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Modulation of K_{2P}3.1 (TASK-1), K_{2P}9.1 (TASK-3) and TASK-1/3 heteromer by reactive oxygen species

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Abstract

Reactive oxygen species (ROS) generated by mitochondria or NADPH oxidase have been implicated in the inhibition of K⁺ current by hypoxia in chemoreceptor cells. As TASKs are highly active background K⁺ channels in these cells, we studied the role of ROS in hypoxia-induced inhibition of TASKs. In HeLa cells expressing TASKs, H₂O₂ applied to inside-out patches activated TASK-1, TASK-3 and TASK-1/3 heteromer starting at ~16 mM. When applied to cell-attached or outside-out patches, 326 mM H₂O₂ did not affect TASK activity. Other K_{2P} channels (TREK-1, TREK-2, TASK-2, TALK-1, TREK) were not affected by H₂O₂ (tested up to 326 mM). A reducing agent (dithiothreitol) and a cysteine-modifying agent (MTSEA) had no effect on basal TASK activity and did not block the H₂O₂-induced increase in channel activity. A TASK mutant in which the C-terminus of TASK-3 was replaced with that of TREK-2 showed a normal sensitivity to H₂O₂. Xanthine/xanthine oxidase mixture used to generate superoxide radical showed no effect on TASK-1, TASK-3 and TASK-1/3 heteromer from either side of the membrane, but strongly activated TASK-2 from the extracellular side. Acute H₂O₂ (32–326 mM) exposure did not affect hSlo1/b1(BK) expressed in HeLa cells and BK in carotid body glomus cells. In carotid body glomus cells, adrenal cortical cells and cerebellar granule neurons that show abundant hypoxia-sensitive TASK activity, H₂O₂ (>16 mM) activated the channels only when applied intracellularly, similar to that observed with cloned TASKs. These findings show that ROS do not support or inhibit TASK and BK activity, and therefore are unlikely to be the hypoxic signal that causes cell excitation via inhibition of these K⁺ channels.

Keywords

Background K⁺ channels; hydrogen peroxide; reactive oxygens species; hypoxia; chemoreceptor cells

Introduction

Acute hypoxia causes rapid depolarization of various types of cells such as carotid body glomus cells, cerebellar granule neurons, adrenal chromaffin cells and pulmonary artery smooth muscle cells (2, 8, 26, 31). The hypoxia-induced depolarization is believed to be mainly due to inhibition of K⁺ channels, particularly those that are active at rest and help to

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set the resting membrane potential (29). One of the targets of hypoxia is a group of background K^+ channels known as TASKs (TASK-1, TASK-3 and TASK-1/3 heteromer) that belong to the K_{2P} channel family (22). Therefore, in cell that express TASKs, inhibition of TASKs is an important mechanism that contributes to the hypoxia-induced depolarization (7, 20, 26).

Of the putative signals involved in hypoxia-induced excitation of chemoreceptor cells, ROS as a hypoxic signal have been the focus of many studies and a subject of controversy (1, 9). The sources of ROS are mitochondria and NADPH oxidase, and the activity of these enzymes during normoxia and hypoxia determines the cellular level of ROS. In airway chemoreceptor cells, NADPH oxidase has been proposed to act as an O_2 sensor, where ROS generated during normoxia help to maintain the basal level of K^+ channel activity (14, 23). It was therefore hypothesized that hypoxia reduces the generation of H_2O_2 , resulting in the decreased background K^+ current and cell depolarization. However, evidence against the role of ROS as hypoxic signal has also been presented. For example, studies using p47phox knockout mice lacking a functional NADPH oxidase showed that ROS produced during hypoxia may instead be activating a K^+ channel to facilitate cell repolarization (12, 18). Mice lacking gp91 subunit of NADPH oxidase also showed no effect on hypoxia-induced inhibition of the whole-cell K^+ current (3). The role of ROS from mitochondria in K^+ channel inhibition also remains unresolved. The question of whether hypoxia increases or decreases the production of ROS is also debated.

To clearly define the role of ROS in hypoxia-induced depolarization in various types of cells, both direct and indirect actions of ROS on individual O_2 -sensitive K^+ channels need to be determined. As ROS are generated inside the cell and presumably act on intracellular targets, it would be important to study the effect of ROS on ion channels from the intracellular side of the membrane. A direct effect of ROS on TASK, a predominant O_2 -sensitive K^+ channel in chemoreceptor cells, was studied by testing the intracellular and extracellular effects of H_2O_2 and superoxide radical on TASK activity in inside-out and outside-out patches. To test for any indirect effect of ROS on TASK, H_2O_2 was applied to cell-attached patches. We also tested the effect of H_2O_2 on the large conductance Ca^{2+} -activated K^+ channel (BK) as BK is expressed in chemoreceptor cells and has been shown to be O_2 -sensitive (27, 37).

