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THE IMPACT OF HYDROGEN SULFIDE (H₂S) ON NEUROTRANSMITTER RELEASE FROM THE CAT CAROTID BODY

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Abstract

Do cat carotid bodies (CBs) increase their release of acetylcholine and ATP in response to H₂S? Two CBs, incubated in a Krebs Ringer bicarbonate solution at 37° C, exhibited a normal response to hypoxia -- increased release of acetylcholine (ACh) and ATP. They were challenged with several concentrations of Na₂S, an H₂S donor. H₂S, a new gasotransmitter, is reported to open K_{ATP} channels. Under normoxic conditions the CBs reduced their release of ACh and ATP below control values. They responded identically to pinacidil, a well-known K_{ATP} channel opener. CB glomus cells exhibited a positive immunohistochemical signal for cystathione-β-synthetase, a H₂S synthesizing enzyme, and for a subunit of the K_{ATP} channel. The data suggest that Na₂S may have opened the glomus cells' K_{ATP} channels, hyperpolarizing the cells, thus reducing their tonic release of ACh and ATP. Since during hypoxia H₂S levels rise, the glomus cells responding very actively to hypoxia may be protected from over-exertion by the H₂S opening of the K_{ATP} channels.

Keywords

Hydrogen sulfide; Carotid body; Glomus cell; ATP; Acetylcholine; K_{ATP} channel; Neuroprotection

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1. Introduction

The carotid body (CB) is an extremely important polymodal sensor of arterial blood in most vertebrates. When arterial oxygen tension, pH, and blood glucose decrease, or arterial carbon dioxide tension increases, the neurotransmitter-containing glomus cells in the CB depolarize. This results in the exocytosis of neurotransmitters and neuromodulators into the synaptic-like cleft between these cells and the abutting sensory afferent nerves. The released neural agents bind to the appropriate receptors both on the nerve and back onto the glomus cells. The action potentials created in the sensory afferent nerve by this action make their way to the nucleus tractus solitarius in the medulla where they are distributed to various loci in the CNS. Multiple cardiopulmonary, endocrine, and renal reflex responses ensue (Fitzgerald and Shirahata, 1997).

Some years ago it was demonstrated that the gasotransmitter, nitric oxide (NO), reduced the hypoxia-induced increase in cat CB neural output (ZZ Wang, et al., 1994a, 1994b). This laboratory reported that from the incubated cat CBs L-arginine- or sodium nitroprusside-induced NO reduced the hypoxia-induced increase in the release of acetylcholine (ACh), a generally accepted excitatory neurotransmitter in the CB (Fitzgerald, et al. 2005a, 2005b).

Recently a new gasotransmitter, hydrogen sulfide (H₂S), was reported to have effects on vascular smooth muscle similar to the effects of hypoxia. Currently, there is an abundance of literature reporting both the relaxing effects of H₂S on vascular smooth muscle (Kimura, 2005, 2009) and H₂S's protective effects on neural tissue (Abe and Kimura, 1996; Barannano, et al., 2001; Boehning and Snyder, 2003; Kimura, 2002, 2005). Indeed, H₂S has been reported to exist at 50–160 μM levels in the CNS (Hughes, et al., 2009; Savage and Gould, 1990; Warenycia, et al., 1989). H₂S also regulates the release of corticotropin-releasing hormone from the hypothalamus (Dello Russo, et al., 2000). This makes it possible that H₂S may modify the release of neurotransmitters (Kimura, 2002).

Given the above, the purpose of the present study was to see if, like hypoxia, H₂S affected the release of either ACh or a second excitatory neurotransmitter in the cat CB, ATP. Though there is much literature on the effect of H₂S on vascular smooth muscle and some recent reports on airway relaxing plus anti-inflammatory effects (Chen, et al., 2009; Fitzgerald, et al., 2010), to our knowledge the effect of H₂S on CB behavior in the cat has never been reported. The question, then, is: “What effect does H₂S have on neurotransmitter release from the specific oxygen sensor, the CB?”

2. Materials and methods

2.1 Carotid body harvesting and preparation for incubation

Male and female cats (2–3 kg) were sedated with 25–30 mg/kg ketamine, ip. Subsequently they were given 25 mg/kg pentobarbital (iv). Two iv injections (2 ml each) of 1 mM tetraisopropylphosphoramidate (isoOMPA; 100 μM/kg per injection, i.v.), a non-specific cholinesterase inhibitor in a Krebs-Ringer bicarbonate solution (KRB), were given. This was followed by a 2000 IU/kg injection of heparin in KRB. Composition of the KRB was as follows (in mM): NaCl, 112; KCl, 4.7; CaCl₂, 2.2; MgCl₂, 1.1; glucose, 5; NaHCO₃, 22. After preparation of the neck area the animals were sacrificed with an injection of pentobarbital (50 mg/kg), and the heads rapidly removed. Bilateral common carotid arteries were perfused with 100 μM neostigmine KRB to remove blood from the CBs. Procedures were approved by the University's Animal Care and Use Committee which follows Federal Guidelines. Carotid bifurcations were removed rapidly and placed in ice-cold 100 μM neostigmine KRB. Fat and connective tissue were removed. The CBs were then placed in

250 μ L of 10 μ M neostigmine KRB at 37° C, bubbled with 30% O₂/5% CO₂, and allowed to recover from surgical procedures for 20 min.

