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THE HUMAN CAROTID BODY

in sensing and signaling of oxygen and inflammation

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Cover picture: Human carotid body type 1 cells expressing HIF-2α and β-III-tubulin.

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TILL FARMOR

ABSTRACT

Oxygen is essential for cell survival and global oxygenation is closely monitored in order to protect tissues from hypoxic damage. The carotid body is an important systemic oxygen sensor responding to hypoxia and a multitude of other blood borne stimuli, including inflammatory mediators. Activation of the carotid body by depolarization of the chemosensitive type 1 cells ultimately leads to appropriate ventilatory and cardiovascular responses. While animal carotid body oxygen sensing and signaling is extensively studied, this is essentially uncharacterized in the human carotid body. The aim of this thesis was to investigate the human carotid body in terms of morphology, global and specific expression of oxygen sensing and signaling genes as well as inflammatory response genes. To assess the response to hypoxia, slices of the human carotid body were exposed to acute or prolonged hypoxia and release of ACh, ATP and cytokines was measured. In order to evaluate the human carotid body gene expression profile it was compared to the carotid body gene expression from two mouse strains as well as functionally related tissue transcriptomes.

The human carotid body revealed a specific tissue gene expression profile with enrichment of genes related to angiogenesis and inflammation when compared to brain and adrenal gland and showed a neurological profile in comparison to adrenal gland. Specific expression of genes related to oxygen sensing was demonstrated such as K^+ channels, enzymes synthesizing gaseous messengers and proteins involved in ROS-turnover and energy status. Despite many important similarities to animals, differences exist, for instance in expression of oxygen sensitive K^+ channels. Our data suggest TASK-1, Maxi-K or both as potential oxygen sensitive K^+ channels in the human carotid body. The Maxi-K splice variant ZERO that is more sensitive to hypoxic regulation than the Strex splice variant is the exclusively expressed isoform in the human carotid body.

Furthermore, the human carotid body expresses nicotinic acetylcholine and $GABA_A$ receptor subunits known as important targets for anesthetic agents, as well as purinergic receptors and the dopamine D_2 receptor. With few exceptions this is similar to the receptor map that we demonstrated in mouse carotid bodies. When exposed to acute hypoxia the human carotid body increases the release of ACh and ATP. This confirms findings in animal models where ACh and ATP are considered excitatory neurotransmitters.

Finally, the human carotid body expresses early and late inflammatory mediators as well as corresponding receptors and shows an overexpression of this group of genes compared to functionally related tissues. During prolonged hypoxia the human carotid body moreover releases pro- and anti-inflammatory cytokines.

In conclusion, we have studied human carotid body morphology, gene expression and hypoxia-induced neurotransmitter and cytokine release. We found similarities but also differences in the expression of key genes in oxygen sensing and signaling compared to the animal carotid body. Furthermore, the human carotid body has a structural and functional capacity to play a role in sensing and mediating systemic inflammation.

Key words: carotid body, oxygen sensing, oxygen signaling, chemosensor, hypoxic ventilatory response, hypoxia, gene expression, inflammatory response, receptor, K^+ channels, acetylcholine, ATP, cytokine release.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Presence of nicotinic, purinergic and dopaminergic receptors and the TASK-1 K^+-channel in the mouse carotid body**
Kåhlin J, Eriksson LI, Ebberyd A, Fagerlund MJ
Respiratory Physiology and Neurobiology, 2010, Jul 31;172 (3):122-8

II. **The human carotid body; expression of oxygen sensing and signaling genes of relevance for anesthesia**
Fagerlund MJ*, Kåhlin J*, Ebberyd A, Schulte G, Mkrtchian S, Eriksson LI
Anesthesiology, 2010, Dec; 113(6):1270-9

III. **The human carotid body transcriptome with focus on oxygen sensing and inflammation- a comparative analysis**
Mkrtchian S*, Kåhlin J*, Ebberyd A, Gonzales C, Sanchez D, Balbir A, Kostuk EW, Shirahata M, Fagerlund MJ*, Eriksson LI*
Journal of Physiology, 2012, Aug 15;590(Pt 16):3807-19

IV. **The human carotid body releases acetylcholine, ATP and cytokines in response to acute hypoxia**
Kåhlin J, Mkrtchian S, Ebberyd A, Hammarstedt L, Nordlander B, Yoshitaki T, Kehr J, Prabhakar N, Poellinger L, Fagerlund MJ*, Eriksson LI*
Manuscript

* These authors contributed equally to the paper

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChR	Acetylcholine receptor
AMPK	AMP-activated protein kinase
ASA	American Society of Anesthesiology
BK	Large conductance potassium channel
CB	Carotid body
CBS	Cystathionine β-synthase
cDNA	Complimentary DNA
CHF	Congestive heart failure
CIH	Chronic intermittent hypoxia
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
CPG	Central pattern generator
CSE	Cystathionine γ-lyase
CSN	Carotid sinus nerve
DA	Dopamine
DAPI	4', 6-diamidino-2-phenylindole
ETC	Electron transport chain
$GABA_A$	γ-amino butyric acid A
GDNF	Glial derived neurotrophic factor
GPN	Glossopharyngeal nerve
H_2S	Hydrogen sulfide
HERG	Human ether-à-go-go
HIF	Hypoxia inducible factor
HMGB1	High-mobility group protein B1

HO-2	Heme oxygenase 2
HPLC	High-performance liquid chromatography
HVR	Hypoxic ventilatory response
IL	Interleukin
Iso-OMPA	Tetra-isopropyl-pyrophosphoramide
KO	Knock out
KRS	Krebs Ringer solution
L-NMMA	L-N^G-monomethyl-L-arginine
LPS	Lipopolysaccharide
MAS	Affymetrix microarray suite software
NAC	N-acetyl-cystein
NADPH	Nicotinamide adenine dinucleotide phosphate
NMBA	Neuromuscular blocking agents
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NTS	Nucleus tractus solitarius
OSA	Obstructive sleep apnea
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SNP	Single nucleotide polymorphism
TASK-1	TWIK-related acid sensitive potassium channel 1
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNF α	Tumor necrosis factor α
TWIK	Two-pore inward rectifying potassium channel

INTRODUCTION

"PUT ON YOUR OWN OXYGEN MASK
BEFORE ASSISTING OTHERS"

Oxygen is the key to life. This molecule is vital for survival of cells and tissues in aerobic organisms and consequently, serious oxygen deprivation results in tissue damage or death. Oxygen is acquired through continuous alveolar gas exchange and transported to ultimately reach the mitochondria within the cell, where it is used in a large number of enzymatic reactions, most importantly ATP synthesis through oxidative phosphorylation. Hence, reliable and rapid sensing and signaling of blood oxygen levels is an essential mechanism to protect the body against lack of tissue oxygen.
In healthy humans, arterial oxygenation is a function of ambient partial pressure of oxygen. Hypoventilation due to disease or pharmacological agents, increased oxygen-consumption and ascent to high altitude are examples of circumstances where arterial oxygenation decreases. This deficit of oxygen in blood (hypoxemia) is sensed by specialized oxygen sensing cells. While all cells to a varying extent react to changes in oxygen, these specialized cells can sense oxygen with a global benefit for the organism. This is achieved through physiological reflexes aiming to restore homeostasis in order to avoid hypoxic tissue damage. The carotid bodies (CBs) are the primary oxygen sensors in mammals, strategically located in the carotid bifurcation bilaterally where they monitor arterial blood destined for the brain, therefore sometimes entitled as "watchdogs of the brain".

THE CAROTID BODY

Peripheral oxygen sensing cells are present across the chain of species from fish and amphibians to mammals. However, the distribution, location and the evoked physiological response are variable between and within species. In fish, oxygen sensors can be dispersed along the entire respiratory passage, including the gills. Hypoxia commonly elicits a reflex bradycardia in fish and the chemosensors related to this reflex are localized in the first gill arch in many species[2]. In mammals, the distribution of chemoreceptors is reduced to a primary location along the major arteries[2]. The main peripheral chemoreceptor involved in regulation of breathing is the CB, responsible for the acute hypoxic ventilatory response (HVR) as well as cardiovascular responses. The third branchial arch, a structure homologous to the first gill arch in fish gives rise to the CBs during embryonic maturation. The fourth branchial arch forms the aortic bodies that are chemo- and baroreceptive units scattered along the cranial part of the vagus nerve, traditionally proposed to be involved in cardiovascular reflexes[3]. Lately, however, the rat aortic body was demonstrated to have similar structural and functional characteristics to

the CB[4, 5]. The human aortic body is not studied in-depth but is generally regarded to have a minor role in hypoxic regulation of breathing, since denervation of the CB bilaterally nearly completely abolishes the HVR[6].

Fig. 1. Corneille Heymans

As early as in the middle of the 18th century Taube and von Haller determined the CB as a gland or a ganglion[7], but it was not until in 1927 that the Belgian scientist Corneille Heymans (Fig. 1) together with his father Jean François Heymans described the role of the CB in regulation of respiration in dogs[8]. This discovery was awarded the Nobel Prize in in Physiology or Medicine 1938 and the CB was thereafter defined as the dominant peripheral chemoreceptor in mammals. Notably, in 1926, one year before Heymans' publication, the Spaniard Fernando de Castro established the novel sensory nature of the organ with studies on both animals and humans[9].

The CB is located bilaterally in the bifurcation of the common carotid artery. In humans the CB is generally situated in the center of the bifurcation whereas the rabbit CB commonly embraces the internal carotid artery [10, 11]. The human CB weighs approximately 15 mg (range 2-90mg)[12] and is a lobulated organ consisting of multiple cell types (Fig. 2). The most abundant cell type is the type 1 cell or glomus cell, which is generally accepted as the chemosensory unit in the CB. Each lobule typically hosts three to five type 1 cells[13, 14]. The CB type 2 cell intimately surrounds a lobule and constitutes less than 20% of the CB[14]. This cell type was previously considered merely a supportive cell, but in the last decade the type 2 cell has been proposed a function of a type 1 cell progenitor in chronic hypoxia and in paracrine signaling in the hypoxia evoked cascade[15-17]. In addition, vascular cells, nerve cells and fibroblasts are also incorporated in the CB, as well as mast cells and lymphocytes[18, 19].

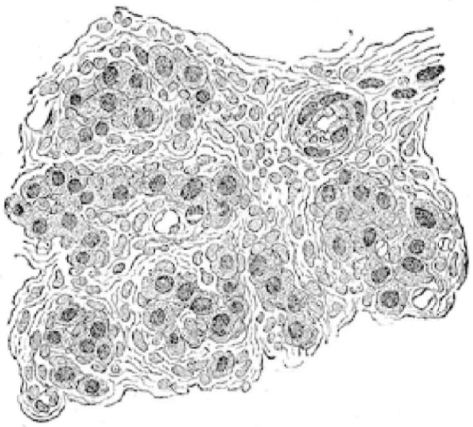


Fig. 2. The lobular structure of the CB.

The glomic arteries stemming from the external or internal carotid artery branch to a dense and complex capillary network in the CB, allowing a close connection between the type 1 cells and the fenestrated capillaries[20]. This virtue combined with the extremely high blood flow for its small weight (approximately 1-2 l/min/100g) provides a base for rapid arterial oxygen sensing and signaling[13, 21, 22]. Considering the high perfusion, the estimated O_2-consumption (1-1.5 ml/min/100g) is remarkably low with only about 3% of the delivered oxygen being consumed[23, 24]. This seemingly excessive perfusion serves to guarantee that the intrinsic metabolic needs of the type 1 cell is protected even in severe hypoxia and to ensure sustained hypoxic activation of the CB[25].

Nerve endings of the afferent carotid sinus nerve (CSN) oppose the type 1 cells in order to facilitate synaptic transmission of oxygen signaling (Fig. 3). Activation of CB type 1 cells causes neurotransmitter release and an increased discharge in the nerve endings. The action potential is further transmitted in the glossopharyngeal nerve, via the petrosal ganglion to the nucleus tractus solitarius (NTS) in the medulla, to ultimately modulate respiration. The CSN also contains baroafferents, mainly from the carotid sinus but with a minor addition of afferents from baroreceptive CB units. The CB receives sympathetic and parasympathetic efferent innervation via the CSN and the glossopharyngeal nerve[26, 27]. Sympathetic activity modulates (and most often then increases) CB sensory activity primarily by altering the CB blood-flow[28]. Parasympathetic efferent nerve activity, on the other hand, is proposed to inhibit CB chemosensory activity through a paracrine ATP effect on P2X-receptors located on efferent nerve endings, resulting in a subsequent NO-mediated inhibition of type 1 cells[26].

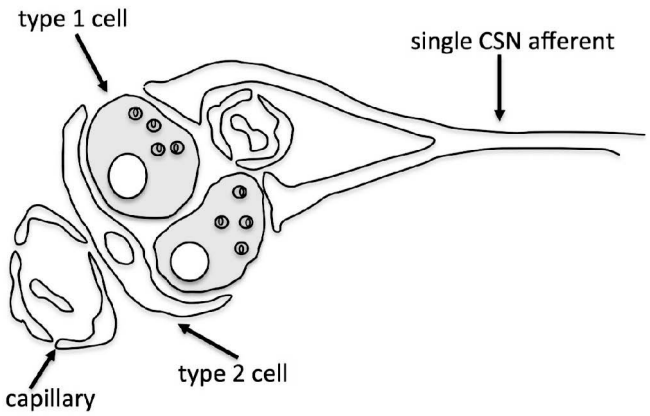


Fig. 3. The cellular arrangement in the CB. The type 1 cell is in synaptic contact with afferent chemosensory nerve fibers and embraced by type 2 cells. The organ is richly vascularized by capillaries that are in close connection with the type 1 cells.

