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Non-additive interactions between mitochondrial complex IV blockers and hypoxia in rat carotid body responses

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Abstract

The metabolic hypothesis of carotid body chemoreceptor hypoxia transduction proposes an impairment of ATP production as the signal for activation. We hypothesized that mitochondrial complex IV blockers and hypoxia would act synergistically in exciting afferent nerve activity. Following a pretreatment with low dosage sodium cyanide (10-20 μ M), the hypoxia-induced nerve response was significantly reduced along with hypoxia-induced catecholamine release. However, in isolated glomus cells, the intracellular calcium response was enhanced as initially predicted. This suggests a cyanide-mediated impairment in the step between the glomus cell intracellular calcium rise and neurotransmitter release from secretory vesicles. Administration of a PKC blocker largely reversed the inhibitory actions of cyanide on the neural response. We conclude that the expected synergism between cyanide and hypoxia occurs at the level of glomus cell intracellular calcium but not at downstream steps due to a PKC-dependent inhibition of secretion. This suggests that at least one regulatory step beyond the glomus cell calcium response may modulate the magnitude of chemoreceptor responsiveness.

Keywords

carotid body; chemoreceptors; hypoxia; calcium; mitochondria

1. Introduction

The carotid body (CB) is the primary sensory organ in mammals for the detection of systemic hypoxia and responds to a decrease in arterial oxygen tension with an increase in action potential generation in the carotid sinus nerve (Gonzalez et al., 1994). The site of initial transduction appears to be the glomus cell – a secretory cell on which many afferent nerve fibers terminate (McDonald and Mitchell, 1975). Work from multiple laboratories demonstrated an increase in intracellular calcium in most glomus cells which occurred at O₂

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levels that did not alter intracellular calcium in other cell types (Buckler and Vaughan-Jones, 1994; Duchen and Biscoe, 1992; Wasicko et al., 2006). Thus, a widely accepted model of CB O₂ transduction is that hypoxia raises intracellular calcium leading to secretion of one or more transmitters that are excitatory to the afferent nerve fibers.

Although the mechanism by which hypoxia is detected by the glomus cell is not completely resolved, strong evidence exists that the initial step occurs at the mitochondrion, i.e., 'The metabolic hypothesis'. This hypothesis dates back to the earliest days of carotid body research where the organ function was partially identified based on the ablation of cyanide-induced respiratory stimulation by sinus nerve section (Heymans et al., 1931). Like hypoxia, mitochondrial poisons such as rotenone (complex I blocker), antimycin A (complex III blocker), cyanide (complex IV) and oligomycin (ATP synthase blocker) increase CB afferent nerve activity, depolarize the glomus cell and increase glomus cell intracellular calcium (Duchen, 1990; Wyatt and Buckler, 2004). In addition, one metabolic stimulus is able to preclude the stimulus-induced calcium change from the other stimulus, suggesting a common mechanism of action (Buckler and Vaughan-Jones, 1994; Duchen and Biscoe, 1992). Taken together, this supports the hypothesis that ATP synthesis is a critical factor in hypoxic chemoreception.

Previous studies employed dosages of mitochondrial poisons which would be expected to completely block mitochondrial function. Here, we asked whether the same interaction between hypoxia and mitochondrial poisons would be observed at low dosages where one would expect a synergism between hypoxia-induced suppression of mitochondrial metabolism and chemically induced suppression of mitochondrial metabolism. For instance, a synergism is observed in adrenal chromaffin cells where mitochondrial poisons attenuate the ability of mitochondria to buffer calcium changes resulting in enhanced intracellular calcium levels and enhanced secretion (Giovannucci et al., 1999). Although we observed the expected synergism at the level of glomus cell intracellular calcium, a negative interaction was observed at the levels of catecholamine secretion and afferent nerve activity. Thus, other regulatory elements downstream of glomus cell calcium levels may exert a major effect on the magnitude of afferent chemoreceptor activity at a given level of hypoxia. Protein kinase C (PKC) may be one of these regulatory elements.

2. Methods

2.1. Ethical approval

Experiments were undertaken with the approval of the Yale Animal Care and Use Committee and Animal Care and Use Committee of the University of Arkansas.

2.2. Experimental preparation for afferent nerve recording

Experiments were conducted on tissue harvested from rats, aged 15-30 d, at which time the chemoreceptor response is mature as judged from afferent nerve recordings and response of glomus cell intracellular calcium levels to hypoxia (Kholwadwala and Donnelly, 1992; Sterni et al., 1995; Wasicko et al., 1999). Prior to tissue harvest, rats were deeply anesthetized by placement in a closed chamber whose atmosphere was gradually replaced with 100% CO₂. While anesthetized, the animals were removed and decapitated. The carotid bifurcation, sinus nerve, glossopharyngeal nerve and petrosal/vagal complex were removed and transferred to cold oxygenated (95% O₂/5% CO₂) saline solution (in mM: 120 NaCl, 5 KCl, 2 CaCl₂, 1 Na₂HPO₄, 1 MgSO₄, 24 NaHCO₃ and 5 glucose). Following an initial cleaning, the complex (carotid body/sinus nerve/glossopharyngeal nerve and ganglia) was transferred to a chamber filled with saline containing collagenase (0.1%, Roche Diagnostics type P) and protease (0.02% Sigma type XIV) at 37°C for 30 min with gentle agitation. The complex was further cleaned and transferred to a perfusion chamber (Warner Instruments

RC-22C, chamber volume approximately 140 μ L) mounted on the stage of an inverted microscope equipped with Hoffman contrast optics (Zeiss Axiovert 10). The complex was superfused (ca 3ml/min) with oxygenated (21% O₂/5% CO₂/balance N₂) saline controlled at 37°C by an in-line heater (TC344 temperature controller; Warner Instruments). Chamber oxygen tension was measured by phosphorescence quenching (Oxy-micro with PST-1 probe, World Precision Instruments).

