



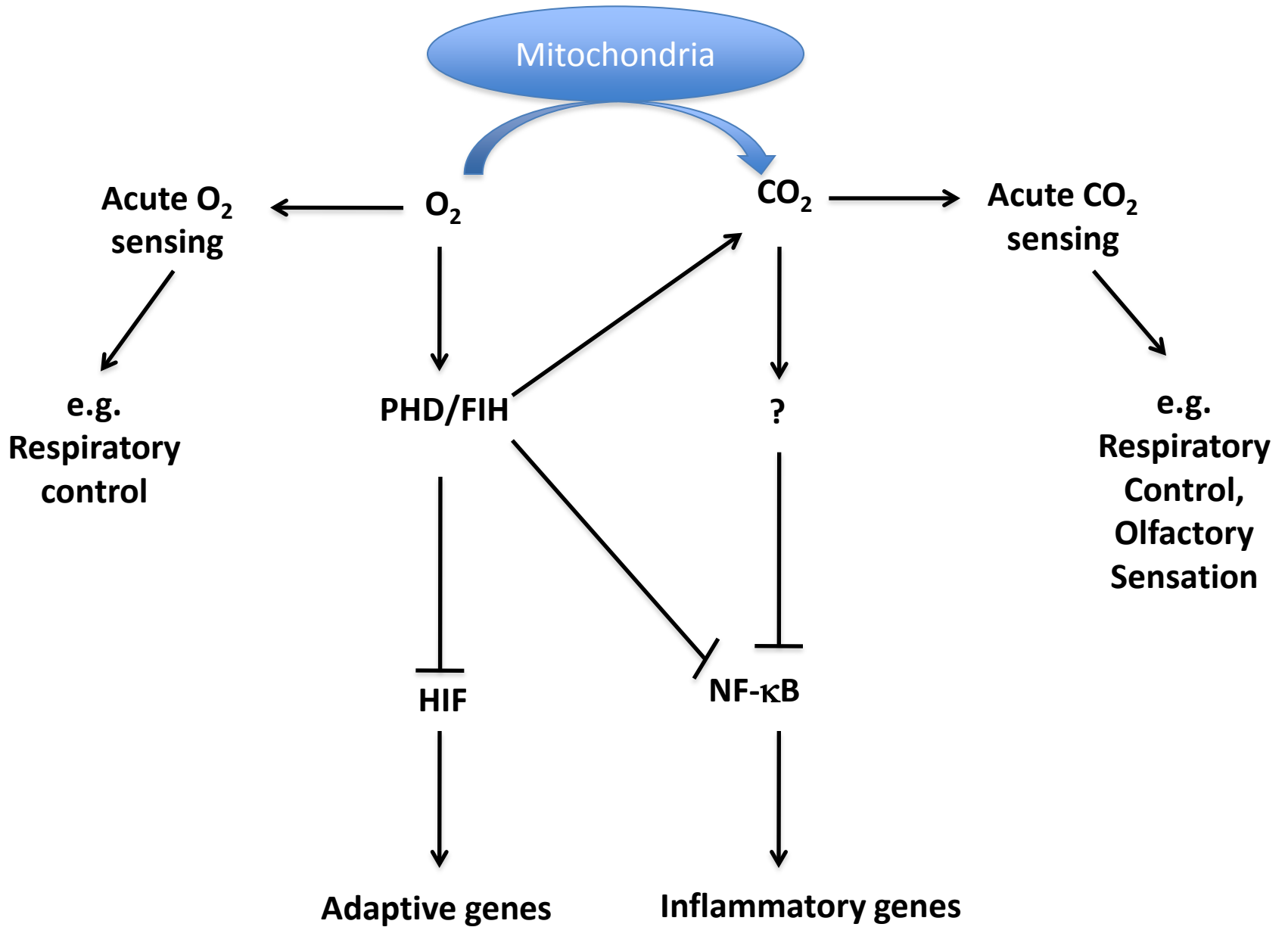
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Regulation of gene expression by carbon dioxide

