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Research Paper

Differential endothelial signaling responses elicited by chemogenetic H_2O_2 synthesis

Seyed Soheil Saeedi Saravi[a,1], Emrah Eroglu[a,2,1], Markus Waldeck-Weiermair[a,b,1], Andrea Sorrentino[a], Benjamin Steinhorn[a], Vselovod Belousov[c], Thomas Michel[a,*]

[a] Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA, 02115, USA
[b] Molecular Biology and Biochemistry, Gottfried Schatz Research Center, Medical University of Graz, Neue Stiftingtalstraße 6/6, 8010, Graz, Austria
[c] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia

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ABSTRACT

Hydrogen peroxide (H_2O_2) modulates critical phosphorylation pathways in vascular endothelial cells, many of which affect endothelial nitric oxide synthase (eNOS) signal transduction. Both intracellular and extracellular sources of H_2O_2 have been implicated in eNOS regulation, yet the specific endothelial pathways remain incompletely understood. Here we exploited chemogenetic approaches and live-cell imaging methods to both generate and detect H_2O_2 in different subcellular compartments (cytosol, nucleus, and caveolae) of cultured EA.hy926 human endothelial cells. We developed novel recombinant constructs encoding differentially-targeted yeast D-amino acid oxidase (DAAO), which generates H_2O_2 only when its D-amino acid substrate is provided. DAAO was expressed as a fusion protein with the new H_2O_2 biosensor HyPer7.2, which allowed us to quantitate intracellular H_2O_2 levels by ratiometric imaging in living endothelial cells following the activation of DAAO by D-alanine. The addition of extracellular H_2O_2 to the HyPer-DAAO-transfected cells led to increases in H_2O_2 throughout different regions of the cell, as measured using the differentially-targeted HyPer biosensor for H_2O_2. The sensor response to extracellular H_2O_2 was more rapid than that quantitated following the addition of D-alanine to transfected cells to activate differentially-targeted DAAO. The maximal intracellular levels of H_2O_2 observed in response to the addition of extracellular H_2O_2 vs. intracellular (DAAO-generated) H_2O_2 were quantitatively similar. Despite these similarities in the measured levels of intracellular H_2O_2, we observed a remarkable quantitative difference in the activation of endothelial phosphorylation pathways between chemogenetically-generated intracellular H_2O_2 and the phosphorylation responses elicited by the addition of extracellular H_2O_2 to the cells. Addition of extracellular H_2O_2 had only a nominal effect on phosphorylation of eNOS, kinase Akt or AMP-activated protein kinase (AMPK). By contrast, intracellular H_2O_2 generation by DAAO caused striking increases in the phosphorylation of these same key signaling proteins. We also found that the AMPK inhibitor Compound C completely blocked nuclear H_2O_2-promoted eNOS phosphorylation. However, Compound C had no effect on eNOS phosphorylation following H_2O_2 generation from cytosol- or caveolae-targeted DAAO. We conclude that H_2O_2 generated in the cell nucleus activates AMPK, leading to eNOS phosphorylation; in contrast, AMPK activation by cytosol- or caveolae-derived H_2O_2 does not promote eNOS phosphorylation via AMPK. These findings indicate that H_2O_2 generated in different subcellular compartments differentially modulates endothelial cell phosphorylation pathways, and suggest that dynamic subcellular localization of oxidants may modulate signaling responses in endothelial cells.

1. Introduction

The stable reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) plays essential roles in endothelial cell biology, including endothelium-regulated vasorelaxation and vascular remodeling [1–4]. Higher concentrations of ROS can cause pathological oxidative stress in the vasculature, but lower concentration of H_2O_2 play important roles in cellular signal transduction. H_2O_2 is differentially localized in subcellular

* Corresponding author.
E-mail address: thomas_michel@hms.harvard.edu (T. Michel).
[1] These authors contributed equally.
[2] Sabanci University, Faculty of Engineering and Natural Sciences, Genetics and Bioengineering Program, Istanbul, Turkey.

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organelles, yet little is known of the consequences of H_2O_2 localization on cellular signaling responses [5–7]. H_2O_2 is generated in diverse pathways that are themselves differentially distributed within cells [2,5–8], including membrane-targeted NADPH oxidases (NOXs), uncoupled nitric oxide synthases, xanthine oxidases, and components of the mitochondrial electron transport chain. Degradation of H_2O_2 is catalyzed by a broad range of oxidoreductases that also are localized in specific subcellular compartments [5,8]. The downstream targets of H_2O_2 include a broad range of differentially-targeted protein kinases, phosphoprotein phosphatases, and oxidant-modulated transcription factors. H_2O_2 also modulates cellular signaling pathways by the dynamic oxidation of specific enzymatic substrates and cofactors. Thus, the differential redox state of specific subcellular locales can significantly affect oxidant-dependent signaling responses in cells [5,9]. In vascular endothelial cells, H_2O_2 regulates phosphorylation pathways that modulate the endothelial nitric oxide synthase (eNOS), a redox-modulated enzyme that generates nitric oxide (NO), a critical determinant of vascular homeostasis [4]. eNOS is dynamically targeted to plasmalemmal caveolae in endothelial cells, and the differential agonist-modulated localization of eNOS affects its role in cell signaling [10]. Two key signaling regulated by H_2O_2 that have been implicated in the modulation of eNOS phosphorylation in endothelial cells are kinase Akt (also known as protein kinase B) and the AMP-associated kinase (AMPK), both of which are phosphoproteins that also undergo dynamic subcellular translocation within cells [1,11–13].

For many years, the biological roles of H_2O_2 were studied principally simply by adding exogenous H_2O_2 to cultured cells or isolated tissues and then measuring one physiological or biochemical response or another. However, the addition of exogenous H_2O_2 to cultured cells does not replicate the more selective spatiotemporal pattern of intracellular endogenous H_2O_2 flux in the regulation of signaling responses [7,14,15]. With this increased appreciation for the intracellular roles of endogenous H_2O_2 as a second messenger, we developed a chemogenetic approach that dynamically modulates H_2O_2 levels in specific subcellular organelles in cultured endothelial cells.