Oxygen sensing

Oxygen is critical for cell survival. In mammals, oxygen enters the body via alveolar gas exchange and is subsequently transported to the periphery primarily bound to hemoglobin-molecules. While all cells may respond to severe hypoxia with a decreased metabolism and eventually apoptosis, specialized oxygen sensing cells react to a moderate decrease in oxygen tension within the physiological range to evoke systemic responses[29]. The smooth muscle cell of the pulmonary artery is one such example. Outward K^+ currents in this cell are dose-dependently inhibited by hypoxia that triggers membrane depolarization and a rapid influx of Ca^{2+}[30]. This ultimately leads to an activation of contractile elements of the myocytes resulting in pulmonary vasoconstriction (also termed hypoxic pulmonary vasoconstriction) that diverts pulmonary blood flow to better-oxygenated parts of the lung[31]. A similar mechanism accounts for hypoxic vasoconstriction in the placental arteries, as well as the catecholamine release from adrenal chromaffin cells in the fetus caused by the hypoxia encountered during delivery[29, 32]. On the contrary, K^+ channels in the smooth muscle cells of the ductus arteriosus are inhibited by the *increase* in oxygen tension at birth when the neonate encounters normoxia, resulting in closure of the shunt[33]. Other oxygen sensing cells are neuroepithelial cells in the airway mucosa and peritubular kidney cells releasing erythropoietin upon hypoxia[29]. Common features for all these cells are involvement of K^+ channels, depolarization and Ca^{2+}-flux, with the appropriate oxygen sensitive K^+ channel differing between species[34].

Although many components of the oxygen sensing and signaling process have been described and are similar between oxygen sensitive tissues, the comprehensive mechanism for sensing oxygen and the key molecules involved in this crucial function are still not defined[35]. Nevertheless, the CB deserves a champion post in oxygen sensing and signaling due to its rapid response (within seconds), sensitivity, endurance and the diversity of respiratory and cardiovascular responses triggered[25].
Hypoxia, or more specifically, reduced partial pressure of oxygen in the blood (PaO_2) is the primary stimulus of the CB type 1 cell. The basal discharge in the CSN begins to increase within seconds of moderate hypoxia (10-11 kPa) and reaches its maximum frequency at a PaO_2 of around 4kPa[36, 37] The discharge is maintained for the entire period of hypoxia but may decrease upon severe hypoxia (PaO_2<4 kPa)[38]. The increased CSN signal modulates brainstem ventilatory output, ultimately resulting in the hypoxic ventilatory response (HVR).
Besides hypoxia the CB may also sense and respond to a multitude of stimuli, for example hypercapnia, acidemia, hyperthermia, hyperkalemia, hypo-osmolarity and hypoglycemia[25]. Thus, the CB is best described as a polymodal chemosensor, providing a comprehensive approach to metabolic stress when commonly more than one parameter is deranged.

Over the years, several research groups worldwide have been involved in the pursuit of finding the exact mechanism for CB oxygen sensing[39, 40]. Currently the primary theories include oxygen sensing through direct inhibition of oxygen sensitive K^+ channels ("the membrane hypothesis"), inhibition of specific complexes in the ETC ("the mitochondrial hypothesis") and finally, altered balance of reactive oxygen species (ROS) or through a change in the energy status of the cell sensed by AMP kinase[13]. Considering the rapidity and stamina of the CB response, as well as the range of PaO_2 the CB senses, the actual sensing mechanism might contain parts of more than one of these hypotheses. Below is an attempt to summarize the prevailing theories:

The membrane hypothesis

The CB type 1 cell contains membrane bound O_2-sensitive K^+ channels that are inhibited during hypoxia, resulting in rapid depolarization of the type 1 cell[41]. The subsequent increase in Ca^{2+} concentration triggers release of neurotransmitters. A multitude of different K^+ channels have been identified in the type 1 cell and only a few of them are modulated by chemostimuli[42]. Furthermore, the role of a specific K^+ channel diverges between species[43] (Table 1) and there is disagreement on the mechanism by which hypoxia inhibits K^+ channels.
The resting membrane potential in type 1 cells is maintained at approximately -60 mV by background or "leak" K^+ channels, admitting a slow and constant outward K^+ flux from the type 1 cell[41]. This two-pore domain acid sensitive K^+ channel (TASK) exists in several isoforms where TASK-1 and TASK-3 are most commonly encountered in the type 1 cell[44]. In some species TASK-1 and TASK-3 are identified as functional heterodimers[45]. Both isoforms are inhibited by hypoxia causing membrane depolarization, however, only TASK-1 knock-out (KO) mice exhibit an impaired ventilatory response to hypoxia, raising doubts about the importance of TASK-3[46]. In rats, TASK-1 and the large conductance K^+ channel Maxi-K (or BK) are inhibited by hypoxia[45, 47]. There is limited knowledge on human CB K^+ channels although K^+ currents similar to in rodents have recently been reported in human CB type 1 cells[48].

K+ channel	Species	References
TASK-1/3	Rat	Buckler (1997)[49], Kim et al. (2009)[45]
Maxi-K (BK)	Rat	Peers (1990)[47]
Maxi-K (BK)	Mouse	Otsubo et al. (2006)[50]
Kv 1.2	Mouse (A/J)	Otsubo et al. (2006)[50]
Kv 3.1-3.3	Mouse (DBA/2J)	Perez-Garcia et al. (2004)[51]
Kv 4.1/4.3	Rabbit	Sanchez et al. (2002)[52]
HERG-like channels	Rabbit	Overholt et al. (2000)[53]

Table 1. Oxygen sensitive K^+ channels in different species.

The mitochondrial hypothesis

Type 1-cell mitochondria are considered to display extraordinary sensitivity to changes in oxygen tension and could thus serve as oxygen sensors[54, 55]. Blockers, or uncouplers of the mitochondrial complexes within the ETC stimulate depolarization and Ca^{2+} entry in the type 1 cell in a manner similar to hypoxia[56], although studies using ETC complex inhibitors have also demonstrated that hypoxia and mitochondrial inhibitors may activate the type 1 cell independently of each other[57]. ETC complex II has been proposed a role in oxygen sensing since mutations of this complex, and in particular the small membrane-anchoring subunit of succinate dehydrogenase (SDHD), give rise to familiar CB tumors with hyperplasia, a histological profile resembling CBs of individuals exposed to chronic hypoxia[12, 58]. In addition, heterozygous SDHD KO-mice ($SDHD^{+/-}$) showed signs of CB hyperplasia[59]. Nonetheless, the type 1 cell hypoxia response was unaltered in these KO-mice, shedding doubt on the theory concerning complex II as being critical for oxygen sensing[59]. Instead, ETC complex IV (the cytochrome oxidase complex) has been suggested as the key oxygen sensitive ETC complex[60]. Cytochrome oxidase has a low affinity for O_2, and it is recently proposed that the closure of TASK-channels (followed by depolarization) by the gaseous messengers and oxygen sensing component hydrogen sulfide (H_2S) are actually a consequence of cytochrome oxidase inhibition[61].

AMPK in oxygen sensing

The role for AMP-activated protein kinase (AMPK) in chemosensing is described as the uniting link between the membrane hypothesis and the mitochondrial hypothesis[62, 63]. AMPK is activated by an increased AMP/ATP ratio appearing in cellular metabolic stress (for example during hypoxia, low glucose or exercise) due to increased ATP consumption or decreased ATP production through insufficient ETC oxidative phosphorylation, hereby monitoring the energy status of the cell[64]. AMPK activation induces depolarization of the type 1 cell through inhibition of TASK and/or Maxi-K[65]. Consequently, if hypoxia inhibits mitochondrial oxidative phosphorylation resulting in diminished ATP production, AMPK embodies the missing link to K^+ channel closure. The enzyme is demonstrated in several species[65, 66] and knocking-out the catalytic AMPK $\alpha 2$ subunit leads to a decreased ventilatory response to hypoxia[66].

ROS

Yet another theory on CB oxygen sensing involves reactive oxygen species (ROS). ROS are generated in the mitochondria when electrons are incompletely transported through the ETC. ROS can also be generated by membrane bound NADPH oxidases (NOX). The general view is

that the levels of ROS increase during hypoxia[67] even though a decrease is reported as well[68]. ROS produced by the NOX-family are favored in this theory, with specific focus on the NOX located in the type 1 cell, notably in the vicinity of the TASK-1 channel[69]. NOX 4 activity is increased during hypoxia and the generated ROS modulate the TASK-1 open probability, acting as negative second messengers in CB chemoreception[70].
In humans, the ROS scavenger N-acetyl-cysteine (NAC) increases the HVR, implying that the redox-state is of importance for hypoxic regulation of breathing[71].

Gaseous messengers in oxygen sensing: CO, H_2S and NO

Gaseous molecules are appealing alternatives (or complements) to the other theories on oxygen sensing, having many characteristics in common with oxygen such as hemoglobin affinity and diffusability across the plasma membrane. The three suggested molecules involved in sensing of oxygen are CO, hydrogen sulfide (H_2S) and nitric oxide (NO)[72].
CO is produced by heme oxygenase 2 (HO-2) with O_2 as a rate-limiting co-factor and increases the open probability for Maxi-K channels in the type 1 cell[44]. CO (in low tensions) thereby has a depressant effect on CB activity and when inhibiting HO-2 the sinus nerve discharge increases[73]. However, as TASK-channels have been shown to be unaffected by CO[74], oxygen sensing through CO turnover may not be an applicable theory across species due to variable expression of TASK-1 and Maxi-K[42]. Furthermore, while some studies in HO-2 KO mice have shown an augmented response to hypoxia[75] others have demonstrated an unaffected type 1-cell response to hypoxia[76]. Nevertheless, a reduced formation of CO by HO-2 under hypoxia is generally considered important in CB oxygen sensing[75, 77], and proposed actions of CO on the afferent nerve ending in cooperation with ATP suggests a wider range of tasks for this gaseous messenger[78].
The emergence of another gasotransmitter involved in oxygen sensing, H_2S, revived the CO-theory and provides a more comprehensive oxygen sensing and signaling approach[79, 80]. It was reported that H_2S is required for CB oxygen sensing and that mice lacking one of the H_2S synthesizing enzymes, cystathionine γ-lyase (CSE) display markedly reduced CB sensitivity to hypoxia and a reduced HVR[79]. H_2S inhibits Maxi-K and TASK-channel activity and thereby depolarizes the type 1 cell, leading to an increased afferent nerve discharge[61, 81]. Interestingly, H_2S-levels are regulated not only by oxygen availability (low levels in normoxia) but also by CO that exerts tonic inhibition on CSE in normoxia. This tonic inhibition is removed in hypoxia when HO-2 activity is slowed due to lack of O_2-molecules, ultimately resulting in increased H_2S-synthesis[79]. Diverse HVR magnitudes in different rat strains is recently reported to be a result of differences in CB oxygen sensing due to varying, strain specific levels of H_2S and CO[82].
In conformity with CO, NO is oxygen-dependently generated and acts as a negative modulator of CB activity[72]. NO synthases (NOS) produce NO in course of the conversion of arginine to citrulline and the three isoforms have been found in the rat and cat CB, although in neurons and endothelial cells and not yet in type 1 cells[83, 84]. NO from adjacent nerve cells exerts a tonic inhibition on the type 1 cell by enhancing K^+ conductance and inhibiting Ca^{2+} entry[85, 86]. Thus, NO is proposed to be an important mediator of efferent CB inhibition[26]. Studies showing that NOS inhibitors increase and NO donors inhibit CB activity further support the role of NO in oxygen sensing and signaling, together with the fact that homozygous neuronal-NOS KO-mice present an enhanced ventilatory response to hypoxia[87, 88]. In line with this, the human HVR was blunted with administration of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA)[89].

Oxygen sensing and redox homeostasis through HIF

Hypoxia inducible factors (HIFs) are central in oxygen sensing and master the reduction-oxidation homeostasis in cells, especially during chronic and intermittent hypoxia[90]. HIF-1 and HIF-2 are heterodimeric transcription factors, consisting of a HIF-1β subunit and a HIF-1α or a HIF-2α subunit, the supply of the two latter being regulated by O_2[91]. In normoxia, HIF-2α is more abundant than HIF-1α in the CB type 1 cell and regulates the expression of anti-oxidant and ROS-scavenging enzymes, such as manganese superoxide dismutase (SOD-2)[72, 92]. During hypoxia the level of HIF-1α in the CB increases, resulting in transcription of pro-oxidant enzymes, for example NOX2[93]. A balance between HIF-1α and HIF-2α is essential for maintaining the redox homeostasis in the type 1 cell, seemingly important for cardiorespiratory regulation[94]. This is demonstrated in studies with KO-mice where HIF-1$\alpha^{+/-}$ mice exhibit a blunted CB chemoreflex function whereas HIF-2$\alpha^{+/-}$ show an excessive CB function affected by oxidative stress, resulting in breathing instability with recurrent apneas, sympathetic activation and hypertension[95, 96]. The phenotype characteristics of the HIF-2$\alpha^{+/-}$ notably resemble key features in obstructive sleep apnea (OSA).

