Extreme Resistance to Hypercapnia-Induced Pulmonary Edema of the African Naked Mole-Rat

BY

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THESIS

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<td>NF-κB</td>
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<td>NK1r</td>
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<td>O(_2)</td>
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<td>PBS</td>
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LIST OF ABBREVIATIONS (continued)

PCO$_2$  Partial Pressure of Carbon Dioxide
pH    Measure of Acidity or Basicity in an Aqueous Solution
PKC  Protein Kinase C
PNS  Peripheral Nervous System
RAR  Rapidly Adapting Mechanoreceptor
PKA  Protein Kinase A
RNS  Reactive Nitrogen Species
ROS  Reactive Oxygen Species
SAR  Slowly Adapting Mechanoreceptor
SP   Substance P
tac1  Tachykinin 1 Gene
tac3  Tachykinin 3 Gene
tac4  Tachykinin 4 Gene
TNF  Tumor Necrosis Factor
Trp  Transient Receptor Potential Channel
TrpV1  Transient Receptor Potential Vanilloid 1 Channel
WT   Wild-Type
SUMMARY

Extreme hypercapnia induces pulmonary edema in many terrestrial mammals. Pulmonary c-fibers that innervate lung tissue are activated by hypercapnia-acidosis. These fibers release the pro-inflammatory neuropeptide substance P (SP) and thus can induce edema in the lungs. The aim of my study was to discover more about the mechanism of hypercapnia-induced pulmonary edema. My working hypothesis was that pulmonary c-fibers are necessary for hypercapnia-induced pulmonary edema. Tissue CO₂ level is an important stimulus for respiration rate control. As part of evaluating the effects of hypercapnia-induced edema, respiration rate responses to moderate hypercapnia were measured in all animal groups.

We tested SP deficient mice, tac¹⁰⁰, to determine the extent SP contributes to respiration rate and hypercapnia-induced edema. We found that these mice had a significantly increased respiration rate in response to 10% CO₂ compared to wild-type (WT) mice. This demonstrated that substance P has a modulatory role in respiration rate. These mice were then tested for hypercapnia-induced edema. Counter to our hypothesis, SP released from c-fibers triggers edema, tac¹⁰⁰ mice had significant increased edema at 15, 20, and 30% CO₂. Hemokinin-1 (HK-1) is found in lung tissue and has similar affinities as SP to the SP receptor NK1r. To date, HK-1 has not been found expressed in neuronal cells but is abundant in immune cells such as alveolar macrophages. We measured the combined SP+HK-1 concentration in lung lavage at 0% and 30% CO₂. Since, tac¹⁰⁰ mice lack SP, HK-1 was the only peptide detected in tac¹⁰⁰ mice. We found no difference between SP+HK-1 measurements between
SUMMARY (continued)

tac\textsuperscript{1KO} and WT mice. Therefore, this indicates that HK-1 is the predominant NK1r ligand released in the lungs at 0 and 30% CO\textsubscript{2}.

To determine the role c-fiber acid sensitivity has in hypercapnia-induced edema, we tested mice that lacked the TrpV1 (TrpV1\textsuperscript{KO}) or TrpV1 and ASIC3 (TrpV1\textsuperscript{KO}/ASIC3\textsuperscript{KO}) ion channels. These channels account for a large portion of the acid sensitivity of pulmonary c-fibers. These mice had no significant difference in moderate hypercapnia induced respiration rate increase. These mice also had no difference in hypercapnia-induced pulmonary edema. These results indicated that pulmonary c-fiber acid sensitivity and subsequent release of SP does not have an effect on hypercapnia-induced pulmonary edema. To confirm this, SP+HK-1 content of TrpV1\textsuperscript{KO} mice was measured at 0 and 30% CO\textsubscript{2}. No difference was observed between these and WT mice.

The volatile anesthetic isoflurane (ISO) is known to have anti-inflammatory effects on induced pulmonary inflammation. We combined ISO with 30% hypercapnia to determine if ISO would attenuate hypercapnia-induced pulmonary edema. The ISO significantly reduced hypercapnia-induced edema and significantly attenuated the release of SP+HK-1 into the lung airways. Thus, ISO has an anti-inflammatory effect on hypercapnia-induced pulmonary edema.

My last aim was to determine if African naked mole-rats, mammals that naturally live in hypoxia and hypercapnia in their burrows, have attenuated response to hypercapnia. We found that naked mole-rats have attenuated response to moderate hypercapnia-induced respiration rate increase. We also found that naked mole-rats had
SUMMARY (continued)

no hypercapnia induced edema at levels as high as 50% CO₂. We then tested SP+HK-1 level of lavage fluid at 0 and 30% CO₂. It was surprising that naked mole-rats had no detectable amount of SP+HK-1 at either of the CO₂ concentrations. The absence of edema and lack of SP+HK-1 in naked mole-rat lavage fluid demonstrates the existence of an adaptation of naked mole-rats.

These findings demonstrate that extreme hypercapnia induces the release of SP+HK-1. The protective effect of ISO may be part of the same pathway that protects naked mole-rats against hypercapnia-induced edema. Overall, pulmonary c-fibers may not have a function in hypercapnia-induced edema.
1. INTRODUCTION

To understand the role of carbon dioxide (CO₂) in normal and pathophysiological conditions, it is important to review how CO₂ interacts with lung tissues and the molecular pathways involved. Elevated blood CO₂, hypercapnia, that occurs in multiple pathological respiratory conditions, such as chronic obstructive lung disease, exacerbates these conditions resulting in increased lung barrier permeability and edema [1]. The normal carbon dioxide level is considered to be within the range of 35-45 mm Hg. Hypercapnia is considered to be anything above 45 mm Hg and mild is up to 50 mm Hg [2, 3]. Moderate hypercapnia range extends from 55 to 70 mm Hg [4] and above 75 is considered extreme hypercapnia [5, 6]. The sections below discuss moderate to severe hypercapnia effects as well as the protection isoflurane (ISO) offers against hypercapnia-induced pulmonary edema. These sections also establish the naked mole-rat as a model animal for studying hypercapnia-induced pulmonary edema in the laboratory.

1.1 Carbon Dioxide

1.1.1 Carbon dioxide transport

Carbon dioxide is an important by-product of cellular respiration. While low in concentration in earth’s atmosphere, 0.04%, it reaches much higher levels within organisms. Detection of CO₂ concentrations is involved in regulating several physiological processes. It is utilized by some organisms in mating as an attractant, as well as a repellant for avoiding predators and harmful substances [7].
Classically, CO₂ is thought to passively diffuse across cell membranes due to it being a small non-polar molecule [8]. Overton’s rule dictates that the less impeded a chemical dissolves in a lipid, the faster it will be transported into a cell. A study used this rule to conclude that passive diffusion could explain CO₂ transport into cells [9]. This study has since been disputed by a more recent study [10]. Likewise, CO₂ transport by only passive diffusion has been challenged by a number of studies. If CO₂ is permeable to all membranes by simple diffusion, the apical membranes of gastric gland cells would not have been found to be impermeable to CO₂ [11]. Red blood cells would also not have reduced permeability to CO₂ by the anion transport inhibitor 4,4’-Diisothiocyanatostilbene-2,2’-disulfonate [12]. Even the discovery of evolutionarily conserved proteins that can serve as CO₂ transporters challenges simple diffusion as the only transport mechanism of CO₂ [13-17]. Despite this, the significance of how CO₂ is transported across membranes is not fully understood. [9]. Once CO₂ is either produced or transported into cells by whatever means, it is quickly and reversibly converted to bicarbonate (HCO₃⁻) and hydrogen (protons) by carbonic anhydrase.

1.1.2 **Carbonic anhydrase**

Carbonic anhydrase is an essential enzyme the greatly speeds up the reaction of CO₂ and H₂O to produce HCO₃⁻ and protons. The increased reaction rate by carbonic anhydrase is estimated to be between 0.5 to 1 X 10⁶ times. There are more than 15 isoenzymes of carbonic anhydrase that have been identified. Of these, carbonic anhydrase II, CAII, is the most efficient and is the most studied [18]. The CAII activity in the lung epithelium is involved in the elimination of CO₂ into the alveolar space [19]. In addition, CAII-deficient mice will develop respiratory acidosis due to CO₂ retention in the
lungs [20]. Since the enzymatic activity of CAII is the first reactive step in several
intracellular cascade reactions, carbonic anhydrase can be viewed as the first biological
sensor of CO₂.

1.1.3 **Respiratory control**

The lungs are the site of most gas exchange in mammals with the outside
environment. As such, they are exposed to rapid fluctuations in oxygen and carbon
dioxide. In mammals, respiratory control is one of the most important physiological
processes regulated by CO₂ detection. Respiration is the most efficient mechanism to
remove excess CO₂. A one mm Hg increase of blood PCO₂ can induce increased
respiratory frequency in many terrestrial mammals [21]. CO₂ sensory cells are located
in both in the brain and the periphery. For the past century rhythmic respiration has
been believed to be regulated primarily by sensing arterial blood PCO₂ / pH changes in
the CNS [22]. A complex network exists in the lower brainstem that is critical for
breathing. This network outputs to the phrenic nerve that stimulates the diaphragm in a
rhythmic pattern [23]. The cellular input of CO₂ detection in the brainstem is still
plagued by controversy. Primarily this has been attributed to no identification of
molecular CO₂ transducers in brain areas regulating breathing if one even exists. Mice
lacking CO₂ chemosensitivity in the CNS by genetic modification can still breathe [24].
An alternative interpretation is that the carotid body, a peripheral source of detection,
can function as a backup CO₂ sensor to drive respiration. During normal breathing, the
carotid chemoreceptors are critical for maintaining normal CO₂ levels [25]. In response
to hypercapnia, the central chemosensitive neurons may become the dominant
contributor to the respiration rate [26]. Even still, the carotid neurons are the first responders to rapid transient changes in arterial CO₂ levels [27].

1.1.4 **Carbon dioxide as a possible intracellular signaling molecule**

Physiological gasses are known to have a large impact in cellular function and intracellular signaling pathways. Nitric oxide alters the activities of guanylate cyclase and cytochrome c oxidase [28, 29], and molecular oxygen alters hydroxylase activity leading to the activation of hypoxia inducible factor [30]. Hypercapnia suppresses pro-inflammatory transcriptional pathways [31] related to the NF-κB pathway [32]. The link of high CO₂ with modulating the inflammatory pathway appears to be evolutionarily conserved, found in both *Drosophila melanogaster* [33] and *C. elegans* [34]. In mice, hypercapnia has been demonstrated to reduce cytokine release and phagocytic activity of macrophages that were activated by lipopolysaccharide (LPS) [35]. Alternatively, hypercapnia has been found to have pro-inflammatory effects on endothelial cells and neutrophil responses [36]. Overall, the effects of carbon dioxide on cells may not be direct. Rather, the CO₂ conversion into other molecules such as reactive species may be the signal for modulating cellular processes.

1.1.5 **Reactive species**

Carbon dioxide and its coupled acidosis can both undergo further reactions that produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). These are considered to be second messengers that may act at different levels of cell signaling cascades [37, 38]. In fact, activating a number of plasma membrane channels causes their intracellular levels to increase, functioning as cellular signals. At high concentrations, these have damaging effects on most if not all cellular components,
such as proteins, carbohydrates, lipids, and DNA, that is kept in check by endogenous antioxidant defenses [39, 40].

