Sensing, physiological effects and molecular response to elevated CO₂ levels in eukaryotes

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Abstract

Carbon dioxide (CO₂) is an important gaseous molecule that maintains biosphere homeostasis and is an important cellular signalling molecule in all organisms. The transport of CO₂ through membranes has fundamental roles in many basic aspects of life including photosynthesis, nutritive transport, oxidative metabolism and signalling. Once transported into the cell, CO₂ interacts with H₂O to produce carbonic acid, which is in equilibrium with H⁺ and HCO₃⁻. At 37°C, this reaction is accelerated 0.5 to 1 × 10⁶ times by the zinc-containing enzyme carbonic anhydrase (CA). Another chemical reaction affecting CO₂ concentration within cells is its interaction with a free R-NH₃⁺ group on proteins to form carbamate. Although work presented below suggests that there are specific transporters that help move CO₂ across membranes, a recent study supports the hypothesis that passive diffusion is sufficient to provide

CO₂ transport into cells

Transport of carbon dioxide (CO₂) through membranes has fundamental roles in many aspects of life including photosynthesis, nutritive transport, oxidative metabolism and signalling. Once transported into the cell, CO₂ interacts with H₂O to produce carbonic acid, which is in equilibrium with H⁺ and HCO₃⁻. At 37°C, this reaction is accelerated 0.5 to 1 × 10⁶ times by the zinc-containing enzyme carbonic anhydrase (CA). Another chemical reaction affecting CO₂ concentration within cells is its interaction with a free R-NH₃⁺ group on proteins to form carbamate. Although work presented below suggests that there are specific transporters that help move CO₂ across membranes, a recent study supports the hypothesis that passive diffusion is sufficient to provide
necessary transport and that facilitated transport of CO₂ is not necessary. This study measured the changes in CO₂ gradients through planar lipid bilayer membranes and through epithelial cell monolayers. Membrane permeability was calculated from measuring small changes in pH and by applying Overton’s rule, which, in lay terms, dictates that the easier it is for a chemical to dissolve in a lipid the faster it will be transported into a cell. This study concluded that passive diffusion could explain CO₂ transport into cells with a rate-limiting step of near-membrane unstirred layer [1]. It should be noted, however, that a recent study questions the validity of Overton’s rule and thus the sufficiency of passive diffusion of CO₂ [2].

The hypothesis that CO₂ is transported solely by passive diffusion through membranes has been challenged by several observations including the impermeability of apical membranes of gastric gland cells to CO₂ [3], the reduction of permeability of red blood cells (RBCs) to CO₂ by the anion transport inhibitor 4,4′-Diisothiocyanatostilbene-2,2′-disulfonate [4], and by the discovery of evolutionarily conserved proteins that can serve as CO₂ transporters [5–8].

One of the major conserved CO₂ transporter-protein families are the aquaporins (AQP). In plants, CO₂ uptake can be a rate-limiting step in photosynthesis. Work in the plant Nicotiana tabacum showed that the water channel AOP could also serve as a CO₂ channel. Overexpression of the aquaporin AQP1 gene increased membrane permeability for both CO₂ and water, and enhanced leaf growth [5], while down-regulation of AQP1 lowered CO₂ permeability in isolated membranes of the inner chloroplast membrane, where AQP1 resides, and caused a 20% lower conductance to CO₂ within leaves in vivo [6].

The rhesus (Rh) protein family is another putative CO₂ channel. Rh proteins are best known as antigens on human RBCs, but they are not restricted to RBCs and are conserved in evolution. In organisms as diverse as human and yeast the Rh proteins function as ammonium and methylammonium transporters [7]. Work in the green alga Chlamydomonas reinhardtii showed that the Rh protein, RH1, can also function as a CO₂ channel [8]. Expression of rh1 is induced under high CO₂ and is suppressed under low CO₂ [9]. Alga, in which the rh1 gene has been down-regulated in vivo grow normally in air, but slowly in high CO₂, apparently because they fail to equilibrate CO₂ rapidly. That RH1 is important for CO₂ responses is demonstrated by the observation that Chlamydomonas reinhardtii genes that are known to be down-regulated by high CO₂ fail to be down-regulated in RH1-null cells [8]. Methylammonium uptake is little changed by the absence of Rh1, suggesting that in Chlamydomonas reinhardtii either RH1 does not function as an ammonium channel or that there are other transporters that can handle ammonium transport in the absence of Rh1.

In mammalian cells there is evidence for both AQP and Rh proteins having roles in CO₂ transport. In AQP1-null RBCs (known as Colton null RBCs) CO₂ transport was reduced by 50% [10]. Inhibition of AQP1 in RBCs by the inhibitor p-chloromercuribenzenesulfonic acid reduced pCO₂ by 60%. These results led to the conclusion that AQP1 is responsible for ~60% of the high pCO₂ in RBCs. Band 3 complex in RBCs includes both AQP1 and the Rh proteins [11, 12]. RBCs lacking Rh protein had only 50% transport of CO₂, similar to the reduction in RBCs cells lacking AQP1 [13], suggesting overlapping functions between the AQP and Rh proteins.

It should be noted that in addition to transporting ammonium/methylammonium, CO₂ and H₂O there is evidence that Band 3, Rh and AQP1 complexes can transport O₂ and nitric oxide, which makes them non-specific gas channels [14, 15]. However, although AQP and Rh channels can transport a broad range of gases, they do not transport all gases equally. In a recent study, genes encoding human AQP1, rat AQP4, rat AQP5, human RhAG and bacterial AmiB were injected into X. oocytes and then surface pH was measured to establish the relative selectivity to CO₂ and NH₃ [16]. It was found that the different channels have different gas selectivities with the AQP channel more efficiently transporting CO₂, while the Rh channel more efficiently transported NH₃.