The term “chemogenetics” is used to describe experimental systems in which the activity of a protein can be dynamically regulated inside a cell by providing or withholding the protein's biochemical stimulus. We recently developed and validated chemogenetic approaches to manipulate H_2O_2 levels in specific subcellular locales using differentially-targeted yeast D-amino acid oxidase (DAAO) constructs. Yeast DAAO is a stereospecific enzyme that generates hydrogen peroxide (H_2O_2) only when D-amino acids are provided as substrate [16]. Since most mammalian tissues contain only L-amino acids, the yeast DAAO is inactive until D-amino acids are made available [14,16]. We constructed a fusion protein between DAAO and the H_2O_2 biosensor HyPer7.2 to allow the simultaneous quantitation (HyPer) and generation (DAAO) of H_2O_2. The new HyPer7.2 ratiometric biosensor is much more sensitive than earlier generations of the HyPer biosensor, and permits the quantitation of intracellular H_2O_2 levels in specific subcellular locales after adding extracellular H_2O_2 to the cells in culture. In these studies, we report experiments performed using the human endothelial cell line EA.hy926, which has been extensively studied from the standpoint of endothelial signaling [4,17]. As we show below, there is a marked discordance in the biochemical responses to extracellular H_2O_2 *vs.* intracellular (chemogenetic) H_2O_2 in the modulation of phosphorylation pathways in endothelial cells.

2. Materials and methods

Reagents: Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT); all other cell culture reagents and media were from Invitrogen. The PI3–K inhibitor wortmannin and AMPK inhibitor Compound C were from Calbiochem. Polyclonal antibodies against phospho-eNOS Ser-1177 and Thr-495, phospho-Akt Ser-473, Akt, phospho-AMPK Thr-172, AMPK, phospho-ACC Ser-79 and ACC, as well as total eNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibodies plus secondary antibodies conjugated with horseradish peroxidase were from Cell Signaling Technologies. Phospho-eNOS Ser-114 and Ser-633 monoclonal antibodies were from EMD-Millipore. Super Signal (Femto) chemiluminescence detection reagents were from Pierce Biotechnology. D-alanine, L-alanine, H2O2 and other reagents were from Sigma Aldrich. The immunoblotting reagents were from Bio-Rad and Boston Bioproducts.

Cell culture: EA.hy926 human endothelial cells were obtained from ATCC (CRL-2922) and cultured in Dulbecco's modified Eagle's medium (DMEM) culture medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin–streptomycin [4]. The cells were studied at 60–70% confluence between passages 30 and 50.

Plasmid and Adenovirus HyPer-DAAO fusion constructs: The generation and characterization of differentially-targeted HyPer1-DAAO constructs have been previously described in detail [14]. We attached subcellular targeting signal sequences to the coding region of HyPer-DAAO to create constructs that are cytosol-targeted (using a nuclear exclusion sequence, termed NES); nucleus-targeted (nuclear localization sequence, termed NLS); or caveolae-targeting (CAV) sequences, as described [14]. The PCR fragment was then ligated into the pC1-CMV vector. The constructs were generated by fusing the cDNA for HyPer1 with the DAAO-NES or -NLS or -Cav with a Gly-Gly-Ser-Gly linker between HyPer1 and DAAO using the NEBuilder HiFi DNA assembly system (New England Biolabs). The resulting fusion constructs were inserted into the adenovirus serotype 5 (AV5) expression vector between the EcoRI and *Hin*dIII restriction enzyme sites. Constructs were verified by whole plasmid sequencing and packaged into AV5 viral vectors by ViraQuest Inc, USA.

For imaging experiments, we used the novel ratiometric HyPer7.2 construct to generate a fusion construct composed of HyPer7.2 [18] and DAAO, which were targeted using subcellular localization sequences as described above. In brief, the HyPer1 sequence from previously-characterized differentially-targeted HyPer-DAAO constructs were exchanged for HyPer7.2. The resulting differentially-targeted HyPer7.2-DAAO fusion constructs were then subcloned in a pCS2+ vector following excision of the original full-length insert using EcoRI and *Xho*I.

Adenoviral transduction: The cells were transduced with adenovirus 5-HyPer-DAAO targeted to the cell cytosol, nucleus or caveolae at a multiplicity of infection of 1000 in serum-free culture media; 5 h later, the media was exchanged for fresh media containing 10% FBS 5h. All cell treatments and experiments were performed 48 h after adenoviral transduction.

Plasmid transfection: EA.hy926 cells at ~70% confluence were transfected with 1 μg plasmid DNA encoding HyPer7.2-DAAO targeted to the cell cytosol, nucleus or caveolae [14] in serum-free culture medium, using the transfection reagent PolyJet according to the manufacturer's instructions (SignaGen Laboratories). After 5h incubation, the media was exchanged for fresh media containing 10% FBS. All treatments and experiments were performed 16–24 h after transfection.

Intracellular H_2O_2 detection by HyPer7.2 imaging: EA.hy926 endothelial cells expressing HyPer7.2-DAAO targeted to specific subcellular locales were treated with D-alanine or H_2O_2 48 h after transfection, and were then imaged in real time as previously described in detail [16,18]. In brief, cells were first washed with PBS and incubated for at least 2 h in a HEPES-buffered solution containing using a custom perfusion system with a peristaltic pump to maintain stable superfusion conditions. The superfusion solution consists of a physiological buffer with the following composition: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose and 1 mM HEPES, pH 7.4. For live-cell fluorescence imaging, the ratiometric HyPer7.2 biosensor was excited at 420 nm and 490 nm, and emission was collected at 530 nm. Detailed imaging protocols using HyPer7.2 have been previously reported [18]. The HyPer7.2 ratio was visualized with a 40X oil immersion objective (Olympus). Images were acquired using a CCD camera (Hammamatsu) and analyzed by Metafluor Software (Molecular Devices). Following

equal background subtraction of the emissions at both wavelengths, ratiometric HyPer7.2 signals were defined as a ratio of the intensities of the emission signals excited by 490 nm or 420 nm and normalized through R0. Ratiometric images of Fig. 3 are displayed in a color lookup table in which the color corresponds to the HyPer1 ratio of AV5-HyPer1DAAO transduced cells.

Cell treatment and immunoblotting: 48 h after viral infection, EA.hy926 cells transduced by differentially-targeted AV5- HyPer-DAAO were incubated with D- or L-alanine (10 mM) or vehicle (H2O). Wortmannin and Compound C were dissolved in dimethyl sulfoxide (DMSO) and used to treat the cells for 30 min before adding D-alanine or H_2O_2. Where indicated, water or DMSO 0.1% (v/v) were used as control. After drug treatments, the cells were washed twice with ice-cold PBS and lysed using the cell radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM Na3VO4, 10 mM NaF, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml soybean trypsin inhibitor, and 2 μg/ml lima trypsin inhibitor). Cell lysates were prepared, resolved by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Immunoblot analyses of protein expression and phosphorylation were probed using phosphospecific primary antibodies (1:1000) as indicated. The membranes were then incubated with a horseradish peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin G secondary antibody (1:2000). The membranes were then re-blotted with total eNOS, Akt, AMPK and ACC antibodies (1:1000) as loading controls. Immunoblots were analyzed by chemiluminescence using a ChemiImager HD4000 (Alpha Innotech, San Leandro, CA) and quantitative densitometric analyses were performed using ImageJ software (NIH, Bethesda).

