 4B), suggesting that eNOS-dependent AMPK activation requires CaMKKβ.

Intracellular H_2O_2 in AMPK activation. We next pursued cellular imaging approaches to detect intracellular H_2O_2 using the H_2O_2 biosensor, HyPer (28) transfected into BAEC. As shown in panel A of Figure 5, exogenous H_2O_2 causes a ~2.5-fold increase in fluorescence in HyPer-transfected endothelial cells relative to vehicle-treated HyPer-transfected cells ($p<0.01$). These results indicate that transfected HyPer can detect an increase in intracellular H_2O_2 in endothelial cells following the extracellular addition of H_2O_2. We next used HyPer to detect endogenous production of H_2O_2. Treatment of BAEC with the NOS inhibitor L-NAME leads to a striking 2.0-fold increase in intracellular H_2O_2 production (Figure 5). Importantly, siRNA-mediated eNOS knockdown increases the production of H_2O_2 to a similar magnitude, again detected as a marked increase in HyPer fluorescence in cells co-transfected with HyPer and eNOS siRNA, as compared to control siRNA-transfected cells (Figure 5). We note that these same interventions (L-NAME treatment and siRNA-mediated eNOS knockdown) also similarly increase AMPK phosphorylation (Figure 4). Conversely, treatment of endothelial cells with PEG-catalase to

degrade intracellular H_2O_2 (27) markedly suppresses both the increase in H_2O_2 generation (Figure 5A-B and 5D-E) as well as the increase in AMPK phosphorylation (Figure 5C and 5F) that are seen after eNOS inhibition with L-NAME (Figures 5 A-C) or following siRNA-mediated eNOS knockdown (Figure 5D-F). Cells cultured in high glucose media are known to show an increase in AMPK phosphorylation as well 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 BAEC 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 (Supplemental Figure S3).

AMPK activation in tissues and cells from eNOS$^{-/-}$ mice. After finding that siRNA-mediated eNOS knockdown markedly enhances AMPK phosphorylation, we studied AMPK phosphorylation and expression in tissues and cells isolated from wild type and eNOS$^{-/-}$ mice. As shown in Figure 6, in the liver and lung of eNOS$^{-/-}$ mice, AMPK phosphorylation is strikingly increased compared to the level of AMPK phosphorylation that is seen in corresponding tissues of wild type mice, with no change in overall levels of AMPK expression. In lung tissue of eNOS$^{-/-}$ mice, AMPK phosphorylation is increased by 4.9 ± 0.3 fold compared to that of wild-type littermates (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 striking 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. We examined AMPK phosphorylation in cultured endothelial cells isolated from lungs of eNOS$^{-/-}$ mice and wild type mice. As shown in Figure 6, AMPK phosphorylation in lung endothelial cells from eNOS$^{-/-}$ mice is significantly increased compared to lung endothelial cells from wild type mice (1.5 ± 0.1 fold increase, n=3, $p<0.01$). H_2O_2-induced AMPK phosphorylation also was enhanced in lung endothelial cells isolated from eNOS$^{-/-}$ mice compared to endothelial cells isolated from wild-type mice (n=3, $p<0.05$; Fig. 6B & C).

The role of AMPK and CaMKKβ in endothelial cell tube formation. H_2O_2 has been reported to play a role in angiogenesis (29). We investigated the consequences of siRNA-mediated knockdown of AMPK and CaMKKβ on H_2O_2 responses in the Matrigel endothelial cell tube formation assay, which is commonly used as an index of angiogenesis (30). As shown in Figure 7A, H_2O_2 treatment enhances endothelial tube formation. siRNA-mediated knockdown of AMPK or CaMKKβ substantially suppresses basal endothelial tube formation and blocks the response to H_2O_2; control siRNA is without effect. Quantitative analyses of tube formation (20) confirm that siRNA-mediated knockdown of AMPK or CaMKKβ significantly reduces both basal and H_2O_2-stimulated endothelial tube formation (Fig. 7B).

Discussion

The present studies have explored the endothelial cell signaling pathways modulated by H_2O_2 in the context of AMPK regulation. Reactive oxygen species are produced by a variety of vascular cells (31-33). The production of superoxide anion ($O_2^{\cdot-}$) in the vasculature has been extensively analyzed, yet the short half-life and small radius of diffusion of $O_2^{\cdot-}$ limit its role as an important paracrine agent in vascular biology (1, 3). The $O_2^{\cdot-}$ metabolite H_2O_2 has been identified as an important cellular signaling agent capable of modulating diverse aspects of endothelial cell physiology and pathophysiology (1, 3, 34). H_2O_2 has been reported to activate AMPK by inducing oxidative stress (13, 27), and H_2O_2 is involved in several receptor-mediated pathways of

AMPK activation (35). We assessed AMPK activation in endothelial cells by measuring AMPK phosphorylation at its critical activating residue and by quantitating phosphorylation of the AMPK substrate acetyl CoA carboxylase (Figure 1). Under all conditions, we saw a striking parallel between the level of AMPK phosphorylation and the phosphorylation of its substrate ACC. This observation suggests that phosphorylation of AMPK can serve as an effective marker for enzyme activation. We found that H_2O_2 induces AMPK phosphorylation in a time-dependent and dose-dependent manner, with an EC_{50} value of 65±15 μM, a concentration within in the range of physiological concentrations of H_2O_2 (6). These studies also help establish the importance of the CaMKKβ/AMPK pathway in angiogenesis: following siRNA-mediated knockdown of AMPK or CaMKKβ, H_2O_2 treatment no longer increases endothelial tube formation (Figure 7), suggesting that H_2O_2-modulated angiogenic responses can be modulated by the CaMKKβ/AMPK pathway.

