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Carbon dioxide-dependent signal transduction in mammalian systems

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Carbon dioxide (CO_2) is a fundamental physiological gas known to profoundly influence the behaviour and health of millions of species within the plant and animal kingdoms in particular. A recent Royal Society meeting on the topic of 'Carbon dioxide detection in biological systems' was extremely revealing in terms of the multitude of roles that different levels of CO_2 play in influencing plants and animals alike. While outstanding research has been performed by leading researchers in the area of plant biology, neuronal sensing, cell signalling, gas transport, inflammation, lung function and clinical medicine, there is still much to be learned about CO_2-dependent sensing and signalling. Notably, while several key signal transduction pathways and nodes of activity have been identified in plants and animals respectively, the precise wiring and sensitivity of these pathways to CO_2 remains to be fully elucidated. In this article, we will give an overview of the literature relating to CO_2-dependent signal transduction in mammalian systems. We will highlight the main signal transduction hubs through which CO_2-dependent signalling is elicited with a view to better understanding the complex physiological response to CO_2 in mammalian systems. The main topics of discussion in this article relate to how changes in CO_2 influence cellular function through modulation of signal transduction networks influenced by pH, mitochondrial function, adenylate cyclase, calcium, transcriptional regulators, the adenosine monophosphate-activated protein kinase pathway and direct CO_2-dependent protein modifications. While each of these topics will be discussed independently, there is evidence of significant cross-talk between these signal transduction pathways as they respond to changes in CO_2. In considering these core hubs of CO_2-dependent signal transduction, we hope to delineate common elements and identify areas in which future research could be best directed.

1. pH

For many years, carbon dioxide (CO_2) has simply been considered as a waste product of aerobic metabolism with few direct signalling consequences. As CO_2 is a weak acid, it has been experimentally challenging to dissect the impact of an increase in CO_2 concentration from the associated change in pH, i.e. hypercapnic acidosis. Recent research has clearly defined roles for CO_2-dependent signal transduction to be both dependent on pH and independent of changes in pH in a context-specific manner [1,2]. CO_2 in a solution can combine with water to form carbonic acid (H_2CO_3), which can then dissociate into bicarbonate (HCO_3^-) and hydrogen (H^+) ions. Thus, there is extensive evidence of CO_2-dependent signal transduction eliciting changes in cellular behaviour through the medium of altered pH. Notably, central chemoreception of CO_2 via pH is particularly important in the control of breathing (discussed below).

1.1. Intracellular pH regulation

Alterations in intracellular pH (pH_i) affect the ionization state of all weak acids and bases. This includes all peptides and proteins and could have dramatic

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Table 1. Acid-loading and acid-extruding ion channels, their associated ions and effects on intracellular pH.

	channel	ion(s) moved into the cell	ion(s) moved out of the cell	intracellular pH
acid loaders	AE1-3	Cl^-	HCO_3^-	decrease
	NBCe1-A (renal proximal tubule)	—	HCO_3^-/Na^+	decrease
acid extruders	V-ATPases	—	H^+	increase
	SLC9/NHE	Na^+	H^+	increase
	NBCe1-B (pancreatic duct)	HCO_3^-/Na^+	—	increase
	NBCe1-C (neuron)	HCO_3^-/Na^+	—	increase
	NBCn1/2	HCO_3^-/Na^+	—	increase
	NDCBE	HCO_3^-/Na^+	Cl^-	increase

effects on a multitude of signalling pathways and mechanisms. The importance of maintaining optimal pH_i is demonstrated by the fact that all examined mammalian cell types actively regulate pH [3,4]. In any cell, the vast majority of protons (H^+ ions) are bound to 'buffers', which are weak acid/base conjugate pairs. The buffering power of a cell allows for the minimization, but not the elimination, of pH_i changes when subject to an acid load. When the buffering power of the cell has been overcome, ion transporters on the plasma membrane act either as acid extruders, removing H^+ and introducing HCO_3^-, as or acid loaders, removing HCO_3^-.

The vacuolar ATPases are proton pumps which require energy from the hydrolysis of adenosine triphosphate (ATP) to expel H^+ ions into the extracellular milieu [5]. The SLC9/NHE family of Na^+/H^+ exchangers, in particular SLC9A1 (NHE1), instead uses the energy from the sodium ion (Na^+) gradient to remove excess protons from the cell [6]. HCO_3^- transporters can function either as acid extruders or as acid loaders. The SLC4A family of genes produces a range of proteins, which regulate pH_i by facilitating the movement of HCO_3^- across the plasma membrane. These include Cl^-/HCO_3^- exchangers (AE1-3), electrogenic and electroneutral Na^+/HCO_3^- cotransporters (NBCe1/2, and NBCn1/2 respectively) and a Na^+, driven Cl^-/HCO_3^- exchanger (NDCBE1) [7]. While these proteins all transport HCO_3^-, they have diverse functions across cell and tissue types. A well-known example of this is the Na^+/HCO_3^- cotransporter NBCe1, which mediates the efflux of HCO_3^- across the basolateral membrane of renal proximal tubule cells [8]. This important function allows for HCO_3^- reabsorption from urine. Thus, in response to a stimulus (e.g. hypercapnia or metabolic acidosis) that disturbs the cellular *status quo* with respect to pH, a series of signal transduction mechanisms are elicited to deal with that stimulus/challenge and restore homeostasis (table 1).

1.2. Neural control of breathing

1.2.1. Central chemoreception

As well as tightly regulating pH_i, the body actively maintains a consistent extracellular pH (pH_o) of 7.35–7.45. There are two main homeostatic mechanisms by which pH_o is controlled, through the renal and respiratory systems. In the renal system HCO_3^- can be added or removed from the blood, using the HCO_3^- transporters mentioned above, primarily by the epithelium of the early and convoluted proximal tubule, but also in distal tubule epithelial cells [9,10]. However, this response, which is often referred to as renal compensation, is considered slow and generally described in relation to chronic acidosis/alkalosis. The elimination of CO_2 via the lungs is a much more rapid response to acute acidosis or hypercapnia. As mentioned above, CO_2 in the solution can combine with water to form H_2CO_3, which can then dissociate into HCO_3^- and H^+ ions. Central and peripheral chemoreceptors sense alterations in pH, by the increased availability of H^+ ions, and transduce this signal into modified breathing mechanics.

Central chemoreceptors were first described as localized to the ventral medulla, but have now been identified in multiples sites in the brainstem, cerebellum, hypothalamus and midbrain. Within the ventral medulla there are three specific sites, which likely confer the chemosensitivity of this region: the retrotrapezoid nucleus (RTN), the medullary raphe and the caudal medulla [11]. Of these, the RTN is the best-characterized neuronal cluster. Neurons in the RTN detect local changes in the partial pressure of CO_2 (pCO_2) of the cerebrospinal fluid via two proton detectors: TASK-2 and GPR4. These two proteins are sparsely expressed in other brain regions, and their deletion leads to a near abolition of the central respiratory chemoreflex [12]. Following activation by elevated pCO_2, the neurons of the RTN signal to four regions of the brainstem are essential for breathing: the ventral respiratory column, the Kölliker–Fuse nucleus, the lateral parabrachial nucleus and the nucleus of the solitary tract. These nuclei subsequently signal to the respiratory muscles, so that through these pathways the RTN regulates both the rate and depth of breathing, allowing for the removal of CO_2 and restoration of pH_o [13].

1.2.2. Peripheral chemoreception

While the central chemoreceptors monitor alterations in pCO_2/pH of the cerebrospinal fluid, the peripheral chemoreceptors in the carotid and aortic bodies instead detect these changes in arterial blood. Hypercapnic acidosis causes closure of both voltage-dependent and -independent potassium (K^+) channels. This leads to depolarization of glomus cells and the entry of Ca^{2+} through L-type Ca^{2+} channels [14]. The subsequent release of excitatory neurotransmitters leads to a signalling loop via the brainstem to stimulate respiratory muscles and increase alveolar gas exchange. A more detailed discussion of CO_2-sensitive central and peripheral chemoreceptors is beyond the scope of this article but has recently been more extensively reviewed [15].

1.3. pH-sensitive/dependent channels and signalling

Systemic surveillance of extracellular pH is necessary for protection, as localized acidification of tissues, such as in inflammation, can cause severe damage. Channels and receptors on the surface of primary afferent neurons respond to alterations in the pH of the extracellular milieu. These include the acid-sensing ion channels (ASICs) [16] and transient receptor potential (TRP) channels [17,18]. While the roles of ASICs and TRPs in pH sensing of neuronal cells have been well characterized, there is increasing evidence that pH-sensitive G-protein-coupled receptors have a role in a variety of tissue and cell types. These include the ovarian cancer G-protein-coupled receptor 1 (OGR1) [19], G-protein-coupled receptor 4 (GPR4) [20], the G2 accumulation (G2A) receptor [21] and the T-cell death-associated gene 8 (TDAG8) receptor [22].

Several of these pH-sensitive channels directly respond to alterations in CO_2 levels. Inhalation of CO_2 decreases brain pH and induces fear behaviour in mice. ASIC1a is expressed in the amygdala and is involved in the fear response to hypercapnia. This behaviour is lost when either ASIC1a is eliminated or pH is buffered [23]. High concentrations of CO_2, such as in carbonated beverages, induce a noxious stimulus, which is due to activation of trigeminal nerve fibres. The TRP channel TRPA1 has been implicated in this response, through detection of intracellular acidification by CO_2 [24].

pH-sensitive G-protein-coupled receptors have also been shown to respond to CO_2. As previously mentioned, GPR4 is crucial in the CO_2 regulation of breathing [12]. Furthermore, TDAG8-dependent acid sensing in microglia has been shown to play a role in the hypercapnic fear response [25].

The divergent roles of pH-sensitive cell surface proteins in various cellular contexts highlight the importance of pH sensing and, moreover, sensing of CO_2-dependent alterations in pH at a cellular, tissue and systemic level.

1.4. Carbonic anhydrases

Carbonic anhydrases (CAs) are a group of zinc-containing enzymes which catalyse the reversible reaction of CO_2 and water to form H_2CO_3 and subsequently HCO_3^- and H^+ ions,

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+.$$

Six families of CAs have been identified to date: α-, β-, γ-, δ-, ζ- and η-CAs, with only α-CAs found in mammals [26]. The conservation and diversity of CAs highlight their importance in the regulation of pH and CO_2 homeostasis [27,28]. The conversion of CO_2 and H_2O to HCO_3^- and H^+ happens spontaneously and rapidly. However, the presence of CA accelerates this process half a million to a million-fold over the uncatalysed rate [29]. CO_2 has the capacity to diffuse across the cell membrane, while HCO_3^- requires transport. As such, the conversion to HCO_3^- can be used to trap excess CO_2 within cells [30]. CAs bind with acid/base transporters to facilitate the shuttling of HCO_3^- ions. The complex formed between a CA and a HCO_3^- transporter is referred to as a bicarbonate transport metabolon. The two main CAs which have been shown to form these complexes are CAII (cytoplasmic) and CAIV (membrane associated). Interactions have been shown between CAII and the Cl^-/HCO_3^- exchanger AE1, and between CAII and the Na^+/HCO_3^- co-transporters NBC1 and NBC3. Interestingly, the Cl^-/HCO_3^- exchanger SLC26A3 (DRA) is the only HCO_3^- transporter to not contain a consensus CAII binding site. AE1 and NBC1 have also been shown to interact with CAIV to increase their HCO_3^- flux [31].

