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Original Contribution

Hypoxia induces K_v channel current inhibition by increased NADPH oxidase-derived reactive oxygen species

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ABSTRACT

There is current discussion whether reactive oxygen species are up- or downregulated in the pulmonary circulation during hypoxia, from which sources (i.e., mitochondria or NADPH oxidases) they are derived, and what the downstream targets of ROS are. We recently showed that the NADPH oxidase homolog NOX4 is upregulated in hypoxia-induced pulmonary hypertension in mice and contributes to the vascular remodeling in pulmonary hypertension. We here tested the hypothesis that NOX4 regulates K_v channels via an increased ROS formation after prolonged hypoxia. We showed that (1) NOX4 is upregulated in hypoxia-induced pulmonary hypertension in rats and isolated rat pulmonary arterial smooth muscle cells (PASMC) after 3 days of hypoxia, and (2) that NOX4 is a major contributor to increased reactive oxygen species (ROS) after hypoxia. Our data indicate colocalization of K_v1.5 and NOX4 in isolated PASMC. The NADPH oxidase inhibitor and ROS scavenger apocynin as well as NOX4 siRNA reversed the hypoxia-induced decrease in K_v current density whereas the protein levels of the channels remain unaffected by siNOX4 treatment. Determination of cysteine oxidation revealed increased NOX4-mediated K_v1.5 channel oxidation. We conclude that sustained hypoxia decreases K_v channel currents by a direct effect of a NOX4-derived increase in ROS.

Introduction

The unique feature of pulmonary circulation is its ability to contract in response to hypoxia. This ability of the lung vasculature helps in minimizing ventilation perfusion mismatch by diverting blood flow from poorly to well ventilated regions of the lung [1,2]. This phenomenon is known as hypoxic pulmonary vasoconstriction (HPV) [3]. In chronic, generalized hypoxia the sense of this regulation is lost, and in addition to vasoconstriction a vascular remodeling process with hypertrophy of the vessel media, mainly driven by smooth muscle cell proliferation, occurs. There is no doubt that K_v channel currents are decreased by hypoxia, leading to depolarization of the smooth muscle cell´s plasma membrane and thus contribute to the development of pulmonary hypertension [4]. However there is current debate by which upstream mechanism the K_v current is modulated. Reactive oxygen species (ROS) have been suggested to mediate this process. However, controversial data exist whether ROS are up- or downregulated by hypoxia and from which sources such species are derived [5–7]. Likely candidates are mitochondria as well as NAD(P)H oxidases [8–11]. The voltage gated K^+ channels (K_v) have been suggested to play an important role in the adaptive response of the pulmonary vasculature to hypoxia [12–15]. There are at least four K_v channel subfamilies: *Shaker* (K_v1), *Shab* (K_v2), *Shaw* (K_v3), and *Shal* (K_v4) consisting of several members [16]. Of these channels, K_v2.1 and K_v1.5 have been shown to conduct a major portion of the hypoxia-sensitive membrane current in pulmonary artery smooth muscle cells (PASMC) and their regulation is responsible for the increased pulmonary vascular tone under acute and chronic hypoxia [17,18]. Moreover, the acute hypoxia-induced pulmonary vasoconstrictor response has been suggested to be mediated by inhibition of K_v channels, leading to membrane depolarization and subsequent calcium influx via L-type Ca^{2+} channels in pulmonary artery smooth muscle cells [19]. In addition, the chronic hypoxia-induced pulmonary vascular remodeling, characterized by increased proliferation

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and hypertrophy of PASMC, has been attributed to decreased K_v channel function and increased calcium influx in PASMC [15,20–22].

With regard to ROS there is multiple evidence that K_v channel currents are reduced and/or that K_v channels are closed under hypoxia by a decrease of ROS [23–26]. However, a variety of reports have shown that there is an upregulation of ROS levels under hypoxia. Although mitochondria are one possible source of such an increase [27–30], NADPH oxidases are alternative ROS generators. However, to the best of our knowledge they have not been investigated yet thoroughly as to their possible role in K_v channel regulation. Their possible role in various cardiovascular diseases such as atherosclerosis, hypertension, myocardial infarction, and ischemia–reperfusion injury has been reported [31,32]. Currently seven homologs of NADPH oxidases are known (NOX1–NOX5 and DUOX1 and DUOX2) [33–35]. We and others have recently shown that the homolog of NADPH oxidase NOX4 is upregulated under chronic hypoxia in the pulmonary vasculature and that it is important for pulmonary artery myocyte and fibroblast proliferation [36–38]. Against this background the current study explored the possible role of NOX4-derived ROS production for the regulation of K_v channels in isolated rat PASMC after sustained hypoxia.

Experimental procedures

Animal experiments

Adult male Sprague-Dawley rats (350 to 400 g body wt) were obtained from Charles River Laboratories. Animals were housed under controlled temperature (22 °C) and lighting (12/12-h light/dark cycle), with free access to food and water. After 3 weeks of hypoxia RVSP, SAP, and heart ratio were measured as described previously [39,40]. After intraperitoneal injection of a lethal ketamine and xylazine dose, the lungs were flushed with Krebs Henseleit buffer (125.0 mM NaCl, 4.3 mM KCl, 1.1 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 13.32 mM glucose, gassed with 5% CO_2, and adjusted with $NaHCO_3$ to a pH range of 7.4) through a catheter in the pulmonary artery at a pressure of 20 cm H_2O at room temperature. During perfusion of the lungs, the buffer was allowed to drain freely from a catheter in the left ventricle. Once the effluent was clear of blood, lungs were dissected from the thoracic cavity and immediately frozen in liquid nitrogen for mRNA analysis.

Cell culture and hypoxic exposure

SMCs were isolated from precapillary pulmonary arterial vessels of adult male Sprague-Dawley rats (200–250 g body weight) as described previously [41]. SMCs were identified by immunohistochemical staining with SMC-specific α-actin and myosin antibodies (data not shown). Rat PASMC were exposed to hypoxia (1% O_2, 5% CO_2, rest N_2) in 1% (v/v) FCS and 1% (m/v) penicillin and streptomycin M199 medium for the indicated period of time. mRNA analysis and Western blotting were done from cells transferred to respective buffers under hypoxic atmosphere. All other measurements were performed under normoxic conditions, starting 30 min after termination of hypoxia.

