Differential endothelial hydrogen peroxide signaling via Nox isoforms: Critical roles for Rac1 and modulation by statins

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Keywords:
Endothelial cells
Oxidative stress
Rac1
Signal transduction
NADPH oxidases

Abstract

Statins have manifold protective effects on the cardiovascular system. In addition to lowering LDL cholesterol levels, statins also have antioxidant effects on cardiovascular tissues involving intracellular redox pathways that are incompletely understood. Inhibition of HMG-CoA reductase by statins not only modulates cholesterol synthesis, but also blocks the synthesis of lipids necessary for the post-translational modification of signaling proteins, including the GTPase Rac1. Here we studied the mechanisms whereby Rac1 and statins modulate the intracellular oxidant hydrogen peroxide (H₂O₂) via NADPH oxidase (Nox) isoforms. In live-cell imaging experiments using the H₂O₂ biosensor HyPer7, we observed robust H₂O₂ generation in human umbilical vein endothelial cells (HUVEC) following activation of cell surface receptors for histamine or vascular endothelial growth factor (VEGF). Both VEGF- and histamine-stimulated H₂O₂ responses were abrogated by siRNA-mediated knockdown of Rac1. VEGF responses required the Nox isoforms Nox2 and Nox4, while histamine-stimulated H₂O₂ signals are independent of Nox4 but still required Nox2. Endothelial H₂O₂ responses to both histamine and VEGF were completely inhibited by simvastatin. In resting endothelial cells, Rac1 is targeted to the cell membrane and cytoplasm, but simvastatin treatment promotes translocation of Rac1 to the cell nucleus. The effects of simvastatin both on receptor-dependent H₂O₂ production and Rac1 translocation are rescued by treatment of cells with mevalonic acid, which is the enzymatic product of the HMG-CoA reductase that is described to treat hypercholesterolemia and prevent cardiovascular disease. In addition to their well-characterized effects on lowering cholesterol, statins also lead to alterations in cellular redox balance that are associated with beneficial cardiovascular outcomes. The molecular mechanisms for these “antioxidant” roles of statins are incompletely understood, and the present studies explore the pathways connecting statins and NADPH oxidase isoforms and reveal an unexpected role for the small GTPase Rac1.

1. Introduction

Many cardiovascular disease states are associated with pathological oxidative stress caused by excessive levels of reactive oxygen species (ROS) [1]. Yet at lower ROS concentrations, intracellular oxidants play key roles in cell survival and growth [2]. The stable ROS hydrogen peroxide (H₂O₂) modulates physiological signaling pathways in vascular endothelial cells [3,4]. However, prolonged and/or high H₂O₂ concentrations in endothelial cells can lead to pathological oxidative stress and vascular dysfunction. Intracellular redox balance is determined by a complex network of oxidases and reductases, and the NADPH oxidase (Nox) isoforms represent a family of differentially-regulated membrane-targeted oligomeric enzymes that play a central role in determining both the concentration and subcellular distribution of oxidants within cells. Cellular redox balance is also influenced by statins [5,6], which are HMG-CoA reductase inhibitors that are widely prescribed to treat hypercholesterolemia and prevent cardiovascular disease. In addition to their well-characterized effects on lowering cholesterol, statins also lead to alterations in cellular redox balance that are associated with beneficial cardiovascular outcomes. The molecular mechanisms for these “antioxidant” roles of statins are incompletely understood, and the present studies explore the pathways connecting statins and NADPH oxidase isoforms and reveal an unexpected role for the small GTPase Rac1.