Oxygen signaling

The signal from type 1 cell depolarization to increased CSN discharge is relayed through neurotransmitter release effectuated by Ca^{2+} influx. The synaptic unit consisting of the CB type 1 cell and the afferent nerve ending of the CSN exhibits features of complex neural signaling with multiple excitatory and inhibitory neurotransmitters as well as a mosaic map of receptors, in order to appropriately orchestrate the response to hypoxia. This contrasts to oxygen sensing and signaling in fish and amphibians, where chemosensation is based on fewer neurotransmitters in separate cells[2]. The increasing complexity of CB oxygen signaling in higher species involves a combination of multiple paracrine and autocrine neurotransmitter effects[39, 97, 98]. Neurotransmitters and receptors differs between species and developmental stages and the specific function of a certain receptor or neurotransmitter may alter during development[99]. Over the years, a substantial number of studies have aimed to clarify the role of a multitude of neurotransmitters involved in CB oxygen signaling. While there are differences in certain species regarding their role in excitation or inhibition of the CB signaling pathway, acetylcholine (ACh) and ATP are generally accepted as primary excitatory neurotransmitters in most species, in addition to dopamine (DA) that has a modulatory or inhibitory role in oxygen signaling throughout species[2, 98, 100]

Acetylcholine

ACh released from CB type 1 cells activates ACh-receptors (AChR) present on opposing nerve terminals, increasing CSN discharge[101]. Yet, it is too simplistic to classify ACh as merely excitatory in CB signaling since its action depends on the receptor targeted, the cellular localization of the receptor and interactions with other neurotransmitters[101]. As an example, while ACh-binding to postsynaptic neuronal nicotinic AChR (nAChR) results in frequency gain in the CSN, ACh-binding to presynaptic muscarinic receptors inhibits type 1 cell activity with a resulting reduced CB output as demonstrated in rabbits[102].

Several subtypes of the neuronal nAChR have been demonstrated in CB type 1 cells and nerve terminals from different species; still, the map is far from being comprehensive[101]. The nAChR is a pentameric receptor with five subunits surrounding a central pore. The subunits α2-6 and β2-4 combines to heteromeric receptors and the α7- α9 to homomeric receptors[103]. In the CB

the α3, α4 and β2 subunits are present in the cat type 1 cell and α7 on cat and rat CB nerve terminals[100, 104, 105]. Furthermore, mRNA of the α3-5, α7, β2 and β4 nAChR subunits has been demonstrated in the mouse CB[106]. Undoubtedly important, this information does not provide information on the cellular location within the CB of these subunits.
Being a ligand-gated cation channel, nAChRs on the type 1 cell contribute to depolarization, indicating an autoregulatory role for ACh on neurotransmitter release. However, as the released neurotransmitters are either excitatory, inhibitory or both, the final result of activation of type 1 cell nicotinic autoreceptors may thus be either an increase or decrease in CSN discharge. In fact, $\beta 2^{-/-}$ mice show an augmented HVR, suggesting a net inhibitory role of the autoreceptors containing this subtype in mice[106]. On the other hand, in humans the HVR is attenuated by systemic administration of a neuromuscular blocking agent (primarily targeting nAChRs)[107] which in animals seems to cause a depression of CB nicotinic transmission[108-111].
Since even high concentrations of nAChRs blockers were not able to completely suppress CB afferent activity, co-transmission with another neurotransmitter, ATP, has been suggested[98, 112, 113].

ATP

ATP is an excitatory CB co-transmitter released in response to hypoxia[1, 114, 115]. ATP activates the inotropic, excitatory P2X and metabotropic, inhibitory P2Y purinergic receptors, available in several isoforms, of which $P2X_2$ and $P2X_3$ are present at postsynaptic nerve terminals in rat and cat CB[116, 117], $P2Y_1$ on type 1 cells[118] and $P2Y_2$ on type 2 cells[15]. P2X-blockade with suramin attenuates the CB response to hypoxia[113] and $P2X_2^{-/-}$ mice exhibit a strikingly blunted HVR[117], providing evidence for the role of ATP in CB oxygen signaling. However, as in the case for ACh, the ATP actions are complex in the CB, activating both inhibitory P2Y-receptors on type 1 and type 2 cells, and excitatory P2X-receptors on nerve afferents[119]. This interplay between excitation and inhibition by neurotransmitters in the CB has been termed a “push-pull” mechanism, aiming at prolonging and modulating the CB afferent discharge[97]. As stated previously, ATP-action on P2X receptors of CB nerve afferents results in NO-formation that exerts inhibition on CB activity, providing another mechanism for regulation of CB chemosensory output[26].
Conflicting results include the demonstration that both cat CB discharge and HVR was unaffected by suramin, reflecting possible species differences in expression of receptors[120].

Additional neurotransmitters in oxygen signaling

Dopamine (DA) is abundant in type 1 cell vesicles and is released in response to hypoxia[121]. Since tyrosine hydroxylase (TH) is the enzyme responsible for DA synthesis, TH has been applied as an indicator for type 1 cells[122].
Whereas DA behaves as the primary neurotransmitter in rabbits and possibly dogs[123, 124], it only deserves recognition as a modulatory transmitter in other species[13]. For instance, DA acting on D_2 receptors on type 1 cells results in a negative feedback mechanism of hypoxic transmitter release[125]. Administering intravenous DA to humans at altitude reverses the previous increase in HVR[126]. Moreover, peripheral chemosensitivity is enhanced in patients with OSA receiving the D_2-antagonist domperidone[127], both observations pointing to the involvement of DA in regulation of breathing at hypoxia. D_2 immunoreactivity is identified in the human CB type 1 cell in autopsy material[128].
GABA released during hypoxia results in postsynaptic inhibitory feedback through ligand gated $GABA_A$ receptors that are demonstrated on CB neurons opposing type 1 cells[129].

$GABA_A$ receptor agonists such as midazolam and propofol markedly depress hypoxia-induced increase in CSN activity[130, 131]. However, $GABA_B$ also seems to be involved in regulating CB activity since selectively blocking $GABA_B$ enhanced CB chemoafference through inhibition of TASK-1[132].

Adenosine and serotonin (5-HT), both excitatory transmitters, operate on type 1 cell $A2_A$ and 5-HT_{2A} receptors, respectively, exercising a positive feedback mechanism through autocrine and paracrine regulation of neurotransmitter release[133, 134]. Hypoxia furthermore evokes the release of histamine acting on H_1, H_2 and H_3 receptors, and substance P acting on neurokinin-receptors, both mediating sensory excitation[135, 136].

Figure 4 summarizes some of the autocrine and paracrine mechanisms in CB oxygen signaling.

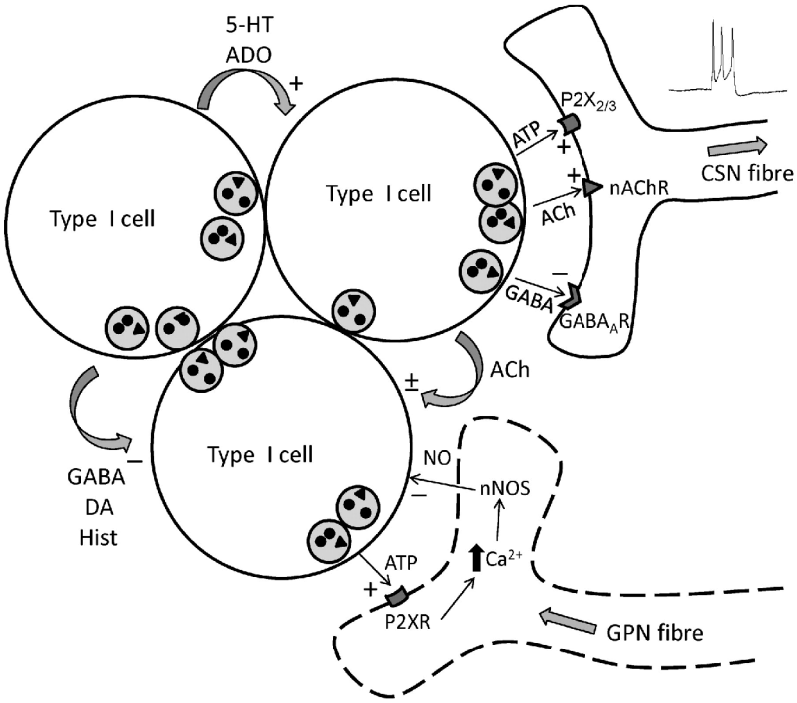


Fig. 4. A schematic overview of the interaction between neurotransmitters and their receptors on chemoafferent nerve fibers as well as autorecptors on type 1 cells or paracrine receptors on efferent nerve fibers, all modulating CB induced discharge in the CSN. From Nurse C[1] with permission.

Inflammation sensing and signaling in the CB

Lately, the CB has gained attention as a possible sensor and modulator of the systemic inflammatory response. Infiltration of inflammatory cells in the CB has previously been recognized with mast cells and lymphocyte infiltration demonstrated in the human CB, the latter increasing with age[18, 19]. Recently, it was also shown that intravenous administration of the endotoxin lipopolysaccharide (LPS) induces tachypnea, which was abolished after bilateral transection of the CSN[137]. Furthermore, the CB is activated by the pro-inflammatory interleukins (IL)-1β and IL-6[138, 139]. The inflammasome activating pathogen zymosan is also demonstrated to increase CB signaling and induce IL-1β production[140]. On the contrary, TNF-α attenuates the CB chemosensory discharge and the suggested mechanism behind this is inhibitory DA-signaling[137, 141]. Accordingly, it has been proposed that the CB could serve as a

rapidly responding connection between the immune system and the CNS, analogously with the afferent limb of the cholinergic anti-inflammatory reflex pathway[142]. Considering that cytokines poorly cross an intact blood-brain-barrier, the peripheral CB location is ideal for this proposed immunosensory function. Notably, rat CB type 1 cells express the cytokine receptors IL-1R, IL-6R, TNF-R and toll-like receptor 4 (TLR 4)[137, 141, 143, 144]. Whether the human CB expresses cytokines and their corresponding receptors is not known.
Ibuprofen is reported to diminish the HVR in chronically hypoxic rats as well as reducing the acclimatization to chronic hypoxia (where inflammatory modulations are central processes)[145]. Animals exposed to chronic intermittent hypoxia (CIH) display resembling inflammatory CB alterations i. e. up-regulation of the cytokine and cytokine receptor gene expression and an increased CB chemoreactivity to hypoxia. These changes were also reversed by ibuprofen, as was the further up-regulation of pro-oxidant enzyme expression in CIH[146, 147].
The CB thus has the potential to participate in immune-to-brain signaling and inflammation seems to be central for the CB chemosensitivity acclimatization in chronic and chronic intermittent hypoxia[148].

REGULATION OF BREATHING

Respiration originates in the medulla of the brain where the central pattern generator (CPG) is located, consisting of multiple interconnected regions and nuclei. In the CPG, the rhythmic breathing pattern is coordinated with behavioral events such as speech, swallowing and coughing and furthermore with input from central and peripheral chemoreceptive areas. The breathing pattern can be overridden voluntarily and is furthermore affected by factors such as degree of wakefulness, emotions and temperature. While breathing is controlled by CO_2, pH and O_2, resting ventilation is primarily maintained by CO_2 that readily crosses the blood-brain-barrier and changes pH through hydration and ionization. An increase in $PaCO_2$ thus results in a proportional decrease in cerebrospinal fluid pH[149]. Central chemoreceptive areas on the surface of the anterior medulla, in proximity of the nuclei of the glossopharyngeal and vagus nerves, sense this decrease in pH with an unknown mechanism.
The CPG also receives input from peripheral mechanoreceptors in the pharynx, larynx and the lung, ultimately coordinating the multitude of demands to an appropriate breathing pattern[150]. The neurons in the medulla are organized in the ventral and dorsal respiratory group. The afferents of the glossopharyngeal and vagus nerves terminate in the dorsal respiratory group, or more specifically, in the NTS. This part of the medulla mainly contains inspiratory neurons and is concerned with timing of respiration.
Excitatory and inhibitory neurotransmitters act in concert to modulate respiratory output from the medulla. The main excitatory neurotransmitter operating in this area is glutamate, acting on N-methyl-D-aspartate (NMDA)-receptors and the primary inhibitory transmitters are GABA and glycine, acting on $GABA_A$ and glycine receptors, respectively[151]. Neuromodulators such as ACh from central chemoreceptor neurons and glutamate from pontine and peripheral chemoreceptor neurons then exert their effects on CPG neurons, fine-tuning the coordination of breathing. Consequently, drugs reaching the CNS and acting on these receptors can affect breathing.

Regulation of breathing during hypoxia and hypercapnia

During hypoxia and hypercapnia ventilation is typically augmented to compensate for these states. The responses to hypoxia and hypercapnia are rapid (within seconds) and originate in peripheral chemoreceptors (CB) and medullary chemoreceptors, respectively.