How important are the Rh and AQP proteins to animals as a whole? In C. elegans, one of the Rh1 proteins (CeRh1) is expressed in all tissues albeit with higher expression in the hypodermis, which is the tissue directly exposed to the surrounding atmosphere. One study showed that mutation of the rh1 gene causes lethality and abnormal development [17].

Another C. elegans study tested the effects of homozygous deletions in each of the two Rhesus genes: rh1 and rh2, as well as in the aquaporin gene aqp-2 on egg-laying in animals grown in 15% or 19% CO₂. They found that for all the single deletions the number of laid eggs was further reduced in CO₂, suggesting that in C. elegans the response to hypercapnia is independent of these genes [18].

Fungi, like C. elegans, do not require Rh1 or AOPs for CO₂ transport. The counterparts for the Rh proteins are the Mep NH₃/NH₄⁺ ion transporters. S. cerevisiae and the two C. albicans and C. neoformans Mep proteins probably do not transport CO₂ (reviewed in [19]). Likewise, the C. albicans aquaporin aqp1 gene is required for freeze-tolerance but not for CO₂-mediated filamentation [20].

In conclusion, despite considerable work in many species, how CO₂ is transported into cells is still a matter of some debate. While passive diffusion through membrane can explain much of the CO₂ transport, the passive diffusion model cannot explain the low permeability of gastric cells and RBCs to CO₂ and the demonstrated roles of AQP and Rh protein complexes in CO₂ transport in plants, some lower organisms and in RBCs. More studies, including simultaneous knockouts of the Rh and AOPs in mice, D. melanogaster and C. elegans are required to understand how CO₂ is transported across cell membranes.

**CO₂ sensing in cells and organisms**

Chemosensation, the recognition of soluble and volatile chemicals by cells, plays an essential role in the behaviour and survival of
most organisms. CO2 is a by-product of metabolism and is an important signal for a variety of animal behaviours, including feeding, ventilation, mating and avoiding predators and harmful substances. The ability to sense CO2 has been described in many eukaryotes ranging from fungi to human beings.

**CO2 sensing in mammalian neuronal cells**

From a physiological standpoint, some of the most important CO2 sensors are those that control breathing in mammals. Changes in CO2 and CO2/H+ levels are sensed in special chemosensitive neurons located peripherally in the carotid body and centrally in the central nervous system. The peripheral carotid neurons detect arterial CO2 and pH, as well as variations in O2 levels in arterial blood [21]. The central neurons reside in several regions of the hindbrain and detect CO2 and pH in the cerebrospinal fluid [22].

**CO2 sensing in peripheral chemoreceptors**

The carotid body (carotid glomus) is located near the fork of the carotid artery. It has two types of cells; catecholamine-containing type I (glomus) cells, which are sensitive to changes in both CO2 and O2 levels in arterial blood [21, 23], and glial-like type II cells. The type I glomus cells are the primary site of peripheral chemoreception, where they act as a sensor that detects changes in CO2 and O2 levels in arterial blood [21, 23]. During normal breathing, the carotid chemoreceptors are critical for maintaining stable and normal CO2 levels [24]. While the central chemosensitive neurons appear to have a quantitatively larger contribution to stimulating ventilation in response to hypercapnia [25], during mild hypercapnia the carotid chemosensitive neurons contribute to roughly 30% of the response (summarized in Table 1 in [21]). Importantly, the response of the carotid body neurons is quicker than that of the central neurons and the carotid neurons react first to rapid transient changes in arterial CO2 levels [26]. Indeed, denervation of the carotid body causes a slower response to changes in CO2 levels and a constant fluctuation in CO2 levels during normal breathing. Carotid body denervation also decreases normal breathing, causing 13–18 mmHg increase in CO2 levels in different mammals including awaked dogs, goats and ponies [27–31].

It is not clear yet what the exact signal sensed by the glomus cells is or what the molecular sensor is. Studies have suggested that it could be elevated CO2 levels, HCO3− concentration, reduced intracellular pH or a pH gradient across the membrane. For example, inhibition of intracellular change in pH by using a permeable CA inhibitor slowed down the firing of the neurons, suggesting that change in pH is important for the response of these cells [22, 32]. On the other hand, hypercapnia-induced acidosis had much greater effect on neuron firing as compared to metabolic acidosis [33], suggesting that CO2 or changes in pH across the membrane are the signals for neuron firing [34]. While the identity of the CO2 sensor is not clear, it has been determined that neuronal firing in response to CO2 requires L-type Ca2+ channels, protein kinase A (PKA) and soluble adenylate cyclase (sAC) (see section ‘Molecular responses in mammalian neuronal cells’).

**CO2 sensing in central chemoreceptors**

The central chemosensitive neurons have a major contribution in mediating increased ventilation in response to hypercapnia. These neurons are found in numerous hindbrain stem regions and have different levels of chemosensitivities. They include the retrotrapezoid nucleus (RTN), the rostral medullary raphe, the caudal nucleus tractus solitarius, the fastigial nucleus of the cerebellum, locus ceruleus, A5 region and the pre-Bötzinger complex [35]. Further, *in vitro* and *in vivo* studies have shown that additional types of neurons can respond to changes in CO2 levels. These neurons show altered firing rate in response to changes in CO2 levels and reside in regions shown to alter ventilation in response to acidification. However, it is not clear yet whether they intrinsically respond to changes in CO2 levels or whether they respond to altered synaptic input from other neurons that are themselves chemosensitive (reviewed in [22]).