2.2. Immunohistochemical techniques

For immunohistochemical determinations the CBs, immediately after recovering from surgical procedures, were placed in 1 mL of a zinc fixative and incubated at room temperature overnight. They were then embedded in paraffin blocks and remained at room temperature for four days. Sections (5 μ m) of the CB were prepared for immunohistochemical staining in the following manner: (1) The CB sections were deparafinized with repeated xylene and alcohol; (2) CBs were next treated with citric acid to reactivate the antigen; (3) CBs were washed with PBS; (4) Next the sections were incubated with AvidinD and Biotin in steps with PBS washes; (5) endogenous peroxidase activity was quenched with 30% H₂O₂; (6) Nonspecific blocking was accomplished by incubating the sections with normal serum from the goat for 1 hr at RT; (7) Primary antibodies for tyrosine hydroxylase (TH), cystathionine- β -synthetase (CBS), and the KIR 6.1 subunit of the K_{ATP} channel (All antibodies were made in rabbit; concentration was 6 μ g/mL of phosphate buffered saline) were applied to the sections; (8) Sections were incubated for 48 hours at 4°C; (9) After PBS wash sections were incubated in the secondary antibody (goat-anti-rabbit) for 24 hours at 4° C; (10) After PBS wash the chromagen (Vecta-SG-Stain from Vector Laboratories, Inc. Burlingame, CA 94010) was applied for 30 min at room temperature; (11) After PBS wash 3 drops of SG in peroxide solution was applied for 90 sec followed by an H₂O wash. Sections upon drying were cover-slipped. The negative controls were manufactured by incubating the sections with IgG but no primary antibody under the same conditions as above before application of the secondary antibody and chromagen.

Figures 9 was taken with an Olympus BX51 W1 microscope using an Olympus PLAN system at 20x/0.40 magnification numerical aperture with a QImaging Retiga-SRV Fast 1394 camera; Hamamatsu Simple PCI Version 6.5.1 was the acquisition software, and Adobe Photoshop 7.1 was used for image cropping and layout.

2.3. Protocol for incubation

The protocol for the incubation studies to determine the impact of hypoxia and H₂S on the release of the excitatory neurotransmitters, ACh and ATP, had three stages: Control (CTL), Challenge (CHL), and Recovery (RCV). For detection of acetylcholine (ACh) released into the KRB incubation medium the CBs were first situated in the incubation medium bubbled with 30% O₂/5% CO₂ for 6 min. This constituted CTL. During the hypoxic CHL the medium-containing CBs was bubbled with 6% O₂/5% CO₂ for 6 min. For RCV the CTL conditions were repeated for 6 min. For an H₂S CHL the CTL and RCV exposures were as stated above. For the H₂S CHL Na₂S, a commonly used donor of H₂S in solution, was dissolved in KRB in concentrations of 1, 10, 20 nM, 5 μ M, and 100 μ M. This solution was bubbled with 30% O₂/5% CO₂ for 6 min. To measure ATP release the same format was followed but exposure durations were 3 min.

2.4. Measurement of ACh and ATP

Basic high-pressure liquid chromatography-electrochemical detection (HPLC-ECD) methodology was used to determine ACh in 20 μ L samples of the incubation medium. Equipment used was the Bioanalytical Systems PM 80 (Bioanalytical Systems, Inc., 2701 Kent Ave., West Lafayette, IN 47906) unit with microbore settings; details have been presented previously (Fitzgerald, et al., 1999). For ATP measurement in 100 μ L of incubation medium the luciferin-luciferase reaction (Enliten ATP Assay System Bioluminescence Detection Kit for ATP, Promega Corporation, Madison, WI 53711-5399)

was used with the Lumat LB9507 luminometer (Berthold Technologies, Oak Ridge, TN 37830).

2.5. Statistical Analysis

Data (both Amounts and Percentages of Control) are presented as Mean $\pm$ SEM. Statistical analysis of the data in Figures 1, 2, 3, 5, 6, 7, and 8 was performed using a One-Way Repeated Measures ANOVA if the data passed the Normality and Equal Variance tests. Post-hoc testing was done with the Tukey test (All-Pairwise Multiple Comparisons procedure). Software was provided by the Sigma Stat programs (Jandel Scientific, SYSTAT SOFTWARE, Inc., Port Richmond, CA 94804-2028). If the data did not pass the Normality and Equal Variance tests, a Friedman's One-Way Repeated Measures ANOVA on Ranks was performed (Friedman, 1937). Post-hoc testing was done using the Tukey tests. But if the sample size was small, the post-hoc testing used the Monte-Carlo Resamplings procedure. Infrequently a Student's paired T-test was used to test one particular comparison. For Figure 4 the regression analysis tried several models, linear, piecewise linear, and spline (De Boor, 1978) (Software provided by R Development Core Team, 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.).