Statistical analysis: Statistical analysis was performed using GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA). Statistical differences were assessed by one-way Analysis of variance (ANOVA) followed by Tukey's multiple comparison post-test. At least three independent triplicated experiments have been performed for each experimental set-up. For live cell fluorescence imaging, the number of experiments is indicated as "n = X/x" where X indicates the number of independent experiments and x indicates the total number of individual cells analyzed. Mean values for individual experiments are expressed as mean ± SEM, and $P < 0.05$ was defined as statistically significant, indicated as *; $p < 0.01$ as **; and $p < 0.001$ as ***.

3. Results

3.1. Quantitation of intracellular H_2O_2 by differentially-targeted biosensors

For the quantification of intracellular H_2O_2 dynamics, we used the recently developed "ultrasensitive" ratiometric H_2O_2 biosensor, HyPer7.2 [18]. Here we investigated H_2O_2 responses in the human vascular endothelial cell line EA.hy926, which has been extensively studied as a robust and informative model system for study of H_2O_2 signaling to eNOS [4,17]. We created a fusion protein between HyPer7.2 and the chemogenetic H_2O_2-generating enzyme DAAO in order to simultaneously generate (DAAO) and detect (HyPer7.2) H_2O_2 in transfected cells [14,18]. We used a signal sequence to direct the recombinant HyPer7.2-DAAO protein to the cell cytosol using a nucleus-exclusion signal sequence (NES, Cyto), as we did previously [4]. We analyzed EA.hy926 cells transfected with the HyPer7.2-DAAO fusion construct in live cell imaging experiments to compare the biosensor responses to the addition of "physiological" levels of exogenous H_2O_2 (25 μM) with the biosensor responses to intracellular H_2O_2 generated by DAAO following addition of D-alanine (10 mM). As shown in Fig. 1, we found that both 10 mM D-alanine and 25 μM H_2O_2 promoted increases in cytosolic H_2O_2, as detected by HyPer7.2. The addition of exogenous H_2O_2 promoted a rapid increase in the HyPer7.2 ratiometric signal, which then decayed; the subsequent addition of D-alanine promoted no further increase in the HyPer7.2 signal. In contrast, the HyPer7.2 response following addition of D-alanine was slower; after the signal reached steady state, the addition of H_2O_2 led to a slight but significant increase in the HyPer7.2 ratio (Fig. 1B). The specificity of DAAO for D-alanine was confirmed by studying the HyPer7.2 response to L-alanine, which did not lead to any H_2O_2 formation (Fig. 1C) as we have previously shown [19]. We confirmed that the cells that were unresponsive to L-alanine still had a robust HyPer7.2 response to exogenous H_2O_2 (Fig. 1C).

Given these differences in the response of cytosolic HyPer7.2 to exogenously added H_2O_2 versus DAAO-generated intracellular H_2O_2, we decided to characterize the H_2O_2 responses of differentially-targeted HyPer7.2-DAAO. We generated HyPer7.2-DAAO constructs targeted to the cell nucleus (Nuc), as we previously described for HyPer1 [14]. We also used a targeting sequence to direct the HyPer7.2-DAAO fusion protein to plasmalemmal caveolae (Cav), which are signal-transducing membrane microdomains in endothelial cells [17]. We then used cell imaging to analyze the H_2O_2 responses of HyPer7.2-DAAO targeted to the cytosol, nucleus or caveolae, once again comparing the HyPer7.2 response to extracellular vs. intracellular H_2O_2 in real time in EA.hy926 cells. For all three differentially-targeted biosensor constructs, we observed similarly rapid HyPer7.2 kinetics upon addition of H_2O_2 to the cells (Fig. 2A–C). Likewise, the response to D-alanine was similar in cytosol, nucleus, and caveolae, and in all cases was slower and of slightly lower magnitude than the response to extracellular H_2O_2. The addition of exogenous H_2O_2 leads to a faster but more transient increase in intracellular H_2O_2 compared to the response to D-alanine (Fig. 2D and E), whereas the response to H_2O_2 endogenously generated by DAAO is more sustained (Fig. 2A-C).

3.2. Differential eNOS phosphorylation responses to intracellular and extracellular H_2O_2

The cell imaging analyses shown in Figs. 1 and 2 were performed using plasmid-based transfection methods; the low plasmid transfection efficiency of these endothelial cells does not preclude analyses in cell imaging approaches. But the study of biochemical responses to chemogenetically-generated H_2O_2 in these cells required more uniform expression of the recombinant proteins, and we decided to use viral transduction with adenovirus-5 (AV5). We generated chimeric AV5 constructs consisting of DAAO fused to the H_2O_2 biosensor HyPer1 targeted to the cytosol, the nucleus, or the caveolae, as was done in Fig. 2; the same DAAO cDNA was used for both plasmid- and adenovirus-mediated studies. We imaged the HyPer signals 48 h following transduction of EA.hy926 cells with recombinant AV5 constructs, and verified a robust and uniform level of expression of differentially-targeted HyPer-DAAO to the correct subcellular compartment (Fig. 3A–C). We also observed similar temporal patterns of HyPer responses following addition of either H_2O_2 or to D-alanine to these adenovirus-transduced cells (Fig. 3D–F). These findings set the stage for biochemical analyses of signaling responses in adenovirus-transduced EA.hy926 endothelial cells.

We observed strikingly different phosphorylation responses to exogenous vs. endogenous H_2O_2 (Figs. 3–5), in contrast to the similar maximal levels of intracellular H_2O_2 that were detected in response to exogenous H_2O_2 treatment and to chemogenetic generation of intracellular H_2O_2 (Figs. 1 and 2). We focused first on studies of the endothelial signaling protein eNOS, a key caveolae-targeted phosphoprotein that undergoes H_2O_2-modulated phosphorylation at multiple sites [11], and which also undergoes dynamic intracellular translocation following agonist activation [8]. We transduced EA.hy926 endothelial cells with the three differentially-targeted AV5-HyPer-DAAO constructs, and then performed cell treatments either with D-alanine or with H_2O_2. We then probed immunoblots using well-characterized phosphospecific antibodies for eNOS and observed strikingly different phosphorylation responses to exogenous vs. by endogenous H_2O_2. As shown in Fig. 4, chemogenetic generation of H_2O_2 in either the cell

