Worm 4:1, e1008898; January/February/March 2015; © 2015 Taylor & Francis Group, LLC

# Pharyngeal pumping inhibition and avoidance by acute exposure to high $CO_2$ levels are both regulated by the BAG neurons via different molecular pathways

Kfir Sharabi, Chayki Charar, and Yosef Gruenbaum*

Department of Genetics; Institute of Life Sciences; Hebrew University of Jerusalem; Jerusalem, Israel

**Carbon dioxide ($CO_2$) is a key molecule in many biological processes. Studies in humans, mice, *D. melanogaster*, *C. elegans*, unicellular organisms and plants have shed light on the molecular pathways activated by elevated levels of $CO_2$. However, the mechanisms that organisms use to sense and respond to high $CO_2$ levels remain largely unknown. Previous work has shown that *C. elegans* quickly avoid elevated $CO_2$ levels using mechanisms that involve the BAG, ASE and AFD neurons via cGMP- and calcium- signaling pathways. Here, we discuss our recent finding that exposure of *C. elegans* to high $CO_2$ levels leads to a very rapid cessation in the contraction of the pharynx muscles. Surprisingly, none of the tested $CO_2$ avoidance mutants affected the rapid pumping inhibition response to elevated $CO_2$ levels. A forward genetic screen identified that the *hid-1*-mediated pathway of dense core vesicle maturation regulates the pumping inhibition, probably through affecting neuropeptide secretion. Genetic studies and laser ablation experiments showed that the $CO_2$ response of the pharyngeal muscle pumping is regulated by the BAG neurons, the same neurons that mediate $CO_2$ avoidance.**

**Keywords:** BAG neurons, $CO_2$ avoidance, *Caenorhabditis elegans*, dense core vesicles, pharynx

*Correspondence to: Yosef Gruenbaum; Email: gru@vms.huji.ac.il

Submitted: 12/05/2014

Revised: 12/23/2014

Accepted: 01/07/2015

http://dx.doi.org/10.1080/21624054.2015.1008898

## Introduction

The respiratory gases, carbon dioxide ($CO_2$) and oxygen ($O_2$), are key molecules in oxidative metabolism. In order to maintain cellular homeostasis, all organisms must adapt to changes in the levels of these gases. The $CO_2$ and $O_2$ homeostasis in our body is mainly achieved by $CO_2$ chemoreceptors in the brain that promote respiratory responses to maintain normal $CO_2$ and $O_2$ levels in the blood. At the cellular level, under normal oxygen conditions, the transcription factor HIF-1α is hydroxylated in a conserved proline residue and subsequently targeted for proteasomal degradation. Under hypoxic conditions (low oxygen levels), HIF-1α is stabilized and orchestrates an adaptive response that maintains normal metabolism.[1]

Whether a master regulator that responds to changes in $CO_2$ levels at the cellular level exists is not known. Several recent studies have shed new light on molecular pathways activated by elevated levels of $CO_2$. In lungs, elevated levels of $CO_2$ are associated with impaired fluid reabsorption as a consequence of Na,K-ATPase endocytosis.[2] The $CO_2$-mediated Na,K-ATPase endocytosis is partially regulated by activation of AMPK and subsequent activation of PKC-ζ.[3] The soluble adenylyl cyclase (sAC) was also found to be activated by $CO_2$/HCO3.[4] In a feedback loop found in alveolar epithelial cells, sAC activation results in elevated cAMP levels which leads to PKA-1α-dependent phosphorylation of the actin cytoskeleton component α-adducin and endocytosis of the Na,K-ATPase.[5] $CO_2$ also inhibits cell proliferation by inducing mitochondrial dysfunction mediated by down regulation of the TCA cycle enzyme isocitrate dehydrogenase-2 (IDH2).[6] In addition, elevated levels of $CO_2$ impair innate immunity responses in mammalian cells. $CO_2$ leads to nuclear translocation of RelB and IKKα, 2 central regulatory