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ABSTRACT

Carbon dioxide (CO₂) is a physiologic gas found at low levels in the atmosphere and produced in cells during the process of aerobic respiration. Consequently, the levels of CO₂ within tissues are usually significantly higher than those found externally. Shifts in tissue levels of CO₂ (leading to either hypercapnia or hypocapnia) are associated with a number of pathophysiologic conditions in humans and can occur naturally in niche habitats such as those of burrowing animals. Clinical studies have indicated that such altered CO₂ levels can impact upon disease progression. Recent advances in our understanding of the biology of CO₂ has shown that like other physiologic gasses such as molecular oxygen (O₂) and nitric oxide (NO), CO₂ levels can be sensed by cells resulting in the initiation of physiologic and pathophysiologic responses. Acute CO₂ sensing in neurons and peripheral and central chemoreceptors is important in rapidly activated responses including olfactory signaling, taste sensation and cardiorespiratory control. Furthermore, a role for CO₂ in the regulation of gene transcription has recently been identified with exposure of cells and model organisms to high CO₂ leading to suppression of genes involved in the regulation of innate immunity and inflammation. This latter, transcriptional regulatory role for CO₂ has been largely attributed to altered activity of the NF-κB family of transcription factors. Here, we review our evolving understanding of how CO₂ impacts upon gene transcription.

The natural history of CO₂

During the history of metazoan evolution in the Phanerozoic eon, atmospheric levels of CO₂ in dry air ranged from over 6000 ppmv (0.6%) around 600-400 million years ago to 284 ppmv (0.0284%) in the mid 1800s (Berner, 2003; Berner and Kothavala, 2001; Beerling & Berner, 2005; Royer *et al.*, 2007; Vandenbroucke *et al.*, 2010). Current atmospheric pCO₂ levels are approximately 387 ppmv (0.0387%), representing an increase of approximately 36% since the advent of human industrial activity. While relatively low, this level of CO₂ is key in regulation of the Earth's temperature and climate (Lacis *et al.*, 2010). The low background level of atmospheric CO₂ can increase dramatically in the presence of respiring organisms or decomposing organic matter (Luo *et al.*, 2009). In fact, a number of species use the presence of environmental CO₂ gradients as a behavioral cue including prey-seeking behavior in mosquitoes and avoidance behavior in fruitflies and nematodes (Bretscher *et al.*, 2008; Turner & Ray, 2009).

In respiring metazoans, the main source of CO₂ is the electron transport chain of mitochondria where the chemical reduction of molecular oxygen is responsible for the generation of CO₂ as a by-product. Thus, in contrast to molecular oxygen, the levels of CO₂ found in tissues of the body are significantly higher than those found in the external atmosphere. A number of enzymes utilise CO₂ during their activity including Carbonic Anhydrases, a family of ubiquitously expressed metallo-enzymes which are responsible for catalyzing the reversible hydration of CO₂ and H₂O to HCO₃⁻ and H⁺ (De Simone & Supuran, 2010). Remaining CO₂ is primarily removed by the blood and is exhaled or diffuses through the skin. Recent advances

have demonstrated that organisms contain distinct mechanisms capable of sensing changes in CO₂ and eliciting distinct acute responses or changes in gene expression through transcriptional regulation.

Acute cellular sensing of CO₂

Many species of animal including nematodes, fruit-flies, rodents and humans possess the ability to sense CO₂ in an acute manner leading to a rapid, neuronally-mediated response (Chandrashekar *et al.*, 2009; Frommer, 2010). For example CO₂ levels can be sensed in *D. melanogaster* by neurons equipped with Gr63a and Gr21a olfactory receptors leading to avoidance behavior (Jones *et al.*, 2007). In *C. elegans*, CO₂ avoidance is also mediated by specialized neurons (Bretscher *et al.*, 2008). Furthermore, in humans, acute carbon dioxide sensing has been identified in the synapses of taste receptor cells in a manner dependent upon carbonic anhydrase IV (Chandrashekar *et al.*, 2009). Furthermore, a diverse range of CO₂ sensitive potassium channels have been shown to be important in CO₂ sensing in maintaining homeostasis in higher species (Tang *et al.*, 2004). The ability of metazoan cells to sense CO₂ acutely and initiate rapid neuronal responses is analogous in nature to the acute oxygen-sensing pathways which exist in specialized tissues such as the carotid body (Weir *et al.*, 2005; Lopez-Barneo *et al.*, 2009) leading to neuronal signaling to control rate and depth of breathing. It is likely that in vivo, such changes in neuronal activity will lead indirectly to CO₂-induced changes in gene transcription as a consequence of altered neuronal activity. Associations between altered neuronal activity and gene expression have been expertly described previously (Kandel, 2001). Furthermore, previous studies have reported that stress responses to hypercapnia in animals results in increased expression of stress hormones (Schaefer *et al.*, 1968) which can also regulate gene

expression (De Kloet, 2004). Thus altered neuronal activity and/or increased stress hormone production in vivo in response to high CO₂ may be an indirect link between CO₂-sensing and gene expression. In this review however, we will focus on direct mechanisms by which CO₂ can regulate gene expression.

