

Carbon dioxide-sensing in organisms and its implications for human disease

Eoin P. Cummins · Andrew C. Selfridge ·
Peter H. Sporn · Jacob I. Sznajder · Cormac T. Taylor

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Abstract The capacity of organisms to sense changes in the levels of internal and external gases and to respond accordingly is central to a range of physiologic and patho-physiologic processes. Carbon dioxide, a primary product of oxidative metabolism is one such gas that can be sensed by both prokaryotic and eukaryotic cells and in response to altered levels, elicit the activation of multiple adaptive pathways. The outcomes of activating CO₂-sensitive pathways in various species include increased virulence of fungal and bacterial pathogens, prey-seeking behavior in insects as well as taste perception, lung function, and the control of immunity in mammals. In this review, we discuss what is known about the mechanisms underpinning CO₂ sensing across a range of species and consider the implications of this for physiology, disease progression, and the possibility of developing new therapeutics for inflammatory and infectious disease.

Keywords Carbon dioxide (CO₂) · Hypercapnia · Physiological gases · Immune regulation · NF-kappaB

Introduction

The natural history of CO₂

In the time since the formation of the planet approximately 4,500 million years ago, the composition of the Earth's terrestrial atmosphere has varied both dramatically and continuously. Furthermore, fluctuations in the gaseous composition of the atmosphere have had significant implications for the evolution of both aquatic and terrestrial life whereby the rise or fall of various atmospheric gases over geological time played a major role in shaping the nature of the planet's biota [1]. For example, rising levels of atmospheric molecular oxygen (O₂), which began approximately 2,500 million years ago as a result of the proliferation of photosynthesizing cyanobacteria (the blue-green algae of the planet's oceans), led to eradication of the majority of life on earth during a period termed the "great oxidation event" while at the same time ultimately allowing the rise and radiation of the metazoans (multicellular organisms) through the provision of the chemical energy by which to fuel oxidative metabolism [1].

Another atmospheric gas, the levels of which have fluctuated over geologic time has heavily influenced the nature of life on earth is carbon dioxide (CO₂). Since the radiation of metazoans, the level of CO₂ in the atmosphere has fluctuated from around 7,000 ppm during the Cambrian period to current levels, which were just recently reported to exceed 400 ppm. Therefore, in the Earth's current atmosphere, the background levels of atmospheric CO₂, although clearly of key importance in climate determination [2], are relatively low when compared to levels reached previously over geologic time. However, it is important to note that local CO₂ levels found in the microenvironments of niches such as in and around respiring organisms or decomposing

E. P. Cummins · A. C. Selfridge · C. T. Taylor (✉)
School of Medicine and Medical Science, UCD Conway
Institute, University College Dublin, Belfield, Dublin 4, Ireland
e-mail: cormac.taylor@ucd.ie

P. H. Sporn · J. I. Sznajder
Division of Pulmonary and Critical Care Medicine, Feinberg
School of Medicine, Northwestern University, Chicago, IL
60611, USA

C. T. Taylor
Systems Biology Ireland, University College Dublin, Belfield,
Dublin 4, Ireland

matter are greatly increased relative to background atmospheric levels.

CO₂ produced in cells during the citric acid cycle is a by-product of oxidative metabolism (respiration) and as such is found at significantly higher concentrations inside a respiring organism than in the external atmosphere. Because CO₂ produced is expired, this leads to the formation of CO₂ gradients away from a respiring organism. Indeed, use of CO₂ gradients in prey-seeking or predator-avoidance behavior has been described for various insects and nematodes, respectively (discussed below). Furthermore, exposure to altered tissue levels of CO₂ due to disturbed homeostasis during disease can lead to the common pathological states of hypocapnia and hypercapnia in mammals. Such conditions can lead to disrupted pH homeostasis, which in turn can result in systemic alkalosis/acidosis, organ failure, and in some cases, death. These are just selected examples of the many reasons why most organisms have evolved mechanisms to sense changes in microenvironmental CO₂ levels and elicit an appropriate physiologic response.

Therefore, like other physiologic gases such as O₂ and nitric oxide (NO), it is important that cells, tissues and organisms retain the ability to sense changes in CO₂ and respond accordingly. Recent advances in our understanding of oxygen-sensing in cells was led by the discovery of the ubiquitous oxygen-sensing mechanisms of the hypoxia-inducible factor (HIF) pathway and the specialized oxygen-sensing mechanisms of the carotid body [3, 4]. Similarly, the discovery that soluble guanylate cyclase can act as a nitric oxide sensor in mammalian cells shed light on how this endogenous gas is sensed [5]. Less, however, is known about cellular CO₂-sensing mechanism(s), particularly as it pertains to transcriptional responses to hypercapnia. In this review, we will discuss both what is known and what remains to be discovered in relation to cellular CO₂ sensing.

The biology of CO₂

CO₂, as a primary by-product of oxidative metabolism is constantly produced during the citric acid cycle within mitochondria. The majority of CO₂ leaves the cell in which it was produced through the cell membrane. The most straightforward way by which CO₂ molecules are capable of traversing the cell membrane is via passive diffusion. In this process, CO₂ dissolved in the lipid bilayer traverses the opposing face of the membrane. This mechanism is passive and is dependent upon the transmembrane concentration gradient of CO₂. Molecular carbon dioxide is suitable for passive diffusion as it is a small non-polar molecule [6].

While it was previously believed that CO₂ moves across biological membranes only by passive diffusion, recent evidence has also proposed the existence of discreet CO₂

channels in biological membranes [7]. To date, aquaporins, rhesus channels and connexins have all been implicated in the selective transport of CO₂ molecules. Currently there are conflicting experimental data regarding the significance of active transport in the passage of CO₂ through membranes [8].

Aquaporins primarily function as water conduits within cells. Yet these membrane intrinsic proteins are also capable of associating with uncharged gases such as CO₂ [9, 10]. The movement of carbon dioxide through aquaporin channels is facilitated by the formation of soluble complexes [11]. It has been demonstrated in *Xenopus* oocytes that injection with carbonic anhydrase (CA) enhances AQ1 (aquaporin 1) expression thus rendering the membrane more porous to CO₂ [12]. Similarly, in plants the tobacco aquaporin (NtAQPI) is responsive to CO₂. Upregulation of NtAQPI increases the permeability of plant membranes to H₂O and CO₂ consequently augmenting leaf development [11]. In contrast, aquaporins do not appear to be of importance to CO₂ transport in mammalian cells. The deletion of *AQ1* in erythrocyte and lung mice models does not alter CO₂ movement [13]. Therefore, it has been hypothesized that aquaporins are only of physiological relevance to carbon dioxide transfer in systems in which the difference between intracellular and extracellular CO₂ levels is slight [11]. However, as stated above the relative contribution of passive diffusion to CO₂ movement versus channel mediated transport of CO₂ is controversial. A recent review discusses this issue in more depth and postulates that channels may be of particular importance in cells that contain a high proportion of membrane proteins, e.g. red blood cells [14].