Methods

Transfection in HeLa cells

Full lengths TASK-1, TASK-3, TREK-2, TRESK, TALK-1 and TASK-2 DNA coding sequences were cloned previously in this laboratory and inserted into the pcDNA3.1 vector. hSlo1 α and hSlo1 β clones were gifts from Dr. H. Toshinori (U. Penn). TASK-3/TREK-2C mutant was constructed by PCR. HeLa cells were seeded at a density of 2×10^5 cells per 35 mm dish 24 h prior to transfection in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. HeLa cells were co-transfected with DNA fragments encoding a K_{2P} channel and green fluorescent protein (GFP) in pcDNA3.1 using LipofectAMINE2000 and OPTI-MEM I Reduced Serum Medium (Invitrogen). Green fluorescence from cells expressing GFP was detected with the aid of a Nikon microscope equipped with a mercury lamp light source. Cells were used 2–3 days after transfection.

Cell isolation

The animal protocol used in this study was approved by the Animal Care and Use Committee of Rosalind Franklin University. Rats (postnatal 16–18 day; 386 rats) were anesthetized with isoflurane before removing the necessary tissues.

Cerebellar granule cells—The cerebellum was isolated from ~16-day old rat and washed with oxygenated physiological buffer solution at 4°C (17). The cerebellar cortex was cut into thin sections and incubated for 15 min in a solution containing papain (12 units/ml; Worthington, Lakewood, NJ, USA), albumin (0.2 mg/ml) and DL-cysteine (0.2 mg/ml). After digestion, the tissue was washed twice with phosphate-buffered saline (PBS) and resuspended in a solution containing DNase I (1000 Kunitz/ml; Worthington). After gentle trituration of the solution using a fire-polished glass pipette, the suspended cells were gently passed through a 3 ml, 25 gauge syringe. The suspension was layered on top of sterilized fetal bovine serum and centrifuged at 100 *g* for 10 min. The pellet was resuspended in plating medium that contained Neurobasal Medium supplemented with B-27 (10 ml; Life Technologies, Rockville, MD, USA), glutamic acid (2.5 mM), glutamine (20 mM), gentamicin (50 mg/ml) and fungizone (2.5 mg/ml). The cells were plated on glass coverslips coated with poly-L-lysine at a density of 1×10^5 cells cm^2 . Cells were kept at 37 °C in a humidified atmosphere of 95% air-5% CO₂, and used between 2–4 days.

Carotid body glomus cells—Carotid body (CB) was removed and placed in ice-cold low-Ca²⁺, low-Mg²⁺ phosphate buffered saline solution (PBS: 137 mM NaCl, 2.8 mM KCl, 2 mM KH₂PO₄, 0.07 mM CaCl₂, 0.05 mM MgCl₂, pH 7.4). Each CB was cut into 3–4 pieces and placed in PBS containing trypsin (400 ug/mL) and collagenase (400 ug/mL) and incubated at 37°C for 20–25 minutes. CBs were gently triturated using a fire polished Pasteur pipette to mechanically dissociate the cells. CB growth medium (Ham's F-12 containing 10% fetal bovine serum, 23 mM glucose, 200 mM L-glutamine, 10K units penicillin/streptomycin, and 300 ug/ml insulin) was added to stop enzyme activity. After brief trituration, the solution containing the digested CBs was centrifuged for 4 min at ~6000 rpm (~2000 × *g*) using a microcentrifuge. After discarding the supernatant, warm CB growth media was added to resuspend the pellet. Suspended CB cells were placed on glass coverslips coated with poly-L-lysine and incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂. Cells were used within 6 hrs.

Adrenal cortical cells—Adrenal glands were removed from ~16-day old rats, and washed with PBS buffer. Cortical region of the adrenal gland was dissected out under microscope, cut into small pieces and placed in DMEM medium containing collagenase type 1A (2.5 mg/ml) and DNase 1 type III (0.1 mg/ml). The tissues were digested for 30 min at 37°C in a shaking water bath. After allowing the digested tissue settle to the bottom of the tube, the supernatant was removed and the tissue fragments were resuspended in 1 ml medium. The cells were mechanically dissociated by trituration. The medium containing the dissociated cells was centrifuged at 3000 rpm for 5 min. After removing the supernatant, the cells were resuspended in culture medium supplemented with 2% fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin. Cells were plated on glass coverslips coated with poly-L-lysine and maintained at 37°C in a humidified atmosphere of 95% air-5% CO₂. The cells were used within 6 h.