Owing to the mechanism of action of the CAs, CA inhibitors such as acetazolamide have been of great interest for the treatment of conditions which result in alterations in blood pH and/or CO_2 content, such as high altitude sickness, chronic obstructive pulmonary disease (COPD) and sleep apnoea [32–34]. CA inhibition is beneficial in the context of hypocapnia. For example, at altitude, inhibition of CAs counteracts hypocapnic alkalosis, which occurs as a result of the hypoxic ventilatory response [33]. Furthermore, CA inhibition reduces the occurrence of augmented breaths, or sighs, in rats exposed to hypocapnic hypoxia (low CO_2, low O_2). Augmented breaths exacerbate sleep-disordered breathing. As such, this finding provides a potential avenue for the treatment of sleep apnoeas. By contrast, CA inhibition does not appear to be effective in hypercapnia. CAII is expressed in alveolar type I and type II cells. Inhibition of CAII delays the decrease in intracellular pH in these cells but does not affect fluid reabsorption in hypercapnia [35]. In addition, while CA inhibition may induce a small reduction in pCO_2 in patients with mild COPD, by stimulating breathing, this is not known to be associated with clinical benefit [36]. Indeed, CA inhibition in severe COPD may be detrimental, causing CO_2 retention [34]. Thus, while CAs are crucial in the regulation of pH and CO_2/HCO_3^- homeostasis, inhibition of CAs is complex and the potential benefits and risks need to be considered in the treatment of respiratory disorders.

2. Mitochondria/metabolism

2.1. Mitochondrial dysfunction

CO_2 is generated from the oxidative decarboxylation of pyruvate (the end product of glycolysis) to acetyl-CoA, the molecule which links glycolysis to the tricarboxylic acid (TCA) cycle in aerobic respiration. CO_2 is also produced at two points in the TCA cycle: the conversion of isocitrate to α-ketoglutarate and the conversion of α-ketoglutarate to succinyl-CoA (figure 1).

While glycolysis occurs in the cytosol, both decarboxylation of pyruvate and the TCA cycle take place within the mitochondrial matrix. The CO_2 produced in these processes has typically been considered a simple waste product. However, as we begin to better understand the complexity of CO_2-dependent signalling, we must consider the potential role of altered CO_2 concentrations on mitochondria, in terms of both respiratory activity and metabolic function.

Evidence that CO_2 can have direct effects on mitochondrial function was observed as early as 1984. In a study of the effects of acidosis on rat brain mitochondrial respiration, Hillered *et al.* [37] altered pH through the introduction of lactate or increased CO_2 and noted a reduction in respiratory activity, measured polarographically as a reduction in state 3 adenosine diphosphate ((ADP) stimulated) respiration and reduced state 3 to state 4 (oxygen (O_2) consumption) respiration in isolated mitochondria. The authors noted that, in hypercapnia, the reduced activity was only partially explained by acidosis, as the rate of O_2 consumption did not return completely when the pH was adjusted [37]. This suppressive effect of hypercapnia on O_2 consumption has also been observed in

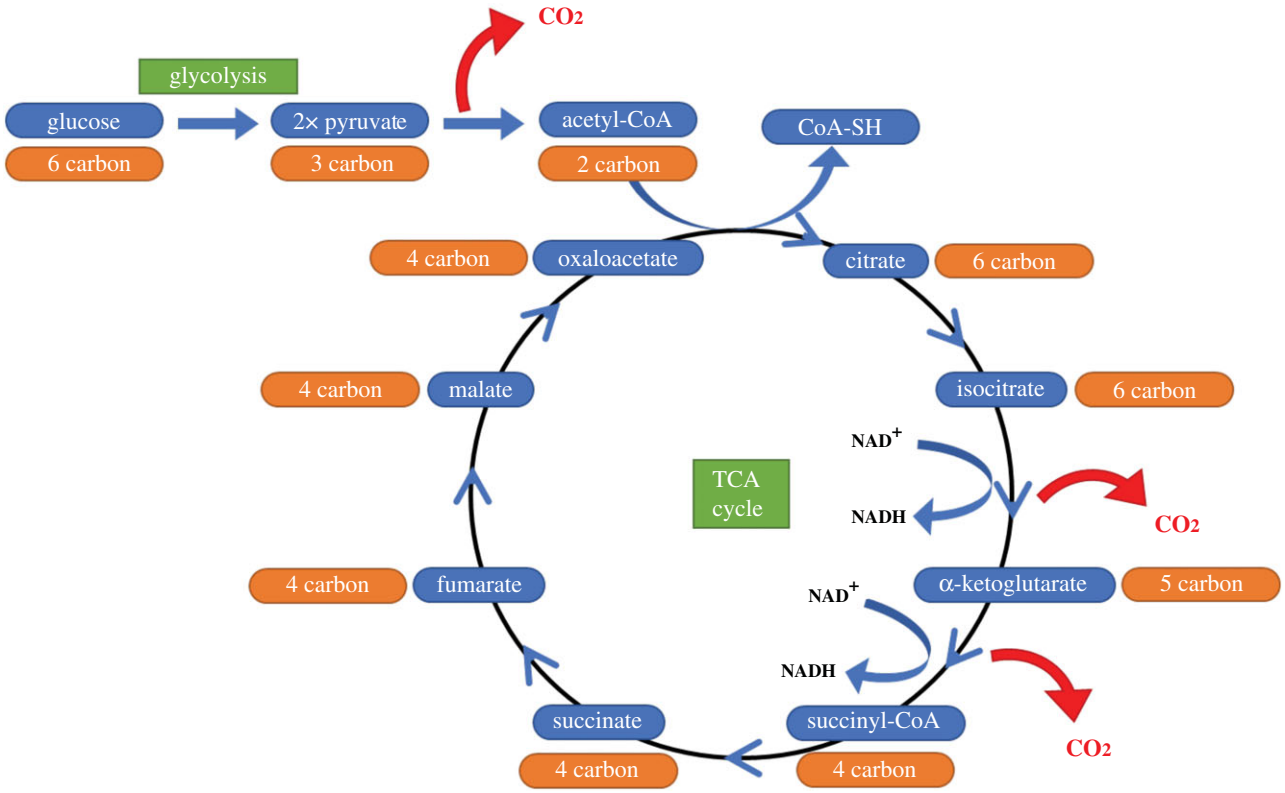

Figure 1. Production of CO_2 in glucose metabolism. A CO_2 molecule is produced by the oxidative decarboxylation of pyruvate to acetyl-CoA, allowing entry to the TCA cycle. CO_2 is also generated at two points in the TCA cycle: the conversion of isocitrate to α-ketoglutarate, and the subsequent conversion of α-ketoglutarate to succinyl-CoA. In both instances, CO_2 is produced as a by-product of the conversion of NAD^+ to NADH.

human cells. Vohwinkel *et al*. [38] observed a CO_2-dependent (40–120 mmHg) decrease in O_2 consumption and ATP production in human fibroblasts and alveolar epithelial cells, which was independent of both acidosis and hypoxia. Decreased cell proliferation was detected in hypercapnia for both cell types, which was not explained by either an increase in cell death or cell cycle arrest. Instead, the authors provide evidence that the cells progressed through cell cycle phases at a slower rate, owing to mitochondrial dysfunction. The role of mitochondria and mitochondrial damage in cell cycle progression has been previously described elsewhere [39–41]. Hypercapnia-dependent mitochondrial dysfunction results from a downregulation of isocitrate dehydrogenase 2 (IDH2) mRNA and protein levels, a key enzyme in the TCA cycle. IDH2 catalyses the conversion of isocitrate to α-ketoglutarate, one of the two CO_2-generating steps of the TCA cycle (figure 1). The authors suggest that CO_2-dependent inhibition of the TCA cycle stimulates a switch to glycolysis as the primary source of energy, as cells were unable to survive when glycolysis was impaired in hypercapnia [38].

The idea that elevated CO_2 leads to mitochondrial dysfunction was further explored in a 2019 study by Fergie *et al*. [42] Using the lipophilic, cationic dye JC-1, the authors saw an attenuation of mitochondrial membrane potential in smooth airway epithelial cells and mesenchymal stem cells, indicating an impaired mitochondrial function in hypercapnic acidosis. This result was comparable both in non-inflammatory conditions and in cells stimulated with a combination of the pro-inflammatory cytokines tumour necrosis factor alpha (TNFα), interleukin-1β (IL-1β) and interferon (IFN)-γ. Furthermore, there was a reduction in the levels of ATP produced by small airway epithelial cells in hypercapnic acidosis, suggesting a decrease in oxidative phosphorylation [42]. The authors further explored the impact of this mitochondrial impairment on wound healing. In hypercapnic acidosis, there was a reduction in the degree of wound closure by small airway epithelial cells co-cultured with mesenchymal stem cells. An important mechanism by which mesenchymal stem cells promote wound healing is through the transfer of mitochondria in extracellular vesicles [43]. Mitochondrial transfer from healthy cells mitigates the injury to damaged cells by improving their bioenergetics [44]. Thus, Fergie *et al*. [42] propose that CO_2-dependent mitochondrial dysfunction may cause the impaired wound-healing capacity observed in their model.

2.2. Reactive oxygen species/metabolism

Oxidative phosphorylation is an important generator of ATP within mammalian cells. This ATP production is achieved through the transfer of electrons through the mitochondrial electron transport chain (complex I–IV). The transfer of protons (H^+) from the mitochondrial matrix to the intermembrane space by complex I, III and IV creates an electrochemical gradient. Complex IV, or cytochrome c oxidase, oxidizes cytochrome c and transfers the electrons to O_2. This O_2 is then converted to water with the addition of H^+.

ATP synthase, also referred to as complex V, uses the electrochemical gradient to generate a molecule of ATP for every four H^+ ions which pass through, returning to the mitochondrial matrix. However, leakage of electrons from the transport chain at complex I and III leads to a partial reduction of O_2, and the production of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [45,46]. ROS production can also occur in the TCA cycle, through α-ketoglutarate dehydrogenase [47] and succinate dehydrogenase (which is also complex II) [48]. While ROS play

important roles in numerous cell signalling pathways [49,50], excess ROS production is cytotoxic, damaging proteins, nucleic acids and lipids. The balance between pro-oxidant and antioxidant pathways is important for cell homeostasis and excessive oxidant activity can induce cell death mechanisms such as apoptosis [51]. Given that hypercapnia has been shown to affect mitochondrial function, it is likely that alterations in CO_2 affect the production of mitochondrial ROS, in turn modulating oxidative stress in cells.

Interestingly, studies to date have provided evidence that elevated CO_2 is protective against oxidative stress. Nichol *et al*. [52] examined the effect of hypercapnic acidosis on endotoxin-induced lung injury in mechanically ventilated mice and used the ROS indicator dihydrorhodamine to detect oxidation in the lung tissue of these mice. Dihydrorhodamine is oxidized to the fluorescent product rhodamine both by H_2O_2 (in the presence of the enzyme myeloperoxidase) and by peroxynitrite, a reactive species generated by the reaction of O_2^- anions with nitric oxide. The study found that hypercapnic acidosis reduced the concentration of rhodamine formed in the lung homogenates of endotoxin-inoculated mice compared with the normocapnic counterparts. Notably, the addition of the nitric oxide synthase inhibitor L-NMMA had no significant effect on rhodamine production in either normocapnia or hypercapnia, indicating that the antioxidant effects of hypercapnic acidosis observed in this study were independent of peroxynitrite production and thus likely resulted from a decrease in O_2^- and/or H_2O_2 [52].

