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Therapeutics

FORUM ORIGINAL RESEARCH COMMUNICATION

Enhanced Depolarization-Induced Pulmonary Vasoconstriction Following Chronic Hypoxia Requires EGFR-Dependent Activation of NAD(P)H Oxidase 2

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Abstract

Aims: Chronic hypoxia (CH) enhances depolarization-induced myofilament Ca^{2+} sensitization and resultant pulmonary arterial constriction through superoxide (O_2^-)-dependent stimulation of RhoA. Because NAD(P)H oxidase (NOX) has been implicated in the development of pulmonary hypertension, we hypothesized that vascular smooth muscle (VSM) depolarization increases NOX-derived O_2^- production leading to myofilament Ca^{2+} sensitization and augmented vasoconstrictor reactivity following CH. As epidermal growth factor receptor (EGFR) mediates Rac1-dependent NOX activation in renal mesangial cells, we further sought to examine the role EGFR plays in this response. ***Results:*** Vasoconstrictor responses to depolarizing concentrations of KCl were greater in lungs isolated from CH (4 wk, 0.5 atm) rats compared to normoxic controls, and this effect of CH was abolished by the general NOX inhibitor, apocynin. CH similarly augmented KCl-induced vasoconstriction and O_2^- generation (assessed using the fluorescent indicator, dihydroethidium) in Ca^{2+}-permeabilized, pressurized small pulmonary arteries. These latter responses to CH were prevented by general inhibition of NOX isoforms (apocynin, diphenylene iodonium), and by selective inhibition of NOX 2 (gp91ds-tat), Rac1 (NSC 23766), and EGFR (AG 1478). Consistent with these observations, CH increased KCl-induced EGFR phosphorylation, and augmented depolarization-induced Rac1 activation in an EGFR-dependent manner. ***Innovation:*** This study establishes a novel signaling axis in VSM linking membrane depolarization to contraction that is independent of Ca^{2+} influx, and which mediates myofilament Ca^{2+} sensitization in the hypertensive pulmonary circulation. ***Conclusion:*** CH augments membrane depolarization-induced pulmonary VSM Ca^{2+} sensitization and vasoconstriction through EGFR-dependent stimulation of Rac1 and NOX 2. *Antioxid. Redox Signal.* 18, 1777–1788.

Introduction

ENDOGENOUS REACTIVE OXYGEN SPECIES (ROS) are physiologically important intracellular second-messenger molecules that regulate vascular smooth muscle (VSM) phenotype (62) and contractility (4) in the normal pulmonary circulation. However, excessive ROS production from various enzymatic sources is considered to be a major contributing factor to both arterial remodeling (27, 36) and vasoconstrictor (21, 30, 36) components of chronic hypoxia (CH)-induced pulmonary hypertension (PH). Interestingly, recent evidence supports an important contribution of superoxide anion (O_2^-)-dependent RhoA activation to enhanced membrane depolarization-

Innovation

This study establishes a novel signaling axis in VSM linking membrane depolarization to contraction that is independent of Ca^{2+} influx, and which mediates enhanced myofilament Ca^{2+} sensitization in the hypertensive pulmonary circulation. The concept of depolarization as a Ca^{2+}-independent effector of EGFR-Rac1-NOX 2-RhoA signaling has potentially broad implications for understanding not only mechanisms of pulmonary vasoconstriction, but also for depolarization and oxidant regulation of cytoskeletal dynamics, motility, proliferation, apoptosis, and myogenicity in other cell systems.

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induced myofilament Ca^{2+} sensitization in hypertensive pulmonary arteries from CH rats (7). Although the signaling mechanism that links depolarization to RhoA-mediated VSM Ca^{2+} sensitization in this setting is unknown, evidence that depolarization stimulates NAD(P)H oxidase (NOX) to produce O_2^- in macula densa (38) and endothelial cells (10; 44; 60) suggests a potential role for NOX in this response.

NOX isoforms are multi-subunit enzymes found in the plasma membrane and on endosomes and have been implicated in the development of PH (27, 36, 46). NOX subtypes 1, 2, and 4, are the most abundant forms in VSM (41). The catalytic subunits of NOX 1 and 2 are activated by phosphorylation of the cytosolic subunits NOXO1 and NOXA1 in the case of NOX 1, and $p47^{phox}$ and $p67^{phox}$ in the case of NOX 2 (6, 12, 41). The small GTP-binding protein, Rac1, is also a critical signaling mediator of both NOX 1 and 2 activation (6, 12).

A potential upstream activator of NOX and Rac1 is the epidermal growth factor receptor (EGFR) (68), which transitions from an inactive monomeric form to an active homodimeric form upon phosphorylation of multiple tyrosine residues. EGFR has previously been implicated in the development of PH in rats (14, 45), and mediates PH in mice that overexpress the EGFR ligand, transforming growth factor alpha (33). Interestingly, depolarization can activate EGFR in both PC12 cells and cardiomyocytes (17, 63, 69). Furthermore, EGFR stimulation leads to Rac1 and NOX activation in glomerular mesangial cells (68), as well as RhoA activation in renal tubule epithelial cells (31). We therefore hypothesized that membrane depolarization increases NOX derived O_2^- following CH though activation of EGFR. We tested our hypothesis by assessing the roles of NOX, Rac-1, and EGFR in membrane depolarization-dependent vasoconstriction and O_2^- production in isolated small pulmonary arteries from CH and normoxic control rats. We also examined the contribution of NOX to depolarization-induced vasoconstriction in isolated lungs. Our findings reveal a unique role for VSM membrane depolarization to activate NOX 2 through EGFR-dependent stimulation of Rac1 in pulmonary arteries from CH rats, but not in those of control animals. Notably, this response represents a novel mechanism of depolarization-induced vasoconstriction occurring independently of changes in global intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Results

CH resulted in polycythemia, as indicated by a greater hematocrit (64 ± 1%) in CH rats than in controls (47 ± 1%). We have previously demonstrated that this 4 wk CH exposure protocol additionally results in PH, arterial remodeling, and right ventricular hypertrophy (3, 55, 56, 59).

CH augments depolarization-induced vasoconstriction but inhibits the Ca^{2+} response to KCl: Role of L-type Ca^{2+} channels

We have previously reported that CH augments vasoconstrictor reactivity to depolarizing concentrations of KCl in isolated lungs (7). To evaluate effects of CH on pulmonary arterial VSM reactivity and vessel wall $[Ca^{2+}]_i$ responses to KCl, and to determine the contribution of L-type voltage-gated Ca^{2+} channels to this response, we performed concentration–response curves to KCl in endothelium-disrupted, pressurized pulmonary arteries [approximately 150–200 μm inner diameter (ID)] from CH and control rats. Arteries were loaded with the ratiometric Ca^{2+} indicator fura 2-AM, and superfused with physiological saline solution (PSS) (pH, Po_2 and Pco_2 of 7.39 ± 0.02, 59.6 ± 1.3, and 32.9 ± 0.9, respectively). KCl-induced constriction was greater in CH compared to control arteries. Whereas KCl produced concentration-dependent increases in $[Ca^{2+}]_i$ in control arteries, Ca^{2+} responses were largely attenuated following CH, with no significant changes occurring over the 30–60 m*M* range of KCl (Fig. 1A and B). Basal $[Ca^{2+}]_i$ was not significantly different between vessels from control and CH rats. Background autofluorescence also was not significantly different between arteries from control [fluorescence emission at 340 nm excitation (F_{340}) = 233 ± 16, fluorescence emission at 380 nm excitation (F_{380}) = 231 ± 17; n = 4) and CH (F_{340} = 268 ± 14, F_{380} = 265 ± 9; n = 4) rats, and was approximately 7—8-fold less than resting emissions following fura 2 loading. Diltiazem, an L-type Ca^{2+} channel inhibitor, significantly lowered basal fura 2 emission ratios and diminished both vasoconstrictor and Ca^{2+} responses to KCl in each group. However, vasoconstrictor reactivity remained elevated in CH arteries compared to controls following L-type Ca^{2+} channel blockade, despite similar levels of $[Ca^{2+}]_i$ between groups.