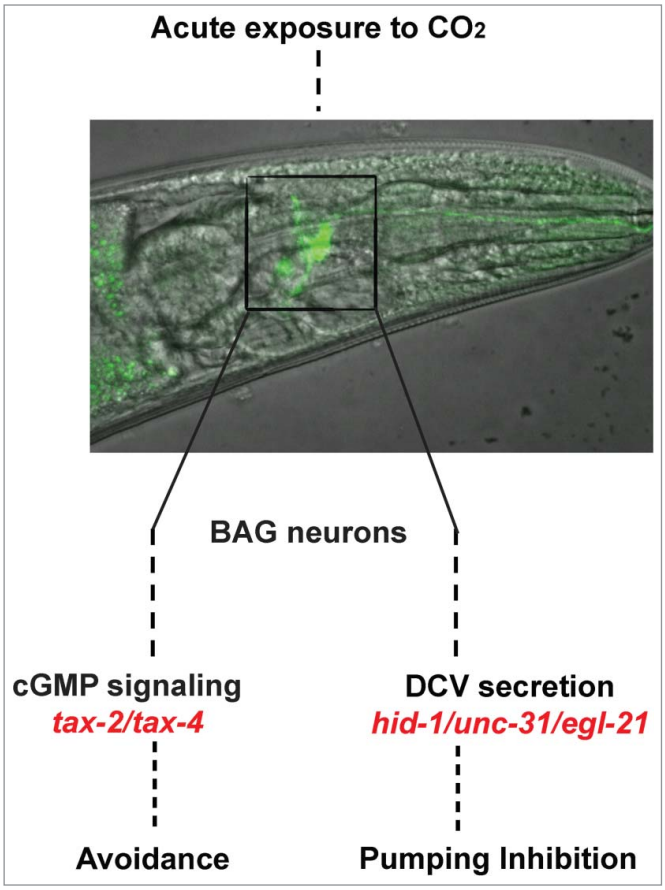


**Figure 1.** Acute exposure of *Caenorhabditis elegans* to elevated $CO_2$ level causes animal avoidance and stops pharyngeal muscle contractions. Both responses are regulated by the BAG neurons but through separate signaling pathways. While avoidance requires cGMP signaling, the inhibition of muscle contraction in the pharynx is mediated by neuropeptide secretion. Both $CO_2$ responses are decreased following starvation.

components of the NF-κB signaling pathway, which leads to significant attenuation of NF-kB signaling and altered inflammatory responses.[7,8] Most importantly, all the effects mentioned above are pH-independent, suggesting specific cellular responses to $CO_2$.

The nematode *C. elegans,* is a good model in which to study the physiological and molecular responses to high levels of $CO_2$.[9] In this model organism, high levels of $CO_2$ induce an avoidance response, which is mediated by cGMP signaling pathway activation in the BAG neurons (**Fig. 1**).[10,11] This response is modulated by the nutritional state of the worm. Starved worms do not avoid $CO_2$ and worms mutated in insulin/IGF signaling, which mimics the starvation response, also do not avoid $CO_2$.[10,11] The homeostatic response to $CO_2$ is also modulated by both temperature and $O_2$ sensing neurons in that $CO_2$ is less aversive to animals acclimated to 15°C compared to animals acclimated to 22°C.[12] This difference requires the activation of temperature sensitive AFD neurons, which are also activated by $CO_2$. In addition, signaling from the oxygen-sensing neuron URX inhibits $CO_2$-mediated avoidance.[12,13] Little is known about how $CO_2$ is actually sensed in the BAG neurons, it was previously suggested that the guanylate cyclase receptor *gcy-9*, whose expression is directly controlled by the transcription factor ETS-5, serves as the $CO_2$ sensor in these neurons.[14] ETS-5 is also required for proper differentiation and proper $CO_2$ responses of the BAG neurons.[15,16] More recently, EGL-13 and EGL-46 were also found to be important for the differentiation of the $CO_2$-sensing BAG neurons.[17,18]

