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Author(s)
Takahashi, Nobuaki; Kuwaki, Tomoyuki; Kiyonaka, Shigeki; Numata, Tomohiro; Kozai, Daisuke; Mizuno, Yusuke; Yamamoto, Shinichiro; Naito, Shinji; Knevels, Ellen; Carmeliet, Peter; Oga, Toru; Kaneko, Shuji; Suga, Seiji; Nokami, Toshiki; Yoshida, Jun-Ichi; Mori, Yasuo

Citation
Nature chemical biology (2011)

Issue Date
2011-08-28

URL
http://hdl.handle.net/2433/145668

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Type
Journal Article

Textversion
author
Kyoto University
TRPA1 underlies a sensing mechanism for O₂

Nobuaki Takahashi¹,², Tomoyuki Kuwaki³, Shigeki Kiyonaka¹,⁴, Tomohiro Numata¹, Daisuke Kozai¹, Yusuke Mizuno¹, Shinichiro Yamamoto¹, Shinji Naito⁵, Ellen Knevels⁶,⁷, Peter Carmeliet⁶,⁷, Toru Oga⁸, Shuji Kaneko⁹, Seiji Suga¹⁰, Toshiki Nokami¹⁰, Jun-ichi Yoshida¹⁰ & Yasuo Mori¹,⁴*

¹Laboratory of Molecular Biology, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, and Laboratory of Environmental Systems Biology, Department of Technology and Ecology, Hall of Global Environmental Studies, Kyoto University, Kyoto 615-8510, Japan

²Advanced Biomedical Engineering Research Unit, Kyoto University, Kyoto 615-8510, Japan

³Department of Physiology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8544, Japan

⁴CREST, JST, Chiyoda-ku, Tokyo 102-0075, Japan

⁵Division of Pathology, Research Laboratory, National Ureshino Hospital, Ureshino 843-0393, Japan

⁶Vesalius Research Center, Katholieke Universiteit Leuven, 3000 Leuven, Belgium

⁷Vesalius Research Center, Flanders Institute for Biotechnology (VIB), 3000 Leuven, Belgium

⁸Department of Respiratory Care and Sleep Control Medicine, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

⁹Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences,
Kyoto University, Kyoto 606-8501, Japan

Laboratory of Synthetic Organic Chemistry, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan

*e-mail: mori@sbchem.kyoto-u.ac.jp
Oxygen (O\textsubscript{2}) is a prerequisite for cellular respiration in aerobic organisms but also elicits toxicity. To understand how animals cope with the ambivalent physiological nature of O\textsubscript{2}, it is critical to elucidate the molecular mechanisms responsible for O\textsubscript{2} sensing. Here, our systematic evaluation of TRP cation channels using reactive disulfides with different redox potentials reveals the capability of TRPA1 to sense O\textsubscript{2}. O\textsubscript{2} sensing is based upon disparate processes: while prolyl hydroxylases (PHDs) exert O\textsubscript{2}-dependent inhibition on TRPA1 activity in normoxia, direct O\textsubscript{2} action overrides the inhibition via the prominent sensitivity of TRPA1 to cysteine-mediated oxidation in hyperoxia. Surprisingly, TRPA1 is activated through relief from the same PHD-mediated inhibition in hypoxia. In mice, \textit{Trpa1} gene disruption abolishes hyperoxia- and hypoxia-induced cationic currents in vagal and sensory neurons, and impedes enhancement of \textit{in vivo} vagal discharges induced by hyperoxia and hypoxia. The results suggest a novel O\textsubscript{2}-sensing mechanism mediated by TRPA1.
Molecular oxygen (O$_2$) plays a paradoxical role in life on the Earth. O$_2$ is the engine of life essential for cellular respiration in all aerobic organisms$^1$. However, O$_2$ also exerts toxicity through the production of reactive oxygen species (ROS), causing aging, respiratory disorders and eventually death in a high-O$_2$ environment, namely hyperoxia$^1$. Due to the ambivalent physiological nature of O$_2$, aerobic life forms must adapt themselves to hyperoxia and hypoxia (low-O$_2$ environment) by sensing surrounding O$_2$ availability and by transmitting this information to effector systems.

In mammals, the carotid bodies, located near the carotid artery bifurcations, and brainstem catecholaminergic neurons detect changes in partial O$_2$ pressure (P$\text{O}_2$) in arterial blood$^{2,3}$. With regard to the mechanisms underlying O$_2$-sensing in the carotid bodies, it is known that BK$_{Ca}$, TASK and K$_v$ K$^+$ channels are involved in arterial O$_2$ sensing$^{2,4}$. Hypoxia inhibits K$^+$ channels through several mechanisms such as carbon monoxide production by hemeoxygenase and depletion of intracellular ATP to depolarize glomus cells, leading to the activation of voltage-dependent Ca$^{2+}$ channels, exocytosis and the excitation of carotid sinus nerves, while hyperoxia reduces depolarization and inhibits exocytosis$^{2,4}$. Sensory and vagal afferent neurons, which project nerve endings throughout the body, have been also proposed to detect hypoxia in organs such as the airway, lungs and heart under ischemia and other conditions of low O$_2$ supply$^{5-8}$. Hypoxia detection by sensory and vagal neurons remains elusive$^8$. In terms of hyperoxia, *Caenorhabditis elegans* has been reported to exhibit a strong avoidance of hyperoxia through detection by sensory neurons$^9$. Furthermore, insects breathe discontinuously to avoid O$_2$ toxicity in hyperoxia$^{10}$. However, compared to invertebrates, physiological relevance of hyperoxia sensing through sensory systems is less established in vertebrates including mammals. Therefore, it is extremely
important to seek hyperoxia-sensing molecular processes in vertebrate sensory systems.

The *Drosophila* transient receptor potential (TRP) protein and its homologues form cation channels activated by sensing diverse stimuli from the extracellular environment and from inside the cell\textsuperscript{11,12}. Recently, a class of TRP channels has been demonstrated as cell sensors for changes in redox status\textsuperscript{13-16}. Certain members of the TRPC and TRPV subfamily, including TRPC5 and TRPV1, are activated by nitric oxide, oxidants and reactive disulfides through modification of cysteine (Cys) free sulfhydryl groups\textsuperscript{14}. Oxidative Cys modification by pungent compounds and inflammatory mediators\textsuperscript{17-20} are important activation triggers of TRPA1 in addition to noxious cold (< 17°C)\textsuperscript{21} and other stimulants\textsuperscript{22,23}. Notably, as polymodal receptors for noxious stimuli at nerve endings in sensory and vagal afferent neurons\textsuperscript{21,24-26}, TRPV1 and TRPA1 sense endogenous algesic substances and environmental irritants including oxidants throughout the body to evoke defensive responses such as pain, coughing and changes in respiration pattern\textsuperscript{17,21,24,25,27}. Considering unique redox reactivities of oxidizing chemical species including O\textsubscript{2}, it is interesting to examine whether respective redox-sensitive TRP channels sense specific ranges of redox status.