Due to the interconnections between CO₂ and its products, the difficulties of identifying which of these molecules that is directly responsible for inducing the observed physiological reflexes has been noted [41]: altering the concentration of one molecule will result in a concentration change in the others. Using buffers to substitute for the intrinsic CO₂/ HCO₃⁻ buffering system reduces the intracellular concentration of HCO₃⁻. This will affect the activation of soluble adenylyl cyclase and numerous transport systems for HCO₃⁻. Hepes and similar buffer compounds can inhibit gap junctions and hemichannels [42, 43]. For example, the presence of CO₂/HCO₃⁻ was essential to see the involvement of ATP in chemosensory neuronal responses [44]. Therefore, determining which among CO₂ and its products is responsible for the effect requires careful experimental planning.

1.2 Alveoli and Neighboring Cells

The alveoli of the lungs are the site of gas exchange between the lung airspace and the circulatory system. As such, the alveoli are exposed to wide fluctuations in CO₂ concentrations and inhaled noxious stimuli. When damaged, the alveoli will repair itself through proliferation of resident stem cells and recruit immune cells to fight against inflections. This requires a complex system of signaling between cells within the lungs and circulatory system.

The alveolus is comprised of an epithelium and endothelium monolayer. In between these layers is a thin interstitial space. The short distance between the
alveolar airspace and the endothelial capillary lumen facilitates gas exchange (20-22). The alveolar airspaces are lined by epithelial cells, alveolar type I (AECI) and alveolar type II (AECII). AECI are flattened, covering 95% of the alveolar surface and are involved in fluid clearance [45-48]. AECII are cuboidal and accounting for 5% of the surface area. Surfactant is secreted by AECII cells for the purpose of surface tension maintenance [49]. Tight junctions exist between the AECs creating a strong barrier that prevents most molecules and pathogens from freely crossing. Residing on the apical surface of AECI and AECII cells are alveolar macrophages. These cells are responsible for initiating the inflammatory reaction of the lung. Under the epithelial layer there are endothelial cells lining the pulmonary capillaries, a scattering of mast cells, dendritic cells, and neuronal fibers.

At the bronchioalveolar duct are bronchioalveolar stem cells responsible for repopulating the bronchioles and alveolar cells after lung injury. Just proximal to these cells are club cells that are nonciliated secretory cells that protect the airways from environmental insults [50]. The bronchioles are lined with ciliated cells that move the mucosa, aiding in the elimination of hazardous particles and debris in the airways. Outside the alveoli and embedded in the epithelium are neuroendocrine bodies (NEBs). NEBs are clusters of cells that are highly innervated by nerve fibers and are thought to function as oxygen detectors [51].

1.3 **Pulmonary Edema**

Extreme hypercapnia has been found to induce pulmonary edema in many terrestrial mammals and this is the focus of the present dissertation. Pulmonary edema
is the condition where the pulmonary epithelium barrier becomes leaky and fluid, plasma and inflammatory cells seep into the alveolar airspace. This fluid coats the respiratory membranes of the lungs, thereby hindering gas exchange. Other complications will then arise including suffocation. Heart failure/circulatory system impairment, and inflammation of lung tissue are known causes for pulmonary edema [52]. While heart failure will cause edema by increasing blood pressure, inflammation and its resulting edema can be triggered by inhalation of noxious stimuli. Examples are ammonia and CO₂. Due to their interaction with pulmonary nerve fibers, the resulting edema has been classified as neurogenic pulmonary edema.

1.4 **Alveolar Epithelial Barrier**

The connections between cells within the alveoli are critical for regulating contents between the alveolar airspace and lung tissue. For hypercapnia to induce fluid and cells to leak into the alveolar airspace, the epithelial barrier of the alveoli must become disrupted. The alveolar epithelial barrier comprises AECs expressing a complex of proteins embedded in the paracellular AEC membranes. Both AECI and AECII express well-organized paracellular adherens and tight junctions and are polarized, with apical epithelial sodium channels and basolateral Na/K-ATPases [53-55].

The adherens junctions are comprised of epithelial cadherins. The extracellular domain of cadherins enables interaction with neighboring cells. The intracellular domain binds with intracellular adhesion molecules linking the junction to the cell actin cytoskeleton [56-60]. Adheren junctions both establish strong cell-cell adhesion and are involved in cell signaling [61].
The paracellular tight junctions account for the low permeability of the epithelial barrier [55, 62-64]. The tight junction composition determines its properties [65]. It is comprised of occludens [66, 67], zonula occludens [64], and members of the claudin family [68]. The most important component of the tight junction protein complex are claudins. The alveolus epithelium expresses mainly claudin 3, 4, and 18 [65]. For inflammatory cells (e.g. leukocytes) to infiltrate the alveolar space, the alveolar epithelial barrier must become disrupted for inflammatory cells to pass from the interstitial space into the alveoli lumen. Several stimuli such as oxidative stress can cause changes in composition, expression, and location of tight junctions [69-71].

1.5 **Intracellular Pathways**

Hypercapnia causes a direct effect on alveolar epithelial cells as evident by hypercapnia-induced endocytosis of Na/K-ATPase from AEC membranes. This mechanism involves the CO2-induced rapid activation of AMP kinase by Ca²⁺/calmodulin-dependent protein kinase-beta and PKC-ζ phosphorylation [72]. AMPK activates energy-generating pathways while reducing energy-consuming pathway. Therefore, endocytosis of Na/K-ATPase eliminates the functioning of this energy-consuming protein that accounts for 40% of the AEC resting energy expenditure. A cell’s need to increase energy-generating pathways may be related to CO₂-induced mitochondria dysfunction [73]. Hypercapnia can produce reactive nitrogen species (RNS) that are toxic to cells and may mediate production of reactive oxygen species (ROS) [74, 75]. Thus, hypercapnia may induce AEC damage leading to pulmonary inflammation.
Activation of cell receptors and ion channels will lead to the activation of intracellular signaling. This includes activation of mitogen-activated protein kinases (MAPKs) that relay exogenous and endogenous signals by phosphorylating intracellular proteins. These proteins can be upstream such as growth factor receptors, g-proteins, and tyrosine kinases; or downstream such as nuclear transcription factors [76]. Therefore, activating MAPKs can have short-term and lasting cellular responses. There are four known MAPK families. They are extracellular-regulated kinases (ERKs), c-jun-NH2-terminal kinase (JNKs), p38 MAPK and the big MAPK-1 (BMAPK-1) [77].

Hypercapnia has previously been demonstrated to activate some of these MAPKs to promote cancer cell proliferation [78].

1.6 **Hypercapnia-Induced Altered Gene Expression**

Hypercapnia has been demonstrated to have anti-inflammatory properties in models of inflammation. Hypercapnia at minimal and moderate levels ultimately alters activation of Nuclear factor-kappaB (NF-kB) [79-81]. This is a family of transcription factors that are intricately involved in the regulation of innate immunity and inflammation [82]. NF-kB are DNA binding proteins that interact with enhancing domains of target genes. NF-kB is arranged as a dimer of two members of the NF-κB/Rel/Dorsal family of proteins [83]. NF-κB are sequestered next to and inhibited in the cell cytoplasm by members of the IκB family. A wide variety of extracellular stimuli activate NF-κB. Once activated, IκB is degraded and NF-κB translocates to the nucleus to induce transcription of genes. RelA, the endotoxin-stimulated NF-κB, translocation in the nucleus is inhibited by hypercapnia in pulmonary-artery endothelial cells [84]. This was not true of
human macrophages [35]. This demonstrates that the decreased activation of NF-κB is cell specific. Alternatively, RelB, another member of NF-κB family, is activated by hypercapnia and translocates to the nucleus in mouse fibroblasts and human pulmonary epithelial cells. Together, the anti-inflammatory action of low to moderate hypercapnia may in part be explained by its effects on NF-κB activity on gene regulation. Despite this, the present dissertation used short 10 minute hypercapnia exposures that may rule out altered activation of NF-κB.

1.7 **Lung Innervation**

Acidosis has been known to be a potent stimulus of lung airways [85]. Its effects have been attributed to activation of a subset of neuronal afferent fibers innervating the lungs. Overall, there are a number of innervating plexuses of the lungs. They either belong to the sympathetic, parasympathetic pathways, or the dorsal root ganglion (DRG). “Sympathetic nerve fibers originate from sympathetic cervical and thoracic ganglia. A minority of sensory nerve fibers originates from dorsal root ganglia” [86, 87]. The parasympathetic, preganglionic neurons are all supplied by the vagus nerve and the majority of sensory nerve fibers innervating the lungs [88, 89].

The vagal pulmonary pathway has two ganglia, the jugular and the nodose. The cells originate from different pools of cells early on in development. The jugular cells originate from neural crest cells, and the nodose cells originate from the epibranchial placodes [90, 91]. This difference defines early on their phenotype of the vagal fibers. The vagal airway afferents are grouped together as rapidly adapting mechanoreceptors (RARs), slowly adapting mechanoreceptors (SARs), and C-fibers. Most SARs are
located in the intrathoracic airways [92]. In guinea pigs, the SARs are only connected to
the jugular ganglion and the RARs are connected to the nodose ganglion of the vagal
nerve [93-95]. The central nerve endings of the airway afferents terminate in the
nucleus tractus solitarii of the medulla oblongata. The vagal efferent fibers control
vasodilation in the lungs whereas the afferent fibers (a-δ and C) convey pain and
stretch sensation to the central nervous system. Degeneration studies have
approximated that 75% of the vagal afferent fibers from the respiratory tract are
unmyelinated C-fibers [96]. The primary hypothesis of the present dissertation was that
a subset of pulmonary fibers, pulmonary c-fibers, would be stimulated by extreme
hypercapnia by hypercapnia acidosis. These fibers would then release peptides that
lead to induced pulmonary edema.

1.7.1 Peptidergic c-fibers

C-fibers are unmyelinated fibers that secrete several pro-inflammatory molecules
when stimulated. They are polymodal, responding to a variety of noxious stimuli.
These include heat, capsaicin, and acid. How they respond is determined by expressed
receptors and channels on their cytoplasm. Acid has been known to activate these
fibers to induce the cough reflex. Additionally, acid can potentiate c-fibers. In
asthmatic reactions, the pH of the airway water vapor condensate reduces from 7.65 to
5.23 [97, 98]. A pH of less than 5 has been demonstrated to induce the c-fiber release
of peptides that are known to initialize pulmonary edema [99, 100].

1.7.2 Acid sensing by c-fibers

Hypercapnia potentiates pulmonary c-fibers to inflammatory mediators by
hypercapnia acidosis [101]. Their acid sensitivity is mediated by a group of receptors
expressed on their peripheral axons. These include the TrpV1 channel and acid-sensing ion channels (ASICs). TrpV1 channel is attributed to the c-fiber sensitivity to a mild decrease of pH to 7.0 and ASICs to a pH decrease to below 6.5. These two channels are thought to make up the majority of the acid sensitivity of pulmonary c-fibers [102].

1.7.3 **Acid-sensing ion channels**

Acid-sensing ion channels are trimeric channels selective for Na⁺ and are predominantly expressed in neuronal cells. Three genes encode 5 major transcripts as components of functional channels. The subunits ASIC1A and ASIC1B both assemble into homomeric channels with high acid sensitivity. The ASIC2A assembles into homomeric channels but has low sensitivity to acid [103]. The ASIC2B channel is silent but the subunit does assemble with other ASIC subunits to modify the channel ion selectivity [104]. The ASIC3 channels are unique where they are biphasic, generating an early fast phase followed by a long prolonged phase. These are the only ASIC subunit not expressed in the CNS. They are also most closely associated with DRG cells [105]. The ASIC4 subunit is insensitive to acid and also modulates the properties when combined with the other subunits [106, 107]. ASICs are expressed in brain regions linked to hypercapnia-induced fear. ASIC1a knockout (KO) mice have a severely blunted fear response to hypercapnia. This is the only genetic evidence for a primary chemosensory transducer for CO₂ [108].