Two separate lines of evidence establish a key role for CaMKKβ in AMPK activation by H_2O_2. Pharmacological inhibition of CaMKKβ by STO-609 completely abolishes H_2O_2-induced AMPK activation (Figure 2). In addition, siRNA-mediated downregulation of CaMKKβ suppresses H_2O_2-induced AMPK activation (Figure 3). These findings suggest that CaMKKβ is critically involved in modulating H_2O_2-induced AMPK activation. We also found that phosphorylation of the AMPK kinase LKB1 also is increased by H_2O_2 treatment (Figure 2). However, the H_2O_2-promoted increase in LKB1 phosphorylation is *not* blocked by the CaMKKβ inhibitor STO-609 under conditions where H_2O_2-promoted AMPK phosphorylation is completely blocked. Taken together, these findings argue against a central role for LKB1 in H_2O_2-induced AMPK phosphorylation.

The mechanisms whereby H_2O_2 modulates CaMKKβ remain to be completely defined. H_2O_2-induced oxidative stress has been reported to lead to an increased level of intracellular AMP, leading to AMPK phosphorylation. However, AMP has no effect on the activity of CaMKKβ (13), so our finding that CaMKKβ inhibition abolishes H_2O_2–stimulated AMPK activation suggests that H_2O_2-induced AMPK phosphorylation does not importantly involve changes in AMP levels, at least at physiological levels of H_2O_2 (Figure 3). The signaling pathways leading to activation of CaMKKβ 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 cyclic GMP-dependent protein kinase undergoes oxidation at key thiol residues, leading to kinase activation independent of cyclic GMP (4,8). An intriguing and entirely speculative hypothesis in the context of these studies is that redox-active cysteine thiols in CaMKKβ might undergo reversible oxidation as well as S-nitrosation, each modification having opposing effects on CaMKKβ activity.

eNOS is a Ca^{2+}/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 CaMKKβ inhibitor STO-609 on AMPK activation, treatment of endothelial cells with the PI3K inhibitor wortmannin failed to suppress AMPK phosphorylation (Figure 2). We found that treatment of endothelial cells with NOS inhibitors leads to a significant increase in basal AMPK and ACC phosphorylation (Figure. 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 H_2O_2 (Figures 2 and 4). siRNA-mediated knockdown of CaMKKβ abolishes the effect of eNOS inhibitors on AMPK activation (Figure 4), suggesting that eNOS-dependent activation of AMPK involves CaMKKβ. Further evidence for an inhibitory role for eNOS in modulating AMPK signaling pathways comes from our studies using eNOS siRNA (Figure 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 (Figure 6).

Our studies document an important relationship between H_2O_2 and eNOS in the reciprocal regulation of the CaMKKβ/AMPK pathway. Our experiments using the HyPer H_2O_2 biosensor have been particularly informative in the analysis of intracellular H_2O_2 generation in endothelial cells. Exogenous H_2O_2 promotes phosphorylation of AMPK (Figure 1) and eNOS (Supplemental Figure S1), associated with a strong intracellular HyPer signal (Figure 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 (Figure 4) as well as marked increases in intracellular H_2O_2 (Figure 5). The effects of siRNA-mediated eNOS knockdown or eNOS enzyme inhibition on enhanced H_2O_2 generation or AMPK phosphorylation are fully reversed by treatment of endothelial cells with PEG-catalase (Figure 5). Catalase catalyzes the decomposition of H_2O_2 into H_2O and O_2, and the striking inhibitory effects of PEG-catalase clearly identify eNOS as a critical determinant of endogenous H_2O_2 synthesis. In addition, stronger HyPer signals were observed in transfected BAEC treated with high glucose (30 mM) compared to cells in lower glucose (5 mM), in parallel with an increase in AMPK phosphorylation (Supplemental Figure S4). Thus, conditions leading to increased generation of intracellular H_2O_2 are associated with enhanced AMPK activation. Importantly, these studies implicate eNOS as a key negative regulator of intracellular H_2O_2 generation, and also as an inhibitor of CaMKKβ-dependent AMPK phosphorylation. The role (if any) of NADPH oxidases in the control of CaMKKβ-regulated responses, as well as the mechanisms whereby eNOS modulates H_2O_2 production and CaMKKβ-dependent AMPK phosphorylation, remain to be definitively explicated. Our evidence points away from cyclic GMP/PKG as a key modulator, in that siRNA-mediated PKG knockdown does not attenuate H_2O_2–promoted AMPK phosphorylation (Supplemental Figure S5). We speculate that suppression of eNOS activity may lead to enhanced mitochondrial respiration (1,2,3,45) and thereby increase H_2O_2 production, which then promotes CaMKKβ activation by oxidation of key thiols (5,8) either in CaMKKβ or in an upstream kinase or phosphatase, leading then to AMPK phosphorylation (13,20) and activation (see model in Figure 8).

These findings contrast with the conclusions of a recent publication, which reported that nitric oxide donors promote an *increase* in AMPK phosphorylation (38). The conclusions of this previous paper were largely based on experiments in which treatment of cultured cells with pharmacological NO donors, such as SNP, increased AMPK phosphorylation in HeLa cells and in human umbilical vein endothelial cells. However, we did not observe AMPK activation in BAEC treated with SNP under the same conditions (Supplemental Figure S4). These discrepant findings may indicate that subtle differences in culture conditions, possibly associated with variations in nitrosative or oxidative stress, may lead to differential effects of eNOS on the AMPK pathway. Our present observations consistently reveal an *inhibitory* role for endogenous eNOS in AMPK activation, an effect seen both in cultured native endothelial cells and in tissues from $eNOS^{-/-}$ mice. It seems plausible that under some conditions, prolonged exposure to high doses of pharmacological NO donors might lead to AMPK activation because of an inhibition of cellular energy flux. The previous observations on the activation of AMPK by *pharmacological NO donors* probably reflect a pathway distinct from the inhibition of AMPK by *endogenous eNOS*. Our finding that eNOS enzyme inhibition as well as siRNA-mediated eNOS knockdown enhances AMPK phosphorylation have lead us to conclude that eNOS *negatively* regulates AMPK activation in endothelial cells.