Yang *et al*. [53] further explored the protective, antioxidant effect of hypercapnic acidosis in 2013. Similar to Nichol *et al*. [52] this study examined the effects of hypercapnic acidosis in lung injury during mechanical ventilation using multiple histological markers of lung damage. Rats in the hypercapnic acidosis group had reduced lung damage compared with the normocapnic group. This protective effect was associated with an increase in superoxide dismutase activity (antioxidant enzyme), as well as decreased malondialdehyde content (a marker of lipid peroxidation) and decreased apoptosis. Taken together, these data indicate reduced oxidative stress and reduced lung damage in hypercapnic acidosis in this model [53].

While attenuated ROS production and reduced oxidative stress may be a beneficial effect of hypercapnia in ventilator-induced lung injury, the opposite can be true in lung cancer. The microenvironment of solid tumours is hypercapnic and acidotic [54]. Cisplatin is a drug widely used in chemotherapy for the treatment of solid tumours. Cisplatin exerts its therapeutic effects via induction of cell death through the damage of both nuclear and mitochondrial DNA. Damage to mitochondrial DNA leads to an increase in ROS production. Chemoresistance to cisplatin is a major problem in cancer treatments. In order to assess the role of the hypercapnic microenvironment in cisplatin resistance, Kikuchi *et al*. [55] performed a study in lung adenocarcinoma A549 cells and lung non-small cell carcinoma H1299 cells. The cells were exposed to cisplatin over a range of 5–15% CO_2. The authors found that CO_2 was cytoprotective against cisplatin, in a concentration-dependent manner, with cells exposed to 15% CO_2 requiring approximately twice the dose of cisplatin to induce the same levels of cell death as 5% CO_2. Furthermore, this cytoprotective effect was independent of pH. To investigate the mechanism of this effect, the authors examined the effect of elevated CO_2 on DNA damage and expression of the pro-

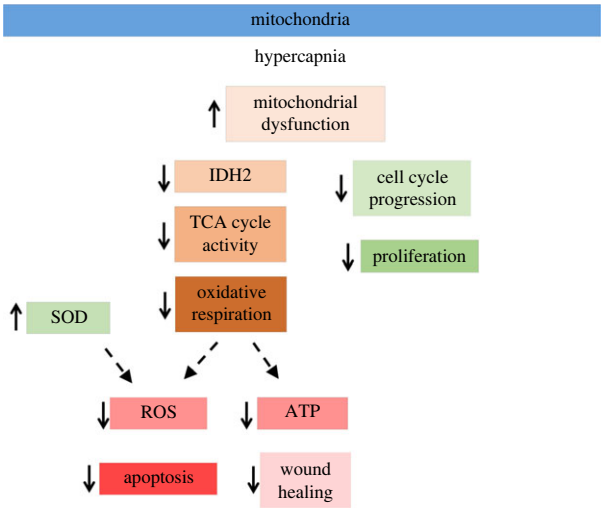

Figure 2. Effects of hypercapnia on mitochondrial activity. Elevations in CO_2 lead to mitochondrial dysfunction, decreased IDH2 and subsequent decreased oxidative respiration. Reduced oxidative respiration results in decreased ATP and ROS production. Increased superoxide dismutase (SOD) activity in hypercapnia also contributes to reduced ROS levels. Reductions in ROS levels limit oxidative stress and apoptosis. Reductions in ATP production can limit wound healing. Mitochondrial dysfunction also inhibits cell cycle progression and proliferation.

apoptotic protein Bax in response to cisplatin, and noted that there was no change in either, suggesting that chemoresistance in hypercapnia is not due to modulation of cisplatin-dependent DNA damage [55]. However, cells cultured in 15% CO_2 had reduced mitochondrial O_2^- and cellular ROS levels, in both the basal and cisplatin-stimulated state, when compared with normocapnic cells. This effect was not mediated by increased antioxidant activity, but rather by a decrease in mitochondrial respiration, with significant inhibition of both glycolysis and oxidative phosphorylation observed in these cells in hypercapnia. Notably, this effect was also shown to be largely independent of alteration in pH.

These studies taken together provide strong evidence that elevation in CO_2 leads to mitochondrial dysfunction, inhibiting mitochondrial respiration and reducing the production of both ATP and ROS. This response is beneficial in the context of ventilator-induced lung injury, where reduced oxidative stress improves outcomes, but is detrimental in the context of wound healing and cisplatin therapy, which rely on functional mitochondria (figure 2).

3. Carbon dioxide-dependent regulation of intracellular calcium

Calcium (Ca^{2+}) is an invaluable second messenger in mammalian cells, with a wide range of physiological functions, including muscle contraction, cell motility and synaptic signalling. Ca^{2+} signalling is largely dependent on fluctuations in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Increases in $[Ca^{2+}]_i$ concentration can arise as a result of an influx of Ca^{2+} through channels on the cell membrane or by the release of Ca^{2+} from intracellular stores, mainly the sarco/endoplasmic reticulum and the mitochondria. Similarly, Ca^{2+} can be sequestered into intracellular stores or expelled from the cell through ion pumps in order to reduce intracellular concentrations. It is important to note that sub-cellular Ca^{2+} concentrations are not uniform, which is a valuable part of

Ca^{2+} signalling. Steep localized gradients of Ca^{2+} are created via an influx of Ca^{2+} through ion channels or release from intracellular stores. Propagation of the Ca^{2+} signal is dependent on an intracellular network involving a number of receptors including the ryanodine receptor and inositol trisphosphate (IP_3) receptors. The mechanisms of Ca^{2+} signalling and the involved intracellular networks have been extensively reviewed elsewhere [56]. The idea that hypercapnia could potentially alter $[Ca^{2+}]_i$ has been explored since as early as 1934 when Forbes [57] examined the effect of breathing air with increased CO_2, and subsequent respiratory acidosis, on Ca^{2+} deposition in growing rats. The results from this study showed no difference in Ca^{2+} retention between the hypercapnic and normocapnic groups. However, it is worth noting that these early experiments did not directly measure Ca^{2+} concentrations within cells or tissues, but rather in the urine and faeces of the animals [57].

Since its development in 1985, Fura-2 has been widely used to visualize and measure $[Ca^{2+}]_i$. Fura-2 is a ratiometric indicator. When used at low concentrations, the ratio between the 340 and 380 nm excitations can be used to analyse $[Ca^{2+}]_i$ concentrations [58]. This method was employed by Nishio and colleagues [59] in a 2001 study examining the effects of hypercapnia and hypocapnia on Ca^{2+} levels in endothelial cells. The authors found that both hypercapnic acidosis and hypocapnic alkalosis led to an increase in $[Ca^{2+}]_i$, with a greater increase seen in hypocapnia. Interestingly, when further exploring the relationship between acidosis/alkalosis and $[Ca^{2+}]_i$, the authors noted no significant effect of intracellular alkalinization on $[Ca^{2+}]_i$. In order to determine the source of the Ca^{2+} for these increases, the authors used the Ca^{2+} chelating agent EGTA and the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (TG) to deplete extra- and intracellular stores of Ca^{2+}, respectively. Intriguingly, there was a striking difference in the mechanisms employed by hypercapnia and hypocapnia to increase $[Ca^{2+}]_i$. Hypercapnic acidosis-related increases in Ca^{2+} were not significantly affected by the use of either EGTA or TG, suggesting that the source of Ca^{2+} in this instance is either from TG-insensitive intracellular stores, such as the mitochondria, or release from Ca^{2+} binding sites. By contrast, both EGTA and TG significantly limited the increase in $[Ca^{2+}]_i$ in response to hypocapnic alkalosis, indicating that both extracellular Ca^{2+} and Ca^{2+} sequestered in the endoplasmic reticulum are involved in this response. Hypercapnia and hypocapnia are associated with vasoconstriction and vasodilation in the pulmonary circulation, respectively. Nishio *et al*. [59] found that hypocapnia significantly increased the production of the vasodilator prostacyclin, whereas hypercapnia had no effect. As prostacyclin is produced by cyclooxygenase, which requires Ca^{2+} for activation, the authors propose that the vasodilatory response to hypocapnia may be due to Ca^{2+}-dependent prostacyclin production, whereas the vasoconstriction seen in hypercapnia is likely to be unrelated to prostacyclin levels [59]. There is some evidence that the findings of Nishio *et al*. may be cell specific. Cook *et al*. [60] also detected increases in $[Ca^{2+}]_i$ in response to hypercapnia; however, the source of the Ca^{2+} in this study varied from those reported by Nishio *et al*. The study by Cook *et al*. examined the effect of elevated CO_2 levels on cyclic adenosine monophosphate (cAMP) signalling in kidney cells and used a variety of pharmacological inhibitors to determine the source of increased $[Ca^{2+}]_i$. The sensitivity of the cellular response to BAPTA-AM ($[Ca^{2+}]_i$ chelator), TG (endoplasmic reticulum Ca^{2+} channel inhibitor) and IP_3 receptor inhibition revealed Ca^{2+} release from intracellular endoplasmic reticulum stores to be the likely mechanism. This is different from the mechanism proposed by Nishio *et al*. in endothelial cells. Interestingly, the use of adenylyl cyclase (AC) inhibitor had no effect on the Ca^{2+} response to hypercapnia, indicating that the Ca^{2+} release was upstream of cAMP, which demonstrated lower accumulation in hypercapnia [60]. The potential for hypercapnia to have cell-specific effects on Ca^{2+} signalling is further demonstrated by Shigemura *et al*. [61], whose 2018 study explored the effect of elevated CO_2 on airway smooth muscle contractility. In accordance with previous studies, Shigemura *et al*. also noted an increase in $[Ca^{2+}]_i$ in response to hypercapnia. This increase was associated with increased cleavage of caspase 7 by the Ca^{2+}-dependent cysteine protease calpain-1. Further exploration by the authors determined that cleaved caspase 7 in turn cleaves myocyte-specific enhancer factor 2D (Mef2D), leading to the downregulation of the micro-RNA miR-133a and subsequent upregulation of Ras homologue family member A (RhoA), a protein which plays an important role in the regulation of smooth muscle contraction [61]. Thus, the Ca^{2+}-dependent signal transduction hub, which is central to the regulation of many physiological processes, responds to conditions of elevated CO_2 by increasing $[Ca^{2+}]_i$ and activating downstream signalling cascades including AC and caspase-dependent signalling.

4. Carbon dioxide signal transduction in the adenylyl cyclase–cAMP signalling axis

Identified in 1958 by Dr Earl Sutherland as the first intracellular second messenger of extracellular ligand action [62], cAMP is widely regarded as a universal regulator of cellular functions in organisms, including amoebas, plants and humans [63]. Biological processes mediated by this second messenger system include memory, metabolism, gene regulation and immune function [63].

The role of AC enzymes in the regulation of cAMP-dependent signal transduction has been expertly reviewed elsewhere [64,65] and the focus of this section will be on components of the AC–cAMP pathway involved in CO_2-dependent signalling.