3. Results

3.1. First measurements

In spite of the abundance of literature showing that H_2S relaxed vascular smooth muscle in several species, it was not clear what concentration of H_2S , or an appropriate precursor, would be necessary to see an effect on the cat CB variable we were using, ACh and ATP release into an incubation medium. Because of this gap, we used a broad spectrum of concentrations of the precursor, Na_2S (1 nM to 100 μM). The experiments were carried out using 17 different pairs of CBs. Figures 1A and 1B present the results of all 34 tests (including all concentrations of Na_2S) of the impact on ACh release, both the raw data (1A) and the data normalized to their respective controls (1B). Friedman's Repeated Measures ANOVA on Ranks (RMANOVAOR) revealed that for both 1A and for 1B the global $P = < 0.001$. All pairwise multiple comparison procedures (Tukey Test) generated the following results: CTL vs Na_2SCHL , RCV vs Na_2SCHL , CTL/CTL vs Na_2SCHL/CTL , RCV/CTL vs Na_2SCHL/CTL – all $P < 0.05$. Control vs Recovery, both raw and normalized values were not significant.

Figures 2A and 2B demonstrate that ATP release was also reduced in the 29 trials (ATP was not measured in the first two sets of CBs). Friedman's RMANOVAOR and Tukey Tests generated the same results as in Figures 1A and 1B.

3.2. Normal functioning of these CBs

Interspersed among the above trials were hypoxic challenges to assure that these CBs were behaving normally (Figs 3A, 3B). That is, we and others (Fitzgerald, et al., 1999; Iturriaga and Alcayaga, 2004; Nurse, 2005; Shirahata, et al., 1996; Varas, et al., 2003; Zhang, et al., 2000) have reported that hypoxia increases the release of ACh and ATP from the CBs. In this study where cat CBs were challenged with Na_2S in 17 experiments, the impact of hypoxia on ACh and ATP release was tested once in each of the following 15 experiments: experiments 3–17. For ACh release (Fig. 3A) Friedman's RMANOVAOR and Tukey Tests were performed. Again the global $P = < 0.001$, and the hypoxia challenge was significantly greater ($P < 0.05$) than either control or recovery while control did not differ from recovery. This was true for both raw and normalized data.

For ATP release (Fig. 3B) results differed somewhat. In an earlier measurement of the impact of hypoxia on ATP release (where “n” was 18) the Friedman’s RMANOVAOR and Tukey Tests gave significant results. But here, matching these 15 ATP experiments with the 15 ACh experiments, the above tests gave $P = 0.074$. However, with these same data the paired t-Test of control *vs* challenge was significant ($P = 0.029$); recovery *vs* challenge, $P = 0.048$; control *vs* recovery, $P = 0.124$. Hence, a new analysis, more sensitive to a smaller number of observations, was employed. The Friedman’s RMANOVAOR was used, but the post-hoc testing was done not with the Tukey Test but with the Monte-Carlo Resamplings procedure. For control *vs* challenge $P = 0.044$; for challenge *vs* recovery, $P = 0.160$; for control *vs* recovery, $P = 0.800$. The cat CBs appeared to be functioning normally in response to hypoxia.

3.3.Dose-Response Relationship?

Following the observation that Na_2S did significantly lower the release of both ACh and ATP from the cat CBs, and establishing that the cat CBs in these 17 experiments were functioning normally, an effort was made to detect a dose-response relationship. The scatter plot of the ATP results in Figure 4 has as the independent variable the concentration (dose) measured in nanomoles of Na_2S so that all concentrations could be put on the same scale. The units of the scale are in natural log units of nM ($5\mu\text{M}$ becomes 5000nM ; $\ln$ of 5000 is 8.52). The dependent variable (response) is the percent ACh (or ATP) release was reduced from control. To integrate the points in a linear regression model was tried first; it was not significant. Next tried was a Natural Cubic Spline regression model. The curve integrating the points is significant ($P = 0.036$). As the concentration of Na_2S increased, so also did the percent reduction in ATP released increase slightly up to a point. Then it began to decrease. This phenomenon will be addressed in the Discussion.

For ACh release none of the regression models tried was significant; for the Natural Cubic Spline regression model $P = 0.530$, though the same curvilinearity was suggested.

3.4 Response to 100 μM Na_2S

Next to be singled out were the responses to separate concentrations of Na_2S , proceeding from high to low concentrations. In six of the experiments where only $100\mu\text{M}$ Na_2S was used, data was evaluated with Friedman’s RMANOVAOR; global $P = 0.052$. Since the sample size was small, the post-hoc P values were evaluated using the Monte-Carlo Resamplings procedure. Control *vs* challenge, $P = 0.022$; recovery *vs* challenge, $P = 0.052$; recovery *vs* control, $P = 0.975$. Figure 5 reveals that at this concentration the effect of Na_2S was to reduce the release of ACh (under relatively normoxic conditions [$30\%\text{O}_2/5\%\text{CO}_2$]) to $69.3 \pm 7.9\%$ of the control value. However, in the same six experiments this concentration of Na_2S did not produce a statistically significant change in ATP release.