Molecular analysis

The RNA was extracted from cells using spin columns (RNeasy, Qiagen, Germany). Relative quantification of NOX4 was done using the Mx 3000 P detection system (Stratagene, Germany) as described previously [40]. The C_t values of NOX4 were normalized to the endogenous control β_2-microglobulin (B2M). Specificity of the products was verified by dissociation curves and by analysis on an ethidium bromide-stained agarose gel. Protein extracts for Western blotting from rat lung were prepared in RIPA buffer (containing 1 mM sodium vanadate, Protease-Inhibitor Mix complete (Roche, Mannheim, Germany), and 0.1 mM PMSF). The proteins were denatured in the presence of β-mercaptoethanol and 1X LDS sample buffer (Nu PAGE, Invitrogen, Karlsruhe, Germany) at 100 °C for 10 min. Equivalent amounts of protein were resolved on 8% SDS polyacrylamide gels and detected with a 1:5000 diluted custom-made rabbit polyclonal anti-NOX4 primary antibody using enhanced chemiluminescence (ECL, Amersham, Freiburg, Germany). The NOX4 antibody was raised in rabbit against a synthetic peptide corresponding to position 556–569: LHKLSNQNNSYGTR of the NOX4 protein (GenBank Accession No. NP_058627) as described previously [42].

RNA interference, apocynin, and mito-TEMPO treatment of cells

Rat PASMC were transfected with approximately 100 nM Cy3-labeled NOX4 siRNA (Biospring-Frankfurt, Germany) using X-tremeGENE siRNA transfection reagent (1:200 dilution, Roche, Mannheim, Germany). The following NOX4 siRNA sequence was used: siNOX4:CGAGAGACUUUACCGAUGCAUCAUGAUGC. Both siRNA and transfection reagent were diluted in OPTI-MEM medium (Gibco, Karlsruhe, Germany). For controls, a similar Cy3-labeled, scrambled siRNA (Biospring-Frankfurt, Germany) was employed. Transfection efficiency was consistently greater than 90% in all the experiments performed. After 5 h of transfection, the medium was changed to low-serum medium containing antibiotics (1% (v/v) FCS, 1% (m/v) penicillin, and streptomycin) in M199 and left under normoxic conditions for 2 days to bring down the level of NOX4 protein. After this 2-day incubation period, the hypoxic group of the cells was transferred to hypoxic conditions (1% O_2, 5% CO_2, rest N_2) for the indicated period of time whereas the normoxic group was incubated at 21% O_2, 5% CO_2, rest N_2.

For apocynin and mito-TEMPO experiments, the rat PASMC were treated with the indicated concentration of apocynin or mito-TEMPO dissolved in DMSO in parallel with the hypoxic or normoxic exposure. PASMC were cultured in M199 medium containing 1% (v/v) FCS and 1% (m/v) penicillin and streptomycin and were incubated under normoxic (21% O_2, 5% CO_2, rest N_2) or hypoxic conditions (1% O_2, 5% CO_2, rest N_2) for 3 days. Controls received dimethyl sulfoxide (DMSO) only.

Immunohistochemistry and colocalization of NOX4 and K_v channels

For immunocytofluorescence, isolated rat PASMC were cultured on chamber slides, fixed in acetone and methanol (1:1), blocked with 3% (m/v) BSA and 0.2% Triton 100 in PBS for 1 h, followed by overnight incubation with the NOX4 antibody in combination with either K_v1.5 or K_v2.1 antibodies, diluted in the blocking buffer. The direct labeling of primary NOX4 (custom made), K_v2.1 (Sigma, Hamburg, Germany), and K_v1.5 (Sigma, Hamburg, Germany) antibodies was done using the rabbit IgG tricolor labeling kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Nuclear counterstaining was performed with Hoechst-33258 (Invitrogen, Karlsruhe, Germany). Immunohistochemistry of NOX4 on paraffin-embedded rat lung sections was performed as described previously [36].

Electrophysiology

Electrodes for whole cell recording were pulled on a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument, Novato, CA, USA) from filamented borosilicate capillary glass (1.2 mm O.D., 0.69 mm I.D., World Precision Instruments, Sarasota, FL, USA). The electrodes were fire-polished, and resistances were 2–5 MΩ for voltage-clamp experiments using the above-noted solutions. Membrane potentials (V_m) were recorded in the current-clamp mode at the moment of establishment of a whole cell configuration. Input resistance (Rm) was calculated from linear regression fitting the current traces between -87.29 and -55.16 mV as 1/slope by a ramp voltage from -160 to 100 mV in the voltage-clamp mode. In all our recordings

between the two former voltages, there was no active channel current except an ohmic current, which yielded a perfect straight line.

Membrane potential and membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Signals were obtained at sampling rates of 5 kHz, filtered at 2 kHz, and stored on the hard disk of a personal computer. Stimulus generation and data acquisition were controlled with the ClampEx program in the pClamp6 software package (Axon Instruments). Before seals were made on cells, liquid junction potentials were nulled for each individual cell with the Axopatch 1 C amplifier. The series resistances were primarily under 10 MΩ. Current traces in voltage clamp were leak-subtracted.

The bath or extracellular solution used for the voltage-clamp experiments contained (in mM) 130 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, and 10 glucose and pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mM) 138 KCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (Na + salt), and 10 EGTA and pH was adjusted to 7.4 with Tris. Osmolarity of all solutions was adjusted to 290 mOsm. All recordings were performed at room temperature (22–24 °C). The cells were transfected with NOX4 siRNA and were exposed to normoxic or hypoxic conditions as described above. At the conclusion of the treatment, the cells were trypsnized, resuspended in extracellular solution, and kept in the incubator at 37 °C for 1–2 h to adhere at the bottom. The cells that were exposed to hypoxia were kept in the hypoxic incubator during this period. For dissecting out the current specific to $K_v1.5$ and $K_v2.1$ channels, the antibodies against both channels were dissolved in the pipette solution at the dilution of 1:100. The control experiments were done without any antibody in the pipette solution. The current specific to K_v channels was analyzed using custom-made software (designed by Dr. Xiang Q. Gu at the University of California San Diego [UCSD]) which averaged 21 data points shortly before the termination of voltage commands.