Rhesus (Rh) proteins are highly conserved constituents of red blood cell plasma membranes. A search for a common biological role for Rh antigens in different organisms has engendered much debate [15]. Expression of the *RH1* gene in the green algae *Chlamydomonas reinhardtii* is increased in hypercapnia (3 % CO₂) in comparison to ambient conditions (0.03 % CO₂) [16]. Growth of *C. reinhardtii* in high CO₂ is hindered by repression of RH1 [15]. For these reasons it was proposed that Rhesus proteins may act as carbon dioxide channels. It has subsequently been shown that Rh antigens in aquatic species function as dual ammonia and carbon dioxide transporters [17, 18].

In vertebrates, connexin proteins accumulate to form inter neuronal channels known as gap junctions. These pathways are involved in chemoreception and exhibit sensitivity to carbon dioxide [19]. Gap junctions link neuronal cells allowing the exchange of small molecules including carbon dioxide [20]. Immunohistochemistry in rats identified the gap junction proteins Cx26 and Cx32 as being possible substrates in cellular communication [21]. Cx26 reacts to increases in CO₂ by opening and closes when CO₂

levels decrease. Furthermore, the release of ATP by Cx26 is reliant on the amount of carbon dioxide present [22].

Therefore, CO₂ is a key by-product of oxidative metabolism, which is generated by respiring cells. CO₂ is transported out of cells primarily by passive diffusion but this transport may also be facilitated by the existence of CO₂ channels, which in turn can be regulated in a CO₂-dependent manner. It has recently become clear, however, that rather than simply being a waste product of oxidative metabolism, CO₂ can also act as a physiologic stimulus for a number of cellular signaling pathways across virtually all species. Selected examples of the mechanisms underpinning the capacity of various species to sense CO₂ are outlined below.

CO₂ sensing across species

Bacteria

The capacity to sense and respond to altered CO₂ allows bacteria to adjust to their environment, thus increasing the likelihood of their persistence. This may be of key importance as bacteria leave the relatively low CO₂ levels of the external atmosphere for the higher CO₂ levels found inside most multicellular host organisms. Bacteria may upregulate virulence factors at host physiologic CO₂ levels (as opposed to atmospheric CO₂ levels) in order to facilitate colonization or infection. Examples of these pathways in a selected number of pathogens are given below.

Many pathogenic bacteria have developed sensing mechanisms to determine the amount of carbon dioxide in their surroundings. These include *Bordetella*, a species of Gram negative Proteobacteria capable of infecting the human respiratory tract. In the external environment, *Bordetella bronchiseptica* has limited antigen expression. Yet it has been shown that this bacterium has increased cytotoxicity and adherence at a CO₂ level consistent with that which exists within a mammalian host. The transcription of antigens such as adenylate cyclase toxin (ACT) and the type III secretion system (TTSS) is also elevated at 5 % CO₂ [23]. ACT and TTSS induce immunological non-responsiveness in dendritic cells by interfering with MAPK signaling [24]. In this way, *B. bronchiseptica* responds to the internal mammalian CO₂ concentration by increasing its capacity for respiratory tract colonization. Subsequent experiments with the whooping cough pathogens *B. pertussis* and *B. parapertussis* confirmed that a CO₂-dependent increase in pathogenicity is a common characteristic of members of the *Bordetella* genus [23].

The food borne pathogen *Bacillus cereus* presents an unusual public health challenge by virtue of the fact that it can survive extreme conditions through sporulation.

Comparative analyses of *B. cereus* under hypercapnic and normocapnic conditions have revealed genomic dissimilarities, which implicate carbon dioxide as a key determinant in the virulence of the bacteria. Pathogenic species display greater quantities of plasmid encoded virulence genes and S-layer protective proteins at elevated CO₂. Additionally the activation of the pleiotropic virulence regulators PICR and AtxA were found to be influenced by oxygen and carbon dioxide concentrations [25]. The AtxA regulon; necessary for capsule and toxin gene transcription was stimulated by increased CO₂ [26]. Conversely, the PICR regulon, which is associated with non-virulence related characteristics such as food supply and cell protection was more prevalent in ambient air [27]. Thus it appears that in order to adapt for colonization, *B. cereus* has evolved a CO₂-sensing system that provokes the differential expression of its virulence factors.

The zoonotic agent responsible for Lyme disease *Borrelia burgdorferi* experiences dramatic oscillations in carbon dioxide availability throughout its complex life cycle. It must first transmit from the external environment to the arthropod vector and onwards to the mammalian reservoir host [28]. It has been shown that carbon dioxide can directly modulate borrelial gene expression. At 5 % CO₂ the alternate sigma factor RpoS is activated and antigen synthesis is promoted. There is also increased translation of the lipoprotein genes *ospC* and *dbpA*, which alter the structure of *B. burgdorferi* to render adherence to the host more viable [29]. CO₂ concentrations in vivo cause the borrelial pathogen to adapt for adherence and enhance its infectivity.

The production of enterotoxin by *Vibrio cholerae*, the etiological agent of cholera increases in association with rising carbon dioxide levels. It has been determined that a hypercapnic atmosphere of 10 % CO₂ maximizes enterotoxin yield [30]. Carbonic anhydrases mediate the interconversion of carbon dioxide and bicarbonate in *V. cholerae*. Bicarbonate has been identified as the first positive effector for ToxT in cholera. ToxT is a regulator that transcriptionally induces the cholera virulence cascade. The introduction of ethoxzolamide, an antagonist of carbonic anhydrase prevents the occurrence of bicarbonate associated pathogenicity [31]. This inhibition highlights the importance of carbonic anhydrases and the conversion of CO₂ to bicarbonate to *V. cholerae*. Therefore *V. cholerae* is reliant upon carbon dioxide and becomes more virulent at higher levels of CO₂.

Pseudomonas aeruginosa is an environmental bacterium that like many of the bacteria described above resides in drastically different CO₂ environments depending on whether it is colonizing a host or not. *P. aeruginosa* infection is a major clinical challenge in a hospital and immunocompromised setting. Recently three functionally active carbonic anhydrase isoforms were identified

in *P. aeruginosa* PAO1 with the most abundant and active (psCA1) playing an important role in PAO1 survival at high CO₂ [32]. Thus, a better understanding of bacterial adaptation and survival across a range of CO₂ environments may be of importance in the development of future antimicrobial therapies.

