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Hydrogen sulfide and hypoxia-induced changes in TASK ($K_{2P}3/9$) activity and intracellular Ca^{2+} concentration in rat carotid body glomus cells

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ABSTRACT

Acute hypoxia depolarizes carotid body chemoreceptor (glomus) cells and elevates intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Recent studies suggest that hydrogen sulfide (H_2S) may serve as an oxygen sensor/signal in the carotid body during acute hypoxia. To further test such a role for H_2S, we studied the effects of H_2S on the activity of TASK channel and $[Ca^{2+}]_i$, which are considered important for mediating the glomus cell response to hypoxia. Like hypoxia, NaHS (a H_2S donor) inhibited TASK activity and elevated $[Ca^{2+}]_i$. To inhibit the production of H_2S, glomus cells were incubated (3 h) with inhibitors of cystathionine-β-synthase and cystathionine-γ-lyase (DL-propargylglycine, aminooxyacetic acid, β-cyano-L-alanine; 0.3 mM). SF7 fluorescence was used to assess the level of H_2S production. The inhibitors blocked L-cysteine- and hypoxia-induced elevation of SF7 fluorescence intensity. In cells treated with the inhibitors, hypoxia produced an inhibition of TASK activity and a rise in $[Ca^{2+}]_i$, similar in magnitude to those observed in control cells. L-cysteine produced no effect on TASK activity or $[Ca^{2+}]_i$ and did not affect hypoxia-induced inhibition of TASK and elevation of $[Ca^{2+}]_i$. These findings suggest that under normal conditions, H_2S is not a major signal in hypoxia-induced modulation of TASK channels and $[Ca^{2+}]_i$ in isolated glomus cells.

1. Introduction

Hypoxia depolarizes carotid body glomus cells by inhibiting the outward K^+ current and elevates $[Ca^{2+}]_i$ by opening voltage-gated Ca^{2+} channels. The rise in $[Ca^{2+}]_i$ increases the secretory event leading to augmented activity of the carotid sinus afferent nerve (Lopez-Barneo et al., 2004; Pardal et al., 2000). Although this general scheme of events is well accepted, the intracellular O_2 sensor and associated signals that mediate the hypoxia-induced excitation of glomus cells *via* modulation of ion channels are still not well defined. A number of recent studies have focused on the potential role of H_2S as an O_2 sensor/signal in the response of the carotid body (CB) to hypoxia. So far, evidence both in support of and against a role for H_2S has been presented, and the issue remains unresolved.

In those studies with positive findings, H_2S increased the carotid sinus afferent nerve activity in carotid sinus nerve-CB preparation and increased $[Ca^{2+}]_i$ in isolated glomus cells (Li et al., 2010; Makarenko et al., 2012; Peng et al., 2010). In these studies, inhibitors of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) that normally produce H_2S in the cell markedly reduced the chemoreceptor afferent nerve activity as well as the rise in $[Ca^{2+}]_i$ in response to hypoxia. Glomus cells from mice with deletion of the CSE gene ($CSE^{-/-}$) also showed a strongly reduced catecholamine secretion in response to hypoxia, compared with cells from wild type mice (Peng et al., 2010). $CSE^{-/-}$ mice also exhibited an impaired ventilatory response to hypoxia (Peng et al., 2010). Together, these findings support the hypothesis that H_2S mediates the ventilatory response to hypoxia by increasing glomus cell $[Ca^{2+}]_i$, the secretion of transmitters and the chemosensory response of the CB.

Arguing against the hypothesis that H_2S is a mediator of the glomus cell response to hypoxia are findings in which NaHS or

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Na_2S (H_2S donors) inhibited the transmitter release from cat CB (Fitzgerald et al., 2011), and reduced mitochondrial function, as assessed by NADH autofluorescence and measurement of intracellular [Mg^{2+}] that was taken as an index of ATP concentration (Buckler, 2012). Any agent that inhibits glomus cell mitochondrial oxidative phosphorylation would be expected to produce effects similar to those of hypoxia. Therefore, it was argued that the effect of exogenously applied H_2S on glomus cells was simply due to an effect on mitochondrial function similar to that produced by hypoxia (Buckler, 2012). In another study, an indirect sequestration of H_2S using methemoglobin (produced by injection of sodium nitrite) did not prevent hypoxia-induced hyperventilation (Haouzi et al., 2011a, 2011b). These findings therefore do not support a role for H_2S as an intracellular signal that mediates the CB glomus cell response to hypoxia.

We hypothesized that if endogenous H_2S were a true mediator of the glomus cell response to hypoxia, it would cause inhibition of K^+ channels known to be involved in hypoxia-induced depolarization and elevate $[Ca^{2+}]_i$ in isolated glomus cells. Such effects of H_2S on TASK and BK have been reported recently (Buckler, 2012; Telezhkin et al., 2010). More importantly, the hypoxia-induced inhibition of the K^+ channels and elevation of $[Ca^{2+}]_i$ should be blocked or strongly reduced when the endogenous H_2S production is eliminated. Reduction of hypoxia-induced rise in $[Ca^{2+}]_i$ by inhibitors of CSE and in $CSE^{-/-}$ mice has been reported recently (Makarenko et al., 2012), but the effect of inhibitors of CSE and CBS on hypoxia-induced inhibition of K^+ channels remains to be determined. We believe that these experiments are crucial tests to show whether H_2S is indeed an intracellular signal mediating the hypoxia response in glomus cells. In this study, we tested the effect of inhibitors of CSE and CBS on hypoxia-induced inhibition of TASK channel, and measured changes in $[Ca^{2+}]_i$ to revisit the earlier finding that the inhibitors of these enzymes reduce the $[Ca^{2+}]_i$ response to hypoxia. We also tested the effect of elevating endogenous production of H_2S using L-cysteine on TASK and $[Ca^{2+}]_i$. To our surprise, our results showed that L-cysteine or inhibitors of CSE and CBS failed to block the hypoxia-induced inhibition of TASK channel and the rise in $[Ca^{2+}]_i$. Our findings therefore do not support the role of H_2S as an O_2 sensor/signal in hypoxia-induced excitation of rat glomus cells.