There may be some interesting implications for organismal energy metabolism that reflect this interplay between eNOS and AMPK pathways. For example, AMPKα2 transgenic mice show reduced maximal exercise capacity, suggesting a critical role for AMPK in physical activity

(39). Likewise, eNOS-dependent responses in both mice and rats appear to affect exercise capacity, oxygen consumption, and aerobic work (40). Interestingly, $eNOS^{-/-}$ mice have show marked reductions in physical work capacity (41). Our current finding that eNOS negatively regulates AMPK activation might suggest that the decreased physical activity in $eNOS^{-/-}$ mice could lead to the activation of AMPK-dependent metabolic pathways, which in turn would suppress the accumulation of energy stores and thereby lead to a decrease in exercise capacity.

In summary, our studies have demonstrated that H_2O_2 activates AMPK in endothelial cells through CaMKKβ, and establish a key role for the CaMKKβ/AMPK pathway in endothelial cell signaling. These studies also identify a previously unrecognized role of eNOS in the inhibition of H_2O_2 generation and AMPK activation in the vascular endothelium.

Materials and Methods

Reagents and siRNA constructs. Fetal bovine serum (FBS) was from Hyclone (Logan, CT); Dulbecco's modified Eagle medium (DMEM), Lipofectamine 2000 transfection reagent and other cell culture reagents were from Invitrogen. The CaMKKβ inhibitor STO-609, was from Calbiochem. Polyclonal antibodies against phospho-AMPK (Thr^{172}), AMPK, phospho-ACC (Ser^{79}), ACC, phospho-eNOS (Ser^{1179}), LKB1, and phospho-LKB1 (Ser^{428}) were from Cell Signaling Technologies (Beverly, MA). eNOS monoclonal antibody was from BD Transduction Laboratories (Lexington, KY). CaMKKβ monoclonal antibody was from Novus Biologicals (Littleton, CO). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody and PEG-catalase were from Sigma. Super Signal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce. Gelatin, hydrogen peroxide and other reagents were from Sigma. Custom-designed duplex siRNA constructs targeting eNOS and CaMKKβ were from Ambion (Austin, TX), as well as a nonspecific control siRNA from Dharmacon (Lafayette, CO) and have been characterized in detail previously (20). The HyPer plasmid constructed was from Evrogen (Moscow, Russia).

Cell culture and immunoblotting. Bovine aortic endothelial cells (BAEC) were from Cell Systems (Kirkland, WA) and cultured in DMEM supplemented with 10% (v/v) fetal bovine serum as described previously (42). Cells were maintained in 0.2% gelatin-coated culture dishes and starved in serum free media overnight prior to treatment. For siRNA transfections, endothelial cells were transfected with specific siRNA targeting constructs as described previously (43), and were analyzed 48 h after transfection. For PEG-catalase treatment (27), endothelial cells were incubated with PEG-catalase (50 units/ml) or vehicle for 18 hours. After treatments, cell lysates were prepared and immunoblotted with specific antibodies using protocols provided by the manufacturers. Immunoblots were analyzed by quantitative chemiluminescence using a ChemiImager 4000 (Alpha-Innotech), and reported in arbitrary units.

Intracellular H_2O_2 detection by HyPer. Endothelial cells were transfected with the cytosol-targeted Hyper plasmid and processed for imaging 48 h after transfection following the protocol as 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 (Downingtown, PA).

Tube formation assay. 100 μl of growth-factor reduced Matrigel (BD Biosciences) was added to wells in a 48-well plate and 10^4 cells were added to each Matrigel-coated well. Quantitative assays of tube formation were performed as we have reported previously (20).

Analyses of tissues and cells from wild-type and eNOS^{null} mice. C57BL/6Jwild type and eNOS$^{-/-}$ mice from the Jackson Laboratory were euthanized, and liver and lung were harvested expeditiously and homogenized using a Polytron homogenizer in a buffer containing 50mM Tris-HCl pH7.4; 5mM EGTA; 2mM EDTA; 100mM NaF; 2mM Na_3VO_3; and Sigma protease inhibitor cocktail. Following determination of protein concentrations, equal quantities of liver or lung lysates were resolved by SDS-PAGE and analyzed in immunoblots as described above. Mouse lung endothelial cells were isolated as reported previously (44) and maintained in DMEM supplemented with endothelial cell growth factor and 20% (v/v) FBS. Cells between passage 3 and 5 were studied.

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

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FIGURE LEGENDS

Figure 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. Panel A shows 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; panel B shows pooled data from five independent experiments, analyzing the intensities corresponding to phospho-AMPK and total AMPK by quantitative chemiluminescence. Panel C shows a representative time course experiment in BAEC treated with 200 μM H_2O_2 for the indicated times and analyzed in immunoblots probed with antibodies as

shown; panel D shows pooled data from five independent experiments. * designates $p<0.05$, and ** indicates $p<0.01$ by ANOVA.

Figure 2. Effects of protein kinase inhibitors and NOS inhibitors on H_2O_2-induced AMPK phosphorylation.
Panel A shows a representative immunoblot analyzed in endothelial cells treated with H_2O_2 (200 μM, 30 min) after first being incubated for 30 minutes with inhibitors as shown: STO-609 (CaMKKβ inhibitor, 10μg/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. Panels B, C, and D show quantitative analyses of pooled data from 3-5 independent experiments, analyzing the phosphorylation responses for AMPK, ACC, and LKB1, respectively.

Figure 3. CaMMKβ inhibitor STO-609 and siRNA-mediated CaMMKβ knockdown: effects on H_2O_2-stimulated AMPK phosphorylation.
This figure shows the results of dose response experiments exploring AMPK phosphorylation in BAEC treated with the CaMKKβ inhibitor STO-609 (panel A), or transfected with CaMKKβ or control siRNA (panel B). The cells were then incubated for 30 minutes 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; the * indicates $p < 0.05$.