In summary, many bacterial pathogens demonstrate increased growth potential and virulence when exposed to the elevated CO₂ levels found within mammalian hosts. Therefore, bacteria express CO₂-sensing mechanisms, which allow them to adapt to the host environment and thrive therein.

Plants

Perhaps the most important biological function for carbon dioxide on our planet is its contribution to photosynthesis whereby CO₂ and water (in the presence of chlorophyll and sunlight) give rise to the production of carbohydrates and oxygen ($6\text{ CO}_2 + 6\text{ H}_2\text{O} + \text{light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{ O}_2$). The initiation of this key biochemical reaction in photosynthetic bacteria altered the course of life and has been shaping evolution ever since. Carbon dioxide enters the leaf via small pores known as stomata where it combines with RuBP as part of the carbon fixing component of the Calvin cycle. This reaction is catalyzed by RUBISCO, (the most abundant protein on the planet) which accounts for up to 50 % of total protein mass in the leaves of certain plants [33]. The consequences of RUBISCO's catalytic activity to fix CO₂ is the production of two molecules of 3-phosphoglycerate, a metabolic intermediate that can be used for metabolic processes and the production of organic molecules such as glucose.

The stomata through which CO₂ enters the leaf to participate in the photosynthetic reaction are also the portals through which H₂O exits the leaf during transpiration. As a consequence the degree of stomatal opening needs to be a tightly regulated process to ensure adequate CO₂ entry without excessive H₂O loss, which is particularly important in hotter climates. Guard cells surround the stomatal aperture and physically regulate the degree of opening (pore size) in response to a number of environmental factors including the plant hormone abscisic acid (ASA) (which is produced in response to drought), light and carbon dioxide [34]. This response to increased CO₂ is likely an evolutionary adaptation to prevent water loss at night when CO₂ levels are relatively elevated and photosynthesis cannot occur due to the absence of light. The mechanisms through which elevated CO₂ can cause stomatal closure have recently been elegantly investigated in *Arabidopsis* using genetic tools [34]. CO₂ in combination with water can form carbonic acid, which in turn can be converted into HCO₃⁻ and H⁺. This reaction is catalyzed by carbonic

anhydrase of which there are several variants. Hu et al. [34] identified a key role for carbonic anhydrase (specifically CA1 and CA4) in mediating the CO₂-dependent closure of stomata. Intriguingly, the introduction of structurally unrelated mammalian carbonic anhydrase was sufficient to restore CO₂-sensitivity to stomata in CA-mutant *Arabidopsis* [34]. HCO₃⁻ is proposed as the likely signal downstream of CO₂ and carbonic anhydrase that results in stomatal closure via effects on anion channels in the guard cells [34, 35]. Taken together, these findings suggest that carbonic anhydrase can function as a CO₂ transponder to facilitate key downstream signaling events in plants [36]. A detailed review of the molecular mechanisms underpinning CO₂-dependent stomatal closure has recently been published [37]. Of note, the authors highlight the convergence of ABA and CO₂-dependent signaling at the level of the *gca* (growth controlled by ASA) gene in the regulation of the stomatal regulatory circuit. *gca* mutant plants that do not respond to ABA with respect to stomatal closure were also strongly impaired in their stomatal response to CO₂ [38].

In summary, CO₂ is a key stimulus in plants that is responsible for the regulation of stomatal closure and as such requires an effective sensing mechanism, which is mediated by CA and integrates with signaling pathways utilized by other mediators of stoma regulation.

Fungi

The fungal kingdom encompasses a diverse array of microorganisms, which have evolved to possess complex growth strategies. During their life cycles fungi encounter a wide range of ecological conditions; including fluctuating carbon dioxide levels. Fungi have developed sensing mechanisms to determine the concentration of CO₂ in their surroundings and respond effectively to this environmental cue. The elevated levels of carbon dioxide present in mammalian tissues (5 %) when compared to the external environment (0.03 %) favor the survival of some pathogenic fungi within animal hosts.

Extensive research has elucidated a complex CO₂-sensing system in the model fungal organism *Candida albicans*. Carbon dioxide has been shown to have numerous tangible effects upon the fungus. In *C. albicans*, the transcription factor Flo8 functions as a carbon dioxide sensor [39]. *C. albicans* switches to filamentous growth in response to CO₂ tension in vivo [39]. Carbon dioxide instigates this transformation in populations of *C. albicans* via its action as a signaling molecule [40]. The carbon dioxide induced filamentous form of *C. albicans* exhibits greater pathogenicity than the monocellular yeast. Elongated filaments attach to generate significant biomasses, which in turn facilitate the initial colonization of tissues and subsequent increase and

dissemination of fungal infection [41]. For example, it has been demonstrated that increased concentrations of carbon dioxide at the skin surface relative to the external atmosphere exacerbate the extent of dermatological candidiasis [42]. In order to mate effectively, *C. albicans* undergoes a transition from a white to an opaque phenotype [40]. Physiological carbon dioxide levels preferentially select the opaque phenotype thus augmenting the fungal rate of reproduction [43].

Like *C. albicans*, the opportunistic human pathogen *Cryptococcus neoformans* also confronts dramatic variations in carbon dioxide availability during its life cycle. It has been documented that a CO₂-sensing system consisting of adenylyl cyclase *Cac1* and carbonic anhydrase *CAN2* is integral to ensure the propagation of *C. neoformans* [44]. However, in areas of plentiful carbon dioxide supplies the beta-carbonic anhydrase generating *CAN2* gene is no longer a prerequisite for persistence of the cryptococcal species [45]. The production of a polysaccharide capsule by *C. neoformans* enhances its virulence in vivo [46]. The process of capsule biosynthesis is partially reliant upon the presence of 5 % CO₂ [47]. Carbon dioxide also exerts an influence upon the growth pattern of *C. neoformans*. The fungus is most likely to be found as a biofilm in the external environment and as planktonic cells in animal tissues. The formation of a biofilm favors the endurance of *C. neoformans* ex vivo [48].

Fungi primarily sense carbon dioxide via the carbonic anhydrase and adenylyl cyclase pathways. This subject matter has previously been expertly reviewed by [49]. HCO₃⁻ acts as a signaling molecule and conveys the CO₂ message; thus allowing fungi to respond to alterations in the carbon dioxide levels of their surroundings [50]. Induction of adenylyl cyclase by carbon dioxide in *C. neoformans* and *C. albicans* is reliant upon carbonic anhydrase activity [45]. Furthermore it has been determined experimentally that bicarbonate is capable of directly activating adenylyl cyclase in *C. albicans* [51]. In fungi, carbonic anhydrases are also active in sexual mating and function to ensure that there are adequate resources of bicarbonate in CO₂ limiting conditions [50]. Thus, CO₂ is sensed by fungi in a manner that elicits growth advantage and promotes fungal infection in host species.