Metazoan cells have evolved the capacity to respond to changes in microenvironmental oxygen through the induction of specific adaptive gene cohorts in a manner governed by the hypoxia-inducible factor (Semenza, 2007; Kaelin & Ratcliffe, 2008). Recent advances have indicated that an analogous transcriptional response to CO₂ may also exist in metazoan cells. The evidence supporting this will be discussed below.

CO₂ and gene expression

In studies investigating the mechanisms underpinning the protective effects of “permissive hypercapnia” in pulmonary disease, gene array analysis experiments were carried out on neonatal mice exposed to atmospheric hypercapnia (Li *et al.*, 2006). This study identified altered levels of **pulmonary** genes related to cell adhesion, growth, signal transduction and innate immunity (Li *et al.*, 2006). Of note in these studies, genes related to innate immunity were broadly suppressed in response to elevated CO₂. In separate studies, exposure of the model organisms *C. elegans* and *D. melanogaster* to high CO₂ also resulted in altered gene expression (Helenius *et al.*, 2009; Sharabi *et al.*, 2009). *C. elegans* exposure to hypercapnia impacted upon motility, fertility and lifespan which was associated with altered expression of over 6% of the transcriptome **in whole animals** including genes encoding proteins involved in the regulation of innate immunity (Sharabi *et al.*, 2009). In *Drosophila*, exposure to hypercapnia impaired embryonic

morphogenesis, egg laying, egg hatching and innate immune responses resulting in increased susceptibility to bacterial infection which was associated with suppression of the NF- κ B homolog Relish in **adult flies** (Helenius *et al.*, 2009). This response was independent of the known neuronal Gr63a CO₂ sensor, changes in extracellular pH or nitric oxide. Interestingly, in both *D. melanogaster* and *C. elegans*, the CO₂ sensing pathway was not directly dependent upon the hydroxylase enzymes known to be important in oxygen sensing mechanisms in the hypoxia-inducible factor (HIF) pathway (reviewed by Kaelin & Ratcliffe, 2008). A further study in mouse macrophages exposed to hypercapnia also demonstrated an immunosuppressive effect of hypercapnia as determined by decreased levels of cytokine release from cells exposed to lipopolysaccharide (Wang *et al.*, 2010). In this study, the authors also demonstrated a suppression of NF- κ B signaling which was independent of acidosis and resulted in decreased phagocytic activity of the cells (Wang *et al.*, 2010). A separate study also demonstrated that hypercapnia results in the suppression of NF- κ B signaling in mouse embryonic fibroblasts and a number of other mammalian cell types (Cummins *et al.*, 2010).

Thus, a common feature of the transcriptional response to elevated CO₂ in mice, fruitflies and nematodes is the suppression of the genes associated with innate immunity. This suppression of immunity has been linked to decreased activity of the NF- κ B signaling pathway. A summary of the studies linking hypercapnia to altered NF- κ B signaling is provided in Table 1. NF- κ B is a master regulator of the genes involved in innate immunity and inflammation. The NF- κ B pathway is complex and has been expertly reviewed recently (Gilmore 2006). Briefly, NF- κ B is sequestered in the cytosol of a cell by inhibitory molecules known as I κ Bs. In

response to a range of inflammatory stimuli, activation of receptor-specific upstream signaling cascades leads to the phosphorylation of I κ B by the IKK complex (composed of three subunits IKK α /IKK β /NEMO). This leads to the degradation of I κ B thus liberating NF- κ B and allowing its translocation to the nucleus where it drives the expression of genes involved in driving innate immune and inflammatory responses.