In brief, ACs are enzymes that catalyse the conversion of ATP to cAMP and pyrophosphate. The cAMP produced by AC serves as a regulatory signal via binding of cAMP binding proteins, transcription factors (TFs), enzymes or ion transporters and is involved in the regulation of diverse biological processes such as oogenesis, embryogenesis, hormone secretion, glycogen breakdown, cardiac contraction and smooth muscle relaxation [60,66–68]. ACs can be classified as being transmembrane bound (tmAC) or soluble (sAC) forms of the enzyme. Both tmACs and sAC have been implicated in sensing/responding to changes in CO_2/HCO_3^- (key forms in which inorganic carbon can exist in the body)

4.1. Soluble adenyl cyclase

As its name suggests, sAC has a variable cellular distribution (within the cytoplasm and specific organelles) and more closely resembles cyanobacterial ACs than tmACs [69–71]. In 2000, Chen *et al*. [70] demonstrated that HCO_3^- can directly

modulate the activity of sAC and, thus, revealed that physiological CO_2/HCO_3^-/pH could be sensed via cAMP second messenger signalling. Using a stable HEK293 cell line expressing sAC, Chen *et al*. examined the effect of HCO_3^- concentrations mimicking those observed in sperm (from ≤5 mM in caudal epididymal sperm pre-ejaculation to ≥25 mM in sperm post-ejaculation) and reported that HCO_3^- increased cAMP production [70]. Furthermore, the authors demonstrated, using a purified sAC assay in the presence of $NaHCO_3^-$ with 10 mM ATP and 40 mM $MgCl_2$, that HCO_3^- stimulation was not due to altered pH as both Mg^{2+}-ATP and HCO_3^--stimulated sAC were insensitive to pH change in the range from 7.0 to 8.5. This observation confirmed that HCO_3^- stimulation was direct, specific and pH independent. Further evidence in support of sAC being activated by CO_2 and HCO_3^- was provided by Townsend *et al*. [72].

Recently, sAC has been proposed as a potential putative CO_2 sensor in alveolar lung epithelia with relevance to multiple cellular processes [73]. Human A549 and Sprague Dawley rat alveolar epithelial type I (AT1) cells were isolated and stimulated with either HCO_3^- or trisaminomethane buffer under normocapnic or hypercapnic conditions. Intracellular pH, cellular cAMP and micropuncture plasma membrane wound resealing were investigated. The experimental results indicated that exposure to hypercapnia for as little as 15 minutes resulted in intracellular acidosis, decreased intracellular cAMP levels and decreased wound repair. While hypercapnia inhibited the AC–cAMP signal transduction axis, buffering of hypercapnia was shown to restore the AC–cAMP axis and the likelihood of injured cells to repair. Taken together these data suggest that the AC–cAMP axis is CO_2 sensitive and pH dependent in lung epithelia.

4.2. Transmembrane adenyl cyclase

tmACs are a family of ACs containing transmembrane spanning domains and are exquisitely sensitive to G-proteins. Under normal conditions, these enzymes are activated secondary to G-protein-coupled receptor activation, e.g. activation of parathyroid receptor (PTHR) by binding of parathyroid and subsequent intracellular signal transduction influencing a tmAC [60]. Downstream of tmAC activation, cAMP may act as a second messenger, leading to activation of effector proteins such as protein kinase A (PKA), cAMP response binding element (CREB), phosphodiesterase domains and cyclic nucleotide-gated ion channels. cAMP produced in this classical system via PTH binding of PTHR is decreased in response to elevated CO_2, resulting in activation of the sodium/proton (Na^+/H^+) antiporter. To demonstrate this, the authors used pharmacological inhibitors of downstream mediators of the cAMP signalling pathway such as the PKA inhibitor H-89 and the IP_3 receptor antagonist xestospongin C to investigate CO_2-responsive pathways in opossum kidney cells [60]. The results concluded that CO_2 suppressed the activity of cAMP signalling through IP_3 receptor-mediated Ca^{2+} release.

The previous example highlighted how tmACs may be indirectly sensitive to CO_2 by acting downstream of PTH. There is also evidence that tmACs are also directly sensitive to CO_2. Recombinant G-protein activated tmACs isolated from mammalian cells and the prokaryote *Mycobacterium tuberculosis* were found to be specifically activated in response to CO_2 but not HCO_3^-. The authors performed *in vitro* experiments under conditions of disequilibrium which exploit the fact that the prevailing form of inorganic carbon in the assay exists in the form in which it was initially added (i.e. CO_2/HCO_3^-) when the temperature of the experimental condition was low (approximately 0°C). The activity of both the mammalian and related prokaryotic tmACs was stimulated by CO_2, which resulted in phosphorylation of CREB [72]. Radiolabelled CO_2 was used to demonstrate CO_2 binding to CREB; however, the specific binding site remains to be described.

Collectively, there is significant evidence implicating sAC, tmACs and the cAMP signalling axis as being important for CO_2 sensing [60,72,73]. Future directives should focus on defining the exact mechanisms through which both CO_2 and HCO_3^- are directly sensed by the AC–cAMP signalling axis.

5. The role of AMP-activated protein kinase in carbon dioxide-dependent signal transduction

The AMP-activated serine/threonine protein kinase (AMPK) is a conserved metabolic sensor charged with regulating anabolic and catabolic pathways in response to reduced energy levels [74,75] that has been shown to be involved in hypercapnia responses in some cell types. Cellular ATP/AMP (and ADP) ratios reflect the energy status of the cell and directly impact the activity of AMPK. AMPK is typically activated when ATP levels drop and AMP binds to the γ subunit of phosphorylated AMPK. AMPK senses AMP levels directly but can respond to other metabolic signals in the absence of AMP binding, such as low glucose or hypoxia [75]. The AMPK protein consists of three subunits, encoded in mammals by 11 genes with specific expression patterns (catalytic subunits α1 and α2, plus β1, β2 and γ1, γ2, γ3 regulatory subunits). The combination of different α, β and γ subunits potentially allows the existence of functionally diverse AMPK proteins with distinct expression, activity and regulation. AMPK has distinct responses that can be associated with specific catalytic subunit isoforms in different cells.

AMPK is activated by phosphorylation of the alpha subunit on Thr172. The liver kinase B1 (LKB1) activates AMPK-α and other (non-AMP-responsive) kinases of the same family in response to multiple stresses and to several pharmacological agents [74]. While LKB1 seems to be responsible for prototypical AMP-dependent regulation of AMPK-α2 in response to energy levels, other kinases can activate AMPK in response to diverse signals, like Ca^{2+}, even without measurable AMP increases. Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK) is reported to activate AMPK in response to increased $[Ca^{2+}]_i$ in several cell types.

5.1. AMP-activated protein kinase in alveolar fluid reabsorption

Lung oedema is a reported negative effect of hypercapnia in lung disease, associated with impaired sodium transport across alveolar epithelia. High CO_2 levels (up to 120 mmHg) cause a fast (less than 5 min) and pH-independent increase in phosphorylated AMPK-α1 (Thr172) in rat alveolar epithelial cells [76,77]. Reduced alveolar fluid reabsorption (AFR) due to hypercapnia is mediated via Ca^{2+} increase, and subsequent activation of CaMKK-β, AMPK-α and PKC-ζ, leading to increased endocytosis of the

basolateral Na^+/K^+-ATPase [76]. PKC-ζ activation of c-Jun N-terminal kinase (JNK) is also required for endocytosis in hypercapnia [77]. JNK promotes the phosphorylation of LMO7b, a scaffolding protein that interacts with Na^+/K^+-ATPase to promote its endocytosis in alveolar epithelial cells [78]. In A549 cells, a cancer-derived model of type II alveolar epithelial cells (AECs) deficient in LKB1, CaMKK, also activates AMPK [76,79]. How elevated CO_2 levels lead to increases in $[Ca^{2+}]_i$ to activate CaMKK is still unclear.

Ca^{2+} levels peak transiently (seconds to minutes) upon hypercapnia exposure, but prolonged exposure of type II AECs to high CO_2 levels (up to 24 hours at 120 mmHg) causes a sustained decrease of membrane Na^+/K^+-ATPase, and AFR was similarly reduced in rat lungs after 1 hour at 60 mmHg CO_2 or after 3 or 7 days under 10% CO_2 atmosphere [76].

Multiple pathways may thus cooperate to sustain AFR reduction during prolonged hypercapnic exposure. Extracellular signal-regulated kinases (ERK1 and ERK2), members of the MAPK family, also seem to be involved in AFR inhibition in hypercapnia. ERK was reported to phosphorylate AMPK after exposure to high CO_2 in alveolar epithelial cells, increasing the phosphorylation of the Na^+/K^+-ATPase [80]. ERK also downregulates the ENaC transporter (epithelial Na^+ channel) activity in response to hypercapnia in AECs. ERK is suggested to mediate phosphorylation of the ENaC complex and to activate AMPK. AMPK and JNK activation results in phosphorylation of the E3 ligase Nedd4-2, leading to polyubiquitination and subsequent endocytosis of the ENaC complex [81].

AMPK activation also occurs in hypoxia, a situation that commonly associates with hypercapnia, e.g. in vascular disruption and solid tumours. Hypoxia-dependent AMPK activation may decrease energy requirements and O_2 demand and promote autophagy; however, the mechanism is not fully elucidated. Severe hypoxia can lead to increased AMP/ATP ratios and AMP-dependent LKB1 activation of AMPK. AMP-independent activation of AMPK has been suggested via multiple pathways, involving rises in Ca^{2+}, mitochondrial ROS, hypoxia-inducible factor (HIF)-hydroxylases, glucose or hormone levels [82]. Hypoxia also inhibits AFR in alveolar cells via Na^+/K^+-ATPase endocytosis, mediated by mitochondrial ROS and PKC-ζ [83]. Na^+/K^+-ATPase downregulation during hypoxia in alveolar epithelial dysfunction was linked to ROS-induced AMPK-α1 activation, requiring Ca^{2+} and CaMKK but not LKB1 [84].

As will be discussed further in this article, hypoxia and hypercapnia often co-occur (§6), and this fast and transient Ca^{2+}-induced AMPK activation may mediate other common responses to transient changes in CO_2 and O_2, but also to sustained hypercapnia.

5.2. AMP-activated protein kinase in muscle wasting in hypercapnia

Sustained (and intermittent) hypercapnia is often a feature in patients with COPD, where AMPK is proposed to mediate hypercapnia-induced muscle atrophy. Mice exposed to 10% CO_2 (versus room air) for 21 days had decreased muscle weight, muscle fibre size and grip strength [85]. Mouse C2C12 myotubes exposed to hypercapnia (24 hours at 120 mmHg CO_2) also presented time- and dose-dependent reduction of fibre diameter, associated with a decrease in protein content and 45S pre-rRNA levels, indicating decreased ribosomal biogenesis and increased proteasomal degradation of muscle proteins. The effects of CO_2 in muscle atrophy are mediated by muscle-specific ring finger protein 1 (MuRF1), an E3-ligase that is upregulated by the TF FoxO3. AMPK and its known target acetyl-CoA carboxylase (ACC) are quickly (15 min) and sustainably phosphorylated in response to CO_2 elevation. AMPK-α2 (not α1) promotes FoxO3 phosphorylation and nuclear translocation in myotubes [85]. AMPK-α2 is the main isoform in skeletal muscle, activated typically by LKB1 and AMP levels. In mouse myotubes, hypercapnia-induced phosphorylation of AMPK was LKB1 but not CaMKK dependent [86].