1.7.4 **Transient receptor potential channels**

Transient receptor potential channels (Trp) have been well studied in the airways. Activation of this class of receptors induces neurogenic inflammation. This
causes cough, airway constriction, and airway hyperreactivity [109-111]. The TrpV1 channel belongs to the family of 28 Trp channels spanning two groups. TrpV1 channels are semiselective calcium channels. Each subunit is a 6 transmembrane protein and they are arranged as a tetramer. The channel subunits can be comprised of 4 identical subunits, or of a mixture of the other 3 Trp subunits (2-4). TrpV1 is polymodal, activating in response to heat, acid, capsaicin, and several pungent substances. This channel can also be activated, sensitized, and desensitized by intracellular signaling.

1.7.5  **Tachykinins**

The effect of tachykinins in response to noxious stimuli has been well documented [112]. Tachykinins are a class of neuroamines encoded by three genes. Tac1 has two splice variants, substance P (SP) and neurokinin A. Tac3 encodes neurokinin B. The most recent tachykinin gene discovered is tac4 that encodes hemokinin-1 (HK-1). In humans, tac4 has four splice variants, endokinin A-D, as opposed to one for the murine gene. While tachykinin lengths vary, their binding domains are fairly well conserved. This enables every tachykinin to bind to all three of the tachykinin receptors NK1, NK2, and NK3. Every tachykinin binding affinity is influenced by their varying C-terminus binding domains and the composition of their N-terminus. For example, SP and HK-1 are both eleven amino acid peptides. Their affinity differences are attributed to the variant amino acid sequence occurring in the N-terminus of 4 amino acid differences. Therefore, SP has a 100 fold higher affinity to NK1 than HK-1.

Substance P is found throughout the CNS and PNS. In the CNS, it is predominately found in areas that regulate emotion. The neurons in the brain
containing SP are also found in close proximity to those containing serotonin and norepinephrine. These are the same areas that are targeted by antidepressant drugs. In the PNS, SP is most known for its involvement in pain perception and inflammation. African naked mole-rats, lack cutaneous SP expression in their c-fibers and as such have no cutaneous inflammatory pain and itch perception from noxious stimuli applied to their skin [113]. However, when SP expression via vector is introduced in their DRGs, pain perception is induced by the same noxious stimuli. SP has also been shown to cause vasodilatation and endothelial leakiness, and migration of neutrophils into the inflamed tissue. These effects occur by its binding to the NK1 receptor, where a NK1 antagonist will reduce or abolish this effect [114]. There is also a link between SP alveolar release and late airway response to allergenic challenge [115].

Substance P has been determined to be the peptide that mediates neurogenic inflammation in the lung [116-118]. Lung permeability increases due to the stimulation of pulmonary c-fibers and is inhibited by TrpV1 and NK1 antagonists [117, 119-121], and exogenous SP mimics the increased pulmonary permeability [122]. Therefore, this dissertation predicted that hypercapnia acidosis would stimulate the pulmonary c-fibers to release SP. The release SP would thus induce pulmonary edema.

1.7.6 Neurokinin signaling

Neurokinin receptors are g-protein-coupled receptors and as such, the cellular effect induced by their activation is a product of the 2nd messenger cascades they initiate. Binding of NKs to NK receptors activates bound g-proteins that leads to PKC activation and $[\text{Ca}^{2+}]$, increases by IP$_3$ receptor activation [123]. PKA can activate PKC that phosphorylates other receptors, thereby desensitizing those receptors. The
extracellular activity of NKs is eliminated by extracellular proteases that break up NKs, thereby making them inactive. After binding of NKs, NK receptors are endocytosed. Neurons expressing NK1r will be completely desensitized to SP within 10 minutes after initial stimulation. The receptors are translocated to early endosomes. This is mediated by \( \beta \)- Arrestins that couples with agonist-occupied receptors and couples the receptors to the clatherin-mediated endocytosis machinery. Once in the endosome, endothelin-converting enzyme-1 degrades neuropeptides, thereby destabilizing the receptor from \( \beta \)- Arrestin and promotes the receptor for recycling back into the cell membrane. Cell sensitivity to SP is almost restored by 60 minutes after initial stimulation [124]. Desensitization is not fully mediated by endocytosis of the receptor but likely due to phosphorylation of the receptor by 2\textsuperscript{nd} messenger kinases. Phosphorylated receptors can be resensitized by protein phosphatase 2A by it translocating to the plasma membrane and dephosphorylating the desensitized receptors [125].

1.8 **Alveolar Macrophages**

Alveolar macrophages are the main resident immune cells in the lungs that initiate and recruit other inflammatory cells. Therefore, alveolar macrophages are likely to be the first immune cells activated by the pathway of hypercapnia-induced pulmonary edema. They express the NK1r and can be activated by released SP [126]. Alveolar macrophages are a subtype of macrophages that strictly reside in the lumen of the bronchioles and alveoli. They have two functions, clearing of cellular debris in a steady-state manner in healthy lungs, and initializing lung inflammation in response to lung assault. They are kept in the stead-state mode by their interaction with the apical
surface of AECs by receptors and anti-inflammatory cytokines. The induction of the inflammatory response in alveolar macrophages is thought to arise from either the destruction of juxtapose epithelial cells, or loss of exposure to the epithelial secreted regulatory ligands. A homeostatic balance exists between pro- and anti-inflammatory signals that once tipped will initiate the alveolar macrophage inflammation.

Different signals to alveolar macrophages will determine whether they respond by maintaining steady-state or pro-inflammatory behavior. In response to cell apoptosis, alveolar macrophages must take an anti-inflammatory response to avoid inflammatory response to self [127]. Alternatively, necrosis of AECs liberates pro-inflammatory cellular constituents. Alveolar macrophages can also be stimulated directly by increasing the pro-inflammatory pathways of alveolar macrophages, thereby overcoming the inflammation suppression by interaction with AECs. Alveolar macrophages have pattern recognizing receptors on their cell membrane. When these receptors come into contact with a pathogen, pro-inflammatory responses will be initialized. For example, oxidative stress enhances alveolar macrophage exocytosis and thus increased surface expression of TLR4 [128]. In addition, peptidergic c-fibers innervate lungs and secrete substance P that can induce inflammation by alveolar macrophages.

1.9 **General Anesthetics**

General anesthetics are a broad group of substances that possess anesthetic activity. There are many ion channels and receptors that anesthetics interact with. Anesthetics can depress the firing of neurons and interfere with components of
chemosensitivity [129-131]. To date, the specific receptor targeted by anesthetics to produce anesthesia is unknown. This is due to the lack of specificity of anesthetics. Gamma-aminobutyric acid and N-methyl-D-aspartate receptors in the CNS are indicated as the likely targets needed to produce anesthesia [132]. General anesthetics are known to have anti-inflammatory effects [133-138]. Therefore, the present dissertation examined the effect of a general anesthetic, isoflurane, has on hypercapnia-induced pulmonary edema.

1.9.1 **Isoflurane**

Isoflurane, a common volatile anesthetic, is known to excite pulmonary c-fibers but also offers protection against induced inflammation. In humans, inhalation of ISO induces tachypnea. The pulmonary c-fibers are thought to be the target cells of ISO to induce this response in dogs [139]. Blocking pulmonary c-fiber conduction using perineural capsaicin treatment of the cervical vagi abolished this response and unblocked ISO increases the firing rate of pulmonary c-fibers. Isoflurane has recently been found to block transient and delayed-rectifier K⁺ channels of the pulmonary c-fibers of the nodose ganglia. This effectively makes the cells hyper excitable [140]. The same authors in another study found that ISO inhibits apnea evoked by a 5-HT₃ agonist on pulmonary c-fibers, suggesting that ISO has an inhibitory effects of the ionotropic 5-HT₃ channels [141]. ISO also excites the TrpV1 receptor [142]. Not all subtypes of a receptor is inhibited or excited by ISO [143]. In addition to cell membrane receptors, it binds to intracellular components. ISO interferes with multiple sides of the electron transport chain [144] including the mitochondrial electron transport chain complex III.
via potentiation of the electron transport chain complex I [146]. This ultimately causes the mitochondria to produce ROS.

Isoflurane, as is the case with other volatile anesthetics, has a pro-inflammatory action in some conditions, and an anti-inflammatory action in others. The difference of effects of ISO has on inflammation appears to be dependent on the cell type and method of induced inflammation. In alveolar macrophages, ISO increase the secretion and gene expression of some cytokines. In AECII, ISO decreases the production of pro-inflammatory cytokines [137, 138]. This is thought to be due ISO potentiating the GABAA receptors [147]. Changes of gene expression by ISO exposure offers protection from later injury known as preconditioning. This is thought to be due to activation of inhibitory g-proteins via receptors [148] and PKC [149, 150]. ROS generated by the mitochondria can also activate PKC. Ultimately, ISO exposure causes increased heme oxygenase-1 (HO-1) activity. HO-1 activity utilizes mitochondria heme-containing proteins in a reaction that is protective against induced cell apoptosis and maintains mitochondrial membrane potential [151]. HO-1 is thought to be a scavenger of heme liberated by oxidants in cellular oxidative stress [152]. Correlated with ISO-induced increase HO-1 was a decrease in nitric oxide production that was mediated by HO-1 activity [133]. This is to be expected as HO-1 inhibits iNOS expression and activation. Of particular interest, ISO has been found to induce transient changes of alveolar epithelial permeability [153].

1.10  **Naked Mole-Rats**
Previous studies in our lab and others have found naked mole-rats possess remarkable insensitivities to noxious stimuli. In their skin, naked mole-rat cutaneous c-fibers lack SP [154]. This absence was found to prevent pain-related behaviors in the naked mole-rat. These behaviors were only observed once SP expression was transfected into their c-fibers [155]. However, they demonstrated insensitivity to acid. Naked mole-rats have been found to possess a special form of the Nav1.7 channel [156]. This sodium channel version is only expressed in c-fibers and is inactivated by acid. The naked mole-rat form has a special motif that causes the channel to be more susceptible to acid inactivation.

Naked mole-rats have an extreme resistance to hypoxia. They were found to survive 6 minutes of anoxia without any apparent lasting detrimental effects [157]. Hippocampal slices also revealed great hypoxia resistance, thereby negating any effects the circulatory system [158]. Naked mole-rats were found to contain a subunit composition similar to that of neonate mice that also have hypoxic resistance [159]. In the tunnels of naked mole-rats, hypercapnia occurs coupled with hypoxia [160]. To date, no studies have evaluated the naked mole-rat pulmonary resistances to hypercapnia.