The central chemoreceptors detect changes in CO2/H+ of cerebrospinal fluid, which are caused by changes in CO2 that diffuse from the plasma to the cerebrospinal fluid and forms carbonic acid [36]. To avoid a potential general inhibitory effect by CO2/H+, most of the studies on central chemosensitive neurons have focused on CO2-excited neurons [37]. However, in studies on slices from the dorsal or ventral medulla, half of the neurons were actually inhibited by hypercapnia [38, 39], suggesting that many features of hypercapnia neuronal responses are missed when studying only CO2-excited neurons. Rat brain stem slices were also used to show the specificity of chemosensitive neurons [40, 41]. Upon exposure to hypercapnic acidosis, neurons from the chemosensitive ventrolateral medulla and the nucleus tractus solitarii regions became acidic and remained acidic during the entire exposure, with pH returning to control values upon return to normocapnic solution. In contrast, neurons from the non-chemosensitive inferior olive and hypoglossal regions recovered from the hypercapnia acidosis-induced acidification during the period of acid exposure [42].

Similar to what has been described for the carotid body, it is not clear whether the central chemosensitive neurons detect pH levels, CO2 levels, HCO3− concentration or a pH gradient across the cell membrane. What is clear is that the ventilatory response to hypercapnia is greater than to metabolic acidosis [43]. In addition, central neurons have greater firing response to hypercapnic acidosis than isocapnic acidosis, where the CO2 levels remain constant and HCO3− concentration and pH are decreased [44, 45]. These observations suggest the existence of CO2 receptors, analogous to receptors for other gases such as O2, nitric oxide and CO [46–49]. It was demonstrated that the L-type Ca2+ channels mediate responses of CO2 receptors, whose identity has not been determined (see details in section ‘Molecular responses in mammalian neuronal cells’). Another important observation is that there is variability in the chemosensitivity of neurons in different areas of the brain, suggesting different patterns of responses by
the different brain areas. In addition, it is likely that there is not a single adequate stimulus for central chemosensitive neurons but rather multiple stimuli responses and targets.

Key insights into the identities of central neurons required for normal physiological regulation of CO2 levels has come from studies of genetic diseases and brain lesions. The rare congenital hypoventilation syndrome (CHHS) is defined by the failure of automatic control of breathing. It is characterized by abnormal control of respiration in the absence of neuromuscular or lung disease, or an identifiable brainstem lesion [50, 51]. The patients have absent or negligible ventilatory sensitivity to hypercapnia and hypoxemia. They breathe normally while awake but hypoventilate with normal respiratory rates and shallow breathing during sleep. Severely affected patients hypoventilate both awake and asleep (reviewed in [52]). Most CHHS patients have mutations in the paired-like homeobox gene PHOX2B [53, 54]. However, in rare cases this disease can be also caused by mutations in the RET, GDNF, EDN3, BDNF and ASCL1 genes (see OMIM # 209880). The RTN expresses PHOX2B. It contains ~2000 neurons that selectively innervate the respiratory centres of the pontomedullary region in a chemosensitive region [52]. These neurons are highly sensitive to acidic pH in vitro and are activated by inputs from the carotid body and from the hypothalamus in vivo [52]. Mice heterozygous for the CHHS-causing expanded alanine tract in the Phox2b gene show irregular breathing, do not respond to an increase in CO2, and die from central apnea soon after birth [55]. Postmortem examination of these mice showed specific loss of Phox2b-expressing glutamatergic neurons in the RTN region, whereas other areas thought to be involved in breathing regulation were anatomically normal. In addition, destruction of 70% of RTN neurons helped demonstrating the role of these neurons in normal breathing in anesthetized rats [56]. These data suggest that RTN neurons regulate CO2 levels by automatic breathing during sleep and also contribute to breathing when awake. Because most patients can breathe at normal rhythm when awake, these data also suggest that the RTN neurons do not directly control breathing rhythm, but rather generate a large portion of the excitatory signal to locate prey [66].

It still remains to be determined whether the RTN neurons have intrinsic response to CO2/H+ in vivo. Also, the precise targets of RTN neurons and the modes of synaptic transmission remain to be defined.

**CO2 sensing by non-neuronal mammalian cells**

In addition to central and peripheral neurons, other mammalian cell types have also been shown to respond to CO2. Given the critical role of kidney tubule cells in HCO3− homeostasis (see section ‘Physiological effects on mammalian tissues’), one might expect kidney cells to be CO2 responsive. Indeed, work by Zhou et al. has provided evidence that there are CO2 and/or HCO3− sensors on the basolateral surfaces of rabbit proximal tubule epithelial cells that regulate HCO3− generation and reabsorption [57]. Further work using small molecule inhibitors has indicated that CO2-induced responses require an as yet unidentified receptor tyrosine kinase that could be the actual CO2 sensor [58].

There is also considerable evidence that cardiovascular and pulmonary cells respond to CO2 levels independently of neuronal input. Work in our laboratories has demonstrated that CO2 levels control cell-surface levels of the Na,K-ATPase in cultured alveolar epithelial cells via a CO2 response pathway that acts independently of extracellular and intracellular pH. This pathway involves Ca2+/Ca2+/calmodulin dependent protein kinase β, AMP-kinase and protein kinase C-ζ (PKC-ζ) (see section ‘Lung cells’ and Fig. 3) [59, 60]. Other in vitro studies have shown that elevated CO2 levels suppress expression of tumour necrosis factor and other cytokines by pulmonary artery endothelial cells [61] and in peritoneal and pulmonary macrophages [62, 63]. The molecular mechanisms of this suppression are not yet clear, but at least for endothelial cells are thought to involve pH-independent suppression of NF-κB activation [61].