Here, our systematic chemical-biological evaluation of redox sensitivity of TRP cation channels reveals that TRPA1 critically contributes to O\textsubscript{2}-sensing mechanisms in sensory and vagal afferent neurons. While prolyl hydroxylases (PHDs) exert O\textsubscript{2}-dependent inhibition on TRPA1 activity in normoxia, direct O\textsubscript{2} action overrides the inhibition via the prominent sensitivity of TRPA1 to Cys-mediated oxidation in hyperoxia. In hypoxia, TRPA1 is activated through relief from the same PHD-mediated inhibition.
RESULTS

High oxidation sensitivity underlies O₂ sensing of TRPA1

Using a congeneric series of reactive disulfides, we systematically compared responses of redox-sensitive TRP channels to electrophiles to evaluate oxidation sensitivity of these channels on a quantitative basis (Fig. 1a,b)²⁸. A strong dependence of the redox potentials on substituents (Fig. 1a) suggests that the resulting changes in the electron density distribution of the aromatic ring affect the reduction propensity of the disulfide bond, indicating that redox potential is an excellent index for electrophilicity of reactive disulfides as electron acceptors (see METHODS). Indeed, plotting of intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) rises in human embryonic kidney (HEK) 293 cells expressing recombinant TRPs against redox potentials of reactive disulfide stimuli revealed positive correlations between these parameters, and threshold redox potentials (x-intercepts) for respective TRPs (Fig. 1c). This strongly indicates that respective redox-sensitive TRP channels have a characteristic redox sensitivity. Among the TRPs tested, only TRPA1 responded to an inert electrophile, diallyl disulfide, with a redox potential of −2,950 mV, revealing the highest sensitivity of TRPA1 to reactive disulfides. When an inert oxidant O₂ (−2,765 mV) was tested, only TRPA1 responded to hyperoxic solution prepared by bubbling with O₂ gas in a concentration-dependent manner (Fig. 1d,e), although O₂ differs from reactive disulfides such that it snatches an electron from a Cys sulfhydryl group rather than electrophilically attacks the group²⁹. Hyperoxia-induced Ca²⁺ responses were suppressed by the TRPA1-specific blocker AP-18 (ref. 30) and omission of extracellular Ca²⁺ in TRPA1-expressing HEK293 cells, indicating that TRPA1 mediates Ca²⁺ entry in response to hyperoxia (Supplementary Results, Supplementary Fig. 1a,b). Other redox-sensitive TRPs (TRPC1, TRPC4,
TRPM2 and TRPM7\textsuperscript{14} that by themselves do not respond to reactive disulfides including 5-nitro-2-PDS (10 µM) failed to respond to hyperoxia (Fig. 1d).

TRPA1 responses were sustained after induced conditions of hyperoxia (86% O\textsubscript{2} for 19 min) when switched back to normoxia (20% O\textsubscript{2}) (Supplementary Fig. 1a). The ROS scavenger N-acetylcysteine (NAC) or the reducing agent dithiothreitol (DTT) but not the inhibitor for nitric oxide synthases \textit{N}G-nitro-L-arginine methyl ester (L-NAME) reversed the TRPA1 response during readministration of normoxia. TRPA1 responses were reversed in normoxia also after mild hyperoxia at a lower O\textsubscript{2} concentration (28%) or after hyperoxia (86% O\textsubscript{2}) for a shorter time period (5 min), whereas TRPA1 responses were not reversed for 5-nitro-2-PDS with the same time protocol (Supplementary Fig. 1c–e). The observed reversibility was unaffected by NAC, DTT or L-NAME (Supplementary Fig. 1d). Diphenylene iodonium (DPI), a potent inhibitor for O\textsubscript{2}˙-producing enzymes, failed to affect hyperoxia-induced TRPA1 responses (Supplementary Fig. 1f), suggesting directness of O\textsubscript{2} action on Cys residues. Thus, TRPA1 during activation by hyperoxia may take at least two oxidized states distinguished by reversibility in normoxia after hyperoxia.

In TRPA1-expressing HEK293T cells, hyperoxia dramatically increased whole cell currents inhibited by AP-18 and DTT (Fig. 2a–c and Supplementary Fig. 2). Hyperoxia-activated currents showed a reversal potential ($E_{\text{rev}}$) characteristic of nonselective cationic channels ($E_{\text{rev}} = 2.2 \pm 0.9$ mV ($n = 37$)). Single-channel currents were significantly enhanced by hyperoxic solution applied from the intracellular side of cell-free excised inside-out patches at a holding potential ($V_h$) of –60 mV (Fig. 2d,e). Unitary conductance was $97.3 \pm 2.0$ pS at –80 - +20 mV and $180.6 \pm 10.7$ pS at +40 - +80 mV, as previously reported\textsuperscript{26}, and the open probability was reduced by AP-18 and
DTT (Supplementary Fig. 3a–d). Single-channel currents were enhanced also by hyperoxic solution applied from the extracellular side of cell-free excised outside-out patches (Supplementary Fig. 3e,f). Thus, TRPA1 is unique in having a prominent susceptibility to Cys oxidation, such that it is directly activated by the weak oxidant O2 to function as a hyperoxia sensor.

In inside-out patches, single-channel currents induced by hyperoxia of a relatively short period (2 min) were maintained after readministration of normoxia, but were reversed by the reduced form of intracellular antioxidant glutathione and DTT (Fig. 2f and Supplementary Fig. 3g). This is consistent with the reversal of hyperoxia-induced single-channel currents by normoxia in cell-attached patches, which maintains intact cellular configuration (Supplementary Fig. 3h,i). 5-nitro-2-PDS-induced single-channel currents were suppressed by DTT but not by glutathione (Fig. 2g and Supplementary Fig. 3j). Thus, glutathione-sensitive oxidation of Cys residues is likely to be involved in TRPA1 activation by hyperoxia.

TRPA1 channel responses showed a characteristic time course: the initial gradually rising phase and the second rapidly rising phase (Fig. 2a and Supplementary Fig. 4). In whole-cell patches, the second phase was followed by a gradual decrease unlike in other measurement methods. This may be due to intracellular dialysis of the Ca2+ chelator BAPTA via the pipette solution: extracellular Ca2+ known to control TRPA1 activity by permeating into the cell26 (Supplementary Fig. 4c,e) is absorbed rapidly by BAPTA, which suppresses the global [Ca2+]i level at 30 nM and localizes [Ca2+]i elevation near the inner mouth of TRPA1. By contrast, global [Ca2+]i increases may sustain TRPA1 activity in [Ca2+]i measurements and cell-attached mode.
Cys residues responsible for hyperoxia sensing of TRPA1

We individually mutated 29 Cys residues in human TRPA1 to serines and tested the responsiveness of these mutant clones. Compared to wild-type (WT), mutants C173S, C192S, C414S, C421S, C633S, C641S, C665S, C786S, C834S and C856S showed significantly suppressed responses to hyperoxia, as well as to diallyl disulfide (10 μM) having a redox potential similar to O₂ (Supplementary Fig. 5a,b). C414S, C421S, C786S and C834S likely have deleterious effects on channel function, because they significantly suppressed responses to 100 μM 2-aminoethyl diphenylborinate (2-APB) (Supplementary Fig. 5c), which activates TRPA1 independently of Cys modification. The mutants with impaired responses to hyperoxia were further assessed by the patch clamp method at fixed membrane potentials and under a defined and optimized intracellular composition, using pipette solution containing polytriphosphate (10 mM) and Ca²⁺ (30 nM), which sensitizes activity and prevents inactivation, respectively, of TRPA1 (refs. 26, 31). Other than those non-functional mutants, C633S, C856S and the double mutant C633S·C856S showed severely suppressed hyperoxia-induced currents, suggesting that Cys633 and Cys856 are main target sites of O₂ in hyperoxia (Fig. 2h and Supplementary Fig. 5a,d). These mutants required stronger reactive disulfides such as 5-nitro-2-PDS and 4-nitrophenyl disulfide at higher concentrations for full activation compared to WT (Supplementary Fig. 5e–h). TRPA1 responses to the membrane-impermeable reactive disulfide 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) were induced only in the presence of a detergent F-127, and were suppressed significantly by C633S and C856S mutations (Supplementary Fig. 6a,b), supporting the cytoplasmic disposition of Cys633 and Cys856. Incorporation of DTNB-2Bio, the DTNB derivative with two biotin groups attached for detection, into green fluorescent
protein-tagged TRPA1 (GFP-TRPA1) was abolished by C633S but not by C856S (Fig. 2i and Supplementary Fig. 6c). Notably, cotransfection of WT with the C633S·C856S construct at the same amount nearly abolished hyperoxia-induced but enhanced 2-APB-induced responses (Supplementary Fig. 7), raising a possibility that all four TRPA1 subunit proteins of tetrameric channel complexes\textsuperscript{11,12} have to carry oxidizable Cys633 and/or Cys856 in responding to hyperoxia. These findings suggest that the free sulphydryls of Cys633 and Cys856 act as nucleophiles to directly attack electrophiles such as O\textsubscript{2} and reactive disulfides, and this oxidative modification is maintained for Cys633 in TRPA1 activation (Fig. 1b).