While the effects of *in vivo* hypercapnia on gene expression likely occurs in part through indirect mechanisms such as altered neuronal activity or the release of stress hormones, recent evidence suggests that CO₂ may also directly regulate gene expression through the NF- κ B pathway (Cummins *et al.*, 2010). Some insight into a possible mechanism underpinning the suppression of NF- κ B activity by hypercapnia was recently provided by the demonstration of CO₂-induced nuclear localization of the IKK α subunit (Cummins *et al.*, 2010). In a non-canonical manner, nuclear IKK α has previously been shown to repress NF- κ B activity (Lawrence *et al.*, 2005) and have a nucleosomal role in the expression of NF- κ B target genes including the repressor protein I κ B α (Yamamoto *et al.*, 2003). Consistent with the studies outlined above, Cummins and colleagues also demonstrated the immunosuppressive effects of high CO₂ were not directly dependent upon the oxygen sensing hydroxylases which regulate HIF, extracellular pH or pathways which mediate acute CO₂-sensing in nematodes and flies (Cummins *et al.*, 2010).

In summary, the studies outlined above provide evidence that metazoan cells possess the capability to sense changes in microenvironmental CO₂ levels and activate a transcriptional response which results in the suppression of innate immunity and inflammatory signalling. While the specific CO₂ sensor mediating this

transcriptional response has yet to be identified, the sensing mechanism appears to be independent of extracellular pH, oxygen sensing enzymes associated with the HIF pathway and mechanisms that mediate acute neuronal CO₂ sensing (Figure 1). The experiments excluding a role for altered pH have relied mainly on the buffering of extracellular pH or the manipulation of pH in the absence of changing CO₂, neither of which elicit a transcriptional response comparable to hypercapnia (Cummins et al., 2010). However, this does not exclude possible roles for changes in intracellular pH in mediating the response to hypercapnia. Furthermore, alternative oxygen sensing mechanisms such as alterations in intracellular reactive oxygen species (ROS) production could also be implicated. Additionally, altered CO₂ levels likely impact upon metabolic processes such as glycolysis. Whether such changes in metabolism mediate transcriptional changes also requires experimental investigation. Finally, whether a role for enzymatically-mediated post-translational modifications such as carboxylation of target proteins (Bandyopadhyay et al., 2002) plays a role in CO₂ sensing remains to be determined. Future studies will be aimed at answering these important questions and identifying the CO₂ sensor which regulates the cellular response to hypercapnia.

Crosstalk between oxygen and carbon dioxide sensing

Because of the close association between oxygen consumption and CO₂ production, the capacity of a cell to sense changes in CO₂ is likely closely linked to its ability to sense changes in oxygen. However, the nature of this crosstalk is poorly understood and is an area in need of further investigation. As outlined

above, the oxygen sensing hydroxylases responsible for conferring oxygen/hypoxia sensitivity to the HIF pathway do not appear to play a direct role on conferring CO₂ sensitivity to the NF-κB pathway. However, it should be noted that these enzymes produce CO₂ during their oxygen sensing enzymatic activity in the regulation of HIF and therefore may contribute to the intracellular levels of CO₂ (Figure 1). Furthermore, studies in hyperoxia have demonstrated a possible role for reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the regulation of NF-κB (reviewed by D'Angio et al. 2000). Because both O₂ and CO₂ play an important role in determining ROS/RNS levels in cells (Dean, 2010), altered intracellular redox potential may be a point of convergence between O₂ and CO₂ in the regulation of gene expression through NF-κB.

CO₂ in health and disease

In normal conditions, pCO₂ levels in the body likely vary between tissues and individual cells. Typical arterial blood pCO₂ values are in the range of 35-45 mmHg. A thorough review of the contribution of CO₂ to physiologic and pathophysiologic processes has recently been published elsewhere (Curley *et al.*, 2010).

Hypocapnia is the condition which arises when the mean arterial pCO₂ is lower than normal. It usually occurs as a consequence of hyperventilation in conditions such as asthma and acute lung injury. While hypocapnia is associated with adverse outcomes in diabetic ketoacidosis (Glaser *et al.*, 2001), therapeutic hypocapnia is used in the management strategy for brain injury associated with increased intracranial pressure (Neumann *et al.*, 2008).

Hypercapnia arises when the mean arterial pCO₂ is elevated above normal levels and can occur as a consequence of respiratory failure (e.g. in chronic obstructive

pulmonary disease) but clinically it is commonly seen as a consequence of a low tidal volume ventilation strategy for acute respiratory distress syndrome (ARDS). Environmental hypercapnia may also occur in the natural habitats of burrowing animals (Lechner, 1976). Mechanical ventilation strategies to elicit normal physiological blood gas levels have been associated with stretch-induced mechanical lung damage. In the Acute Respiratory Distress Syndrome Network (ARDSnet) multi-centre trial examining lower tidal volumes as compared to traditional tidal volumes there was a significant decrease in patient mortality in the lower tidal volume group (ARDSnet, 2000). Hypercapnic acidosis (HCA), which can be a consequence of patient hypoventilation, was also identified as being associated with decreased mortality in a subset of the ARDSnet patient cohort (patients receiving 12ml/kg tidal volumes who were defined as having hypercapnic acidosis on day 1 of the study) independent of changes in mechanical ventilation (Kregenow *et al.*, 2006). Taken together these data are suggestive of elevated CO₂ levels being protective in the critically ill patient.