HVR

At normoxic resting ventilation, the CBs contribute to 10-20% of the respiratory drive[152]. This is established by "Dejours' test", a brief period of hyperoxia (100% for 1-2 minutes) that results in a ventilation decrease. The magnitude of ventilatory depression by hyperoxia is often used as an index of CB chemosensitivity[153].
The HVR is the ultimate and signature consequence of CB activation in acute hypoxia. Within seconds ventilation is increased, dependent on both increase in tidal volume and frequency, constituting the acute HVR, solely controlled by the CBs (Fig. 5). Ventilation increases for 5-10 minutes before a decline begins, the hypoxic ventilatory decline, thought to be caused by central GABA-mediated inhibition[154]. This phase resides for 20-30 minutes and takes minute ventilation to a plateau, still above resting ventilation[155]. If hypoxia is sustained, ventilation continues to increase for up to 24 hours.
Hypoxia also has a direct effect on central respiratory neurons causing ventilatory depression and consequently, severe hypoxia in the medulla may result in apnea or severely depressed breathing in both animals and humans[150, 156]. In line with this, if the peripheral chemoreceptors are denervated, phrenic motor activity is abolished upon hypoxia[150].

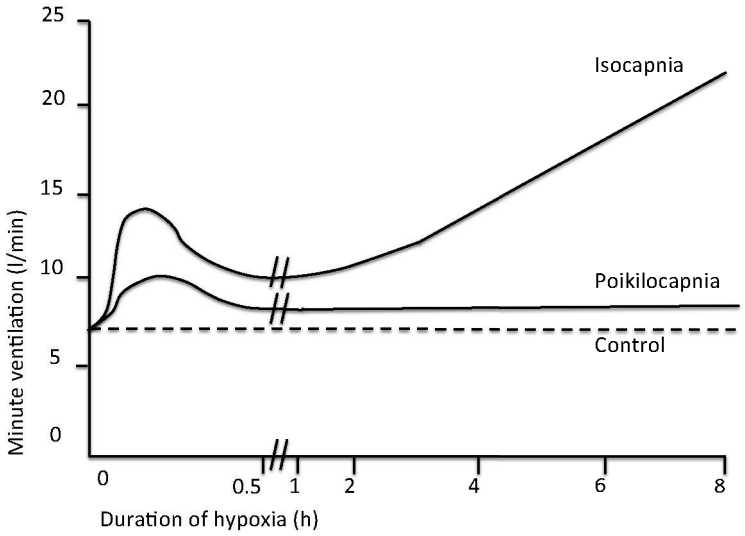


Fig. 5. The HVR during isocapnic and poikilocapnic conditions.

HVR variability

The HVR is an individualized response and about 10% of humans are even lacking a measurable HVR[157]. The genetic background for this inter-individual HVR variability is supported by twin-studies and the presence of familial clusters. However, the responsible genes involved have not yet been identified[157]. Two mutations and their influence on HVR further

strengthens the theory of genetic involvement, namely the heterozygous missense mutation of SDHD (as described above) that resulted in a slight decrease in HVR, and a mutation in the von Hippel-Lindau tumor suppressor protein gene that causes Chuvash polycythemia with HIF-accumulation and an augmented HVR[157, 158].
The HVR matures in the development from fetus to newborn and is considered complete around 2 months of age in humans. Notably, fetal hypoxia results in abolished breathing movements and neonates exposed to hypoxia exhibit only a slight increase in ventilation but more importantly, a marked reduction of metabolism indicating a lack of protective mechanisms against hypoxia when the HVR is immature[159]. Interestingly, the fetal response to hypoxia resembles the centrally mediated response to hypoxia in adults described above. Circadian rhythm, hormones, psychological factors, and pregnancy further affect HVR as well as pharmacological agents, as discussed below.

HVR in humans and animals

The HVR shows a wide variation between species as well as between different strains of species, where the magnitude of HVR can vary with a factor up to 7 between rat strains[160]. In mice, the DBA/2J strain has a substantially larger HVR than A/J mice. The genetic background to this phenotype difference includes a reduced expression of Maxi-K channels in A/J mice[161]. In humans no such phenotype-genotype studies are available and since differences in terms of HVR exist between humans and animals, further human studies of this kind are needed.
In humans, the magnitude of the HVR is conserved throughout life[157]. In animals, on the contrary, age markedly reduces the HVR by more than 50%[162]. Since the CBs of aging humans exhibit fibrosis, loss of type 1 cells and infiltration of inflammatory cells, the conservation of HVR supports a redundancy of CB chemoreceptive capacity not seen in animals[12]. While animals show restoration of the lost hypoxic drive upon bilateral CB resection in weeks-years which may be due to a chemoreceptive activation of the aortic bodies[163] there is a lack of HVR recovery is observed in humans 4-8 years after such surgery[6, 152]. Accounting for these differences, the human CB appears to be more specialized and indispensible for hypoxic regulation of breathing than in animals.

Hypercapnic ventilatory response

Briefly, a rise in $PaCO_2$ results in a rapid ventilation augmentation through gain in tidal volume and frequency of breathing due to an increased discharge in the phrenic nerve. A steady state is reached after a few minutes, but with sustained hypercapnia ventilation continuously increases for about an hour before reaching a plateau level. The slope of the hypercapnic ventilatory response (HCVR) curve steepens with addition of hypoxia. The HCVR is used to evaluate the response of the entire respiratory system, including respiratory muscle function. Although the HCVR originates from central chemoreception, the CBs contribute with up to 30%[150], and the sensing mechanism is recently suggested to be dependent on a decrease in pH[164].

HYPOXIC VENTILATORY CONTROL IN ANESTHESIA

General anesthetics are known to impose central depression of breathing. Having multiple targets throughout the respiratory system, drugs used in anesthesia exhibit central actions as well as effects on airway muscles and peripheral chemoreceptors. Even at low drug concentrations when central depression of breathing is not apparent, these agents may depress hypoxic CB chemosensitivity resulting in a reduced or abolished HVR.

Volatile anesthetic agents induce a dose-dependent depression of ventilation characterized by a reduction in tidal volume that is not compensated by the concurrent increase in respiratory frequency[165]. This depression is mainly generated through central $GABA_A$, glycine and nACh-mediated inhibition, but to what extent volatile anesthetics affect peripheral chemoreception is still under debate[166, 167]. In humans, subanesthetic concentrations of halothane are suggested to reduce the HVR whereas isoflurane and sevoflurane have less or no effect on hypoxic ventilation[168, 169]. Anesthetic levels of volatile anesthetics greatly depress both the HVR and HCVR, possibly by a combination of central and peripheral chemosensitivity depression.

In animals, the effect of volatile anesthetics on the HVR varies between and sometimes even within species. Halothane, isoflurane and enflurane in cats, goats and dogs depress HVR markedly, paralleled by a HCVR depression, in contrast to the rabbit HVR that seems resistant to isoflurane[167, 170].

Hence, in common for human and animal hypoxic regulation of breathing under influence of volatile anesthetics is that the choice of anesthetic is the main determinant, with the greatest HVR depression caused by halothane and the least depression by sevoflurane[167].

Neuromuscular blocking agents (NMBA) interfere with hypoxic regulation of breathing by reducing the CB response to hypoxia[108]. This is most likely accomplished through inhibition of neuronal nAChR in the CB[111]. Accordingly, residual effects of NMBAs postoperatively can attenuate the CB response to hypoxia and thereby the HVR, without changes in resting ventilation.

Propofol is a commonly used agent for anesthesia and sedation. Propofol causes central respiratory depression via $GABA_A$ and neuronal nACh-receptors but is also shown to potently depress the HVR and CB chemoreactivity[171, 172]. The hypoxia-induced CB response is abolished by propofol through impairment of nicotinic transmission in the CB[131].

In summary, residual effects of anesthetic drugs such as general anesthetics and NMBAs impair CB chemosensation and the subsequent HVR. This is of crucial importance, since impaired peripheral chemosensation during hypoxia could induce apnea due to an unopposed central hypoxia response[150]. Consequently, residual effects of anesthetic agents leaves the patient at risk for acute hypoxia and thereby postoperative respiratory complications[173]. Since serious respiratory complications are common and expensive[174], understanding of the underlying mechanisms behind impaired regulation of breathing during hypoxia is essential.

HYPOXIC REGULATION OF BREATHING IN DISEASE

Hypoxia can be both chronic as in exposure to high altitude or in hypoxemic disease, and intermittent with repeated hypoxic episode as in OSA. Chronic hypoxia and chronic intermittent hypoxia induce changes in the CB that culminate in an increased HVR and development of cardiovascular disorders, albeit with different pathophysiological mechanisms. Exposure to chronic hypoxia as in chronic obstructive pulmonary disease, congestive heart failure (CHF) and at high altitude induces structural and functional plastic changes in the CB.

The CBs turns severely hyperplastic, plausibly due to stem cell activation and differentiation where type 2 cells in adult CBs are demonstrated to convert into intermediate progenitor cells that subsequently differentiate into mature type 1 cells[12, 16]. The morphological CB changes are accompanied by an increased CB hypoxic sensitivity. This results in augmentation of the HVR, which is reversible on return to normoxia[157].
Furthermore, chronic hypoxia induces inflammatory changes in the CB. Macrophage infiltration is increased and mRNA expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) with corresponding receptors is induced whereas anti-inflammatory drugs are shown to reduce this up-regulation[144, 175]. Inflammation is thus an element in adaptation to chronic hypoxia.
Chronic hypoxia as experienced by high altitude residents living at >2000 meters increases the risk for CB tumors with a factor of ten, related to the vigorous hyperplasia[176].
Obstructive sleep apnea (OSA) is characterized by intermittent hypoxia due to repeated apneas and hypopneas during sleep caused by upper-airway obstruction. Patients with severe OSA have >30 apneas or hypopneas per hour, typically causing subsequent desaturations. OSA patients may thus be exposed to chronic intermittent hypoxia that induces an increased CB chemosensitivity, contributing to sympathetic activation and increased catecholamine synthesis ultimately causing hypertension and cardiovascular disorders[177, 178]. This is proposed to be due to the imbalance between HIF-1α and HIF-2α resulting in accumulation of ROS and thereby oxidative stress, as well as activation of pro-inflammatory pathways in the CB, ultimately resulting in enhancement of CB activity[146, 179]. Thus, one of the important mechanistic differences between chronic hypoxia and chronic intermittent hypoxia is the CB down-regulation of HIF-2α in chronic intermittent hypoxia, which is proposed to be the key to systemic hypertension through increased sinus nerve discharge[179]. Interestingly, no morphological CB alterations are seen in rats exposed to chronic intermittent hypoxia[180]. Whether this is true for humans is not known at present.

STUDIES ON THE HUMAN CAROTID BODY

After De Castro's and Heymans's discoveries of the CB chemosensing function, these organs received a (well-deserved) place in the spotlight. In the 50's and 60's many anatomists studied the human CB during necropsies and defined the gross morphology as well as cellular structures using conventional and electron microscopy. But even before this, a Japanese pioneer surgeon, Nakayama, started to perform bilateral removal of the CBs to treat chronic bronchial asthma[181]. From 1942 and on, he treated 4000 patients with this surgical technique and gained disciples worldwide that adopted his method, although mostly in a unilateral manner. In a report on 350 glomectomized patients in North Carolina, 80% showed immediate relief of asthma symptoms (i. e. breathlessness and "wheezing") and very few complications and/or side effects were noted[182]. However, in the next decades it became obvious that these patients exhibited an irreversibly blunted or nearly abolished HVR after unilateral and bilateral CB resection, respectively, as demonstrated by Severinghaus and co-workers studying patients after unilateral and bilateral carotid endarterectomy as well as unilateral and bilateral CB resection to treat asthma symptoms[152, 183]. They also reported a reduced ventilatory response to hypercapnia in all patient groups.
The human HVR has furthermore been investigated in the presence of pharmacological interventions. Besides the anesthetic agents discussed above, the human HVR increases with administration of the DA antagonists haloperidol and domperidone and the anti-oxidant N-

acetylcystein[71, 184, 185]. Conversely, treatment with DA and L-NMMA attenuates the human HVR[89, 184]. Moreover, endogenous adenosine levels showed a direct correlation with HVR[186]. Without disregarding the importance of these studies on CB resected patients and HVR interventions, they are after all indirect demonstrations of human CB function. Until 2013, there was no further advancement in terms of direct human CB function confirmations. But recently, Lopez-Barneo and co-workers could show that human CB cells exhibit voltage-dependent Na^+, Ca^{2+} and K^+ currents, Ca^{2+} influx and quantal release of neurotransmitters upon hypoxia, as investigated on human CB tissue from deceased patients during or after organ donation surgery[48]. Although performed on tissue severely affected by inotropic drugs and diminished or abolished circulation, it was certainly a first step towards knowledge on human CB function.