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

Figure 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 hours later; cells were incubated with PEG-catalase or vehicle, as noted, and processed either for imaging or immunoblot analyses. Panels A-C show the results of treatments with H_2O_2 (200 μM) or L-NAME (100 μM) following incubation of cells with PEG-catalase or vehicle. The results in panels D-F are from endothelial cells transfected with control or eNOS siRNA constructs and then treated with PEG-catalase or vehicle, as shown. Panels A and D show representative images of HyPer-transfected cells that were treated as shown, then fixed and stained with Hoechst 33342, and analyzed for HyPer fluorescence. Panels B and E show pooled data from 15 individual HyPer transfected cells from three experiments, quantitated for the determination of fluorescence intensity using MetaMorph software. Panels C and F show representative immunoblot experiments in which endothelial cells were treated with PEG-catalase or vehicle, and either treated with L-NAME or H_2O_2 (panel C) or transfected with control or eNOS siRNA (panel F) as described in the text, and then analyzed in immunoblots probed with antibodies as shown. ** designates $p < 0.01$.

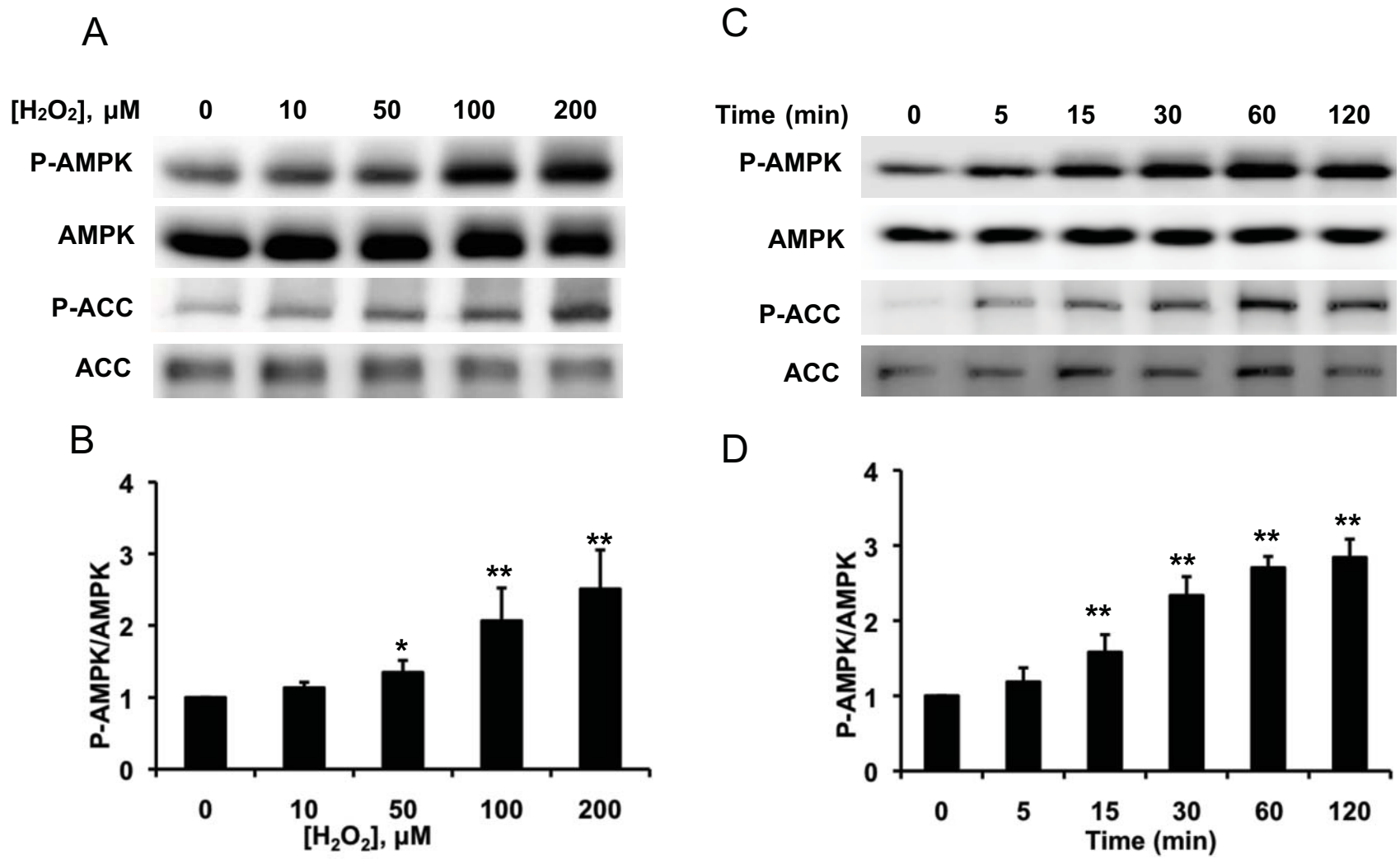
Figure 6. AMPK phosphorylation in tissues and cells from $eNOS^{-/-}$ mice.

This figure shows immunoblot analyses of liver, lung, or endothelial cells from wild-type (wt) or $eNOS^{-/-}$ mice. In Panel A, liver, lung tissues and isolated lung endothelial cells (MLEC) 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; *** signifies $p<0.001$. In Panel B, MLEC from wild-type or $eNOS^{-/-}$ mice were treated with H_2O_2 (100 μM for 30 minutes). The experiment shown is a representative of five similar experiments showing that MLEC from $eNOS^{-/-}$ mice have increased basal as well as H_2O_2-stimulated AMPK phosphorylation relative to MLEC from wild-type mice; pooled data are also shown, with * indicating $p<0.05$.

Figure 7. siRNA-mediated knockdown of AMPK and CaMKKβ impairs H_2O_2 -induced endothelial cell tube formation.
Endothelial cells were transfected with siRNA constructs targeting AMPK or CaMKKβ, and analyzed in the Matrigel tube formation assay. Panel A presents representative images of endothelial Matrigel tube formation in H_2O_2–treated endothelial cells transfected with siRNA constructs as shown. Panel B shows pooled data from three independent tube formation experiments, plotting the total tube length normalized for the different treatments relative to the total tube length as measured for untreated endothelial cells transfected with control siRNA (*, $p< 0.05$, **, $p< 0.01$).