**CO2 sensing in Drosophila and other insects**

**Neuronal-mediated CO2 sensing**

Many arthropods have anatomical features dedicated to CO2 sensing (reviewed in [64]). Between species, these structures differ greatly at all levels of organization, but all of them are types of olfactory sensilla that can in some cases form specialized CO2-sensing organs. Insects, and *Drosophila* in particular, have been useful models for the study of chemoreception because there is notable functional and anatomical similarity in smell and taste pathways between vertebrates and *Drosophila* (reviewed in [65]). In some herbivorous adult arthropods, such as beetles and grasshoppers, no CO2-sensing structures have been identified. Indeed, these structures are most commonly found in blood-feeding insects such as mosquitoes, which presumably use CO2 as a signal to locate prey [66].

*D. melanogaster* are able to detect both gaseous CO2 [67, 68] and CO2 in water [69]. This distinction serves to illustrate the division of *D. melanogaster* chemoreception into two separate modalities – the detection of volatile molecules (olfaction or smell) and the detection of soluble molecules (gustation or taste). Thus, the fruit fly can ‘smell’ CO2 in air, and ‘taste’ carbonation.

In adult flies, the olfactory sensilla are located on two appendages on the fly head: the maxillary palp and the third segment of the fly antenna [70]. The antenna contains the olfactory receptor neurons (ORNs), organized morphologically into three sensilla used for smell: the basiconic, trichoid and coeloconic sensilla [71]. In contrast, several locations on the fly body contain gustatory sensilla, including the labial palps of the proboscis, sense organs inside the pharynx and taste bristles located on the legs and anterior wing margin.

The antennal basiconic (ab) sensilla can be grouped into three types (ab1, ab2, ab3) based on their odour responses, and the ab1 sensillum contains four different types of ORNs [72]. The sensilla protrude from the antennal cuticle, making electrophysiological measurements relatively easy. In an electrophysiological analysis...
of odour coding of these ORNs, the C neuron of the ab1 sensillum (ab1C) was found to respond specifically and strongly to CO₂. These findings were confirmed in a separate study [73].

In *D. melanogaster* adults, gaseous CO₂ elicits an avoidance behaviour [74]. This discovery was made after it was observed that flies preferred to avoid containers in which other flies had previously been exposed to stressful conditions, such as vigorous shaking or electric shock. This behaviour was inhibited by surgical removal of the third antennal segment, indicating that the stressed flies may have emitted a ‘stress gas’ that was detected by other flies new to the container. Mass spectrometry and gas chromatography revealed that CO₂ was a component of the ‘stress gas’, and CO₂ alone could elicit the avoidance behaviour in a T-maze assay. This behaviour was dose dependent, with flies avoiding CO₂ concentrations of 0.1% and above. Further, stressed flies were shown to emit 3- to 4-fold more CO₂ than unstrressed flies.

This study also found that CO₂ (0.05% and above) causes activation of a single glomerulus, the V glomerulus, in the fly antennal brain lobe [74]. This glomerulus is innervated by ORNs that had previously been shown to express the candidate gustatory receptor Gr21a [75], and which project specifically from the region of the antenna where the ab1 sensilla are located. Subsequent experiments showed that CO₂ activates these Gr21a-expressing neurons, and that they are required for the CO₂ avoidance behaviour. These findings were corroborated by Faucher et al. [76]. The importance of Gr21a-expressing neurons to CO₂ responsiveness has been further demonstrated by the finding that in larvae, Gr21a is expressed in a single neuron in the terminal organ, with genetic ablation of this neuron resulting in loss of CO₂ avoidance.

That Gr21a-expressing neurons respond to CO₂ suggests that Gr21a could in fact be a receptor for CO₂. As detailed in section ‘Molecular responses in *D. melanogaster*’, recent molecular work presents strong evidence that Gr21a, along with the related Gr63a receptor, is a CO₂ receptor in the *Drosophila* olfactory system [67, 68] see section ‘Molecular responses in *D. melanogaster*’.

In contrast to gaseous CO₂, which is repellent, carbonation is attractive to *D. melanogaster* adults [69]. The E409 neurons in the gustatory sensilla of the fly proboscis are responsive to both beer and the supernatant of growing yeast, but not to over 50 other tested compounds [69]. After testing two common products secreted by yeast, CO₂ and ethanol, it was found that the neurons could detect carbonation specifically and robustly from 0.2% upwards. However, E409 neurons are also able to respond to high levels of gaseous CO₂, with 100% CO₂ gas causing a response equal to 0.4% CO₂ in water, which is a saturating stimulus. Although the molecular mechanism of gustatory detection of carbonation is not yet known, detection of carbonation is likely not dependent on pH because these neurons do not respond to acids or solutions of varying pH, and pH does not change the attractiveness of PBS solutions to flies. It is possible, however, that the neurons could be detecting carbonic acid. The ability to differentially detect gaseous and aqueous CO₂ may allow the fly to appropriately fine-tune responses to local and global CO₂ levels, or to distinguish between a danger signal and a good meal at the pub.

**Non-neuronal CO₂ sensing**

As CO₂ is an important environmental cue, one would expect the sensory system to respond to CO₂. One might further expect that because CO₂ is a ubiquitous metabolite, that non-neuronal cells would also have mechanisms for sensing physiological CO₂ levels. The existence of a non-neuronal CO₂-sensing system has recently been demonstrated by showing that in CO₂ levels as low as 7%, both wild-type and adult flies lacking the Gr63a receptor become immune suppressed and lay fewer eggs [77]. Further, 13% CO₂ suppresses the expression of anti-microbial peptides in the immune-responsive S2* cell line. This non-neuronal CO₂ response pathway appears to be a novel response pathway because its activity is independent of pH, nitric oxide, heat shock and hypoxia responses [77]. These results demonstrate that *Drosophila* cells have responses to CO₂ that are independent of the neuronal CO₂ responses. However, it remains to be determined to what extent these neuronal CO₂-sensing impacts responses to CO₂ that can be regulated without neuronal input.