Rac1 is a multifunctional protein that subserves many roles in
endothelial cells, including cellular differentiation, adhesion, angiogenesis, migration, vascular permeability, and redox signaling [7–9]. Rac1 undergoes posttranslational modifications that modulate its activity and subcellular localization. Covalent lipid modification of Rac1 by prenylation at a specific site near the protein’s carboxyl terminus modifies Rac1 function and is required for its membrane localization. The prenylation of Rac1 provides an important connection between Rac1 and statins: in addition to their effects on cholesterol synthesis, statins also block the synthesis of isoprenoid lipids that are necessary for Rac1 and statins: in addition to their effects on cholesterol synthesis, statins also block the synthesis of isoprenoid lipids that are necessary for Rac1 prenylation of signaling proteins, including Rac1. A broad range of cell surface receptors modulate Rac1 activation, which activate distinct downstream signaling pathways. Our lab has previously shown that siRNA-mediated knockdown of Rac1 in cultured vascular endothelial cells (EC) affects key endothelial signaling pathways and modulates the endothelial response to various receptor agonists, including vascular endothelial growth factor (VEGF), sphingosine-1-phosphate (S1P), epinephrine, and angiotensin-II [10–15]. Notably, Rac1 also serves as a modulator of some (but not other) NADPH oxidase (Nox) isoforms.

Members of the Nox protein family catalyze the reduction of molecular oxygen (O2) either to superoxide (Nox1, Nox2, Nox3, Nox5) or to H2O2 (Nox4). NADPH oxidase isoforms are targeted to diverse cellular membranes [16], and serve as cell type-specific modulators of redox balance [17]. The composition and localized expression of the diverse Nox isoforms are critical determinants of receptor-dependent redox responses in different cell types. Even among vascular endothelial cells, Nox isoforms are differentially expressed in different vascular beds [18] and are differentially regulated by Rac1 [19]. The development of genetically-encoded biosensors for H2O2 [20,21] has permitted the study of receptor-modulated H2O2 in real time using live cell imaging. The novel genetically-encoded H2O2 biosensor HyPer7 [22] represents the most sensitive and selective probe for intracellular H2O2 [23]. In the present studies, we have exploited HyPer7 to define the pathways whereby statins exert antioxidant effects on vascular endothelial cells and have implicated specific Nox isoforms and Rac1 in the control of redox balance in these cells.

2. Materials and methods

2.1. Materials

Histamine dihydrochloride (histamine), PP2, simvastatin, (R)-mevalonic acid lithium salt (mevalonic acid) and imaging buffer components were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vascular endothelial growth factor (VEGF) was obtained from R&D Systems (Minneapolis, MN, USA). NSC23766 and LB42708 were from APEXBio Inc (Houston, TX, USA). Lipofectamine™ 3000 Transfection Reagent (LF3000) were purchased from Thermo Fisher Scientific (Waltherm, MA, USA). GKT137831 was obtained from Cayman Chemical (Ann Arbor, MI, USA) and PepMute™ siRNA Transfection Reagent was from SignaGen (Frederick, MD, USA).

2.2. Cell culture

The entire study was performed with a pooled human umbilical vein endothelial cell (HUVEC) strain CC-2519 lot #18TL14996 (Lonza, Walkersville, MD, USA) at passages 3–7. Optimum cell culture conditions are critical for primary endothelial cells to ensure vital functions in cell growth and proper endothelial signaling in imaging procedures in particular with respect to VEGF signaling. Accordingly, we used various cell culture media varying in serum and VEGF composition. HUVEC at cell confluence below 40% were cultured in Endothelial cell growth medium-V2 (ECGM-V2, #213–500, Cell Applications Inc., San Diego, CA, USA) containing serum and growth factors for accelerated growth. Alternatively endothelial basal medium (EBM, #EC-3121, Lonza) with Endothelial Cell Growth Medium SingleQuots™ Suplements (#{CC-4133, Lonza} had been used below 60% cell confluence. HUVEC were placed at least 40 h before experiments in ECGM without serum and VEGF (#{211–500, Cell Applications Inc.}). Cells were maintained under standard conditions in a humidified incubator (37 °C, 5% CO2, 95% air). Small molecule inhibition with GKT137831 (20 μM), or PP2 (100 nM) was performed in ECGM for 1–2 h prior live cell imaging experiments. For overnight treatments with other chemical compounds (200 μM NSC23766, 10 μM simvastatin, 400 μM mevalonic acid, 10 μM LB42708) respective substances were added to ECGM and cells were incubated for at least 16 h before imaging experiments. For live-cell imaging experiments presented in the Supplementary Movie 1 with mevalonic acid administration were applied to ECGM without phenol red (#211 PR-500, Cell Applications Inc.). The small molecule inhibitors (GKT137831 and NSC23766) were tested for cytotoxic effects using the Cell proliferation Kit I (MTT, Roche Diagnostics, Germany) according to the manufacturer’s protocol. Neither GKT136731 nor NSC23766 had cytotoxic effects on the HUVEC under the conditions used in these experiments (Supplementary Fig. 5).