3.5. Response to 5 μM Na_2S

In five of the experiments a $5\mu\text{M}$ Na_2S concentration in the incubating KRB ($30\%\text{O}_2/5\%\text{CO}_2$) generated a significant reduction in the release of ACh from 3.01 ± 0.860 to 1.58 ± 0.568 pmoles/ $20\mu\text{L}$ KRB (Figure 6), a 51.2% reduction. Friedman’s RMANOVAOR and Tukey Testing of the raw data (Fig 6A) generated a global $P = 0.026$; control *vs* challenge, $P = 0.025$; recovery *vs* challenge, $P = 0.089$, recovery *vs* control, $P = 0.683$. Testing the normalized data (Figure 6B) produced virtually identical results. The values for ATP release again showed significant variability resulting in no significant change in ATP release under conditions of this challenge.

3.6. Response to low concentrations of Na₂S

Next the analysis of a series of three Na₂S challenges at 1, 10, and 20 nM concentrations in four experiments was performed. Since Friedman's RMANOVAOR of these three low concentrations with an "n" of only 4 did not produce a significant change, the three low concentrations were grouped. Figure 7A presents the normalized control, challenge, and recovery values for ACh release at these three low concentrations. The data passed the Normality and Equal Variance tests. The standard RMANOVA produced a significant change ($P = 0.002$). Tukey Testing yielded: CTL/CTL vs Na₂SCHL/CTL, $P = 0.013$; RCV/CTL vs Na₂SCHL/CTL, $P = 0.002$; RCV/CTL vs CTL/CTL, $P = 0.648$.

The release of ATP in response to these very low concentrations of Na₂S in the four experiments was always less but never significantly so. However, when combining the release values for three low concentrations of Na₂S (1, 10, 20 nM) in the four experiments for CTL, CHL, and RCV, the decrease in CHL reached statistical significance. Figure 7B presents the normalized data. Again the data passed the Normality and Equal Variance tests. One-Way RMANOVA produced a global $P = 0.041$. Tukey Tests resulted in: CTL/CTL vs Na₂S CHL/CTL, $P = 0.348$; RCV/CTL vs Na₂SCHL/CTL, $P = 0.032$; CTL/CTL vs RCV/CTL, $P = 0.407$. The CTL/CTL vs Na₂S CHL/CTL value was somewhat puzzling since 9/12 values were less with 2/12 being ~100% CTL. Further, a Paired t-Test gave this individual comparison a significant value ($P = 0.030$).

3.7. Response to Pinacidil, K_{ATP} channel opener

To determine if the Na₂S might be functioning in the incubated cat CBs by opening K_{ATP} channels, its role in the relaxation of vascular smooth muscle, the CBs were incubated with low concentrations of pinacidil (nM). Pinacidil is a well recognized K_{ATP} channel opener. Application of a One-Way RMANOVA or a Friedman's RMANOVAOR and Tukey Testing or Monte-Carlo Resamplings post-hoc comparisons produced statistical significance between CTL/CTL vs CHL/CTL and RCV/CTL vs CHL/CTL for both ACh and ATP. Figure 8A presents the normalized data of a decrease in ACh from 9.58 ± 1.65 to 6.87 ± 1.01 pmoles ACh/20 μ L KRB. The reduction in ATP (Figure 8B) is even greater (111.82 ± 35.16 to 26.03 ± 5.92 fmoles ATP/100 μ L KRB).

3.8. Components needed for CB genesis and detection of H₂S

Finally to determine if the components for endogenously generated H₂S were available and if the K_{ATP} channel was present in the cat CB, immunohistochemical studies were carried out. Figure 9 (A, B, C, D) presents the results of testing for TH, the commonly accepted marker for glomus cells in the CB (A), for CBS, an H₂S synthesizing enzymes found mostly in neural tissue (B), for the KIR 6.1 subunit of the K_{ATP} channel (C), and a negative control, rabbit IgG (D). Sections were made from the same CB. The signal for TH is much stronger than for CBS and for KIR6.1. However, the pattern of the signal in A, B, and C is similar. Glomus cells are gathered in clusters (glomeruli) in which the individual glomus cell has a large nucleus occupying >75% of the volume of the cell. Nuclei appear as unstained or lightly stained (arrows indicate glomus cells in figures). Signal is least strong in the KIR 6.1 sections, but still clearly present. Some nerves also seem to be stained in this section. The negative control was the same for panels A, B, and C. Panels C and D exhibit some artifactual staining on the periphery of the section.