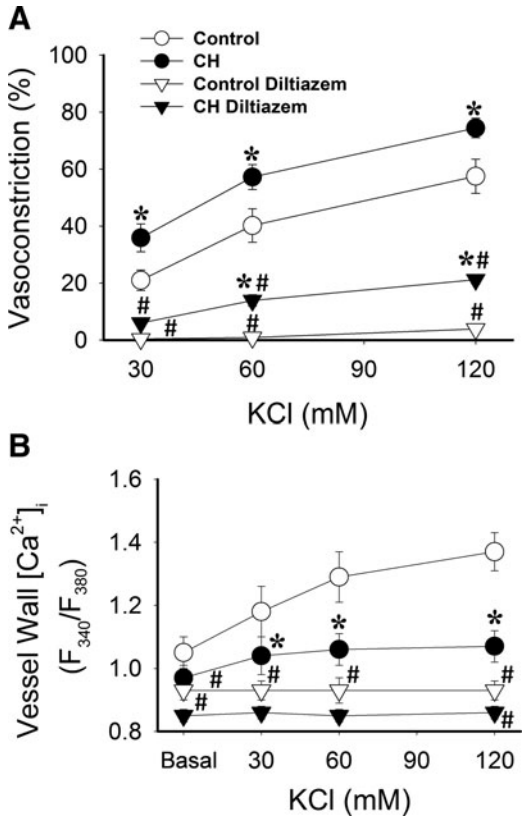

FIG. 1. CH increases vasoconstriction to depolarizing concentrations of KCl in pressurized pulmonary arteries, but attenuates L-type Ca^{2+} channel-mediated increases in vessel wall $[Ca^{2+}]_i$. KCl-induced (30–120 m*M*) **(A)** vasoconstriction and **(B)** vessel wall $[Ca^{2+}]_i$ [expressed as fura 2 340/380 nm emission ratios (F_{340}/F_{380})] responses in isolated, pressurized (12 Torr), endothelium-disrupted CH and control pulmonary arteries in the presence or absence of the L-type Ca^{2+} channel inhibitor diltiazem (50 μM). Values are means ± SE of n = 6–7 rats/group; *$p < 0.05$ vs. respective control; #$p < 0.05$ vs. respective vehicle.

O_2^-, but not H_2O_2, mediates CH-dependent increases in vasoconstrictor reactivity and VSM Ca^{2+} sensitization in response to KCl

Previous work from our laboratory has demonstrated that the O_2^- spin trap agent, tiron, prevents enhanced vasoconstriction to KCl following CH exposure (7). However, by scavenging O_2^-, tiron should also decrease H_2O_2 levels. Therefore, we sought to determine the relative contributions of O_2^- and H_2O_2 to enhanced depolarization-induced Ca^{2+} sensitization following CH. Endothelium-disrupted arteries were prepared as above and permeabilized to Ca^{2+} with ionomycin to clamp vessel wall and presumably VSM $[Ca^{2+}]_i$, allowing assessment of vasoconstriction independent of changes in $[Ca^{2+}]_i$ as described previously (7, 53). Ca^{2+}-clamp conditions were confirmed using fura-2. There were no differences in basal ID (control = 162 ± 4, CH = 169 ± 7 μm) or $[Ca^{2+}]_i$ (control = 1.23 ± 0.05, CH = 1.17 ± 0.05; F_{340}/F_{380}) between groups. Experiments were conducted in the presence of the O_2^- scavenger, polyethylene glycol (PEG)-superoxide dismutase (SOD), or the H_2O_2 scavenger, PEG-catalase. Similar to tiron (7), PEG-SOD attenuated reactivity to KCl in pulmonary arteries from rats exposed to CH, while having no effect in control vessels (Fig. 2A). In contrast, PEG-catalase did not alter vasoconstriction to KCl in either group (Fig. 2B).

NOX is required for enhanced depolarization-induced vasoconstriction and myofilament Ca^{2+} sensitization following CH

The role of NOX, as a source of O_2^-, in mediating enhanced membrane depolarization-dependent vasoconstriction following CH was tested in both nonpermeabilized pulmonary arteries and isolated, saline-perfused lungs using the NOX inhibitor apocynin. Apocynin decreased reactivity to KCl in CH arteries, while having no effect in control arteries (Fig. 3A).

Consistent with responses in isolated arteries, vasoconstrictor responses to depolarizing concentrations of KCl were greater in isolated lungs from CH rats than from control lungs (Fig. 3B), as previously reported (7). Apocynin decreased vasoreactivity to KCl in CH lungs, while having no effect in those of control rats. Perfusate Po_2, Pco_2, and pH were not different between groups and treatments (data not shown).

To address more directly the role of NOX in enhanced depolarization-dependent VSM Ca^{2+} sensitization following CH, we further examined effects of NOX inhibitors on KCl-dependent vasoconstriction in Ca^{2+}-permeabilized arteries. Similar to effects of PEG-SOD (Fig. 2A), the nonselective NOX inhibitors apocynin (Fig. 4A) and diphenyleneiodonium (DPI) (Fig. 4B) attenuated KCl-dependent vasoconstriction in arteries from CH, but not control rats, and normalized responses between groups. Similar inhibitory effects were observed using the specific NOX 2 inhibitory peptide, gp91ds-tat [also known as Nox2ds-tat (13)], compared to the scrambled peptide (Fig. 4C).

CH-induced increases in basal and KCl-stimulated O_2^- levels are NOX dependent

O_2^- production was measured by fluorescence detection of dihydroethidium (DHE) oxidation in endothelium-disrupted, pressurized, Ca^{2+}-permeabilized arteries from control and CH rats using previously described methods (7, 30, 53). CH exposure resulted in elevated basal and KCl-induced O_2^- levels in pressurized pulmonary arteries (Fig. 5) as previously described (7). In contrast, KCl did not alter O_2^- production in control arteries. In arteries from CH animals, apocynin lowered basal DHE fluorescence to the level of controls, while both apocynin and DPI prevented KCl-dependent increases in O_2^- production (Fig. 5A and 5B, respectively). gp91ds-tat also decreased basal DHE fluorescence compared to the scrambled peptide in arteries from CH rats, and prevented the effect of KCl to elevate DHE fluorescence in these vessels (Fig. 5C), suggesting that NOX 2 plays a primary role in depolarization-induced O_2^- generation and pulmonary vasoconstriction.