1.11 **Current Study**

The purpose of the current dissertation was to gain a better understanding of hypercapnia effects on lung physiology and possible adaptations naked mole-rats have that desensitize them to the hypercapnia in their burrows. We hypothesize that pulmonary c-fiber acid sensitivity contributes and possibly induces the pulmonary
inflammatory reaction of hypercapnia (Figure 1). The naked mole-rat lack of SP and acid sensitivity in their DRG c-fibers may be part of their adaptations to hypercapnia. An alternative hypothesis was that pulmonary c-fiber acid sensitivity is not involved in hypercapnia-induced pulmonary edema and hypercapnia may directly induce pulmonary edema through its effects directly on alveolar macrophages.
Figure 1. Hypothesized mechanism of hypercapnia-induced pulmonary edema. Hypercapnia causes tissue acidosis (1) that stimulates pulmonary c-fibers (2). The c-fibers release substance P (3) that binds with and increases permeability of the epithelial barrier (4). The increased permeability allows for blood plasma to exude out of the pulmonary blood capillaries and fill the alveolar airspace (5).
The following studies will determine (1) How resistant naked mole-rats are to hypercapnia; (2) the level of SP+HK-1 in the lungs and whether it changes in response to hypercapnia; (3) whether reducing pulmonary c-fiber acid sensitivity in mice alters the level of inflammation induced by hypercapnia; (4) if eliminating SP expression alters hypercapnia-induced respiration rate increase and pulmonary edema; (5) and finally if isoflurane has a pro- or anti-inflammatory effect on hypercapnia-induced inflammation.
2. EXTREME HYPERCAPNIA RESISTANCE OF THE AFRICAN NAKED MOLE-RAT

2.1 Abstract

African naked mole-rats have several adaptations for living together in large colonies underground. In their tunnels, oxygen content is reduced and carbon dioxide builds up to noxious levels. Naked mole-rats have insensitivity to several noxious inflammatory stimuli attributed as adaptations to overcome living in chronic hypercapnia. The purpose of this study was to evaluate hypercapnia effects on naked mole-rat respiration and CO₂-induced pulmonary edema that occurs in other terrestrial mammals. We observed naked mole-rats to have insensitivity to moderate hypercapnia-induced respiration increase. At higher levels of hypercapnia, respiration rate increased but no pulmonary edema ever developed. This is the first time an animal has demonstrated a complete absence of pulmonary edema from extreme hypercapnia. We measured SP+HK-1 levels in alveolar lavage fluid. These are neuropeptides that are released in lungs, induce inflammation within lungs. No detectable amounts were observed at basal or after acute hypercapnia exposure of naked mole-rats. The absence of these two neuropeptides released into the alveolar airspace of naked mole-rats indicates a sensory-inflammmatory adaptation to overcome the physiological challenges of living in hypercapnia.

2.2 Introduction

Hypercapnia in most mammals induces several physiological responses. At low levels, respiration rate will increase which can compensate for the retention of too much tissue CO₂. At extreme hypercapnia, pulmonary edema will occur, resulting in impeded
gas exchange. The threshold for pulmonary edema induction is about the same amongst most mammals tested to date, indicating a shared inflammatory pathway. Mammals that normally live in a noxious hypercapnia environment are thought to have adaptations to overcome their physiological challenges. However, only a few studies have evaluated the pulmonary adaptations in these resistant mammals. African naked mole rats, *Heterocephalus glaber*, chronically live in hypercapnia. This study evaluates their physiological responses to acute hypercapnia.

The naked mole-rat is well adapted to its indigenous life in the semi-arid landscape of north-eastern Africa. In this environment, naked mole-rats live completely subterranean, avoiding the heat and desiccating effect of the surface. To remain hydrated, they intake moisture from the roots and tubers they eat, and their kidneys excrete little but highly concentrated urine [21]. Maximum urine concentration is about 1,500 mmol/kg [22]. Naked mole-rats display an unusual social structure called eusociality. Their colonies consist of workers, soldiers, few male breeders and one to two breeding females. Besides from breeding, no sexual bias exists for the roles performed by each naked mole-rat. All individuals work together to aid in their survival and child rearing in colonies as large as 300 individuals [23-26]. A consequence of so many naked mole-rats in their narrow, enclosed tunnels is that oxygen levels decrease (hypoxia) and carbon dioxide increases (hypercapnia). CO$_2$ dissipation from the tunnels varies depending on soil composition, recent rain fall, and depth of the tunnel systems [161]. In the natural habitat of naked mole-rats, the soil has been described as being “like cement” [21] and “dark red sandy clay loams” [1]. In such conditions, it would be adaptive to be resistant to hypoxia and hypercapnia.
Naked mole-rats have several adaptations for living in their hypoxic tunnels. Their metabolic rates are low compared to their size [162, 163]. Their blood has high affinity for oxygen [164]. Under anoxia, naked mole-rat brain hippocampal slices survive 3 to 4 times longer than mice [158]. Peterson et al. found that these slices have reduced internal calcium that builds up from hypoxia compared to mice [165]. In conjunction with this, they found that naked mole-rats retain a greater portion of GluN2D NMDA subunits into adulthood than mice [159]. These subunits are known to be protective during hypoxia.

Less is known of how naked mole-rats are adapted to hypercapnia. A known physiological effect of hypercapnia is the coupled tissue acidosis caused by the conversion of CO₂ into HCO₃⁻ and protons. Naked mole-rat blood has a higher buffering capacity of CO₂ but it does not prevent acidosis in naked mole-rats from occurring in their tissues. Naked mole-rats are insensitive to cutaneous acid application [154, 155]. The inflammatory response induced by acid is mediated by cutaneous c-fibers, which in other animals rely on voltage-sensitive sodium channels (Nav1.7) to propagate action potentials. The naked mole-rat Nav1.7 has increased inhibition by acid [156]. This difference may explain the significant decreased activity of the trigeminal pain nucleus from 50% acetic acid instead of the increase seen in mice [166].

Hibernating animals also need to cope with a hypercapnia. The same Nav1.7 amino acid motif responsible for the increased acid inhibition seen in naked mole-rats has been found in these animals, occurring in both a parallel and divergent evolution [167].
Another striking feature about naked mole-rats concerns SP and other pro-inflammatory molecules that are usually released by c-fibers. Naked mole-rats lack SP in their cutaneous c-fibers [154]. This was confirmed by transfecting fibers with SP, which enabled a behavioral response to the TrpV1 channel agonist capsaicin. This also demonstrated that naked mole-rats do contain the functional receptor for SP [168]. These physiological differences in naked mole-rats are suggested to be adaptations for living in a chronic hypercapnia.

The acidosis resulting from hypercapnia is believed to induce pulmonary edema. As it is in the skin, pulmonary c-fibers detect acid. For the majority of mammals tested so far, most pulmonary c-fibers originate from the vagus nerve and the rest are from the DRG. Once activated, these c-fibers release pro-inflammatory molecules. SP and Neurokinin A, members of the tachykinin family, are the major contributors of inflammation released by c-fibers. SP preferentially binds to the NK1 receptor and NKA to the NK2 receptor. Hemokinin-1, the most recent discovered tachykinin, also preferentially binds with the NK1 receptor [169, 170]. Unlike SP that is expressed heavily in areas of the brain and in peripheral c-fibers [171], HK-1 is widely expressed throughout immune and inflammatory cells [172-176]. As such, HK-1 has necessary roles in their functions [169, 177-181]. Even though SP and HK-1 preferentially bind to the NK1r, several studies have found different effect induced by SP and HK-1 [177, 182-184]. Recently, HK-1 was found to potentially act as a NK1 antagonist where SP is present [185], although the existence of a proper HK-1 receptor has been suggested [184].
Respiration rate is predominantly controlled by PCO₂ in the blood. An increase of 1 mm Hg of arterial PCO₂ will increase the respiration rate by 30 percent in humans [21]. This is an example of the sensitivity most terrestrial mammals have for regulating their arterial PCO₂. However, the same level of sensitivity might not be advantageous for naked mole-rats who live in a chronic hypercapnia. If naked mole-rats have comparable sensitivity to elevated CO₂, then this would imply that they live normally in a state of hyperventilation.

We predicted that naked mole-rats would have reduced physiological responses to hypercapnia. We measured respiration rate in moderate hypercapnia at levels representing those in their natural habitat. Additionally we measured respiration rate during extreme hypercapnia exposure to determine if respiration rate is modulated in naked mole-rats at these levels. Mice were used as a comparison species to the naked mole-rats because they have similar physiological responses to hypercapnia with other mammals and are closer to the naked mole-rat body size.

Due to the naked mole-rats known cutaneous adaptation of acid inhibition of their Nav1.7 channels and reduced or absent SP, hypercapnia-induced pulmonary edema was measured in the naked mole-rats. Part of the inflammatory response in the lungs of most mammals is the release of pro-inflammatory molecules SP+HK-1 into the alveolar airspace. The absence of SP in the naked mole-rat cutaneous c-fibers and the c-fiber acid insensitivity may indicate abnormal expression or release of SP in their lungs. Therefore, we measured the release of SP+HK-1 in their lungs at extreme hypercapnia in vivo.
2.3 Materials and Methods

2.3.1 Animals

The present study used BL/6 mice that were bred from stock obtained from Charles River Laboratories, Wilmington, MA. All mice were housed at a constant temperature at 22°C (+/-2) in a 12-hour light-dark cycle. Food and water were available ad libitum. The naked mole-rats were born and maintained in our colonies at the University of Illinois at Chicago. Naked mole-rats were kept at 29°C (+/-1) and fed ad libitum. Mice and naked mole-rats were of both sexes. The mice were at least 3 months old and no older than one year. The naked mole-rats were older than 1 year. Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

2.3.2 Environmental chamber

The environmental chamber for isolating each animal in a set gas mixture of oxygen, nitrogen, and carbon dioxide was a circular clear plastic container (16 cm X 5.5 cm) with a lid that sealed and locked to the container. On one end of the container lid, a small hole had the gas tube fed into it. The opposite end of the lid had a small open hole, functioning as the exhaust for the container. Gas after passing through regulators was mixed together before entering the environmental chamber. Between the oxygen regulator and the environment chamber was an ISO vaporizer that was used to add ISO to the oxygen stream for some experiments.

2.3.3 Respiration rate

The environmental chamber was washed with distilled water, dried, and wiped with 70% ethanol before each animal was placed into the chamber. Gas was turned on
at a 20% O2/80% N₂ ratio at 4 liters/minute. Four animals were used for each testing condition and no animals were used in more than one condition. The environmental chamber with the animal inside was placed on stilts that were 30 cm tall. A video camera was placed underneath facing up towards the underside of the environmental chamber. Recording was started five minutes into setting up the animal into the chamber. After 5 minutes of recording, the nitrogen flow was reduced equal to the CO₂ flow increase. CO₂ concentrations used were 5%, 10%, 20%, and 40%. Recording was carried out for an additional 15 minutes. At the end of the recording, animals were removed and the gas was turned off. The recordings were later analyzed.

2.3.4 **Respiration rate recording analysis**

Each recording was played at half speed and number of breaths for the first 20 seconds of every minute was counted. These 20 seconds were used to calculate the breaths per minute. The average of the first 5 minutes of recordings corresponded to the 0% CO₂ condition. Thus the first 5 respiration rate calculations were averaged together to get a basal respiration rate for each animal. The next 15 respiration rate calculations corresponding to the hypercapnia exposure period were averaged together to determine the respiration rate in the hypercapnia condition.

2.3.5 **Pulmonary edema measurement**

Three animals were used for each testing condition. No animal was used more than once. As it was for the respiration rate, the environmental chamber was washed with distilled water, dried, and wiped with 70% ethanol before each animal was placed into the chamber. Gas was turned on at a 20% O₂/80% N₂ ratio at 4 liters/minute. The N₂ flow was reduced by the amount of CO₂ increase. CO₂ concentrations used were 0,
10, 20, 30, and 50%. After 10 minutes, animals were anesthetized with ISO for 90 seconds and the lungs were removed from their chest. The lobes were individually removed from the trachea and placed into saline. Each lobe was then patted dry using a Kimwipe and all lobes from an animal were weighed together in an aluminum foil weigh boat. The weigh boat with the lungs was placed into an oven set to 55 degrees Celsius for drying. After 24 hours, the lungs were reweighed. The ratio of wet weight to dry weights was calculated.