2.3. Generation and transduction of genetically encoded molecules

Plasmid construction of OFP-Rac1 and OFP-Rac1C189S mutant were performed with standard cloning procedures. In brief, the coding
sequence of Rac1 was amplified from HUVEC cDNA by PCR using a Q5® High-Fidelity DNA Polymerase (New England Biolabs Inc., NEB, Ipswich, MA, USA) with recognition site primer overhangs (Supplementary Table 1). The PCR product was digested with indicated restriction enzymes (NEB) and C-terminally fused using the Quick Ligation™ Kit (NEB) to an orange fluorescent protein (OFP) in a pcDNA3.1(+) vector [24]. Mutagenesis of the CAAX domain of Rac1 from cysteine to serine was alternately amplified and subcloned in the same vector. In addition, we used a plasmid encoding a biosensor for H₂O₂ targeted to the cytosol, HyPer7-NES [22]. All plasmids were transiently (co-)transfected in HUVEC using LF3000 in Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific) according to the manufacturers’ protocol. For the knockdown of target proteins, we used pools of 2 different siRNA for each target mRNA (Horizon Discovery, Waterbeach, UK) with sense strands (Supplementary Table 1): All siRNAs were transfected in HUVEC at a concentration of 40 nM by the reverse transfection method as described for the PepMute siRNA transfection protocol (SignaGen). Transfection mixture of plasmid as well as of siRNA was replaced with ECGM 8–10 h after transfection. Alternatively, HyPer7-NES was transduced with an adenovirus AV5 (Viraquest, North Liberty, IA, USA) at a multiplicity of infection of 10 in ECGM 42–48 h before imaging experiments either 24 h before adding chemical inhibitors or 24 h after siRNA transfection.

2.4. Quantitative real time PCR

We determined the mRNA levels of all NADPH oxidase isoforms (Nox1-Nox5) as well as of Rac1. Total RNA was isolated using the RNeasy Plus Universal Mini Kit (Qiagen Inc., Germantown, MD, USA), and reverse transcription was performed in a EdvoCycler™ Jr. Personal PCR Machine (Edvotek Inc., Washington, DC, USA) using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). An iTaq™

Fig. 1. Histamine- and VEGF-induced H₂O₂ responses are mediated by different Nox pathways. This figure shows ratiometric live cell imaging in HyPer7-transfected HUVEC in response to (A) histamine (30 μM; n = 21) or (B) VEGF (40 ng/ml; n = 17). In panels C and D, HUVEC were first treated with the selective Nox1/4 inhibitor GKT137831 prior to agonist addition. Panels E and F show agonist-modulated H₂O₂ responses in HyPer7-transfected HUVEC following treatment with control duplex siRNA (siControl, black or blue curve); siRNA targeting Nox2 (siNox2, orange curve); or siRNA targeting Nox4 (siNox4, gray curve). Validation of the HyPer7 ratiometric response to agonist treatments is shown in Supplementary Fig. 1. Statistical analysis in the effects of GKT137831 is shown in Supplementary Fig. 2. Validation of the Nox2 and Nox4 siRNA is shown in Supplementary Fig. 3. * indicates P < 0.05 and *** indicates P < 0.001, calculated by ANOVA.
Universal SYBR® Green One-Step Kit (Bio-Rad, Hercules, CA, USA) was used to perform real time PCR on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Target genes were quantified using the primers with indicated transcript positions (Supplementary Table 1) and the relative expression was normalized with RNA polymerase II subunit A (Rpol2) as a housekeeping gene.