Therapeutic hypercapnia has been reported to be of benefit in ischemia/reperfusion injury in the mesentery (Laffey *et al.*, 2003) and recently in the liver (Li *et al.*, 2010). The mechanisms for this protection are not yet fully elucidated *in vivo* however the latter study reports attenuated IRI-mediated pro-inflammatory gene expression (TNF α), enhanced anti-inflammatory cytokine production (IL-10), decreased apoptosis and decreased immunohistochemical staining for NF- κ B in the hypercapnia treated groups. These studies are consistent with the observations described above for CO₂ (independent of extracellular pH) having a suppressive effect on NF- κ B signaling (Cummins *et al.*, 2010; Wang *et al.*, 2010) and of hypercapnic acidosis blunting endotoxin-stimulated NF- κ B signaling, resulting in

decreased ICAM-1 and IL-8 expression in pulmonary endothelial cells (Takeshita *et al.*, 2003).

The role of intracellular acidosis in the protective effects of hypercapnia remains to be fully elucidated. In vitro experiments provide evidence that CO₂ driven anti-inflammatory gene expression is independent of changes in extracellular pH (Cummins *et al.*, 2010; Wang *et al.*, 2010), while in vivo studies indicate the protective effects of HCA to be dependent on acidosis in experimental models of lung injury (Laffey *et al.*, 2000).

Hypercapnic acidosis is associated with better prognosis in inflammatory conditions such as ARDS (Amato *et al.*, 1998; ARDSnet, 2000), ischemia-reperfusion injury (Li *et al.*, 2010) and models of acute lung injury (Laffey *et al.*, 2004). Conversely, experiments examining bacterial-induced lung injury in mice (Nichol *et al.*, 2009) and in pathogen-challenged *Drosophila* (Helenius *et al.*, 2009) demonstrate that exposure to high CO₂ results in a worse prognosis. Furthermore, the initial benefit of HCA in the case of attenuated lung damage may be negated through a reduced ability of pulmonary epithelial cells to promote wound healing (via NF- κ B) (O'Toole *et al.*, 2009). Taken together with the recent data demonstrating the blunting of NF- κ B pathway dependent genes with elevated CO₂ these outcomes are perhaps not as unexpected as they first seem. CO₂ through its modulation of NF- κ B signaling has the ability to both suppress inflammatory signaling as well as diminish innate immune responses. Depending on the nature of the challenge, CO₂ and/or HCA can both blunt inflammation driven tissue damage as in the case of LPS-induced lung injury and exacerbate lung damage in response to pathogen infection.

This has clear implications for the potential therapeutic applications of CO₂ in the clinic where CO₂ suppresses inflammation but also the ability to fight infection.

Therapeutic applications of CO₂

Given the increasing evidence for a potent effect of CO₂ on gene expression and in directing the course of human and animal models of disease it is important to consider the opportunities for the therapeutic manipulation of CO₂ levels.

The ARDS net tidal volume study provides clear evidence for the benefit lower tidal volumes in patients with acute lung injury and ARDs (ARDSnet, 2000). Use of lower tidal volumes in the ICU results in associated permissive HCA and evidence from models of HCA point to the importance of elevated CO₂ in contributing to the beneficial patient outcome. However, concerns over the potential deleterious effects of CO₂ with respect to immune suppression and wound healing have stunted the progression of trials for therapeutic hypercapnia (where CO₂ is actually administered via the ventilator).

With this in mind there is perhaps more potential for therapeutic strategies directed towards controlled and localized delivery of CO₂ to an acute inflammatory locus e.g. to the gut in IBD or where organ injury can be predicted and controlled e.g. in IRI or transplantation. Recent studies have pointed to the beneficial effects of CO₂ pneumoperitoneum post-laparoscopy (Ikechebelu *et al.*, 2005) and in animal models of LPS-induced sepsis (Hanly *et al.*, 2006). This suggests that laparoscopic surgery with CO₂ used as the insufflating gas as may be of particular benefit in surgical procedures with a high risk of developing sepsis e.g. stomach trauma.