Figure 8. Model for eNOS regulation of CaMKKβ/AMPK pathway via H_2O_2. This figure presents a plausible if speculative model for eNOS modulation of CaMKKβ/AMPK via H_2O_2 in endothelial cells. The present studies have shown that H_2O_2 promotes the phosphorylation of AMPK in endothelial cells (Figure 1); the response is blocked by the CaMKKβ inhibitor STO-609 (Figure 2) or by siRNA-mediated CaMKKβ knockdown (Figure 3). Conversely, the NOS inhibitor L-NAME potentiates AMPK phosphorylation (Figure 2), as does siRNA-mediated eNOS knockdown (Figure 4); both of these effects are inhibited by CaMKKβ inhibition (Figure 4). siRNA-mediated eNOS knockdown or enzyme inhibition with L-NAME leads to a marked increase in intracellular H_2O_2 generation (Figure 5); the increase in intracellular H_2O_2 generation is blocked by PEG-catalase, which also suppresses the increase in AMPK phosphorylation. Since previous work have shown that eNOS modulates mitochondrial function, we propose that suppression of the eNOS-NO pathway (by siRNA or enzyme inhibition) enhances H_2O_2 production and thereby leads to CaMKKβ –dependent phosphorylation of AMPK. We propose that H_2O_2 directly or indirectly activates CaMKKβ. The phosphorylated AMPK in turn phosphorylates and activates eNOS, representing a feedback mechanism controlling this pathway.

Fig. 1.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AMPK
P-ACC
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[H2O2], μM
C
Time (min)
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AMPK
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ACC
D
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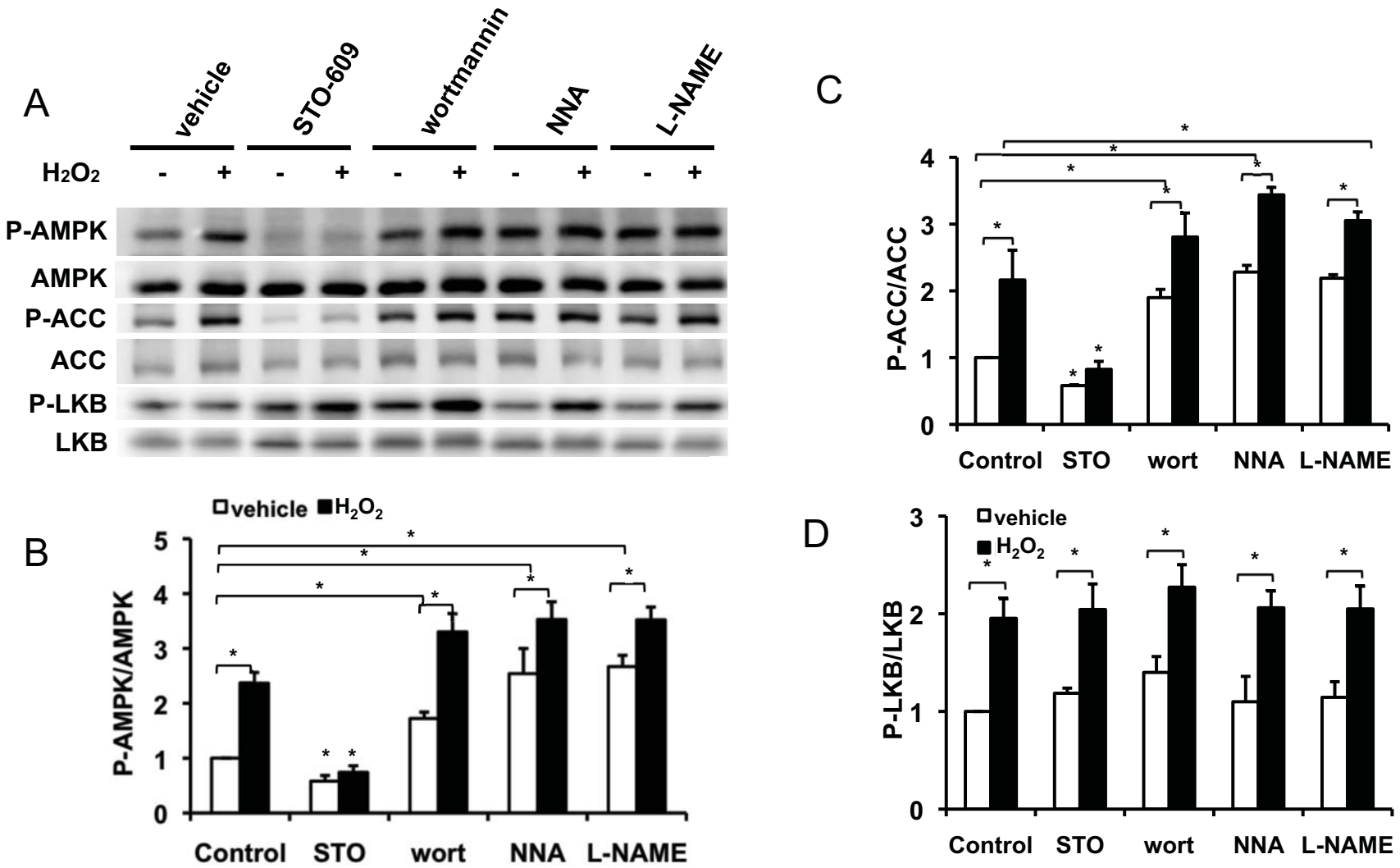
Time (min)