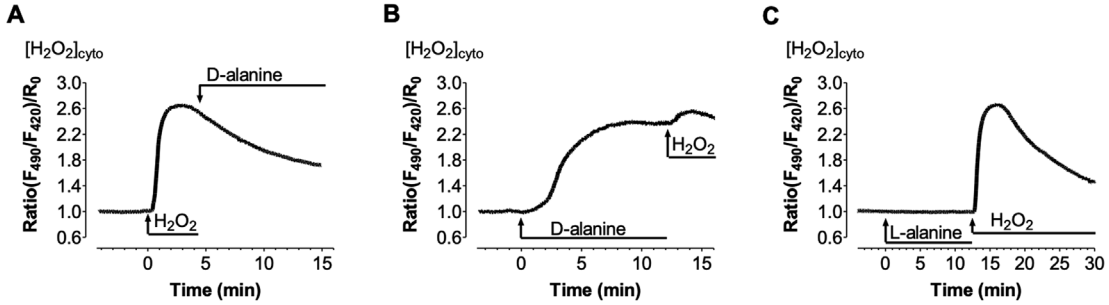

Fig. 1. Sequential ratiometric responses of the cytosol-targeted chimeric biosensor HyPer7.2-DAAO: Representative curves of ratiometric live-cell H_2O_2 imaging in EA.hy926 cells transfected with the cytosol-targeted biosensor/chemogenetic construct HyPer7.2-DAAO-NES, analyzed in real time in response to the sequential addition of (**A**) 25 μM H_2O_2 followed by 10 mM D-alanine, (**B**) D-alanine followed by H_2O_2 or (**C**) L-alanine (10 mM) followed by H_2O_2. Arrows indicate the times of drug addition. These experiments were independently repeated three times with similar results.

cytosol (DAAO-NES), nucleus (DAAO-NLS), or caveolae (DAAO-Cav) led to a robust increase in the phosphorylation of eNOS at Ser^{1177}, Thr^{495} following addition of D-alanine to the cells, whereas eNOS Ser^{114} underwent dephosphorylation, as has been previously seen in response to agonist treatment of endothelial cells [20–22]. In contrast to the robust eNOS phosphorylation responses to the generation of intracellular H_2O_2 by DAAO, the addition of exogenous H_2O_2 to the cells led only to a nominal phosphorylation response (Fig. 4D and E). Indeed, Ser^{1177} was the only eNOS phosphorylation site that underwent a significant if weak phosphorylation in response to extracellular H_2O_2 (25 μM). We also performed D-alanine and H_2O_2 dose response experiments analyzing eNOS phosphorylation at Ser^{1177}, and again found a much more robust phosphorylation response to DAAO-generated H_2O_2 compared to the response to extracellular H_2O_2 (Supplementary Fig. 1). Adenoviral infection has no effect on D-alanine- or H_2O_2-dependent phosphorylation responses in these cells. (Supplementary Fig. 3). We also did immunoblot experiments in EA.hy926 cells expressing differentially-targeted DAAO following L-alanine (10 mM) treatment and probed eNOS phosphorylation at Ser^{1177} (Fig. 1); treatments with L-alanine did not elicit eNOS phosphorylation in any subcellular compartment or any time point (Supplementary Fig. 2).

3.3. Differential kinase Akt and AMPK phosphorylation responses to intracellular and extracellular H_2O_2

Multiple protein kinases have been found to be involved in H_2O_2-modulated eNOS phosphorylation [11,12]. Two of the key eNOS kinases are kinase Akt and the AMP-activated protein kinase (AMPK) [22,23]. These kinases are known to be activated in response to H_2O_2 in multiple cell types including endothelial cells, and undergo dynamic translocation inside cells [24,25]. We again used a chemogenetic approach to generate H_2O_2 in EA.hy926 endothelial cells that had been transduced with differentially-targeted AV5-HyPer-DAAO constructs. After adding D-alanine or H_2O_2 to transduced cells, we analyzed immunoblots probed with phosphospecific antibodies for kinase Akt (phosphorylated at Ser^{473}) and AMPK (Thr^{172}). For each of the three differentially-targeted DAAO constructs, D-alanine treatment elicited a robust 30-40-fold increase in kinase Akt phosphorylation. D-alanine treatment also promoted phosphorylation of AMPK, but the response for each of the differentially-targeted DAAO was an order of magnitude less than that seen for kinase Akt phosphorylation (Fig. 5A–C and E). In marked contrast, addition of extracellular H_2O_2 elicited no phosphorylation response whatsoever for either kinase Akt nor AMPK (Fig. 5D