Measurement of reduced/oxidized $K_v1.5$ channel

For the measurement of oxidation state of $K_v1.5$ channels, rat PASMC were cotransfected with either overexpressing plasmids for human $K_v1.5$ and NOX4 or $K_v1.5$ and inactive human NOX4 plasmids. Cotransfection (each 4 μg) was performed using Lipofectamine (2.5 μl per μg of plasmid). After 24 h of cotransfection, the cells were lysed in the presence of EZ-Link maleimide-PEG-biotin (Pierce, Rockford) and subsequently immunoprecipitated using avidin agarose beads and analyzed by immunoblotting with $K_v1.5$ channel.

Coimmunoprecipitaton of NOX4 with $K_v1.5$ channel

For coimmunoprecipitation, rat PASMC were transfected with NOX4 and $K_v1.5$ overexpressing plasmids using Lipofectamine as described above. After 24 h of transfection $K_v1.5$ was immunoprecipitated according to the protocol described previously [40].

ROS measurement

ROS measurement was done in isolated PASMC using 2′7′-dichlorofluorescein diacetate (DCFH-DA) or luminol. The treatment of rat PASMC with NOX4 siRNA was done as described above. At 30 min after termination of hypoxic or normoxic incubation the cells were subjected to DCFH-DA or luminol measurements under normoxic conditions. For DCFH-DA measurements the cells were incubated with 10 μM DCFH-DA dissolved in Hepes-Ringer buffer (HRB [in mM]: 136.4 NaCl, 5.6 KCl, 1 $MgCl_2$, 2.2 $CaCl_2$, 10 Hepes, 5 glucose, 0.1% BSA, pH 7.4) in the dark for 5 min at room temperature. The cells were subsequently washed twice and DCF fluorescence was measured with Infinite 200 microplate reader (TECAN, Crailsheim, Germany) at excitation/emission wavelength of 502/530, respectively.

For ROS measurement using luminol PASMC were made permeable with 10 μg/1 million cells digitonin (Sigma-Aldrich) and measured in on 96-wells plates containing 200 μl of 0.1% BSA/HBSS buffer and 1 million cells each. Ten microliters of 1 mM luminol (Sigma-Aldrich) and 2 μl (8 U) HRP (Sigma-Aldrich) were added to each well and luminescence was read after 30 min of incubation at 37 °C by multimode microplate reader Infinite M200 (Tecan Group Ltd.).

Statistics

Values are given as mean ± SEM (standard error of the mean). For the comparison of two groups, a *t* test was used. For more than two groups one-way ANOVA followed by Dunnett's post hoc test was applied. A *P* value of less than 0.05 was considered significant.

Results

NOX4 is a major contributor to increased ROS levels under chronic hypoxia

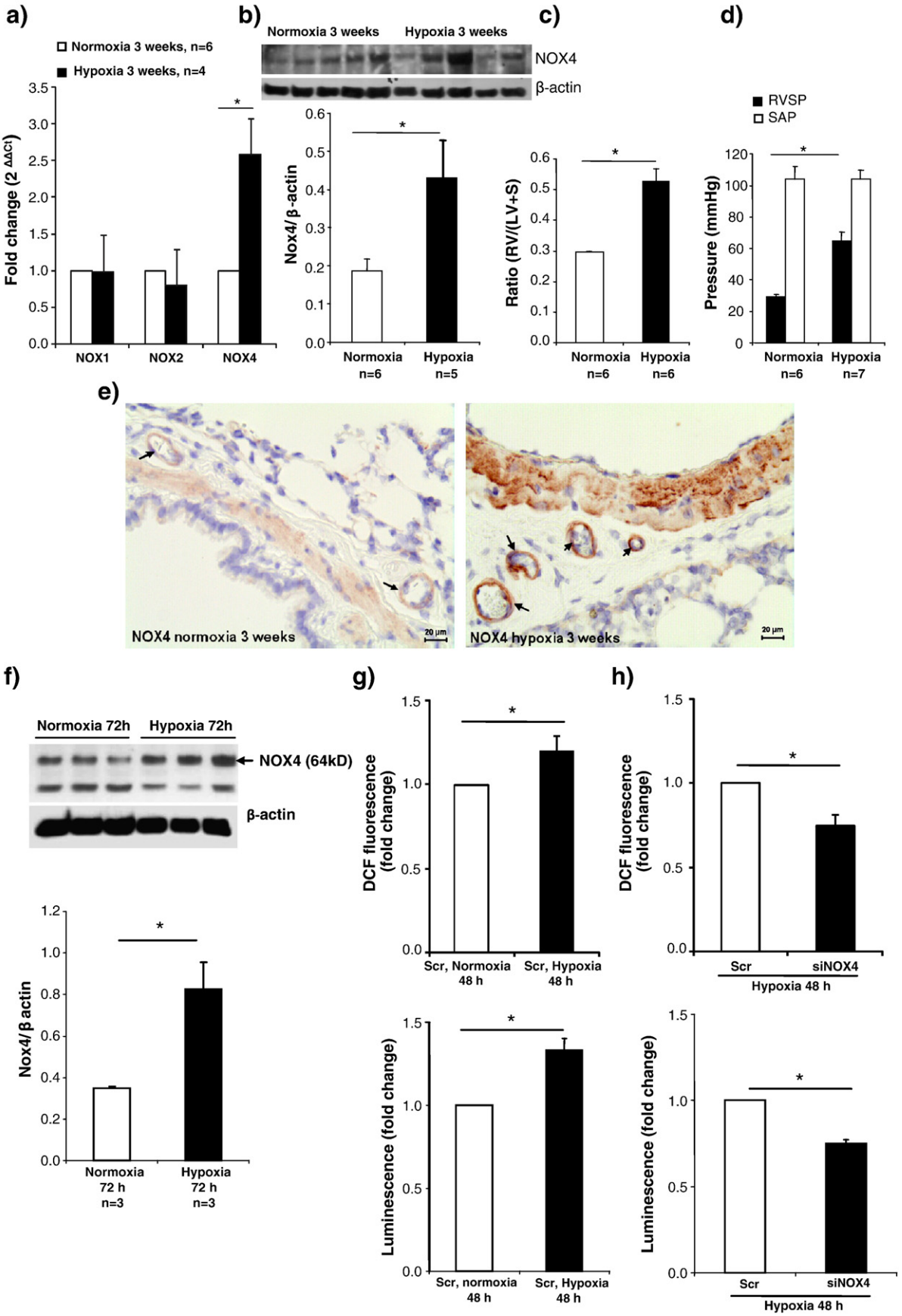
Our previous investigations have shown in the mouse model of hypoxia-induced pulmonary hypertension that NOX4 is selectively upregulated in the media of pulmonary arteries and may contribute to the pathogenesis of pulmonary vascular remodeling [36]. Similar to our findings in the mouse model, we here demonstrate a significant upregulation of NOX4 mRNA and protein in the lung homogenate of rat exposed to 3 weeks of chronic hypoxia, whereas NOX1 and NOX2 mRNA did not undergo any significant regulation (Fig. 1a and b). The right ventricular hypertrophy was significantly higher in rats exposed to 3 weeks of hypoxia compared to the normoxic rats which was paralleled by a significant rise in right ventricular systolic pressure (RVSP), confirming the validity of hypoxic exposure (Fig. 1c and d). No increase in systemic arterial pressure (SAP) was observed in chronic hypoxic rats compared to normoxic control (Fig. 1d). Immunohistochemistry additionally revealed an increased NOX4 immunoreactivity in the smaller pulmonary vessels (<20 μm) of rats exposed to hypoxia compared to normoxic control (Fig. 1e). A similar upregulation of NOX4 protein as in the lung homogenate was found in isolated rat pulmonary arterial smooth muscle cells exposed to 72 h of hypoxia (Fig. 1f). In parallel to the hypoxia-induced upregulation of NOX4, a significant increase in DCF fluorescence and luminol chemiluminescence was observed after 48 h of hypoxia (Fig. 1g). Treatment of PASMC with NOX4 siRNA resulted in attenuation of the hypoxia-induced increase of DCF fluorescence or luminol luminescence (Fig. 1h).