Studies on the human CB biochemical structure have also been performed on tissue obtained during autopsies, 3-78 hours post mortem. In patients who deceased from heroin intoxication, protein stainings of HIF-1α, vascular endothelial growth factor and neuroglobin were stronger compared to trauma-deaths, indicating exposure to extended hypoxia in the first group[187]. There was, however, no up-regulation of NOS-2 demonstrated. With regards to oxygen signaling, TH, the D_2 receptor, catecholamines and histamine H1 and H3 were demonstrated with immunohistochemistry[128, 188]. Interestingly, in the human CB there is a limited expression of TH compared to the mouse and rat CB[189, 190]. Likewise, other components such as spexin, cytoglobin, leptin, adrenomedullin, bombesin, neurofilament, serotonin, ERK, enkephalins and several neurotrophic factors have filled the otherwise fairly empty map of human CB-resident proteins[187, 189, 191-194].

Attempts have been made with stereotactic autoimplantation of CB cells in the striatum of patients with severe Parkinson disease based on the high CB expression of glial derived neurotrophic factor (GDNF), diligently tested as protection for nigrostriatal pathways in Parkinson disease[195]. A modest, clinical improvement was noted, yet not regarded as enough to consider CB autotransplantation a realistic therapeutic option, according to the authors.

In summary, control of respiratory and cardiovascular homeostasis is a prioritized physiological process and therefore rapid sensing and signaling of the chemical composition of arterial blood is essential. The CB is the primary peripheral chemosensor, responding to hypoxia and several other blood-borne stimuli that are transduced to an increased firing in the sinus nerve, ultimately resulting in a respiratory and cardiovascular response[25]. The mechanism of CB oxygen sensing and signaling has been extensively explored in different species in the past 25 years[1, 97]. Still, a consensus on the exact oxygen sensing mechanism is missing and, notably, knowledge on the human CB function and molecular structure in relation to sensing and signaling of oxygen and inflammation is limited. Moreover, both important similarities and differences in CB molecular structure and HVR is seen between species, further emphasizing the need for CB studies targeting the human CB, since animal data may not be readily translatable to humans[196].

Finally, respiratory disease and anesthetic agents can affect CB oxygen sensing and signaling and there is consequently a need for detailed knowledge on the human CB in order to improve our understanding of the underlying mechanisms behind impaired hypoxic regulation of breathing by anesthetic agents and diseases.

AIMS

The overall aim of this thesis was to describe the human carotid body in terms of gene expression in oxygen sensing and signaling pathways and the inflammatory response, solely and in comparison with other species, and furthermore to investigate the response of the human carotid body to hypoxia. The specific aims were:

- To characterize the expression of cholinergic, purinergic and dopaminergic receptors and potassium channels in the mouse carotid body
- To map the expression of key genes in sensing and signaling of oxygen and inflammation in the *human* carotid body on mRNA and protein level.
- To comprehensively define the human carotid body transcriptome and compare it to transcriptomes of other human tissues and of the mouse carotid body with focus on oxygen sensing and signaling as well as inflammation
- To investigate human carotid body function under hypoxic challenge by analyzing neurotransmitter and cytokine release during acute and prolonged hypoxia.

MATERIALS AND METHODS

The following sections provide a brief description and discussion of materials and methods used in this thesis. Detailed information on all materials and methods is presented in paper I-IV.

PATIENTS

Patients were recruited following study approval from the Regional Ethics Committee on Human Research at Karolinska Institutet, Stockholm, Sweden. The trials were performed in agreement with the 2008 revision of the Declaration of Helsinki. All patients entered the study after informed and written consent.
A total of 14 patients (13 male and 1 female) scheduled for a modified radical neck dissection due to head and neck malignancy (cancer of the parotid gland or the tongue) were included. None of the patients had a tumor involving the carotid body, nor had they received radiation or chemotherapy prior to surgery. One patient (patient no. 4 in study IV) was using snuff but the remainder was nicotine-free. For patient demography, see table 2 below.

Patient	**Study**	**Gender (M/F)**	**Age (y)**	**ASA**	**BMI (Kg/m^2)**
1	II	M	67	1	26
2	II	M	36	1	21
3	II, III	M	68	1	20
4	II, III	M	64	2	31
5	II	M	38	1	26
6	II, III	M	56	1	24
7	III	M	43	1	28
8	III	F	57	2	26
9	IV	M	71	3	26
10	IV	M	42	1	26
11	IV	M	80	3	20
12	IV	M	57	1	28
13	IV	M	46	1	30
14	IV	M	58	2	32

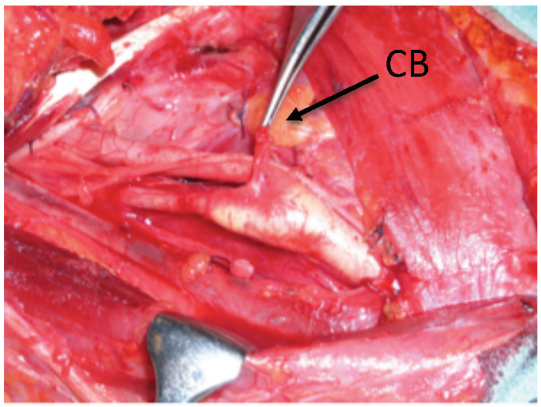


Fig. 6. The human CB demonstrated in the bifurcation of the common carotid artery during surgery.

Anesthesia was induced with propofol or thiopental and maintained with sevoflurane and opioids during normoxic and normocapnic mechanical ventilation. During the modified radical neck procedure, the CB was removed unilaterally (Fig. 6). In the operating room, the tissue was immediately taken for preparation by members of the research group. In study II and III the CB was divided into pieces and put in liquid nitrogen or paraformaldehyde and in study IV the intact CB was immediately placed in pre-oxygenated, ice-cold buffer for transfer and further experimentation at the laboratory. The CB samples that were fixed in paraformaldehyde were then cryoprotected in 30% sucrose and rapidly frozen in Tissue Tek® OCT compound to be stored in -80° C for later sectioning.

Because of lack of human tissue, the human CB is poorly studied compared to animals. During a modified radical neck dissection as currently performed in Sweden a substantial amount of tissue, including lymph nodes, related nerves, blood- and lymphatic vessels is excised in order to prevent secondary spread of malignancy[197]. With this procedure the CB sinus nerve is obliterated, implicating denervation of the CB and thereby a non-functional CB on the corresponding side[6]. Consequently, the CB can be removed unilaterally by a skilled ENT-surgeon with no added impairment for the patient.

ANIMALS

Animal studies were approved by the local animal ethics committee at Karolinska Institutet. In paper I a mouse model was used to provide a map of CB receptors in one species and as a platform for further human studies.
Male C57BL/6 mice with a body weight of 20-33g were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (1.0 mg/kg). The carotid bifurcation with the CB was surgically removed en bloc bilaterally. The bifurcation was immediately fixed in 4% zinc-paraformaldehyde for 1-2h and then cryoprotected in 30% sucrose in PBS for 1-3 days. Finally, the bifurcation was embedded in Tissue Tek® OCT compound and frozen to -80°C for later sectioning.

METHODS

Below is a presentation (Table 3) of the methods used in paper I-IV.

Method	Paper
Cryostat sectioning	I, II, III, IV
Hematoxylin-Eosin staining	I, II, III, IV
Immunohistochemistry	I, II, IV
Confocal microscopy	I, II, IV
Microarray analysis	II, III
PCR	II, III
HPLC	IV
Luminometric assay	IV
Multiplex assay	IV

SECTIONING AND HEMATOXYLIN-EOSIN STAINING

Serial sections of 14 µm of both mouse and human CBs were cut using a cryostat and mounted onto microscope slides. Selected slides were stained with hematoxylin and eosin to determine the histology of the sections and to provide a correlate for the immunohistochemistry images.

IMMUNOHISTOCHEMISTRY

In order to demonstrate localization of a selected protein in the type 1-cell of the CB, double-staining with tyrosine hydroxylase (TH) or β-III-tubulin, established markers of the type 1 cell was carried out[198]. After incubation in PBS and blocking in PBTA (consisting of PBS, bovine serum albumin and Triton-X) the primary antibodies in appropriate dilution in PBTA were applied and incubation overnight in 4 °C ensued. The slides were then washed in PBS before secondary antibodies conjugated with the flourochromes Alexa 488, 555 and 647 were applied for a 30-minute incubation in room temperature. The antibody excess was washed off with PBS and the coverslips were mounted using cover medium containing 4', 6-diamidino-2-phenylindole (DAPI) for visualization of the cell nuclei.

CONFOCAL MICROSCOPY

The fluorescence of the spectrally separated secondary antibodies was detected with a confocal microscope (Zeiss LSM 710). The flourochromes were excited with an appropriate wavelength of light (laser) in the confocal microscope and the emitted light was then detected and transformed into an electrical signal, interpreted by the computer software (Zen 2009 and Zen 2012).

MICROARRAY ANALYSIS

In order to determine the human CB gene expression in study II and III, mRNA was identified with microarray analysis. Prior to this, the RNA was extracted and purified from the samples with an RNeasy kit (Invitrogen) after homogenization and subsequent extraction with chloroform and ethanol. The samples were then pre-labeled and hybridized using the Affymetrix oligonucleotide microarray. The mRNA hybridization signal intensity was analyzed with Microarray Suite Software (MAS) to estimate the expression values for each transcript. Transcripts above the threshold of detection (defined by a statistical detection algorithm in MAS) were considered present and transcripts below the threshold of detection were considered absent.
In study III, the number of expressed genes was also estimated with the Gene Expression Barcode software (Affymetrix Software) as described by others[199]. In study III, raw global CB gene expression data from C57BL/6 mice and DBA/2J normoxic control mice from two previous studies[161, 200] were further analyzed in the same manner as the human data to determine the expression profiles of the genes in mouse CB oxygen and inflammation sensing and signaling. The resulting lists of genes were thereafter compared to our human data.

DIFFERENTIAL GENE EXPRESSION ANALYSIS

In order to evaluate the unique specific gene expression profile of the human CB the raw transcriptome data in Affymetrix CEL files were compared to the pre-computed transcriptomes of other tissues using the Barcode online analysis tool[199]. Differential gene expression analyses of the human CB versus the transcriptomes of functionally related human and mouse tissues, specifically brain and adrenal gland, were accomplished using yet another online resource, WebArrayDB[201]. The transcriptomics data for these tissues were obtained from the public repositories (NCBI's GEO and ArrayExpress). A fold change of 5 and $p<0.01$ were arbitrary chosen as cut-off values for up- or down regulation of gene expression.
The resulting lists of up- and down regulated genes in the human CB were further analyzed for gene ontology (GO) term enrichment with additional online tools, the DAVID online bioinformatics resource[202] and WEB-based Gene Set Analysis Toolkit (WebGestalt) (www.bioinfo.vanderbilt.edu/webgestalt).

PCR

Two different PCR techniques were used, conventional PCR and real-time PCR. In study II and III, Real-time PCR was used to evaluate the expression levels of the selected genes. Briefly, complimentary DNA (cDNA) was produced using oligo(dT) primers in a reverse transcription system (Invitrogen), followed by amplification with the 7500 Real Time PCR System (Applied Biosystems). For this reaction, cDNA template and TaqMan Gene Expression Assays for the selected genes were used. The values acquired according to the ΔC_t method were related to the expression of TH.
In study III, we used conventional PCR to amplify the human cDNA of a putative Maxi-K K^+ channel splice variant.

HUMAN CB SLICE PREPARATION

To prepare for hypoxic and hyperoxic challenges in study IV the CB was cut in 400 µm slices with a tissue chopper (McIlwain). The slices were placed in cell plate wells, followed by a 30-minute rest in pre-oxygenated Krebs Ringer solution (KRS) of 37°C, pH 7.40 and containing specific and unspecific cholinesterase inhibitors (neostigmine bromide and teraisopropylpyrophosphoramide, Iso-OMPA). The cell plates were then placed in different incubators according to the protocol.

Acute hypoxia challenge

In order to study the release of neurotransmitters, the cell plate with the CB slices was exposed to a brief 5-minute period of hypoxia. The hypoxic challenge was achieved by placing the cell plates in a sealed chamber, with an efferent tube for continuous gas flow into the chamber and by exchanging the medium for the different hypoxic and hyperoxic challenges. At the end of the challenge the slices were frozen for later morphological analysis. For a schematic presentation of the acute hypoxic challenge, see figure 7 below.

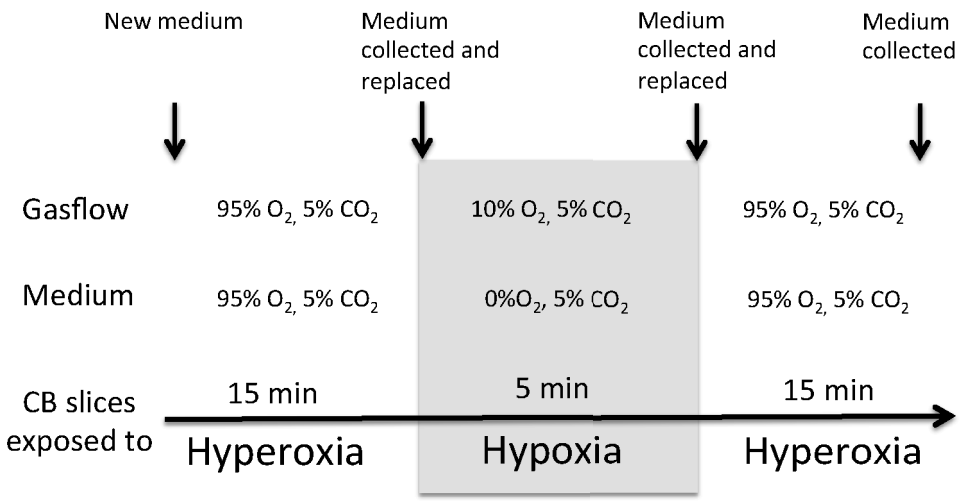


Fig. 7. Schematic protocol of the acute hypoxia challenge, with the chamber gas flow and the gas composition the medium was bubbled with stated.