**CO₂ sensing in *C. elegans***

Two recent papers have demonstrated that wild-type *C. elegans* (N2) acutely avoid CO₂ [78, 79]. Worms subjected to a 0–5% CO₂ gradient in a microfluidic chamber quickly avoid the area where the CO₂ level is high. Similarly, when the head of a forward-moving worm is exposed to an air stream containing 10% CO₂, within few seconds the animal reversed its direction. In both studies 1% CO₂ was measured as the threshold level of avoidance. This avoidance behaviour is probably independent of any CO₂-induced pH changes, since exposing the animals to different media with pH levels ranging from 4.9 to 7.1 did not affect the avoidance behaviour. cGMP signalling probably contributes to the CO₂ avoidance as *tax*-2 and *tax*-4 mutants, encoding two subunits of a cGMP gated ion channel, do not avoid CO₂. This result also indicates that *TAX-2* and *TAX-4* are essential for CO₂ avoidance behaviour. Expression of *TAX-4* in BAG neurons alone was sufficient to recover the CO₂ avoidance defect of *tax*-4 mutants, thus implicating the involvement of these neurons in CO₂ avoidance and demonstrating the sufficiency of *TAX-4* in mediating CO₂-responsive behaviours [78, 79].

Notably, *C. elegans* do not avoid CO₂ in all situations. For example, starved animals do not avoid CO₂, which implicates the involvement of metabolism as a regulator. Indeed, animals with a reduced *daf-2* signalling, which mimics starvation conditions, do not avoid CO₂ [78, 79]. In addition, animals defective in the transforming growth factor (TGF)-β pathway, which is another key regulator of starvation, do not avoid CO₂ [78, 79]. Interestingly, feeding behaviour is intimately linked with CO₂ avoidance behaviour. For example, different wild-type isolates of *C. elegans* show different feeding behaviour. Animals with solitary feeding behaviour strongly avoid CO₂, while animals with social feeding behaviour do not. Genetically interfering with the feeding behaviour can change the way wild-type (N2) animals respond to CO₂. Animals with a null mutation in the *npr-1* gene, a neuropeptide Y receptor, change
their feeding behaviour from solitary to social and accordingly change the way they respond from CO2 sensitive to CO2 insensitive. Mutations in NPR-1 or the neuronal globin domain protein, GLB-5, affect the responses to both CO2 and O2, implicating a potential crosstalk between the CO2 and O2 response pathways. Genetic variation in these genes is probably responsible for the different responses exhibited by the laboratory wild-type strain N2, which avoids elevated CO2 but is almost indifferent to O2 levels, while another wild-type strain CB4856 avoids elevated O2 levels and is attracted to high CO2 levels [80].

**CO2 sensing in fungi**

Certain fungi pathogenic to human beings sense and adapt to the widely varying CO2 concentrations of the environments they inhabit, which can have significantly different CO2 levels. For example, the mammalian blood stream is at ~5% CO2 which is >100 times higher than ambient air at 0.039%. In C. neoformans [81] and C. albicans [82, 83] elevated CO2 levels have strong effects on growth, and induce virulence factors such as capsule biosynthesis and filamentation, (reviewed in [19, 84]). Because virulence factors in these organisms are regulated by adenylyl cyclases and cAMP signalling [85, 86], possible roles for adenylyl cyclases in CO2 sensing were investigated. Purified adenylyl cyclases from both these species are activated by bicarbonate in vitro [20, 87], and appear to act as sensors of elevated CO2 levels. However, adenylyl cyclases do not act alone in CO2 sensing in these organisms as CAs, which convert gaseous CO2 to bicarbonate, were also shown to be involved. The CAs Nce103 of C. albicans [20] and Can2 of C. neoformans [87] are required for growth and pathogenicity in low CO2 conditions. However, strains mutant for these CAs can still infect and proliferate in a mammalian host, where CO2 levels are high and presumably sufficient levels of bicarbonate are produced by spontaneous conversion without the need for catalysis. It is currently unknown how these results generalize to other fungi.

**Physiological effects of elevated CO2**

**Physiological effects on mammalian tissues**

CO2 is produced by the body's metabolism at approximately the same rate as oxygen consumption (at rest ~3 ml/kg/min.). CO2 diffuses readily from cells into the bloodstream, where it is carried partly as HCO3-, partly in chemical combination with haemoglobin and plasma proteins, and partly in solution at a partial pressure of about 46 mmHg in mixed venous blood. CO2 is eliminated from the body by the lung, where it is normally exhaled at the same rate at which it is produced, leaving a partial pressure (paCO2) of about 40 mmHg in the alveoli and arterial blood [88].

CO2 plays a major role in pH homeostasis as part of the CO2/HCO3- buffer system that regulates plasma pH [89]. Unlike the other buffer systems in the body, where addition or loss of hydrogen ions changes the concentration of the weak acid, in the CO2/HCO3- system, the concentration of the weak acid (CO2) is essentially constant. This is because the paCO2 is regulated by our respiratory system to be about 40 mmHg. Any rise or fall in pCO2 resulting from the addition or loss of hydrogen ions is sensed by the respiratory centres in the brainstem that alter the rate of ventilation to restore the concentration (see section ‘CO2 sensing in mammalian neuronal cells’). However, adding or removing hydrogen ions from a source other than CO2 changes the concentration of HCO3-. Adding hydrogen ions by diet or some physiological process reduces HCO3- on a nearly mole-for-mole basis. Removing hydrogen ions drives the reaction to the right and raises HCO3- in the same way. The problem of maintaining hydrogen ion balance becomes one of maintaining HCO3- balance. For every hydrogen ion added to the body, one HCO3- disappears; therefore, to maintain balance it is necessary to generate new HCO3- to replace the one that was lost. Generation of new HCO3- is the responsibility of the kidneys. The kidney has two major responsibilities in maintaining acid–base balance. The first is to reabsorb any HCO3- that is filtered at the glomerulus and to return it to the plasma. The second is to generate HCO3- through non-bicarbonate buffer systems and by metabolizing glutamine [90].