Colocalization of NOX4 with $K_v1.5$ and $K_v2.1$ channels

Immmunofluorescence staining of NOX4 with $K_v1.5$ and $K_v2.1$ in isolated rat PASMC (Fig. 2) revealed a colocalization of NOX4 with $K_v1.5$ but not with $K_v2.1$. This colocalization was further confirmed by coimmunoprecipitation of NOX4 with $K_v1.5$ in rat PASMC transfected with plasmids overexpressing NOX4 and $K_v1.5$ proteins (S5).

NOX4 regulates K_v currents in PASMC under chronic hypoxia

We next used the whole cell patch clamp technique to measure the whole cell K_v current (IK_v). Preincubation of PASMC for 48 and 72 h under hypoxia significantly decreased the whole cell IK_v current at all positive V_m compared to normoxic cells (Fig. S1 and Fig. 3). Apocynin treatment of the cells during hypoxia reversed the decrease of the K_v current density compared to DMSO (solvent control)-treated cells (Fig. 3a–c). The K_v current density at 90 mV on the DMSO-treated hypoxic cells was 15.2 ± 1.8 pA/pF ($n = 11$), which was increased by approximately 2.7-fold and reached 41.4 ± 8.7 pA/pF ($n = 8$) ($P < 0.05$) with apocynin treatment (Fig. 3b). To isolate the current specific to $K_v1.5$ and $K_v2.1$ channels, whole cell K_v current was


a)
Normoxia 3 weeks, n=6
Hypoxia 3 weeks, n=4
Fold change (2 ΔΔCt)
NOX1
NOX2
NOX4
b)
Normoxia 3 weeks
Hypoxia 3 weeks
NOX4
β-actin
Nox4/β-actin
Normoxia n=6
Hypoxia n=5
c)
Ratio (RV/(LV+S)
Normoxia n=6
Hypoxia n=6
d)
RVSP
SAP
Pressure (mmHg)
Normoxia n=6
Hypoxia n=7
e)
NOX4 normoxia 3 weeks
NOX4 hypoxia 3 weeks
20 μm
f)
Normoxia 72h
Hypoxia 72h
NOX4 (64kD)
β-actin
Nox4/β actin
Normoxia 72 h n=3
Hypoxia 72 h n=3
g)
DCF fluorescence (fold change)
Scr, Normoxia 48 h
Scr, Hypoxia 48 h
Luminescence (fold change)
Scr, normoxia 48 h
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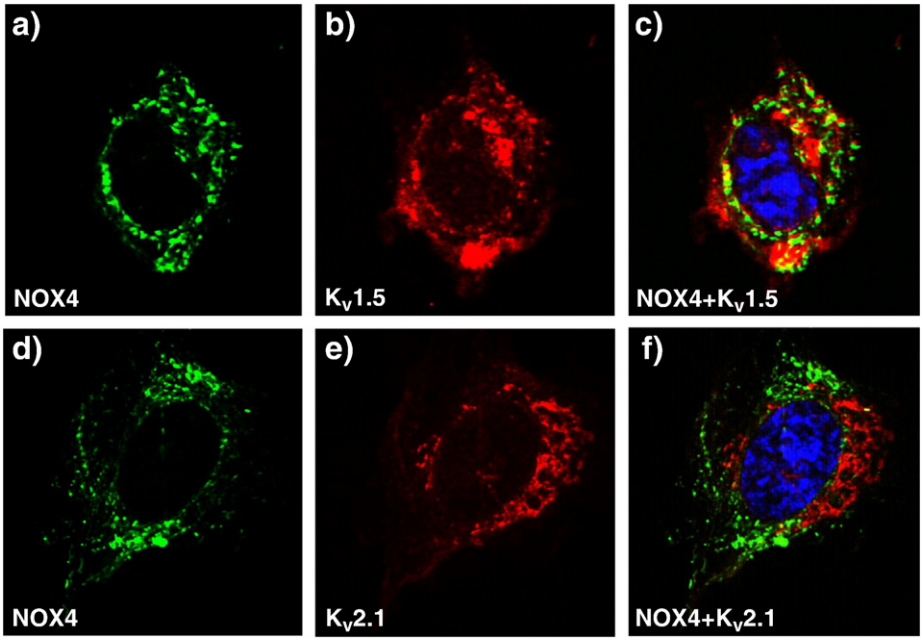

Fig. 2. Colocalization of NOX4 with K_v1.5 channels in isolated rat PASMC. Confocal immunofluorescence microscopy of isolated precapillary pulmonary arterial smooth muscle cells stained with antibodies against NOX4 (green; a, d) and K_v1.5 and K_v2.1 (red; b, e) antibodies; c and f are the merged images of NOX4 and with K_v1.5 and K_v2.1, respectively.