Further studies are required to fully understand the molecular mechanisms induced by high levels of $CO_2$ and how these mechanisms regulate physiologic responses to elevated $CO_2$. We discuss here our recent study of a previously uncharacterized behavioral response of *C. elegans* to elevated $CO_2$, and a new component that is involved in *C. elegans* $CO_2$ signaling.[19]

## High Levels of $CO_2$ Halt Pharynx Contractions

We previously described the effects of wild type *C. elegans'* exposure to chronic, high $CO_2$ levels.[20] These studies were performed to establish the potential use of *C. elegans* as a model organism for investigating the molecular mechanisms, at the whole organism level, that are activated in response to elevated levels of $CO_2$. We found that when wild type *C. elegans* are maintained at high $CO_2$ conditions they have a smaller brood size, delayed development, reduced motility that is coupled with striated muscle deterioration and a significant increase in life span. To gain a better insight into how worms respond to high levels of $CO_2$ we set to study the immediate responses elicited when worms are exposed to high $CO_2$ levels. We designed a small chamber, connected to a $CO_2$ tank, and used it to expose wild type *C. elegans* to elevated levels of $CO_2$ ranging from 5–20% $CO_2$.[21] We noticed that in $CO_2$ concentrations higher than 10%, the pharynx, which normally contracts ~200 times/min, almost completely stopped contracting after a few seconds of $CO_2$ exposure. The contraction cessation is probably counteracted by other response mechanisms since after 2 min of continuous exposure we started to observe a recovery in the pharynx response. However, even after prolonged exposure to high levels of $CO_2$ (30 min) a complete recovery of the pharynx contractions was not seen, suggesting a sustained response. Since $CO_2$ might potentially change the pH of the growth medium we also tested the response of the pharynx under different media conditions. The pharynx response was not affected by the growth medium pH, suggesting the effect of elevated $CO_2$ is probably not mediated

by pH changes in the medium. It is well established that the nutritional state of the worm can significantly alter its responses to external environmental cues. Similarly, the nutritional state of the worm partially modulated the response of the pharynx to high levels of $CO_2$. In 10% $CO_2$, starved worms were able to partially contract the pharynx in contrast to well-fed animals where the pharynx contraction completely halted. However, in 20% $CO_2$ both starved and well-fed animals had a complete cessation of the pharynx contractions. This dose dependency suggests the existence of several response mechanisms that are activated at different $CO_2$ concentrations.

## $CO_2$-Mediated Pumping Inhibition Regulation is not Shared with $CO_2$-Mediated Avoidance Components

We hypothesized that since the $CO_2$-mediated pumping inhibition is quick and robust, similar to the $CO_2$-mediated avoidance, the 2 behavioral responses probably share common components. However, all the $CO_2$ avoidance mutants that we tested showed a similar inhibition of the pumping in response to an acute high $CO_2$ exposure.[19] The mutants that we tested included mutants in chemo sensation (*tax-4*), nutritional state of the worm (*daf-2*), ciliated neuron development (*osm-3*, *che-10*) and $CO_2$ neuron specification (*gcy-9,ets-5*). To identify new components that mediate the response of the pharynx to high $CO_2$, we performed a forward genetics screen after EMS mutagenesis. Specifically, we searched for mutants in which pumping inhibition is impaired when exposed to 10% $CO_2$. One of the genes identified in this screen was *hid-1*. HID-1 was previously identified in a screen for mutants that induce dauer formation under high temperature conditions.[22] HID-1 was also found to be an important component of dense core vesicle secretion that controls neuropeptide release.[23-25] Interestingly, the response of this mutant to high $CO_2$ is dose dependent. After exposure to 5% $CO_2$, the pumping inhibition is completely rescued and pumping continues at the same rate as under normal atmospheric conditions. After exposure to 20% $CO_2$, this mutation can no longer rescue the $CO_2$-mediated pumping inhibition and pumping completely halts as in wild type worms.