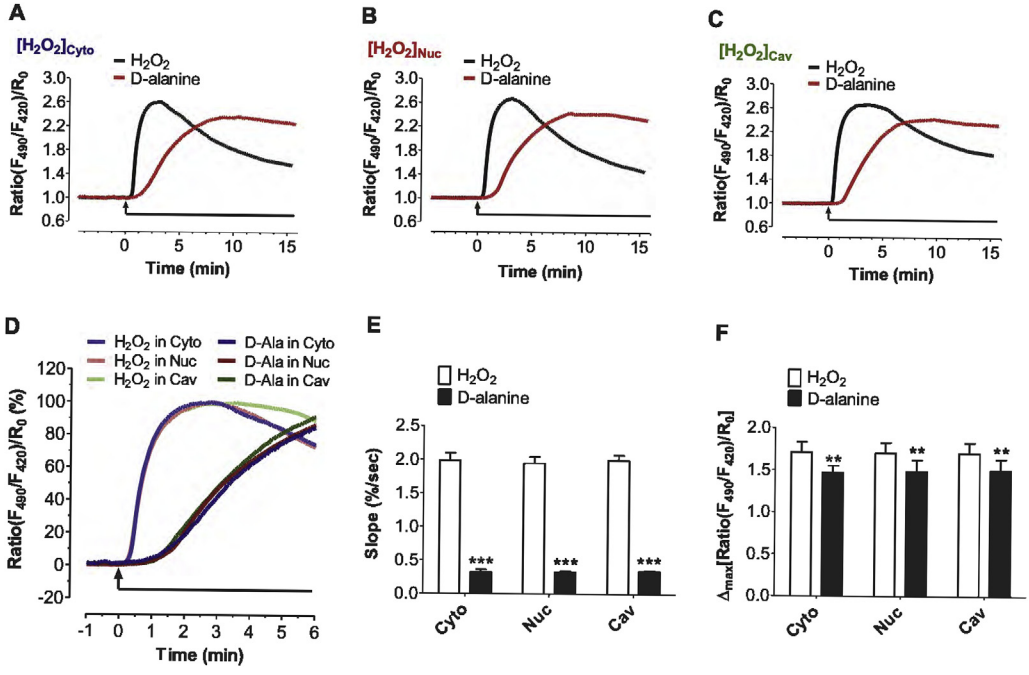

Fig. 2. Simultaneous generation and detection of intracellular H_2O_2 in different subcellular locales using differentially-targeted HyPer7.2-DAAO. (**A-C**) Average curves HyPer7.2 ratio measured in response to 25 μM H_2O_2 (black curves) or 10 mM D-alanine (red curves) of EA.hy926 expressing HyPer7.2-DAAO in (**A**) cell cytosol (Cyto), (**B**) nucleus (Nuc) or (**C**) plasmalemmal caveolae (Cav). (**D**) Zoom in overlaid average curves presented in A-C after calculation of maximum responses of all individual cells treated with either H_2O_2 or D-Alanine defined as 100%. Time point 0 indicates time of application. (**E**) Statistical evaluation of average slopes expressed in %/s within the 10–70% interval of maximum cell responses to extracellular H_2O_2 or endogenously generated H_2O_2 by DAAO after adding D-alanine, in the cytosol (white bar, n = 8/18 and gray bar, n = 8/12); nucleus (white bar, n = 8/14 and gray bar, n = 9/21); or caveolae (white bar, n = 9/23 and gray bar, n = 8/18). (**F**) Columns represent maximal responses after application of exogenous H_2O_2 (white bars) vs. D-alanine (gray bars) of same cells and subcellular locales analyzed in E. All values are presented as mean ± S.D; $^{**}P < 0.01$, $^{***}P < 0.001$. Responses to D-Alanine vs. extracellular H_2O_2 application was analyzed by one-way ANOVA and Tukey's multiple comparison test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

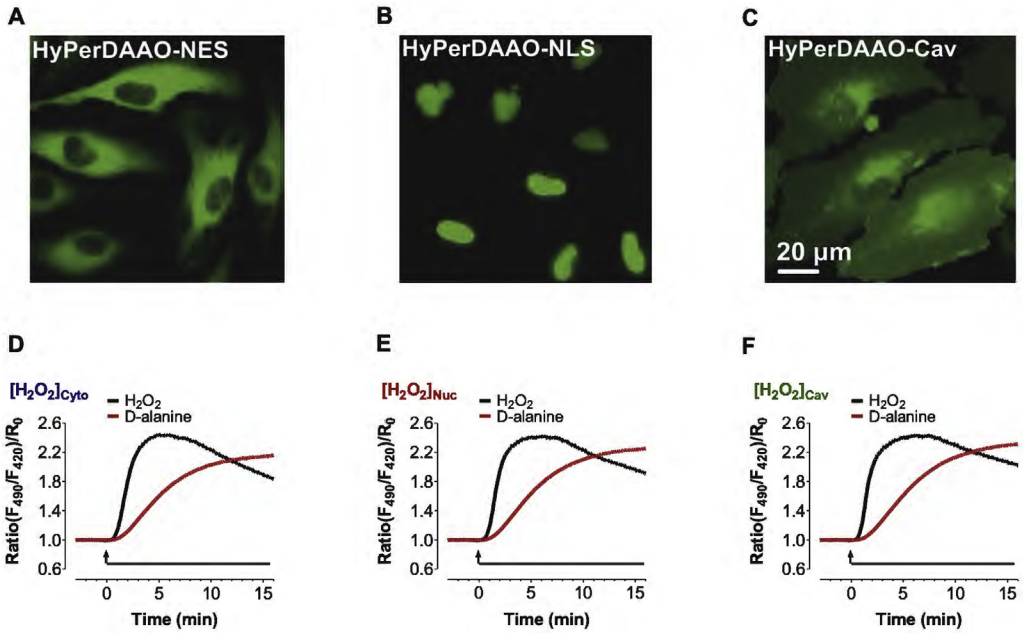

Fig. 3. Representative images of localized HyPer-DAAO expression. The upper panels show representative widefield ratiometric HyPer images of EA.hy926 endothelial cells transduced with adenoviral AV5 vectors encoding HyPer-DAAO constructs targeted to (**A**) the cell cytosol (HyPerDAAO-NES); (**B**) the cell nucleus (HyPerDAAO-NLS) and (**C**) plasmalemmal caveolae (HyPerDAAO-Cav). The lower panels (**D–F**) show representative HyPer responses quantitated in real time after treatment with H_2O_2 (25 μM, black lines) or D-alanine (10 mM, red curves) of EA.926hy cells transduced with recombinant AV5 constructs expressing HyPer1-DAAO in different subcellular regions, as indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and E). Thus, as we found for H_2O_2-promoted eNOS phosphorylation (Fig. 4), neither AMPK nor kinase Akt phosphorylation is affected by exogenous H_2O_2, in contrast to the striking phosphorylation responses seen following intracellular generation of H_2O_2 by DAAO expressed in different subcellular organelles.

3.4. Differential phosphorylation pathways activated by H_2O_2 in the cell nucleus

We next used a series of small molecular protein kinase inhibitors in order to probe the pathways leading from H_2O_2 to eNOS phosphorylation. We used the well-characterized PI3-kinase inhibitor wortmannin to block kinase Akt phosphorylation, and used the AMPK inhibitor Compound C in order to probe the effects of AMPK on downstream phosphorylation responses. Again, we studied EA.hy926 endothelial cells that had been transduced with AV5-DAAO constructs targeted to cytosol, nucleus or caveolae. After briefly pre-incubating the transduced cells either with the PI3–K inhibitor wortmannin or the AMPK inhibitor Compound C, we added D-alanine, harvested the cells at various times, and then analyzed phosphorylation responses in immunoblots probed with a series of phosphospecific antibodies.