Single-unit activity was recorded using a suction electrode advanced into the petrosal ganglion. Electrode tip size was approximately 30 μ m in diameter which allowed individual ganglion cells to enter the tip. The pipette potential was amplified with an extracellular amplifier (Dagan Ext-1), filtered (0.03-5 kHz), displayed on an oscilloscope, digitized (10 kHz sample rate) and stored on computer (Axoscope, Axon Instruments). Unit chemoreceptor activity was discriminated and timed, post-hoc, using an event detection program which identified the timing and magnitude of action potential events (CLAMPFIT9.0, Axon Instruments). The number of individual spikes per second was calculated and graphed as a function of time and fit to a 3-sec moving average for determining peak firing frequencies (Origin 7.5; Microcal Corporation).

As an aid for identification of nerve fibers projecting to the carotid body, a stimulus electrode (pipette filled with 1M NaCl; 1 M Ω impedance) was advanced into the carotid body. A constant-current stimulus (200 μ A)(BSI-2, BAK Instruments) was delivered at 1 Hz $\times$ 0.1 msec (BAK Instruments, BPG-1) and the success of the stimulus in initiating an orthodromic action potential was determined in the post-stimulus period (Donnelly et al., 2011; Donnelly and Rigual, 2000).

In some cases, free tissue catecholamine was measured using a Nafion-coated, carbon fiber electrode placed into the carotid body and polarized at constant voltage (ie, amperometric mode) above the oxidation potential for catecholamine (ca. 120mV vs. AgCl), as previously described (Donnelly, 1993, 1996). At the end of the experiment, the electrode was removed from the tissue and calibrated during superfusion with 0 and 2 μ M dopamine.

2.3. Experimental preparation for glomus cell isolation and measurement of intracellular calcium

For glomus cell intracellular calcium ([Ca²⁺]_i) measurements, rat pups of the same ages as above were anesthetized with isoflurane, the preferred method of the University of Arkansas IACUC and decapitated. The carotid bifurcations were removed and placed in ice-cold saline. The carotid bodies were dissected free, cut in half and placed in saline containing 1mg/ml trypsin (Sigma) and 1mg/ml collagenase (Worthington Biochemical, type 1). Cells were dispersed by gentle trituration and pelleted for 4min at 2000g. Cells were resuspended in Ham's F12 with 10% fetal calf serum and plated on coverslips coated with poly-D-lysine. Greater details are given elsewhere (Wasicko et al., 2006). Cells were loaded with the calcium-sensitive dye, FURA-2 by incubation with 4 μ M FURA-2 acetoxymethyl ester for 30 min at 37 °C in saline equilibrated with 21% O₂/5% CO₂/bal N₂. FURA-2 fluorescent emission was measured every 8 seconds at ~510nm in response to alternating excitation at 340 and 380nm. Images were acquired and stored using a Nikon TE300 inverted microscope and cooled CCD camera (Photometrics) under computer control (Metafluor, Universal Imaging, West Chester, PA, USA).

2.4. Experimental protocol

The same protocol for the interaction between complex IV blockers and hypoxia was used for the afferent nerve recording, catecholamine measurements and glomus cell calcium measurements. After an initial stabilization period, the response to acute, progressive

hypoxia was elicited by switching the perfusate to a small (60ml) reservoir, initially equilibrated with 21% O₂/5% CO₂/bal N₂ and residing in a heated (37°C) water bath. The reservoir PO₂ was decreased by switching the gas source for bubbling to 0% O₂/5% CO₂/bal N₂ resulting in a slow decrease in PO₂. The hypoxic stimulus was terminated upon reaching a maximal discharge rate for the nerve recording or upon reaching <0.5 torr PO₂ using isolated cells. Upon return to normoxia, chemical blockers were applied for a pre-treatment period of 10 min followed by progressive hypoxia in the presence of the chemical agents. Because cyanide can escape during bubbling (Wyatt and Buckler, 2004), gas flow was stopped after addition of cyanide to the reservoir and during the pre-treatment period. Gas flow was resumed with low-oxygen gas to produce the graded reduction in PO₂ during the test period. Upon return to normoxia, blockers were washed out for a 10min post-treatment period following by a stimulus period using progressive hypoxia.

2.5. Data analysis

Unit spiking rates and calcium levels were graphed against PO₂ for the trials before, during and following treatment with blockers. The points were best-fit to a Boltzman function which yielded a mid-point of maximal response and a slope factor. The variables were analyzed using repeated measures ANOVA. If ANOVA was significant for drug effects then a post-hoc comparison was made using Student's t-test. For all statistical testing, significance was established at $p < 0.05$. All results are expressed as mean $\pm$ SEM.

3. Results

3.1. Afferent nerve response to cyanide and hypoxia

Bubbling the small volume reservoir with 0% O₂/5% CO₂/bal N₂ caused a progressive decrease in superfusate PO₂ and an increase in afferent nerve activity (Fig 1). When nerve activity reached a perceived plateau or decreased in rate, the perfusate was returned to 21% O₂. A time-dependent decrease in activity has been previously observed during strong stimulus presentations (Donnelly, 1993; Donnelly et al., 2005; Eyzaguirre and Koyano, 1965; Mulligan and Bhide, 1989). Addition of a low-concentration of sodium cyanide (10 μ M) to the superfusate caused a small increase in discharge rate initially (Fig 1, **) followed by a partial return towards baseline. This dose was chosen as producing nerve excitation below that produced by hypoxia indicating sub-maximal excitation. Progressive hypoxia in the presence of cyanide caused an increase in discharge rate but the peak rate was considerably lower than that produced by hypoxia in the absence of cyanide (Fig 1). Washout of the cyanide produced a partial recovery towards the initial response (Fig 1).