**Pathophysiological effects of elevated levels of CO2**

There are several pathological conditions in which the pulmonary, blood or tissue levels of CO2 increase and cause hypercapnia. Hypercapnia may result from decreased ventilation and non-metabolic generation of CO2 as occurs during tissue hypoxia. The physiological responses to hypercapnia depend on the concentration of CO2 and the duration of elevated CO2 exposure [91]. Low concentrations of CO2 in the inspired air are tolerated, with an increase of respiratory rate as the main effect. Higher levels cause dyspnoea (shortness of breath), headaches, which are thought to result from cerebral vasodilation caused by the elevated paCO2, restlessness, faintness, dulling of consciousness, greatly elevated alveolar ventilation, muscular rigidity and tremors occur at inspired CO2 concentrations greater than 15%. At 20% to 30% inspired CO2, generalized convulsions can be produced [92].

In several lung diseases there is inadequate gas exchange which results in accumulation of CO2 in the body to levels above 50 mmHg. The effects of high pCO2 on the lung epithelium and how hypercapnia contributes to disease progression have not been fully elucidated. A significant number of patients with chronic obstructive pulmonary disease have elevated pCO2 levels, which is associated with worse outcome [93–96]. In patients with pulmonary oedema and acute respiratory distress syndrome, there is impairment in pulmonary gas exchange and poor patient outcome unless corrective measures are taken to resolve the oedema and re-establish normal epithelial barrier function [97–100]. During respiratory failure an important measure is the use of mechanical ventilation. However, ventilation at levels that restore pulmonary CO2 levels to normal frequently results in ‘ventilator-induced lung
injury'. There is significant evidence that ventilator-induced lung injury can be reduced through the use of 'permissive hypercapnia' regimes in which less aggressive ventilation is used and CO2 levels are allowed to rise as high as 100 mmHg [101–106]. paco2 levels as high as 250 mmHg have been reported in patients with uncontrolled asthma [107, 108]. The use of 'permissive hypercapnia' has been supported by results from experimental models where hypercapnic acidosis has been reported to be protective against ischemia reperfusion and ventilator-induced lung injury [104, 109–111]. However, more recent studies have suggested that hypercapnia may have deleterious effects on the lungs such as impaired alveolar fluid absorbance [112–116], which questions the clinical use of the 'permissive hypercapnia' [112–117].

Hypercapnia leads to impaired lung oedema clearance, a major function of the alveolar epithelium [59]. The changes triggered by the alveolar epithelium appear to be independent of pH, as metabolic acidosis does not have an effect on fluid clearance, whereas hypercapnia with normal pH levels lead to decreased fluid clearance. The deleterious effects of hypercapnia on the alveolar epithelia are not due to hypoxia, nor are they mediated by CAs [118]. Effects of hypercapnia on alveolar epithelia have been observed for up to 7 days in rats and at least in the short term, are reversible upon normalization of CO2 levels [59]. The effects of hypercapnia on alveolar fluid clearance are also reversed by the treatment of rats with β-adrenergic agonists [60].

Mechanical ventilation that alters paCO2 and paO2, may also affect vascular dynamics via activation or inactivation of vasoactive factors such as nitric oxide, angiotensin II, endothelin and bradykinin [119]. Hypercapnia is inversely correlated with renal blood flow (RBF) and causes renal constriction. The direct mechanisms include activation of the sympathetic nervous system through release of norepinephrine. The increased sympathetic activity reduces RBF and glomerular filtration rate and contributes to a non-osmotic release of vasopressin. The indirect mechanism is a decrease in systemic vascular resistance due to systemic vasodilatation. The decrease leads to further release of norepinephrine and stimulation of the renin–angiotensin–aldosterone system, causing decreased RBF. These hypercapnic effects occur independently of paO2 and determine the renovascular response to changes in arterial blood gas parameters [119].

Physiological effects on D. melanogaster

Drosophila and other insects are routinely anesthetized by using elevated CO2 levels. Exposure to 100% CO2 for a few seconds is sufficient to render adult flies and larvae immobile and easy to handle. In this paralysed state, the animals are non-responsive to mechanical sensory stimuli [120]. Therefore, the effects of CO2 on fly physiology have primarily been considered in the context of potential side effects resulting from its use as an anaesthetic, usually an acute pulse of 100% CO2. Fewer studies have explored the physiological effects of chronic exposure (hours to weeks) to non-anaesthetic (<30%) levels of CO2.

Physiological effects on C. elegans

In response to CO2 levels of 9% and above, C. elegans show specific phenotypes that are independent of pH changes in the growth media [77]. These phenotypes include reduced number of laid eggs, which developed with normal morphology but at a reduced rate, and a reduced pharyngeal pumping rate, which returned to normal when the animals were returned to normocapnia. In addition, chronic exposure to CO2 caused a reduction in motility, probably due to the deterioration of striated muscle (Fig. 1). The effect of chronic exposure to elevated CO2 levels on muscle cells are especially intriguing, because patients with
chronic obstructive pulmonary disease show reduction in muscle mass and increased ubiquitination and proteolysis [127, 128]. The C. elegans phenotypes were more severe with increasing levels of CO2 (up to 19%), and were accompanied with specific and dynamic changes in transcription (see section ‘Molecular responses in C. elegans’).