2.5. Immunochemical procedures

For Western blot experiments siRNA transfected HUVEC remained either untreated or were stimulated with 30 μM histamine and 40 ng/ml VEGF, respectively for 10 min. Protein sample collection and Western blotting procedures were then performed as previously described [25]. Immunoblot analysis were incubated using primary antibodies (1:1000) for phosphorylated Akt kinases (Akt) and extracellular signal-regulated protein kinase (Erk) (CST, Cell Signaling Technologies, Danvers, MA, USA) and labeled with a secondary anti-rabbit or anti-mouse antibody (CST, 1:2000). The blots were then re-probed for total, Akt or Erk protein abundance. Blots were then incubated with an enhanced chemiluminescent horse radish peroxidase substrate, either a SuperSignal™ West Femto or Pico substrate (Thermo Fisher Scientific) before analysis by chemiluminescence using a Chemidoc Imaging System (Bio-Rad). Protein knockdown efficiencies of Nox2 and Rac1 was done 3 days after siRNA transfection using primary antibodies for Nox2 (ABclonal, Woburn, MA, USA). Densitometric quantification of protein bands was performed using ImageJ software. (NIH, Bethesda, MD, USA).

2.6. Live-cell imaging procedures

Live-cell fluorescent recordings were performed on an inverted wide-
field fluorescent microscope (IX80, Olympus, Waltham, MA, USA) equipped with a motorized sample stage (Prior, Rockland, MA, USA), a Lumen 200 Fluorescence Illumination System (Prior), and a charge-coupled device camera (Hamamatsu, Bridgewater, NJ, USA). Real-time H$_2$O$_2$ measurements were performed with a 20x oil immersion objective (PlanSapo, Olympus). Ratiometric HyPer7 excitation was alternately performed at 420 and 490 nm (Semrock, Rochester, NY, USA) using an optical filter wheel (Sutter Instruments, Novato, CA, USA) and emission was recorded at 530 nm (Semrock) using Metafluor Software (Molecular Devices, San Jose, CA, USA). OFP-Rac1 variants were monitored with a dichroic filter (SP Gold-B OMF, Semrock) at 600 nm.

2.7. Statistical analysis

Data were analyzed using GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard error of mean (SEM) of independent experiments (N) throughout the whole manuscript. For comparisons between two groups, two-tailed Student t-test was used, and for comparison across multiple groups, one-way analysis of variance (ANOVA) with Tukey’s Multiple Comparison was utilized. A p value between 0.01 and 0.05 was considered significant and indicated with **, p between 0.001 and 0.01 as very significant with *** and p < 0.001 as highly significant with ****. Data shown are either average or representative curves of at least three independent experiments, including analyses from imaging, immunoblot and qRT-PCR experiments.

3. Results

3.1. Differential receptor-modulated H$_2$O$_2$ generation via Nox2 and Nox4 in endothelial cells

We transfected human umbilical vein endothelial cells (HUVEC) with plasmids encoding the H$_2$O$_2$ biosensor HyPer7, and then treated the transfected cells either with the G-protein coupled receptor (GPCR) agonist histamine or with the receptor tyrosine kinase (RTK) agonist VEGF. We performed real-time HyPer7 ratiometric imaging experiments to quantitate H$_2$O$_2$ formation following agonist addition. Both histamine and VEGF stimulate robust increases in intracellular H$_2$O$_2$ (Fig. 1A and B; Supplementary Fig. 1). We next explored the role of NADPH oxidases in receptor-stimulated H$_2$O$_2$ formation in these cells. Quantitative mRNA profiling using a series of validated Nox isoform-specific RT-PCR primers showed that both Nox2 and Nox4 transcripts are present in these cells, but transcripts for Nox1, Nox3, or Nox5 were not detected (Supplementary Fig. 3A). We pre-treated the cells with the selective Nox1/Nox4 small molecule inhibitor GKT137831 [26], and found that the H$_2$O$_2$ response to histamine was entirely unaffected, but the VEGF-promoted H$_2$O$_2$ signal was completely blocked by GKT137831 (Fig. 1C and D, Supplementary Fig. 2). We then developed and validated duplex siRNA constructs targeting Nox2 and Nox4, which are the only Nox isoforms that we detected in HUVEC (Supplementary Figs. 3B and 3C). siRNA-mediated knockdown of Nox4 completely blocked the VEGF-promoted H$_2$O$_2$ response, but Nox4 knockdown had no effect on the H$_2$O$_2$ response to histamine. In contrast, siRNA-mediated knockdown of Nox2 blocked the H$_2$O$_2$ response to both agonists (Fig. 1E and F, Supplementary Figs. 3D and 3E). Taken together, these results indicate that Nox2 is critical for both histamine- and VEGF-stimulated H$_2$O$_2$ signaling, whereas Nox4 is involved in the VEGF response but not in the histamine-stimulated H$_2$O$_2$ response.