Nematodes

Nematodes including *Caenorhabditis elegans* demonstrate an acute avoidance response following exposure to elevated levels of CO₂. This is likely an evolutionarily conserved survival mechanism to control internal CO₂ levels and determine attraction/avoidance from prey/decaying food sources, however, CO₂ avoidance does vary between strains of *C. elegans* and within different species

of free-living nematode [52]. The avoidance behavior is primarily governed by a mechanism involving cGMP signaling within BAG neurons (ciliated neurons in the head whose expression is regulated by the transcription factor ETS-5 [53]) via Tax-2/Tax-4. Interestingly, the CO₂-avoidance is affected by both nutritional feeding patterns (solitary feeding species *C. elegans* are CO₂-sensitive) and nutritional status (starved *C. elegans* have an attenuated CO₂ avoidance response), which is suggestive of a mechanism that re-balances the necessity to avoid predators in times of nutrient deprivation [52]. Subsequent studies identified a receptor-type guanylate cyclase *GCY-9* (which is enriched in BAG neurons) as being required for CO₂-dependent *C. elegans* avoidance. *GCY-9* is proposed as being direct CO₂ or CO₂ metabolite sensor but a downstream role for *GCY-9* could not be excluded [54]. Intriguingly, *C. elegans* in the dauer phase of development display the opposite response to CO₂ of adults and are in fact attracted to elevated levels of CO₂ again with a requirement for BAG neurons to mediate CO₂-sensitivity [55]. Taken together, this demonstrates a key role for the BAG neurons in regulating *C. elegans* responsiveness to CO₂ irrespective of whether CO₂ is mediating repulsive or attractive responses. While central to CO₂ sensitivity in *C. elegans*, BAG neurons are not the only CO₂-sensitive neurons that govern avoidance behavior in the worm. A recent study identified AFD and ASE neurons (previously characterized as being involved in temperature and salt ion detection, respectively) as being primary CO₂ sensors in addition to BAG neurons (which are also involved in O₂ detection) in *C. elegans* [56]. Interestingly, the nature of the neuronal response to CO₂ in each case is unique and differs from the pattern of neuronal activation elicited by non-CO₂ stimuli, e.g. temperature in AFD neurons. The authors speculate that given their sensitivity to both CO₂ and O₂, these signals could be integrated at a molecular level within BAG neurons [56].

High CO₂ levels are associated with gross changes in normal physiology in *C. elegans*. Exposure of worms to CO₂ levels in excess of 9 % had marked effects on motility, fertility and lifespan. Impaired motility was associated with age-dependent deterioration of muscle organization, brood size was significantly attenuated in a CO₂ dose-dependent fashion from 0.03 to 19 % CO₂ but interestingly lifespan was extended in animals grown at 19 % CO₂ compared to ambient air. Gene expression analysis of *C. elegans* exposed to 19 % CO₂ over a time course revealed specific effects of hypercapnia on sub-sets of genes including those associated with 7-transmembrane domain proteins, nuclear hormone receptors, E3 Ub ligases and innate immunity [57]. Together these reports point to both acute neuronal sensing of CO₂ governing attraction/repulsion and a more chronic non-neuronal sensing of CO₂ affecting distinct

subsets of genes governing key physiological processes in the nematode.

Insects

Many species of insect have been reported to demonstrate CO₂ sensitivity, however, here, we will focus on those species where the potential mechanism underpinning the CO₂ sensitivity has been described. Indeed, the nature of anatomical adaptations to CO₂ sensing in insects has been comprehensively reviewed elsewhere [58]. The CO₂-dependent prey-seeking behavior of female mosquito species is an area of interest with respect to developing strategies to limit the spread of malaria. Mosquitoes are attracted to exhaled CO₂ from potential hosts for the purpose of obtaining a blood meal [59] and in some species CO₂ can also sensitize the mosquito to detect human skin odor [60]. Interestingly, ultra-prolonged activation of CO₂-sensing neurons can disorient mosquitoes [61], an effect that is being used to develop strategies to disrupt host seeking behavior.

Drosophila melanogaster demonstrates CO₂-sensitive behaviour that, depending on the circumstance can be both attracted to or repulsed by increased CO₂ levels in the local environment. Furthermore, elevated CO₂ elicits a change in whole organism gene expression in *Drosophila*. Because of the genetic tools available, this organism has the best characterized insect CO₂-sensing pathways. *Drosophila* in contrast to their mosquito counterparts are repelled by elevated CO₂ levels (in walking assays). The mechanism underpinning this repulsion involves two chemosensory receptor genes *Gr21a* and *Gr63a*, which are necessary for neuronal CO₂ sensing. These genes are highly conserved in insects including mosquito but are absent in certain insects that retain CO₂ sensitivity, e.g. honeybee, which is suggestive of the evolution of different CO₂-sensing pathways in insects [62]. The *Gr21a* and *Gr63a* receptors are thought to work in concert to mediate CO₂ avoidance behavior in *Drosophila* [59]. CO₂ is a component of *Drosophila* stress odorant [63] and this in part explains the aversion of *Drosophila* to CO₂. However, it is not obvious why an insect that feeds on ripening fruit should be repulsed by CO₂. A recent study has made an intriguing observation that *Drosophila* will actively seek a narrow CO₂ plume in flight (as opposed to in a walking assay). Interfering with the *Gr21a* transduction pathway surprisingly had no significant effect on *Drosophila* CO₂ tracking in flight, suggesting an alternative mechanism for CO₂ sensing during flight [64]. Several signaling components have been implicated as being required for CO₂ tracking in flight including octopamine (a flight modulated biogenic amine [65]), the expression of an acid receptor Ionotropic receptor 64a (*Ir64a*) and an olfactory co-receptor *Orco* [64].

In addition to the neuronally mediated responses of insects to CO₂ described above, there are a number of non-neuronal effects of CO₂ of interest. Elevated CO₂ causes defects in developmental morphogenesis, egg laying and hatching in *Drosophila* [66]. These extraneuronal effects of CO₂ in *Drosophila* occur even in the absence of the *Gr63a* expression. It was also shown in this study that hypercapnia down-regulates expression of multiple antimicrobial peptides, key innate immune effectors in the fly that are regulated by the NF-kappaB analogue Relish. This effect was not mediated by acidosis, nitric oxide signaling or the heat shock response. Moreover, hypercapnia caused striking increases in mortality due to bacterial infections, an effect that was also independent of *Gr63a* [66]. Notably, *Drosophila* do not express soluble adenylyl cyclases (sACs) [67], which may function in CO₂ sensing in other systems (see below). Thus, the immunoregulatory effects of hypercapnia in *Drosophila* appear to be mediated by mechanism(s) distinct from other well-characterized neuronal and non-neuronal CO₂-sensing pathways. In summary, a range of insects display the ability to sense carbon dioxide and elicit a range of responses including prey-seeking, avoidance and immune suppression.