Importantly, there was also a significant increase in lifespan [18]. This lifespan increase is probably independent of the IGF-1 pathway, because both the short-lived daf-16(mu86) and the long-lived daf-2(e1370) mutants still show significant CO2-dependent extension in lifespan. The lifespan extension was also independent of mitochondria-mediated aging, egg laying, diet restriction or diet deprivation, because clk-1(e2519), eat-2(ad1116) and glp-1 (or178) mutant animals all showed extension in lifespan [18] (K.S. and Y.G., unpublished observations).

Molecular responses to elevated CO2 levels

Molecular responses in mammalian neuronal cells

It is not clear yet whether mammalian chemosensitive neurons sense cellular pH, a pH gradient across the membrane, CO2 or HCO3. Therefore, it is not surprising that the receptors that directly sense CO2 levels have not yet been defined. HCO3− (but not CO2 or change in pH) causes direct activation of a 48 kD sAC protein [129]. In mammalian cells, soluble sAC is targeted to well-defined intracellular compartments including mitochondria, centrioles, mitotic spindles, mid-bodies and nuclei, where it responds to intrinsic cellular signals and can regulate cell metabolism [130, 131]. Under conditions of elevated CO2 levels, there is an increase in HCO3− levels leading to increase in sAC and cyclic AMP (cAMP) levels [130] (Fig. 2). The cAMP activates PKA, which in turn leads to opening of L-type Ca2+ channels and influx of Ca2+ into cells. Indeed, in the glomus cells of the carotid nucleus, increased CO2 levels cause the activation of L-type Ca2+ channels and an elevated Ca2+ influx [132–134]. Experiments in isolated glomus cells showed that the activation of Ca2+ channels and the elevated Ca2+ influx are independent of changes in pH [133] (Fig. 2).

Molecular response to elevated levels of CO2 in mammalian non-neuronal cells

Lung cells

Although the sensing of CO2 and the mechanisms by which high CO2 levels lead to alveolar dysfunction (i.e. impaired alveolar fluid clearance) have not been fully elucidated, significant progress has been made in the understanding of some of the signalling pathways. Acute hypercapnia leads to impaired function of the alveolar epithelial Na,K-ATPase due to its endocytosis [59, 60]. The signalling pathway leading to Na,K-ATPase endocytosis starts with an increase in cytosolic Ca2+, which leads to activation of the Ca2+-calmodulin dependent protein kinase β (Fig. 3). This kinase phosphorylates the AMP-activated protein kinase at Thr172 in the activation loop of its α subunit, which in turn activates the...
atypical PKC-ζ. Phosphorylation of the Na,K-ATPase α1-subunit at Ser 18 by PKC-ζ promotes its endocytosis resulting in decreased Na,K-ATPase activity in alveolar epithelial cells and accompanying pulmonary oedema [59, 60] (Fig. 3).

Kidney cells
A major task of the kidney is to secrete H+ into the urine, and thus hypercapnia rapidly stimulates renal H+ secretion [135, 136]. The renal proximal tubule reabsorbs ~80% of the HCO3− filtered by the glomerulus by secreting H+ into the proximal tubule lumen and using this H+ to titrate luminal HCO3− to CO2 and H2O. After entering the cell across the apical membrane, the CO2 and H2O recombine to produce H+ and HCO3−. Extrusion of H+ into the lumen across the apical membrane is carried out by Na-H exchangers [137] and H+ pumps [138] and HCO3− is transported across the basolateral membrane via the electrogenic Na+−HCO3− co-transporter NBCe1-A [139]. Although there is not much information on the molecular mechanisms involved in renal H+ secretion induced by high levels of CO2, it has been described that respiratory acidosis stimulates activity of renal H+ ATPases [136], probably by stimulating their recruitment towards the plasma membrane [140]. Other work has indicated that an as yet unidentified receptor tyrosine kinase is required for responses of rabbit proximal tubule cells to changes in CO2 levels [58].

Molecular responses in D. melanogaster

Olfactory responses
A critical clue to the molecular mechanisms mediating CO2 responses came from observations that there is a single population of neurons expressing the candidate gustatory receptor Gr21a, which is required for sensing gaseous CO2 in both adult flies and larvae [74, 76]. Subsequent genetic and molecular experiments showed that the Gr21a receptor is not merely a marker of CO2-responsive neurons, but is required for sensing CO2 [67, 68]. The ab1C neurons also express another gustatory receptor, Gr63a. Ectopic expression of both the receptors together, but not either alone, conferred CO2 responsiveness to neurons that were previously unresponsive to CO2. The response is highly specific and dose dependent, with ab1C neurons starting to fire immediately upon increasing CO2 concentration above ambient, while in the ectopic system 3% CO2 was readily detected. Flies lacking Gr63a fail to avoid CO2 in a T-maze assay, similar to flies in which the Gr21a-expressing neurons had been silenced. Thus, a Gr21a/Gr63a heterodimer is thought to be necessary and sufficient for sensing CO2 [67, 68]. Gr63a and Gr21a are highly conserved across Drosophilid genomes, and paralogs of Gr21a are present in mosquitoes, the silk moth and red flour beetle [141]. Although ancient, the Gr21a and Gr63a gene lineages are absent in the other hexapods whose genomes have been sequenced, even though some of these species are known to sense CO2.

It is not yet known how Gr21a and Gr63a detect CO2, and what the role of each receptor is. For example, it is unclear if one acts as a signalling or localization cofactor or chaperone, while the other binds ligand or if both receptors are required for both functions. Likewise, it is not yet known if the signalling ligand is CO2 itself, bicarbonate or perhaps CO2 bound to some other as yet unknown factor. Neither study in which the CO2 receptors were identified examined the pH-sensitivity of the receptors. In addition, at present nothing is known about the signal transduction cascade downstream of these receptors.