recorded in the presence of antibodies against K_v2.1 and K_v1.5 channels in the intracellular pipette solution [17,18]. The initial recording of the whole cell K_v current was made immediately after the establishment of a whole cell configuration and is represented by T-0 (Fig. S2). Further recordings at different time intervals revealed a gradual decrease in the K_v current density with increasing time. A 55% inhibition of K_v current was observed after 22 min which could indirectly represent the percentage of K_v1.5- and K_v2.1-specific current in the whole cell K_v current (Fig. S2a). No effect of run down on the K_v current was observed in the normoxic rat PASMC even after 25 min into the whole cell configuration in the absence of antibodies (Fig. S2b). Under hypoxic conditions inhibition of K_v current by K_v1.5 and K_v2.1 antibodies was observed only in PASMC after apocynin treatment but not in DMSO-treated hypoxic rat PASMC (Fig. 3d). The difference in current before and after antibody blockage revealed a specific and a significant increase in the current density of K_v1.5 and K_v2.1 channels on rat PASMC treated with apocynin under hypoxia at all positive V_m (Fig. 3e).

To address specifically the role of NOX4 in the regulation of hypoxia-sensitive K_v channels, we suppressed the NOX4 protein levels using NOX4 specific siRNA and recorded the whole cell K_v current. *I–V* curves revealed a 5-fold increase of K_v current after NOX4 siRNA treatment at V_m of 90 mV as compared with scrambled siRNA-treated cells after hypoxic conditions (Fig. 4a–c). The mean K_v current density of scrambled siRNA-treated hypoxic rat PASMC was 19.9 ± 5.4 pA/pF ($n = 10$) at V_m of 90 mV which increased up to 105.9 ± 30.2 pA/pF ($n = 12$, $P < 0.05$) on siNOX4 treatment. Hypoxia significantly decreased the K_v current density at all positive V_m on the scrambled siRNA-treated hypoxic cells as compared to scrambled siRNA-treated normoxic cells (Fig. 4a–c). The siRNA treatment restored the decrease in current density induced by hypoxia, whereas no effect of NOX4 siRNA was observed on K_v current from normoxically incubated cells (Fig. S3). In contrast to the effects of siRNA against NOX4 or apocynin treatment, mito-TEMPO did not increase the whole cell K_v current under hypoxia in rat PASMC (Fig. S4). The reduced levels of NOX4 protein in siNOX4 transfected rat PASMC was confirmed by immunoblotting (Fig. 4d). The treatment of rat PASMC with NOX4 siRNA under chronic hypoxia resulted in a significant hyperpolarizing shift of the voltage-dependent activation and a reduction in the slope factor *K* (Fig. 4e). The Boltzman distribution of scrambled siRNA-treated hypoxic cells had a $V_{1/2} =$ 11.43 mV ($n = 5$) which was shifted to hyperpolarizing direction of $V_{1/2} =$ -9.89 mV ($n = 9$) by siNOX4 treatment in hypoxia. This hyperpolarizing shift was accompanied by a decrease in the value of slope factor *K* from 15.80 in scrambled siRNA-treated cells to 12.77 in NOX4 siRNA-treated cells under hypoxia (Fig. 4e). Although, the protein levels of K_v1.5 and K_v2.1 were significantly reduced in rat PASMC at 48 and 72 h of hypoxia (Fig. 4f), no significant effect of siNOX4 was observed on the expression levels of these channels (Fig. 4g).

NOX4-derived ROS lead to oxidation of cysteine residues in K_v1.5 channels

The function of redox-sensitive K_v channels is modulated by oxidation/reduction of redox-sensitive cysteine residues. Therefore, we investigated if NOX4-mediated inhibition of K_v1.5 channel is due to the oxidation of cysteine residues in the K_v1.5 channel. The content of the oxidized or reduced thiol residues (-SH) in K_v1.5 in isolated PASMC was measured in the lysed rat PASMC by labeling of the proteins with biotin polyethylene oxide maleimide. This compound selectively labels the reduced sulfhydryl group (-SH) in the proteins with biotin [43]. Isolated rat PASMC were cotransfected with the plasmids overexpressing NOX4 and K_v1.5 or K_v1.5 and inactive NOX4 and the content of the reduced form of K_v1.5 channels was measured by pulling down biotinylated K_v1.5 protein with avidin-agarose beads and subsequent

Fig. 1. NOX4 is a major contributor to increased reactive oxygen species after chronic hypoxia. (a, b) Quantitative real-time PCR for NOX1, NOX2, and NOX4 and immunoblotting for NOX4 from homogenized rat lungs exposed either to 3 weeks of chronic hypoxia (10% O_2) or to normoxia. (c, d) Right ventricular hypertrophy given as the ratio of the right ventricle (RV)/left ventricle + septum (LV + S) and right ventricular systolic pressure (RVSP), systemic arterial pressure (SAP). (e) Immunoreactivity of NOX4 in lung sections of rat exposed to chronic hypoxia (10% O_2, 3 weeks) or normoxia. Arrows indicate increased immunoreactivity in small vessels (outer diameter ≤20 μm). (f) Upregulation of NOX4 protein in isolated pulmonary arterial smooth muscle cells on exposure to 72 h of hypoxia (1% O_2). (a–d, f) $n =$ number of rats. (g) Measurement of ROS in isolated PASMC treated with scrambled siRNA exposed to 48 h of hypoxia or normoxia using a DCFH-DA or luminol-based assay. (h) Attenuation of DCF fluorescence or luminol luminescence on NOX4 siRNA treatment compared to scrambled siRNA after hypoxia. Values in g and h are referenced to the scrambled siRNA values (= 1.0) and data are derived from $n = 3$ parallel measurements for DCF and $n = 8$ for luminol measurements. Cells were derived from at least 3 cell isolations.