For a population of chemoreceptor units, cyanide (10-20 μ M) decreased the peak frequency in hypoxia from 23.4 ± 3.9 Hz to 8.6 ± 1.6 Hz ($N=10$, $p=0.001$, paired t-test). The PO₂ at half maximal response was not significantly changed (Control 94.7 ± 7.6 ; Cyanide: 96.4 ± 13.1 torr, $N=10$). Half-maximal values varied among chemoreceptor units, likely due to the differing diffusion distance between the O₂ sensor site and superfusate. The slope factor in the Boltzman fit was also not significantly changed, but there appeared to be a trend to a larger slope factor with cyanide, indicating a flatter response curve during cyanide exposure (Control: 13.8 ± 1.7 ; Cyanide: 20.8 ± 6.0 , $N=10$).

3.2. Glomus cell intracellular calcium response to cyanide

Cells isolated from carotid bodies were loaded with the calcium dye, FURA2, and glomus cells were identified by a positive stain for rhodamine tagged peanut agglutinin (Katz et al., 1995; Kim et al., 2009). Increasing concentrations of cyanide produced increasingly greater [Ca²⁺]_i responses. Low concentration cyanide, 2-10 μ M, produced no change in intracellular calcium level and 20-100 μ M cyanide produced a transient increase followed by a partial

return towards baseline (Fig 2). A high concentration of cyanide, 1mM, produced an initial transient increase in $[Ca^{+2}]_i$ followed by a secondary increase (Fig 2).

Application of progressive hypoxia at the end of a ten minute exposure to cyanide produced an increase in $[Ca^{+2}]_i$ with a peak value greater than that observed in the absence of cyanide (Fig 3). Baseline (pre-hypoxia) calcium increased from 19.7 ± 1.4 nM to 29.7 ± 2.8 nM ($N=15$, $p<0.001$) in $10\mu M$ cyanide. In hypoxia, peak calcium levels were increased from 123 ± 17 nM (control) to 225 ± 28 nM (with cyanide) ($N=15$, $p<0.001$). The PO_2 at which half maximal response was achieved increased from 7.7 ± 0.8 torr (control) to 34.8 ± 5.9 torr (with cyanide) ($N=15$, $p<0.001$). As speculated in our original hypothesis, the interaction between cyanide and hypoxia appears to be synergistic. For instance, a PO_2 of 20-80 torr produced no increase in glomus cell calcium but did produce an increase in the presence of low-dose cyanide, while low-dose cyanide, alone, produced no change in calcium. The synergism between hypoxia and low-dose cyanide may not necessarily involve identical transducing elements. For instance, cyanide may alter calcium buffering by mitochondria which may contribute to the enhanced calcium response when superimposed with hypoxia (Yan et al., 2012).

3.3. Effect of cyanide on glomus cell catecholamine secretion

The cyanide enhancement of the intracellular calcium response and reduction in the carotid body nerve response appeared to be inconsistent with the postulate that an increase in intracellular calcium is directly causal to the nerve activity increase. We examined an intermediate step – hypoxia-induced catecholamine release – to determine if the secretory response was enhanced or reduced by cyanide ($N=2$). During graded hypoxia without cyanide there was an increase in afferent nerve activity and an increase in tissue catecholamine release (Fig 4). Cyanide ($20\mu M$) produced a transient increase in nerve activity and catecholamine release (Fig 4) which was consistent with that expected from the observed transient increase in glomus cell calcium (Fig 2). Progressive hypoxia in the presence of cyanide produced less nerve excitation and less catecholamine release, an impairment which partially reversed following cyanide washout (Fig 4).

3.4. Dependence of cyanide effect on protein kinase C (PKC)

We initially postulated that the explanation for the reduced catecholamine secretion in the carotid body during hypoxia in the presence of cyanide may be due to a reduction in activated PKC. In adrenal medullary cells, activated PKC enhances the sensitivity of the secretory process to calcium (Knight, 1987); thus, a cyanide-induced loss of PKC activity could explain the reduced secretion despite increased calcium levels. We attempted to reduce PKC activity by using pharmacologic blockers. The response to hypoxia in the presence of cyanide was elicited in the presence of PKC blocker, Go-6983 ($1\mu M$, $N=4$) or protein kinase blocker H-7 ($10\mu M$, $N=2$, Supplementary figure 1). Rather than producing a further reduction in the response to hypoxia, these blockers nearly ablated the cyanide-induced impairment of hypoxia sensitivity (Fig 5). In other experiments, Go-6983 did not alter the response to hypoxia in the absence of cyanide ($N=2$, Supplementary figure 2).

3.5. Effects of other complex IV blockers on the afferent nerve response to hypoxia

In order to determine whether the effect of cyanide was specific to cyanide or was present with other complex IV blockers, we observed the effects of sodium azide ($500\mu M$, $N=4$) and H_2S ($50\mu M$, $N=6$) on the afferent nerve response to hypoxia. Azide did not produce nerve excitation but did reduce the afferent nerve response to hypoxia (Fig 6). From preliminary experiments, the threshold for an effect of azide (e.g., nerve excitation or alteration of the hypoxia response) was considerably higher than for cyanide and may reflect the tissue or cell permeation characteristics of this larger molecule. H_2S ($50\mu M$) produced a moderate

nerve excitation but the excitatory effect was considerably below that produced by hypoxia. In addition, H₂S nearly ablated the response to hypoxia (Fig 7).