Prolonged hypoxia challenge

With a 1-hour hypoxia challenge we investigated the release of cytokines from the CB slices. The cell plate with CB slices in hypoxic KRS was placed in a hypoxic chamber (H35 Hypoxystation, Don Whitley Scientific) set at 10% O_2, 5% CO_2 and 37°C. At the end of the 1-h hypoxic challenge, the medium was collected and the CB slices frozen or fixated for further analysis. A control cell plate with CB slices was exposed to hyperoxia (95% O_2 and 5% CO_2) at 37°C for 1h.

HPLC

ACh release from the CB slices was detected using high-performance liquid chromatography (HPLC). The samples were run through a centrifugal filter and ACh was determined by HPLC linked to a post column immobilization enzyme reactor followed by electrochemical detection with a Pt-electrode as described by Yoshitake et al.[203].

LUMINOMETRIC ASSAY

ATP levels in the medium following acute hypoxia and in hyperoxic controls before and after the challenge were determined using the ATP Bioluminescence Assay Kit (Sigma) containing luciferin-luciferase. Luciferase oxidizes luciferin in the presence of ATP. Photons are produced in this reaction and are detected with a luminometer. ATP values were calculated using the standard ATP calibration curve.

MULTIPLEX ELISA ASSAY

After prolonged 1-h hypoxic and hyperoxic challenges, the levels of ten cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α) were determined with the Human Ultrasensitive Cytokine 10-Plex Panel (Life technologies). The assay was performed using the 200™ dual laser detection system (Luminex Corporation).

STATISTICS

In study VI, the data on neurotransmitter and cytokine release were analyzed with Student's t-test and Wilcoxon's signed rank test (Prism 6.0 GraphPad), respectively. A P-value of <0.05 was considered significant.

SUMMARY OF RESULTS

PAPER I

In paper I we investigated the mouse CB with focus on receptors and K^+ channels central for oxygen sensing and signaling. We demonstrated presence of the α3, α4, α7 and β2 nAChR subunits, the ATP-receptors $P2X_2$ and $P2X_3$, the dopamine D_2 receptor and the TASK-1 K^+ channel with immunohistochemistry (Fig. 8). This paper provided a map of CB oxygen signaling components and served as a platform for further studies on the human CB.

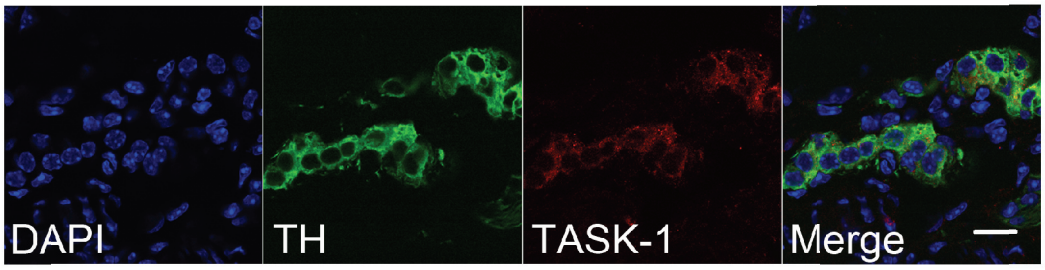


Fig. 8. TASK-1 (red) demonstrated in CB type 1 cells by co-localization with TH (green). Scale bar is 10 µm.

PAPER II

In paper II we characterized the human CB in terms of morphology (Fig. 9) and oxygen sensing and signaling proteins of relevance for anesthesia. We found gene expression of the oxygen-sensing enzyme HO-2, the K^+ channels TASK-1 and BK, the α2, β3 and γ2 $GABA_A$-receptor subunits, the α3, α7 and β2 nAChR subunits, the purinoreceptors $A2_A$ and $P2X_2$ along with the dopamine receptor D_2 (Fig. 10), all important receptors and subunits in oxygen signaling and common targets for anesthetic agents. With this work we demonstrate similarities but also differences in human and animal CB gene expression.

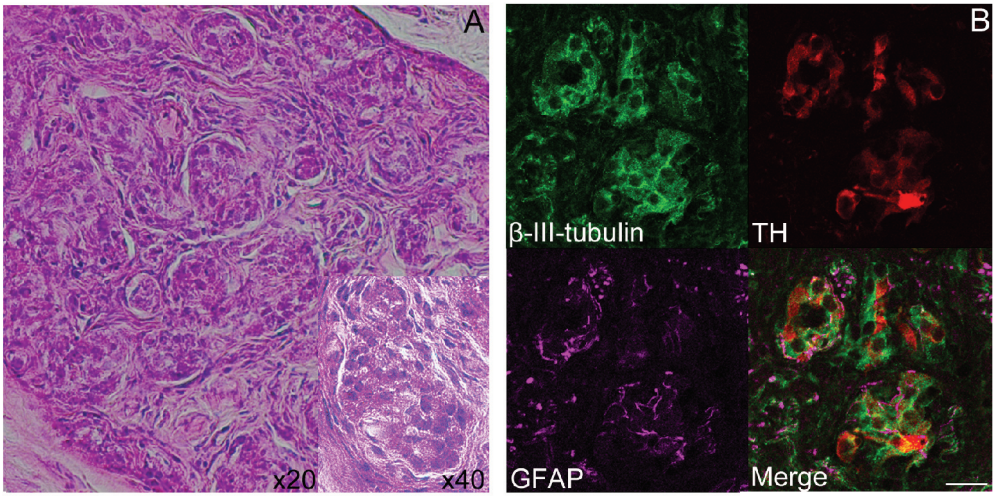


Fig. 9. A. Hematoxylin-eosin staining demonstrating the human CB morphology. B. The localization of different cell types in the human CB demonstrated with immuno-histochemistry: type 1 cells marked with β-III-tubulin (green) and TH (red), type 2 cells with glial fibrillary protein (GFAP) (purple). Scale bar 20 μm.

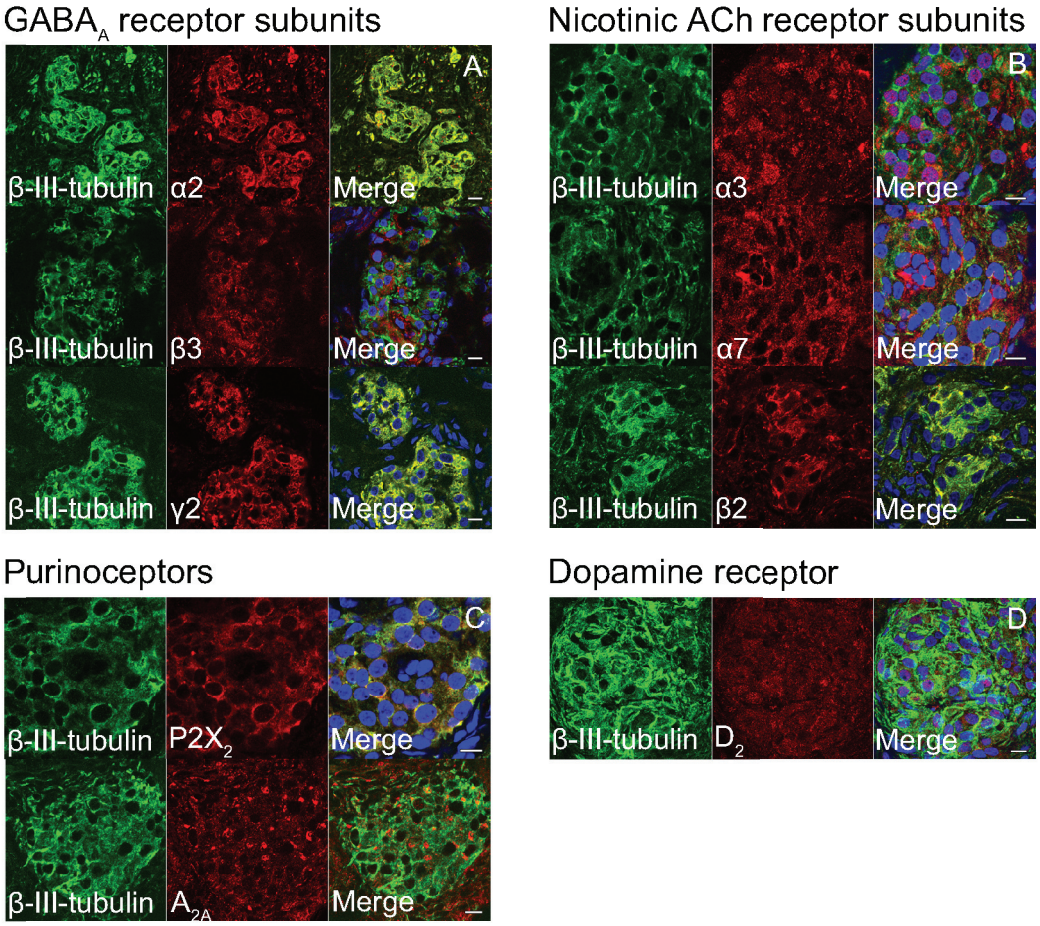


Fig. 10. Expression of receptors and receptor subunits in the human CB. Co-localization of β-III-tubulin (green) with GABA$_A$ receptor subunits (A), nAChR subunits (B), purinoreceptors (C) and the D$_2$ receptor (D) (red) in the type 1 cell is demonstrated. Scale bar is 10 μm.

PAPER III

In paper III we aimed at a more global description of human CB gene expression. In comparison with brain and adrenal gland the human CB overexpressed genes of the inflammatory response, angiogenesis and neurological processes, the latter in comparison with adrenal gland. CB gene expression differed between the human and mouse CB on several genes involved in chemosensory function, for instance CSE, NOX2, HIF-2α, Maxi-K and TASK-1 and TASK-3.

In the human CB we demonstrated expression of a number of oxygen sensing genes such as AMPK, NOX2 and 4, CSE, HIF-1α and HIF-2α, specific K^+ channels (Fig. 11). We also noted expression of genes of the inflammatory response, both inflammatory mediators (IL-6, high-mobility group protein B1 (HMGB1)) and receptors (TLR1, TLR4, IL-1R1, IL-6R, IL-10R, TGFβR1). In table 4 the inflammatory response gene expression in the human and mouse CB are shown.

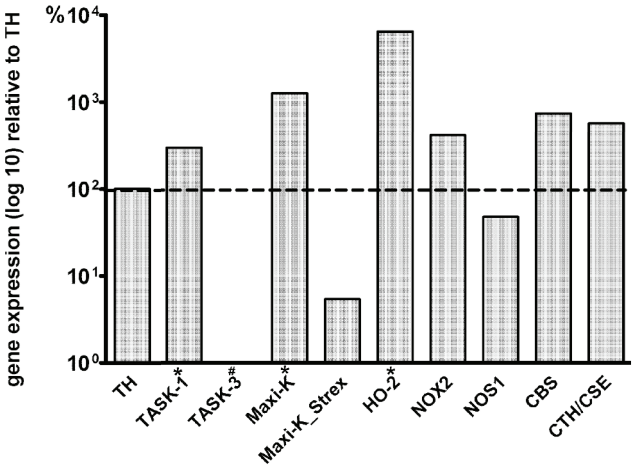


Fig. 11. Expression of selected chemosensory genes in the human CB determined by PCR. The values are expressed as mean± SEM, n=5, with the exception of Maxi-K_Strex, which was expressed only in the CBs of patients B and E. *, combined data results from patients A-C (from study II) and data from two patients D, E (Study III) #, data from patients B and C.

Gene symbol (HGNC)	Human					Mice	
	Patients						
Inflammatory response	A	B	C	D	E	C57BL/6J	DBA/2J
TLR1	p	p	p	p	p	a	a
TLR4	p	p	p	p	p	p	p
IL1R1	p	p	p	p	p	a	a
IL1A	a	a	a	p	a	a	a
IL6R	p	p	p	p	p	u	a
IL6	p	p	p	p	p	a	a
IL10RA	p	p	p	p	p	p (IL10RB)	p (IL10RB)
IL10	a	a	a	a	a	a	a
NOS2 (iNOS)	a	a	a	a	a	a	a
TNFRSF1A	p	p	p	p	p	p	p
TNFRSF1B	p	p	p	p	p	p	p
TNF (TNF-α)	a	a	a	a	a	a	a
TGFBR1	p	p	p	p	p	p	p
TGFB1 (TGF-β)	a	a	a	p	p	a	a
HMGB1	p	p	p	p	p	p	p
NFKB1	p	p	p	p	p	p	p

Table 4. Expression of inflammatory response genes in the human and mouse CB. p=present, a=absent, u=uncertain (due to conflicting results from multiple probe sets), HGNC, human genome name classification.