Possible molecular functions of the CO2-responsive gustatory receptors (Grs) Gr21a and Gr63a can be derived from advances in understanding the function of the fly olfactory receptors (Ors). Grs
Nonetheless, G-protein signalling may still be involved in fly olfaction, although membrane topology is inverted compared to GPCRs [147]. Nucleotide cAMP appears to mediate effects of elevated CO2 levels on gated channels [149]. Also, as discussed above, the cyclic nucleotides gate the Or ion channel. Production of cyclic nucleotides is independent of extracellular pH. Critically, the deleterious in vivo effects of CO2 on fecundity and bacterial resistance are still observed in Gr63a mutant flies.Taken together, these results suggest that the fly possesses as yet unidentified CO2 detection mechanisms, which are non-neuronal and can be cell autonomous. These mechanisms may be conserved, because CO2 may also suppress innate immune responses in mammalian systems.

Molecular responses in C. elegans

The TAX-2 and TAX-4 subunits of a cGMP gated ion channel are essential for the CO2 avoidance behaviour [80], because tax-2 and tax-4 mutants do not avoid CO2 [78, 79]. Expression of TAX-4 in the BAG neurons alone was sufficient to recover the CO2 avoidance defect of tax-4 mutants, which implicates the involvement of these neurons in CO2 avoidance (Fig. 4). Importantly, C. elegans do not avoid CO2 in all conditions; starved animals do not avoid CO2, N2 animals with null mutation in the neuropeptide Y receptor npr-1 change their feeding behaviour from solitary to social and accordingly change the way they respond to CO2 from CO2 sensitive to CO2 insensitive [80]. The npr-1 signalling and the neuronal globin domain protein, GLB-5 also affect the response to carbonation equally well to wild-type flies [69], indicating that the molecular mechanisms of sensing gaseous and soluble CO2 are distinct.

**Gustatory responses**

The molecular basis for detection of carbonation is not presently known. The E409 neurons that respond to CO2 in solution do not express Gr21a or Gr63a [67, 75], and Gr63a mutants respond to carbonation equally well as they have no homology to GPCRs and their membrane topology is inverted compared to GPCRs [147]. Nonetheless, G-protein signalling may still be involved in fly olfaction as with the GLB-5 and the insulin and TGF-β starvation pathways.

and Ors are distantly related [142, 143] and together define a novel superfamily of insect chemoreceptors [144]. Two recent papers showed that in a heterologous in vitro system, Drosophila Ors function as ligand-gated ion channels that respond to odorants [145, 146]. Although Grs and Ors, like GPCRs, are 7-transmembrane spanning proteins, it is not obvious that they transduce signals via G proteins as they have no homology to GPCRs and their membrane topology is inverted compared to GPCRs [147]. Nonetheless, G-protein signalling may still be involved in fly olfaction as with the GLB-5 and the insulin and TGF-β starvation pathways.

Could cyclic nucleotide-gated ion channels represent a general conserved mechanism for CO2 sensing? Such a possibility is supported by evidence that in C. elegans, tax-2 and tax-4 are subunits of an ion channel gated by cyclic GMP required for the avoidance behaviour to CO2 (see below). Further, a recent study show that bicarbonate can activate guanylyl cyclases in CO2-responsive neurons in mice [148], and the responses to CO2 are known to be dependent on the opening of cyclic GMP-sensitive cyclic nucleotide-gated channels [149]. Also, as discussed above, the cyclic nucleotide cAMP appears to mediate effects of elevated CO2 levels in fungi [19], and adenyl cyclases can be activated by bicarbonate in mammalian sperm [129, 150] and by molecular CO2 in cyanobacteria [151].
O$_2$, which implicates a potential relationship between the CO$_2$ and O$_2$ responses; however, soluble guanylyl cyclases (sGC) which serve as oxygen sensors in *C. elegans* do not seem to be involved at the avoidance response from CO$_2$ because animals mutants in sGC genes avoid CO$_2$ normally [80].

The development, fertility, motility and aging phenotypes detected in *C. elegans* grown in >9% CO$_2$ levels conditions are accompanied with changes in the expression of specific genes (Fig. 5). These changes are dynamic and a large number of genes are already changed more than 2-fold after 1-hr exposure to 19% CO$_2$. After a 6-hr exposure to hypercapnia, most of the genes affected after a 1-hr exposure returned to their baseline-expression levels, but expression of many other genes not affect by the 1-hr exposure were now changed.

**Fig. 5** Hypercapnia induces change in gene expression in *C. elegans*. Fold change in log$_2$ scale of gene expression during 1, 6 or 72 hrs exposure to air containing 19% CO$_2$ of innate immunity (A), heat shock (B), 7-transmembrane domain (C), major sperm proteins (D), nuclear hormone receptor (E) and several other genes of interest. The data were taken from [18].
Following a 72-hr exposure to 19% CO₂, over 6% of *C. elegans* genes were either up-regulated or down-regulated at least 2-fold. Many of the genes that were up-regulated following 1-hr exposure to hypercapnia are probably involved in coordinating the initial response of the animal to hypercapnia, including several 7-transmembrane domain and hormone receptors, sGC and ubiquitin ligase genes (Fig. 5).

In summary, we review here the effects of hypercapnia on mammals, *Drosophila* and *C. elegans*. High CO₂ levels appear to be sensed by cells independent of pH or O₂ via specific signalling pathways, which results in distinct effects (phenotypes). Studies in mice, *Drosophila* and *C. elegans* may provide valuable insights into the effects of hypercapnia on human health.

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