2. Methods

2.1. Cell isolation

Rats (postnatal 18–24 day; Sprague-Dawley) were anesthetized with isoflurane and used according to the animal protocols approved by the Animal Care and Use Committees of Rosalind Franklin University and University of Arkansas for Medical Sciences. The carotid bodies were removed and placed in ice-cold low-Ca^{2+}, low-Mg^{2+} phosphate buffered saline solution (low Ca^{2+}/Mg^{2+}-PBS: 137 mM NaCl, 2.8 mM KCl, 2 mM KH_2PO_4, 0.07 mM $CaCl_2$, 0.05 mM $MgCl_2$, pH 7.4). Each carotid body was cut into 2–3 pieces and placed in a solution containing trypsin (400 mg/ml) and collagenase (400 mg/ml) in low Ca^{2+}/Mg^{2+}-PBS and incubated at 37 °C for 20–25 min. Carotid bodies were gently triturated using a fire polished Pasteur pipette to mechanically dissociate the cells. Growth medium (Ham's F-12, 10% fetal bovine serum, 23 mM glucose, 2 mM L-glutamine, 10 K units penicillin/streptomycin, and 300 mg/ml insulin) was added to stop enzyme activity. After brief trituration, the solution containing the digested carotid bodies was centrifuged for 4 min at ~6000 rpm (~2000 × *g*) using a microcentrifuge. The supernatant was removed and warm growth medium added to gently resuspend the pellet. Suspended cells were placed on glass coverslips coated with poly-L-lysine, and incubated at 37 °C in a humidified atmosphere of 95% air-5% CO_2. Cells were used 3 h later.

2.2. Electrophysiological studies

Electrophysiological recording was performed using a patch clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA). Patches were formed using borosilicate glass pipettes with 3–5 megaohm tip resistance. The pipette solution contained (mM) 150 KCl, 1 $MgCl_2$, 5 EGTA, 10 glucose and 10 HEPES (pH 7.3), and the bath perfusion solution contained (mM) 117 NaCl, 5 KCl, 23 $NaHCO_3$, 1 $CaCl_2$, 1 $MgCl_2$ and 11 glucose (pH 7.3). 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 (NP_o, where N is the number of channels in the patch, and P_o is the open probability of a channel). NP_o was determined from 15 to 30 s of current recordings. Because glomus cells express both ~16-pS (TASK-1) and ~35-pS (TASK-3 and TASK-1/3) channels, analysis was done to detect all three isoforms by setting the open levels as multiples of ~16-pS channel. Single-channel current tracings shown in figures were filtered at 1 kHz. All electrophysiological experiments were performed at ~35 °C.

2.3. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured by quantitative fluorescence imaging using the calcium-sensitive dye fura-2. Cells plated on a coverslip were incubated with 4 μM fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes) for 30 min at 37 °C. Fura-2 fluorescence emission was measured at 510 nm in response to alternating excitation at 340 and 380 nm. Images were acquired and stored using a NIKON Eclipse TE300 microscope (with 40× oil immersion objective) and CCD (CoolSNAP HQ_2) camera under computer control (MetaFluor: Molecular Devices). For each coverslip, the background light levels were determined and subtracted from each image before measurement of the fluorescence intensity ratio. $[Ca^{2+}]_i$ was determined using the 340 nm/380 nm fluorescence ratio as described previously (Grynkiewicz et al., 1985). Calibration was performed using cell-free solutions. The perfusion solution used for $[Ca^{2+}]$ measurements contained (mM): 118 NaCl, 23 $NaHCO_3$, 3 KCl, 2 KH_2PO_4, 1 $MgCl_2$, 1.2 $CaCl_2$, 11 glucose. The temperature of the perusion solution at the recording chamber was 35 °C.

2.4. SF7 fluorescence recording

Endogenous production of intracellular [H_2S] was estimated using SF7-AM (sulfide fluor-7-acetoxymethyl ester; gift from Dr. Christopher Chang, University of California, Berkeley), a recently developed fluorescence probe for H_2S (Lin et al., 2013). Cells plated on a glass coverslip were incubated with SF7-AM (2.5 μM) for 10 min at 37 °C. Excitation and emission wavelengths were 490 nm and 525 nm, respectively. Images were acquired and stored using a Nikon Eclipse TE300 microscope (with 40× oil immersion objective) and CCD (CoolSNAP HQ_2) camera under computer control (NIS Element, Nikon). The data are shown as fluorescence unit. The perfusion solution (35 °C) used for measurement of SF7 signal contained (mM): 118 NaCl, 23 $NaHCO_3$, 2 KH_2PO_4, 3 KCl, 1 $MgCl_2$, 1.2 $CaCl_2$, 11 glucose.

2.5. Hypoxia studies

Cell-attached patches were formed on glomus cells and perfused with a bicarbonate-buffered solution containing (mM) 117