4. Discussion

The principal conclusion from the present work is that low levels of mitochondrial complex IV blockers enhance the hypoxia-induced increase in intracellular calcium of rat glomus cells, but reduce both the hypoxia-induced increase in catecholamine secretion and the hypoxia-induced increase in afferent nerve activity of rat carotid body. Since the impairment of the afferent nerve response to hypoxia is greatly reduced by the presence of PKC blockers, complex IV blockers may activate a PKC-dependent pathway which impairs the coupling between intracellular calcium and neurotransmitter release. This strongly suggests that in carotid body chemoreceptors there is at least one important regulatory step between the level of glomus cell intracellular calcium and the excitation of afferent nerve activity.

It has long been known that peripheral chemoreceptors are stimulated by both hypoxia and mitochondrial poisons. These observations and those like them forms the basis of the 'metabolic hypothesis' which postulates that chemoreceptor afferent nerve activity is determined by the level of impairment of mitochondrial metabolism (Anichkov and Belen'kii, 1963; Joels and Neil, 1962; Joels and Neil, 1963). However, the interactions between the mitochondrial poisons and hypoxia have generally not been rigorously considered beyond statements that the excitatory action of cyanide appeared to be reduced by hyperoxia (Euler et al., 1939). Mulligan and Lahiri (Mulligan and Lahiri, 1981; Mulligan et al., 1981) performed the first systematic examination of stimulus interactions and demonstrated that nerve excitation by a bolus injection of cyanide and other mitochondrial poisons is potentiated by hypoxia and reduced by hyperoxia. Later they demonstrated that oligomycin, (ATP synthase blocker), and antimycin A (complex III blocker) (Mulligan and Lahiri, 1982) delivered in bolus form, ablated the chemoreceptor response to hypoxia, again showing a dependence between mitochondrial metabolism and hypoxia sensing. These results were consistent in supporting the metabolic hypothesis of chemoreception.

Several studies have extended these observations of the effects of metabolic poisons to the cellular level, but most have employed relatively high levels of poisons. For instance, cyanide (2 mM) enhances NADH fluorescence (i.e., an increase in NADH/NAD⁺) consistent with a large reduction in electron transport and no further increase in NADH fluorescence is observed with superimposed anoxia (Duchen and Biscoe 1992). Similarly, cyanide (2mM) was demonstrated to depolarize glomus cells and ablate any further depolarization with superimposed hypoxia (Wyatt and Buckler, 2004). Recent results by Buckler and Turner show that mitochondria in rat Type I cells are highly and unusually sensitive to hypoxia and mitochondrial poisons (Buckler and Turner, 2013). These results suggest that cyanide and hypoxia act through a common pathway leading to a reduction in ATP synthesis. This argument is strengthened by the indirect demonstration that the ATP level decreases in glomus cells during hypoxia and that ATP enhances K⁺ channel activity in excised patches (Varas et al., 2007). Both results are consistent with an oxygen sensor working through a reduction in mitochondrial ATP production.

In contrast, Ortega-Sáenz et al. observed a major inhibitory effect of complex I blockers on hypoxia-induced catecholamine release from carotid body slices but little effect of complex III and IV blockers (Ortega-Sáenz et al., 2003). They conclude that complex I is the critical hypoxia –sensing site. However, this conclusion is dependent on achieving complete blockade of electron transport, which was not demonstrated in their paper. In our study, 100uM cyanide (the dose used in the Oretga-Saenz study) produced a transient increase in calcium which was considerably below that reached at higher levels of cyanide (Fig 2).

Thus, the failure of cyanide to reduce or ablate hypoxia-induced catecholamine release in the study of Ortega- Sáenz et al., may be due to inadequate cyanide concentration, or, alternatively, due to the relatively short application time used in their study (Ortega-Sáenz et al., 2003).

Other data suggest a degree of independence between impaired ATP production and excitation of afferent nerve activity. For instance, oligomycin, a blocker of F₀/F₁ ATP synthase, increases intracellular calcium levels of glomus cells and no further increase is observed when anoxia is superimposed (Duchen and Biscoe, 1992). However, oligomycin fails to sustain stimulation of afferent nerve activity at levels achieved during severe hypoxia. For instance, 50-500 µg oligomycin, in vivo, increased baseline nerve activity from 0.2-1.6 Hz to 3.5-6.3 Hz, but this increase was less than that observed during moderate hypoxia (9.6-14 Hz) (Mulligan et al., 1981). In another study, oligomycin applied in bolus form, caused only a small (<20%) afferent nerve excitation despite ablating the response to hypoxia (Mosqueira and Iturriaga, 2002). This suggests that impairment of mitochondrial metabolism is not the sole factor in determining chemoreceptor afferent activity.

Based on the postulate that mitochondrial metabolism is the transducing element for chemoreception, we would expect a synergism between mitochondrial poisons and hypoxia, both of which impair mitochondrial metabolism. In our study, the expected synergy was confirmed at the level of glomus cell $[Ca^{+2}]_i$ (Fig 3). This is generally consistent with the model proposed by Buckler and colleagues in which a reduction in ATP production inhibits a leak K⁺ channel leading to cell depolarization and activation of voltage-dependent calcium currents (Wyatt and Buckler, 2004). The transient calcium changes observed at low doses of cyanide are generally expected since metabolic control analysis of other cell types demonstrates that small impairments of mitochondrial metabolism have no steady-state effect on oxygen consumption or, presumably, ATP production (Davey and Clark, 1996).