Fig. 2.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Control STO wort NNA L-NAME
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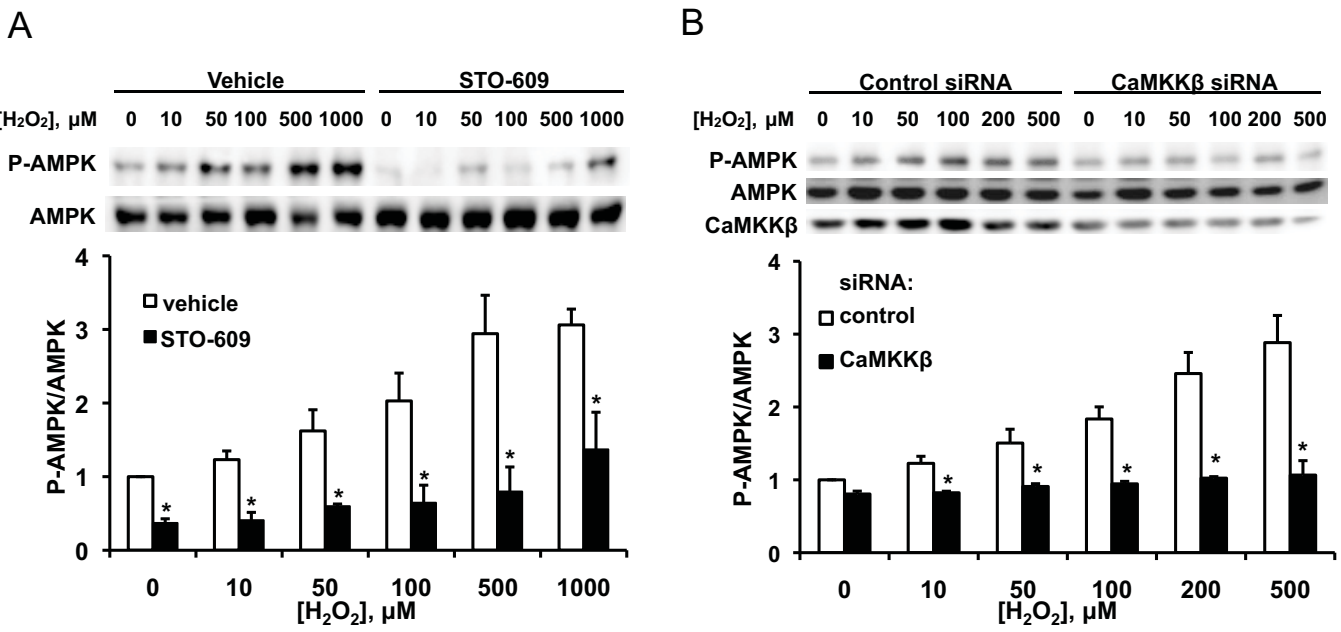
Fig. 3.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vehicle
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[H2O2], μM
B
Control siRNA
CaMKKβ siRNA
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[H2O2], μM

Fig. 4.