Importantly, *hid-1* is probably a component specifically involved in mediating the response of the *pharynx* to $CO_2$ but not of other responses induced by high $CO_2$. The development, fertility and brood size of *hid-1* mutants are still impaired when chronically exposed to high levels of $CO_2$.

HID-1 functions in *C. elegans* to regulate neuropeptide secretion by dense core vesicles. We therefore investigated whether neuropeptide secretion constitutes a fundamental component of *C. elegans'* response to acute high $CO_2$ exposure. We tested 2 mutants, *unc-31*, an essential player in the dense core vesicle secretion machinery and *egl-21*, which encodes a carboxypeptidase required for neuropeptide precursor processing. Like in *hid-1*, the response of these mutants to high $CO_2$ was significantly impaired compared to that of wild type worms. In contrast, *unc-13* and *rab-3* mutants (**Fig. 1**), specifically involved in synaptic vesicle secretion, responded to high $CO_2$ like the response of the wild type. Unfortunately, all of our attempts to identify single neuropeptide-encoding mutants in which the response to high $CO_2$ is impaired failed, suggesting that there is probably more than one neuropeptide/receptor involved in mediating this response.

## HID-1 Function is Required in the BAG Neurons

*hid-1* was previously shown to be expressed in the gut and also in the nerve system of *C. elegans*.[23,25] By tissue specifically expressing HID-1 in a *hid-1* null background, either in the gut (under a *ges-1* promoter) or in the nervous system (under a *rab-3* promoter), we demonstrated that *hid-1* is only needed in the nervous system to mediate pumping inhibition.[19] We next searched for the neuronal subtype in which *hid-1* mediates the effect of $CO_2$ on the pharynx. We used the *nlp-3* promoter, which is expressed in neurons that secrete DCVs, to drive the expression of *hid-1*. Indeed, overexpression of *hid-1* in this subset of neurons in a *hid-1* null background restored the $CO_2$-mediated pumping inhibition to almost wild type levels. In addition, overexpression of *hid-1* in the BAG neurons either under an *flp-17* promoter or under a *gcy-33* promoter restored the $CO_2$-mediated pumping inhibition to wild type levels. Laser ablation of BAG neurons in *hid-1*-null strains overexpressing HID-1 under a *gcy-33* or *flp-17* promoter eliminated the $CO_2$-mediated pumping inhibition. This further supports that the expression of *hid-1* is indeed required in BAG neurons. Our data support a model in which $CO_2$ elicits 2 different responses in the BAG neurons, the first induces avoidance and the second, mediated by *hid-1*, induces pumping inhibition.

## Concluding Remarks

Studying *C. elegans'* response to both chronic and acute high $CO_2$ levels has already yielded many insights into how cells react to this stressor and implications for patients suffering from pulmonary diseases. It is becoming clear that the nematode, *Caenorhabditis elegans,* presents a valuable model system to study the molecular sensing and response to changing levels of $CO_2$. This response involves specific neurons that sense the changes in $CO_2$ levels, neuropeptide secretion and activation of specific signaling pathways. Both the response and the recovery depend on the cell type and its metabolic state.

Many properties of organisms' and cells' response to elevated $CO_2$ levels have yet to be determined. In *C. elegans*, these include the identification of the sensing molecules, understanding why the BAG neurons require 2 different responding pathways that react to high $CO_2$ levels, discovering the responsible neuropeptides and their targets that respond to elevated $CO_2$ levels and how the adaption to longer and chronic exposures to elevated $CO_2$ levels occurs.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Funding

This work was supported by NIH grant HL085534.