Electrophysiological studies

Electrophysiological recording was performed using a patch clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA). Channel current was filtered at 3 kHz using 8-pole Bessel filter (−3 dB; Frequency Devices, Haverhill, MA) and transferred to a computer using the Digidata 1320 interface at a sampling rate of 20 kHz. Single-channel currents were analyzed with the pCLAMP program (Version 10). Channel openings were analyzed to obtain channel activity (NPo, where N is the number of channels in the patch, and Po is the probability of a channel being open). NPo was determined from ~20 s of current recording. The single channel current tracings shown in the figures were filtered at 1 kHz. In experiments using cell-attached and inside-out patches, pipette and bath solutions contained

(mM): 140 KCl, 1 MgCl₂, 5 EGTA, 10 glucose and 10 HEPES (pH 7.3). Outside-out patch was formed from the whole-cell configuration by gently lifting up the pipette. For statistics, Student's *t*-test (for comparison of two sets of data) and one-way analysis of variance (ANOVA) with Bonferroni correction (for comparison of more than two data sets) were used with $p < 0.05$ as the criterion for significance. Data were analyzed using the Origin program and represented as mean $\pm$ S.D. All experiments were performed at $\sim 24^{\circ}\text{C}$.

Materials

H₂O₂, xanthine, xanthine oxidase, dithiothreitol, glutathione, NADPH were purchased from Sigma. H₂O₂ was purchased in liquid form (30% = 9.79M) and diluted to desired final concentration just before use. MTSEA (2-Aminoethyl Methanethiosulfonate Hydrobromide) was purchased from Toronto Research Chemicals.

Results

Activation of TASK by high concentrations of H₂O₂

TASK-1, TASK-3 and TASK-1/3 heteromer are referred as TASK, unless indicated otherwise. Intracellular action of H₂O₂ on TASK were studied by applying H₂O₂ to inside-out patches containing TASK-1, TASK-3 or TASK-1/3 heteromer expressed in HeLa cells. Inward current was recorded by setting the pipette potential at +100 mV with pipette and bath solutions containing 150 mM K⁺. H₂O₂ was applied starting at 1.63 mM (0.01% solution), and the concentration increased progressively. H₂O₂ had no significant effect at concentrations up to 16.3 mM H₂O₂, but produced significant increases at 32.6 mM and above. For each concentration of H₂O₂, the effect of TASK-1 was rapid, with the maximal activation occurring within ~ 15 seconds. H₂O₂ did not alter the pH of the perfusion solution. Fig. 1A shows a typical single-channel recording in the presence and absence of 326 mM H₂O₂. Based on amplitude and duration histograms, the single-channel conductance (16 ± 1 pS) and the mean open time (0.6 ± 0.1 ms) were not significantly altered by H₂O₂ (Fig. 1B). The relative TASK-1 activity as a function of [H₂O₂] is shown in Fig. 1C. At the highest [H₂O₂] used (1632 mM), TASK activity increased markedly for ~ 20 – 30 sec but the membrane soon became unstable for current recording. The activation of TASK-1 by H₂O₂ could not be washed off easily when [H₂O₂] was 326 mM or less (Fig. 1D). However, additional activation produced by higher [H₂O₂] was easily reversible upon washout. Thus, low millimolar levels of H₂O₂ had no effect on TASK activity, but higher concentrations strongly activated TASK-1.

Similar experiments were performed using TASK-3 and TASK-1/3 heteromer expressed in HeLa cells. The results were qualitatively similar to those observed with TASK-1 (Fig. 2). The single-channel conductance and mean open time values were 32 ± 1 pS and 0.8 ± 0.1 ms for TASK-3, and 31 ± 1 pS and 0.8 ± 0.1 ms for TASK-1/3 heteromer. These values remained unchanged after exposure to H₂O₂. Again, the maximal [H₂O₂] tested was 1.6 M that briefly caused strong activation of both TASK-3 and TASK-1/3 heteromer. A current tracing showing the strong activation of TASK-1/3 by 1.6 M H₂O₂ is shown in Fig. 2B. Like TASK-1, partial reversibility was observed only when [H₂O₂] was high (326 mM–1.6 M). Sodium stannate (0.5 ppm = $2.35 \mu\text{M}$ in 30% [H₂O₂]) that is used to stabilize H₂O₂ did not cause any significant effect even at $5 \mu\text{M}$ on all three TASK isoforms. These results show that high concentrations of H₂O₂ can increase the activity of all three TASK isoforms in a similar way.