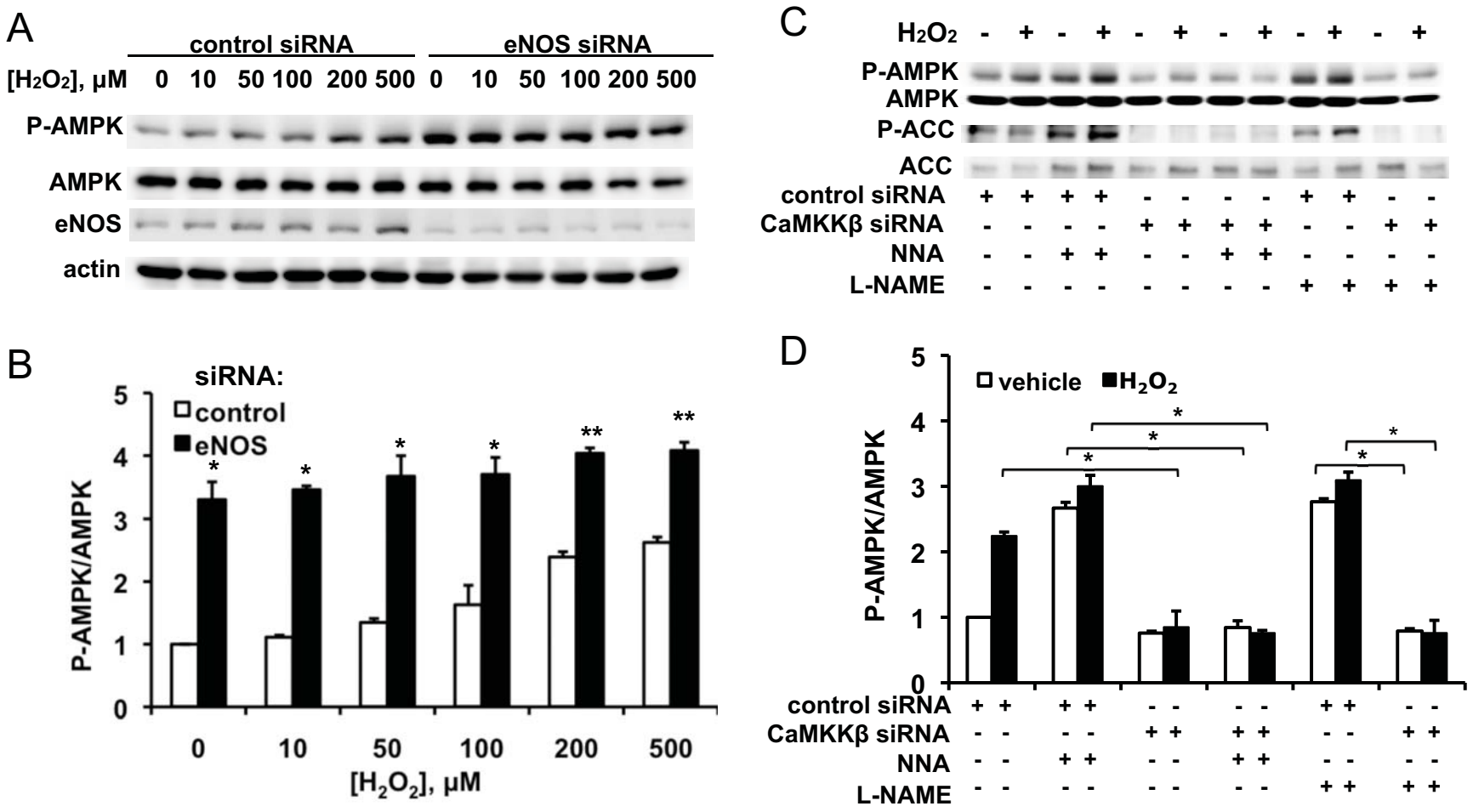
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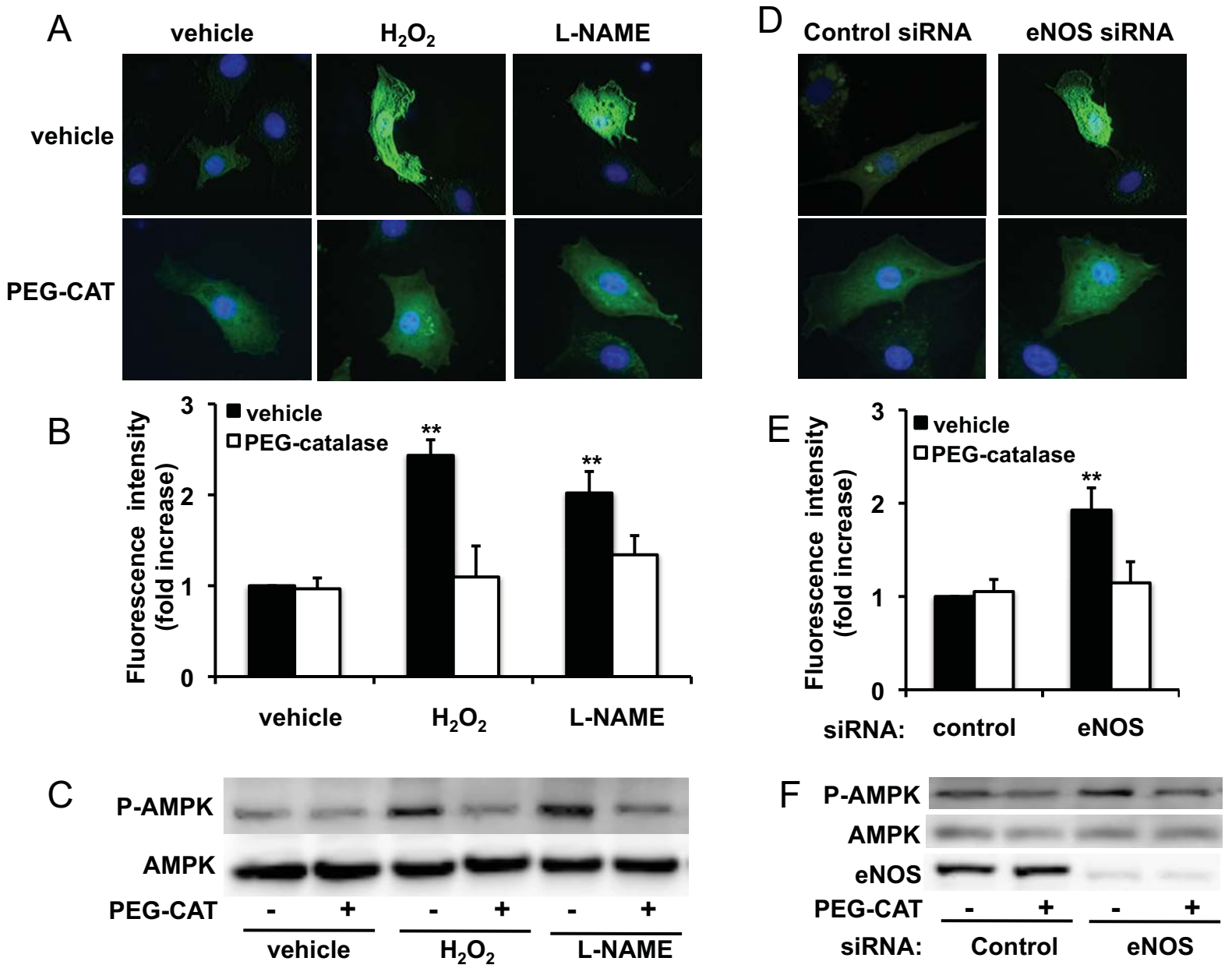
Fig. 5.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vehicle
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L-NAME
E
Fluorescence intensity (fold increase)
vehicle
PEG-catalase
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siRNA: control eNOS
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AMPK
PEG-CAT - + - + - +
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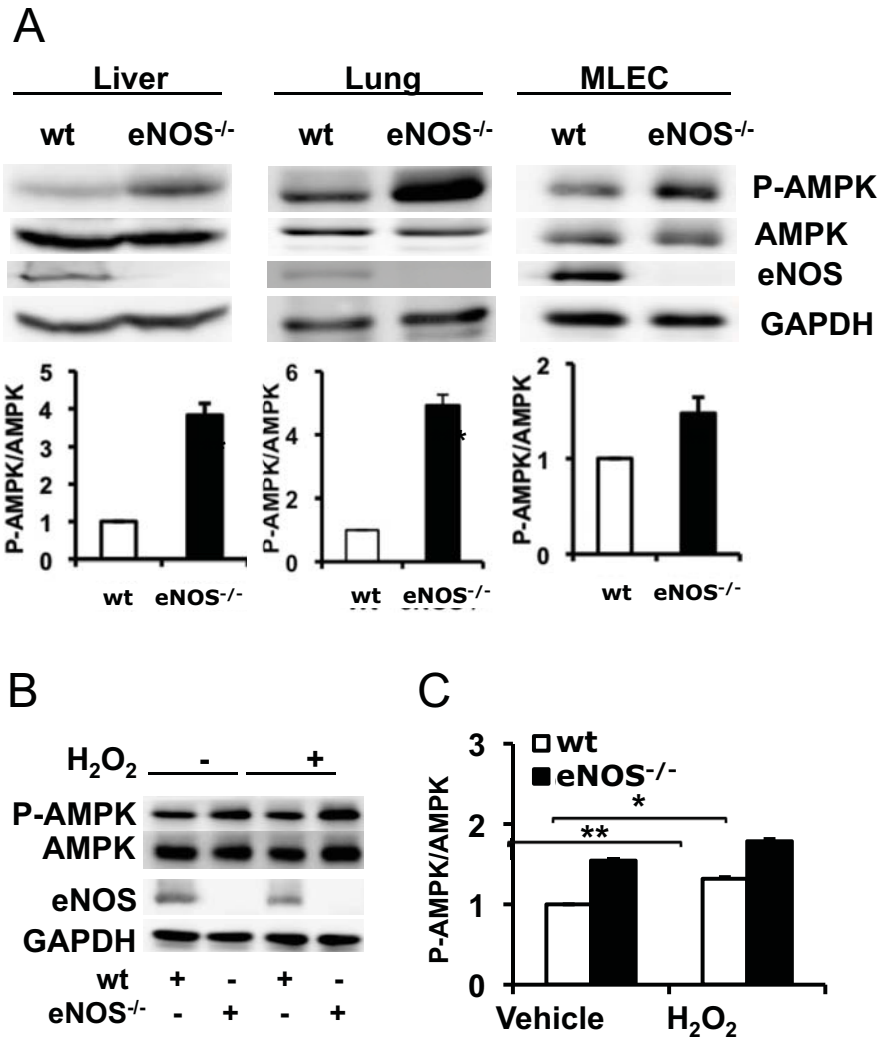
Fig. 6.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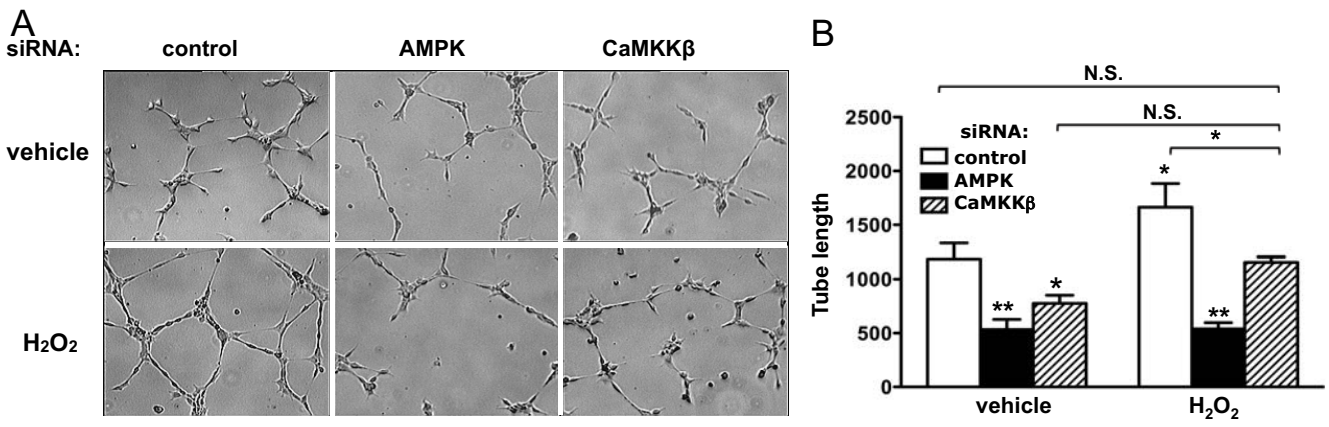
H2O2