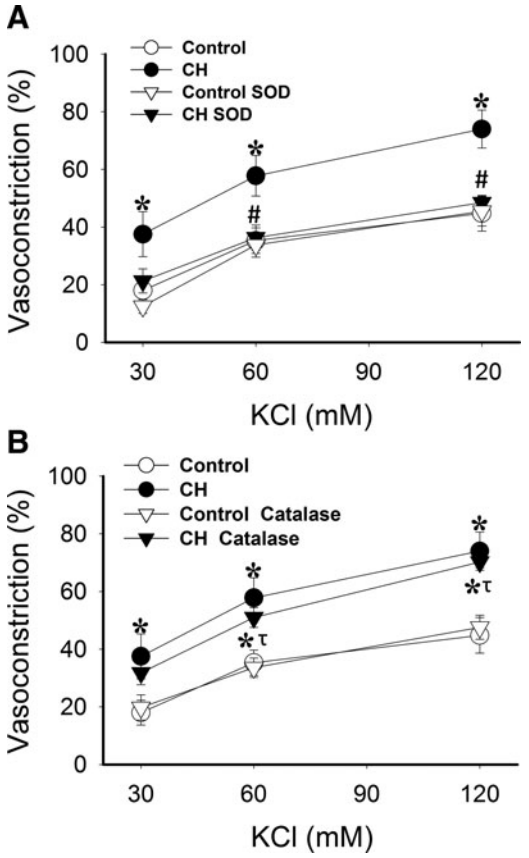

FIG. 2. O_2^-, but not H_2O_2, mediates enhanced depolarization-induced myofilament Ca^{2+} sensitization and constriction in Ca^{2+}-permeabilized arteries from CH rats. Vasoconstrictor responses to KCl in pressurized, endothelium-disrupted, Ca^{2+} permeabilized (3 μM ionomycin) pulmonary arteries from CH and control rats in the presence of **(A)** PEG-SOD (120 U/mL) or **(B)** PEG-catalase (250 U/mL). Values are means ± SE of $n = 4$–5 rats/group; *$p < 0.05$ vs. control; #$p < 0.05$ CH SOD vs. CH vehicle; $\tau p < 0.05$ CH catalase vs. CH SOD.

VSM membrane potential is not altered by NOX inhibition

To confirm that inhibitory influences of gp91ds-tat on KCl-induced vasoconstriction and O_2^- production in CH arteries do not result from potential effects of the peptide to cause VSM membrane hyperpolarization, we next measured membrane potential with sharp electrodes in pressurized arteries from each group in the presence of gp91ds-tat or its

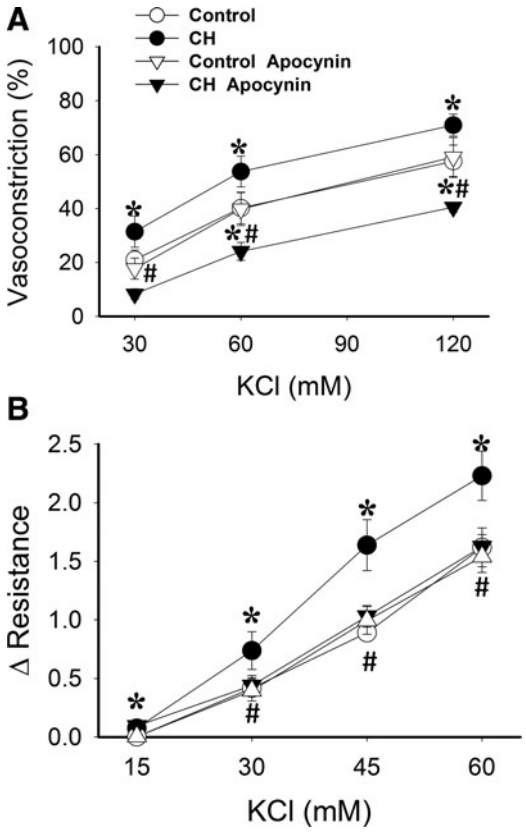

FIG. 3. Augmented KCl-induced vasoconstriction following CH is NOX-dependent in both nonpermeabilized arteries and isolated perfused rat lungs. (A) Vasoconstrictor responses to KCl in pressurized, endothelium-disrupted pulmonary arteries from CH and control rats in the presence or the NOX inhibitor apocynin (30 μM) or vehicle. **(B)** Vasoconstrictor responses to KCl (15–60 mM) in the presence of vehicle or apocynin (30 μM) in isolated, saline-perfused lungs from CH and control rats. Values are means ± SE of $n = 5$–7 rats/group; *$p < 0.05$ vs. respective control; #$p < 0.05$ CH apocynin vs. CH vehicle.

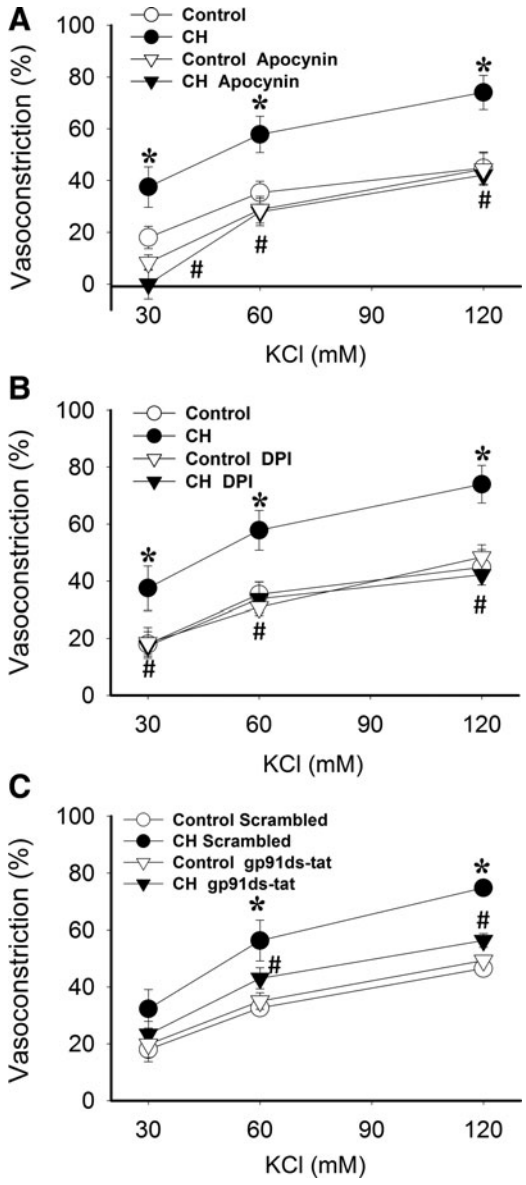

FIG. 4. NOX2 is required for greater KCl-mediated Ca^{2+} sensitization and constriction in Ca^{2+}-permeabilized arteries from CH rats compared to controls. Vasoconstrictor responses to KCl in pressurized, Ca^{2+} permeabilized pulmonary arteries from CH and control rats in the presence of the NOX inhibitors **(A)** apocynin (30 μM), **(B)** DPI (10 μM), **(C)** gp91ds-tat (50 μM), their respective vehicles, or the scrambled control peptide for gp91ds-tat. All arteries were endothelium-disrupted. Values are means ± SE of $n = 4$–5 rats/group. *$p < 0.05$ vs. control; #$p < 0.05$ vs. CH vehicle.

scrambled control peptide. VSM membrane potential was depolarized in CH arteries compared to controls under both basal and KCl-stimulated conditions (Fig. 6) as previously reported (7). However, NOX 2 inhibition was without effect on membrane potential in either group.