We also tested the intracellular effect of [H₂O₂] on several other K₂P channels such as TREK-1, TREK-2, TALK-1, TASK-2 and TRESK. None of these channels were activated by 326 mM [H₂O₂] (Fig. 3). Therefore, the activating effect of H₂O₂ on TASK was selective

among K_{2P} channels. Interestingly, H_2O_2 (326 mM) applied to outside-out patches did not activate TASK-1, TASK-3 or TASK-1/3 heteromer during ~3 min of perfusion (Fig. 3B), suggesting that an intracellular site for H_2O_2 is present ($n=5$ each). H_2O_2 (326 mM) applied extracellularly failed to affect other K_{2P} channels in outside-out patches (Fig. 3B). As predicted from these findings, application of 326 mM H_2O_2 to the solution perfusing cell-attached patches for 5 min failed to activate TASK-1/3 (NP_o : 0.21 ± 0.03 vs 0.23 ± 0.03 ; $n=5$; $p>0.05$).

Effect of xanthine/xanthine oxidase mixture on TASKs

To test the effect of superoxide radical on TASKs, inside-out patches were formed and a xanthine (50 μ M)/xanthine oxidase (50 milliunit/ml) mixture applied to the cytosolic side of the membrane. This mixture showed no significant effect on all K_{2P} channels tested (Fig. 4A). The xanthine/xanthine oxidase mixture applied to outside-out patches did not affect the activity of K_{2P} channels except TASK-2 that was strongly activated (Fig. 4B–C). Xanthine alone did not cause activation of TASK-2, but xanthine oxidase alone produced a small but significant increase in TASK-2 activity. The control solution used to dissolve xanthine oxidase contains ammonium sulfate in sodium phosphate buffer. This control solution without the enzyme also produced a small activation of TASK-2 ($NP_o=0.42 \pm 0.05$) similar to that produced by xanthine oxidase solution ($NP_o=0.36 \pm 0.04$), but the activation produced by xanthine/xanthine oxidase was much greater (Fig. 4C). These results suggest that superoxide radical does not affect the activity of TASK-1, TASK-3 and TASK-1/3 heteromer.

Agents that modify the redox state do not affect H_2O_2 -induced activation of TASK

To test the possibility that H_2O_2 augments TASK activity by oxidation of –SH groups, we repeated the experiments similar to those described in Fig. 1 in the presence of 1 mM dithiothreitol (DTT), a reducing agent. DTT was present in the bath solution ~5 min prior to formation of inside-out patches and remained present thereafter. H_2O_2 (326 mM) applied 5 min after DTT application increased the activity of TASK-1/3 heteromer 2.4 ± 0.5 -fold, compared to 2.3 ± 0.3 -fold observed in control patches without DTT ($n=4$ each). H_2O_2 (326 mM) also increased TASK-1.3 activity 2.5 ± 0.4 -fold ($n=4$) when glutathione (1 mM), an antioxidant, was present. Perfusion of inside-out patches with 200 μ M MTSEA, a cysteine-modifying agent showed no significant effect on the channel activity of TASK-1/3, and also failed to affect the H_2O_2 -induced increase in TASK-1 activity (2.6 ± 0.4 -fold; $n=4$). Similarly, glutathione (GSH; 1 mM), NADH (1 mM), and NADPH (1 mM) each showed no effect on TASK-1/3 activity, and did not significantly affect the H_2O_2 -induced increase in TASK-1/3 activity ($P>0.05$; $n=4$ each). The range of increase in channel activity elicited by H_2O_2 (326 mM) was 2.4–2.6-fold.

The finding that H_2O_2 activates TASKs from the intracellular side of the membrane suggests that this ROS could be acting directly on the channel protein. To test whether the C-terminus of TASKs mediates the activation by H_2O_2 , we constructed a chimeric channel in which the entire carboxyl-terminus of TASK-3 was replaced with that of TREK-2. H_2O_2 (326 mM) activated this mutant 2.7 ± 0.5 -fold ($n=5$), similar to the degree of activation of wild-type TASK-3 (2.5 ± 0.4 fold). Thus, H_2O_2 activates TASKs via a mechanism unrelated to oxidation of the channel and without the involvement of the carboxyl terminus.