Our single-channel recording in cell-excised membrane patches suggests a role of glutathione in regulation of TRPA1 activation by hyperoxia (Fig. 2f and Supplementary Fig. 3g). In fact, glutathionylation of TRPA1 detected in normoxia was augmented in hyperoxia after 5 min, but became undetectable after 20 min (Supplementary Fig. 8a). C633S·C856S disrupted glutathionylation, implying changes in oxidative modifications at Cys633 and Cys856 during hyperoxia in terms of glutathione sensitivity. Interestingly, when glutathione was perfused via patch pipette solution, hyperoxia-induced whole-cell currents were nearly abolished for those Cys mutants, which showed impaired responses to hyperoxia in [Ca\textsuperscript{2+}]\textsubscript{i} measurements but intact responses to 2-APB (Supplementary Fig. 8b; compare to Fig. 2h). Thus, Cys173, Cys192, Cys641, Cys665, Cys786 and Cys834 may protect O\textsubscript{2} reactivity of Cys633 and Cys856 from glutathione, exerting an antioxidant action to reverse TRPA1 activation by O\textsubscript{2}.

Central roles of PHDs in hypoxia sensing of TRPA1
Strikingly, Cys oxidation is not the only mechanism that underlies O₂ sensing in TRPA1 channels. Indeed, hypoxic solutions prepared by bubbling with N₂ gas induced robust TRPA1 responses; TRPA1 activation showed an inverted bell-shaped O₂-dependency curve with a minimum at the PO₂ of 137 mmHg (18%), which is slightly below the atmospheric PO₂ of 152 mmHg (20%) (Fig. 3a,b), regardless of the presence of bicarbonate/CO₂ (Supplementary Fig. 9a). Such O₂-dependency enabled TRPA1 to respond to subtle changes (from 18% to 20% O₂) in the physiological range of PO₂ at sea level (Supplementary Fig. 9b) (see also DISCUSSION below). Hypoxia-induced TRPA1 responses were suppressed by AP-18 and omission of extracellular Ca²⁺, indicating that TRPA1 mediates Ca²⁺ entry in response to hypoxia (Supplementary Fig. 9c,d). Other redox-sensitive TRPs failed to response to hypoxia, except for TRPM7 causing marginal responses¹⁶ (Fig. 3a). Hypoxia dramatically increased whole cell TRPA1 currents inhibited by AP-18 (Fig. 3c–e and Supplementary Fig. 9e,f). Hypoxia-activated TRPA1 currents showed E_{rev} characteristic of nonselective cation channels (E_{rev} = 1.7 ± 0.6 mV (n = 34)). Single-channel currents were not enhanced by hypoxic solution in excised inside-out patches (Supplementary Fig. 9g,h), suggesting intracellular components required for TRPA1 activation in hypoxia.

The PHD family is comprised of subtypes PHD1, PHD2 and PHD3, which are central to hypoxia-sensing pathways leading to hypoxia inducible factor-1α (HIF-1α) activation as a result of their absolute requirement upon O₂ as a cofactor for enzymatic activity³²,³³. Importantly, because Michaelis constant (K_m) values of PHDs for O₂, representing the substrate concentration at which half of the enzyme active sites are occupied, are close to the atmospheric O₂ concentration, physiological reductions in O₂ concentration result in decreased levels of protein hydroxylation by PHDs.
Interestingly, alignment of the consensus sequences for the prolyl hydroxylation motif\textsuperscript{32,33} with the amino-acid sequence of TRPA1 revealed conservation for flanking amino acid residues of Pro394 in the N-terminal cytoplasmic ankyrin repeat of TRPA1 (Fig. 4a). When PHD2, the ubiquitous PHD isoform\textsuperscript{32,33}, was coincubated with the TRPA1(386–405) peptide substrate which includes the motif, mass spectrometry analysis of the resultant peptide product showed a mass increase of 16 daltons, consistent with hydroxylation of Pro residues (Fig. 4b). This was undetectable in TRPA1(386–405) incubated with a pan-hydroxylase inhibitor dimethyloxalylglycine (DMOG), TRPA1(386–405)P394A carrying alanine substitution for Pro394, and TRPA1(983–1002) (Fig. 4b and Supplementary Fig. 10a,b). Prolyl hydroxylation detected using antibody against a synthetic TRPA1 subfragment carrying hydroxylated Pro394 was suppressed by DMOG, hypoxia and the P394A mutation (Fig. 4c and Supplementary Fig. 10c,d). Furthermore, GFP-TRPA1 showed coimmunoprecipitation with PHD1–3-Flag, which was reduced in GFP-P394A (Fig. 4d and Supplementary Fig. 10e). Interaction between native PHD2 and TRPA1 proteins was confirmed by coimmunoprecipitation using mouse dorsal root ganglia (DRG) cell extract (Fig. 4e and Supplementary Fig. 11).

Recombinant TRPA1 channels were activated by inhibition of endogenous PHDs by DMOG in HEK cells (Fig. 5a,b and Supplementary Fig. 12). This DMOG-induced TRPA1 activity was suppressed by intracellular application of purified PHD2 through patch pipette solution (Fig. 5b–d), suggesting that an increase of absolute quantity of PHD2 free from DMOG inhibition inhibits TRPA1. Overexpression of PHD2 suppressed TRPA1 responses to mild hypoxia with PO\textsubscript{2} at 14% (Supplementary Fig. 13). Furthermore, overexpression of catalytically dead mutants
for PHD1 (PHD1-Mut), PHD2 (PHD2-Mut) and PHD3 (PHD3-Mut), which showed coimmunoprecipitation with GFP-TRPA1, elevated basal [Ca\(^{2+}\)], indicative of constitutive TRPA1 activation, and abolished TRPA1 responses to hypoxia and DMOG (Fig. 5e,f and Supplementary Fig. 14a–d). By contrast, PHD-Muts failed to affect TRPA1 responses to both allyl isothiocyanate (AITC) and hyperoxia (Supplementary Fig. 14e,f). Cotransfection of siRNAs for PHD1–3 abrogated TRPA1 responses to hypoxia but not to hyperoxia (Fig. 5g and Supplementary Fig. 15). P394A showed significantly elevated basal activity, and abrogated responses to hypoxia and DMOG but not to AITC and hyperoxia (Fig. 5h and Supplementary Fig. 16). These results suggest that hydroxylation of Pro394 by PHDs inhibits TRPA1 channels in normoxia, while a decrease in O\(_2\) concentration diminishes PHD activity, to relieve TRPA1 from inhibition, leading to channel activation in hypoxia.

On the other hand, hyperoxia-induced TRPA1 activation was observed even in the presence of excess intracellular PHD2 applied through patch pipette solution (Supplementary Fig. 17a–d). Single channel behavior of WT TRPA1 and the P394A mutant in hypoxia was compared with that in hyperoxia or normoxia using cell-attached patch clamp method (Supplementary Fig. 17e,f and Supplementary Table 1). Open times were fitted to three components with different ranges of time constants: \(\tau_1 = 0.53–3.04\) ms, \(\tau_2 = 3.35–22.01\) ms and \(\tau_3 = 31.01–136.12\) ms). Interestingly, hypoxia significantly enhanced opening events with \(\tau_2\) compared to normoxia in WT, whereas opening events with \(\tau_1\) and \(\tau_2\) were observed similarly in normoxia and hypoxia for P394A, suggesting that hydroxylation of Pro394 prevents TRPA1 from dwelling in a state with longer open time (\(\tau_2\)). Hyperoxia, however, induced open states with \(\tau_1\), \(\tau_2\) and \(\tau_3\) regardless of hydroxylation at Pro394, which is supported by responses to
hyperoxia in the presence of DMOG (Supplementary Fig. 17g). Therefore, it is quite conceivable that direct O$_2$ action overrides the PHD-mediated inhibition via prominent sensitivity of TRPA1 to Cys-mediated oxidation in hyperoxia.