Pulmonary arterial NOX 2 expression following CH

To address whether greater KCl-dependent pulmonary vasoreactivity and O_2^- production following CH are associated with an increase in NOX 2 expression, we measured levels of the catalytic subunit NOX 2 ($gp91^{phox}$) in intrapulmonary arteries from CH and control rats. However, we observed no difference in NOX 2 levels between CH and control arteries (Fig. 7).

EGFR-induced Rac1 activation contributes to augmented depolarization-induced vasoconstriction and O_2^- generation following CH

The contribution of EGFR and Rac1 to augmented depolarization-induced Ca^{2+} sensitization and O_2^- generation following CH was assessed by measuring KCl-dependent vasoconstriction and DHE fluorescence in arteries from each group in the presence or absence of the Rac1 inhibitor, NSC 23766 (39), or the EGFR inhibitor, AG1478 (68). Consistent with our hypothesis, both Rac1 and EGFR inhibition prevented KCl-mediated increases vasoreactivity (Fig. 8A and C) and DHE fluorescence (Fig. 8B and D) in arteries from CH rats, while having no effect in control arteries. Similar to effects of O_2^- scavenging (7, 30) and NOX inhibition (Fig. 5), Rac1 inhibition reduced basal O_2^- levels in CH arteries (Fig.

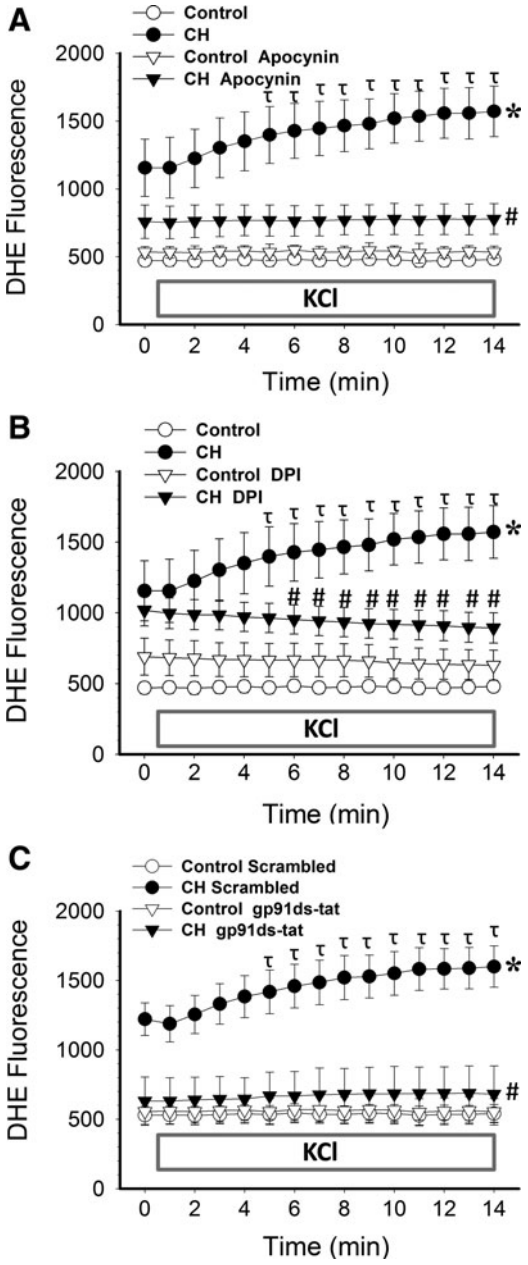

FIG. 5. CH-induced increases in basal and depolarization-stimulated O_2^- levels in pressurized arteries are NOX 2-dependent. DHE fluorescence under basal conditions and following administration of KCl (60 m*M*) in pressurized, endothelium-disrupted, Ca^{2+}-permeabilized pulmonary arteries from CH and control rats in the presence **(A)** apocynin, **(B)** DPI, **(C)** gp91ds-tat, their respective vehicles, or the scrambled control peptide for gp91ds-tat. KCl was administered 1 min into recording. Values are means ± SE of $n = 4$–5 rats/group; *$p < 0.05$ vs. control at all time points. #$p < 0.05$ vs. CH vehicle; $\tau p < 0.05$ vs. CH vehicle at time 0.

8B). In contrast, EGFR inhibition was without effect on basal DHE fluorescence in either group (Fig. 8D).

We next sought to define the signaling relationship between EGFR and KCl-dependent Rac1 activation in ionomycin-treated intrapulmonary arteries from control and CH rats using a Rac1-GTP pull-down assay. Consistent with effects of Rac1 inhibition on vasoreactivity and O_2^- production (Fig. 8A and B),

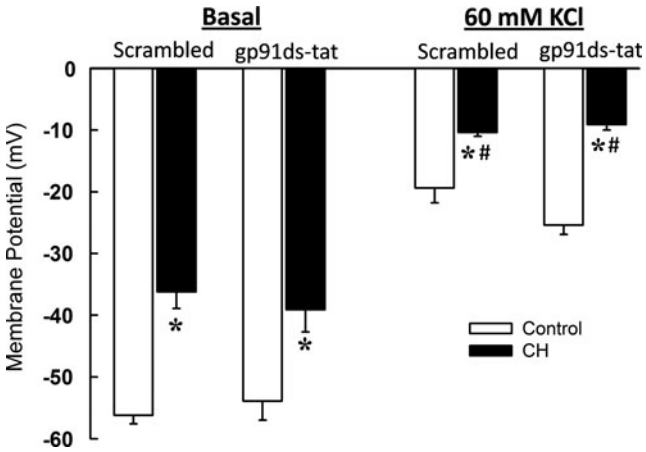

FIG. 6. NOX 2 inhibition does not alter vascular smooth muscle (VSM) membrane potential in pressurized arteries from either control or CH rats. VSM membrane potential responses to KCl (60 m*M*) in the presence of the NOX 2 inhibitory peptide, gp91ds-tat (50 μM), or its scrambled control in pressurized, endothelium-disrupted, Ca^{2+}-permeabilized pulmonary arteries. Values are means ± SE of $n = 4$ rats/group; *$p < 0.05$ vs. respective control; #$p < 0.05$ vs. corresponding basal measurement.