Summary and Conclusions

Metazoans evolved to consume oxygen and generate carbon dioxide during the process of aerobic metabolism to maximize the efficiency of metabolic processes. As a consequence, it is not surprising that animals have evolved the ability to sense and respond to alterations in the availability of these important physiologic gasses via altered gene transcription in order to maintain metabolic homeostasis. The ability of cells to sense oxygen deprivation (hypoxia) via reduced activity of hydroxylase enzymes resulting in the induction of the hypoxia-inducible factor (HIF) have been well characterized (Semenza, 2007; Kaelin & Ratcliffe, 2008). However, the mechanisms by which cells sense changes in CO₂ leading to altered gene transcription remain less well understood. Recent advances have identified the repression of the NF-κB transcriptional pathway by CO₂ in a manner which may be of therapeutic benefit in chronic inflammatory disease. Interestingly, NF-κB is also a hypoxia-responsive transcription factor (Figure 1). Future studies will be aimed at identifying the CO₂ sensor and determining whether crosstalk between O₂ and CO₂ sensing pathways exist at the level of transcription factors such as NF-κB.

Figure Legends:

Figure 1: Transcriptional regulation by Oxygen and carbon dioxide. Metazoan cells have evolved to be capable of sensing levels of physiologic gasses in the microenvironment through highly conserved pathways in the control of gene expression. Acute O₂ and CO₂ sensing leads to neuronally mediated changes in processes such as respiratory control and olfactory sensation respectively. Molecular oxygen (O₂) is sensed by prolyl and aspariginyl hydroxylases which confer oxygen-dependent instability upon the HIF transcription factor. Activation of this pathway in hypoxia leads to the expression of adaptive genes. The sensor for regulation of NF-kappaB-dependent gene expression in response to changes in CO₂ has yet to be defined. However, elevated CO₂ leads to repression of the NF-κB pathway and decreased levels of genes which promote innate immunity and inflammation.

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Experimental model	Cellular Effect	Evidence of NF- κ B involvement	Reference
Rat Hepatic IRI	<ul style="list-style-type: none"> • \downarrow TNFα • \uparrow IL-10 • \downarrow Apoptosis • \downarrow Liver injury 	<ul style="list-style-type: none"> • \downarrow NF-κB staining by IHC 	Li et al.
In vitro buffered hypercapnia (MEF, A549 lung epithelial cells & others)	<ul style="list-style-type: none"> • \downarrow TNFα, ICAM-1, & CCL2 • \uparrow IL-10 	<ul style="list-style-type: none"> • \downarrow NF-κB Luciferase promoter reporter • \downarrow Nuclear p65 accumulation • \downarrow IκBα degradation • \uparrow Nuclear IKKα 	Cummins et al.
In vitro hypercapnic acidosis (Pulmonary endothelial cells)	<ul style="list-style-type: none"> • \downarrow ICAM-1, IL-8 • \downarrow Neutrophil adherence 	<ul style="list-style-type: none"> • \downarrow Nuclear p65 binding (EMSA) • \downarrow IκBα degradation 	Takeshita et al.
In vitro Hypercapnia (Macrophages)	<ul style="list-style-type: none"> • \downarrow IL-6, TNFα • IL-10 unaffected • \downarrow phagocytosis 	<ul style="list-style-type: none"> • No change in p65 or IκBα • \downarrow IL-6 promoter activity 	Wang et al.
In vitro Hypercapnia Acidosis (wound healing model in A549 lung epithelial cells)	<ul style="list-style-type: none"> • \downarrow wound healing • \downarrow cell migration 	<ul style="list-style-type: none"> • \downarrow IκBα degradation • \downarrow NF-κB Luciferase promoter reporter • Effect of HCA lost when NF-κB inhibited 	O'Toole et al.
Drosophila (Flies +/- pathogen at a range of CO ₂ concentrations)	<ul style="list-style-type: none"> • \uparrow Mortality • \downarrow Antimicrobial peptide genes 	<ul style="list-style-type: none"> • Proteolytic cleavage of Relish unchanged • Hypercapnia inhibits Rel targets in parallel or downstream of proteolytic activation of Rel 	Helenius et al.

Table 1: Summary table of the evidence for NF- κ B involvement in response to CO₂