Fish

Fish detect CO₂ in their environment and are sensitive to small changes in CO₂ concentrations caused by anthropogenic or natural events. CO₂ sensing in fish has been comprehensively reviewed elsewhere [68]. Fish use CO₂-sensitive chemoreceptors located mainly in the gill to respond to changes in ambient CO₂ levels and initiate cardiorespiratory reflexes including bradycardia and hyperventilation. In this sense, the fish gill and the mammalian carotid body are remarkably similar, acting as sensing centers for both O₂ and CO₂ in fish and mammals, respectively. Fish have a significantly lower circulating pCO₂ than air breathers with normal levels in the region of ~2–3 mmHg as compared to the ~40 mmHg in mammalian circulation [69]. As a consequence they demonstrate a more sensitive response to CO₂ commensurate with their relatively lower normocapnic pCO₂. Zebrafish hyperventilate at environmental CO₂ levels of ~1 mmHg. It is thought that this acute sensitivity to CO₂ in the fish is a reflection of the relatively higher arterial pH changes elicited in response to a small change in CO₂ in fish as compared to mammals. In the zebrafish gill, neuroepithelial cells (NEC) sense CO₂ (as well as O₂) with a resultant inhibition of background K⁺ channels and subsequent depolarization at increasing CO₂ levels. Piscine carbonic anhydrase is implicated in this response as pharmacological inhibition of CA with acetazolamide blunted electrophysiological indices of NEC-CO₂ sensitivity. Thus, the

authors propose a CO₂-dependent sensing mechanism that may be sensitive to changes in intracellular acidification/pH [69]. Finally, given the acute nature of the fish response to elevated CO₂ it is likely that the continued rise of anthropogenic CO₂ will have marked effects on fish physiology and behavior [70] and in particular by those species that are most sensitive to changes in pCO₂.

Mammals

Mammals exhibit a host of responses to alterations in CO₂ levels, including modulation of ventilation, alveolar fluid re-absorption, olfactory and gustatory responses, cell proliferation, muscle function, inflammation and innate immune responses. These effects will be dealt with in separate sections below:

Breathing

A number of mechanisms have evolved to sense changes in arterial pO₂ and pCO₂ and elicit alterations in the rate and depth of breathing. Central chemosensation of carbon dioxide by neurons located within the brainstem is the predominant mechanism through which changes in arterial pCO₂ are detected and affect breathing [71]. The contribution of the carotid body in modulating CO₂-dependent respiratory control is relatively smaller than the brainstem in general but appears to have increasing importance at lower levels of hypercapnia [72]. The importance of the carotid body in sensing changes in arterial pO₂ has been expertly reviewed elsewhere [4]. There is significant ongoing research into the mechanisms of central chemoreception of CO₂ and this topic has been also been reviewed elsewhere [71, 73]. A current challenge in the field is to consolidate new data suggesting increasing numbers of CO₂-sensitive areas (within the brain) as well as several molecular detectors for CO₂ with pre-existing theories on CO₂ sensing [73]. Furthermore, there is emerging evidence for cross-talk between central and peripheral chemoreceptors whereby the carotid body can fine tune the sensitivity of central chemoreceptors under conditions of hypercapnia [74]. Intriguingly, recent work challenges the long-held view that CO₂-sensitive central chemoreceptors are essential to maintain the drive for rhythmic breathing [75]. In mice that conditionally express a mutant PHOX2B gene associated with central hypoventilation syndrome in humans, resulting in loss of the murine central chemoreceptor locus, Ramanantsoa et al. [76] report that respiratory rhythm and normal gas exchange are maintained. This maintenance of respiratory rhythm and normal gas exchange is due to compensation by O₂-sensitive peripheral chemoreceptors and occurs even in the absence of the murine central chemoreceptor locus.

Lung epithelial function

One of the most extensively investigated molecular signaling events in response to CO₂ is in the assessment of alveolar epithelial function and endocytosis of the Na,K-ATPase. The Na,K-ATPase plays a key role in the active transport of Na⁺ and K⁺ across membranes and epithelia, thus maintaining cellular ion homeostasis and in alveolar epithelial cells. Exposure of alveolar epithelial cells to high levels of CO₂ is associated with impaired alveolar fluid reabsorption [77]. Impaired fluid reabsorption is a consequence of CO₂-dependent Na,K-ATPase endocytosis from the cell plasma membrane. The mechanism governing this response involves sequential CO₂-induced activation of AMP kinase (AMPK) by Ca²⁺/calmodulin-dependent protein kinase kinase-beta, and PKC-ζ phosphorylation, resulting in the endocytosis of the Na,K ATPase [78]. More recently ERK [79], JNK [80] and PKA1a [81] have also been identified as playing a role in Na,K-ATPase downregulation and thus epithelial dysfunction. However, carbonic anhydrase II, which is expressed in the alveolar epithelial cells and is important in CO₂ metabolism, does not play a role in regulation of alveolar fluid reabsorption [82]. Interestingly, the contribution of JNK to CO₂-dependent signaling is evolutionarily conserved, with RNAi targeted to *Drosophila* JNK preventing CO₂-dependent downregulation of Na,K ATPase in fly S2 cells [80]. With respect to the role of PKA1a, a novel pathway was proposed whereby hypercapnia via a CO₂/HCO₃⁻-sensitive sAC increases the production of cAMP, activates PKA1a and α-adducin, culminating in Na,K ATPase endocytosis in epithelial cells [81].

Smell

CO₂ is odorless to humans but is keenly sensed by rodents [83]. The reasons why rodents retained the ability to smell CO₂ is likely associated with an advantage to detect food sources and predators. The mechanism whereby mice can smell CO₂ is via a subset of olfactory sensory neurons that use bicarbonate (downstream of carbonic anhydrase II) to produce cGMP via direct activation of the intracellular cyclase domain of guanylyl cyclase-D [84]. This mechanism of CO₂ detection is similar to the way bicarbonate can act as on sACs in other systems.