## References

1. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, et al. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998; 394:485-90; PMID:969777; http://dx.doi.org/10.1038/28867
2. Briva A, Vadász I, Lecuona E, Welch LC, Chen J, Dada LA, Trejo HE, Dumasius V, Azzam ZS, Myrianthefs PM, et al. High CO2 levels impair alveolar epithelial function independently of pH. PLoS ONE 2007; 2: e1238; PMID:18043745; http://dx.doi.org/10.1371/journal.pone.0001238
3. Vadász I, Dada LA, Briva A, Trejo HE, Welch LC, Chen J, Tóth PT, Lecuona E, Witters LA, Schumacker PT, et al. AMP-activated protein kinase regulates CO2-induced alveolar epithelial dysfunction in rats and human cells by promoting Na,K-ATPase endocytosis. J Clin Invest 2008; 118:752-62; PMID:18188452; http://dx.doi.org/10.1172/JCI29723
4. Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science 2000; 289:625-8; PMID:10915626; http://dx.doi.org/10.1126/science.289.5479.625
5. Lecuona E, Sun H, Chen J, Trejo HE, Baker MA, Sznajder JI. Protein kinase A-Ialpha regulates Na,K-ATPase endocytosis in alveolar epithelial cells exposed to high CO(2) concentrations. Am J Respirat Cell Mol Biol 2013; 48:626-34; PMID:23349050; http://dx.doi.org/10.1165/rcmb.2012-0373OC
6. Vohwinkel CU, Lecuona E, Sun H, Sommer N, Vadász I, Chandel NS, Sznajder JI. Elevated CO2 Levels Cause Mitochondrial Dysfunction and Impair Cell Proliferation. J Biol Chem 2011; 286:37067-76; PMID:21903582; http://dx.doi.org/10.1074/jbc.M111.290056
7. Cummins EP, Oliver KM, Lenihan CR, Fitzpatrick SF, Bruning U, Scholz CC, Slattery C, Leonard MO, McLoughlin P, Taylor CT. NF-kappaB links CO2 sensing to innate immunity and inflammation in mammalian cells. J Immunol 2010; 185:4439-45; PMID:20817876; http://dx.doi.org/10.4049/jimmunol.1000701
8. Oliver KM, Lenihan CR, Bruning U, Cheong A, Laffey JG, McLoughlin P, Taylor CT, Cummins EP. Hypercapnia induces cleavage and nuclear localization of RelB protein, giving insight into CO2 sensing and signaling. J Biol Chem 2012; 287:14004-11; PMID:22396550; http://dx.doi.org/10.1074/jbc.M112.347971
9. Sharabi K, Lecuona E, Helenius IT, Beitel GJ, Sznajder JI, Gruenbaum Y. Sensing, physiological effects and molecular response to elevated CO2 levels in eukaryotes. J Cell Mol Med 2009; 13:4304-18; PMID:19863692; http://dx.doi.org/10.1111/j.1582-4934.2009.00952.x
10. Bretscher AJ, Busch KE, de Bono M. A carbon dioxide avoidance behavior is integrated with responses to ambient oxygen and food in Caenorhabditis elegans. Proc Natl Acad Sci 2008; 105:8044-9; PMID:18524954; http://dx.doi.org/10.1073/pnas.0707607105
11. Hallem EA, Sternberg PW. Acute carbon dioxide avoidance in Caenorhabditis elegans. Proc Natl Acad Sci 2008; 105:8038-43; PMID:18524955; http://dx.doi.org/10.1073/pnas.0707469105
12. Kodama-Namba E, Fenk LA, Bretscher AJ, Gross E, Busch KE, de Bono M. Cross-modulation of homeostatic responses to temperature, oxygen and carbon dioxide in C. elegans. PLoS Gen 2013; 9:e1004011; PMID:24385919