Protein translocation has been reported to regulate activation of TRP channels, including TRPA1 (refs. 34,35). GFP-TRPA1 showed discontinuous overlaps with the plasmamembrane marker DsRed-monomer-F in confocal laser microscopy (Supplementary Fig. 18a). Evanescent wave microscopy, which illuminates only the subcellular area from the surface of the cell to a depth of less than 100 nm by total internal reflection fluorescence (TIRF), revealed instantaneous augmentation of GFP-TRPA1 near the cell surface upon hypoxia (Fig. 5i,j and Supplementary Fig. 18b). Chlorpromazine (CPZ) or dynasore, which are inhibitors for clathrin-dependent endocytosis, and brefeldin A, the inhibitor for ER-Golgi-dependent exocytotic protein translocation, revealed GFP-TRPA1 insertion and internalization, respectively, in the plasmamembrane (Fig. 5k and Supplementary Fig. 18c–e). The observed internalization and insertion of GFP-TRPA1 were unaffected by hypoxia. In this protocol, GFP-TRPA1 is most likely in a hydroxylated form in the plasmamembrane after inhibition of insertion by brefeldin A. Interestingly, DMOG enhanced plasmamembrane localization of GFP-TRPA1 (Supplementary Fig. 18f). The data suggests that internalization of TRPA1 with unmodified Pro394 is subjected to deceleration in hypoxia, because insertion was unaffected in hypoxia (Fig. 5k and Supplementary Fig. 18d,e). Filipin, the inhibitor of caveolae-mediated endocytosis, failed to affect localization levels of GFP-TRPA1 in the plasmamembrane (Supplementary Fig. 18g). Cell surface labeling experiments support these microscopy data (Supplementary Fig. 18h,i). In $[\text{Ca}^{2+}]$, measurements, brefeldin A
suppressed TRPA1 responses to hypoxia, while CPZ and dynasore but not filipin induced TRPA1 responses in normoxia (Fig. 5l,m and Supplementary Fig. 18j–m). Thus, turnover of TRPA1 proteins in the plasmamembrane is actively maintained to regulate TRPA1 activity in normoxia and hypoxia, suggesting that insertion of unmodified TRPA1 and internalization of hydroxylated TRPA1 underlie the relief of TRPA1 channel activity from PHD-mediated inhibition.

TRPA1 responses were sustained after induced conditions of hypoxia (10% O2 for 19 min) when switched back to normoxia (20% O2) (Supplementary Fig. 19a). NAC or DTT but not L-NAME reversed the TRPA1 response during readministration of normoxia. Hypoxia-induced TRPA1 responses were also reversed in normoxia after hypoxia for a shorter time period (5 min) or after mild hypoxia at a higher O2 concentration (14%) (Supplementary Fig. 19b,c). The observed reversibility was unaffected by NAC, DTT or L-NAME. Washout of DMOG partially reversed TRPA1 responses (Supplementary Fig. 19d), indicating that restoration of PHD activity inhibits TRPA1. Thus, time length and degree of preceding hypoxia and ROS are critical to induce irreversibility of TRPA1 activation in normoxia.

Receptor stimulation via phospholipase C (PLC) activates TRPA1 (ref. 36). However, PLC inhibitor edelfosine (ET) failed to affect responses of TRPA1 to hypoxia and hyperoxia (Supplementary Fig. 20a,b), suggesting that PLC activity is not required for O2-mediated activation of TRPA1. Activation of TRPA1 through receptor stimulation by bradykinin at low concentrations (1–100 nM) was significantly enhanced by hypoxia (Supplementary Fig. 20c). P394A showed bradykinin-induced responses similar to those observed in hypoxia (Supplementary Fig. 20c,d), suggesting that prolyl hydroxylation is not necessary for activation of TRPA1 via PLC-mediated
receptor signaling pathways. Thus, PHD and PLC-mediated receptor signaling pathways may operate independently, but can act synergistically to better adapt TRPA1 activation by humoral factor agonists to hypoxia.

**Hyperoxia and hypoxia excite peripheral nerves via TRPA1**

As polymodal receptors for noxious stimuli at nerve endings in sensory and vagal afferent neurons\textsuperscript{21,26}, TRPA1 senses endogenous algesic substances and environmental irritants\textsuperscript{17,21,24,27}. Notably, TRPA1 protein was identified by immunohistochemistry in a subset of nodose ganglion neurons projecting to the lung and airway in mice\textsuperscript{27} (Supplementary Fig. 21a). We tested whether TRPA1 detects changes in O\textsubscript{2} availability in the mouse nodose ganglion (Fig. 6a–h), in which cell bodies of vagal nerves are located and PHDs are abundantly expressed (Supplementary Fig. 21b). Robust [Ca\textsuperscript{2+}]\textsubscript{i} increases were induced by hyperoxia and hypoxia in a subset of cells responsive to 60 mM KCl, which activates voltage-dependent Ca\textsuperscript{2+} channels in neurons regardless of the presence of bicarbonate/CO\textsubscript{2} (Fig. 6a,b and Supplementary Fig. 21c–f). The majority of hyperoxia- and hypoxia-responsive cells responded to the C-fiber-specific sensory irritant capsaicin\textsuperscript{27} (Fig. 6a,b, Supplementary Fig. 21c,d and Supplementary Table 2), suggesting that a TRPA1-expressing subset of C-fibers senses O\textsubscript{2} availability changes. Whole-cell current amplitudes were also augmented by hyperoxia and hypoxia in nodose neurons (Fig. 6c,f and Supplementary Fig. 21g,h). Hyperoxia- and hypoxia-induced currents showed E\textsubscript{rev} characteristic of nonselective cation channels (hyperoxia; \( E_{\text{rev}} = 0.9 \pm 1.0 \) mV \((n = 31)\), hypoxia; \( E_{\text{rev}} = -0.8 \pm 1.3 \) mV \((n = 15)\)). Ca\textsuperscript{2+} responses and ionic currents induced by hyperoxia and hypoxia were substantially blocked by AP-18 in nodose neurons (Supplementary Fig. 21c–h).
Importantly, nodose neurons prepared from *Trpa1* knockout (KO) mice were largely unresponsive to hyperoxia and hypoxia, but they retained responsiveness to capsaicin and 60 mM KCl (Fig. 6a–h). In *Trpa1* KO mice, it is interesting to note that statistically significant impairments were observed for responses to hypoxia (10, 13 and 15% O₂) when capsaicin-sensitive nodose neurons were selected for analyses, but only for responses to relative mild hypoxia (15% O₂) when all nodose neurons were subjected to analyses (Supplementary Fig. 21i,j). DRG neurons from WT mice exhibited robust responses to hyperoxia and hypoxia via Ca²⁺ influx blocked by AP-18, while those from *Trpa1* KO mice exhibited impaired responses to hyperoxia and hypoxia (Fig. 6i, Supplementary Fig. 22a–h and Supplementary Table 2). *Trpa1* KO also abrogated ability of DRG to respond to subtle O₂ changes (from 18% to 20%) within the physiological range at sea level (Supplementary Fig. 22i). These results suggest that hyperoxia and hypoxia activate native TRPA1 channels in mouse vagal neurons and sensory neurons.

To establish the role of PHDs in activation of native TRPA1 by hypoxia, we employed siRNA strategy for PHD1–3 and *Phd* KO mice. Abundance of expression was PHD2 > PHD1 > PHD3 in DRG and nodose neurons (Supplementary Fig. 23a). Antibody to TRPA1 hydroxylated at Pro394 revealed immunostaining localized near the plasmamembrane compared to the total population of TRPA1 proteins in DRG neurons (Supplementary Fig. 23b). Cotransfection of siRNAs for PHD1–3 elevated basal [Ca²⁺]ᵢ levels and abolished responses of capsaicin-sensitive DRG neurons to hypoxia (Fig. 6j and Supplementary Fig. 23c,d). In capsaicin-sensitive DRG neurons isolated from *Phd1* and *Phd3* KO mice, basal [Ca²⁺]ᵢ levels were elevated and responses to hypoxia were suppressed, while responses to hyperoxia were intact compared with WT
mice (Fig. 6k and Supplementary Fig. 23e,f). Thus, PHDs are essential for TRPA1 responses to hypoxia.