Despite the increase in calcium responsiveness, the afferent nerve activity did not follow suit. The initial nerve response to cyanide was a transient excitation which partially returned towards baseline, quite similar to the transient change in intracellular calcium (Fig 2). However, after the 10 min wash-in period for cyanide, progressive hypoxia produced a much lower nerve excitation, an inhibition which partially reversed following a 10 min washout of cyanide (Fig 1). Since the reversal was not more complete, it suggests that cyanide is altering some intracellular factor which takes a period of time to reverse as the recovery of the metabolic state of the cell from cyanide is rapid (seconds) (Wyatt and Buckler, 2004). Alternatively, the lower response following drug washout may reflect a progressive run-down of hypoxia responsiveness following multiple hypoxia trials, as previously noted (Donnelly et al., 2011).

This unidentified intracellular factor may be related to protein kinase C since the inhibition of the afferent nerve response to hypoxia caused by exposure to cyanide is largely reversed by PKC blockers (Fig 5). PKC falls into three general categories depending on if it is activated by diacylglycerol and calcium ("Conventional PKC"), diacylglycerol alone ("Novel PKC") or neither factor ("Atypical PKC"). In the present study we did not try to discriminate among these categories, but would note that Go-6983 is primarily effective against the Conventional PKC isoforms PKC α and PKC β . H-7 is a relatively broad spectrum protein kinase blocker and, thus, does not discriminate among isoforms or between PKA and PKC. Nevertheless, we were initially surprised by the experimental result since PKC has been shown to potentiate calcium-induced catecholamine release from adrenal medullary cells (Knight, 1987; Pocotte et al., 1985), but, here, PKC appeared to inhibit glomus cell neurotransmitter release because blocking it during cyanide exposure enhanced the afferent nerve response to hypoxia. This is similar to PKC actions in platelets and pancreatic β cells.

In pancreatic β cells, inhibition of Conventional PKC enhances potassium-induced secretion (Zhang et al., 2004) and in platelets sub-maximal application of a pan-PKC inhibitor enhances dense granule secretion in response to ADP (Unsworth et al., 2011).

In order to better identify the cause for the lower nerve response to hypoxia in the presence of cyanide, we measured extracellular catecholamine release within the carotid body using voltammetry. Although catecholamine is unlikely to be the excitatory transmitter in the carotid body, it is likely that it is co-stored in the same secretory vesicles as the excitatory agent(s) and, thus, may serve as a proxy for the release of the excitatory agent(s). As with the nerve activity, low dose cyanide produced a transient release of catecholamine which returned towards baseline (Fig. 4). Therefore the impaired nerve response to hypoxia mirrored the corresponding reduction in catecholamine release. Thus, despite the enhancement of the glomus cell calcium response to hypoxia by low-concentration cyanide, glomus cell neurotransmitter secretion was reduced, perhaps accounting for the lower nerve excitation. This response is, to some extent, different from that observed with an acute application of mitochondrial poisons. Both cyanide and the uncoupler CCP are demonstrated to impair calcium clearance in glomus cells and to enhance the magnitude of vesicular release (Yan et al., 2012). However, these effects were observed a short time after application of mitochondrial poisons and, presumably, the inhibitory response had insufficient time to develop.

The inhibitory effect on the response to progressive hypoxia is not restricted to cyanide. Azide, another mitochondrial complex IV blocker, also produced a lower nerve excitation when combined with hypoxia (Fig 6), suggesting the inhibitory action was not directly related to the effect of cyanide, per se, but due to its actions on the mitochondria. Hydrogen sulfide (H_2S) is another molecule which can potentially inhibit cytochrome oxidase (Cooper and Brown, 2008). It also nearly ablated the afferent nerve response to hypoxia but failed to cause as much nerve excitation as caused by hypoxia alone (Fig 7). However, interpretation of results with H_2S are more complicated since H_2S is proposed to be a major mediator of carotid body hypoxia transduction (Peng et al., 2010). A reduced production of H_2S due to genetic knockout or drug application is correlated with a significant reduction in CB organ function in several laboratories (Li et al., 2010; Olson, 2011; Peng et al., 2010). In contrast, others argue that the excitatory action of H_2S on chemoreceptor activity is solely due to its action on cytochrome oxidase and is not unique when compared to other complex IV blockers (Buckler, 2012). Another study has suggested that H_2S may also be inhibitory to the release of excitatory transmitters by hyperpolarizing the glomus cell via activation of a $K(ATP)$ type channel (Fitzgerald et al., 2011). Finally, H_2S exerts effects on other ion channels such as L-type calcium channels, chloride channels and several TRP channels (Tang et al., 2010). We do not offer any resolution to these competing claims other than to point out that in our model H_2S produced similar effects to cyanide, including a relatively small increase in baseline nerve activity and a considerable diminution in the nerve response to hypoxia.