4. Discussion

4.1. Summary of Results

These data are the first to describe the impact of a donor of this new gasotransmitter, H₂S, on the operations of the cat CB. The experiments showed: (1) A broad spectrum of

concentrations of the H₂S donor, Na₂S, significantly reduced the release of both ACh and ATP from 17 pairs of cat CBs. (2) The cat CBs involved in this study were responding to hypoxia in the same manner as they have previously been reported to do. (3) Regression analysis produced a statistically significant fit of all ATP data in the scatter plot to a Natural Cubic Spline Regression model. The ACh data did not fit to any regression model. (4) But ACh release in response to 100 μ M Na₂S was significantly less. The same concentration did not significantly change ATP release. (5) ACh release was significantly depressed in response to 5 μ M Na₂S, whereas ATP release was not consistently depressed. (6) When the results of the three lowest concentrations of Na₂S used (1, 10, 20 nM) were combined, the release of ACh was significantly depressed; a paired t-Test showed that the combined results of these concentrations of Na₂S significantly depressed ATP release below control value. (7) Pinacidil, the K_{ATP} channel opener, significantly reduced both ACh and ATP release, suggesting the possibility of the presence of K_{ATP} channels in the cat CBs' glomus cells, where the neurotransmitters are stored. (8) Immunohistochemical studies revealed the presence of an H₂S synthesizing enzyme, cystathionine- β -synthetase, as well as the presence of a subunit of the K_{ATP} channel in the cat CB's glomus cells.

4.2. Critique of the Preparation

We have used this preparation (incubation of harvested cat CBs in a constant temperature water bath) for several years. And once again, the response of the CBs to a hypoxic challenge (increased release of ACh and/or ATP) occurred (Fig 3), as previously reported by ourselves and others (Fitzgerald, et al., 1999, 2005a, 2005b; Iturriaga and Alcayaga, 2004; Nurse, 2005; Shirahata, et al., 1996; Varas, et al., 2003; Zhang, et al., 2000). We therefore have no reason to believe that the CBs treated with Na₂S in the same manner as they have been treated with hypoxia in previous studies and in the present study would respond to the Na₂S challenge in the way they did due to some feature of the tissue preparation or the above-described set-up.

Each of the several preparations used to study the carotid body has strengths and limitations. Time is a critical factor with this preparation. What amount is appropriate for the best measurement of ATP? The molecule must be released from the glomus cells, diffuse through the surrounding tissues and out into the incubation medium. Certainly enzymatic degradation is occurring in this process. ACh must do the same, but enzymatic degradation of ACh was attenuated or perhaps even abolished with the neostigmine. We did not use an inhibitor of 5' ectonucleotidase. Further, the system for ATP measurement is, according to the manufacturer, very sensitive to salts. And with this system we found that ATP standards dissolved in KRB measured only about 23% of the value for the same standards dissolved in ATP-free water.

Additional to the limitations imposed by the measuring system itself, the smaller amounts of ATP released could be due to a smaller amount available for release or the shorter time allowed in the interests of avoiding an even greater enzymatic degradation. However, in this study we found that three minutes was a sufficient amount of time to measure a significant increase in the ATP response to hypoxia. Thus, the collected data do not suggest the preparation and technique are the source of the Na₂S-generated reduction in ACh and ATP release compared to their respective controls.

4.3. Critique of/Comments on Data

The failure of the Na₂S-generated decrease in ATP to reach statistical significance on several occasions could in part be due to the rather large variability in the control (CTL) values. With the use of 5 μ M Na₂S (Figure 6) the CTL values were 44.25 $\pm$ 15.19 fmoles ATP/100 μ L, a large SEM. Further, in our previous reports the variability of ATP

measurements with this preparation tended to be larger than with ACh measurements. Additionally, the number of measurements was limited. This could affect the statistical evaluation, as seen when the effect of the three low concentrations of Na₂S in the four experiments on ACh and ATP were combined. This increase in sample size to 12 resulted in statistical significance.

Finally, ATP is known to be co-released with ACh. But given the extensive heterogeneity of the 100,000 neurotransmitter-containing glomus cells in the cat CB (Gonzalez, et al., 1994), reported by both McDonald (1981) and Heath and Smith (1992), the two transmitters just might not be released in exactly a parallel or identical fashion.

Insofar as nM concentrations of Pinacidil, the recognized K_{ATP} channel opener, had the same effect on ACh and ATP release as Na₂S, the data suggested that the Na₂S/H₂S molecule might well be working to open the K_{ATP} channels in the cat CBs' glomus cells. Much is currently understood as to how this channel operates (Jiang, et al., 2009; Liss and Roeper, 2001). As the concentration of Na₂S increased, the percent *reduction* of ATP released into the incubation medium *slightly increased* up to a point. This could mean that the Na₂S was opening an increasing number of K_{ATP} channels, tending to hyperpolarize more of the neurotransmitter-containing glomus cells, and reducing the release of ACh and ATP. As the concentration of Na₂S increased further, it could be that other K⁺ channels were being blocked (cf. below), tending to depolarize the glomus cells. This would offset the hyperpolarizing impact of Na₂S via the K_{ATP} channel, and perhaps start to depolarize the glomus cells. Such an action would tend to release ACh and ATP, reducing the percent decrease from control. Such a pattern could be behind the natural cubic spline regression.