We evaluated in vivo TRPA1 responses that regulate vagal activities under systemic hyperoxia and hypoxia. Exposure of WT mice to hyperoxic (100% O₂) or hypoxic gas (10, 13 and 15% O₂) via a tracheal cannula significantly enhanced discharges of afferents in the cervical vagal trunk and in the superior laryngeal vagal branch innervating the mucosa of the larynx as shown by multifiber neurogram (Fig. 7 and Supplementary Figs. 24 and 25). In Trpa1 KO mice, the enhancement of nerve discharges by hyperoxia and mild hypoxia (15% O₂) was abolished, while that by severe hypoxia (10 and 13% O₂) was delayed. Basal activity in normoxia (20% O₂) of vagal afferents in Trpa1 KO mice was indistinguishable from that in WT mice. Thus, TRPA1 is essential for regulation of discharges in vagal afferents in hyperoxia and mild hypoxia (15% O₂) but plays less critical roles in severe hypoxia (10 or 13% O₂), suggesting that other hypoxia-sensitive channels such as K⁺ channels contribute to vagal discharges in severe hypoxia²⁴.
DISCUSSION

The present study reveals critical roles played by TRPA1 in O2 sensing of vagal and sensory neurons. TRPA1 is activated by hyperoxia and hypoxia through mechanisms involving Cys oxidation and Pro hydroxylation, respectively.

Our results suggest that free Cys sulfhydryls are the key to O2-sensing ability of TRPA1 in hyperoxia. A high reactivity of Cys633 and Cys856 as electron donors enables TRPA1 to respond to O2, which is a relatively poor electron acceptor, by overriding the inhibition by Pro hydroxylation to activate TRPA1. Contribution of multiple Cys residues to oxidation sensitivity of protein function has also been reported for other proteins41–43. Because sensitivities to O2 and reactive disulfides are reduced efficiently and similarly in single mutants C633S and C856S and are nearly abolished in the double mutant C633S·C856S, these Cys residues are more likely to contribute to the same molecular determinant for O2 sensitivity than to separate sites with characteristic oxidation sensitivities. Importantly, to activate TRPA1, our mutation studies suggest that relatively weak electron acceptors such as diallyl disulfide and 4-tolyl disulfide target the same Cys residues as O2, while reactive disulfides such as 4-nitrophenyl disulfide and 5-nitro-2-PDS with higher potency also act on additional Cys residues (Supplementary Fig. 5a,b,f–h). For sensing α,β-unsaturated carbonyls, multiple but different Cys residues have been identified18–20. The difference is attributable to different stability of immediate reaction products: O2 may lead to disulfide bond formation via an unstable oxidized product (Fig. 8), while α,β-unsaturated carbonyls give stable Michael addition adducts.

Reversibility of TRPA1 activation in normoxia depends on time and degree of preceding hyperoxia (Supplementary Fig. 1). This together with sensitivity to
inhibitors for ROS and antioxidants suggests that a transition of oxidation state is induced in Cys633 and Cys856 (Fig. 8): sulfhydryl groups of Cys633 and Cys856 are initially oxidized by hyperoxia into glutathione-sensitive sulfenic acid, which are subsequently converted into relatively stable, glutathione-insensitive disulfide bonds. The dominant inhibitory effect of the C633S·C856S mutant implies that oxidation is introduced in all four subunits of the TRPA1 channel complex for activation, although it is unclear whether disulfide bonds are formed intermolecularly or intramolecularly by TRPA1 proteins. Our data also suggest extracellular Ca\textsuperscript{2+} plays an important role in the later phase of TRPA1 activation.

Multiple stimuli activate TRPA1\textsuperscript{17–24}. Our preliminary experiments have suggested that cold-sensitive activation of TRPA1 is in part due to the well known effect of cold temperature to enhance dissolution of O\textsubscript{2} (data not shown). Zn\textsuperscript{2+}, known well for binding to Cys residues, activates TRPA1\textsuperscript{45}. Interestingly, in TRPV1, Cys S-nitrosylation significantly enhances sensitivity to protons\textsuperscript{14}, which also activate TRPA1\textsuperscript{20,46}. Therefore, redox-sensitivity may be coupled with other modes of sensitivity to finely regulate TRPA1, depending on the environment.

In the adaptation to hypoxia, elevated levels of HIF-1\textalpha increase red blood cell mass and stimulate new blood vessel growth\textsuperscript{33,47}. Prolyl hydroxylation of HIF-1\textalpha by PHD1–3, which utilizes molecular O\textsubscript{2} and 2-oxoglutarate as substrates, and subsequent ubiquitination and targeting of HIF-1\textalpha to the 26S proteasome for degradation control HIF-1\textalpha levels\textsuperscript{32}. Notably, TRPA1 represents the first example of a channel where the activation is controlled by prolyl hydroxylation. PHDs hydroxylate conserved Pro394 within the N-terminus ankyrin repeat of TRPA1 and inhibit TRPA1 channels in normoxia, while a decrease in O\textsubscript{2} concentrations diminishes PHD activity and relieves
TRPA1 from inhibition, leading to its activation in hypoxia. Importantly, O₂ dependence of enzymatic activities of PHDs is consistent with the major role played by PHDs in inhibiting TRPA1 activity: normoxic O₂ concentration of 200 μM (152 mmHg) is comparable to $K_m$ values of 230–250 μM (175–190 mmHg) by O₂ concentrations for PHDs. The relief can be achieved by rapid insertion of unmodified TRPA1 proteins to the plasmamembrane or by dehydroxylation through an unidentified molecular mechanism. Our data demonstrate that turnover of TRPA1 proteins is maintained in the plasmamembrane and that inhibition of PHDs by DMOG decelerates their internalization (Fig. 5i–m and Supplementary Fig. 18), supporting that insertion of unmodified TRPA1 proteins contribute to instantaneous TRPA1 responses to hypoxia. Different gating behaviours of single TRPA1 channels observed in normoxia and hypoxia (Supplementary Fig. 17e,f and Supplementary Table 1) is more compatible to the TRPA1 channel activation via dehydroxylation in hypoxia, although this data does not necessarily contradict the translocation scenario, in which TRPA1 inserted in the plasmamembrane remain dehydroxylated in hypoxia but immediately become hydroxylated in normoxia. For triggering hypoxic inhibition of K⁺ channels, the importance of mitochondrial function has been suggested, because mitochondria are the largest consumers of O₂ and control cytosolic O₂ concentration. Depletion of intracellular ATP and carbon monoxide production by hemeoxygenase have been also suggested in K⁺ channel inhibition. These mechanisms can also contribute to hypoxia-induced activation of TRPA1.

It is known that hypoxia is involved in some pathophysiological reactions such as nociceptive pain in sensory neurons. For example, chest pain occurs when the heart is exposed to hypoxia because of coronary artery blockade, disease or inhalation.
of hypoxic gas, while surgical dissection of cardiac sensory neurons relieves angina\textsuperscript{48}. We find that hypoxia potently excites DRG neurons in a TRPA1-dependent manner (\textbf{Fig. 6i} and \textbf{Supplementary Fig. 22}), suggesting that hypoxia-activated TRPA1 together with other mechanisms such as K$^+$ leak\textsuperscript{7} may underlie chest pain via cardiac sensory neurons.

Vagal nerve conveys sensory information about the state of the body’s organs to the central nervous system besides output to the various organs in the body. Enhanced discharges in vagal afferents induce respiratory, cardiac and vascular responses\textsuperscript{8,48,49}. In the airway and lungs, chemicals encountered are detected by bronchopulmonary C-fibers and superior laryngeal nerve, a branch of vagal nerve\textsuperscript{49}. Recently, TRPA1 has been shown to sense environmental irritants, initiating defensive reflexes such as coughing and respiratory depression in bronchopulmonary C-fibers\textsuperscript{17,24,27}. In this context, TRPA1 expressed in the airway and lungs may detect O$_2$ toxicity at the frontline of defense against noxious chemical challenges.