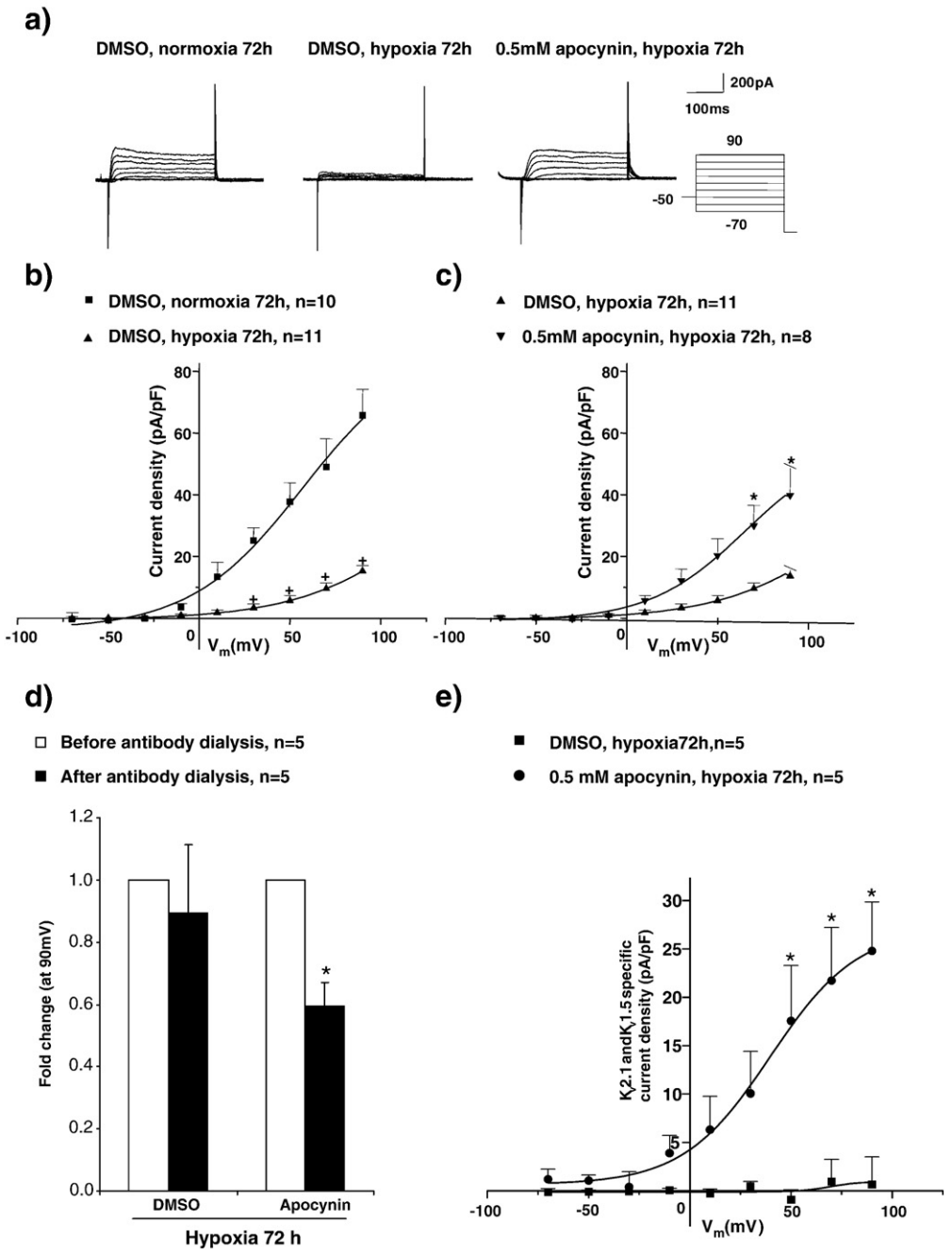

Fig. 3. Apocynin reversed the hypoxia-induced decrease of the whole cell K_v current in rat pulmonary arterial smooth muscle cells. (a) Representative recordings of whole cell K_v currents. (b,c) *I–V* curve expressed as the whole cell K_v current density against V_m. (b) Effects of hypoxia (1% O_2, 72 h) on K_v current density in dimethyl sulfoxide (DMSO)-treated hypoxic cells compared to normoxic controls. (c) Effect of apocynin on the hypoxia-induced diminished K_v current density. (d) Inhibition of the K_v current by antibodies directed against $K_v2.1$ and $K_v1.5$ in apocynin and DMSO (=control)-treated hypoxic rat PASMC. No inhibition was observed in the DMSO-treated hypoxic rat PASMC. (e) Apocynin treatment under hypoxia increased the current density of $K_v2.1$ and $K_v1.5$ channels on rat PASMC at all positive V_m. *n* = number of the cells investigated. The cells were from two independent cell isolations.* $P<0.05$ compared to DMSO-treated hypoxic cells. $^{+}P<0.05$ compared to DMSO-treated normoxic cells.

immunoblotting with $K_v1.5$ antibody. Overexpression of NOX4 protein along with $K_v1.5$ channels resulted in a significant decrease in the reduced thiol residues of $K_v1.5$ channels compared to inactive NOX4 plasmid transfected control, indicating that $K_v1.5$ channels were oxidized at cysteine residues by overexpression of the NOX4 protein (Fig. 5a and b).

Discussion

A variety of reports have suggested that vascular remodeling in chronic hypoxia-induced pulmonary hypertension involves a reduction in K_v channel currents and a subsequently increased calcium influx in PASMC [22,25,44]. The mechanisms of inhibition of K_v channels in the pulmonary vasculature have been addressed in several studies and a possible link between redox alterations and K_v channel function has been suggested [5,15,17,23,24,45–50]. However, (1) there is current discussion whether ROS are decreased or paradoxically increased by hypoxia [10], (2) from which sources a hypoxia-dependent ROS formation is derived, and (3) how ROS formation is linked to K_v channel regulation in the pulmonary arterial vasculature. Among the sources of ROS for such processes mitochondria as well as NAD(P)H oxidases have been discussed. In this regard we recently provided evidence for an important role of the nonphagocytic