PAPER IV

In paper IV, we studied the human CB response to hypoxia. CB slices were exposed to brief or prolonged hypoxia and the release of neurotransmitters and cytokines, respectively, was analyzed. We determined release of ACh and ATP in response to acute hypoxia (Fig. 12). Furthermore, pro- and anti-inflammatory cytokines (IL-1β, IL-4, IL-6, IL-8 and IL-10) were released in response to prolonged hypoxia (Fig. 13). The expression of the corresponding receptor proteins IL-1R1, IL-6R and IL-10R, as well as the transcription factors HIF-1α and HIF-2α was demonstrated with immunohistochemistry (Fig. 14).

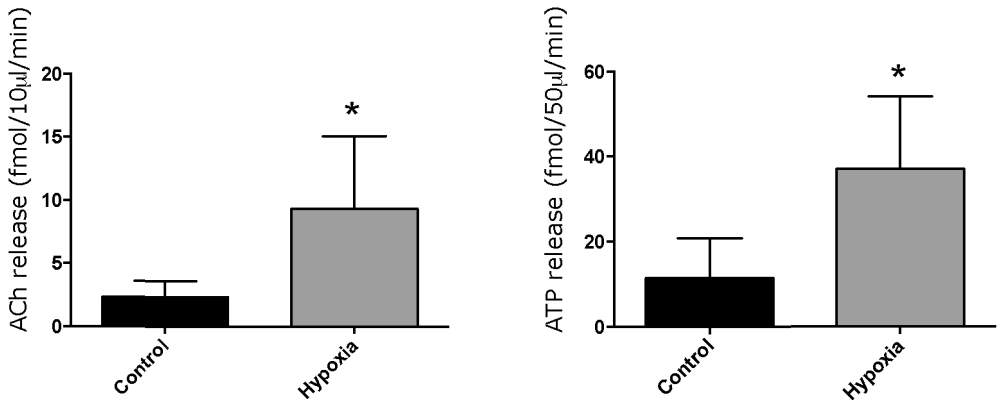


Fig. 12. Acute hypoxia increases the release of ACh and ATP from human CB slices. Data are presented as mean ± SEM. * P<0.05.

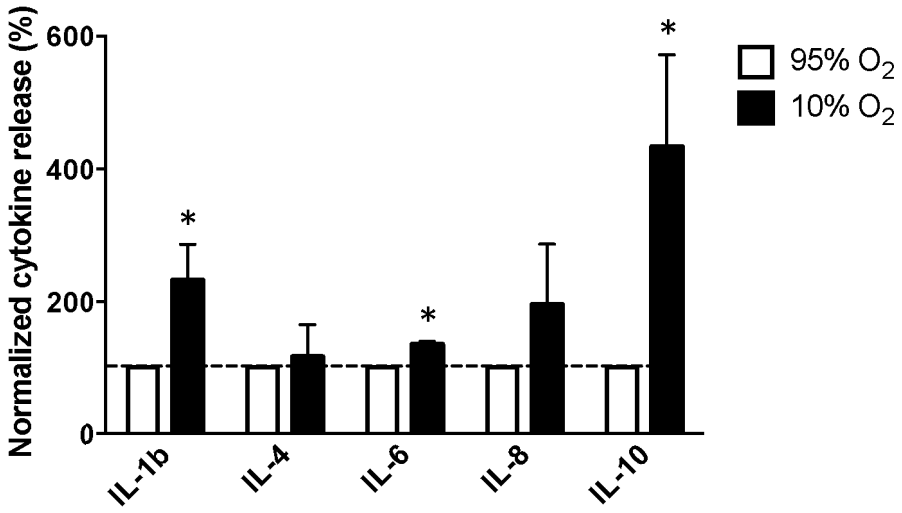


Fig. 13. The effect of prolonged hypoxia on the release of cytokines from human CB slices. The cytokine levels were measured before and after 1 h incubation under hypoxic (10% O_2) and hyperoxic conditions (95% O_2). The relative changes in cytokine levels during hypoxia (close bars) were divided by corresponding changes during hyperoxia (open bars). Data are presented as mean ± SEM. * P<0.05.

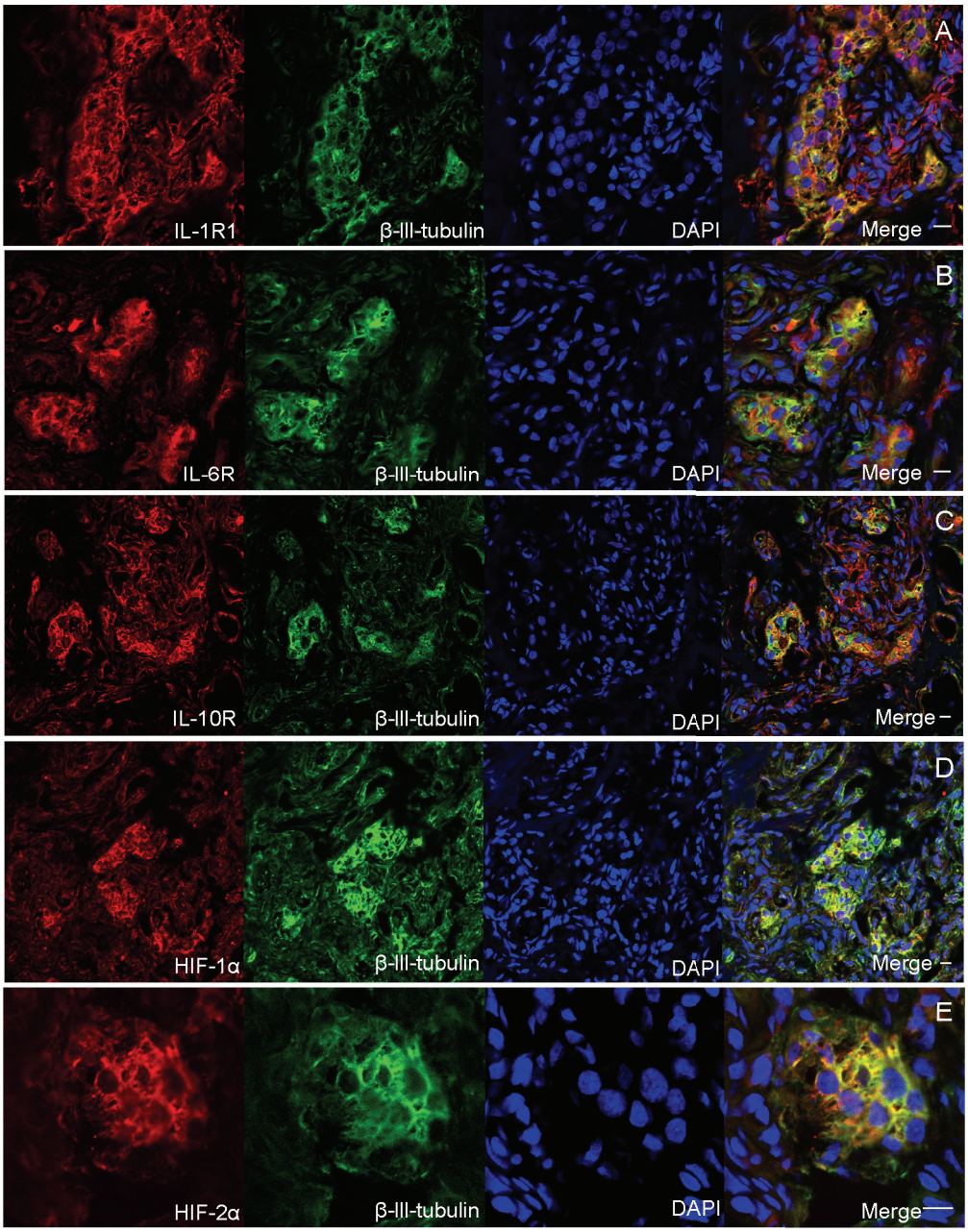


Fig. 14. Demonstration of the cytokine receptors IL-1R1 (A), IL-6R (B) and IL-10R (C) and the transcription factors HIF-1α (D) and HIF-2α (E) (red) in the human CB type 1 cells. Type-1 cells were labeled with antibodies against β-III-tubulin (green) and nuclei with DAPI (blue). Scale bar is 10 μm.

SUMMARY

We have systematically characterized the human CB, previously insufficiently investigated. The studies were performed on surgically removed CBs received in the operating room from patients undergoing elective head- and neck cancer surgery. In this unique series of human CBs we show detailed gene expression on the mRNA and protein level as well as functional data on neurotransmitter and cytokine release upon hypoxic challenge. We conclude that the rodent and human CB largely exhibits a similar oxygen sensing and signaling gene expression patterns and functional traits albeit with some differences. Expression of the selected genes and functional data of the human CB demonstrated in study II-IV is summarized in table 5.

Functional group	Gene		mRNA		Protein	Hypoxia induced release
			Microarray	PCR	IHC	HPLC,ATP-assay, multiplex
Oxygen sensing	AMPK		p	p		
	HO-2		p	p	p	
	NOX-2		p	p		
	NOX-4		p			
	SOD-2		p			
	CAT		p			
	CSE		p	p		
	CBS		u	p		
	HIF-1α		p		p	
	HIF-2α		p		p	
	NOS-1		u			
	NOS-2		a			
	NOS-3		a			
K+-channe	TASK-1		p	p	p	
	BK (Maxi-K)		p	p	p	
	Kv 1.5		p			
	Kv 2.1		p			
Oxygen signaling	ACh					↑
	ATP					↑
	nAChR	α3	p	p	p	
		α7	a	p	p	
		β2	a	p	p	
	GABAA	α2	p	p	p	
		β3	p	p	p	
		γ2	a	a	p	
	GABAB1		p			
	A2A		p	p	p	
	P2X2		a	p	p	
	D2		p	p	p	
Inflammatory response	Mediators	IL-1α	a			
		IL-1β	p			↑
		IL-4	a			↑
		IL-6	p			↑
		IL-8	p			↑
		IL-10	a			↑
		HMGB1	p			
		NFkB	p			
	Receptors	IL-1R1	p		p	
		IL-6R	p		p	
		IL-10RA	p		p	
		TLR1	p			
		TLR4	p		a	
		TNF-R1A	p			
		TNF-R1B	p			
		TGFB-R1	p		a	

Table 5. Summary of the findings in the human CB. p, present, a, absent, u, uncertain (due to conflicting results from multiple probe sets), unfilled, not studied or not applicable.

DISCUSSION

In this thesis we sought to characterize the human CB oxygen sensing and signaling gene expression, neurotransmitter release and genes and molecules involved in the inflammatory response. To our knowledge this is the first time that human CB tissue from healthy surgical patients has been investigated during normoxia, acute and prolonged hypoxia. We disclosed many similarities but also differences in human CB gene expression compared to that of other species. We also found that the human CB expresses receptors and receptor subunits that are targets for potent anesthetic agents, findings that provide a base for a more fundamental understanding of possible mechanisms behind anesthesia-related impairment of regulation of breathing during hypoxia.

THE HUMAN CB IN OXYGEN SENSING

Despite an impressive number of investigations using sophisticated animal models aiming to uncover fundamental components in CB oxygen sensing, there is still no general consensus on the mechanism by which oxygen is being sensed by the CB. Rather, there is an array of theories prevailing and the true oxygen sensor(s) may include components from more than one of these concepts. On the other hand, there is more of a general agreement on the transduction steps following oxygen sensing, namely inhibition and closure of oxygen sensitive K^+ channels resulting in depolarization of the type 1 cells, Ca^{2+}-entry and a subsequent neurotransmitter release. Importantly, the expression of these specific K^+ channels is species-dependent, suggesting somewhat divergent CB oxygen sensing mechanisms in different species.

This work is an attempt to describe the human CB on a morphological, gene expressional and functional level. Ultimately, the profiles of the human and rodent CB are compared in order to determine the validity of the rodent CB platform for further studies on oxygen sensing with existing animal- and cell models and for extrapolation of the results to humans CB oxygen sensing.

A substantial part of the large family of K^+ channels is sensitive to oxygen and essential for oxygen sensing and signaling. The composition of oxygen sensitive K^+ channels varies between species as well as their role within the oxygen-sensing pathway. We speculate that out of the

four oxygen sensitive K^+ channels expressed in the human CB (Kv 1.5, Kv 2.1, TASK-1 and Maxi-K) the primary candidates for a key role in oxygen sensing are TASK-1 and Maxi-K, considering the wealth of data supporting the importance of one or the other or both in animal CB oxygen sensing[42]. TASK-1 and TASK-3 are reported to exist as functional heterodimers in rats, but in human CBs TASK-1 was expressed as mRNA and protein without assembly of any of its potential dimerization partners TASK-3 and TASK-5. Interestingly, it has been shown that the open probability of TASK-1 in hypoxia is greatly increased by halothane (but not by isoflurane) in a rat CB cell preparation, thereby rendering the type 1 cell relatively resistant to depolarization[204]. Thus, TASK-1 in the human CB type 1 cell is a possible target for volatile anesthetics, providing a potential mechanism for depression of the CB response to hypoxia by some of the volatile anesthetics. In addition, considering its acid-sensitive nature, human CB TASK-1 channels may be directly inhibited by acidemia in exercise or disease, triggering ventilation and sympathetic activation.