Taste

The discovery of the pleasing taste of soda water by Joseph Priestley in the 1760s paved the way for the advent of carbonated beverages, production of which has grown into an enormous industry. Carbonation elicits both somatosensory and chemosensory responses in mammals that include activation of taste receptors [85] although the exact

reasons why such a sensing mechanism may have evolved is not clear. Using electrophysiological techniques in mice with genetically impaired taste sub-type receptor cells, the authors found that selective ablation of sour sensing cells abolished the ability to taste carbonation. A carbonic anhydrase *Car4* was found to be selectively expressed in sour taste cells and mice deficient in *Car4* had a significantly blunted response to CO₂. An extracellular increase in proton production downstream of carbonic anhydrase is the proposed messenger given that bicarbonate alone was not able to stimulate taste receptor cells [36, 85].

Cell proliferation

In a manner independent of acidosis or hypoxia, hypercapnia has been shown to inhibit proliferation of fibroblasts and alveolar epithelial cells [86]. This is due to mitochondrial dysfunction, resulting from induction by CO₂ of miR-183, which in turn down-regulates the TCA cycle enzyme, isocitrate dehydrogenase-2 (IDH2). By inhibiting cell proliferation in this manner, hypercapnia might interfere with tissue homeostasis and inhibit tissue regeneration and wound repair. In a separate model, exposure of pulmonary epithelial cells to hypercapnia/acidosis impaired epithelial wound repair through an NF-kappaB-dependent mechanism [87].

Muscle function

Mice exposed to high CO₂ demonstrate myocyte degradation and muscle wasting. The mechanisms underpinning this effect involve activation of the energy sensor AMPK and upregulation of the ubiquitin ligase MuRF1, resulting in proteasomal degradation of muscle cells [88]. Interestingly, the detrimental effects of elevated CO₂ on muscle appear to be conserved across species as high levels of CO₂ caused slower locomotion in *C. elegans*, which was associated with and probably due to disturbed muscle morphology [57].

Inflammation and innate immunity

The effects of alterations in the level of CO₂ on mammalian inflammatory and immune responses have been explored in vitro and in vivo. Seeking to understand the basis of reduced peritoneal inflammation associated with laparoscopic surgery when CO₂ was used for abdominal insufflation, West et al. [89] observed that culture of peritoneal macrophages in 80 % CO₂ inhibited lipopolysaccharide (LPS)-induced secretion of tumor necrosis factor (TNF) and interleukin (IL)-1beta. Subsequently, hypercapnia at lower CO₂ concentrations (10–20 %) was found to inhibit LPS-stimulated release of TNF by rat alveolar

macrophages [90]. It was then shown that hypercapnia inhibited IL-6 and TNF mRNA and protein expression in human and mouse macrophage cell lines, as well as alveolar macrophages from both species; the effect of elevated CO₂ was rapid, reversible, noncytotoxic, selective, and independent of extracellular and intracellular acidosis, nitric oxide signaling, and heat shock or hypoxia-inducible gene expression [91].

NF-kappaB is a family of transcription factors that play a key role in the regulation of innate immunity and inflammation [92]. Recent studies into the effects of hypercapnia on NF-kappaB signaling have provided evidence that this pathway may represent a hub of key importance in the hypercapnia-induced signaling response. Hypercapnia inhibited endotoxin-stimulated NF-kappaB RelA nuclear translocation and DNA binding in pulmonary artery endothelial cells [93], although this was not the case in human macrophages [91]. It has also been reported that elevated CO₂ was associated with increased pulmonary inflammation in an NF-kappaB-dependent manner [94]. As discussed above, hypercapnic acidosis suppressed wound healing in A549 lung cells via suppression of NF-kappaB signaling [87] and in vivo NF-kappaB activation was reduced in a rat hepatic ischemia reperfusion injury model examining the effect of therapeutic hypercapnia [95]. Consistent with the concept that the NF-kappaB signaling pathway represents an important hub of CO₂ sensitivity, it was demonstrated that elevated CO₂ levels in cultured cells also significantly impacts upon non-canonical NF-kappaB family members through the regulation of IKK α and RelB signaling [96–98]. In terms of regulating IKK α signaling, it was found that in response to hypercapnia, IKK α rapidly and reversibly translocates to the nucleus in a manner independent of the known components of the cellular oxygen-sensing pathway, intra- or extracellular pH or pathways associated with acute CO₂-sensing in lower species [97]. Furthermore, hypercapnia induces cleavage and nuclear translocation of RelB, a second key component of the non-canonical NF-kappaB pathway [96]. The net effect of these hypercapnia-induced events is a modulation of LPS or cytokine-induced NF-kappaB activity. This effect on NF-kappaB signaling may at least in part, underpin the anti-inflammatory and immunomodulatory effects of hypercapnia [98]. Furthermore, these studies implicate the existence of a CO₂-sensing mechanism in mammalian cells that is independent of changes in intracellular or extracellular pH and which links changes in extracellular CO₂ with transcriptional events. A comparable ubiquitous system that is responsible for the sensing of microenvironmental oxygen levels has been well described in mammalian cells [3]. This system utilizes the oxygen-dependence of a family of cellular hydroxylases to regulate the hydroxylation and subsequent stability of a transcription factor termed the HIF [99, 100].

The effects of hypercapnia have also been studied in rodent models of inflammatory lung injury. Hypercapnia attenuated acute lung injury induced by mechanical ventilation with high tidal volumes in rabbits [101, 102] and by endotoxin in rats [103]. On the other hand, the effects of hypercapnia in mechanically ventilated rats with *E. coli* lung infection were variable, depending on experimental conditions [104–108]. In one of these studies [105], rats were allowed to breathe spontaneously following infection, then mechanically ventilated at the end of the protocol; in this case, hypercapnia worsened lung injury, decreased bacterial clearance from the lungs, and inhibited the neutrophil phagocytic capacity. More recently, it was shown that hypercapnia increased the mortality of *Pseudomonas* pneumonia in spontaneously breathing mice, an effect associated with dysregulated expression of lung cytokines and chemokines, impaired bacterial phagocytosis and reactive oxygen species generation by lung neutrophils, and an increased burden of bacteria in the lungs, liver and spleen [109]. Interestingly, in the latter study, hypercapnia did not worsen lung injury associated with pneumonia, suggesting that the increase in mortality may have been due to systemic sepsis and extrapulmonary organ dysfunction, rather than respiratory failure per se. Taken together, these studies indicate that while hypercapnia can ameliorate lung injury triggered by noninfectious inflammatory insults, by suppressing innate immune function, it worsens the outcome of bacterial lung infections in spontaneously breathing animals.

In summary, elevated carbon dioxide modulates mammalian inflammatory and innate immune responses in vitro and in vivo. This may be of benefit in situations where inflammation is triggered by a sterile insult, but would be deleterious in the setting of infection due to host immunosuppression. This, in combination with the ability of elevated CO₂ to enhance bacterial and fungal virulence and survival, suggests that hypercapnia may predispose to or worsen outcomes of infections in humans. Understanding the molecular signaling pathways involved will be of key

importance in the identification of new approaches to control infection and inflammation in the clinical setting.