Fig. 7.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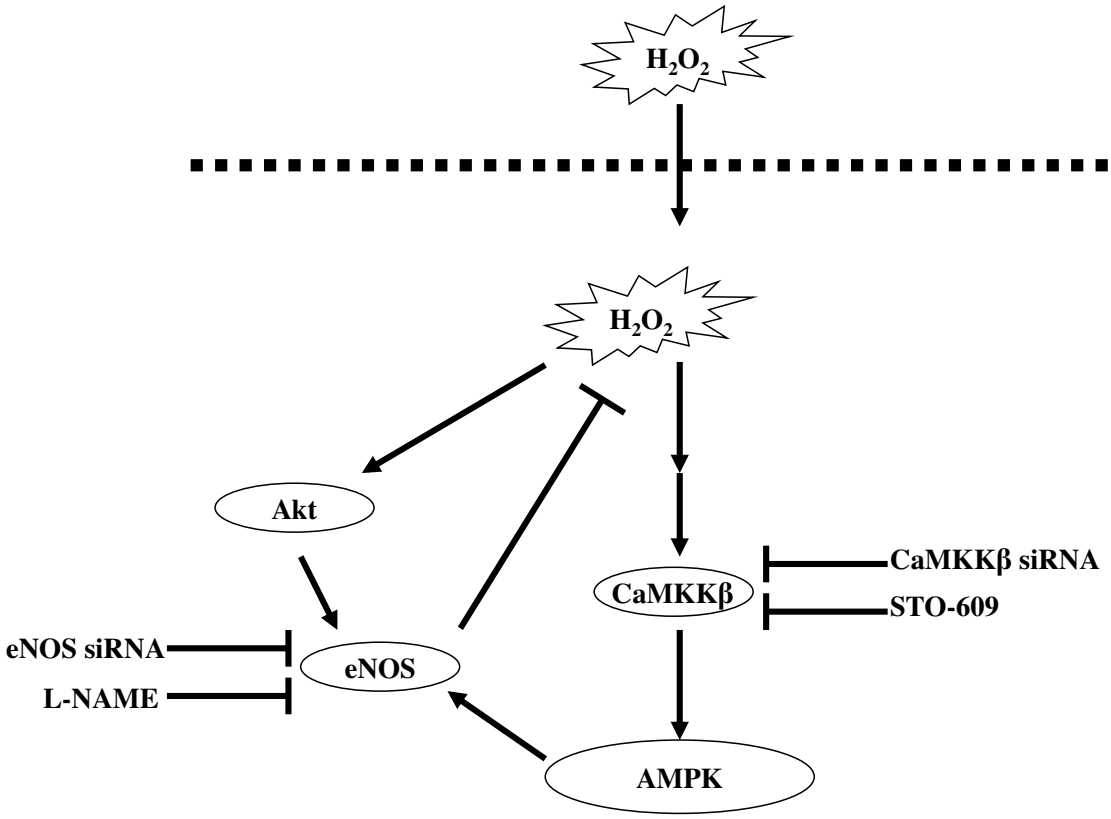
vehicle
H2O2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