2.3.6 **Lung lavage**

Three animals were used for each condition. Animals were exposed to 10 minutes of a gas mixture followed by 20% O₂/80% N₂ with 2% ISO. After the animal was anesthetized, it was transferred from the environmental chamber to a respiratory mask that had the same 20% O₂/80% N₂ with 2% ISO flowing through it. A tracheotomy was done and a 15 gauge needle was inserted into the tracheostomy. Surgical thread was tied around the combined trachea and needle to create a seal. One ml of cold PBS was infused into the lung and then aspirated. Peptidase inhibitors, phenylmethysulfonyl fluoride (PMSF, 1 mM) and DL-thiorphan(5mg/ml), were added to the lavage fluid as was done by a previous study [115]. Samples were then centrifuged for 10 minutes at 1600 rpm. The supernant was removed and stored at -20 degree Celsius for later testing.

2.3.7 **Enzyme-linked Immunosorbent Assay**

Lung lavage fluid SP+HK-1 content was measured using a commercially available enzyme-linked immunosorbent assays (ELISA) in accordance with the manufacturer’s directions (R&D System, Mn, USA).
2.3.8 **Statistical analysis**

The respiration rate percent increase due to CO$_2$ treatment was compared using the one-way ANOVA test. Tukey post hoc test was used to determine significance between respiratory rate groups. One-way ANOVA followed by Tukey post hoc test was used to test for significance between wet/dry lung weight ratios at 0% CO$_2$ and 10, 15, 20, 30, and 50% treatments for both naked mole-rats and mice. No statistical analysis was done for SP+HK-1 lung lavage concentration between naked mole-rats and mice as no detectable amount was found in naked mole-rat lavage fluid.

2.4 **Results**

2.4.1 **Respiration rate**

Respiration rates for mice at 0 and 5% CO$_2$ and naked mole-rats at 0, 5, 10, 20, and 40% CO$_2$ concentration was done (Figure 2). Consistent with known mouse respiratory responses to CO$_2$, mouse respiration rate significantly increased by 29.9% (SE=5.38) at 5% CO$_2$ and 31.5% (SE=4.01) at 10% CO$_2$. No significant change was observed for mice between 5% and 10% CO$_2$ (t(6)=0.3371, p=0.7475). Naked mole-rats had significant difference among the CO$_2$ treatments (F(3,12)=5.846, p=0.0106). Naked mole-rats had no significant change between 0% and 5% or 0% and 10% CO$_2$ (at p<0.05). A significant increased respiration rate was observed for the naked mole-rats at 20% CO$_2$ compared to 5% CO$_2$ (p=0.0248).
Figure 2. Respiration rate of naked mole-rats and mice at increasing CO₂ concentrations. Naked mole-rats (NMRs) did not have a significant increased respiratory rate response at 5% and 10% CO₂. Naked mole-rats significant increased their respiratory rate at 20% CO₂. Error bars are standard error of means. *=p<0.05 (one-way ANOVA, Tukey post hoc test, N = 4).
2.4.2 **Lung edema**

The wet/dry weight ratio of mouse and naked mole-rat lung tissue was calculated after each animal was exposed to one of the following CO$_2$ percent concentrations: 0, 20, 33, and 50 (Figure 3). A significant difference existed amongst the mice treatment groups (F(3,8)=87.76, p<0.0001). Tukey post hoc analysis revealed significant difference between 0% CO$_2$ (M=4.384, SD=0.2185) and 20 (M=7.738, SD=0.1936), 33 (M=7.079, SD=0.2232), and 50% CO$_2$ (M=7.265, SD=0.4223) (p<0.0001). No change was observed between 0% and 10% for either mouse or naked mole-rat. No significant difference was observed for the lung weight ratio at all concentrations of CO$_2$ exposure for the naked mole-rats (F(3,8)=1.473, p=0.2934).
Figure 3. Lung weight ratio of naked mole-rats and mice after hypercapnia treatments. Mice had significant increased wet/dry lung weight ratio at 20%, 33%, and 50% compared to 0% CO₂. No significant difference was found between naked mole-rat (NMR) CO₂ treatments. Error bars are standard error of means. ****=p<0.0001 (one-way ANOVA, Tukey post hoc test, N = 3).
2.4.3 Lung lavage

An ELISA kit was used to quantify the SP+HK-1 amount released in the lungs of the mice and naked mole-rats (Figure 4). SP+HK-1 was measured in the naked mole-rats and mice at 0% and 30% CO₂. A substantial and significant increase was observed for the mice at 0% CO₂ (M=0.7217, SD=0.2021) and 30% CO₂ (M=39.33, SD=19.29) (t(4)=3.466, p=0.0257). There was no detectable amount of SP+HK-1 at either level of CO₂ in the naked mole-rats.
Figure 4. SP+HK-1 concentrations in lung lavage fluid of naked mole-rats and mice after CO₂ treatments. Mice had a significant increase of SP+HK-1 in lung lavage fluid after the 30% CO₂ treatment compared to 0% CO₂. No detectable amount of SP+HK-1 was found in naked mole-rat (NMR) lavage fluid after 0% or 30% CO₂ treatments. Error bars are standard error of means. n.d. = not detected.
2.5 Discussion

Naked mole-rats demonstrate a remarkable resistance to the effects of hypercapnia. Resistance to moderate hypercapnia-induced increase in respiration rate may reflect their insensitivity to hypercapnia in their natural environment. Likewise, absence of edema from extreme hypercapnia may represent this adaptation. Undetectable hypercapnia-induced SP and HK-1 in alveolar lavage fluid could explain the absence of edema. Extreme hypercapnia would reduce blood pH to acidic levels, and the greater inactivation of their Nav1.7 channels could prevent the activation of their c-fibers from releasing SP. However, undetectable basal levels may indicate a more involved adaptation to hypercapnia than just their c-fiber insensitivity to acid. These results set naked mole-rats as a model organism to further our understanding of CO₂ detection and the mechanisms involved in pulmonary inflammation.

Respiration is controlled predominantly by a network of medulla oblongata nuclei in the brainstem. This network is modulated by sensory input of the arterial PCO₂ changes. A number of central and peripheral sensitive cells have been identified, but the sensory transducer that detects PCO₂ remains elusive. The coupled acidosis of increased PCO₂ is considered to be the stimulus that activates sensory input of the respiratory network. Studies have induced increased respiration rate by increasing blood acidity. However, increasing blood PCO₂ while maintaining constant pH also increases respiration. Decreasing blood pH could shift the CO₂ equilibrium towards increased free CO₂. Thus, teasing out the physiological effects of CO₂ from its coupled acidosis has proven difficult. We propose that studying the respiration network in naked mole-rats can reveal critical aspects to further our understanding of respiration control.
The SP found in alveolar airspaces of most mammals has been considered to be from peptidergic c-fibers innervating the lungs. There are two fiber populations, one originating from the vagus [186], the other from the DRG [187]. Naked mole-rats lack SP in their DRG and it is for this reason and their insensitivity to noxious stimuli that we examined SP release in the lungs. Substance P positive neuronal cells have been identified within the lungs of naked mole-rats, presumably of vagal origin (unpublished data). Despite SP presence in lung tissue, we did not detect any SP+HK-1 in naked mole-rat lavage fluid. In respect to the naked mole-rat vagal c-fibers, this may indicate these cells are not stimulated as they are in other mammals. Alternatively, the DRG c-fibers could be the fibers that would normally release SP in other mammals but the naked mole-rat DRG c-fibers do not express SP. Comparing functional differences of the pulmonary nerve fibers between species is problematic because of different innervation patterns between species. Several studies have tried to demonstrate that the SP in the lavage fluid of other mammals is from these c-fibers. Over stimulating these fibers can cause desensitization or degeneration. This results in reduction or absence of SP in lavage fluid. However, these experimental conditions could have effects on other pulmonary fibers beyond the c-fibers.

We did not differentiate the release of SP and HK-1 in the lungs since both are endogenous NK1r agonists. In another study (see Ch 3), we found HK-1 as the dominant NK1 agonist released in the murine lungs. HK-1 is known to be expressed in immune cells and alveolar macrophages but has not been found in neurons. This eliminates HK-1 lung lavage fluid as not originating from pulmonary c-fibers. SP is expressed to a lesser extent in alveolar macrophages than HK-1. These expression
differences of HK-1 and SP in alveolar macrophages may be related to the dominance of HK-1 in alveolar lavage fluid. The absence of detectable alveolar SP or HK-1 in naked mole-rat, even at basal levels, suggests a common source. Alveolar macrophages are potential candidates as they express SP and HK-1 in humans and mice.

Recently, alveolar macrophages have been implicated as the initiator of inflammation induced by hypoxia [188]. Acute alveolar hypoxia has also been demonstrated to induce systemic inflammation, independent of the systemic PO2. Often, hypercapnia occurs with hypoxia as is the case for naked mole-rats. Hypercapnia has been shown to induce gene expression changes in alveolar macrophages [31] and independent of intracellular and extracellular pH [35]. Therefore, a critical difference may exist in the naked mole-rat alveolar macrophages that may make them desensitized to hypercapnia or perhaps lack expression of SP+HK-1.

Substance P is only still considered to be a neuropeptide for historical purposes. SP is expressed by numerous immune cells. HK-1 is also expressed in immune cells but to a much greater amount. HK-1 is necessary for the maturation of B cells and enhances antibody production [177, 181, 189]. The naked mole-rat is considered to have a strong immune system [190] but no cellular immune functions of the naked mole-rat have been studied thus far. Naked mole-rats infected with a recombinant herpes simplex virus type 1 show 100% mortality that was avirulent in mice. This was suggested to be in part from the absence of SP expression in naked mole-rat cutaneous c-fibers [191]. However, the present study indicates a greater deficit for the naked mole-rat immune system. The frequency of alveolar macrophages is disputed as two
studies have described them as numerous [192] and rare [193]. In either case, naked mole-rats do have alveolar macrophages. The absence of expressed SP in cutaneous c-fibers of naked mole-rats suggests the possibility that naked mole-rat macrophages do not express SP+HK-1, thereby explaining the absence of basal SP+HK-1 in lavage fluid. Such difference would be remarkable as SP and HK-1 are encoded by two separate genes.

Naked mole rats have several physiological differences from other terrestrial animals that make them well suited for their subterranean environment. This study adds to the known adaptations they possess. With the recent effort in sequencing the naked mole rat genome, more detailed explanations can be discovered for these adaptations. Future studies could look for expression of HK-1 in the naked mole-rat lungs and other tissue. Differences of SP+HK-1 expression could have substantial impact of the naked mole-rat immune system development and regulation. This can further our understanding the relationship between the nervous and immune systems in vertebrates. Additionally, research in naked mole-rat CO2 detection can lead to insights of CO2 detection in mammals.
3. HYPERCAPNIA-INDUCED PULMONARY EDEMA EXACERBATION BY TAC1 KNOCKOUT MICE AND INHIBITION BY ISOFLURANE.

3.1 Abstract

Extreme hypercapnia induces pulmonary edema. The sensing of hypercapnia in lungs was thought to be by pulmonary c-fibers that detect the hypercapnia-induced tissue acidosis. To better understand the role c-fibers have in hypercapnia-induced pulmonary edema, we exposed mice to several levels of extreme hypercapnia and measured the SP levels of the lung airways. We reduced the acid sensitivity of pulmonary c-fibers by using mice deficient in TrpV1 or TrpV1 and ASIC3 channels. Isoflurane, known to attenuate pulmonary c-fibers to stimuli, was added to the hypercapnia to determine if anesthetic can inhibit hypercapnia-induced pulmonary edema. We found that hypercapnia at 15% CO₂ and above but not 10% and below caused increasing severity of pulmonary edema. The combined quantity of SP and hemokinin-1 in lavage fluid matched the rise of edema from increasing CO₂ concentrations. Tac1KO mice had increased level of edema than WT mice but no difference was observed for TrpV1KO or TrpV1KO/ASIC3KO mice. Similar levels of HK-1 in lavage fluid of tac1KO mice compared to WT were observed after hypercapnia, indicating a dominance of HK-1 alveolar release in mice. Concurrent exposure of mice to ISO with hypercapnia attenuated the hypercapnia-induced pulmonary edema and SP+HK-1 increase in lavage fluid. These results indicate pulmonary c-fibers may not be the instigators of hypercapnia-induced pulmonary edema.