Candidate molecular CO₂ sensors

Carbonic anhydrase

Given the prevalence of carbonic anhydrase enzymes in the sensing of CO₂ across a number of species this family of enzymes will be discussed in more detail below. Carbonic anhydrases are zinc-containing metalloenzymes that mediate the reversible hydration of carbon dioxide [110]. $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$. This process is extremely rapid with rate constants in the region of 10⁵–10⁶ per second. The reversible hydration of CO₂ in the absence of a catalyst is relatively slow [111]. Five distinct classes of carbonic anhydrases exist within nature (α , β , γ , δ and ζ). α CAs are found in mammals and the three main classes of CA α , β , and γ are structurally dissimilar suggestive of independent evolution [112]. The contribution of carbonic anhydrase enzymes to CO₂ sensing is summarized in the table below (Table 1). Given that carbonic anhydrases facilitate a reaction that would otherwise occur more slowly, it is something of a philosophical question as to whether they should be defined as true CO₂ sensors or rather transducers of CO₂. Similarly, given that the downstream effects of carbonic anhydrase activity are elicited independently by both bicarbonate [84] and changes in pH (protons)[82, 85] and can be mimicked by a structurally unrelated CA isoform from another species [34] suggests that carbonic anhydrases are transducing a change in CO₂ rather than sensing it directly.

Adenylyl cyclase pathway

The second messenger 3',5'-cyclic adenosine monophosphate (cAMP) is a key signaling molecule in biology affecting a range of processes including sensitivity to carbon

Table 1 Carbonic anhydrases as carbon dioxide sensors

Species	Carbonic anhydrase isoform	Evidence	Downstream effector	References
Fungi: <i>Cryptococcus neoformans</i>	Can2	Genetic	Bicarbonate	[44]
Fish		Pharmacological: <i>acetazolamide</i>	Protons	[69]
Plants	CA1, CA4, unrelated mammalian CA	Genetic	Bicarbonate	[34]
Bacteria: <i>Vibrio cholerae</i>		Pharmacological: <i>ethoxzolamide</i>	Bicarbonate	[31]
Mammals: mouse smell	CAII	Genetic	Bicarbonate	[84]
Mammals: mouse taste	Car4	Genetic	Protons	[85]

dioxide. The enzyme responsible for cAMP production is adenylyl cyclase (AC) and catalyses the cyclization of ATP to produce cAMP [81, 113]. cAMP-dependent signaling is prominent in animals and lower eukaryotes, as well as having a proposed role in plant signaling systems [114]. AC and cAMP signaling has been reviewed elsewhere [115], and this section will focus exclusively on the components of the pathway that are related to carbon dioxide signaling.

Transmembrane adenylyl cyclases (tmACs)

tmACs as the name suggests have transmembrane spanning domains and are sensitive to G-proteins. Normally these enzymes are activated secondary to ligand- GPCR activation, e.g. parathyroid hormone (PTH) activation of the PTH receptor with the ligand's signal being transduced into the cell and influencing a transmembrane AC. Downstream of tmAC activation cAMP can act as a second messenger to activate effector proteins including protein kinase A (PKA), cAMP response element binding protein (CREB), phosphodiesterase domains and cyclic nucleotide-gated ion channels. Recently it has been shown that cAMP production that is induced in this classical way via PTH activation of PTHR is suppressed by elevated CO₂ and results in activation of the sodium-proton exchanger isoform 3 (NHE3). The authors ascribe this effect to CO₂-dependent alterations in Ca²⁺ signaling downstream of IP3R activation [116]. The specific mechanism through which CO₂ modulates Ca²⁺ release through the IP3 receptor is yet to be defined.

There is, however, evidence that tmACs are also directly CO₂ sensitive as opposed to acting downstream of PTH as exemplified above. A mammalian recombinant G-protein-activated AC was found to be activated specifically in response to CO₂ and not HCO₃⁻. These in vitro experiments were carried out under conditions of disequilibrium that exploits the fact that the predominant form of inorganic carbon (Ci) that exists in the assay is the form in which it is added, i.e. CO₂ or HCO₃⁻ when the temperature is low (approximately 0 °C). The activity of a mammalian tmAC and a related tmAC from *Mycobacterium tuberculosis* was stimulated by CO₂ resulting in downstream CREB phosphorylation. Radiolabeled CO₂ was also used to demonstrate CO₂ binding to the protein, however, the precise site of incorporation is yet to be described [117].

Soluble adenylyl cyclases

sACs are distributed within the cytoplasm and in specific organelles and are more closely related to cyanobacterial ACs than to tmACs. In contrast to tmACs, which are activated by G proteins, sACs are not and instead are activated by intracellular signals including bicarbonate, calcium and ATP [118]. Recently sACs have been identified to be

directly stimulated by HCO₃⁻, which is a key step in sperm cell maturation [119]. This response was independent of intracellular pH and evident in vitro and in vivo. Experiments designed to examine what species of Ci are responsible for eliciting sAC activation demonstrated that both CO₂ and HCO₃⁻ stimulated sAC_T under condition of Ci disequilibrium [117].

Taken together, there is significant evidence for ACs and the cAMP signaling pathway as being important for CO₂ sensing with both sACs demonstrating sensitivity to HCO₃⁻ and CO₂ [81, 113] and a specific mammalian tmAC demonstrating selective sensitivity to CO₂ [117]. Furthermore, the evidence for direct incorporation of CO₂ into a CO₂-sensitive tmAC is a key finding in the search for molecular insight into the effects of CO₂ signaling and sensing.

Clinical implications

Hypercapnia has long been recognized as a marker of poor prognosis in patients with chronic obstructive pulmonary disease [120–123]. More recent studies have identified hypercapnia as an independent risk factor for mortality in patients hospitalized with community-acquired pneumonia [124, 125] and cystic fibrosis patients awaiting lung transplantation [126]. Despite these strong associations, clinicians have generally viewed hypercapnia in chronic lung disorders solely as a marker of advanced disease, without considering the possibility of a causal link between elevated CO₂ and adverse clinical outcomes.