Weather records\textsuperscript{50} suggest that atmospheric PO$_2$ at sea level fluctuates in a range of between approximately 170 mmHg and 137 mmHg. Because our O$_2$ dependence data reveals that minimal TRPA1 activity at 137 mmHg is approximately 30\% of maximum levels at 170 mmHg (\textbf{Fig. 3b}), so-called “normoxia” can be hyperoxic for mammalian TRPA1 channels, as reported in the context of O$_2$ avoidance in \textit{Caenorhabditis elegans}\textsuperscript{9} and insects\textsuperscript{10}.
METHODS

Electrochemical characterization of reactive disulfides. Half-wave potential ($E_{1/2}$) values of reactive disulfides and $O_2$ were determined by rotating disk-electrode voltammetry. The measurements were carried out with a BAS 100B (Bioanalytical Systems) and a RDE-3 rotating disk electrode (Nikko Keisoku) with a SC-5 controller (Nikko Keisoku). Reactive disulfides and $O_2$ were dissolved in dehydrated DMSO (SIGMA). Reactive disulfides were then bubbled with Ar gas through the solution. The voltammetry was measured in 0.1 M $Bu_4NBF_4$/DMSO using a glassy carbon working electrode, a platinum wire counter electrode and an Ag/Ag$^+$ reference electrode at 2,500 rpm. $E_{1/2}$ is an empirical value that is defined as the midpoint of the rise of current in voltammogram and, as such, it differs from the standard reduction potential ($E^o$) of the compound. As previously reported$^{28}$, the relative nature of $E_{1/2}$ values presented in this study was deemed to be a relevant descriptor of the redox potential of these agents.

Cell cultures and cDNA expression. HEK293 and HEK293T cells were cultured similarly as previously described$^{14}$, and were cotransfected with recombinant plasmids and pEGFP-F (Clontech) as a transfection marker using SuperFect Transfection Reagent (Qiagen) and Lipofectamine 2000 (Invitrogen), respectively. $[Ca^{2+}]_i$ measurement, TIRF microscopy, DTNB-2Bio labeling assay and immunoprecipitation were performed 32–48 h after transfection$^{14}$. Electrophysiological measurements were performed 32–72 h after transfection.

$[Ca^{2+}]_i$ measurements. $[Ca^{2+}]_i$ was measured as described previously$^{20}$. Hyperoxic
solution was achieved by bubbling with 22, 24, 26, 28, 30, 32, 34, 36, 80 or 100% O₂ (balanced with N₂) gas for at least 20 min before cell perfusion. Hypoxic solution was achieved by bubbling with 0, 8, 10, 12, 14, 16 or 18% O₂ (balanced with N₂) gas at least 20 min before cell perfusion and by blowing the respective gas over the surface of the experimental chamber using a modified dish. The concentration of dissolved O₂ in the chamber solution was determined with an O₂ microelectrode (InLab 605; METTLER TOLEDO). pH of 7.4 was maintained in the buffers after bubbling with N₂ and/or O₂ gas. Unless otherwise indicated, dissolved PO₂ measured in hypoxic, normoxic and hyperoxic solutions were 10% O₂, 20% O₂ and 86% O₂, respectively. See Supplementary Methods for more detailed description.

**Electrophysiology.** Currents from cells were recorded at room temperature (22–25°C) using patch-clamp techniques. Ramp pulses were applied every 5 or 10 sec from −100 mV to +100 mV or from +100 mV to −100 mV at a speed of 1.1 mV ms⁻¹ from a V₉ of 0 mV. For the conventional whole-cell recordings, the external solution contained (in mM) 100 NaCl, 2 Ca-gluconate and 10 HEPES (pH 7.4 adjusted with NaOH, and osmolality adjusted to 320 mmol kg⁻¹ with D-mannitol). The pipette solution contained (in mM) 100 Cs-aspartate, 5 BAPTA, 1.4 Ca-gluconate, 2 Na₂ATP, 2 MgSO₄, 1 MgCl₂, 10 HEPES and 10 Na₅P₃O₁₀ (pH 7.4 adjusted with CsOH, and osmolality adjusted to 320 mmol kg⁻¹ with D-mannitol; 30 nM calculated free Ca²⁺). For inside-out patch recordings, the external solution contained (in mM) 50 Cs-aspartate, 50 CsCl, 1 MgCl₂, 1 CaCl₂, 10 EGTA, 10 Na₅P₃O₁₀ and 10 HEPES (pH 7.4 adjusted with CsOH, and osmolality adjusted to 300 mmol kg⁻¹ with D-mannitol). The pipette solution contained (in mM) 100 CsCl, 1 MgCl₂, 1 EGTA and 10 HEPES (pH 7.4
adjusted with CsOH, and osmolality adjusted to 300 mmol kg\(^{-1}\) with d-mannitol). pH of 7.4 was maintained in the buffers after bubbling with N\(_2\) and/or O\(_2\) gas. Unless otherwise indicated, dissolved PO\(_2\) measured in hypoxic, normoxic and hyperoxic solutions were 10% O\(_2\), 20% O\(_2\) and 86% O\(_2\), respectively. See Supplementary Methods for more detailed description.

**Peptide hydroxylation assay.** Forty µM of synthetic peptide, PYGLKNLRPEFMQMQQIKEL corresponding to TRPA1(386–405) was incubated with 2 µM of purified PHD2 in a final volume of 10 µl in buffer solution (pH 7.4 adjusted with CsOH) containing (in mM) 50 CsCl, 50 CsOH, 50 L-aspartic acid, 10 HEPES, 3 ascorbic acid, 0.1 FeCl\(_2\), 0.3 2-oxoglutarate, 1 DTT and 0.3 mg ml\(^{-1}\) catalase (SIGMA) at 25°C overnight. For matrix-assisted laser desorption ionization/time of flight mass spectrometric analyses, α-cyano-4-hydroxycinnamic acid (CHCA) solution was prepared in acetonitrile/water containing 0.1% trifluoroacetic acid [50:50 (v/v)] at a concentration of 10 mg ml\(^{-1}\). Assay solution (1 µl) was mixed with CHCA solution (5 µl) and spotted onto the target plate. Mass spectrometric analyses of the samples were performed with autoflex III (Bruker Daltonics).

**Isolation of mouse nodose ganglion and DRG neurons.** Nodose ganglion and DRG neurons were prepared from adult WT, Trpa1 KO, Phd1 KO and Phd3 KO mice as described previously\(^{20}\). The isolated cells were subjected to [Ca\(^{2+}\)]\(_i\) and electrophysiological measurements 8–24 h after plating.

**Recording of multifiber vagal afferent discharges.** Mice were anesthetized with
intraperitoneal injection of urethane, artificially ventilated through L-shaped tracheal cannula perforated at the corner and then paralyzed with 0.15 mg kg\(^{-1}\) panchronium bromide. To record vagal afferent activities, the distal cut end was placed on a pair of silver hook electrodes. Multifiber vagal nerve discharges were amplified (10,000×, AVB-8, Nihon Kohden), full-wave rectified, leaky integrated (time constant = 1 sec, EI-601G, Nihon Kohden) and stored in a hard disk through an analog-to-digital converter (PowerLab, ADInstrument) together with original nerve discharges. Rectified and integrated vagal nerve activities were subtracted by noise level to obtain each nerve activity. Normoxic and hyperoxic gas challenges lasted 40 sec, and hypoxic challenge lasted 30 sec, respectively. See Supplementary Methods for more detailed description.

**Statistical analyses.** All data are expressed as means ± s.e.m.. We accumulated the data for each condition from at least three independent experiments. The statistical analyses were performed using the Student’s \(t\)-test. A value of \(P < 0.05\) was considered significant.