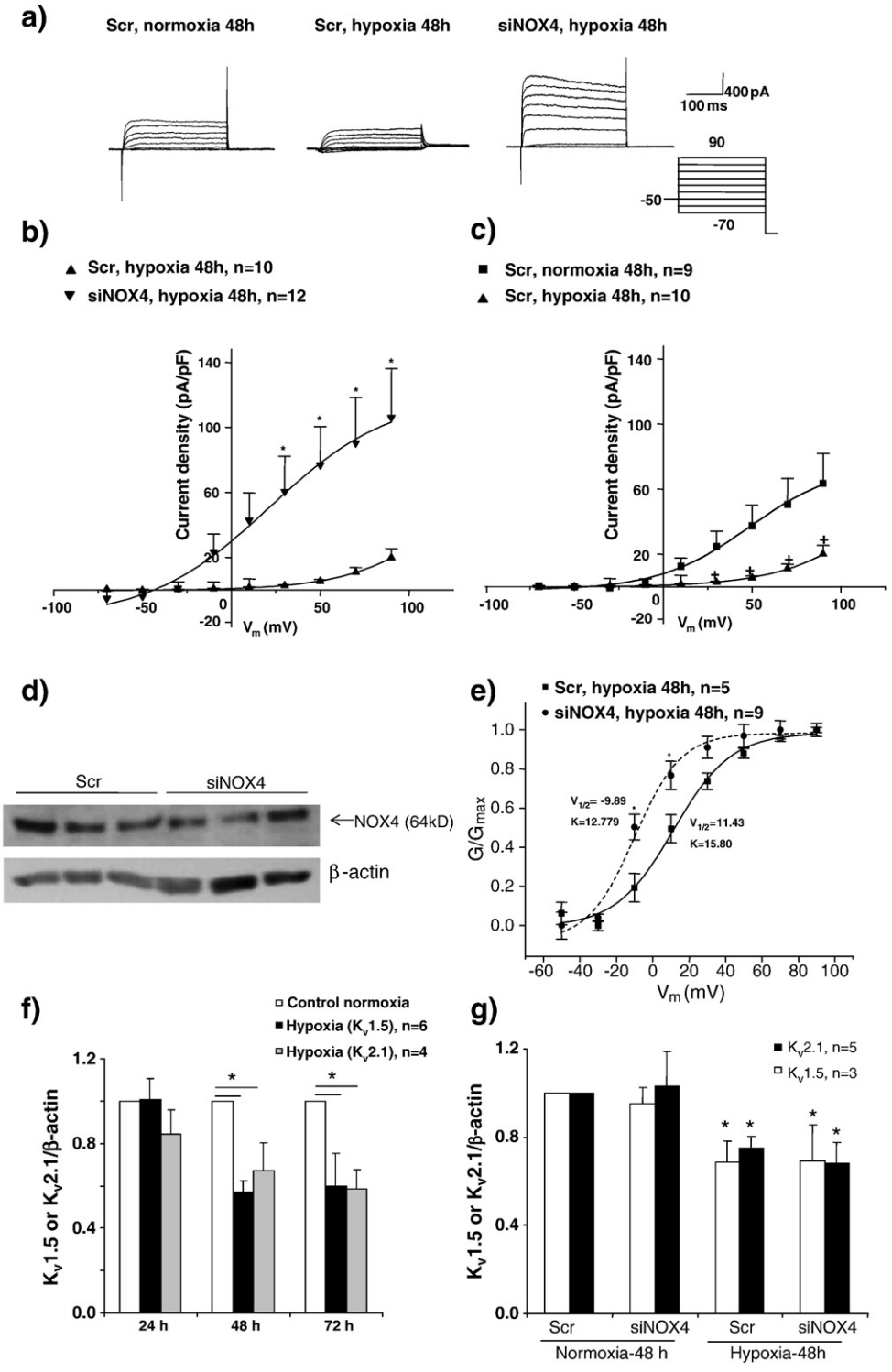

Fig. 4. siRNA targeting of NOX4 increased the K_v current density of rat PASMC exposed to sustained hypoxia without affecting the expression levels of K_v1.5 and 2.1 channels. (a) Representative recordings of the whole cell K_v current from the cells treated with scrambled (scr) and NOX4 siRNA. (b) Effect of NOX4 siRNA on the hypoxia-induced (48 h, 1% O_2) decrease in the K_v current density in rat PASMC compared to scrambeled siRNA transfected cells under hypoxic conditions. (c) Decrease of K_v current density by hypoxia (1% O_2, 48 h) in scrambled siRNA-treated PASMC as compared to normoxia. (d) Representative immunoblot demonstrating the decreased NOX4 protein levels after siRNA-mediated targeting NOX4 in PASMC. (e) Conductance voltage relationship of K_v current in rat PASMC treated with NOX4 siRNA under chronic hypoxia. The conductance was calculated from the peak current amplitude divided by the K^+ ion driving force calculated from the Nernst equation and normalized with the maximal conductance in a given series of voltages. Curves were fit with Boltzmann equation. n = number of the cells from 3 independent cell isolations. $^*P<0.05$ compared to scrambled siRNA-treated hypoxic cells. $^+P<0.05$ compared to scrambled siRNA-treated normoxic cells. (f) Effect of hypoxia (1% O_2) on K_v1.5 and K_v2.1 protein expression after 24, 48, and 72 h of hypoxia. (g) NOX4 siRNA and scrambled siRNA treatment did not affect the expression levels of K_v1.5 and K_v2.1 channels.$^*P<0.05$.

NADPH oxidase NOX4 for the development of pulmonary hypertension whereas others favor a mitochondrial mechanism [23]. Similar to our previous work in hypoxia-induced pulmonary hypertension in mice we here show in rats that chronic hypoxia leads to an upregulation of NOX4 mRNA and protein levels in the pulmonary vasculature and that NOX4 substantially contributes to an increase in

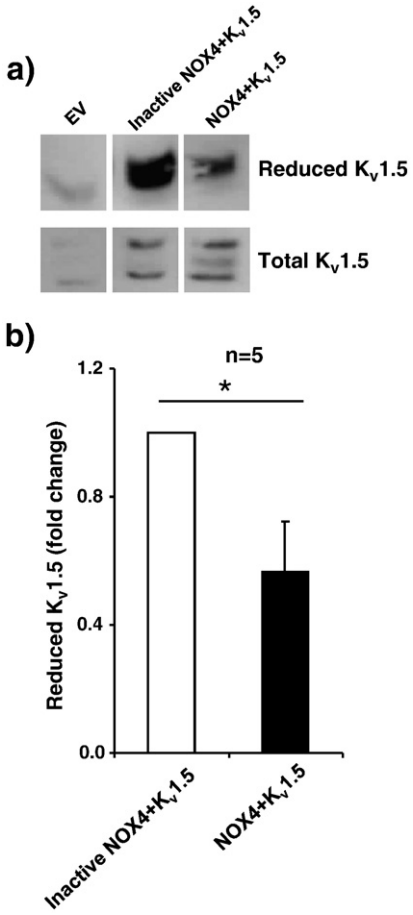

Fig. 5. NOX4 overexpression oxidizes $K_v1.5$ channel cysteine residues. (a) Rat PASMC were cotransfected with NOX4 and $K_v1.5$ plasmids or inactive NOX4 plasmids and $K_v1.5$ plasmids for 24 h and were lysed in the presence of maleimide biotin and subsequently immunoprecipitated with neutravidin agarose beads followed by Western blotting with a $K_v1.5$ antibody. The band represents the reduced form of $K_v1.5$ channel. (b) Bar graph depicting the fold change in the reduced form of $K_v1.5$. n = number of independent experiments from 3 different cell isolations. $P<0.003$.