CH tended to increase basal Rac1 activity (GTP-bound Rac1/total Rac1), although this effect did not achieve statistical significance (Fig. 9). Furthermore, KCl produced an increase in Rac1-GTP levels in arteries from CH but not control rats, and this response to KCl was abolished by EGFR inhibition. EGFR

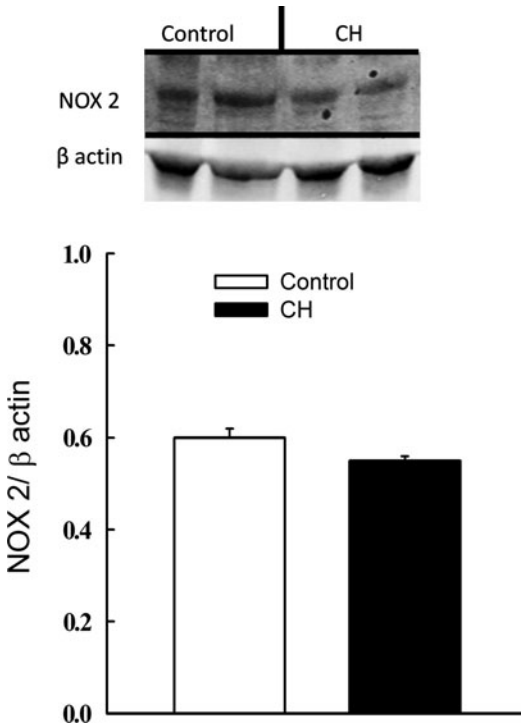

FIG. 7. Pulmonary arterial NOX 2 expression is unaltered following CH. Representative Western blots and mean densitometric data for NOX 2 and β actin in intrapulmonary arteries from CH and control rats. $n = 4$ rats/group. There are no significant differences.

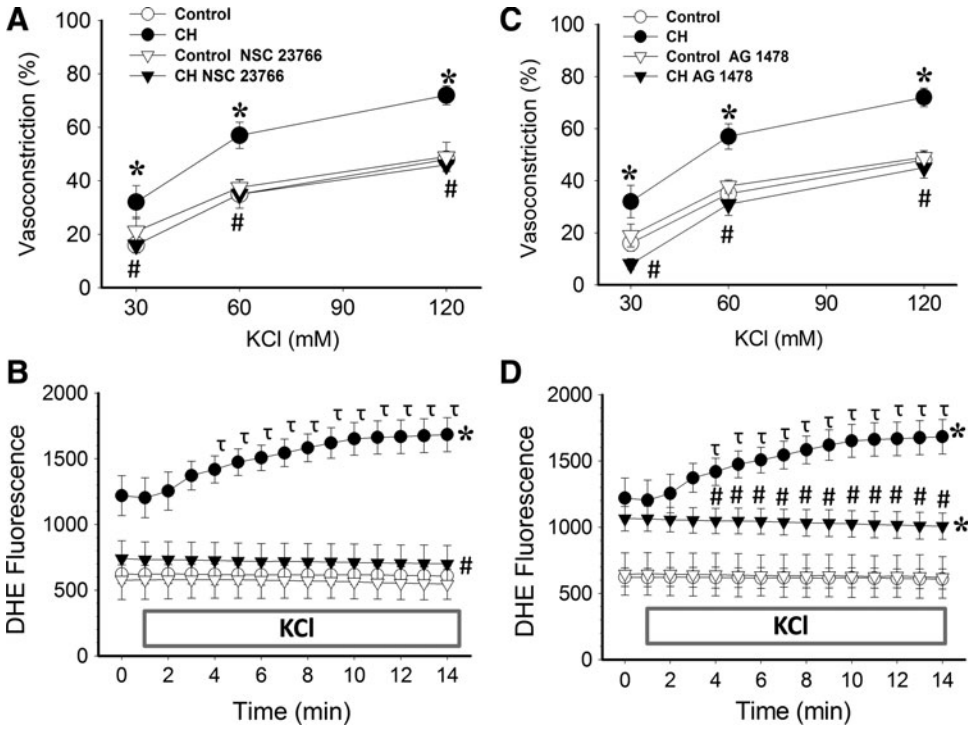

FIG. 8. Rac1 and EGFR contribute to increased KCl-induced Ca^{2+} sensitization, vasoconstriction and O_2^- generation following CH. Vasoconstrictor responses to KCl in pulmonary arteries from control and CH rats in the presence of **(A)** the Rac1 inhibitor, NSC 23766 (50 μM), **(C)** the EGFR inhibitor, AG 1478 (1 μM), or their respective vehicles. DHE fluorescence responses to KCl (60 mM) in the presence of **(B)** NSC 23766 or **(D)** AG 1478 in pressurized CH and control arteries. KCl was administered 1 min into recording. Values are means ± SE of $n = 4$ rats/group; *$p < 0.05$ vs. control; #$p < 0.05$ vs. CH vehicle; $\tau P < 0.05$ vs. CH vehicle at time 0.

activity, assessed as the ratio of phosphorylated to total EGFR levels, was similarly increased by KCl only in CH arteries (Fig. 10). Basal levels of phosphorylated EGFR, however, were not different between control and CH arteries. No differences in either total Rac1 or total EGFR (each normalized to β actin) were observed between groups (data not shown).

Discussion

The goal of the present study was to identify signaling mechanisms linking VSM membrane depolarization to O_2^- production and enhanced myofilament Ca^{2+} sensitization in the hypertensive pulmonary circulation of CH rats. The major findings from this study are that: 1) enhanced membrane depolarization-induced O_2^- production, myofilament Ca^{2+} sensitization, and pulmonary vasoconstriction following CH require NOX 2; 2) CH augments KCl-dependent O_2^- generation and vasoconstriction through EGFR-mediated activation of Rac1; and 3) Rac1, but not EGFR, contributes to elevated basal O_2^- levels following CH. Together, these findings demonstrate a novel mechanism of CH to facilitate membrane depolarization-induced activation of NOX 2 through EGFR-dependent Rac1 signaling in pulmonary VSM, which may contribute to augmented vasoconstrictor sensitivity and PH in this setting.

Exposure to CH increases basal pulmonary arterial tone (8, 49) and augments vasoconstrictor sensitivity to both receptor-mediated agonists (1, 30, 52) and depolarizing stimuli (7, 49). Although mechanisms involving enhanced Ca^{2+} influx may contribute to greater pulmonary vasoreactivity following CH (28, 34, 64), it is clear that myofilament Ca^{2+} sensitization mediated by RhoA provides a major contribution to these vasoconstrictor responses (7, 8, 30, 49, 65). Interestingly, the effect of depolarization to activate RhoA in hypertensive pulmonary arteries is thought to occur through a mechanism that is independent of L-type Ca^{2+} channel stimulation or global changes in VSM $[Ca^{2+}]_i$ (7). Consistent with this possibility are our current findings that enhanced vasoconstrictor reactivity to KCl following CH is associated with a markedly blunted vessel wall Ca^{2+} response compared to control arteries, as previously reported (7). Furthermore, although Ca^{2+} influx through L-type Ca^{2+} channels contributes to KCl-mediated vasoconstriction and vessel wall $[Ca^{2+}]_i$ in arteries from both control and CH rats, KCl-mediated vasoconstriction persisted following L-type Ca^{2+} channel blockade in hypertensive arteries, despite similar $[Ca^{2+}]_i$ levels between groups. Diltiazem also significantly lowered basal $[Ca^{2+}]_i$ in arteries from both control and CH rats, suggesting that L-type Ca^{2+} channels contribute to resting $[Ca^{2+}]_i$ in pressurized small pulmonary arteries. It is noteworthy that, whereas the magnitude of vasoconstrictor responses to KCl in Ca^{2+}-clamped arteries was much greater than the residual vasoconstrictor responses to KCl following L-type Ca^{2+} channel blockade in nonpermeabilized vessels, this difference is likely the result of the higher $[Ca^{2+}]_i$ levels achieved in Ca^{2+}-permeabilized compared to diltiazem-treated nonpermeabilized arteries. Therefore, under physiological conditions, where resting $[Ca^{2+}]_i$ levels are not clamped at a higher value, and where depolarization is only comparable to that achieved with 30 mM KCl, the contribution of enhanced Ca^{2+} sensitivity to depolarization-induced vasoconstriction may be less.