4.4. H₂S Data from Studies Pertinent to the Interpretation of the Data from this Study

This must begin with an appreciation of the fact that H₂S is found in a wide variety of vertebrate tissues. Some investigators (Hosoki, et al., 1997; Savage and Gould, 1990; Warenycia, et al., 1989) have reported H₂S at a level of 50–160 μM in a homogenate of brain tissue. But later studies question such high amounts and the amount remains a controversial issue (Olson, 2009; Kimura, 2009).

4.4.1. H₂S in vascular tissue—A review of the H₂S literature reveals a significant amount of study of the impact of H₂S on vascular tissue over the last 15–20 years where it seems to mimic hypoxia's relaxing action on the vasculature (Kimura, 2009; Kimura, et al., 2005; Olson, et al., 2006). The abundant literature, contributed to and reviewed by Kimura and Wang (Kimura, et al., 2005; Kimura, 2009; Wang, 2002, 2003, 2010), finds that H₂S functions as a vascular smooth muscle relaxant. However, Lim, et al. (2008) report a vasoconstrictive action of H₂S in vascular smooth muscle. The multiple studies of Olson and his colleagues find a biphasic, even multiphasic, action of relaxing and constricting, depending on the vascular smooth muscle involved (Olson, 2005, 2008; Olson, et al., 2006; Olson and Whitfield, 2010).

4.4.2. H₂S influences neural tissue—In neural tissue H₂S seems to have multiple roles. It can inhibit synaptic transmission (blocking sEPSPs), but it can also enhance NMDA receptor-mediated responses, and facilitate long term potentiation in the hippocampus (Abe and Kimura, 1996; Kimura, 2002). Further, it seems to function as an overall neuromodulator in the brain (Abe and Kimura, 1996; Kimura, 2002). The recent study of Peng, et al. (2010) using preparations in which one of the endogenous synthesizing enzymes, cystathionine-γ-lyase, is blocked, reports that H₂S stimulates neural output from the CBs in rat and mice.

Insofar as it is commonly agreed that the principal, if not exclusive, mode of CB excitation involves the glomus cell release of excitatory neurotransmitters which attach to appropriate post-synaptic cholinergic and purinergic receptors on the sensory afferent fibers abutting the glomus cells, the Peng et al. (2010) set of data would seem to be at variance with the data from this study in the cat. The release of those stimulating transmitters could be the result of H₂S blocking the K_{Ca} channel and depolarizing the glomus cell (Li, et al., 2010, Telezhkin, et al., 2009), or H₂S opening the L-type Ca⁺⁺ channel in the glomus cell (Garcia-Bereguian, et al., 2008).

Given the above CB studies, which seem at least partially to be contradictory, can they somehow be resolved? A key consideration is to recall that H₂S acts on a wide variety of ion channels sometimes with opposing effects (Tang, et al., 2010). In the vasorelaxant studies the action proposed was the opening of K_{ATP} channels. This allowed the escape of K⁺ ions, the hyperpolarization of the smooth muscle cells, and the relaxation of the muscle. The constrictive action in the Lim, et al. (2010) study finds that blocking the K_{ATP} channel with glibenclamide did not attenuate the vasoconstrictive effect. The effect was attributed to an alteration in the adenylyl cyclase/cAMP pathway. However, the important study of Telezhkin, et al. (2009) reported that H₂S inhibits human BK_{Ca} channels. They also showed both H₂S synthesizing enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS), in HEK 293 cells and in the rat CB. Inhibiting the BK channel would depolarize the cell and allow the entry of extracellular calcium. This could promote a vasoconstrictive effect, and in the CB the release of neurotransmitters from the glomus cells. But the recent study of Sitdikova, et al. (2010) finds an opposite effect on the BK channels, namely, that H₂S increases calcium-activated potassium channel activity in rat pituitary tumor cells.

This brief overview of the several effects of H₂S on vascular and neural tissue would seem to make a definitive interpretation of the mechanisms involved in our cat CB study extremely difficult, if not impossible, at present.

Perhaps the behavior of the vascular smooth muscle to H₂S and the role of the K channels in this behavior is a function of the distribution, density, orientation, and affinity of the K_{ATP} and K_{BK} channels in the different tissues. On the other hand, the behavior might also involve H₂S impacting a cascade totally outside the realm of K channels.

Regarding CB studies, Shirahata and her colleagues (Balbir, et al., 2007) obtained virtually contradictory ventilatory, neuroanatomical, and neurophysiological results from two different inbred strain of mice, apparently related to the subunit composition of the K_{BK} channel. The above reports suggest that extrapolation from one tissue to another or from one species to another can only be done with great care, or perhaps not at all.