It is currently unclear why hypoxia, per se, does not appear to produce an inhibition of hypoxia sensitivity. In cats, single-fiber chemoreceptor activity is maintained at elevated levels during continuous hypoxia lasting 2-3 hours (Barnard et al., 1987). In goats, chemoreceptor activity remains elevated for the first hour and then increases further (Nielsen et al., 1988). Both hypoxia and cyanide at lower concentration produce an increase in intracellular calcium which is an initial step in PKC activation. However, PKC inhibitory agents had no effect on nerve response to hypoxia in the absence of cyanide. One possibility is that there may be a substrate requirement for oxygen which is present at higher concentrations during the initial cyanide exposure and, of course, present at low concentration during hypoxia. It is also possible that a longer treatment period (longer than

used in the present study) with hypoxia may be needed to activate the pathway. For instance, the hypoxic ventilatory response is significantly impaired following repeated short exposures to hypoxia (Waters and Gozal, 2003); in piglets, exposure to 6% O₂ for 3 minutes reduces all subsequent ventilatory responses to hypoxia (Waters et al., 1996); in humans, ventilatory responses to the same hypoxic stimulus takes up to 60min to recover (Easton et al., 1988). Although the cause for the respiratory impairment could occur at multiple points, a PKC-dependent mechanism identified here could potentially be one important site.

In conclusion, the present results demonstrate the anticipated synergy between mitochondrial complex IV blockers and hypoxia in the determination of glomus cell intracellular calcium, but this synergy does not extend to hypoxia-induced catecholamine release or increased afferent nerve activity. Low level cyanide and other complex IV blockers produce an inhibitory effect on the hypoxic response of afferent nerve activity. Since this inhibition of the hypoxic neural response which is downstream to changes in [Ca²⁺]_i is sensitive to PKC blockers, it suggests that in the expression of carotid body hypoxic chemoreception, an important regulatory step is present between the level of intracellular calcium and the secretion of an excitatory agent which results in an increase in afferent nerve activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Anichkov, SV.; Belen'kii, ML. Pharmacology of the carotid body chemoreceptors. Pergamon Press; London, England: 1963.
- Barnard P, Andronikou S, Pokorski M, Smatresk N, Mokashi A, Lahiri S. Time-dependent effect of hypoxia on carotid body chemosensory function. *J Appl Physiol.* 1987; 63:685–691. [PubMed: 3654428]
- Buckler KJ. Effects of exogenous hydrogen sulphide on calcium signalling, background (TASK) K channel activity and mitochondrial function in chemoreceptor cells. *Pflugers Arch.* 2012; 463:743–754. [PubMed: 22419174]
- Buckler KJ, Turner PJ. Oxygen sensitivity of mitochondrial function in rat arterial chemoreceptor cells. *J Physiol.* 2013 in press.
- Buckler KJ, Vaughan-Jones RD. Effects of hypoxia on membrane potential and intracellular calcium in rat neonatal carotid body type I cells. *J Physiol (London).* 1994; 476:423–428. [PubMed: 8057251]
- Cooper CE, Brown GC. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance. *Journal of bioenergetics and biomembranes.* 2008; 40:533–539. [PubMed: 18839291]
- Davey GP, Clark JB. Threshold effects and control of oxidative phosphorylation in nonsynaptic rat brain mitochondria. *J Neurochem.* 1996; 66:1617–1624. [PubMed: 8627318]
- Donnelly DF. Electrochemical detection of catecholamine release from rat carotid body, in vitro. *J Appl Physiol.* 1993; 74:2330–2337. [PubMed: 8335564]
- Donnelly DF. Chemoreceptor nerve excitation may not be proportional to catecholamine secretion. *J Appl Physiol.* 1996; 81:657–664. [PubMed: 8872631]