As shown in Fig. 6, wortmannin inhibited all of the phosphorylation

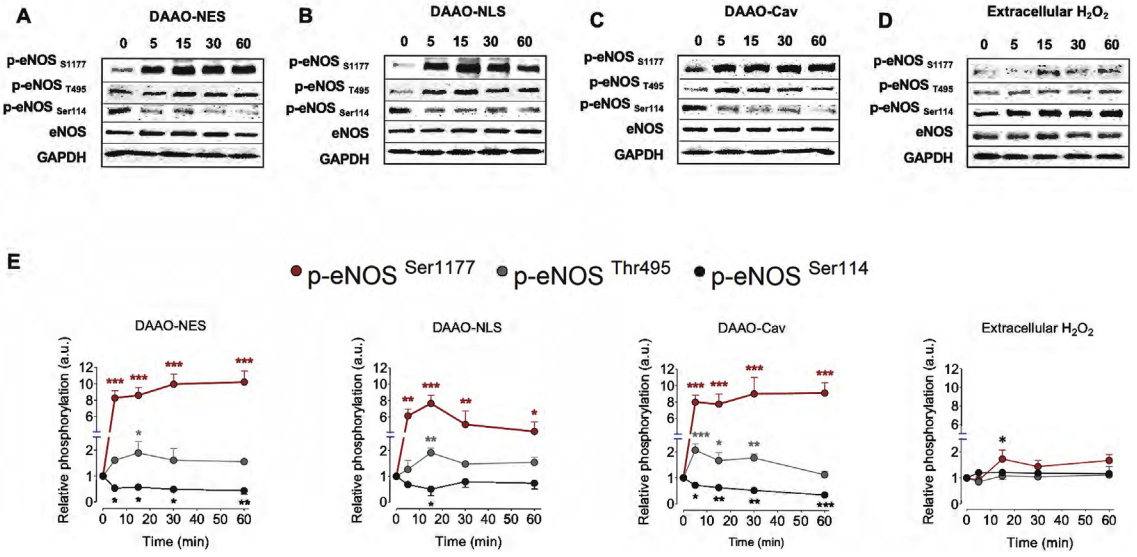

Fig. 4. Differential eNOS phosphorylation responses to intracellular vs. extracellular H_2O_2. (**A-D**) The upper row shows representative immunoblots of time course experiments in EA.hy926 endothelial cells transduced with differentially-targeted DAAO-cells were then treated with D-alanine (10 mM) or H_2O_2 (25 μM). Cells were harvested at the time points indicated, and cell lysates were resolved by SDS-PAGE and probed with phosphospecific antibodies directed against eNOS phosphorylation sites Ser^{1177}, Ser^{114}, and Thr^{495}. The cell lysates were also probed for total eNOS as a loading control to confirm equal loading. (**E**) These graphs present pooled data from three independent times course phosphorylation experiments, each performed in triplicate, showing the quantitative analysis of the intensities corresponding to phospho-eNOS at Ser^{1177}, Ser^{114}, and Thr^{495} by chemiluminescence. In constrast to the robust phosphorylation responses to D-alanine, addition of extracellular H_2O_2 promoted eNOS phosphorylation at a single phosphorylation site at a single time point (Ser114), a finding of uncertain biological significance. All values are presented as mean ± S.D. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to control analyzed by one-way ANOVA and Tukey's multiple comparison test.

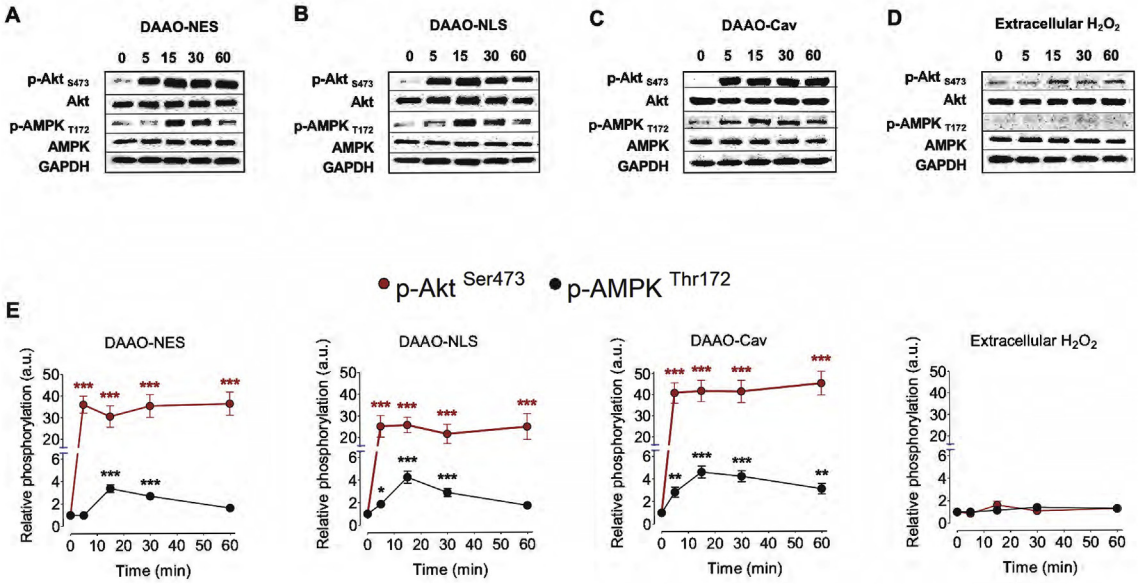

Fig. 5. Differential Akt or AMPK phosphorylation in response to intracellular or extracellular H_2O_2. (**A-C**) shows representative immunoblots of time course experiments in EA.hy926 cells expressing differentially-targeted DAAO, incubated with 10 mM D-alanine, or with 25 μM extracellular H_2O_2 (**D**) Cell lysates were probed with phosphospecific antibodies directed against phosphorylated protein kinases Akt at Ser^{473} and AMPK at Thr^{172}. The lower panel (**E**) shows data from three independent experiments in triplicate represent the quantitative analysis of the intensities corresponding to phospho-Akt^{Ser473} and -$AMPK^{Thr172}$ by chemiluminescence. The values are presented as mean ± S.D. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control analyzed by one-way ANOVA and Tukey's multiple comparison test.

responses elicited by D-alanine-dependent activation of DAAO, including eNOS phosphorylation at Ser^{1177} and Ser^{633}, and also blocking kinase Akt and AMPK phosphorylation. The inhibitory effect of wortmannin was seen to be independent of the intracellular source of H_2O_2. But in contrast to the inhibitory effects of wortmannin, we found that the AMPK inhibitor Compound C entirely failed to block the effects of cytosol- or caveolae-generated H_2O_2 on phosphorylation responses for all of these proteins. We are confident that Compound C was effective