**Additional methods.** Further details of materials, cDNA cloning and plasmid construction, synthesis of AP-18, DTNB-2Bio labeling assay, expression and purification of recombinant PHD2, coimmunoprecipitation, TIRF microscopy, siRNA suppression, cell surface labeling experiment, mice, RNA isolation and RT-PCR, immunohistochemistry, and semi-quantitative RT-PCR analysis and recording of superior laryngeal discharges are in Supplementary Methods online.
Acknowledgements
We thank D. Julius, T. Yoshida and M. Wakamori for experimental advice, T. Miki and J. Ikenouchi for helpful discussions and T. Morii and I. Hamachi for their support in DTNB-2Bio synthesis. We are also grateful to T. Niidome, H. Shirakawa and T. Nakagawa for their support in mouse experiments.

Author contributions
N.T., S. Kiyonaka, Y. Mizuno and Y. Mori initiated and designed the project. N.T., S. Kiyonaka, T. Numata, D.K., Y. Mizuno, S.Y., S.N., T.O., S. Kaneko and T. Nokami performed experiments and analysed data. T.K. supervised in vivo studies. S.S. and J.Y. supervised the electrochemical experiments. E.K. and P.C. established Phd1 KO and Phd3 KO mouse lines subjected to the experiment. N.T., T.K., S. Kiyonaka, T. Numata, D.K. and Y. Mori wrote the manuscript. Y. Mori directed the research. All authors discussed and commented on the manuscript.

Competing financial interests
The authors declare no competing financial interests.

Additional information
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References


Figure legend

Figure 1. The prominent oxidation reactivity confers O₂ sensitivity on TRPA1. (a) Redox potentials (E₁/₂) of reactive disulfides determined by rotating disk-electrode voltammetry. E₁/₂ values of 5 mM reactive disulfides dissolved in dehydrated DMSO are defined as the midpoint of the rise of current in voltammogram. Compounds with less negative redox potentials have stronger electrophilicity. PDS stands for pyridyl disulfide. (b) A chemical mechanism underlying the action of a reactive disulfide compound, 5-nitro-2-PDS, on Cys sulfhydryls. (c) Oxidation sensitivity of TRP channels. Plots of maximum [Ca²⁺]ᵢ rises (Δ[Ca²⁺]ᵢ) induced by 10 μM reactive disulfides (see Figure 1a) in HEK293 cells expressing redox-sensitive TRP channels against redox potentials of respective substances (left) (n = 13–33). Data points are fit to straight lines by the least squares method. Threshold redox potentials for activation of respective TRP channels (right). (d) [Ca²⁺]ᵢ rises evoked by hyperoxic solution (86% O₂) in HEK293 cells expressing redox-sensitive TRP channels. Averaged time courses and Δ[Ca²⁺]ᵢ (n = 16–40). ***P < 0.001 compared to vector. (e) The relationship between PO₂ and maximum Ca²⁺ responses mediated by TRPA1 (n = 24–41). In examining in vitro cellular response to O₂, dissolved PO₂ measured in normoxic (left arrowhead) and hyperoxic solutions (right arrowhead) are 152 ± 1 mmHg (20% O₂) and 655 ± 32 mmHg (86% O₂), respectively. Data points are mean ± s.e.m.

Figure 2. O₂ directly activates TRPA1 through Cys modification. (a,b) Representative time courses of whole cell currents recorded at +100 and −100 mV under ramp clamp in hyperoxia in TRPA1- (a) or vector-transfected HEK293T cells (b). Corresponding I-V relationships at the time points 1 and 2, and those of evoked currents
(2–1) are shown. (e) Peak current densities in normoxia and hyperoxia ($n = 6–18$). ***$P < 0.001$. (d) Single channel activities evoked by hyperoxia at –60 mV in inside-out patches excised from TRPA1- or vector-transfected HEK293T cells. Time-expanded current traces before (1) and during (2) application are also shown. Arrowheads represent the closed state. (e) Averages of $N P_O$ ($N$, number of channels; $P_O$, open probability) representing single TRPA1 channel activity in normoxia and hyperoxia ($n = 20–35$). (f,g) Percentage suppression of hyperoxia-induced (f) or 10 µM 5-nitro-2-PDS-induced $N P_O$ of TRPA1 (g) by 5 mM reduced glutathione and 10 mM DTT at –60 mV in inside-out patches in HEK293 cells ($n = 5–23$). Representative current traces are in Supplementary Figure 3g,j. ***$P < 0.001$ compared to cells maintained in hyperoxia or 5-nitro-2-PDS without the agents. (h) Changes in current density evoked by hyperoxia ($\Delta I$) for TRPA1 Cys mutants ($n = 5–18$). *$P < 0.05$ and **$P < 0.01$ compared to WT. Data points are mean ± s.e.m. (i) Effects of mutations C633S and C856S on DTNB-2Bio incorporation into TRPA1 proteins. Western blotting (WB) of total lysates indicates comparable TRPA1 expression (lysate). See Supplementary Figure 6c for full gels and blots.

**Figure 3. Hypoxia activates TRPA1.** (a) $[\text{Ca}^{2+}]_i$ rises evoked by hypoxic solution in HEK293 cells expressing redox-sensitive TRP channels. Dissolved $P_O_2$ measured in the hypoxic solution is $79 \pm 3$ mmHg (10% O$_2$). Averaged time courses and $\Delta[\text{Ca}^{2+}]_i$ ($n = 20–37$). *$P < 0.05$ and ***$P < 0.001$ compared to vector. (b) The relationship between $P_O_2$ and $\Delta[\text{Ca}^{2+}]_i$ mediated by TRPA1 ($n = 21–41$). Arrowheads indicate, from left to right, $P_O_2$ values for hypoxia, normoxia and hyperoxia. (c,d) Representative time courses of outward and inward whole cell currents in hypoxic
solution in HEK293T cells transfected with TRPA1 (c) or vector (d). Corresponding
$I-V$ relationships at the time points 1 and 2, and those of evoked currents (2–1) are also
shown.  (e) Peak current densities at $-100$ mV in normoxic and hypoxic solutions ($n =
4–8$).  ***$P < 0.001$.  Data points are mean ± s.e.m..

Figure 4.  **TRPA1 protein is susceptible to Pro hydroxylation by PHDs.**  (a)
Alignment of amino acid residues 384–398 of human TRPA1 with conserved prolyl
hydroxylation motif within N-terminal and C-terminal $O_2$-dependent degradation
domain (NODD and CODD, respectively) of HIF-1α and HIF-2α.  (b) Mass
spectrometry analysis of TRPA1 peptides incubated with purified recombinant PHD2.
Mass increase of 16 daltons by PHD2 in the TRPA1(386–405) peptide is abolished by 3
mM DMOG.  (c) Hydroxylated Pro394 of TRPA1 is detectable under normoxia but is
reduced under hypoxia.  TRPA1-Flag-expressing HEK293 cells are treated with
hypoxia for 20 min or 3 mM DMOG for 3 h.  Immunoprecipitates (IP) with antibody
to Flag are subjected to WB with antibody to TRPA1(hydroxylated Pro394).  (d)
Coimmunoprecipitation of GFP-TRPA1 or GFP-P394A with PHD1–3.  IP with
antibody to Flag are subjected to WB with antibody to GFP.  (e) Coimmunoprecipitation of native TRPA1 with PHD2 from DRG extract.  In (c)–(e),
full gels and blots are available in Supplementary Figures 10d,e and 11b.