3.2. Rac1 and agonist-dependent H$_2$O$_2$ responses

We used small molecule inhibitors and siRNA approaches to probe the role of Rac1 in agonist-modulated H$_2$O$_2$ responses in HUVEC. We first used the Src kinase blocker PP2, which is a known inhibitor of receptor-mediated Rac1 activation in endothelial cells [14,27]. As shown in Fig. 2, PP2 blocked both histamine and VEGF-promoted H$_2$O$_2$ responses (Fig. 2A and B, Supplementary Figs. 4A and 4B). The Rac1 small molecule inhibitor NSC23766 [28] completely blocked both histamine- and VEGF-promoted H$_2$O$_2$ signals (Fig. 2C and D, Supplementary Figs. 4C and 4D). siRNA-mediated Rac1 knockdown totally abrogated histamine- and VEGF-stimulated H$_2$O$_2$ signals (Fig. 2E and F, Supplementary Figs. 4E and 4F). Taken together, these experiments suggest that Rac1 has a central role in receptor-modulated H$_2$O$_2$ responses in endothelial cells.

We next studied the roles of Rac1, Nox2 and Nox4 in regulation of receptor-modulated pathways involving the signaling proteins Akt and Erk, both of which are protein kinases that undergo dynamic phosphorylation in response to the activation of cell surface receptors in endothelial cells [29-33]. As shown in Fig. 3, histamine promoted the phosphorylation of Erk in HUVEC, but had no effect on Akt phosphorylation. siRNA-mediated knockdown either of Nox2 or Rac1 blocked the histamine phosphorylation response, while siRNA-mediated knockdown
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of Nox4 had no effect (Fig. 3). In contrast, VEGF treatment of HUVEC promoted the phosphorylation of both kinase Akt and Erk. All of the VEGF phosphorylation responses were blocked following knockdown of either Nox2 or Nox4 or Rac1 (Fig. 3). These observations are consistent with the differential involvement of Nox2 and Nox4 in receptor-dependent signaling, and also underscore the critical role of Rac1.

3.3. Statin treatment affects Rac1 prenylation and targeting

To visualize the subcellular localization of Rac1 in HUVEC, we generated a N-terminal orange fluorescent protein-tagged Rac1 plasmid (OFP-Rac1) and transfected endothelial cells with this construct. As shown in Fig. 4A, OFP-Rac1 is targeted to the cell membrane and cytosol in transfected endothelial cells. Rac1 undergoes C-terminal prenylation, which is a post-translational modification involving a complex lipid that is downstream of the HMG-CoA reductase, which is the enzyme that is inhibited by statin drugs [34]. When endothelial cells are treated with simvastatin (a statin drug widely used to treat hypercholesterolemia), the OFP-Rac1 is now found to be localized exclusively in the cell nucleus (Fig. 4B). A key control to establish the specificity of statin effects is to add the enzymatic product of the HMG-CoA reductase, mevalonic acid, to statin-treated cells. When we added mevalonic acid to statin-treated cells, the OFP-Rac1 is once again found in the cell membrane and cytosol (Fig. 4C and D; see Supplementary Movie 1 in the Supplementary materials), as was observed in untreated cells (Fig. 4A). We next treated HUVEC with the prenylation inhibitor LB42708, and we again observed nuclear targeting of Rac1 (Fig. 4F). In contrast to simvastatin treatment, the effect of the prenylation inhibitor LB42708 was not reversible with mevalonic acid (Fig. 4G and H), showing that the effects of statins on Rac1 targeting are due to the inhibition of Rac1 prenylation and are not a consequence of other statins effects. To further establish the importance of Rac1 prenylation on its subcellular localization, we constructed an OFP-Rac1 C189S mutant in which the cysteine of the C-terminal prenylation consensus sequence CAAX was mutated to serine. This prenylation-deficient Rac1 was exclusively localized in the cell nucleus (Fig. 4E), again confirming that prenylation of Rac1 is necessary for its proper subcellular targeting to cell membrane and cytosol.