Effect of H_2O_2 on TASK-like channels in native cells

To show that the stimulatory effect of H_2O_2 on TASK is also observed in native cells, we tested the effect of H_2O_2 on TASK channels from three different cell types. They are cerebellar granule neurons, carotid body glomus cells and adrenal cortical cells, all of which express TASK-1 and TASK-3 (7, 11, 17). TASK-like channels were present in most cell-attached patches, as judged by their single-channel conductance (32 ± 2 -pS), mean open

times ($\sim 1.0 \pm 0.1$ ms) and the mildly inwardly rectifying I–V relationships. Patches containing channels other than TASKs were discarded. In outside-out patches with 2 mM ATP in the pipette, lowering the extracellular pH from 7.3 to 6.0 inhibited the activity TASK-like channels by $82 \pm 4\%$, $83 \pm 4\%$ and $79 \pm 3\%$ ($n=5$ each) in cerebellar granule neurons, glomus cells and adrenal cells, respectively, indicating that TASK-like channels for present in all three cell types. Addition of 326 mM H_2O_2 to cell-attached patches of cerebellar granule neurons and glomus cells did not alter TASK activity during ~ 5 min of perfusion ($n=5$ each; $p>0.05$). In inside-out patches containing 2 mM ATP that keeps TASK channels in the active state, addition of H_2O_2 (326 mM) produced a significant increase in channel activity in cerebellar granule neurons and glomus cells (Fig. 5A–C). Similar to that observed with TASKs expressed in HeLa cells, H_2O_2 steeply increased TASK activity at very high concentrations (Fig. 5B). H_2O_2 did not alter the main single channel conductance (31 ± 1 pS) or the mean open time (0.8 ± 0.1 ms). We observed qualitatively similar results using adrenal cortical cells that express a high level of TASK-like channels. For example, 32.6 mM H_2O_2 had no effect on TASK channel when applied to inside-out patches (with 2 mM ATP), but 326 mM produced a 2.8 ± 0.5 -fold ($n=4$) increase in TASK activity.

In inside-out patches without ATP in the bath perfusion solution, TASK channel activity is generally very low. Nevertheless, application of high $[\text{H}_2\text{O}_2]$ to these inside-out patches increased TASK channel activity, indicating that ATP was not required for the H_2O_2 -induced activation of TASK. An example of such an effect is shown in Fig. 5D for a carotid body glomus cell. In glomus cells, pretreatment of inside-out patches (with 2 mM ATP in the bath solution) with DTT (1 mM; 3 min) or MTSEA (100 μM ; 3 min) showed no effect on TASK activity, and did not block the activation of TASK by subsequent application of H_2O_2 in the glomus cell (2.5 ± 0.5 -fold increase). These results show that high $[\text{H}_2\text{O}_2]$ activates native TASK-like channels in different cell types, similar to that observed with cloned TASKs expressed in HeLa cells.

Lack of acute effect of H_2O_2 on large conductance Ca^{2+} -activated K^+ channel (BK)

Like TASKs, BK is expressed in chemoreceptor cells and has been shown to be inhibited by hypoxia (27, 35, 37). Inhibition of BK has also been proposed to underlie the hypoxia-induced depolarization of glomus cells (25). Therefore, we tested the effect of H_2O_2 on BK (hSlo1/ $\beta 1$) expressed in HeLa cells. Inside-out patches were formed and perfused with solution containing 10 μM Ca^{2+} . Application of 326 mM H_2O_2 showed no significant effect on BK activity during ~ 3 minute of perfusion (NPo: 3.3 ± 0.4 and 3.6 ± 0.5 at the beginning and end of 3 min, respectively; $n=4$). In a different set of inside-out patches, perfusion with 326 mM H_2O_2 also produced no inhibition of BK activity (NPo: 2.8 ± 0.5 and 3.2 ± 0.6 ; $n=4$). In inside-out patches of glomus cells held at -30 mV (pipette potential of $+30$ mV), perfusion with solution containing 10 μM Ca^{2+} activated BK. Further addition of 32 mM and then 326 mM H_2O_2 for ~ 2 min each produced no significant change in channel activity (NPo: 1.3 ± 0.3 (control), 1.4 ± 0.3 (32 mM) and 1.6 ± 0.3 (326 mM); $n=4$). These results suggest that any rapid inhibition of BK by acute hypoxia is unlikely to be mediated by H_2O_2 .