Within the realm of acute pulmonary disease, over 20 years ago animal studies suggested that mechanical ventilation with high tidal volumes was injurious to the lung, and that ventilation with lower volumes had beneficial effects [127, 128]. It was subsequently confirmed that mechanical ventilation with low tidal volumes decreased mortality in humans with acute respiratory distress syndrome [129, 130]. In these studies, some patients ventilated with low tidal volumes developed hypercapnia, however, there was no difference in mortality in hypercapnic patients randomized to low tidal volume [130]. This observation, combined with some of the aforementioned animal studies [101–103] was interpreted that hypercapnia was not harmful, or even that elevated CO₂ levels might account for some of the benefit of low tidal volume ventilation. Terms such as “permissive” and “therapeutic” hypercapnia were coined to reflect the idea that high levels of CO₂ might have salutary effects in acute lung injury and sepsis [131]. However, none of the clinical studies on which this concept was based actually tested whether hypercapnia per se had beneficial effects. On the other hand, a recent report on a cohort of more than 14,000 mechanically ventilated patients from

40 countries found strong associations between hypercapnia (pCO₂ >50 mmHg) and multiple adverse clinical outcomes, including pneumonia, sepsis and most importantly mortality (Nin et al., Hypercapnia is associated with worse outcome of mechanically ventilated patients, in review).

As reviewed in detail above, recent studies demonstrate that hypercapnia inhibits alveolar fluid clearance, cell proliferation, muscle function, innate immune responses and host defense. Hypercapnic suppression of these essential physiologic functions and protective responses likely underlies, at least in part, the negative impacts of elevated CO₂ in patients with severe acute and chronic lung disease. Further studies are needed to define which of these (or other) effects of hypercapnia actually leads to adverse outcomes clinically. Pending such studies, debate will continue regarding potential benefits and harms of

hypercapnia, and whether the concept of “permissive” or “therapeutic” hypercapnia should continue to hold sway or be abandoned. This topic was recently discussed in a cross-talk feature, in which two groups of researchers laid out the arguments for and against the use of “permissive” hypercapnia in the treatment of ARDS. Of note, however, both groups of authors sounded words of caution with respect to potential detrimental effects of hypercapnia, particularly in the context of infection [132–135].

Conclusions and perspectives

It is now clear that carbon dioxide, like other physiologic gases such as nitric oxide and molecular oxygen is sensed by cells in a manner which involves the activation of an

Fig. 1 Schematic summarizing some of the key outcomes for CO₂ sensing across species (*outer circle*) and examples of the mechanisms underpinning these effects (*inner circle*). *AC* adenylyl cyclase, *CA* carbonic anhydrase, *GC* guanylate cyclase, *gca* growth controlled by abscisic acid, *NECs* neuroepithelial cells, *miR* microRNA, *NF-kappaB* nuclear factor kappa B

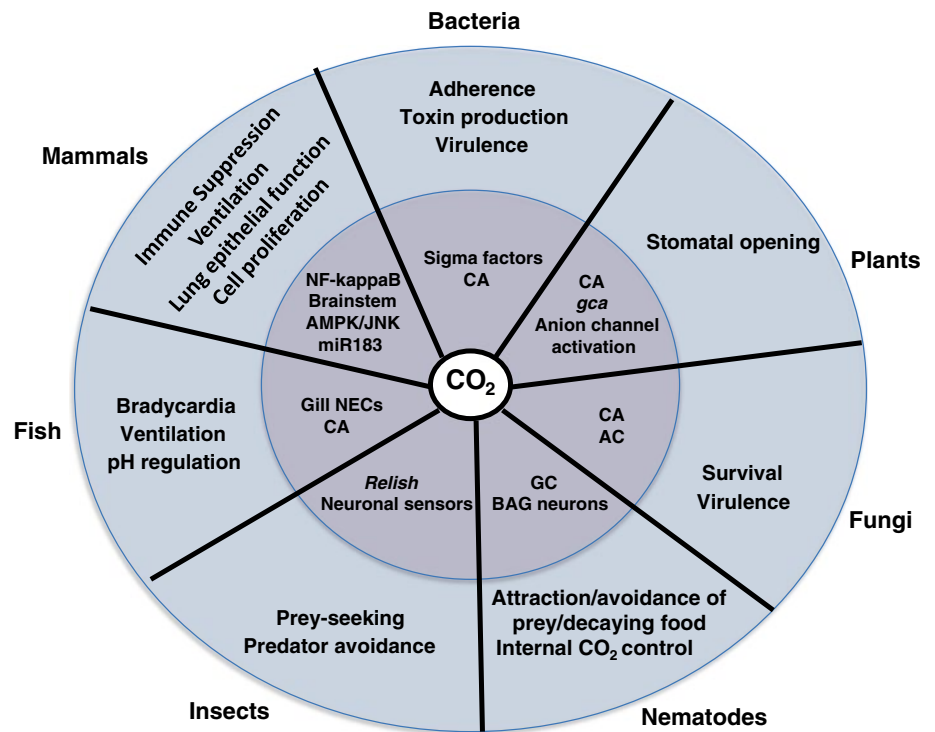
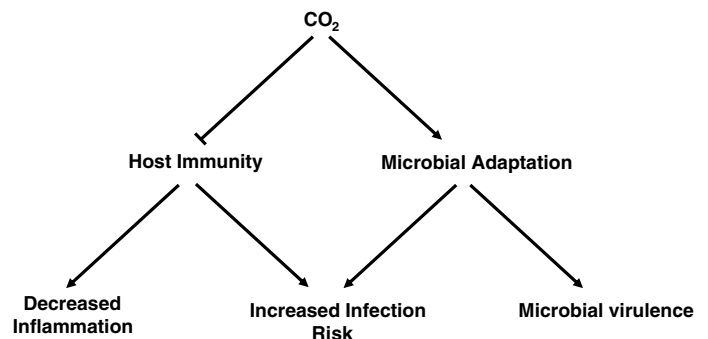


Fig. 2 Key outcomes of CO₂ signaling with respect to inflammation, infection, and microbial virulence. Elevated CO₂ leads to suppression of host immunity and enhances microbial adaptation leading to a state where an increased risk of infection is favored by environmental conditions



adaptive response. The signaling mechanisms involved include CA and AC but there are also likely other, as yet unidentified CO₂ sensors within cells. The consequences of activation of CO₂-sensitive pathways are diverse across species and are summarized in Fig. 1. From a disease progression point of view, the immunosuppressive effects of hypercapnia combined with the promotion of virulence and survival in fungal and bacterial pathogens (see Fig. 2) implicates tissue hypercapnia as a potential contributor to poor patient prognosis in infectious pathologies. It is of interest to consider that the relatively low pO₂ and high pCO₂ found within the mammalian body (in comparison with the external environment) are reminiscent of the ancient atmospheres that existed when prokaryotes were the dominant life forms on the planet. This may explain the successful survival of certain infectious agents and microbiota within the internal environment of the mammalian body due to the existence of levels of carbon dioxide at which they are more likely to thrive. Furthermore, the survival and virulence enhancing effects for pathogens as well as the host immune suppressing effects may represent potential new therapeutic targets in combating acute and chronic infectious diseases.

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