While muscle atrophy is induced by hypercapnia via AMPK–FoxO3–MuRF1, a different pathway can be involved in downregulating protein synthesis. Puromycin incorporation rates and ribosomal gene expression are reduced in hypercapnia [87]. This reduction in protein synthesis seems to be also dependent on AMPK-α2, but not on the proposed mediators mTOR, TIF-1A and KDM2A. Hypercapnia modulation of protein anabolism in muscle cells is mediated by a different pathway that has not yet been fully elucidated [86,87].

Many questions remain unanswered about the role of AMPK in the responses to hypercapnia. The pathways involved in hypercapnic signalling are not only cell type dependent but specific to different targets and potentially to the global cellular environment (other stressors, stimulus and metabolic states). Different diseases like COPD and sleep apnoea induce distinct patterns of elevated CO_2 that may trigger diverse outcomes via different pathways. Our current knowledge of the signal transduction pathways responsible for observed clinical effects of hypercapnia is still very limited and additional tools and research are required to get a clear picture on the diversity and complexity of these mechanisms figure 3.

6. Transcriptional responses to carbon dioxide

The previous sections of this article have focused mainly on rapid responses to CO_2 that are reliant on changes in pH, mitochondrial function, $[Ca^{2+}]_i$, cAMP, AMPK etc. In this section we will address the transcriptional response to hypercapnia, focusing on key CO_2-responsive TFs.

6.1. Hypercapnia and disease—a role for transcriptional regulators

In a clinical setting, hypercapnia is frequently observed in patients with lung pathologies resulting directly from poor gas exchange in diseases such as cystic fibrosis and COPD or in low tidal volume ventilation strategies (permissive hypercapnia). Hypercapnia has been associated with improved prognosis in some settings, but may be a risk factor in other pathologies [88]. Indeed, the clinical management of patients experiencing hypercapnia is still under intense debate. Abundant clinical and experimental research links hypercapnia to reduced lung inflammation in ventilated patients (permissive hypercapnia) and much work has been done to try to better understand whether we can exploit the anti-inflammatory effect of hypercapnia further for clinical benefit. On the other hand it is clear that patients with hypercapnia have a worse outcome when exposed to bacterial infections [89], have impaired wound healing in hypercapnia [90,91], experience muscle wasting [85] and

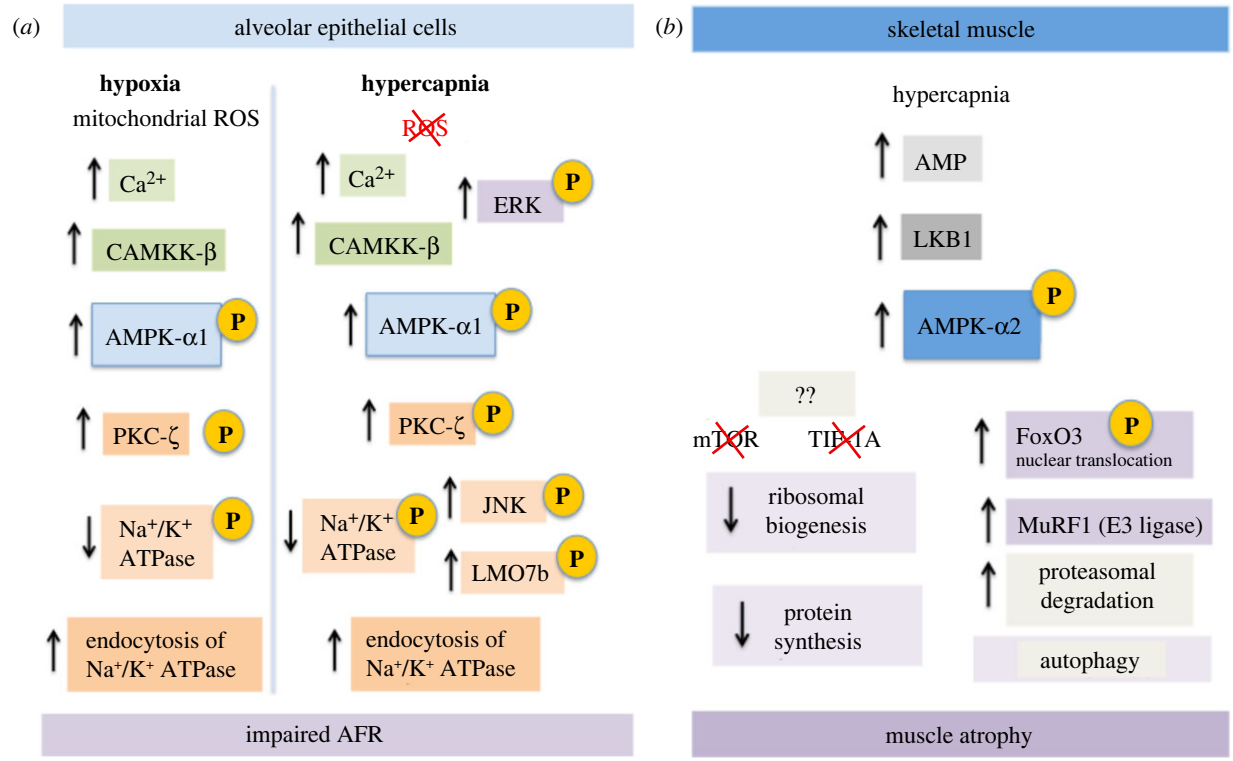

Figure 3: AMPK signalling in hypercapnia. (*a*) In alveolar epithelial cells, the α1 subunit of AMPK promotes endocytosis of the Na^+/K^+-ATPase in response to both hypoxia and hypercapnia, leading to impaired AFR. Responses to hypoxia and hypercapnia leading to reduced Na^+/K^+-ATPase activity are similar, but ROS do not seem to be an initial trigger in hypercapnia. (*b*) In muscle cells, hypercapnia promotes muscle atrophy. It is the α2 subunit of AMPK that activates separate pathways to reduce protein synthesis (anabolism) and increase autophagy (catabolism).

have increased airway smooth muscle contractility [61]. Thus, in the clinical context, hypercapnia has been likened to a 'double-edged sword' where any potential beneficial effects must be balanced against known deleterious effects. These clinical effects are expertly reviewed elsewhere [92,93]. The current view is that the immuno-suppressive effects of hypercapnia are believed to be dependent at least in part on the CO_2-dependent regulation of gene expression. While a full understanding of the mechanism(s) through which elevated CO_2 levels exert their effects in immunity are not yet clear, there is ample evidence to suggest multiple levels of TF (including nuclear factor kappa B (NF-κB)) involvement. These effects are summarized in tables 2 and 3.

6.2. NF-κB

6.2.1. Background

NF-κB is a family of TFs involved in immune and inflammatory responses, but also in cell survival, differentiation and proliferation. In the years following its discovery, a large number of studies have elucidated multiple mechanisms of NF-κB signalling, serving as a model for other TFs. The NF-κB pathway has been reviewed extensively elsewhere [109,110]. Briefly, homo- or heterodimers of the five members of the Rel homology domain family of TFs (RelA/p65, RelB, c-Rel, p100/p52, p105 /p50 in humans) regulate transcription of specific gene subsets [111,112]. RelA, RelB and c-Rel each have a DNA-binding domain and a transcription activation domain (TAD), unlike p52 and p50, which lack a TAD and may function as repressors when not associated with a TAD-containing protein. The complexity of the NF-κB pathway allows temporal control of the expression of a wide range of target genes in a cell-and context-specific manner [111–113].

The most frequently studied NF-κB dimer, the RelA/p50 dimer, is the main TF of the 'canonical' NF-κB pathway. The canonical pathway is regulated by phosphorylation of the IKK complex (a heterotrimeric complex of subunits IKKα, IKKβ and IKKγ). Phosphorylation of the inhibitory protein IκBα removes its repressor function (through ubiquitylation and proteolytic degradation) and allows for the activation and nuclear translocation of RelA. Canonical NF-κB activation is associated with classical pro-inflammatory gene expression, e.g. TNFα, IL-1, other cytokines and chemokines. The second best-characterized pathway is the 'non-canonical' pathway, where the inactive RelB/p100 dimer is activated via IKKα homodimer phosphorylation, leading to proteasomal processing of p100 to p52, independently of IκB. The RelB/p52 dimer then moves to the nucleus to activate gene expression and regulates a discrete set of genes compared with those regulated by RelA/p50 dimers, e.g. those involved in lymphogenesis. NF-κB activity has been fundamentally associated with the immune system, where it induces the expression of TNFα and other pro-inflammatory genes including cytokines, chemokines and adhesion molecules. Initially considered mainly to be a pro-inflammatory signalling pathway, current views highlight a variety of additional roles in development, proliferation and survival of multiple cell types, adding the complexity of this pathway that has both pro- and anti-inflammatory roles. Thus, appropriate NF-κB-dependent signalling is crucial for the coordinated and effective functioning of the innate immune response.

6.2.2. Hypercapnia and the canonical NF-κB pathway

Several studies have implicated key components of the canonical NF-κB pathway, mainly RelA and IκB, in the cellular

Table 2. Evidence relating to CO_2-dependent regulation of the members of the NF-κB family and/or NF-κB-dependent gene expression.

transcription factor/pathway	hypercapnia conditions	cell/tissue (model)	co-stimulus	CO_2 dose	response to hypercapnia	physiological effect of hypercapnia	reference	year
NF-κB	hypercapnic acidosis isocapnic acidosis-buffered hypercapnia	human pulmonary artery endothelial cells	lipopolysaccharide (LPS) 1 μg/ml.	75 mmHg (up to 24 h)	↓ IκBα degradation ↓ ICAM-1 and IL-8 ↓ lactate dehydrogenase release	anti-inflammatory ↓ neutrophil adherence ↓ NF-κB activation pH - synergystic	[94]	2003
NF-κB	hypercapnic acidosis buffered hypercapnia	primary small airway epithelial cells (SAECs), alveolar A549 cells and human bronchial epithelial (HBE) cells	wound injury	10% CO_2 (pH 7.15, PCO_2 8 kPa) or 15% CO_2 (pH 7.0, PCO_2 11 kPa) for 24 h or 48 h (0, 60 and 180 min for IκBα WB)	↓ MMP1 ↑ tissue inhibitor of metalloproteinase-2	↓ wound closure ↓ NF-κB activation ↓ epithelial cell migration pH-independent	[90]	2009
NF-κB	hypercapnic acidosis buffered hypercapnia	differentiated human THP-1 macrophages and human and mouse alveolar macrophages	LPS and other toll-like receptor (TLR) ligands (Pam3CSK4, *Listeria*, poly(I:C), flagellin, FSL-1, imiquimod, ssRNA40 and ODN2006)	9% CO_2 (PCO_2 64 mmHg), 12.5% CO_2 (PCO_2 88 mmHg) or 20% CO_2 (PCO_2 140 mmHg), for different times (to 24 h)	↓ TNFα ↓ IL-6 ↔ IL-10 ↔ IFN-β IκBα degradation, ↔ nuclear RelA/p65	↓ NF-κB activation ↓ phagocytosis pH-independent	[95]	2010
NF-κB	hypercapnic acidosis buffered hypercapnia	mouse embryonic fibroblasts (MEFs), human PBMCs, A549 and other cell lines	LPS treatment (10 μg/ml, 24 h)	10% CO_2 for different times (1 h, 4 h, 6 h), reversible by brief exposure to air	↑ nuclear IKKα ↓ nuclear RelA/p65 ↓ TNFα ↓ CXCL2 ↓ ICAM1 ↑ IL-10	anti-inflammatory ↓ NF-κB activation pH-independent	[96]	2010
NF-κB	hypercapnia (live animal exposure)	mechanically ventilated Wistar rats	hepatic ischaemia–reperfusion injury	45%N_2–50%O_2–5%CO_2, for 15 min before 1 h ischaemia and 4 h reperfusion	↓ liver enzymes ↓ TNFα ↓ apoptotic index ↓ histopathological scores ↑ IL-10	anti-inflammatory ↓ NF-κB expression	[97]	2010
NF-κB	hypercapnia (live animal exposure)	Sprague Dawley rats	mechanical stretch-induced injury	5% CO_2 in air	↓ IL-6, IL-8 ↓ neutrophil infiltration ↓ IκBα degradation	↓ NF-κB activation ↑ oxygenation ↑ compliance ↓ histological injury ↓ cell death pH-dependent	[98]	2012

(*Continued*.)