- Donnelly DF, Kim I, Carle C, Carroll JL. Perinatal hyperoxia for 14 days increases nerve conduction time and the acute unitary response to hypoxia of rat carotid body chemoreceptors. *J Appl Physiol*. 2005; 99:114–119. [PubMed: 15731397]
- Donnelly DF, Kim I, Yang D, Carroll JL. Role of MaxiK-type calcium dependent K⁺ channels in rat carotid body hypoxia transduction during postnatal development. *Respir Physiol Neurobiol*. 2011; 177:1–8. [PubMed: 21356332]
- Donnelly DF, Rigual R. Single-unit recordings of arterial chemoreceptors from mouse petrosal ganglia in vitro. *J Appl Physiol*. 2000; 88:1489–1495. [PubMed: 10749846]
- Duchen M. Effects of metabolic inhibition on the membrane properties of isolated mouse sensory neurones. *J Physiol (London)*. 1990; 424:387–409. [PubMed: 2391654]
- Duchen MR, Biscoe TJ. Relative mitochondrial membrane potential and [Ca²⁺]_i in type I cells isolated from rabbit carotid body. *J Physiol (London)*. 1992; 450:33–61. [PubMed: 1432712]
- Easton PA, Slykerman LJ, Anthonisen NR. Recovery of the ventilatory response to hypoxia in normal adults. *J Appl Physiol*. 1988; 64:521–528. [PubMed: 3372409]
- Euler Uv, Liljestrand G, Zotterman Y. The excitation mechanism of the chemoreceptors of the carotid body. *Skand Arch Physiol*. 1939; 83:139–152.
- Eyzaguirre C, Koyano H. Effects of hypoxia, hypercapnia and pH on the chemoreceptor activity of the carotid body in vitro. *J Physiol (London)*. 1965; 178:385–409. [PubMed: 5890156]
- Fitzgerald RS, Shirahata M, Chang I, Kostuk E, Kiihl S. The impact of hydrogen sulfide (H₂S) on neurotransmitter release from the cat carotid body. *Respir Physiol Neurobiol*. 2011; 176:80–89. [PubMed: 21292043]
- Giovannucci DR, Hlubek MD, Stuenkel EL. Mitochondria regulate the Ca²⁺-exocytosis relationship of bovine adrenal chromaffin cells. *J Neurosci*. 1999; 19:9261–9270. [PubMed: 10531430]
- Gonzalez C, Almaraz L, Obeso A, Rigual R. Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol Rev*. 1994; 74:829–898. [PubMed: 7938227]
- Heymans C, Bouckaert JJ, Dautrebande L. Sinus carotidien et reflexes respiratoires: sensibilite des sinus carotidiens aux substances chimiques. Action stimulante respiratoire reflexe du sulfure de sodium, du cyanure de potassium, de la nicotine et de la lobeline. *Arch Int Pharmacodyn, Ther*. 1931; 40:54–91.
- Joels N, Neil E. Carotid glomus chemosensory excitation. *J Physiol (London)*. 1962; 164:11P–12P.
- Joels N, Neil E. The excitation mechanism of the carotid body. *British medical bulletin*. 1963; 19:21–24. [PubMed: 13957800]
- Katz DM, White ME, Hall AK. Lectin binding distinguishes between neuroendocrine and neuronal derivatives of the sympathoadrenal neural crest. *Journal of neurobiology*. 1995; 26:241–252. [PubMed: 7535839]
- Kholwadwala D, Donnelly DF. Maturation of carotid chemoreceptor sensitivity to hypoxia: in vitro studies in the newborn rat. *J Physiol (London)*. 1992; 453:461–473. [PubMed: 1464840]
- Kim I, Yang DJ, Donnelly DF, Carroll JL. Fluoresceinated peanut agglutinin (PNA) is a marker for live O₂ sensing glomus cells in rat carotid body. *Adv Exp Med Biol*. 2009; 648:185–190. [PubMed: 19536480]
- Knight DE. Calcium and diacylglycerol control of secretion. *Biosci Rep*. 1987; 7:355–367. [PubMed: 3315029]
- Li Q, Sun B, Wang X, Jin Z, Zhou Y, Dong L, Jiang LH, Rong W. A crucial role for hydrogen sulfide in oxygen sensing via modulating large conductance calcium-activated potassium channels. *Antioxidants & Redox Signaling*. 2010; 12:1179–1189. [PubMed: 19803741]
- McDonald DM, Mitchell RA. The innervation of glomus cells, ganglion cells and blood vessels in the rat carotid body: a quantitative ultrastructural analysis. *J Neurocytol*. 1975; 4:177–230.
- Mosqueira M, Iturriaga R. Carotid body chemosensory excitation induced by nitric oxide: involvement of oxidative metabolism. *Respir Physiol Neurobiol*. 2002; 131:175–187. [PubMed: 12126919]
- Mulligan E, Bhide S. Non-sustained responses to hypoxia of carotid body chemoreceptor afferents in the piglet. *Faseb J*. 1989; 3:A399.
- Mulligan E, Lahiri S. Dependence of carotid chemoreceptor stimulation by metabolic agents on PaO₂ and PaCO₂. *J Appl Physiol*. 1981; 50:884–891. [PubMed: 6266995]

- Mulligan E, Lahiri S. Separation of carotid body chemoreceptor responses to O₂ and CO₂ by oligomycin and by antimycin A. *Am J Physiol.* 1982; 242:C200–206. [PubMed: 6801997]
- Mulligan E, Lahiri S, Storey BT. Carotid body O₂ chemoreception and mitochondrial oxidative phosphorylation. *J Appl Physiol.* 1981; 51:438–446. [PubMed: 7263450]
- Nielsen AM, Bisgard GE, Vidruk EH. Carotid chemoreceptor activity during acute and sustained hypoxia in goats. *J Appl Physiol.* 1988; 65:1796–1802. [PubMed: 3141364]
- Olson KR. Hydrogen sulfide is an oxygen sensor in the carotid body. *Respir Physiol Neurobiol.* 2011; 179:103–110. [PubMed: 21968289]
- Ortega-Sáenz P, Pardal R, Garcia-Fernandez M, Lopez-Barneo J. Rotenone selectively occludes sensitivity to hypoxia in rat carotid body glomus cells. *J Physiol (London).* 2003; 548:789–800. [PubMed: 12626666]
- Peng YJ, Nanduri J, Raghuraman G, Souvannakitti D, Gadalla MM, Kumar GK, Snyder SH, Prabhakar NR. H₂S mediates O₂ sensing in the carotid body. *Proc Natl Acad Sci U S A.* 2010; 107:10719–10724. [PubMed: 20556885]
- Pocotte SL, Frye RA, Senter RA, TerBush DR, Lee SA, Holz RW. Effects of phorbol ester on catecholamine secretion and protein phosphorylation in adrenal medullary cell cultures. *Proc Natl Acad Sci U S A.* 1985; 82:930–934. [PubMed: 3156373]
- Sterni LM, Bamford OS, Tomares SM, Montrose MH, Carroll JL. Developmental changes in intracellular Ca²⁺ response of carotid chemoreceptor cells to hypoxia. *Am J Physiol.* 1995; 268:L801–L808. [PubMed: 7762681]
- Tang G, Wu L, Wang R. Interaction of hydrogen sulfide with ion channels. *Clinical and experimental pharmacology & physiology.* 2010; 37:753–763. [PubMed: 20636621]
- Unsworth AJ, Smith H, Gissen P, Watson SP, Pears CJ. Submaximal inhibition of protein kinase C restores ADP-induced dense granule secretion in platelets in the presence of Ca²⁺. *J Biol Chem.* 2011; 286:21073–21082. [PubMed: 21489985]
- Varas R, Wyatt CN, Buckler KJ. Modulation of TASK-like background potassium channels in rat arterial chemoreceptor cells by intracellular ATP and other nucleotides. *J Physiol.* 2007; 583:521–536. [PubMed: 17615104]
- Wasicko MJ, Breitwieser GE, Kim I, Carroll JL. Postnatal development of carotid body glomus cell response to hypoxia. *Respir Physiol Neurobiol.* 2006; 154:356–371. [PubMed: 16466972]
- Wasicko MJ, Sterni LM, Bamford OS, Montrose MH, Carroll JL. Resetting and postnatal maturation of oxygen chemosensitivity in rat carotid chemoreceptor cells. *J Physiol.* 1999; 514(Pt 2):493–503. [PubMed: 9852330]
- Waters KA, Beardsmore CS, Paquette J, Meehan B, Cote A, Moss IR. Respiratory responses to rapid-onset, repetitive vs continuous hypoxia in piglets. *Respir Physiol.* 1996; 105:135–142. [PubMed: 8897659]
- Waters KA, Gozal D. Responses to hypoxia during early development. *Respir Physiol Neurobiol.* 2003; 136:115–129. [PubMed: 12853004]
- Wyatt CN, Buckler KJ. The effect of mitochondrial inhibitors on membrane currents in isolated neonatal rat carotid body type I cells. *J Physiol (London).* 2004; 556:175–191. [PubMed: 14724184]
- Yan L, Lee AK, Tse FW, Tse A. Ca²⁺ homeostasis and exocytosis in carotid glomus cells: role of mitochondria. *Cell calcium.* 2012; 51:155–163. [PubMed: 22209034]
- Zhang H, Nagasawa M, Yamada S, Mogami H, Suzuki Y, Kojima I. Bimodal role of conventional protein kinase C in insulin secretion from rat pancreatic beta cells. *J Physiol.* 2004; 561:133–147. [PubMed: 15388777]