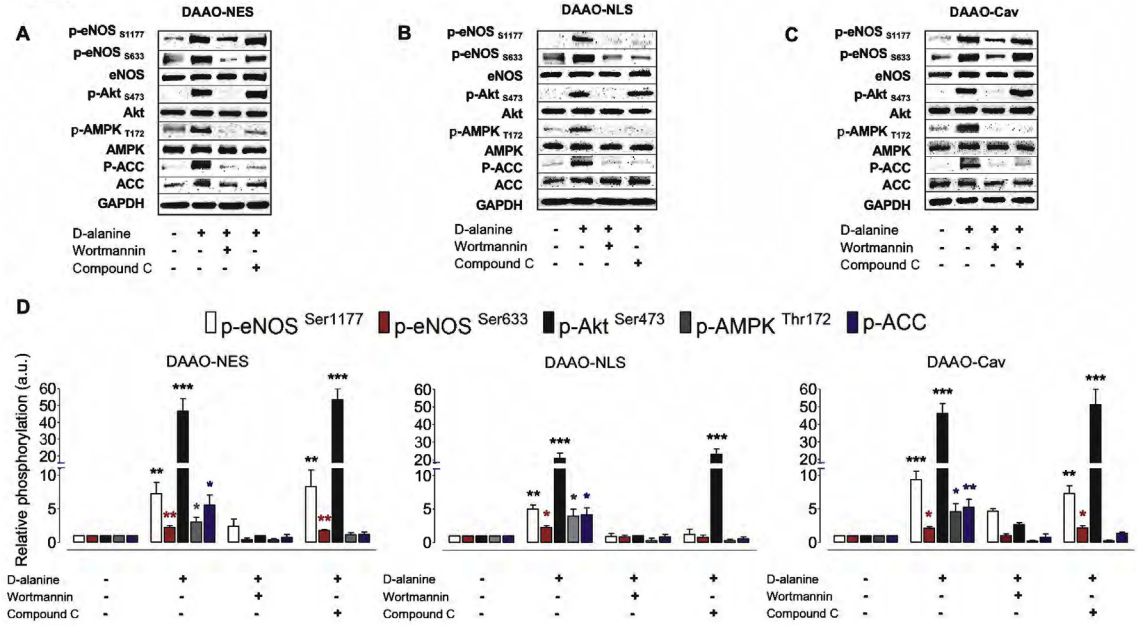

Fig. 6. Effects of kinase inhibitors on eNOS phosphorylation following H_2O_2 generation by differentially-targeted DAAO. Panels (**A-C**) show immunoblots represent the differential effects of PI3–K inhibitor, wortmannin or AMPK inhibitor, Compound C on cytosolic vs. nuclear vs. caveolar H_2O_2-mediated eNOS phosphorylation at stimulatory sites of Ser^{1177} and Ser^{633}, as well as protein kinase AMPK Thr^{172} and its downstream acetyl-CoA carboxylase (ACC). The differentially-targeted DAAO-expressing EA.hy926 endothelial cells were individually pretreated with either wortmannin (1 μM) or Compound C (20 μM) for 30 min followed by incubation with D-alanine or L-alanine (10 mM) for 15 min. Cell lysates were probed with the phosphospecific antibodies directed against phosphorylation sites of eNOS at Ser1177 and Ser633, as well as AMPK at Thr172 and ACC at Ser79. Equal loading was confirmed by immunoblotting with antibodies directed against total eNOS, AMPK and ACC. (**D**) Shown are the pooled data analyzing the intensities corresponding to phospho-eNOS at Ser1177 and Ser633 plus phospho-AMPK at Thr172 and phospho-ACC by chemiluminescence from the independent experiments which were performed in triplicate at the same time. The values are presented as mean ± S.D. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to wortmannin- or Compound C-pretreated group analyzed by one-way ANOVA and Tukey's multiple comparison test.

under these conditions since phosphorylation of the AMPK substrate acetyl-CoA carboxylase (ACC) was completely blocked by Compound C. Importantly, we found that the effects of Compound C on phosphorylation pathways activated by H_2O_2 generated in the cell nucleus were quite different than was observed for cytosol- or caveolae-generated H_2O_2. When H_2O_2 was generated by the nuclear-targeted DAAO, Compound C completely abrogated eNOS phosphorylation at Ser^{1177} and Ser^{633}, and also blocked ACC phosphorylation–while yet leaving kinase Akt phosphorylation unaffected (Fig. 6).

4. Discussion

These studies have exploited chemogenetic approaches to probe H_2O_2-modulated signaling responses in cultured vascular endothelial EA.hy926 cells. Our findings raise important caveats about the interpretation of signal transduction experiments in which H_2O_2 is added directly to cells. The signaling responses following addition of H_2O_2 directly to cultured cells ("extracellular H_2O_2") are remarkably different from the responses seen following the intracellular chemogenetic generation of H_2O_2. We found that the key endothelial signaling phosphoproteins Akt, AMPK, and eNOS exhibit robust and rapid phosphorylation responses following the chemogenetic generation of intracellular H_2O_2 by adding D-alanine to cells transfected with DAAO. In striking contrast, the effects of extracellular H_2O_2– added at "physiological" concentration (25 μM) – had a modest to nil effect on these phosphoproteins. Our data suggest that the protein phosphorylation responses promoted by either exogenous or endogenous H_2O_2 may depend more on the divergent kinetics and reactivity of localized intracellular H_2O_2 than on the absolute amount of H_2O_2. This disparity is reminiscent of findings made 40 years ago in the early years of studying cyclic AMP second messenger signal transduction [26,27]. Back in the day, many papers analyzed cellular signaling responses after adding high concentrations of cyclic AMP to cultured cells. Only with later study did it become clear that the cellular responses that are elicited by treatments with extracellular cyclic AMP are strikingly different (and usually much less robust) that the responses seen following intracellular cyclic AMP generation in response to receptor-dependent activation of adenylate cyclase. There are important parallels between these observations in cyclic AMP- and H_2O_2-mediated signal transduction, and our current studies raise important concerns about the many hundreds of reports (including our own [28,29]) that have relied upon analyses of cellular responses to extracellular oxidants [7]. The current findings provide evidence that studies of signaling responses to extracellular H_2O_2 should be interpreted with caution.

Despite the markedly divergent signaling responses to extracellular vs. intracellular H_2O_2, the maximal levels of H_2O_2 measured in different subcellular locales were more similar than different. We expressed differentially-targeted fusion proteins between the yeast enzyme DAAO and the H_2O_2 biosensor HyPer, a chimeric protein that generates and detects H_2O_2 at levels found in mammalian cells [30]. These fusion proteins were directed to specific subcellular locales using signal sequences in order to generate (DAAO) and detect (HyPer) H_2O_2 either in the cell cytosol, nucleus, or caveolae, and we then quantitated intracellular levels of H_2O_2 in real time using fluorescence microscopy. Extracellular H_2O_2 led to an increase in the HyPer ratio within 1–2 min; a similar time course was found whether H_2O_2 was quantitated in the cell cytosol, nucleus, or caveolae following addition of H_2O_2 to the cell culture media. These observations are consistent with there being similar rates of intracellular transport of H_2O_2 once it crosses the plasma membrane after being added to cultured cells [15]. The time course of intracellular H_2O_2 detection following D-alanine addition to cells was slower than the response to extracellular H_2O_2, presumably reflecting both the time required for transport of D-alanine across the cell membrane and for its intracellular diffusion to reach the differentially-targeted DAAO constructs, as well as the time required for generation of H_2O_2 by the enzyme once its D-alanine substrate arrives (along with the enzyme's co-substrates). By contrast, the detection of exogenous H_2O_2 by HyPer is limited only by the intracellular diffusion/transport of H_2O_2 to the biosensor, which is then rapidly oxidized [18]. Despite the disparate factors affecting H_2O_2 vs. D-alanine transport, diffusion, and metabolism– as well as the complexities of DAAO catalysis– we found that the HyPer response patterns were similar in different subcellular locales no matter whether H_2O_2 was added to cells extracellularly or generated within the cell by DAAO targeted to the cytosol, nucleus, or caveolae. Yet the consequences for cell signaling were strikingly divergent.