13. Carrillo M, Guillermin M, Rengarajan S, Okubo R, Hallem E. O2-Sensing Neurons Control CO2 Response in C. elegans. J Neurosci 2013; 33:9675-83; PMID:23739964; http://dx.doi.org/10.1523/JNEUROSCI.4541-12.2013
14. Hallem EA, Spencer WC, McWhirter RD, Zeller G, Henz SR, Rätsch G, Miller DM, Horvitz HR, Sternberg PW, Ringstad N. Receptor-type guanylate cyclase is required for carbon dioxide sensation by Caenorhabditis elegans. Proc Natl Acad Sci 2011; 108:254-9; PMID:21173231; http://dx.doi.org/10.1073/pnas.1017354108
15. Brandt JP, Aziz-Zaman S, Juozaityte V, Martinez-Velazquez LA, Petersen JG, Pocock R, Ringstad N. A single gene target of an ETS-family transcription factor determines neuronal CO2 chemosensitivity. PLoS One 2012; 7:e34014; PMID:22479504; http://dx.doi.org/10.1371/journal.pone.0034014
16. Guillermin ML, Castelletto ML, Hallem EA. Differentiation of carbon dioxide-sensing neurons in caenorhabditis elegans requires the ETS-5 transcription factor. Genetics 2011; 189:1327-39; PMID:21954162; http://dx.doi.org/10.1534/genetics.111.133835
17. Gramstrup Petersen J, Rojo Romanos T, Juozaityte V, Redo Riveiro A, Hums I, Traunmuller L, Zimmer M, Pocock R. EGL-13/SoxD specifies distinct O2 and CO2 sensory neuron fates in Caenorhabditis elegans. PLoS Gen 2013; 9:e1003511; PMID:23671427; http://dx.doi.org/10.1371/journal.pgen
18. Rojo Romanos T, Gramstrup Petersen J, Redo Riveiro A, Pocock R. A novel role for the zinc-finger transcription factor EGL-46 in the differentiation of gas-sensing neurons in caenorhabditis elegans. Genetics 2014; PMID:25395666
19. Sharabi K, Charar C, Friedman N, Mizrahi I, Zaslaver A, Sznajder JI, Gruenbaum Y. The response to high CO2 levels requires the neuropeptide secretion component HID-1 to promote pumping inhibition. PLoS Gen 2014; 10:e1004529; PMID:25101962
20. Sharabi K, Hurwitz A, Simon AJ, Beitel GJ, Morimoto RI, Rechavi G, Sznajder JI, Gruenbaum Y. Elevated CO2 levels affect development, motility, and fertility and extend life span in Caenorhabditis elegans. Proc Natl Acad Sci 2009; 106:4024-9; PMID:19237558; http://dx.doi.org/10.1073/pnas.0900309106
21. Zuela N, Friedman N, Zaslaver A, Gruenbaum Y. Measuring the effects of high CO(2) levels in Caenorhabditis elegans. Methods 2014; 68:487-91; PMID:24650565; http://dx.doi.org/10.1016/j.ymeth.2014.03.008
22. Ailion M, Thomas JH. Isolation and characterization of high-temperature-induced dauer fFormation mutants in caenorhabditis elegans. Genetics 2003; 165:127-44; PMID:14504222
23. Mesa R, Luo S, Hoover CM, Miller K, Minniti A, Inestrosa N, Nonet ML. HID-1, a new component of the peptidergic signaling pathway. Genetics 2011; 187:467-83; PMID:21115972; http://dx.doi.org/10.1534/genetics.110.121996
24. Wang L, Zhan Y, Song E, Yu Y, Jiu Y, Du W, Lu J, Liu P, Xu P, Xu T. HID-1 is a peripheral membrane protein primarily associated with the medial- and trans-Golgi apparatus. Protein Cell 2011; 2:74-85; PMID:21337012; http://dx.doi.org/10.1007/s13238-011-1008-3
25. Yu Y, Wang L, Jiu Y, Zhan Y, Liu L, Xia Z, Song E, Xu P, Xu T. HID-1 is a novel player in the regulation of neuropeptide sorting. Biochem J 2011; 434:383-90; PMID:21250940; http://dx.doi.org/10.1042/BJ20110027