Discussion

The purpose of this study was to determine the direct effects of superoxide radical and H_2O_2 on TASK function, and thus to assess the role of ROS as potential mediators of hypoxia-induced inhibition of TASK and depolarization that occurs in cerebellar granule neurons and glomus cells. Our results show that superoxide radical (generated by the xanthine/xanthineoxidase mixture) does not affect TASK-1, TASK-3 and TASK-1/3 heteromer, and H_2O_2 activates them at relatively high concentrations that may not be achieved under physiological conditions. No inhibition of TASK was observed at all concentrations of H_2O_2

that were used. Therefore, our results suggest that superoxide radical and H_2O_2 that are generated by NADPH oxidase and mitochondria are unlikely to serve as signals for the hypoxia-induced inhibition of TASK and depolarization. Instead, H_2O_2 could help to oppose the hypoxia-induced depolarization by stimulating TASK activity, if the intracellular level of H_2O_2 can reach levels above ~ 32 mM.

Activation of TASK by H_2O_2

The concentration of H_2O_2 used in various studies to measure ion channel current ranges from ~ 1 μM to ~ 50 mM. For example, 2 mM H_2O_2 caused an increase in K^+ current in NEB cells (19), 3–5 mM H_2O_2 increased the cardiac SR Ca^{2+} release channel activity (6), 300 μM –2 mM H_2O_2 increased BK current in coronary artery smooth muscle cells (5), and 50 mM H_2O_2 inhibited the ATP-sensitive K^+ channel but had no effect on BK in the mouse skeletal muscle cells (33). Therefore, the ~ 32 mM H_2O_2 required to begin to increase TASK activity in HeLa cells expressing TASKs and in three different cell types expressing endogenous TASKs is high. The activation of TASK or any other ion channels by such high levels of H_2O_2 would not be meaningful, if the physiological level of H_2O_2 is in the micromolar range. Because we do not know the true local concentration of H_2O_2 near the membrane, it is difficult to know how much of the effect produced by H_2O_2 is physiologically relevant.

Our studies show that the presence of a basal level of H_2O_2 is not necessary for TASK activity under control conditions, as TASK is active without H_2O_2 . Because H_2O_2 either has no effect (< 32 mM) or activates (> 32 mM) TASKs, it is not possible that H_2O_2 would be involved in hypoxia-induced inhibition of TASKs in the cell types that we studied. Thus, H_2O_2 generated from either NADPH oxidase or mitochondria is unlikely to serve as a signal in hypoxia-induced inhibition of TASKs. The lack of effect of H_2O_2 on TASK-1 has been observed as well in HEK cells expressing TASK-1 (24). This does not mean that H_2O_2 is not involved in other aspects of hypoxia-induced excitation of CB cells, as H_2O_2 may act on K^+ channels other than TASK. For example, hypoxia increased ROS and activated a K_V current, and H_2O_2 (100 μM) decreased the hypoxic inhibition of K_V current in mice glomus cells (12). At such low $[\text{H}_2\text{O}_2]$, there would be no effect on TASKs. It is possible that $[\text{H}_2\text{O}_2]$ is high within a localized cellular space such that its concentration reaches 16–300 mM during hypoxia. In such a case, $[\text{H}_2\text{O}_2]$ would be activating TASK and help in repolarization. Our results support some of the earlier studies suggesting that ROS is not the hypoxic signal for excitation of glomus cells and contraction of pulmonary smooth muscle cells (15, 16, 32, 34).

Mechanism of activation of TASK by H_2O_2

The finding that high concentrations of H_2O_2 are able to activate TASKs is interesting. One possible mechanism involves a change in the sensitivity of TASKs to intracellular ATP. TASKs expressed in HeLa cells (and in Cos-7 or HEK cells) are active in cell-attached patches and remain active when inside-out patch is formed. Thus, in these cells, intracellular molecules are not required to support TASK activity. In contrast, TASK-like channel expressed in glomus cells undergo rapid rundown upon formation of inside-out patches, and ATP reactivates the channels. TASK-like channels in cerebellar granular cells and adrenal cortical cells also undergo rapid rundown upon formation of inside-out patch configuration (unpublished observation; D. Kim). Thus, the property of the TASK residing in the plasma membrane of “native cells” is different from those of mammalian cell lines. What causes the difference in the ATP sensitivity of TASK in native vs. transfected cells remains a mystery. Regardless of this difference, H_2O_2 can increase TASK activity in the absence of ATP in glomus cells (Fig. 5D), indicating that the H_2O_2 -induced increase in TASK activity is not due to an increased sensitivity of TASK to ATP.