Highlights

Impaired mitochondrial metabolism is the likely sensor for detecting hypoxia by the carotid body

If so, then mitochondrial poisons should synergistically enhance the sensitivity to hypoxia.

As predicted, hypoxia and cyanide synergistically enhanced the rise in glomus cell calcium caused by either agent.

Unlike the calcium response, cyanide reduced the nerve response to hypoxia, an effect reduced by a PKC blocker.

This suggests the presence of a modulatory step between glomus cell intracellular calcium and afferent nerve activity

Fig. 1.

Cyanide impairs the chemoreceptor response to graded hypoxia. (top) Polygraphic trace of action potentials recorded extracellularly from the soma of a petrosal neuron with projections to the carotid body. (middle) Rate tracing and superfusate oxygen level vs. time for unit pictured at top. Cyanide (NaCN, 10 μM) was applied (solid bar) for 10 min starting around minute 7 prior to the start of a hypoxia challenge. Note transient increase in discharge frequency with cyanide and accommodation to the stimulus (**). (lower) Rate vs. O₂ levels for the three hypoxia-trials. Note discharge frequency during hypoxia was lower in the presence of cyanide. Lines were fit to a Boltzman function.

Fig. 2.

Low dosages of cyanide cause little or transient increases in glomus cell calcium.

Intracellular calcium responses of glomus cells to anoxia and different dosages of cyanide.

At low dosages of cyanide (2-20 μ M) calcium either did not change or increased transiently.

High dosages (>1mM) produced a maximal change in intracellular calcium.

Fig 3.

Cyanide enhances the glomus cell calcium response to hypoxia. (top) Example of glomus cell $[Ca^{+2}]_i$ response to graded hypoxia before, during and following cyanide (10 μ M) application. (lower) Mean $\pm$ SEM calcium response for 15 glomus cells. Note the right-shift in the calcium/ O_2 relationship during cyanide exposures.

Fig. 4.

Cyanide impairs hypoxia-induced catecholamine release in addition to the afferent nerve activity. (top) Polygraphic trace of action potentials from a chemoreceptor unit recorded from the soma in the petrosal ganglion. (middle) Action potential rate and superfusate PO₂ vs. time for unit pictured at top. Note reduced nerve response to hypoxia, similar to that presented in figure 1. Cyanide application indicated by a bar. (lower) Tissue catecholamine vs. time. Note reduced hypoxia-induced catecholamine release in the presence of cyanide.

Fig. 5. Cyanide induced inhibition of the nerve response to hypoxia is reduced by pharmacologic PKC inhibition. (top) Polygraphic tracing of unit chemoreceptor AP activity recorded from the soma in the petrosal ganglia. (middle) AP rate and superfusate oxygen level vs. time. PKC blocker, Go-6983, and cyanide were applied at the same time for 10min before the hypoxia challenge. (lower). AP rate vs superfusate oxygen level for the three trials. Note that AP activity due to hypoxia was not reduced by cyanide in the presence of Go-6983, a PKC blocker as it was without the PKC blocker (see Fig 1).

Fig. 6.

Azide, like cyanide, reduces the afferent nerve response to hypoxia. (top) Polygraphic tracing of unit chemoreceptor activity recorded from the petrosal ganglion. (middle) AP rate and superfusate oxygen level vs. time. Azide application indicated by bar. (lower) AP rate vs. superfusate oxygen level for the three trials. Note that AP activity in response to hypoxia was reduced by azide.

Fig. 7.

H₂S, like cyanide, reduces the afferent nerve response to hypoxia. (top) Polygraphic tracing of unit chemoreceptor activity recorded from the petrosal ganglion. (middle) AP rate and superfusate oxygen level vs. time. H₂S application indicated by bar. (lower) AP rate vs. superfusate oxygen level for the three trials. Note that baseline AP activity was slightly enhanced by H₂S but the response to hypoxia was greatly reduced.