Endogenous ROS derived from multiple enzymatic sources and cell types are widely considered to play an important role in the pathogenesis of various forms of PH (11, 25, 52), including CH-induced PH (21, 27, 36). While the mechanisms by which ROS contribute to PH are likely multifaceted, it is clear that ROS signaling is central to the vasoconstrictor component of CH-induced PH. Such ROS-dependent vasoconstrictor mechanisms may involve endothelial dysfunction (22, 67), scavenging of NO by O_2^- (29), and direct effects of O_2^- and

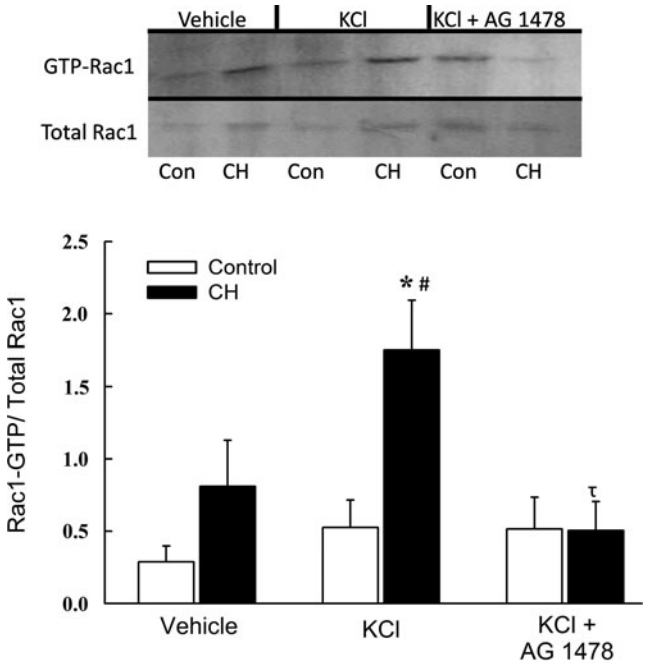

FIG. 9. Depolarization mediates EGFR-dependent Rac1 activation in arteries from CH but not control rats. Representative Western blots and mean densitometric data for GTP bound (active) Rac1 normalized to total Rac1 in homogenates from control and CH intrapulmonary arteries. Active Rac1 was measured under baseline conditions and following 10 min stimulation with KCl (60 m*M*) or KCl plus the EGFR inhibitor, AG1478 (1 μM). Values are means ± SE of $n = 4$ rats/group; *$p < 0.05$ vs. control KCl; #$p < 0.05$ vs.CH vehicle; $\tau p < 0.05$ vs. CH KCl.

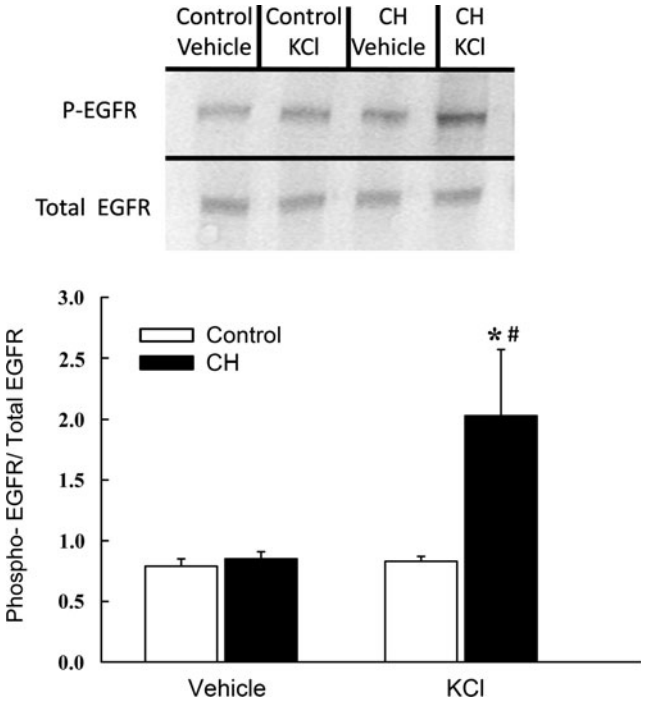

FIG. 10. EGFR phosphorylation is induced by KCl in arteries from CH but not control rats. Representative Western blots and mean densitometric data for phosphorylated (active) EGFR normalized to total EGFR in homogenates from control and CH intrapulmonary arteries. Phosphorylated and total EGFR were measured under baseline conditions and following 10 min stimulation with KCl (60 m*M*). Values are means ± SE of $n = 7$ rats/group; *$p < 0.05$ vs. control KCl; #$p < 0.05$ vs.CH vehicle.

H_2O_2 to promote VSM contraction through intracellular second messenger pathways (7, 30, 32, 35). Indeed, recent studies from our laboratory have demonstrated a link between O_2^- (or reactive products of O_2^-) and myofilament Ca^{2+} sensitization in mediating CH-induced increases in pulmonary vasoconstrictor reactivity to both receptor-mediated agonists and depolarizing stimuli (7, 30). However, neither the specific ROS involved, the enzymatic source of O_2^-, nor the signaling mechanisms linking depolarization to O_2^- generation have been previously addressed.

O_2^- and H_2O_2 are the primary ROS known to be involved in regulation of pulmonary vascular tone. In pulmonary VSM, O_2^- mediates a contractile effect through activation of Rho kinase and subsequent myofilament Ca^{2+} sensitization (32), whereas H_2O_2 is reported to have both contractile (35) and relaxant (9) properties. We have previously demonstrated that tiron, a spin-trap agent that selectively scavenges O_2^- and thus would be expected to lower both O_2^- and H_2O_2 levels, prevents enhanced depolarization-induced vasoconstriction, O_2^- production, and RhoA activation following CH (7). Therefore, we sought to determine the relative contributions of these ROS species to enhanced myofilament Ca^{2+} sensitivity following CH. Our observation that greater vasoreactivity to KCl in Ca^{2+}-permeabilized arteries from CH rats is prevented by PEG-SOD, but not PEG-catalase, supports a role for O_2^- in this response.