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Table 2. (*Continued.*)

transcription factor/pathway	hypercapnia conditions	cell/tissue (model)	co-stimulus	CO_2 dose	response to hypercapnia	physiological effect of hypercapnia	reference	year
NF-κB	hypercapnic acidosis buffered hypercapnia	alveolar epithelial A549 cells and MEF	basal or TNFα or LPS stimulation	10% CO_2 for different times (1 h, 4 h, 24 h)	↑ RelB cleavage ↑ nuclear RelB ↑ nuclear IKKα ↔ nuclear IκBα ↓ TNFα (RelB independent) ↓ COX-2 (RelB independent)	anti-inflammatory ↓ NF-κB activation pH-independent	[99]	2012
NF-κB	hypercapnia (live animal exposure)	mouse skin transplantation model	skin allografts	5% CO_2 in air for 1 h daily, up to 10 days	↓ NF-κB activity in draining lymph nodes ↓ spleen weight on day 3 ↓ inflammatory cell infiltration ↓ epidermal necrosis ↓ NF-κB p65 phosphorylation on days 1 and 3 ↓ CD8 T-cell infiltration at day 7 ↓ TNFα on days 1 and 3 ↓ CXCL2 on days 1 and 10	↑ skin allografts survival	[100]	2015
NF-κB	hypercapnia normocapnic acidosis	normal human bronchial epithelial cells (NHBE), bronchial epithelial cell line, BEAS-2B	Pam3CSK4 (TLR2 ligand), LPS (TLR4 ligand) and a complex stimulus (barn dust extract)	5–9% CO_2, for 24 h	TLR ligands ↓ IL-6 ↓ IL-8 ↑ MCP-1 barn dust ↑ IL-6 ↑ IL-8 ↑ MCP-1 ↑ several chemokines ↔ TLR receptor RNA ↓ NF-κB activation	↓ NF-κB activation ↑ cytokines and chemokines pH-independent	[101]	2015
NF-κB	hypercapnic acidosis metabolic acidosis buffered hypercapnia	human bronchial (HBE and BEAS-2B) and alveolar (A549) epithelial cells	high cyclic stretch (22% strain) for 24 h and 120 h	15% CO_2	↓ stretch-induced degradation of IκBα ↓ NF-kB activation	↓ IL-8 ↓ lactate dehydrogenase release ↑ cell survival pH-dependent	[102]	2016

Table 2. (*Continued.*)

transcription factor/pathway	hypercapnia conditions	cell/tissue (model)	co-stimulus	CO_2 dose	response to hypercapnia	physiological effect of hypercapnia	reference	year
NF-κB	hypercapnic acidosis buffered hypercapnia	HEK, MEF and A549 cells	basal	10% CO_2 for 75 min	↑ RelB cleavage ↑ nuclear RelB ↑ nuclear p100 altered RelB interactome	↓ NF-κB activation pH-independent	[103]	2017
NF-κB	hypercapnia (live animal exposure)	C57BL/6 mice	LPS-induced lung injury (2 mg kg^{-1} intratracheal LPS)	hypercapnia (5% CO_2 in air) for 10 min or 60 min before LPS	pre-treatment with CO_2 for 10 min, but not for 60 min: ↓ oxidative stress ↓ lactate dehydrogenase ↓ TNFα, CXCL2 TLR4 surface expression ↓ NF-κB signalling (↓ nuclear p65, ↑ cytosolic IκBα)	↓ LPS-induced pulmonary oedema ↓ inflammation (BALF protein) ↓ lung injury	[104]	2019
NF-κB	hypercapnia	mouse and human epithelial cell lines (A549, MLE12, primary alveolar epithelial cells, BEAS-2B), murine model and lung resection patients	wound healing on cell lines, murine orthotopic tracheal transplantation, lung resection	10% CO_2, 21% O_2 for up to 13 days for animal experiments. *In vitro* experiments hypercapnia up to 72 h	↓ NF-κB activity (reporter) ↓ CXCL12 ↓ GTP-Rac1 ↓ cell migration	↓ wound closure	[91]	2020

Table 3. Literature relating to CO_2-dependent regulation of TFs CREB, CEBP, PPAR, FoxO3, HIF and HSF1.

transcription factor	hypercapnia conditions	cell/tissue (model)	co-stimulus	CO_2 dose	response	physiological effect	reference	year
CREB	buffered media at 5% and 10% CO_2 (pH 7 HEPES-media)	HEK 293 T cells	G-protein-regulated adenylyl cyclase stimulation (with 50 nM isoproterenol)	5% and 10% CO_2 (versus air 0.03% CO_2)	↑ conversion of ATP to cAMP ↑ phospho-Ser^{133} CREB (only at 5% CO_2)	↑ CREB phosphorylation	[72]	2009
CREB C/EBPβ PPARγ	hypercapnic acidosis buffered hypercapnia	visceral pre-adipocytes	pre-adipocyte differentiation	10% CO_2 (versus 5% CO_2) buffered to pH 7.4 for 1 day, or for 5 min for cAMP levels, 4 days for adipogenesis	↑ PPARγ expression ↑ DNA-binding of CREB, of C/EBPβ and of PPARγ ↑ intracellular cAMP ↑ phosphorylated PKA substrate ↑ GTP-bound Rap1	↑ adipogenesis pH-independent	[105]	2017
FoxO3	buffered hypercapnia	adult maleC57Bl/6, matched *MuRF1*$^{-/-}$ and *MuRF1* $^{+/+}$ mice	none	10% CO_2 for up to 21 days	↑ MuRF1 (muscle ring finger-1) ↓ myotube diameter ↓ muscle weight ↓ muscle strength ↓ nuclear centralization	↑ muscle atrophy	[85]	2015
FoxO3	buffered hypercapnia	cultured myotubes from C2C12 mouse myoblasts	none	120 mmHg CO_2 for up to 24 h	(via AMPK α2) ↑ nuclear FoxO3 ↑ phospho-FoxO3a ↑ phospho-AMPK ↑ phospho-ACC ↑ MuRF1	↑ myotube atrophy ↓ myotube diameter	[85]	2015
HIF	buffered hypercapnia, with/out hypoxia	HEK293 and other cell lines (A549, HeLa, HCT116, RCC4, 786-0 cells)	basal and hypoxia (DMOG, chemical hypoxia or 1% O_2)	10%, 15%, 20% CO_2 for 4 h (versus 5% or 7.5% CO_2)	↓ HIF-1 α HIF-2 α ↓ HIF-dependent transcriptional activity	HIF suppression pH-dependent	[106]	2016
HIF	hypercapnia DMOG-hypoxia	live mice	hypoxia (DMOG, chemical hypoxia)	10% CO_2 for 6 h (versus room air)	↓ liver HIF-2α ↓ serum EPO		[106]	2016

(*Continued*.)

Table 3. (*Continued.*)

transcription factor	hypercapnia conditions	cell/tissue (model)	co-stimulus	CO_2 dose	response	physiological effect	reference	year
HIF	hypercapnic hypoxia (versus permissive hypercapnia, normobaric hypoxia and normoxia)	hippocampal extracts and derived astrocytes from Wistar male rats	hypoxia (*in vitro* chemical hypoxia using sodium iodoacetate) and basal	$PCO_2 \approx 50$ mmHg (with $PO_2 \approx 150$ or 35 mmHg), 7 or 15 days 'training' (30 min daily) before euthanasia	↑ astrocytes HIF-1α (*in vivo* and *in vitro* exposure, using sodium iodoacetate for 30 min) ↑ astrocytes HIF-1α (*in vivo* exposure, basal) ↓ astrocytes HIF-1α (*in vitro* exposure, basal)		[107]	2020
HIF	hypercapnic hypoxia (versus permissive hypercapnia, normobaric hypoxia and normoxia)	male Wistar rats	focal ischaemia-induced brain damage (by transcranial photochemical thrombosis)	$PCO_2 \approx 50$ mmHg (with $PO_2 \approx 150$ or 35 mmHg), 15 days 'training' (30 min daily) before ischaemia	↓ HIF-1α neurons (in hypercapnic hypoxia and permissive hypercapnia)		[107]	2020
HSF1	hypercapnia	primary mouse alveolar macrophages and MH-S cells	heat shock (42°C for 1 h) plus LPS stimulation (1 ng ml^{-1} for 6 h)	15% CO_2 for 16 h	↑ HSF1 ↑ HSP70 ↓ IL-6 ↓ TNFα ↔ IκBα, nuclear p65	inhibition of NF-κB-associated cytokine production	[108]	2018
HSF1	hypercapnia	C57BL/6 mice	none	10% CO_2 for 3 days, 7 days	↑ HSF1 in lung, in bronchoalveolar lavage ↑ HSP70 in alveolar macrophages ↑ proteomic stress	inhibition of NF-κB-associated cytokine production	[108]	2018
HSF1	hypercapnia	male *Hsf1*$^{+/-}$ and control wild-type mice	*Pseudomonas* pneumonia (7 h)	10% CO_2 for 3 days before infection	(only in wild-type mice) ↓ IL-6 ↓ TNFα ↓ lung IL-1β	inhibition of NF-κB-associated cytokine production	[108]	2018

response to hypercapnia. Looking at pulmonary endothelial cells, a study by Takeshita *et al*. [94] was one of the first to observe altered IκBα expression in hypercapnia that was linked to decreased inflammatory gene expression (ICAM1 and IL-8). In models of mechanical stress-induced injury, relevant to ventilator-induced lung injury, hypercapnic acidosis reduced 'classical' NF-κB activation and cytokine production [98,102]. In this pulmonary epithelial cell culture model, reduced IL-8 secretion and (luciferase-reporter) NF-κB activity responses were attributed to acidosis only, via prevention of IκBα breakdown. Hypercapnic acidosis-dependent NF-κB modulation might also be beneficial in transplantation. In mice skin allografts, hypercapnia suppressed RelA/p65 phosphorylation and thus reduced serum TNFα and CXCL2 levels, resulting in improved transplant survival [100]. Other studies have demonstrated suppression of nuclear RelA [96], impaired degradation of IκBα [90], reduced NF-κB reporter luciferase activity [90,91,96,101] and reduced expression of factors known to be regulated by NF-κB under conditions of elevated CO_2 [91,95–97,99,101]. Interestingly, the impact of CO_2 on cytokine expression appears to be affected by the nature of the stimulus [101], with barn dust and the toll-like receptor 2 (TLR2) agonist Pam3CSK4 having differential effects on IL-6 secretion at elevated CO_2, for example.