Previously Chou and Shirahata (1996) have shown in patch clamp studies that the cat glomus cells do not exhibit behavior consonant with the presence of K_{BK} channels. Since the H₂S-synthesizing enzyme, cystathionine-β-synthetase (CBS), is said to be found primarily in the central nervous system, it is understandable that Peng et al. (2010) make no mention of it in the CBs of their mice and rats. However, Telezhkin et al. (2009) found CBS in the CBs of rats, and Li et al. (2010) did so in mice. And since the CB is a neural crest derivative we were not surprised that CBS was found in the cat CB (Figure 9). After detecting the presence of CBS in the cat CB (Figure 9B) and recording the functional results of Na₂S, our study looked for and found evidence of the K channel thought to be involved in H₂S neural actions, the K_{ATP} channel (Figure 9C). Hence, it seems reasonable to expect CB neural output to depend on the presence, type, density, distribution, orientation, and affinity of the various K channels in the structure of that species or strain. Differences in data may be due to variability in K channels and their characteristics. Though there is evidence that

K_{Ca} channels are not operating in the cat CB (Chou and Shirahata, 1996), there is yet to appear a report that K_{ATP} channels are present and/or operate in rat and mice CBs.

4.4.3. Interaction of H₂S and NO, a second CB neural output attenuator—Since we have reported that NO also reduces the release of ACh, the interaction of NO with H₂S should be considered. But any detailed consideration of such goes beyond the purview of this report. In brief, interacting NO and H₂S creates opposing effects (Hosoki, et al., 1997; Zhao, et al., 2001; Zhao and Wang, 2002). How NO and H₂S might interrelate in the cat carotid body remains to be explored.

Finally, H₂S is an endogenous reducing agent, and is produced by oxidative stress. H₂S alone increases the levels of the reduced form of glutathione (GSH), which can protect cells from oxidative stress. It seems more likely that H₂S's function as an antioxidant is via the glutathione pathway. As such it could function as a neuroprotective agent in the highly active glomus cells during a hypoxic challenge, when [H₂S] increases (Olson, 2008, Olson and Whitfield, 2010).

The above complexity surrounding the action of H₂S – multiple channels involved, species of animal, type and condition of tissue, etc. – would seem to preclude the possibility that a linear dose-response curve would capture all the data. Further complicating the matter is the sulfhydrating potential of H₂S. Sulfhydration of proteins changes their biological activity (Mustafa, et al., 2009), and the number of proteins involved in CB chemoreception is considerable.

4.4.4. Possible role for H₂S action in the cat CB—With caution, then, we propose that our Na₂S data lends itself to the K_{ATP} channel activation mechanism. Under normoxic control conditions a certain amount of ACh and ATP is tonically released from the glomus cells. The inclusion of Na₂S under the same normoxic incubation conditions might open the K_{ATP} channels in the glomus cells, hyperpolarizing them. This would attenuate the release of ACh and ATP. In some systems hypoxia increases the level of H₂S (Olson, 2008, Olson and Whitfield, 2010). So whereas hypoxia blocks several K⁺ channels (leak, voltage-dependent, as well as BK-maxi) to depolarize the glomus cells and allow the entry of extracellular calcium, precipitating an increased release of neurotransmitters, H₂S does the opposite; in its hyperpolarizing capacity it could attenuate the depolarization. This suggests a possible role for H₂S and K_{ATP} channels in the cat CBs (Figure 10), a negative feedback mechanism to balance electrical signaling and energy consumption. Although K_{ATP} channels are opened when the intracellular ATP/ADP ratio falls (e. g., during metabolic stress and energy demand, hypoxia), this is not always the case. K_{ATP} channels can be opened in a non-ATP-dependent way (Muller et al., 2002; Zhao and Wang, 2002; Zhao et al., 2001). Since our cat CB experiments were executed in 30% oxygen, there was probably little stress on the structures and much attenuated metabolic loss of ATP, if any.

H₂S operating in the CB is an entirely new concept, and presents an interesting as well as a challenging field for future investigation. Future results might suggest some new strategies for the management of CB activity in such clinical and public health pathologies as Obstructive Sleep Apnea and Chronic Heart Failure.

In fact the beneficial cardiovascular effects of dietary garlic are thought to be due to the H₂S contained therein (Lefer, 2007). And some therapeutic uses of H₂S have already been proposed (Bannenberg and Vieira, 2009; Chen, et al., 2007).

5. Conclusions

This study has revealed that several concentrations of Na₂S, a common donor of H₂S, reduce the release of ACh from incubated cat CBs under normoxic conditions. The release of a second excitatory neurotransmitter, ATP, in this preparation is less consistent, but clearly present. Though it appears this effect may be due to the opening of glomus cells' K_{ATP} channels, the fact that H₂S can act on other ion channels, including other K channels (e.g., BK channels) with opposing effects, sometimes dependent on the concentrations involved, may account for the Cubic Spline Regression rather than the more classic linear dose-response curve.

The phenotypic response of the many tissues responsive to H₂S probably depends critically on the presence, type, density, distribution, orientation, and affinity of the various K channels, as well as the prevalence of L-type calcium channels in a particular tissue.

The fact that the components for generating and sensing H₂S were found in the cat's glomus cells supports the notion that H₂S may play a negative feedback, modulatory role during the CB's high activity under hypoxic conditions.

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FIGURE 1.