NAD(P)H oxidases have been implicated in the development of both systemic (23, 43) and pulmonary (27, 36, 46) hypertension, and thus represent a potential source of O_2^- mediating enhanced depolarization-induced pulmonary VSM Ca^{2+} sensitization following CH. In agreement with this possibility is evidence that CH-mediated increases in pulmonary vasoreactivity, ROS production and PH are attenuated in mice deficient in the catalytic subunit of NOX 2 (36). Additionally, NOX 2 contributes to impaired endothelium-dependent pulmonary vasodilation in mice (22) and neonatal piglets (21) with CH-induced PH. Furthermore, CH increases NOX-derived ROS production, NOX 1 expression, and $p67^{phox}$ levels in the membrane fraction of small pulmonary arteries from piglets, suggesting elevated NOX 1 activity in this neonatal model of PH (16, 21). NOX 4 gene and protein expression are also elevated following CH exposure in murine pulmonary arteries and lung tissue (46, 51) and are similarly upregulated by CH in cultured human pulmonary arterial endothelial cells (40). However, the contribution of NOX 4 to the development of PH remains poorly defined. A role for NOX in augmented depolarization-induced Ca^{2+} sensitization following CH is demonstrated by our present findings that apocynin decreased the vasoconstrictor response to KCl in nonpermeabilized arteries and isolated lungs from CH rats, but not controls. This argument is further supported by evidence from Ca^{2+}permeabilized arteries that nonspecific NOX inhibition with apocynin or DPI attenuated both vasoreactivity and O_2^- generation in response to KCl selectively in arteries from CH rats. Similar results were observed using the highly selective NOX 2 inhibitor, gp91ds-tat, which competitively inhibits $p47^{phox}$ binding to the catalytic subunit of NOX 2, and thus

prevents assembly of the active enzyme complex (58). These data therefore support a distinct contribution of NOX 2-derived O_2^- to enhanced depolarization-induced vasoconstriction independent of changes in global vessel wall $[Ca^{2+}]_i$ following CH. Although we cannot exclude the possibility that localized, subsarcolemmal Ca^{2+} events contribute to the observed responses, we are confident that, within the sensitivity limits of the system, global $[Ca^{2+}]_i$ is effectively clamped by ionomycin in this preparation. An additional limitation of this system is the inability to selectively measure VSM $[Ca^{2+}]_i$, as other cell types within the vascular wall (*e.g.*, fibroblasts) likely contribute to the fura 2 signal. Furthermore, adventitial remodeling resulting from CH exposure may increase the contribution of this layer to the overall signal.

It is unlikely that greater basal and depolarization-stimulated O_2^- production following CH are a function of increased arterial NOX 2 expression, since levels of the NOX 2 catalytic subunit pg91phox were similar between arteries from control and CH rats. Indeed, the lack of effect of NOX inhibition on either vasoreactivity or O_2^- levels in control arteries suggests CH facilitates a coupling of depolarization to NOX 2 activation that is not present in the normal pulmonary circulation. Nevertheless, we cannot exclude a possible contribution of altered expression of other components of the NOX 2 complex to this response.

Rac1 is an important mediator of NOX 1 and 2 activation (6, 12), and thus represents a potential signaling factor that links membrane depolarization to stimulation of NOX 2 following CH. Consistent with this possibility is evidence that depolarization activates Rac1 and NOX in endothelial and macula densa cells (10, 38, 39, 44, 60). Here we demonstrate that KCl-mediated membrane depolarization activates Rac1 selectively in arteries from pulmonary hypertensive rats. Our findings that basal and KCl-mediated O_2^- generation and associated vasoconstriction are diminished by NSC 23766 only in CH arteries further suggest that Rac1 activation contributes to both elevated resting O_2^- levels and depolarization-stimulated O_2^- production and vasoconstriction following CH. However, it is additionally possible that the phosphorylation state of the NOX subunits $p47^{phox}$ or $p67^{phox}$ is altered by membrane depolarization following CH, as phosphorylation of these subunits can also contribute to NOX activation (6, 12, 41).

Based on evidence that depolarization leads to EGFR activation in PC12 cells, and that membrane stretch stimulates EGFR-dependent NOX activation in mesangial cells (68), we next examined EGFR as a proximal mediator of depolarization-induced, Rac1-dependent NOX 2 activation in CH arteries. A role for EGFR in this response is supported by our present findings that EGFR phosphorylation was induced by a depolarizing stimulus only in CH arteries, and that selective EGFR inhibition, like Rac1 and NOX 2 inhibition, prevented effects of CH to augment both depolarization-induced vasoconstriction and vascular O_2^- production. We additionally found that depolarization-mediated Rac1 activation in intrapulmonary arteries from CH rats was prevented with AG 1478, suggesting EGFR signals upstream of Rac1 to promote O_2^- generation and vasoconstriction following CH. However, unlike responses to Rac1 and NOX 2 inhibition, EGFR inhibition had no effect to reduce basal production in vessels from CH rats, suggesting that whereas EGFR is required for KCl-mediated O_2^- production, CH elevates basal O_2^- levels through an EGFR-independent mechanism. Further supporting this possibility is our present finding that basal levels of phosphorylated EGFR were unaltered following exposure to CH. The mechanism by which CH increases basal O_2^- production in pulmonary arteries is unknown, but may involve coupling of transmural pressure-induced VSM stretch to NOX activation.

Although the mechanism by which depolarization mediates EGFR activation in hypertensive pulmonary arteries remains to be established, it is possible that CH alters the composition of membrane signaling platforms, including caveolae or other lipid rafts, to permit regulation of EGFR by voltage-sensitive proteins. Metabotropic signaling by ion channels (5, 20), G protein-coupled receptors (37, 42), and voltage-sensitive phosphatases (19, 26, 47, 48) represent possible mediators of depolarization-induced VSM Ca^{2+} sensitization. It is alternatively possible that EGFR expresses a membrane potential-sensitive domain that imparts direct voltage-sensitive enzymatic activity.

As previously reported following inhibition of Rho kinase, PKC, or scavenging of O_2^- (7), a persistent vasoconstrictor response to KCl was observed even after inhibition of NOX, Rac1, or EGFR in arteries from both groups. Although the mechanism of this residual vasoconstriction is unknown, it is possible that a Ca^{2+}-independent myosin light chain kinase, such as integrin-linked kinase (15) or ZIP kinase (50), contributes to this response. Future studies are also needed to determine the contribution of membrane depolarization-induced NOX 2 activation and subsequent Ca^{2+} sensitization to enhanced agonist-induced pulmonary vasoconstrictor sensitivity (1, 30) and myogenic vasoconstriction (8) following CH, and the potential role of dysregulation of endogenous antioxidant systems (54) in these responses.

In conclusion, we have identified a unique signaling pathway in pulmonary vascular smooth muscle involving EGFR-induced Rac1 and NOX 2 activation that couples membrane depolarization to myofilament Ca^{2+} sensitization and vasoconstriction. Furthermore, whereas this pathway is not functional in the normotensive pulmonary circulation, it is induced by long-term exposure to hypoxia, and may provide a basis for understanding mechanisms of vasoconstriction that contribute to CH-induced PH.

Methods

All protocols and surgical procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center (Albuquerque, NM).

Experimental groups

Male Sprague-Dawley rats (body wt. 250–350 g, age 3–4 mo; Harlan Industries, Indianapolis, IN) were exposed to 4 wk CH by placement in a hypobaric chamber maintained at ~380 Torr as previously described (8, 30, 57).