The generally irreversible effect of H_2O_2 ($<326\text{mM}$) on TASK suggests that a covalent modification of the channel might have occurred in response to H_2O_2 . An earlier study reported that intracellularly applied H_2O_2 inhibits BK via oxidation of a cysteine residue at the carboxyl terminus, as a cysteine-modifying reagent (MTSEA) produced a similar inhibitory effect (30). Our finding that neither DTT nor MTSEA blocked the H_2O_2 -induced increase in TASK activity suggests that oxidation was probably not involved. In support of this conclusion, substitution of the entire carboxyl terminus of TASK containing cysteine residue with that of TREK-2 (that does not respond to H_2O_2) had no effect on H_2O_2 -induced increase in the activity of this mutant.

Another mechanism for H_2O_2 -induced increase in TASK activity could involve alterations in lipid interaction with the TASK channel to modulate gating. Perhaps, this could be why enormously high concentrations of H_2O_2 ($326\text{ mM}–1.6\text{ M}$) still manage to activate TASK until the membrane seal breaks. Although TASK is considered to be a background K^+ channel with a relatively high basal activity compared to other K^+ channels, the finding that TASK can be activated >5 -fold above its basal level by H_2O_2 suggests that the basal open probability is low at rest. Therefore, the range of modulation of TASK activity (and thus the excitability of the cell) is wide, as TASKs can both be inhibited (i.e., by receptor agonists via Gq (10) and stimulated (i.e., by volatile anesthetics and H_2O_2).

As H_2O_2 could activate TASK from the intracellular side of the membrane, we anticipated that H_2O_2 would also activate from the extracellular, as H_2O_2 is believed to be membrane permeable. No activation of TASK by H_2O_2 from the extracellular side during the course of the exposure that lasted ~ 5 min, suggests that the diffusion is very slow and that it may take longer than 5 min to reach the critical concentration to activate the channel. We did not further test the effect of longer exposure of TASK to extracellular H_2O_2 , as hypoxic inhibition of TASK occurs very rapidly (<10 seconds) and generation of H_2O_2 would be intracellular.

Role of BK

In glomus cells, the hypoxia-induced depolarization has been attributed partly to inhibition of BK (25). The signals involved in BK inhibition by hypoxia is still unclear, although some evidence for the role of AMP kinase has been presented (36). Could ROS from mitochondria be involved in this inhibition? The lack of effect of H_2O_2 on BK in our experiments suggests that the hypoxia-induced inhibition of BK does not involve H_2O_2 . In an earlier study, application of 1.7 mM H_2O_2 to inside-out patches was found to produce a strong inhibition of BK expressed in HEK cells (30). However, the inhibitory effect of H_2O_2 on BK was very slow, and essentially no inhibition was present during the first $\sim 1–2$ min, arguing against the involvement of H_2O_2 in hypoxia-induced depolarization of glomus cells that occurs within ~ 3 sec of exposure to hypoxia. Interestingly, $300\text{ }\mu\text{M}$ H_2O_2 was found to stimulate BK activity in coronary smooth muscle cells via phospholipase A_2 -arachidonic acid signaling pathway when H_2O_2 was applied extracellularly (5). Therefore, intracellular and extracellular effects of H_2O_2 may be different, and need to be studied separately, as we have done in this study.

Indirect action of H_2O_2 on TASK

Our study does not completely rule out the possibility that hypoxia inhibits TASKs via indirect actions of ROS. When cell-attached patches are perfused with H_2O_2 , we suspect that intracellular $[\text{H}_2\text{O}_2]$ rises and could start to generate second messenger signals. In COS-7 cells, for example, $5–10$ min of treatment with 5 mM H_2O_2 was found to activate PKC (21). Therefore, our finding that extracellular application of a very high concentration of H_2O_2 has no effect on TASK activity in cell-attached patches suggests that signals that

are potentially generated by H₂O₂ are ineffective in inhibiting TASK at least for the short duration of the experiments. Studies in the past have shown that prolonged exposure of cells to H₂O₂ can activate various type of protein kinases including PKC, ERK1/2 and MAP kinases (4, 28, 38). The activity of these kinases are generally associated with long-term effects such as cell proliferation and death. Therefore, it seems highly unlikely that hypoxia-induced inhibition of TASK that occurs within ~2–3 sec would involve changes in the activity of such kinases.