These studies have shown that the chemogenetic intracellular generation of H_2O_2– whether from the cell cytosol, nucleus, or caveolae promotes quantitatively similar phosphorylation responses for kinase Akt, AMPK and for multiple different phosphorylation sites on eNOS. Yet we still hypothesized that H_2O_2 formed in different subcellular locales might activate distinct downstream signaling pathways on the way to promote the phosphorylation of eNOS. Following on our studies of kinase Akt and AMPK in eNOS phosphorylation (Figs. 4 and 5), we decided to use small molecule inhibitors of these two kinases to try and dissect the intracellular phosphorylation pathways connecting intracellular H_2O_2 generation and eNOS. We found that the PI3–K/Akt inhibitor wortmannin blocked the entirety of all of the phosphorylation responses that were seen following activation of H_2O_2 produced by D-alanine in transduced cells. Whether the H_2O_2 was generated in the cell cytosol, nucleus, or caveolae, wortmannin completely abrogated the phosphorylation of eNOS, kinase Akt, and AMPK, as well as blocking the phosphorylation of the well-characterized AMPK substrate acetyl-CoA carboxylase (ACC). This finding establishes PI3–K/Akt as proximal determinant of H_2O_2-dependent phosphorylation responses in these cells. By contrast, the AMPK inhibitor Compound C inhibited phosphorylation responses *only* to nucleus-derived H_2O_2, and had no effect whatsoever on the phosphorylation response to caveolae- or cytosol-derived H_2O_2. Like other small molecule protein kinase inhibitors, Compound C is not absolutely specific for AMPK inhibition in all cells, but Compound C appears to be a highly selective AMPK inhibitor in endothelial cells [31,32]. We are confident that the added Compound C was effective in blocking AMPK activity because phosphorylation of the AMPK substrate acetyl-CoA carboxylase (ACC) was completely blocked. Thus, AMPK does not appear to modulate eNOS phosphorylation in response to the generation of H_2O_2 in cytosol or caveolae, yet AMPK is essential for eNOS phosphorylation in response to H_2O_2 that is generated in the cell nucleus (Figs. 6 and 7). Since AMPK is dynamically targeted to the cell nucleus [23], these observations may identify a new target for AMPK-dependent signaling in response to oxidants in the cell nucleus.

Our observations indicate that endothelial signaling pathways are differentially modulated by H_2O_2 generated in different subcellular locales. A critical question unanswered by these studies is the identity of the specific enzymes and pathways that modulate intracellular H_2O_2 levels in distinct organelles. It has long been known that the metabolic pathways that modulate intracellular oxidants are differentially distributed within cells [1], Much has been written about this topic, but our understanding remains incomplete (see reviews in Ref. [2,5,6]). There are multiple intracellular sources of H_2O_2, including NADPH oxidase isoforms targeted to diverse intracellular organelles, the mitochondrial electron transport chain, and a broad range of oxidoreductases. The catabolism of H_2O_2 in endothelial cells is also complexly determined, and involves glutathione/glutathione reductases, thioredoxin/thioredoxin reductases, catalase, and peroxiredoxins. These enzymes have distinct catalytic properties and are differentially distributed in cellular organelles. The disparate pathways involved in the generation and catabolism of H_2O_2 lead to an inhomogeneous distribution of the redox potential in specific subcellular compartments, and to distinct patterns of protein oxidative modifications in disparate locales of endothelial cells. Regional differences in redox state have direct implications for the regulation of eNOS, which undergoes

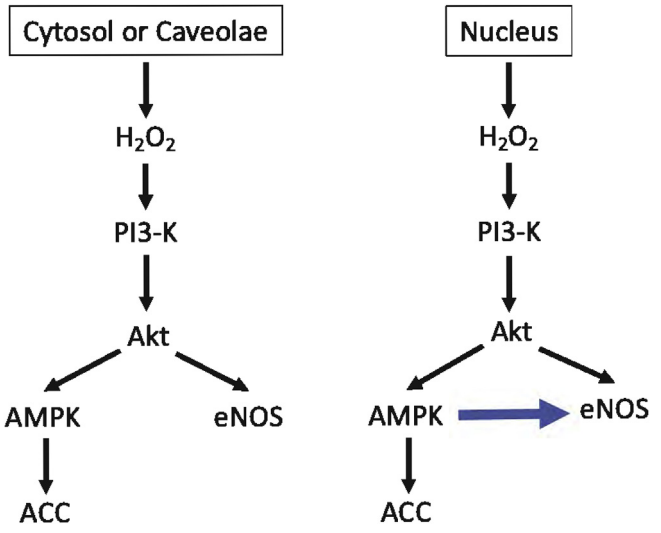

Fig. 7. Schematic model for eNOS phosphorylation pathways via differentially-targeted H_2O_2. This figure presents a model showing pathways for modulation of eNOS phosphorylation pathways by H_2O_2 generated in different subcellular compartments (cytosol vs. nucleus vs. caveolae) in endothelial cells. We propose that H_2O_2 generated in the endothelial cell nucleus, but not in the cytosol or caveolae, leads to eNOS phosphorylation via AMPK. Extracellular H_2O_2 has only a nominal effect on these phosphorylation pathways. See text for details.

dynamic translocation to and from plasmalemmal caveolae [8]. Chemogenetic approaches may allow us to better understand the role of subcellular localization of H_2O_2 in the regulation of endothelial signaling pathways both in health and disease states characterized by oxidative stress.

Author contributions

S.S., E.E., M.W.-W., A.S., B.S. and T.M. designed the experiments. S.S., M.W–W., and E.E. performed and analyzed the experiments. S.S., M.W.-W. and T.M. wrote the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101605.

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