Isolated lung protocol: Contribution of NOX to KCl-dependent vasoconstriction

Lungs were isolated from rats, perfused with PSS containing 4% bovine serum albumin as a colloid, and ventilated with a 21% O_2, 6% CO_2 gas mixture as previously described (57). Following stabilization of baseline pressure, a cumulative concentration–response relationship to depolarizing

concentrations of KCl (15–60 m*M*) was assessed (7). This PSS had the same osmolality as the control solution achieved by exchanging NaCl for equimolar concentrations of KCl. Experiments were conducted in the presence or absence of the general NOX inhibitor apocynin (30 μM, Sigma, St. Louis, MO) (61) or vehicle (PSS).

Isolated pulmonary artery protocols: Assessment of vasoreactivity, vessel wall $[Ca^{2+}]_i$, O_2^- levels, and membrane potential

Small pulmonary arteries were isolated and cannulated for simultaneous assessment of vasoreactivity and VSM $[Ca^{2+}]_i$, O_2^- or membrane potential, as previously described (7). All arteries were studied at a transmural pressure of 12 Torr, as we have previously demonstrated that reactivity to KCl following CH is similarly augmented at 12 or 35 Torr (7). All isolated arteries were studied under normoxic conditions for the pulmonary arterial circulation (equilibrated with a 10% O_2, 6% CO_2, and balance N_2 gas mixture) and endothelium disrupted to directly evaluate effects of CH on VSM reactivity to KCl independent of endothelial influences. Background-subtracted fura-2 F_{340}/F_{380} emission (510 nm) ratios were calculated with IonOptix Ion Wizard software and recorded continuously throughout the experiment to measure vessel wall $[Ca^{2+}]_i$, with simultaneous measurement of ID from red wavelength bright-field images. Disruption of the endothelium was confirmed by lack of response to ACh following a UTP constriction (8, 30). To directly assess mechanisms of myofilament Ca^{2+} sensitization independent of changes in vessel wall $[Ca^{2+}]_i$, we clamped vessel wall $[Ca^{2+}]_i$ in some arteries by permeabilizing with the Ca^{2+} ionophore, ionomycin (3 μM, Sigma), as previously described (30). All Ca^{2+} permeabilized vessels were equilibrated with PSS containing a calculated free Ca^{2+} concentration of 300 n*M*. This concentration of Ca^{2+} was chosen to provide optimal vasoreactivity to KCl while having minimal effects on resting tone based on preliminary studies.

Contribution of L-type Ca^{2+} channels and NOX to KCl-dependent vasoconstriction. KCl-induced (30, 60, and 120 m*M*) vasoconstrictor and Ca^{2+} responses were measured in nonpermeabilized arteries from CH and control rats in the presence of the L-type Ca^{2+} channel inhibitor diltiazem (50 μM) (7), the NOX inhibitor apocynin (30 μM) or their vehicles. All inhibitors used for isolated vessel experiments were added to the superfusate 20 min prior to the beginning of experimentation and were present throughout the protocol.

Role of O_2^-, H_2O_2, NOX, Rac1, and EGFR in KCl-dependent VSM Ca^{2+} sensitization. Vasoconstrictor responses to increasing concentrations of KCl (30, 60, and 120 m*M*) were assessed in Ca^{2+} permeabilized arteries from CH and control rats in the presence of PEG-SOD (120 U/mL, Sigma) (22), PEG-catalase (250 U/mL, Sigma) (16), the NOX inhibitors apocynin (30 μM) (61), DPI (10 μM, Sigma) (66), or their vehicle controls. Parallel protocols were conducted in the presence of the specific NOX 2 inhibitor gp91ds-tat (50 μM, Tufts, Boston, MA) (58), its corresponding scrambled control peptide, the Rac1 inhibitor NSC 23766 (50 μM, Cayman, Ann Arbor, MI; a guanine nucleotide exchange factor inhibitor chosen for its selectivity for Rac1 over the similar G-protein RhoA) (39), the selective EGFR inhibitor AG1478 (1 μM, Cayman) (68), or appropriate vehicles. Fura-2 ratios were monitored throughout all experiments to confirm calcium clamp.

Role of NOX, Rac1, and EGFR in KCl-dependent O_2^- production. DHE (10 μM, Molecular Probes, Eugene, OR) fluorescence was used to assess depolarization-induced O_2^- generation in endothelium-disrupted, pressurized, Ca^{2+}-permeabilized arteries from each group, as previously described (7). After establishing a baseline value, 60 m*M* KCl was administered and DHE fluorescence was measured over 12 minutes. Arteries were exposed to DPI, apocynin, gp91ds-tat, NSC23766, AG1478, or the respective vehicle to determine the role of NOX, Rac1, and EGFR in KCl-stimulated O_2^- production.

Effects of NOX 2 inhibition on VSM membrane potential. VSM cell membrane potential was measured in arteries prepared as above in the presence of gp91ds-tat or scramb-tat using sharp electrodes, as previously described (7).

Western blot analysis

NOX 2 expression. NOX 2 expression was compared between intrapulmonary arteries of CH and control rats by Western blotting, using methods similar to those previously described (7). Pulmonary arterial NOX 2 catalytic subunit expression was measured using a mouse monoclonal NOX 2 ($gp91^{phox}$) antibody (1:2000, BD Biosciences, Lexington, KY) (18). NOX 2 expression was normalized to β actin expression.

Rac1 and EGFR activation. Intrapulmonary arteries from CH and control rats were isolated and incubated with the Ca^{2+} ionophore ionomycin (37°C; 30 min). Some arteries were additionally treated with KCl (60 m*M*) or KCl plus the EGFR inhibitor AG 1478 (1 μM). Arteries were homogenized as previously described (7). Rac1 activity was assessed using a Rac1 activation assay kit (Cytoskeleton, Denver, CO) that detects levels of GTP-bound Rac1 (68). Levels of GTP-bound Rac1 were normalized to total Rac1 protein levels determined from separate Western blots. Homogenates were also probed for levels of phosphorylated EGFR. A polyclonal anti-phospho EGFR antibody (1:500, Cell Signaling, Beverly, MA) (68) was used to detect EGFR phosphorylated on the tyrosine 1068 residue (2, 24). Phosphorylated EGFR levels were normalized to total EGFR levels (1:500, Cell Signaling) for comparison.

Calculations and statistics

For isolated lung experiments, total pulmonary vascular resistance was calculated as the difference between pulmonary arterial and pulmonary venous pressures (P_a and P_v respectively) divided by flow (30 ml·min^{-1}·kg body wt^{-1}). Vasoconstrictor responses were calculated as a percentage of baseline ID. All data are presented as means ± SE, and n refers to the number of animals in each group. A t-test, one-way ANOVA, two-way ANOVA, or one-way repeated measures ANOVA was used to make comparisons when appropriate. If differences were detected by ANOVA, individual groups were compared using the Student-Newman-Keuls or Bonferroni test. A probability of $p < 0.05$ was considered significant for all comparisons.

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Author Disclosure Statement

The authors have no competing financial interests.

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Abbreviations Used

$[Ca^{2+}]_i$ = free intracellular Ca^{2+} concentration
CH = chronic hypoxia
DHE = dihydroethidium
EGFR = epidermal growth factor receptor
ID = inner diameter
NOX = NAD(P)H oxidase
O_2^- = superoxide anion
PH = pulmonary hypertension
PSS = physiological saline solution
ROS = reactive oxygen species
VSM = vascular smooth muscle