3.2 Introduction
Retention of CO₂ in body tissue is a frequent, debilitating symptom in patients with severe chronic pulmonary diseases. The elevated CO₂ can induce several pathophysiological changes presumably from the resulting tissue acidosis. CO₂ is quickly and reversibly converted to HCO₃⁻ and protons and the free protons make the tissue acidic. Acid has been demonstrated by several studies to activate c-fibers [194, 195] and these fibers release peptides that promote pulmonary inflammation [196]. The ability of c-fibers to detect acid is mediated by ion channels that respond to protons.

There are a multitude of acid transducers found on sensory neurons [197]. Evidence suggests there are two distinct ion channel populations responsible for the c-fiber acid sensitivity. C-fibers have two acid-induced electrophysiological currents. The first is a rapidly inactivated Na⁺ influx at pH slightly lower than normal homeostatic level. The second is a mixed Ca²⁺/Na⁺ influx occurring at more acidic levels. The ASIC family of channels seems to be responsible for the first and the second by TrpV1 receptor. This implies that the TrpV1 and the ASIC channels are responsible for most of the c-fiber acid sensitivity. Acid chemosensitive c-fibers are necessary for the inflammatory response to noxious stimuli because they secrete the pro-inflammatory molecules. Of those secreted, tachykinins have many effects.

Tachykinins have received much attention due to their critical role in pain perception and tissue inflammation. They are encoded by three genes, tac1, tac3, and tac4. Tac1 encodes the splice variants substance P and neurokinin A. Neurokinin B and hemokinin-1 are encoded by tac3 and tac4 respectively. In humans, there are 4 splice variants of HK-1 whereas one conserved variant exists in rats and mice. Tachykinins constitute one of the largest families of neuropeptides. They are expressed
abundantly throughout an animal, with specific cell type expressions. Substance P is predominantly expressed in neuronal tissue and in the PNS this is in peptidergic c-fibers. The physiological effects they exert are determined in part by three g-protein-coupled tachykinin receptors: NK1, NK2, and NK3.

Every tachykinin binds to all three of the NK receptors. However, each has a preferred receptor based on its higher affinity for each receptor. SP and HK-1 preferentially binds with NK1, NK A with NK2, and NK B with NK3 receptor. The function of the tachykinins has largely been determined by observing the effects of each tachykinin and blocking it with an antagonist to its corresponding receptor. However, this does not work with SP and HK-1 that both bind preferentially to the NK1 receptor. Recently, HK-1 has been demonstrated to have a competitive antagonist effect to SP [185]. Since this is the first demonstration of competition between SP and HK-1, it is unknown if HK-1 has a competitive inhibitory effect on SP always where the two coexist.

Substance P is the most studied tachykinin and is considered to be one of the major initiators of neurogenic inflammation. In the CNS, it is found in high concentrations in the medulla and is involved in the vomiting pathway and respiration [198, 199]. In the PNS, peptidergic c-fibers will release SP, acting as a potent vasodilator, smooth muscle contractor, and a leukocyte chemoattractant. The wide assortment of its effects is due to the NK1r expression in epithelial, endothelial, macrophages, lymphocytes, neutrophils, dendritic cells, mast cells [200-202]. Alternatively, the other endogenous NK1r ligand HK-1 is expressed in endothelial, inflammatory and immune, glial, and placental cells [172, 176, 177, 180]. Its role is thought to be similar to SP as activating the NK1 receptor by either ligand should elicit
the same physiological response. However, several studies have found different effects between the two. The discovery of HK-1 has produced a renewed interest in trying to find other yet to be discovered tachykinins and their receptors.

Of the tachykinins, SP, NK A, NK B, and HK-1 are all expressed within lung tissue. SP has been implicated in a number of neurogenic-induced pulmonary inflammation studies. This is due to observed increase of SP release in the lungs after noxious stimuli and the inflammation is inhibited by NK1r antagonists [203, 204]. In asthmatic patients, lung inflammation is potentiated that corresponds with SP levels in their airways.

The purpose of this study was to determine the effect hypercapnia on SP+HK-1 levels in alveolar airspace, and whether reducing the c-fiber acid sensitivity can attenuate hypercapnia-induced pulmonary edema. These goals were pursued by determining the following: (1) the dose response of hypercapnia on pulmonary edema formation, (2) amount of hypercapnia-induced SP release into the lung airspace, (3) the effect of tac1 gene elimination has on hypercapnia-induced edema, (4) the effect that gene elimination of the acid-sensing ion channels TrpV1 and ASIC3 have on hypercapnia-induced pulmonary edema, and (5) the effect of ISO has on hypercapnia-induced pulmonary edema and SP+HK-1 release.

3.3 Materials and Methods

3.3.1 Animals

All mice were housed at a constant temperature at 22°C (+/-2) in a 12-hour light-dark cycle. Food and water were available ad libitum. Wild-type BL/6 mice used in the
experiments were bred from stock obtained from Charles River Laboratories, Wilmington, MA. The tac1KO mice were obtained from Jackson laboratories, Bar Harbor, ME. The TrpV1KO and the TrpV1/ASIC3KO mice were obtained from collaborators. All mice were at least 3 months old and no older than one year. Genetic verification of knockout mice was done by Transnetyx, Cordova, TN. Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

3.3.2 Environmental chamber

The environmental chamber was a circular plastic container (16 cm X 5.5 cm) with a lid that sealed and locked to the container. On one end of the container lid, a small hole had the gas tube fed into it. The opposite end of the lid had a small open hole, functioning as the exhaust for the container. The air tube fed from a gas mixing chamber to the environmental chamber. Gas regulators were connected via tubing to the gas mixing chamber on outlet end, and the gas tanks on the inlet end. The gasses used were oxygen, nitrogen, and carbon dioxide. Between the oxygen regulator and the environmental chamber was an ISO vaporizer that was used to add ISO to the oxygen stream for some experiments.

3.3.3 Respiration rate

The environmental chamber was washed with distilled water, dried, and wiped with 70% ethanol before each animal placed into the chamber. Gas was turned on at a 20% O2/80% N2 ratio at 4 liters/minute. The environmental chamber with the animal inside was placed on stilts that were 30 cm tall. A video camera was placed underneath facing up towards the underside of the environmental chamber. Four animals were
used per group. Recording was started five minutes into setting up the animal into the chamber. After 5 minutes of recording, the nitrogen flow was reduced by the amount CO₂ flow was set to. CO₂ concentrations used were 5%, 10%, 20%, and 40%. Recording was carried out for an additional 15 minutes. At the end of the recording, animals were removed and the gas was turned off.

3.3.4 Respiration rate recording analysis

Each recording was played at half speed and scored for twenty seconds starting at the beginning of every minute of the recording. The number of breaths was counted and then multiplied by 3 to obtain the breaths per minute. The average of the first 5 minutes of recordings corresponded to the 0% CO₂ duration. Thus the first 5 respiration rate calculations were averaged together to get a basal respiration rate for each animal. The next 15 respiration rate calculations corresponding to the hypercapnia exposure period were averaged together to determine the respiration rate in each hypercapnia condition.

3.3.5 Pulmonary edema measurement

As it was for the respiration rate, the environmental chamber was washed with distilled water, dried, and wiped with 70% ethanol before each animal was placed into the chamber. Gas was turned on at a 20% O₂/80% N₂ ratio at 4 liters/minute. The environmental chamber was placed onto a table. The N₂ flow was reduced as CO₂ was increased by the same amount, thereby maintaining a 4 liter/minute flow rate. CO₂ concentrations used were 0, 10, 20, 30, and 50. After 10 minutes, animals were anesthetized with 2% ISO for 90 seconds and the lungs were removed from their chest. The lobes were individually removed from the trachea and placed into saline. Each
lobe was then patted dry using a Kimwipe and all lobes from an animal were weighed together in an aluminum foil weigh boat. The weigh boat with the lungs was placed into an oven set to 55 degrees Celsius for drying. After 24 hours, the lungs were reweighed. The ratio of the wet weight to dry weight was then calculated.

3.3.6 Lung lavage

A group size of 8 animals was used for WT and each type of KO mice. Four mice were used per group of the 30% CO₂ with 2% ISO part of the study. After 10 minutes of gas exposure, the air was restored to a 20% O₂/80% N₂ and 2% ISO was added to the gas mixture. After the animal was anesthetized, it was transferred from the environmental chamber to a respiratory mask with the gas mixture attached to it. A tracheotomy was done and a 15 gauge needle was inserted into the tracheostomy. Surgical thread was tied around the combined trachea and needle to create a seal. One ml cold PBS was infused into the lung and then aspirated. Peptidase inhibitors, phenylmethylsulfonyl fluoride (PMSF, 1 mM) and DL-thiorphan(5mg/ml), were added to the lavage fluid as was done by a previous study [115]. Samples were then centrifuged for 10 minutes at 1600 rpm. The supernant was removed and stored at -20 degree Celsius for later testing.

3.3.7 Enzyme-linked immunosorbent assay

Lung lavage fluid concentration of SP / HK-1 was measured using a commercially available enzyme-linked immunosorbent assays (ELISA) in accordance with the manufacturer’s directions (R&D System, Mn, USA).

3.3.8 Statistical analysis
Respiration rates were compared between tac1KO, TrpV1KO, and TrpV1KO/ASIC3KO mice and WT mice using the one-way ANOVA with Dunnet multiple comparisons test. Lung wet/dry weight ratios were compared at 0, 10, 15, 20, 30, and 50% CO2 using the one-way ANOVA test and 0% CO2 was compared post hoc to the other CO2 treatments using Tukey post hoc test. Tac1KO, TrpV1KO, and TrpV1KO/ASIC3KO mice lung wet/dry weight ratios were compared to WT mice ratios using two-way ANOVA with Dunnet’s post hoc analysis. The SP+HK-1 lung lavage concentration at 10, 15, 20, 30, and 50% CO2 was compared to 0% CO2 using one-way ANOVA test with Dunnett’s multiple comparisons test. Lung lavage fluid SP+HK-1 concentrations of TrpV1KO and tac1KO mice were compared to WT mice using multiple t test corrected with Holm-Sidak method of multiple comparisons. Both the lung wet/dry weight ratios and the SP+HK-1 lung lavage fluid concentration of WT mice with 0%, 30% CO2, and 30% CO2 with ISO treatments were compared using the one-way ANOVA with Tukey post hoc test.