Thus, there is substantial evidence to implicate changes in 'canonical' NF-κB signalling under conditions of hypercapnia. It appears that hypercapnic acidosis is not required for the canonical NF-κB response to CO_2; however, this does not preclude the possibility of CO_2-dependent and pH-dependent signalling pathways acting synergistically.

6.2.3. Hypercapnia and the non-canonical NF-kB pathway

In addition to the effects of hypercapnia on canonical NF-κB-dependent signalling, we have also reported several important changes in proteins associated with the non-canonical pathway to be affected by CO_2 [114].

Processing of several proteins involved in the non-canonical NF-κB pathway is altered under elevated CO_2 levels (RelB, IKKα, p100), pointing to the involvement of the non-canonical pathway in hypercapnia-induced immunosuppression [96,103,115]. Notably, these CO_2-dependent changes on non-canonical family members are clearly evident in the basal state, independent of an exogenous inflammatory stimulus. This was not the case for the canonical NF-κB protein RelA when the cellular localization of RelA and RelB was compared using immunofluorescent staining and western blot [99]. RelB is cleaved and translocates to the nucleus in response to hypercapnia in mouse fibroblasts and in human pulmonary epithelial cells, in a process requiring p100 and proteasome activity [103,115]. Increased nuclear levels of the non-canonical pathway proteins RelB, p100 and IKKα in response to hypercapnia have also been reported in multiple cell lines [96,103,115]. Thus, there is evidence linking cellular levels of CO_2 affecting the expression, localization and function of non-canonical NF-κB family members over a range of CO_2 concentrations. This effect of CO_2 modulates signalling/signal transduction via these 'non-canonical' NF-κB proteins and has the potential to cross-talk with other related signalling networks, e.g. 'canonical' NF-κB signalling and beyond. It remains unclear how CO_2 is sensed by the NF-κB pathway, and the precise mechanism through which NF-κB target genes are modulated by CO_2. A conserved Rel family member, *Relish*, which is expressed in flies, downregulates antimicrobial peptide expression following exposure of *Drosophila melanogaster* to elevated CO_2 [116]. These data point to a potential evolutionarily conserved mechanism of CO_2-dependent immune regulation through the NF-κB pathway.

6.2.4. Hypercapnia and toll-like receptor 4 expression

An intriguing recent study has added new insights into our understanding of the immune response to CO_2 and signalling through the TLR4 pathway (which can act upstream of NF-κB activation). Mice exposed to hypercapnia for only 10 min before lipopolysaccharide (LPS) stimulus were protected from lung oedema and NF-κB-induced inflammation, while 60 min of exposure had no effect [104]. Ten minutes of pretreatment with inhaled CO_2 prior to LPS reduced IκBα degradation and RelA nuclear translocation. The authors observed a transient reduction in TLR4 protein expression following a 10 min exposure to CO_2 that had resolved by 60 min of exposure. Thus, this study adds additional insight into the effect of CO_2 on immune signalling, particularly in the context of LPS-dependent signalling. Reduced expression of this key TLR4 receptor by CO_2 could potentially explain several of the downstream signalling consequences on the NF-κB pathway. Furthermore, the rapid and transient kinetics of the response may help to explain apparently conflicting results in the literature. While some studies focus on short (acute, minutes) exposures others use weeks or months (chronic, including intermittent repeated short exposures) in a diverse set of circumstances, e.g. with or without injury, stretch, cytokine stimulation and TLR stimulation. Additional research is required to extend our knowledge of the dose dependence of many hypercapnic responses. Controlling the intensity and duration of exposure to elevated CO_2 levels might be the key to achieving clinical benefit.

In conclusion, the pathways linking elevated CO_2 levels to a downregulation of the immune response are not yet fully elucidated but elements of both the 'canonical' and 'non-canonical' NF-κB pathway appear to play roles (table 2). The acidosis that accompanies elevated CO_2 levels *in vivo* also has the potential to modulate NF-κB-dependent signalling. However, this does not mean that pH is the main route for CO_2-dependent signal transduction to the NF-κB pathway, with several studies observing pH-independent components of the NF-κB response to altered CO_2. Further research is required to reveal the global transcriptional changes induced by hypercapnia and to clarify the roles played by different components of the NF-κB pathway in this response. A better understanding of the contribution of pH changes, the extent to which some responses may be cell type and stimulus specific as well as the temporal control of the immune response in hypercapnia will help to advance the field.

6.3. Heat-shock factor 1 and non-heat-shock responses

Heat-shock factor 1 (HSF1) is a well-characterized and conserved TF that is considered to be a main regulator of the heat-shock protein response, binding to conserved heat-shock elements and inducing the expression of heat-shock proteins (HSPs). In addition to its roles in the regulation of stress response and cellular proteostasis, multiple additional roles of HSF1 are being revealed in cell growth, development, immunity, longevity and other stresses [117,118].

HSF1 can regulate transcription of genes that are not heat-shock proteins, independently of heat-shock elements. One example is the suppression by heat-shock of LPS-induced IL-6 expression. HSF1 induced activating TF (ATF) 3, a negative regulator of IL-6, to inhibit IL-6 gene expression, in mouse embryonic fibroblasts and macrophages [119].

In alveolar macrophages, HSF1 and HSP70 increased in response to 16 h of hypercapnia at 15% CO_2 [108]. LPS-stimulated secretion of IL-6 and TNFα was reduced in hypercapnia, but no changes in IκBα or nuclear p65 were detected. HSF1 knockdown abolished the hypercapnia-induced decrease of TNFα and IL-6 after LPS stimulation, implicating the TF HSF1 in this process. Hypercapnia also induced inhibition of IL-6, TNFα and IL-1β in a mouse model of *Pseudomonas* pneumonia [108].

6.4. Hypoxia-inducible factor and hypoxia

HIF is a family of TFs (HIF-1α, -2α, -3α and HIF-1β) with a basic helix–loop–helix–per/ARNT/Sim (bHLH-PAS) domain, a transactivation domain and (in HIF-α) an O_2-dependent degradation domain (ODDD). O_2-dependent hydroxylation of the ODDD by prolyl hydroxylases in normoxia promotes HIF-α degradation, while low O_2 stabilizes HIF-α and promotes nuclear accumulation of the HIF dimer (α + β subunits), leading to transcription of a large number of hypoxia-responsive genes. HIF is considered the master regulator of cellular responses to hypoxia and has been widely reviewed elsewhere [120–122]. Hypercapnia often co-occurs with hypoxia *in vivo* and, given the central roles of O_2 and CO_2 in metabolism [15,114], some cross-talk between hypercapnia and hypoxia responses is expected and is under investigation. Hypercapnia suppresses serum levels of the prototypical HIF-target gene erythropoietin (EPO) in mice exposed to hypercapnia for 6 h [106]. Hypercapnia was also reported to reduce HIF protein stabilization (HIF-1α, HIF-2α, but not -1β) after exposure to the hypoxia-mimetic dimethyloxallyl glycine (DMOG) in multiple cell lines and (for HIF-2α) in mouse liver. After 4 h of hypercapnia (10% CO_2), basal and DMOG-induced levels of HIF-2α decreased in HEK293 cells. DMOG-induced HIF-1α levels were also reduced after 4 h at 10%, 15% and 20% CO_2, but not 7.5%, indicating a CO_2 dose-dependent response. Hypercapnic suppression of HIF-α was proposed not to be due to changes in HIF hydroxylation but rather via modulation of lysosomal degradation of HIF-α, possibly involving CO_2-dependent changes in pH [106].

Tregub *et al*. [107] have recently provided further evidence for hypercapnia affecting HIF-1α and the hypoxia response. In a rat model of focal ischaemia-induced brain damage, hypercapnic hypoxia demonstrated reduced HIF-1α expression in the peri-infarct area compared with normocapnic hypoxia. This study also studied HIF-1α expression in astrocytes *in vitro*, again providing evidence of cross-talk between CO_2 and HIF-1α expression, but, in this case, hypercapnic hypoxia was associated with increased HIF-1α expression compared with normocapnic hypoxia in an unusual model of chemical hypoxia (using sodium iodoacetate) [107]. Taken together there is evidence for CO_2 levels modulating the degree of HIF-α activation/stabilization depending on the context.

6.5. FoxO3a

FoxO3a, a forkhead box O family TF, regulates a variety of cellular processes, including apoptosis, proliferation, cell cycle progression, DNA damage and oxidative stress. FOXO TFs respond to growth factors, oxidative stress, inflammation and nutritional abundance [123]. Hypercapnia promotes muscle atrophy in live mice and reduced fibre diameter in cultured myotubes. FoxO3a has been implicated in this process, along with muscle-specific ring finger protein 1 (MuRF1), acting downstream of AMPK. Elevated CO_2 increases AMPK-dependent phosphorylation of FoxO3a. FoxO3 then translocates to the nucleus, leading to MuRF1 increase and associated muscle atrophy [85]. The role of AMPK in CO_2-dependent signal transduction is discussed in more detail in §5.

6.6. cAMP response binding element

CREB is a ubiquitous basic leucine zipper TF, which binds the cAMP response element to induce expression of numerous target genes. CREB can respond to multiple factors (like cAMP, Ca^{2+}, growth factors and cytokines), regulating a wide range of biological processes related to cell differentiation and growth. CREB is activated by phosphorylation (primarily at Ser133) by multiple protein kinases (like PKA, calmodulin-dependent protein kinase (CaMK) or mitogen-activated protein kinases (MAPK)) [124]. Thus, CREB acts downstream of multiple important signalling pathways, not just AC–cAMP–PKA (§4).

Hypercapnia is currently an underappreciated component of sleep apnoea [125], which is strongly correlated with obesity. Furthermore, hypercapnia may promote adipogenesis. In cultured human visceral pre-adipocytes, hypercapnia (10% CO_2, pH-buffered) increased the DNA-binding activity of CREB and of its downstream proadipogenic TFs, CCAAT/enhancer-binding protein (C/EBP) β, and peroxisome proliferator-activated receptor (PPAR) γ, leading to increased adipogenesis. In addition, sustained or intermittent hypercapnia could increase adipogenesis independently of hypoxia or acidosis, while hypocapnia had the opposite effect [105]. This effect of CO_2 on adipogenesis was mediated by HCO_3^--regulated sAC (§4), via elevated cAMP, leading to the activation of PKA and EPAC (exchange proteins directly activated by cAMP, also known as the cAMP-regulated guanine nucleotide exchange factors) [105].

In HEK293T cells, exposure to 5% or 10% CO_2 increased cAMP production and CREB ser133 phosphorylation after G-protein-regulated AC stimulation compared with exposure to room air [72]. This could indicate a role in CO_2 sensing for a G-protein-regulated AC that binds CO_2 directly. As detailed in §4, sAC is responsive to both CO_2 and HCO_3^- and is a good candidate for CO_2 sensing. Hypercapnia could then stimulate cAMP production via both sAC and tmACs, leading to CREB activation.