Amount of (A) and Percent of (B) the amount of ACh (normalized to the control amount) recovered from the incubation medium of two incubated cat carotid bodies during the control, Na_2S challenge, and recovery stages (Mean $\pm$ SEM). Seventeen pairs of CBs (from 17 animals) were used in 34 trials. CTL: control; CHL: challenge; RCV: recovery. Concentrations of Na_2S , a precursor of H_2S , ranged from 1nM to 100 μM . ANOVA and Tukey tests showed in both A and B that the challenge was significantly less than both control and recovery ($P < 0.05$). Recovery was statistically indistinguishable from control. * = significantly less than CTL

FIGURE 2.

Impact of Na_2S on the release of ATP from the same CBs as in Figure 1. Both the actual value and the value normalized to the control value of the 29 trials are presented. ANOVA and Tukey tests showed in both A and B that the challenge was significantly less than both control and recovery ($P < 0.05$). As above recovery did not differ from control. * = significantly less than CTL

FIGURE 3.

Impact of hypoxia on these CBs, tested 15 times throughout the trials cited above in Figures 1 and 2. ANOVA and post-hoc tests showed in both A and B that the challenge was significantly greater than both control and recovery ($P < 0.05$ for ACh; $P = 0.044$ for ATP). In both A and B recovery did not differ from control. * = significantly greater than CTL

FIGURE 4.

Regression Analysis for the ATP data. This Natural Cubic Spline Regression model significantly ($P = 0.036$) integrates the data points (solid line). Dashed lines represent 95% confidence limits.

FIGURE 5.

Impact of 100 μM Na_2S on ACh release into the incubation medium from two cat carotid bodies in six different experiments. CTL = control; CHL = challenge; RCV = recovery. ANOVA and post-hoc testing showed in both A and B that the challenge was significantly less than both control ($P = 0.022$) and recovery ($P = 0.052$). In both A and B recovery did not differ from control ($P = 0.975$). * = significantly less than CTL

FIGURE 6.

Impact of 5 μ M Na₂S on ACh release into the incubation medium from two cat carotid bodies in five different experiments. ANOVA and post-hoc testing showed in A that the challenge was significantly less than the control ($P = 0.025$) but not the recovery ($P = 0.089$). In A recovery did not differ from control ($P = 0.683$). In B challenge was significantly less than control ($P = 0.019$) but not the recovery ($P = 0.262$). Recovery did not differ from control ($P = 0.222$). * = significantly less than CTL

FIGURE 7.

Impact of combining the CTL, CHL, and RCV values of the three low concentrations of Na₂S (1, 10, 20 nM) on ACh (A) and ATP (B) release into the incubation medium from two cat carotid bodies in four different experiments (n=12). ANOVA and post-hoc testing of the normalized values showed in A that challenge was significantly less than either control (P = 0.013) or recovery (P = 0.002). Recovery did not differ from control (P = 0.648). In B challenge was significantly less than recovery (P = 0.032) but not control (P = 0.348). However, a paired t-Test of this particular comparison showed challenge to be significantly less (P = 0.030). Recovery did differ from control (P = 0.407). * = significantly less than CTL

FIGURE 8.

Impact of low concentrations of Pinacidil on ACh and ATP release into the incubation medium from two cat carotid bodies in six experiments. Values normalized to the control value. CTL, CHL, RCV as above. ANOVA and post-hoc testing showed that for ACh release challenge was less than either control ($P = 0.052$) or recovery ($P = 0.020$). Control did not differ from recovery ($P = 0.948$). For ATP release challenge was less than either control ($P = 0.009$) or recovery ($P = <0.001$). Control did not differ from recovery ($P = 0.191$). * = significantly less than CTL

FIGURE 9.

A. Positive immunohistochemical signal for the antibody to tyrosine hydroxylase, the commonly accepted marker for glomus cells in the CB. The diameter of glomus cells is approximately 10 μ m. Nuclei are large; arrows designate several glomus cells having either unstained or lightly stained nuclei. B. Signal for cystathionine- β -synthetase, an H₂S synthesizing enzyme found in neural tissue; seen here in the glomus cells of the cat carotid body; again arrows designate several glomus cells having either unstained or lightly stained nuclei. C. Positive immunohistochemical signal for the antibody to the KIR 6.1 subunit of the K_{ATP} channel in the glomus cells of the cat carotid body; arrows, as above. D. The negative control, made with goat IgG free of any positive antibody.

FIGURE 10.

Drawing of the glomus cell of the cat carotid body with two oxygen-sensitive K⁺ channels whose conductance is reduced/blocked by hypoxia (Leak and Voltage-dependent), producing an increase in the intracellular [K⁺], activating voltage gated Ca⁺⁺ channels, and ultimately producing the release of glomus cells' neurotransmitters. The K_{ATP} channel, thought to be opened by H₂S, would allow K⁺ ions to escape and attenuate the depolarizing effects of hypoxia. This would attenuate the release of the glomus cells' store of neurotransmitters. Hypoxia is reported to increase [H₂S] in tissues.