Figure 5.  **TRPA1 is activated by relief from $O_2$-dependent inhibition by Pro
hydroxylation.**  (a) TRPA1 responses to DMOG.  Averaged time courses of $[Ca^{2+}]_i$
changes evoked by DMOG or its vehicle (0.01% DMSO) and dose dependence of
average $[Ca^{2+}]_i$ rises during 1,080–1,200 sec ($n = 22–36$).  (b–d) Whole cell TRPA1
currents evoked by 300 μM DMOG with and without intracellular application of recombinant PHD2 (0.67 μM). Representative time courses and corresponding I-V relationships (b,c). Percentage increment of peak currents after DMOG administration ($n = 7–10$) (d). (e,f) TRPA1 responses to hypoxia (e) and 300 μM DMOG (f) are affected by cotransfection of PHD1-Mut, PHD2-Mut or PHD3-Mut. Basal $[Ca^{2+}]_i$ levels (e) and average $[Ca^{2+}]_i$ rises during 1,020–1,140 sec of stimulation (e,f) ($n = 13–50$). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to cotransfection with vector. (g) TRPA1 responses to hypoxia after treatment with combined siRNAs for PHDs (siPHD1&2&3). Basal $[Ca^{2+}]_i$ levels and average $[Ca^{2+}]_i$ rises during 1,020–1,140 sec of stimulation ($n = 80–147$). (h) Representative time courses of whole cell P394A currents in hypoxia. Corresponding I-V relationships. Peak current densities in normoxia ($n = 23–38$) and percentage increment of the peak current in hypoxia relative to that in normoxia ($n = 6–24$). (i,j) Cell surface expression of GFP-TRPA1 observed by TIRF microscopy is enhanced by hypoxia. In (i), representative images are shown. The bar indicates 10 μm. In (j), average fluorescence changes in TIRF images ($\Delta F/F_0$) during 680–780 sec of stimulation ($n = 12–20$). (k) Protein translocation inhibitors affect $\Delta F/F_0$. After 3 min incubation with 5 μg ml$^{-1}$ CPZ, 100 μM brefeldin A or their vehicle (0.1% DMSO), cells are exposed to hypoxia. Average $\Delta F/F_0$ measured 0–50 sec before and 500–600 sec after start of hypoxia (light blue and light red for CPZ and brefeldin A, respectively; blue and red open squares are corresponding time courses in Supplementary Figure 18d) or measured at the same timing in control experiment (blue and red for CPZ and brefeldin A, respectively; blue and red solid squares are corresponding time courses in Supplementary Figure 18d) are shown ($n = 12–16$). (l) Brefeldin A inhibits hypoxia-induced TRPA1 responses. Three hour prior to and
continuing during hypoxia, cells are incubated with 100 µM brefeldin A. \( \Delta \text{[Ca}^{2+}]_i \) is shown \((n = 27–28)\). (m) TRPA1 responses to CPZ. After 7 min incubation with 1 µg ml\(^{-1}\) CPZ or its vehicle (0.1% DMSO), cells are exposed to hypoxia. \( \Delta \text{[Ca}^{2+}]_i \) measured 0–420 sec before and after start of hypoxia (light blue; blue open squares indicate the corresponding time course in Supplementary Figure 18k) or measured at the same timing in control (blue; blue solid squares indicate the corresponding time course in Supplementary Figure 18k) are shown \((n = 17–33)\). * \( P < 0.05 \), ** \( P < 0.01 \) and *** \( P < 0.001 \). Data points are mean ± s.e.m.. Corresponding time courses for (e)–(g), (j) and (l) are in Supplementary Figures 14c,d, 15b and 18b,j.

**Figure 6.** TRPA1 mediates hyperoxia- and hypoxia-induced cationic currents in vagal and sensory neurons. (a,b) Hyperoxia- (a) and hypoxia-induced \( \text{Ca}^{2+} \) responses (b) are ablated in capsaicin-sensitive Trpa1 KO nodose ganglion neurons. Representative \( \text{Ca}^{2+} \) responses evoked in hyperoxia (a) or hypoxia (b) and by 10 µM AP-18 (0.01% DMSO), 3 µM capsaicin (0.01% DMSO) and 60 mM KCl, and \( \Delta \text{[Ca}^{2+}]_i \) at 60–1,800 (a) or 60–900 sec (b) \((n = 13–46)\). (c–h) Whole cell currents recorded in WT and Trpa1 KO nodose ganglion neurons. Representative time courses and corresponding \( I-V \) relationships in hyperoxia (c,d) and hypoxia (f,g). Current densities evoked by hyperoxia (e) and hypoxia \((n = 16–19)\) (h). (i) Ablated \( \text{Ca}^{2+} \) responses to hyperoxia and hypoxia in capsaicin-sensitive Trpa1 KO DRG neurons. \( \Delta \text{[Ca}^{2+}]_i \) during 0–1,440 sec of hyperoxic stimulation and 0–840 sec of hypoxic stimulation \((n = 12–49)\). (j) \( \text{Ca}^{2+} \) responses to hypoxia in capsaicin-sensitive DRG neurons treated with siPHD1&2&3. Basal \( \text{[Ca}^{2+}]_i \) levels and average \( \text{[Ca}^{2+}]_i \) rises during 740–840 sec of hypoxic stimulation and 0–100 sec after start of costimulation with capsaicin \((n = \) 37
16–27). (k) Ca\(^{2+}\) responses to hypoxia in capsaicin-sensitive Phd1 or Phd3 KO DRG neurons. Basal [Ca\(^{2+}\)]\(i\), levels and Δ[Ca\(^{2+}\)]\(i\), during 0–840 sec of hypoxic stimulation \((n = 22–59)\). *\(P < 0.05\), **\(P < 0.01\) and ***\(P < 0.001\). Data points are mean ± s.e.m.

Corresponding time courses for (i)–(k) are in Supplementary Figures 22e,f and 23d,e.

**Figure 7.** Defects of discharges in vagal afferents of Trpa1 KO mice under systemic hypoxia and hyperoxia. (a,b) Comparison of percentage changes in rectified and integrated vagal nerve activity in response to inhalation of hypoxic (10, 13 and 15% O\(_2\)) or hyperoxic (100% O\(_2\)) gas between WT and Trpa1 KO mice in the sustained phase (20–30 sec) (a) or in the rising phase (0–10 sec) (b) during changes of O\(_2\) availability \((n = 4–7)\). Values denote percentage changes from basal activities recorded during normoxic gas (20% O\(_2\)) exposure. Representative tracings of vagal afferent discharges and rectified and integrated vagal nerve activities are shown in Supplementary Figure 24. *\(P < 0.05\) and **\(P < 0.01\) compared to WT. Data points are mean ± s.e.m.

**Figure 8.** Molecular mechanism underlying O\(_2\)-sensing in TRPA1 channel. PHDs hydroxylate conserved Pro394 within the N-terminus ankyrin repeat of TRPA1 in normoxia, while a decrease in O\(_2\) concentrations diminishes PHD activity and relieves TRPA1 from the prolyl hydroxylation, leading to its activation in hypoxia. The relief can be achieved by insertion of unmodified TRPA1 proteins to the plasmamembrane or by dehydroxylation through an unidentified molecular mechanism. In hyperoxia, O\(_2\) oxidizes Cys633 and/or Cys856, thereby activating TRPA1. TRPA1 may at least take two oxidized state upon hyperoxia: a relatively unstable oxidized state (state 1) readily
reversed by glutathione and a relatively stable oxidized state (state 2). Sulfhydryl group(s) (SH) of the key cysteine residue(s) Cys633 and/or Cys856 may be modified to sulfinic acid (S-OH) in the former state of TRPA1, while that in the latter state of TRPA1 may form a disulfide bond(s) (S-S). These oxidation mechanisms override the inhibition by prolyl hydroxylation to activate TRPA1.
Figure 1

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Figure 2

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Figure 3

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Figure 5

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**Figure 6**

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**Figure 8**

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**Hypoxia**

- O2 / PHD dehydroxylation (?)
- Cys-SH to Cys-SOH (?)
- Na⁺, Ca²⁺ plasmamembrane insertion

**Normoxia**

- Cys-SH
- Na⁺, Ca²⁺

**Hyperoxia (State 1)**

- Na⁺, Ca²⁺ internalization by clathrin dependent pathway
- Cys-SH

**Hyperoxia (State 2)**

- Na⁺, Ca²⁺
- Cys-SH (State 1)
- Hyperoxia (State 2)

Excess glutathione