3.4 Results

3.4.1 Respiration rate

Mice were exposed to 0% CO2 followed by either 5% or 10% CO2 (Figure 5). A significant increase in respiration rate was observed between tac1KO (M=378.2, SD=24.36) mice compared to WT (M=304.6, SD=29.29) mice at 10% CO2; F(3,12)=4.547, p=0.0238.
Figure 5. Respiration rates of wild-type, \(\text{tac}^{1\text{KO}}\), \(\text{TrpV1}^{\text{KO}}\), and \(\text{TrpV1}^{\text{KO}}/\text{ASIC3}^{\text{KO}}\) mice during CO\(_2\) treatments. Only a significant difference was found between the \(\text{tac}^{1\text{KO}}\) mice and WT mice at 10\% CO\(_2\). Error bars are standard error of means. * = p<0.05 (One-way ANOVA, Dunnet post hoc test, N = 4).
3.4.2 **Lung edema**

Wild-type, TrpV1\(^{KO}\) and Tac1\(^{KO}\) mice were exposed to increasing CO\(_2\) concentrations at 0, 10, 15, 20, 30, and 50% CO\(_2\) (Figure 6). As was previously observed, WT mice had a significant increase in lung wet/dry ratios from basal levels at 15% CO\(_2\). This progressively increased from increasing CO\(_2\) concentrations up to 30% CO\(_2\). A significant decrease was found from 30 to 50% CO\(_2\).

No significant difference (p<0.05) was observed for wet/dry ratio at each CO\(_2\) concentration between WT and TrpV1\(^{KO}\) and TrpV1\(^{KO}/ASIC3^{KO}\) mice (Figure 6C and D). A similar increase and then decrease was observed for the tac1\(^{KO}\) mice as was observed with the WT mice (Figure 6B). However, a significant increased wet/dry ratio was observed between WT and tac1\(^{KO}\) mice at 15, 20, and 30% CO\(_2\) (F(3,168)=12.88, p<0.0001).

When ISO was added to the duration of 30% CO\(_2\) treatment for wild-mice (Figure 7), a significant difference existed amongst the wet/dry lung weight ratios (F(2,13)=77.49, p<0.0001). Tukey post hoc analysis found a significant attenuation of the CO\(_2\) with ISO (M=4.97, SD=0.2612) compared to CO\(_2\) (M=6.832, SD=0.4361) treatments (p<0.0001). Isoflurane did not abolished the hypercapnia-induced edema as a significant difference remained between 0% CO\(_2\) (M=4.483, SD=0.1101) and 30% CO\(_2\) with ISO (p=0.0408).
Figure 6. Lung weight ratios of mice after hypercapnia treatments. A. Significant increase was observed in WT mice for 15, 20, 30 and 50% CO2. B. Significant difference existed between tac1 mice and WT mice at 15, 20, and 30% CO2. C. No significant difference existed between TrpV1KO and WT mice at any CO2 level. D. No significant difference existed between TrpV1KO/ASIC3KO mice at any CO2 level. Error bars are standard error of means. * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 (One-way ANOVA, Dunnet post hoc test, N = 8).
Figure 7. Lung weight ratios of mice after hypercapnia and hypercapnia with isoflurane. The addition of ISO with the CO₂ treatment significantly attenuated the induced edema but was not abolished. Error bars are standard error of means. * = p<0.05; **** = p<0.0001 (One-way ANOVA; Tukey post hoc test; N = 4).
3.4.3 **Lung lavage of wild-type mice**

Lung lavage fluid was measured for SP+HK-1 content using an ELISA. A CO\textsubscript{2} dose response of SP/K-1 lung airway content was done in WT mice (Figure 8). This is the first publication of a CO\textsubscript{2} dose response for SP+HK-1 in pulmonary airways. A significant difference existed amongst the treatment groups (F(4, 10)=10.07, p=0.0016). A significant difference was found between mice at 0% CO\textsubscript{2} (M=0.7217, 95% CI [0.22, 1.22]) and 50% CO\textsubscript{2} (M=81.9, 95% CI [-15.18, 179]). Due to the small sample size, no other significance was detected (at p<0.05). However, the mean SP+HK-1 did show a trend of increased SP+HK-1 concentrations at 15 (M=2.973, SD=1.558) and 30% (M=39.33, SD=19.29) CO\textsubscript{2} but not at 10% (M=0.7370, SD=0.2183) CO\textsubscript{2}. Despite a decrease in wet/dry ratio of mice from 30% to 50% CO\textsubscript{2}, an even greater amount of SP+HK-1 was observed at 50% than 30% CO\textsubscript{2}.
Figure 8. SP+HK-1 concentration in lung lavage fluid of wild-type mice after CO₂ treatments. Means are reported above each treatment group. Significant difference exists between 0% and 50% CO₂. Error bars are standard error of means. * = p<0.01 (one-way ANOVA, Tukey post hoc test; N = 3).
3.4.4 **Lung lavage of tac1<sup>KO</sup> and TrpV1<sup>KO</sup> mice**

The SP+HK-1 content of lung lavage fluid was measured in tac1<sup>KO</sup> (Figure 9A) and TrpV1<sup>KO</sup> (Figure 9B) mice exposed to 0% and 30% CO<sub>2</sub>. Due to the lack of SP expressed by tac1<sup>KO</sup> mice, the concentration measured in these mice were determined to be only HK-1. No significant difference was measured between tac1<sup>KO</sup> and TrpV1<sup>KO</sup> compared to WT (F(2,12)=1.067, at p<0.05). This indicated that most SP+HK-1 measured in WT mice is actually just HK-1 and absence of TrpV1 had no significant effect on SP+HK-1 released in the lung (at p<0.05).
Figure 9. SP+HK-1 concentration in lung lavage fluid of tac1\(^{\text{KO}}\) and TrpV1\(^{\text{KO}}\) mice after CO\(_2\) treatments. A. No significant difference was observed between tac1\(^{\text{KO}}\) and WT mice. B. No significant difference was observed between TrpV1\(^{\text{KO}}\) and WT mice. Error bars are standard error of means. N = 3.
3.4.5  **Isoflurane attenuation of hypercapnia-induced SP+HK-1 release**

Isoflurane was added to the course of the 30% CO₂ exposure of WT mice. A significant decrease of SP+HK-1 concentration in lung lavage fluid (Figure 10) was also observed amongst the groups (F(2,6)=11.66, p=0.0086). Tukey post hoc analysis revealed significant difference between 0% (M=0.7127, SD=0.2021) and 30% (M=39.33, SD=19.29) CO₂ (p=0.0129) but not between 30% CO₂ with ISO (M=1.770, SD=1.027) (at p<0.05).
Figure 10. SP+HK-1 concentration in lung lavage fluid with CO₂ and CO₂ with isoflurane. ISO addition to CO₂ treatment significantly reduced SP+HK-1 concentration in lung lavage fluid. Error bars are standard error of means. * = p<0.05 (One-way ANOVA; Tukey post hoc test; N = 3).
3.5 **Discussion**

Pulmonary c-fibers are able to detect hypercapnia-induced tissue acidosis. However, the present study indicates that pulmonary c-fibers may not be involved in the hypercapnia-induced pulmonary edema. Additionally, this study demonstrates that hypercapnia induces HK-1 and not SP release in large amounts into the alveolar airspace. The attenuation of both pulmonary edema and release of SP+HK-1 by ISO during hypercapnia treatment implicates that hypercapnia-induced pulmonary edema is mediated by activation of pulmonary cellular receptors.

Acidosis is a common feature of microenvironments of local inflammatory sites. It induces inflammatory pain through acid-sensing molecules on sensory c-fibers. Acidosis also potentiates c-fibers to other irritants [205]. The present study utilized the reduced c-fiber acid sensitivity of TrpV1\(^{KO}\) and TrpV1\(^{KO}/ASIC3^{KO}\) mice. No difference of lung wet/dry weight ratios were observed between these and WT mice from hypercapnia-induced pulmonary edema. Therefore, this result suggests that acid sensing by the pulmonary c-fibers does not mediate the hypercapnia-induced pulmonary edema.

All tachykinins are found in the lungs as well as their corresponding receptors NK1, NK2, and NK3. By eliminating the encoding genes of the tachykinins, their receptors, or by adding exogenous tachykinins and selective antagonists, the functions of the tachykinins have been ascertained. To date, SP has not been eliminated without the elimination of the splice variant NKA. Even still, their differential preferred affinities to NK1r and NK2r has enabled their functions to be determined by addition of selective antagonists. Gene elimination of HK-1 has only recently been done [178]. The present
study for the first time demonstrates that HK-1 is the predominant tachykinin agonist associated with hypercapnia-induced pulmonary edema for the NK-1r in the alveolar airspace. Pulmonary c-fibers or AECs have not been found to express HK-1 but HK-1 has been found in alveolar macrophages. We predict that the HK-1 we detected in lung lavage fluid originates from alveolar macrophages at unstimulated states. Previous studies have induced pulmonary physiological effects by administering exogenous SP into airways or cell cultures. We propose that such effects may instead be that of HK-1 in vivo. Even still, their results can still be interpreted as NK1r activation effects.

The mode of action of inhaled anesthetics is not specific and can both excite and inhibit neurons [140]. Inhaled anesthetics [206], intravenous barbituates [207], and opioids [208] are known to reduce sensitivities to CO₂. Anesthetics bind and deactivate receptors and ion channels. The present study suggests inhibition of cells due to decreased SP+HK-1 in the alveolar space after adding ISO with the hypercapnia exposure. We do not know what is the specific target cell ISO inhibits to prevent hypercapnia-induced edema and the SP+HK-1 release or whether the HK-1 cells are downstream of the target cells in the inflammatory pathway. Another inhaled anesthetic, sevoflurane, has shown to increased pulmonary leukotriene C4, NO3-, and NO2- in lavage fluid of pigs [209]. The present study demonstrates ISO offers protection against the inflammatory effects of hypercapnia.

We expected attenuation of hypercapnia-induced edema in tac1KO mice. Previous studies have found a decreased pulmonary inflammation induced by sepsis in these mice [210, 211]. They suggested a protective modulatory role of SP/NKA and our results support this protective role. They later observed increased pro- and anti-
inflammatory cytokine expression in these mice [212, 213]. Perhaps in the present study, increased edema observed in tac1KO mice is a result of the increased cytokine production of these mice. Likewise, selective destruction of capsaicin afferents with resiniferatoxin pretreatment enhanced pulmonary inflammation [214]. The effects of eliminating SP content in the lungs may represent increased alveolar macrophage activity.

A similar increase in edema was observed after 72 hours of hyperoxia in NK1rKO mice [215]. Hyperoxia is known to be toxic to lung tissues [216-218]. Consistent with our results, they found no difference between TrpV1KO and WT mice. Hyperoxia and hypercapnia may induce lung inflammation by the same mechanism. Hyperoxia and hypercapnia can induce the formation of ROS and RNS respectively. Therefore, both may cause cellular stress inducing pulmonary edema. If hyperoxia and hypercapnia induces pulmonary edema by the same mechanism, eliminating SP and NK1r influences the involved cells in the same manner. NK1rKO mice in hyperoxia had neutrophil accumulation in the subepithelial space with impaired transversion. This is consistent of studies where activation of NK1r enables this transversion. Perhaps these also have increased cytokine expression seen in tac1KO mice.

The cell inducers of pulmonary inflammation by hypoxia is considered to be alveolar macrophages [188]. Often in environmental and pathophysiological conditions, hypoxia occurs in conjunction with hypercapnia. In alveolar macrophages, CO2 directly suppresses genes involved in regulating inflammation and innate immunity [31]. As such, alveolar macrophages can also be the beginning inducers of hypercapnia-induced
pulmonary edema. Further supporting this is our observation of HK-1 as the dominant NK1r agonist released into the alveolar airways after hypercapnia exposure.