3.4. Effects of simvastatin on receptor-modulated H2O2 responses

We returned to HyPer7 imaging to explore the effects of simvastatin on receptor-modulated H2O2 responses. We treated HUVEC with 10 μM simvastatin for 16 h in the presence and absence of mevalonic acid (400 μM), and then performed live cell HyPer ratemaging imaging after adding histamine (Fig. 5A) or VEGF (Fig. 5B). Simvastatin completely blocked both histamine- (Fig. 5A and C) and VEGF-stimulated H2O2 responses (Fig. 5B and D). The inhibitory effect of simvastatin was completely abrogated by adding mevalonic acid to the statin-treated cells (Fig. 5). These findings establish that the blockade of agonist-stimulated H2O2 responses by simvastatin occurs via inhibition of farnesylation.

4. Discussion

Statins are among the most widely-prescribed drugs in the world, and their beneficial effects on LDL cholesterol levels and on cardiovascular mortality have been extensively studied [5,6]. The beneficial effects of statins on cardiovascular morbidity and mortality are seen soon after the initiation of statin therapy in patients, often weeks before serum LDL levels have changed. Moreover, statins have a beneficial effect even in patients who have normal serum LDL levels yet show signs of systemic inflammation [35]. Numerous studies have reported an

Fig. 4. Effects of simvastatin and prenylation on Rac1 localization. This figure shows representative live cell fluorescence imaging in HUVEC transfected with OFP-labeled Rac1. Panel A shows untreated cells, which exhibit OFP-Rac1 distributed between the cytosol and the cell membrane. In Panel B, HUVEC were treated with simvastatin (10 μM, 16 h), and the OFP-Rac1 is found exclusively in the cell nucleus. In panel C, cells were treated with simvastatin plus the HMG-CoA reductase product mevalonic acid (400 μM), which restores OFP-Rac1 subcellular targeting to the pattern seen in untreated cells. In Panel D, simvastatin-treated cells (16 h, 10 μM) were then treated with mevalonic acid for another 16 h, restoring OFP-Rac1 localization to the pattern seen in control cells (see Supplementary Movie 1). Panel E shows the subcellular distribution of a farnesylation-null mutant OFP-Rac1 in which the prenylated Cys residue in Rac1 is mutated to Ser (C189S), resulting in nuclear targeting of the mutated OFP-Rac1. Panel F shows that HUVEC transfected with OFP-Rac1 and then treated with the farnesyltransferase (FTase) inhibitor LB42708 (10 μM) demonstrate OFP-Rac1 localization to the cell nucleus. (G and H) Addition of mevalonic acid does not alter the nuclear localization of OFP-Rac1 in HUVEC that were treated with the FTase inhibitor LB42708. The cells shown here are representative of >50 cells studied in >10 independent experiments. In addition, prenylation-dependent subcellular targeting of Rac1 has been further verified by co-imaging experiments using the Hoechst nuclear stain (see Supplementary Fig. 6).
HUVEC express both Nox2 and Nox4, but do not show significant responses elicited by the RTK agonist VEGF or the GPCR agonist histamine whereby statins modulate redox balance in the vascular wall. The present studies provide new information that provide a plausible mechanism whereby statins modulate redox balance in the vascular wall.