6.7. Other regulators of protein expression: micro-RNAs

In addition to TFs, micro-RNAs can also mediate changes in protein expression in response to CO_2. Buffered normoxic hypercapnia impaired cell proliferation and decreased O_2 consumption and ATP production in fibroblasts (N12) and alveolar epithelial cells (A549) by mitochondrial dysfunction [38] (§2). High CO_2 levels were found to reduce expression of micro-RNA-183 (miR-183), leading to downregulation of the TCA enzyme IDH2. This micro-RNA is part of a highly conserved cluster, comprising miRs-183, -96 and -182, important in normal development but also associated with cancer and neurological and auto-immune disorders [126].

In mouse airway smooth muscle cells, another micro-RNA, miR-133a, is downregulated in hypercapnia. Increases in CO_2 levels activate Ca^{2+}-calpain signalling and muscle cell contraction, via non-apoptotic activation of Caspase-7, Mef2D cleavage, miR-133a inhibition and RhoA phosphorylation of myosin light chain [61]. This micro-RNA, expressed as a bicistronic miR-1/miR-133a cluster in skeletal and cardiac muscle, is regulated by several myocyte differentiation factors [127]. How CO_2 regulates these micro-RNAs is still unclear, but these examples reinforce the notion that CO_2 levels can simultaneously impact a large variety of regulatory mechanisms (via micro-RNAs, TFs, RNA and protein stability, protein activation, trafficking, etc.) to exert cell-specific, well-defined and context-dependent physiological effects on cells.

6.8. Transcriptional responses to carbon dioxide: summary

In summary, several TFs have been implicated in responses to hypercapnia, and hypercapnia is known to induce transcriptional responses [128,129] in a variety of organisms including mammals. In most cases, we have no direct knowledge of the specific TF pathways involved in these hypercapnia responses (e.g. through the use of TF-deficient cells/animals and/or pharmacological inhibitors). However, extensive research on the immuno-modulatory roles of hypercapnia has highlighted the role of the NF-κB pathway in particular. The contribution of additional TFs and transcriptional regulators that are already known but not yet fully understood and/or TFs and transcriptional regulators that are yet to be discovered require further investigation. Notably, the transcriptional regulators discussed in this section are central regulators of critical cellular functions under conditions of stress and in normal metabolism. This points to a major effect of hypercapnia in regulating growth and development in general, as well as impacting key nodes of normal cellular activity. Furthermore, the observed physiological effects of hypercapnia in immunosuppression, wound healing, hypoxia sensitivity, muscle wasting and adipogenesis support the argument for CO_2 levels exerting a global impact on metabolism. The coming years will doubtless expand our understanding of the global relevance of hypercapnia on modulating cell responses to other stimuli.

7. CO_2-dependent post-translational modifications

In addition to the indirect effects of CO_2 on cellular signal transduction mechanisms, research has outlined a direct influence of CO_2 on protein biochemistry via the CO_2-mediated post-translational modification of target proteins. It has been shown that CO_2 can reversibly bind proteins via the nucleophilic attack of a protein's primary amine group upon CO_2 to result in the formation of a covalent bond between the neutral amine and the carbon of CO_2. This covalent bond causes a + 44 Da mass shift and can take place on lysine side chain amino groups [130] or at the protein's N-terminus [131] to result in the formation of a labile carbamate compound. Carbamate formation, or carbamylation, is an intrinsically rapid chemical reaction that has been shown to take place on proteins such as haemoglobin [132,133], ribulose bisphosphate carboxylase-oxygenase (RuBisCO) [134], peroxisome proteins [135] and select connexin hemichannels [136,137]. Carbamylation of these proteins has been shown to play important functional roles in regulating their activity. In vertebrates, carbamate formation has been described to be necessary for mediating the effects of CO_2 on haemoglobin O_2 affinity (Bohr effect) [131], in regulating the activation of class II peroxidase PRX34 [135] and in promoting an open conformation in the CO_2-sensitive hemichannel connexin 26 (Cx26) [136]. Protein carbamylation has also been implicated in regulating the activity of β-lactamase microbial enzymes [138] and in regulating RuBisCO activation in plants; an enzyme important for catalysing photosynthesis and photorespiration [134,139]. These findings together indicate that protein activity can be directly influenced by CO_2 via the formation of carbamates in the regulation of an array of biological processes.

While the readily reversible, labile nature of carbamate formation may be beneficial from a biological regulation standpoint, the lability of this reaction has posed particular difficulties in accurately detecting the extent of carbamate formation in mammalian cells under physiological and non-physiological conditions. It has therefore been postulated, given the ubiquity of CO_2, that carbamate formation in biological systems has been greatly underestimated [139], and there exists a large cohort of proteins whose carbamate formation capacity remains to be uncovered by more elegant detection methods, such as those established in recent years by Linthwaite *et al*. [135]. This *de novo* mechanism described by Linthwaite *et al*. involves the covalent trapping of CO_2 bound to proteins under physiological conditions of pH and pCO_2 [135]. This approach is discussed in more detail elsewhere in this theme issue. Briefly, CO_2 trapping, carried out by *O*-ethylation with triethyloxonium tetrafluoroborate (TEO), allows for the stabilization of the labile carbamate in order to allow for downstream proteomic analysis [135]. This method of CO_2 trapping has thus far provided confirmation that it is possible to form carbamates on the α- and ε-amino groups of peptides under physiologically relevant conditions of temperature and pH *in vitro* and has provided a mechanism by which we may identify further putative targets of protein carbamylation in future proteomic analyses to help better elucidate the mechanisms by which a cell detects and responds to environmental CO_2.

Interestingly, in addition to binding to proteins, CO_2 also has the capacity to react with oxidant species. For example, CO_2 has the capacity to react with peroxynitrite to form another RNS, nitrosoperoxocarboxylate ($ONO_2CO_2^-$). The potential role for this reactive species in neuronal modulation and chemosensing was previously reviewed by Dean [140].

8. Summary and perspectives

In this article, we have sought to review some of the central signalling hubs involved in sensing and transducing the cellular response to hypercapnia (figure 4). There is significant evidence detailed in this article linking increased levels of CO_2 to decreased intracellular pH, altered mitochondrial metabolism and increases in $[Ca^{2+}]_i$. These key nodes of cellular CO_2 sensitivity can act upstream of and parallel to other prominent signalling pathways, e.g. AMPK and AC signalling, contributing to and modulating the cellular response to hypercapnia. Together these signalling events can contribute to a range of physiological responses, some of which are

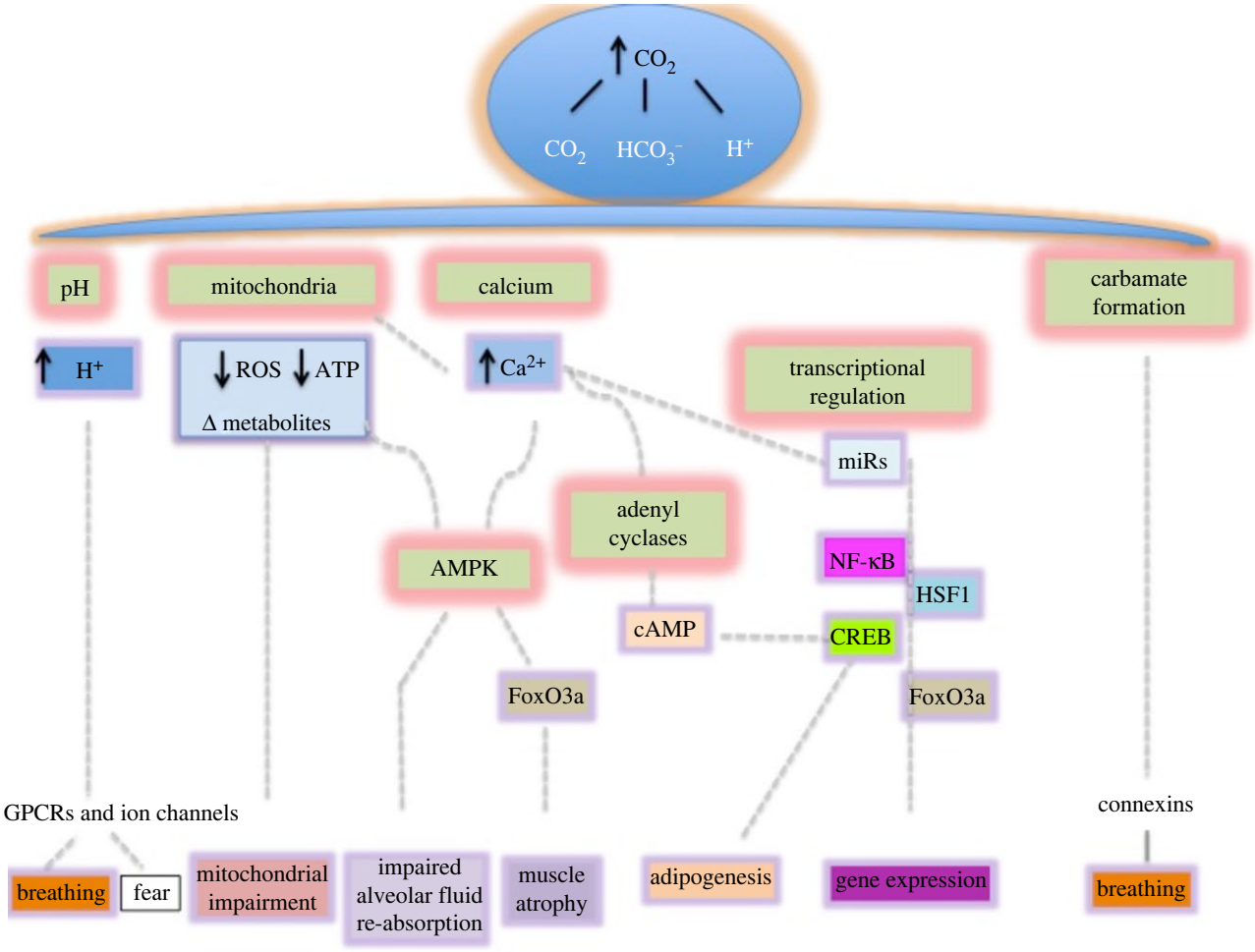

Figure 4. Summary of CO_2-dependent regulation of signal transduction in mammalian cells. Schematic outlining some of the main CO_2-sensitive signalling hubs and consequences of hypercapnic exposure. Dotted lines highlight some of the interconnectivity between the different signalling hubs.

rapid and short-lived, e.g. CO_2-dependent modulation of breathing, and some of which are sustained and involve CO_2-dependent transcriptional regulation, e.g. suppression of pro-inflammatory NF-κB-dependent cytokines. The outcome of hypercapnic exposure for an organism is thus highly context dependent with the overall phenotypic response influenced by a plethora of factors including cell type, duration of exposure, degree of hypercapnia, local pH changes, energy status of the cell, O_2 levels and co-stimuli. The recent Royal Society meeting on the topic of 'Carbon dioxide detection in biological systems' showcased the state of the art with respect to CO_2 signalling and sensing in both the plant and animal kingdoms. Strikingly, while both plant and animal research communities have made steady progress in understanding CO_2-dependent signal transduction, much work remains for both research groups, particularly with respect to understanding the most proximal signalling events in response to an elevation in CO_2 and the precise mechanisms that follow this event. With new technologies and techniques for the measurement and detection of CO_2 and CO_2-dependent signalling, e.g. using mass spectrometry, it will be very exciting to see whether novel, direct CO_2 protein targets are uncovered within the key signal transduction hubs discussed in this review.

Data accessibility. This article has no additional data.

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