intracellular ROS levels in isolated PASMC when exposed to sustained hypoxia. As a possible link of NOX4-derived ROS release and K_v channel function has not been investigated, we, in this regard, focused on $K_v1.5$ and $K_v2.1$ channels because both are abundantly expressed in resistance PASMC and their expression levels are downregulated under chronic hypoxia at mRNA and protein level [21,51]. $K_v1.5$ and 2.1 channels can constitute heteromeric tetramers and $K_v1.5$ has been shown to be essential for hypoxic pulmonary vasoconstriction [17,52]. $K_v2.1$ channels have been shown to be an important determinant in maintaining the resting E_m in resistance PASMC and application of $K_v2.1$ antibody in electrophysiological preparation abolished further hypoxic inhibition of I_{Kv} in PASMC [17,18]. Previous findings were confirmed by our study, demonstrating that the attenuation of PASMC membrane current in sustained hypoxia is predominantly regulated by $K_v1.5$ and $K_v2.1$. In a pharmacological approach we then showed that apocynin treatment of rat PASMC under such hypoxic conditions resulted in the reappearance of $K_v1.5$- and $K_v2.1$-specific current. As this agent has been described to be a ROS scavenger or antioxidant and/or NADPH oxidase inhibitor [53–55], and NOX4 may not be inhibited by this agent [53,56], these experiments were indicative of the concept that an increase in ROS leads to a reduction in K_v currents in PASMC. Our data with apocynin are well in line with findings from Cogolludo and colleagues investigating non-hypoxia-induced regulation of K_v currents [57]. This finding was further substantiated by the fact that siRNA directed against NOX4 also restored the hypoxia-induced reduction in K_v currents in parallel with a decrease in DCF fluorescence and luminol luminescence. The DCF and luminol measurements indicate an increased ROS production when PASMC were preexposed to hypoxia. Our ROS and patch clamp measurements were done 30 min after exposure to hypoxia, avoiding acute reoxygenation effects. As the increased ROS release as well the decrease in K_v current density was inhibited by NOX4 siRNA as well as apocynin, such effects are most likely attributable to the increased NOX4 protein levels induced by hypoxia. To address the question if the NOX4-mediated reduction in K_v currents is attributed to a possible downregulation or a gating of the channels, we determined the expression levels of $K_v1.5$ and 2.1 in the course of sustained hypoxia exposure. Although a significant reduction of the $K_v1.5$ and 2.1 protein occurred after 48 h of hypoxia, Nox 4 siRNA treatment did not restore the expression level to normoxic values, allowing the conclusion that NOX4-derived ROS have a direct impact on the gating of K_v channels rather than on the expression level of channels. Intriguingly, the stimulatory effect of apocynin and NOX4 siRNA on the whole cell K_v current was observed only for hypoxic cells, whereas under normoxia apocynin and NOX4 siRNA had no significant effect on the K_v current. These findings further substantiate that NOX4-mediated inhibition of K_v channels occurs by an upregulation of ROS. The specific impact of NOX4-derived ROS on K_v channel gating is supported by the fact that mito-TEMPO did not restore the hypoxia-induced reduction of K_v currents. This agent has been described to scavenge superoxide and to at least lower stimulated mitochondrial H_2O_2 release [58], although its effectiveness in this regard was not proven in our study. The action of oxidants on the K^+ channel activity is conferred via the oxidative modification at key cystine and methionine residues at the N-terminal inactivation ball domain and pore domain. Using the Boltzman equation $K_v1.5$ channels have been reported to have $V_{1/2}$ and K values of -10 and 7 mV, respectively, whereas for $K_v2.1$ and $K_v2.2$ channels these values are 10 and 5 to 19 mV [16, 59]. Accordingly, we have observed a shift in the activation kinetics of K_v channels toward more hyperpolarized potentials by siNOX4 treatment under chronic hypoxia which resembles the individual activation profile of $K_v1.5$ channel. This shift in activation profile is in agreement with our observation of increased oxidation of cysteine residues in $K_v1.5$ channel on NOX4 protein overexpression in rat PASMC. These data are indicative for a direct effect of NOX4-derived ROS on K_v channels and are in line with previous investigations of ROS effects on cloned $K_v1.5$ channels [60]. Although our data do not exclude a role of mitochondria for K_v channel gating they are in direct contrast to the concept that a decrease in ROS reduces K_v currents in PASMC. For such discrepancies a complex interaction of mitochondria and NADPH oxidases may be responsible. Moreover, one has to discriminate between the effects of very acute hypoxia (lasting seconds to minutes) and sustained hypoxia (lasting hours to days, leading over to chronic hypoxia). Different effects of the duration of hypoxia have been found in the pulmonary circulation [61]. The general discrepancy between studies reporting a decrease or an increase of ROS in hypoxia may also be attributable to uncertainties with regard to the techniques available for ROS measurements. In our study we focused on the effect of several days of hypoxic incubation leading to an upregulation of the ROS producing enzyme NOX4. As our ROS measurements and electrophysiology were performed in subsequent normoxia, 30 min after termination of hypoxia, these studies only allow the conclusion that a NOX4-mediated increase in ROS decreases the K_v currents. Further studies should address the question whether the here documented increase in NOX4-derived ROS after hypoxia already increases ROS levels during the hypoxic phase as well.

In conclusion, we have shown that NOX4 is a major contributor to increased ROS production in PASMC after exposure to sustained hypoxia and that an upregulation of NOX4 and a NOX4-derived increase in ROS leads to a reduction in K_v currents by a direct gating and not via the regulation of K_v channel expression levels.

Supplementary materials related to this article can be found online at doi:10.1016/j.freeradbiomed.2011.12.004.

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