The differential expression of SP and HK-1 demonstrates the function of distinct cell types despite SP and HK-1 shared preference of NK1r binding. As has previously been considered, some of the functions of SP have falsely been applied [175]. Therefore, evaluating the release of each tachykinin from a specific cell type will identify their respective functions. Discovering the complexities of pulmonary cell signaling can aid in the development of treatments of lung diseases.
4. DISCUSSION

4.1 Hypercapnia-Induced Cell Signaling

The premise of hypercapnia-induced pulmonary edema was that hypercapnia acidosis would activate pulmonary c-fibers. These fibers would release SP into the alveolar airspace and induce pulmonary edema. Additionally naked mole-rats, which naturally lives in hypercapnia, would have attenuated response to hypercapnia-induced pulmonary edema. The combined studies in this dissertation indicate that the c-fibers are not the source of SP in the alveolar airways and that their acid sensitivity does not contribute to the hypercapnia-induced edema. The combination of our observed inhibition of hypercapnia-induced pulmonary edema by isoflurane and its attenuated SP+HK-1 release in lung airways during a short 10 minute period indicates that ISO is possibly inhibiting the activation of alveolar macrophages. This is could be due to inhibition of cellular processes of the AECs leading to inhibited communication with alveolar macrophages. Naked mole-rat absence of induced-edema and SP+HK-1 in their lavage fluid indicates that naked mole-rats and ISO inhibition may share a common pathway. Hypercapnia has known anti-inflammatory effects at low to moderate levels [84]. However, extreme concentration of CO$_2$ induces pulmonary edema, demonstrated by others and by this dissertation. How the two can coexist may be explained by their effects on specific cell types and their interactions together. CO$_2$ in tissues is part of a bigger balance of molecular species, some of which are toxic.

High levels of CO$_2$ can lead to increased ROS and RNS. These molecules have numerous damaging effects to cells. In particular, they will cause mitochondrial dysfunction in AECs. Thus O$_2$ consumption and ATP production will decrease [73].
The AECs respond by reducing their energy expenditure by removing their Na/K-ATPase from their plasma membrane [219]. ERK [220], JNK [221], and PKA1a [222] have also been identified in the CO₂-induced down regulation of Na/K-ATPase in AEC membranes. AECs can also respond to oxidative stress by disassembling their tight junctions and adherins junction protein complexes [223]. ROS can also depolymerize epithelial F-actin, perhaps due to F-actin turnover [224]. Perhaps these effects are the AECs means of either trying to protect itself during oxidative stress or preparation of apoptosis. Whether hypercapnia induces directly the AEC barrier permeability has yet to be determined.

Alveolar macrophages are the major sentinel cells of the alveolar airspace that initiates inflammatory reactions to invading organisms and damaged tissue [225]. Alveolar macrophages are in a constant balance of signaling molecules that keeps them in a steady-state mode. However, when activated, they can release many pro-inflammatory cytokines that can activate epithelial permeability. This indicates alveolar macrophages as the potential target of hypercapnia induced inflammation. However, hypercapnia reduces alveolar macrophage inflammatory functions as demonstrated as reduced phagocytic activity and reduced pro-inflammatory gene expression. This coincides with the observed reduced inflammation in studies of inflammation when hypercapnia is added. Perhaps the hypercapnia concentrations in the present dissertation overcome any potential reduction of inflammatory reaction of alveolar macrophages. The current dissertation did not find reduced SP+HK-1 concentrations due to 0% CO₂ and up to 10% CO₂ in the lung. This indicates that 10 minutes of hypercapnia at sub-edema levels does not affect SP+HK-1 release. The increased
hypercapnia-induced edema in tac$^{1}$KO mice coincides with the increased cytokine release by these mice [212, 213]. SP has been suggested to function as an autocrine signal for macrophages [226]. Therefore, eliminating SP may disrupt this regulation and cause macrophages to induce a greater level of inflammation in tac$^{1}$KO mice. Further investigation will be needed to determine what is altered in the alveolar macrophages of these mice.

Naked mole-rat extreme resistance to hypercapnia-induced pulmonary edema may uncover the mechanism by which hypercapnia induces pulmonary edema. A simple explanation would be that naked mole-rats have greater protection from ROS and RNS and thus are protected by hypercapnia effects. This was found to not be the case and they even have reduced levels of antioxidants [227] and their proteins are riddled with oxidative damage [228]. To compensate for the damaged proteins, naked mole-rats have a much higher rate of cellular protein recycling, mediated by their proteasome activity [229].

Recently Naked mole-rats have been found to contain a high-molecular-mass hyaluronan (HA) that is five times larger than the human or mouse form [230]. This is a disaccharide glucuronic acid/N-acetylglucosamine polymer and one of the main components of the extracellular matrix [231]. This polymer is involved in cell signaling and is dependent on its molecular length. Naked mole-rats express low levels of HA degrading enzymes and a unique sequence of HA synthase 2 that is responsible for their HA size.

Naked mole-rats do not have more HA than mice in their lungs [230]. However, high-molecular-mass HA has anti-inflammatory properties and represses mitogenic
signaling [232]. Hyaluronan binds with the CD44 receptor [231, 233, 234]. When HA is fragmented, it induces chemokine gene expression in alveolar macrophages [235]. Naked mole-rat cells have a twofold higher affinity for HA than mouse and human cells, suggesting higher sensitivity of naked mole-rat cells to HA signaling [230]. Intercellular domain of the CD44 receptor interacts with NF2 that mediates contact inhibition of cell proliferation [236].

Hyaluronan and LPS both activate alveolar macrophages through toll-like receptor 4. This is attributed to structural similarities HA and LPS have. LPS is expressed by gram-negative bacteria and is part of the pathogen-associated molecular patterns recognized by immune cells by TLR4 binding [237]. TLR4 activation induces the generation of intracellular ROS. These ROS and cytokines secreted by alveolar macrophages can induce epithelial membrane permeability and neutrophil recruitment. Previous studies have found that ISO pretreatment attenuates the inflammation induced by LPS. Our finding that ISO inhibits the pulmonary inflammation induced by hypercapnia is supported by ISO anti-inflammatory action in macrophages. This is mediated by ISO upregulating Ho-1 activity. In AECs, ISO was found to decrease the transcription level of key tight junction protein ZO-1, indicating ISO having an effect on AECs [238]. This effect may be mediated by ISO activating AECII GABAA receptors [147].

The idea that AECs may act as the inducers of alveolar macrophage inflammatory response from hypercapnia is supported by the anti-inflammatory properties hypercapnia has directly on alveolar macrophages. Hypercapnia (10-20%) reduces LPS-stimulated release of TNF by rat alveolar macrophages [239]. Following
this experiment, the IL-6 and TNF mRNA and protein expression was inhibited by hypercapnia in both mouse and human cell lines. All of this was independent of acidosis [35]. The protection hypercapnia provides in LPS stimulation may be due to the CO₂-sensitive RelB. This member of the NK-kB family is cleaved and translocates to the nucleus in response to hypercapnia. This elevated nuclear RelB correlated with the hypercapnia-induced protection [240].

Hypercapnia may have the same effect as hypoxia in inducing HA fragmentation. Hypoxia can up-regulate hyaluronidase that can increase the transformation of HA into fragments [241]. Naked mole-rats reduced expression of hyaluronidase may counteract the hypoxia-induced up-regulation of hyaluronidase. However, whether hypercapnia up-regulates hyaluronidase or if naked mole-rats up-regulate this enzyme in response to hypoxia or hypercapnia is still unknown. We would predict that naked mole-rats would not have increased hyaluronidase activity in response to hypoxia and hypercapnia, thereby not developing pulmonary inflammation. Likewise, hypercapnia in mice would increase hyaluronidase activity and thus activate a pro-inflammatory response in mouse alveolar macrophages.

The increased size of HA in naked mole-rats is hypothesized to play a role in cancer resistance of naked mole-rats and thus part of their longevity [230]. However, we propose that naked mole-rats have evolved high-molecular-mass HA to inhibit constant inflammatory reaction of hypercapnia in their burrows. An alternative hypothesis is that this is an adaptation to prevent excessive inflammatory reactions to subterranean parasites in their burrows. Naked mole-rats are constantly being exposed to pathogens in their subterranean burrows. Naked mole-rats are known to have
several parasites that are present in their fecal pellets [242-245]. Reduced inflammatory reactions to these pathogens would be advantageous for avoiding a constant state of inflammation in naked mole-rat skin as well as their respiratory system. Naked mole-rats dig with their teeth and could easily breathe in these parasites. They are also coprophagic, thereby re-ingesting the parasites present in their feces. Both hypotheses are supported by our finding that SP+HK-1 was undetectable in naked mole-rat lavage fluid. This could either indicate a strong repression of pro-inflammatory genes and/or their release by naked mole-rat alveolar macrophages. Naked mole-rats may not even express SP+HK-1 in their alveolar macrophages. Whether HA is involved in alveolar macrophage basal levels of SP+HK-1 requires further investigation.

4.2 **Future Directions**

Hypercapnia may be activating inflammation directly by activating alveolar macrophages but the anti-inflammatory properties on alveolar macrophages suggests extracellular signals perhaps from AECs. We propose to determine if hypercapnia induces HA fragmentation as the pro-inflammatory cue. The threshold of hypercapnia-induced pulmonary edema may represent a threshold of HA fragment concentration or HA length before initiating inflammation. Perhaps this would be representative of hyaluronidase activity or ROS concentration. HO-1 activity induced by ISO in AECs is necessary to determine whether ISO only inhibits hypercapnia-induced alveolar macrophage activation. The ISO-induced activation of HO-1 may be receptor specific and thus has no effect on AECs. Alternatively, maybe activation of GABAA receptors is the trigger for the downstream activation of HO-1. If AECs are unaffected by ISO, then
hypercapnia-induced AEC permeability is likely attributed to alveolar macrophage cytokine release.

Naked mole-rats have several adaptations that are attributed to living in their natural environment. Therefore, their HA may not be the only difference that prevents hypercapnia-induced pulmonary edema. The naked mole-rat HA is attributed to being the only difference that explains their resistance to developing cancer [230]. This study also found high-molecular-mass HA in the blind mole-rat. However, blind mole-rats have been found to possess another mechanism to prevent cancer. They have a mutation in their tumor suppressor protein p53 to prevent cells from undergoing apoptosis. To counter over-proliferation, their cells secrete interferon-beta to trigger cells to undergo necrosis [246]. This study did not find this to be the case for naked mole-rats but they did take note of the increased contact inhibition. Blind mole-rats are subterranean and also naturally live in hypoxia/hypercapnia. Thus these animals could be used to confirm that high molecular mass HA protects lungs from hypercapnia-induced inflammation.

Many pathophysiological conditions develop cellular hypoxia/hypercapnia environments and most mammalian cells respond to these environments. As such, these conditions can induce inflammation in a wide variety of tissues. Understanding the complex extracellular and intracellular signaling of inflammation will contribute to developing effective pathophysiological treatments. Naked mole-rats possess a unique set of adaptations to cope with their natural environment. How extensive their differences are could reveal more about the signal cascades of hypercapnia-induced edema.
CITED LITERATURE


103. Bassilana, F., G. Champigny, R. Waldmann, J.R. de Weille, C. Heurteaux, and M. Lazdunski: The acid-sensitive ionic channel subunit ASIC and the mammalian


7/17/2013

Thomas J. Park
Biological Sciences
M/C 067

Dear Dr. Park:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and will be renewed on 7/17/2013.

Title of Application: Effects of Hypoxia, Anoxia, and Hypercapnia on the Naked Mole-Rat
ACC NO: 12-120
Original Protocol Approval: 8/18/2012 (3 year approval with annual continuation required).
Current Approval Period: 7/17/2013 to 7/17/2014

Funding: Portions of this protocol are supported by the funding sources indicated in the table below.
Number of funding sources: 1

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This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Bradley Merrill, PhD
Chair, Animal Care Committee

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