Here we have used the novel genetically-encoded H$_2$O$_2$ biosensor HyPer7 to study receptor-modulated H$_2$O$_2$ responses in cultured primary human endothelial cells. These experiments show that the H$_2$O$_2$ response to histamine involve different Nox activation pathways (Fig. 1). HVEC express both Nox2 and Nox4, but do not show significant effects on protein levels for other Nox isoforms (Supplementary Fig. 3). The H$_2$O$_2$ response to histamine is not affected either by the Nox1/Nox4 small molecule inhibitor GKT137831, nor by siRNA-mediated Nox4 knockdown. In contrast, the H$_2$O$_2$ response to VEGF is blocked both by this small molecule inhibitor and also by Nox4 knockdown. For both agonists, siRNA-mediated Nox2 knockdown completely blocks the receptor-modulated H$_2$O$_2$ response (Fig. 2). The effects on agonist-modulated H$_2$O$_2$ responses following siRNA-mediated Nox2 and Nox4 knockdown reveal a similar pattern when agonist-modulated phosphorylation responses are explored. We found that VEGF-dependent Erk1/2 phosphorylation is blocked by both Nox2 and Nox4 knockdown, whereas the phosphorylation response to histamine is not affected by siRNA-mediated Nox4 knockdown (Fig. 3). This is determined principally by its level of protein expression [37,38]. But the rapid Nox4-dependent H$_2$O$_2$ response to VEGF observed here (Fig. 1) is incompatible with a change in the abundance of Nox4 protein, and plausibly reflects dynamic (and incompletely-understood) post-translational mechanisms that regulate Nox4 activity. It is intriguing that both Nox2 and Nox4 appear to be required for the VEGF H$_2$O$_2$ response, while the histamine response does not appear to involve Nox4. The key role for Nox2 is consistent with our findings implicating Rac1 (Fig. 2), which is a known component of Nox2, but not of Nox4.

The central role of Rac1 in receptor-modulated responses is established by several lines of investigation in these studies. We found (Fig. 2) that the small molecule Rac1 inhibitor NSC23786 completely blocks both histamine and VEGF-promoted H$_2$O$_2$ responses, as does the Src inhibitor PP2- which has been shown to block Rac1-mediated responses in these cells [14,27]. Knockdown of Rac1 by duplex siRNA targeting constructs completely blocks H$_2$O$_2$ responses to both histamine and VEGF, and also totally abrogates agonist-dependent phosphorylation responses (Fig. 3). These studies also confirm that Rac1 targeting plays a key role in modulating receptor-dependent H$_2$O$_2$ responses. In untreated HVEC, Rac1 localizes to the plasma membrane and is also found in the cell cytosol. But when HVEC are treated with a Rac1-activating agent, Rac1 is once again localized in the cell nucleus, yet following treatment with a Rac1-activating agent, the addition of mevalonic acid, is added to statin-treated cells. And when HVEC are treated with the farnesyltransferase inhibitor LB42708, Rac1 is found exclusively in the cell nucleus (Fig. 4). Targeting of Rac1 targeting to plasma membrane and cytosol is restored when the HMG-CoA reductase product, mevalonic acid, is added to statin-treated cells. And when HVEC are treated with the farnesyltransferase inhibitor LB42708, the addition of mevalonic acid does not restore proper Rac1 targeting. We note that the farnesyltransferase inhibitor blocks the final step leading to Rac1
localized in the cell nucleus (Fig. 4E) also helps to establish that Rac1 thus mevalonate would not be expected to recover the phenotype. M. Waldeck-Weiermair et al.

nied by the abrogation of agonist-modulated H$_2$O$_2$ responses (Fig. 5). These studies have used multiple complementary experimental approaches to establish that receptor-modulated H$_2$O$_2$ responses to hista-
mime and VEGF involve distinct N ox isoforms, both of which are completely dependent on Rac1 prenylation. These studies indicate that the salutary cardiovascular effects of statins may be explained at least in part by statin-dependent inhibition of the Rac1/Nox axis, thereby attenuating endothelial oxidative stress.

Author contributions

M.-W.-W., S.Y., J.K., V.R.T., A.P., T.C., A.A.D. and C.K. performed experiments. T.M. and M.-W.-W. designed studies and prepared the manuscript. All authors analyzed data and discussed experimental results at all stages of these studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

We thank Mr. Batur Gultekin, BSc for technical assistance. This work was supported by National Institutes of Health Grants R21 AG063073, R33 HL157918, and R01 HL152173 (to T.M.); by ST32HL07609-34 (to F.S.); and by the Austrian Science Foundation (FWF) Grant J4466 (to M. W.-W.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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