Significance of the finding

How hypoxia inhibits TASK or other K⁺ channels is not yet known. One hypothesis is that mitochondrial hemeproteins act as an O₂ sensor and produce a number of metabolic changes that are coupled to inhibition of K⁺ channels. ROS (from mitochondria and NADPH oxidase) as potential signals that mediate hypoxia-induced excitation of chemoreceptors has been studied for over 20 years and many reviews have been written on this subject. Unfortunately, the role of ROS in O₂ sensing has been controversial, due to the difficult nature of studying the effect of ROS in intact cells. In glomus cells, evidence against the role of ROS in acute hypoxic excitation of glomus cells has been accumulating slowly, and our study confirms this view. In chronic intermittent hypoxia, ROS is clearly an important signal for remodeling of glomus cell function, and our findings suggest that direct ROS effects on TASK and BK are unlikely to be involved. Our findings should apply to not only glomus cells but also other O₂-sensitive cells such as pulmonary smooth muscle cells and adrenal chromaffin cells where TASK and BK channels are well expressed. What other signals could be mediating the inhibition of TASK by acute hypoxia? Recent studies suggest that a reduction of cytosolic [ATP] and/or activation of AMP kinase could play such a role (7, 13).

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Figure 1. Activation of TASK-1 by H₂O₂

A. H₂O₂ was applied to inside-out patches of HeLa cells expressing TASK-1. Pipette potential of patches was set at +100 mV to record inward TASK-1 current. Dotted line indicates the first open level.

B. Amplitude and duration histograms from TASK-1 openings in inside-out patches were fitted with gaussian and exponential functions, respectively.

C. [H₂O₂]-TASK activity relationships shown. Each point is the mean±SD of 5 determinations. For asterisks, ANOVA followed by post-hoc testing based on unpaired t-test with Bonferroni correction was used to determine significance from the control value observed with no H₂O₂ ($p < 0.05$).

D. The stimulatory effect of H₂O₂ was not reversible if [H₂O₂] was 326 or below. Additional effect at higher [H₂O₂] was reversible.

Figure 2. Activation of TASK-3 and TASK-1/3 by H_2O_2

A–B. H_2O_2 was applied to inside-out patches of HeLa cells expressing TASK-3 or TASK-1/3. Pipette potential of patches was set at +60 mV to record inward TASK-1 current. Note the large activation by 1.6 M H_2O_2 . Dotted lines indicate open channel levels. C–D. $[\text{H}_2\text{O}_2]$ -channel activity relationships for TASK-3 and TASK-1/3. See legend to Fig. 1C for statistical analysis. Each point is the mean $\pm$ SD of 6 determinations

Figure 3. Effect of H₂O₂ on K_{2p} channels

A. H₂O₂ was applied to inside-out patches from HeLa cells expressing various K_{2p} channels as shown. Pipette potential was set at +60 mV.

B. Same experiment as in A except that H₂O₂ was applied to outside-out patches. Each bar represents channel activity determined after H₂O₂ application divided by activity before application (mean of 6 determinations). Asterisk indicates a significant effect of H₂O₂ ($p < 0.05$) based on unpaired t-test.

Figure 4. Effect of xanthine/xanthine oxidase on K_{2p} channels

A. Xanthine-xanthine oxidase (X-XO) mixture was applied to inside-out patches from HeLa cells expressing various K_{2p} channels. Pipette potential was set at +60 mV.

B. Same experiment as in A except that X-XO was applied to outside-out patches. Each bar represents channel activity determined after X-XO application divided by activity before application (mean of 6 determinations). Asterisk indicates a significant effect of X-XO ($p < 0.05$) based on unpaired t-test.

Figure 5. Activation of TASK-like channels by H_2O_2 in cerebellar granule neuron and carotid body glomus cell

A–B. H_2O_2 was applied to inside-out patches from cerebellar granule neuron or a carotid body glomus cell that express TASK channels. Pipette potential was set at +60 mV. ATP was present in the perfusion solution.

C. $[H_2O_2]$ -activity relationships for the two types of cells. Each point is the mean $\pm$ SD of 5 determinations. Significance is indicated by asterisks. See legend for Fig. 1C for statistical analysis.

D. H_2O_2 was applied to inside-out patches from glomus cells in the absence of ATP. Expanded single channel tracings are shown (a,b).