Splice variants of the Maxi-K α-subunit respond differently to AMPK-induced channel inhibition[205]. The human CB exclusively expresses the ZERO isoform, short of the stress-regulated exon (Strex) that reduces AMPK inhibition of Maxi-K, as opposed to the rat CB that expresses both variants, albeit only the ZERO isoform in the CB type 1 cell[205]. There is reason to believe that this isoform is also expressed specifically in the type 1 cell of the human CB, being the only expressed isoform shown by the microarray and PCR analyses, and since Maxi-K is demonstrated to co-localize with β-III-tubulin in human CB type 1 cells. AMPK transcripts are, as expected considering its distribution in different cell types, present as mRNA in the human CB, although not yet demonstrated as protein in the type 1 cell. We also demonstrate mRNA and protein expression of HO-2 in the human CB. This CO-synthesizing enzyme is known to co-localize with Maxi-K, likewise present in the human CB, further providing two key components to possibly orchestrate the human oxygen-sensing cascade.

The counterbalance between the two gaseous messengers CO and H_2S seems to be of importance for CB oxygen sensing in animals, H_2S being a stimulating mediator and CO an inhibiting mediator of CB activity[79]. H_2S synthesis is evoked by hypoxia and H_2S is considered to be synthesized mainly by CSE in the CB, whereas cystathionine β-lyase (CBS) is the primary H_2S synthesizing enzyme in the brain. $CSE^{-/-}$ mice show a severely impaired CB response to hypoxia which indicates a role for H_2S in CB activation[79]. Furthermore, CSE is proposed to be the primary H_2S-synthesizing enzyme in three different rat strains[82]. While we found expression of CSE in the human CB with both microarray and PCR, CBS was only detected by PCR and with uncertain expression by microarray. Surprisingly, in the two mouse strains (C57BL/6 and DBA/2J) used for comparison in study III, CSE was absent. As CSE has been demonstrated in mice previously we speculate whether this is due to strain specificity or due to low-resolution signals in the microarray. We propose that CSE may be the primary catalyzer in H_2S formation in the human CB, resulting in enhancement of the hypoxia-evoked response.

Finally we found that NOS-1, one of the enzymes synthesizing NO, another gaseous transmitter proposed to be involved in CB chemosensing was expressed in limited amounts as detected with PCR and with an uncertain expression with microarray due to conflicting results from the multiple probe sets interrogating the same gene. We speculate that NOS-1 is expressed in the human CB, albeit in limited amounts during normoxia, whereas NOS-2 and NOS-3 was absent. Whether NOS-1 is expressed in human CB type 1 cells or on adjacent nerve endings was not determined in this thesis.

In the human CB, there is capacity for ROS-mediated oxygen sensing with the established expression of NOX 2, NOX 4, SOD-2 and catalase. Membrane bound NOX-enzymes are previously shown to be located closely to TASK-1 in oxygen sensing cells in culture[69], and since both of these molecules are expressed in the human CB there is a possibility for NOX 4-induced inhibition of CB activity through ROS-production. This is in line with the fact that the ROS-scavenger NAC increases the HVR in humans[71].
As expected, both HIF-1α and HIF-2α were expressed as mRNA and proteins in the human CB type 1 cell. More insight into up-regulation or down-regulation of the two transcription factors in chronic hypoxia and chronic intermittent hypoxia is needed to further elucidate the impact of human CB HIF-imbalance and sympathetic activation in OSA behind the development of hypertension and increased catecholamine levels[179].

In conclusion, gene expression of the components in the different oxygen sensing theories emerging from animal studies is also present in the human CB. Although differences in e.g. K^+ channel splicing and identity of the H_2S-synthesizing enzyme, many similar genes in oxygen sensing are expressed between species, suggesting that with our present foundation of knowledge there is most likely a comparable CB oxygen sensing mechanism in humans and rodents.
In figure 15, the oxygen sensing elements demonstrated in the human CB in this thesis are shown.

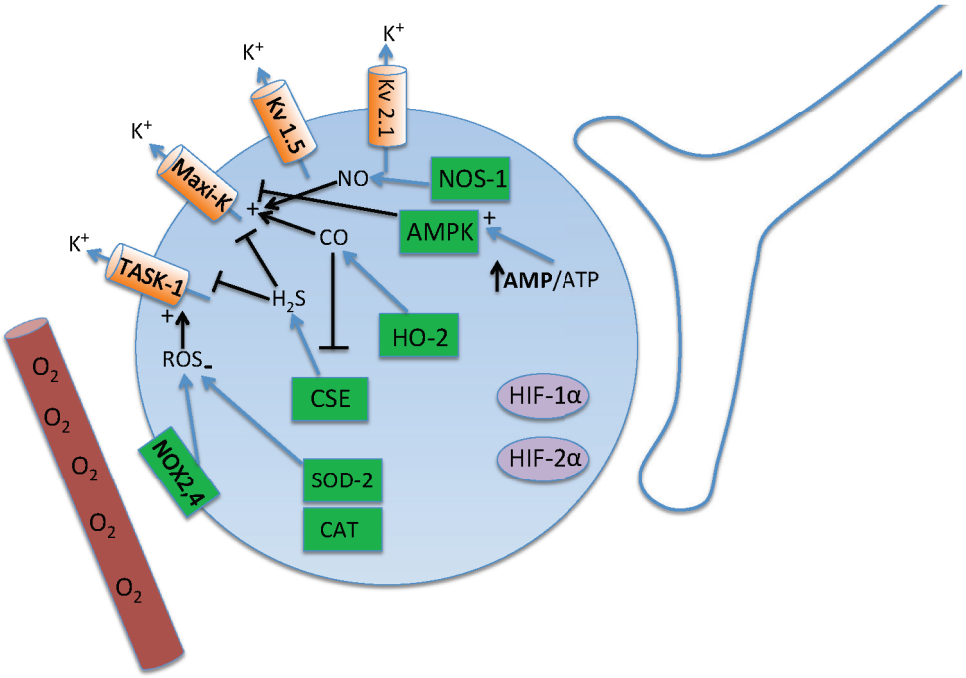


Fig. 15. Human CB oxygen sensing molecules described in this thesis.

OXYGEN SIGNALING IN THE HUMAN CB

The oxygen-signaling cascade is to a large extent not established in the human CB. Recently, however, it was shown that human CBs from brain dead organ donors exhibit quantal release of neurotransmitters in response to hypoxia and hypoglycemia, after type 1-cell depolarization and inward Ca^{2+} currents[190]. The human CB type 1 cell secretion rate in this study was comparable to the secretion rate in rodent models, although at levels in the lower range, whereas the charge

of the average released human quanta was higher than in the rat and rabbit (see[190] and references therein). However, the nature of the neurotransmitters was not revealed.
In this thesis we demonstrate a release of ACh and ATP as well as cytokines in response to hypoxia, and furthermore gene and protein expression of key nicotinic, purinergic, dopaminergic and GABA-ergic receptors and receptor subunits.
When exposing human CB slices to acute hypoxia we found a distinctly increased release of ACh and ATP compared to baseline release, and thereafter return to baseline after the hypoxic exposure. The relative increase in ACh and ATP release was comparable, with an approximately four-fold increase for both transmitters. On the other hand, while the absolute levels for ATP-release was similar to cat and rat ATP-release[114, 206], the absolute human CB ACh-release was 10-fold lower than reported in cats, although the range of release between the different studies was strikingly wide[115, 207]. We speculate that this is related to the fact that the cat sinus nerve discharge is higher than in other species[208], or that the released ACh in the human CBs is amplified through autocrine and paracrine effects. Another explanation could be different set of nACh and mACh receptors in human and cat CBs.
Because ACh and ATP are generally regarded as the primary excitatory neurotransmitters based on an array of studies in multiple species, we hypothesized that the human CB would operate with the similar set of neurotransmitters. From a functional perspective, it should be noted that CB discharge is almost completely abolished by concurrently blocking nAChR and P2X receptors[113], indicating the relevance of ACh and ATP exclusively and in concert for CB neurotransmission. Confirming our hypothesis, we demonstrate that this set of neurotransmitters is rapidly released from the human CB during acute hypoxia.

In study II, the corresponding receptors and receptor subtypes for both ACh and ATP were demonstrated in the human CB.
The α3 nAChR subunit was demonstrated in all patients on both mRNA and protein level. The protein expression of the α7 subunit was inconclusive whereas PCR provided enough data to confirm its expression. The β2 nAChR subunit was detected with PCR and immunohistochemistry but not with microarray, perhaps an effect of the sensitivity of the microarray technique where transcript levels are commonly underestimated. α3 and β2 nAChR subunits co-localized with β-III-tubulin in the type 1 cell in concordance with previously characterized animal CB nAChR subunits[105] as well as with the mouse CB in study I. We note that the α7 nAChR subunit is previously only demonstrated in animal CB nerve terminals, whereas we showed the α7 nAChR subunit in the mouse and human CB type 1 cell. We speculate that besides regular cholinergic signaling between the type 1 cell and the sinus nerve afferents, heteromeric neuronal nAChRs and the homomeric α7 nAChR on the type 1 cell mediate autocrine induction, further increasing the neurotransmitter signal intensity. A possible role of the α7 nAChR in inflammatory signaling is further discussed below.
Although α4 mRNA and protein is demonstrated in CBs of several species, including the mouse CB in study I, we could not detect this subunit in the human CB, indicating that an α3-containing nAChR subtype and the homomeric α7 nAChR may be the functional nAChRs in cholinergic signaling within the human CB. This is in line with that nAChRs containing the α3 (i.e. α3β2 and α3β4) and α7 subunits are present in cholinergic ganglia of the autonomic nervous system.
In the human CB we found that $P2X_2$ was expressed as mRNA and protein and was localized to the type 1 cell, which we also demonstrated in the mouse CB (study I). However, $P2X_3$ was not present in the human CB but present in the mouse CB type 1 cell. As $P2X_2^{-/-}$ but not $P2X_3^{-/-}$ mice show a profoundly attenuated HVR, a genetic abolition of $P2X_3$ could be part in an

evolutionary refinement of the human CB. Indeed, the human CB transcriptome is approximately 30-40% larger than that of the mouse CB[200], indicating a higher complexity.

$GABA_A$ receptor agonists inhibit CB activation through a proposed action on postsynaptic $GABA_A$ receptors[129]. The human CB expresses the α2, β3 and γ2 $GABA_A$ receptor subunits, coincidentally also being the subunits forming the common α2β3γ2 $GABA_A$ receptor subtype in the CNS. The α2 subunit was previously reported as absent in the cat CB while present in the rat, indicating species differences in GABA-ergic CB signaling[129, 209].
Propofol is a positive modulator and agonist at the $GABA_A$ receptor and the β-subunit is essential for binding of propofol, as demonstrated in a study where point mutations in the human $GABA_A$ β2 and β3 subunits resulted in a reduced effect of propofol[210]. Besides $GABA_A$ potentiation, propofol inhibits the neuronal α4β2 nAChR in relevant concentrations[211] while higher doses were needed for inhibition of the α3β2 and α7 subtypes (unpublished data, Jonsson Fagerlund). The propofol-induced depression of the CB, attributed partly to inhibition of nAChRs in the CB[131], may be caused by an activation of the α2β3γ2 $GABA_A$ receptor and inhibition of neuronal nAChRs in the human CB, as well as activation of the TASK-1 K^+ channel. Since the α7 nAChR is unaffected by propofol (unless in very high concentrations) we regard propofol inhibition of CB signaling via this receptor less likely[212].
The importance of GABA-ergic transmission in regulation of breathing in hypoxia is further underlined by the demonstration that a change in the NTS-expression of $GABA_A$ receptor subunits from α3 to α1 in a critical period of respiratory development results in a markedly reduced acute HVR in the rat pup. Whether this is true in human development of respiratory reflexes to hypoxia is not known[99].
In the human CB, the expression of the $GABA_{B1}$ subunit was also confirmed. This metabotropic receptor is present in the rat CB and potentiation of $GABA_B$ with an agonist results in activation of TASK-1, thereby depressing CB activity[132].
DA released in the CB in response to hypoxia is suggested to exert negative feedback on excitatory neurotransmitter release through activation of type 1-cell D_2 receptors, demonstrated in cat and rat CBs[213-215]. We further show the D_2 receptor in CB type 1 cells in mice and humans. However, whether DA is released in the human CB in response to hypoxia is not shown in this thesis.
Adenosine is released from the rat CB in response to hypoxia and acts as an excitatory neurotransmitter by activating A_{2A} receptors on type 1 cells and nerve endings[216, 217]. As in the rat CB[217], the A_{2A} receptor is demonstrated in the type 1 cell in the human CB.

In summary, the human CB releases ACh and ATP as well as cytokines in response to hypoxia. We have furthermore demonstrated nicotinergic, purinergic, GABA-ergic and dopaminergic receptors in the human CB (Fig. 16) and it may thus be argued that a combination of excitatory and inhibitory neurotransmitters exert effects on their corresponding receptors in a direct, autocrine and paracrine manner, resulting in the balanced “push-pull” mechanism in CB activity proposed in mammals.

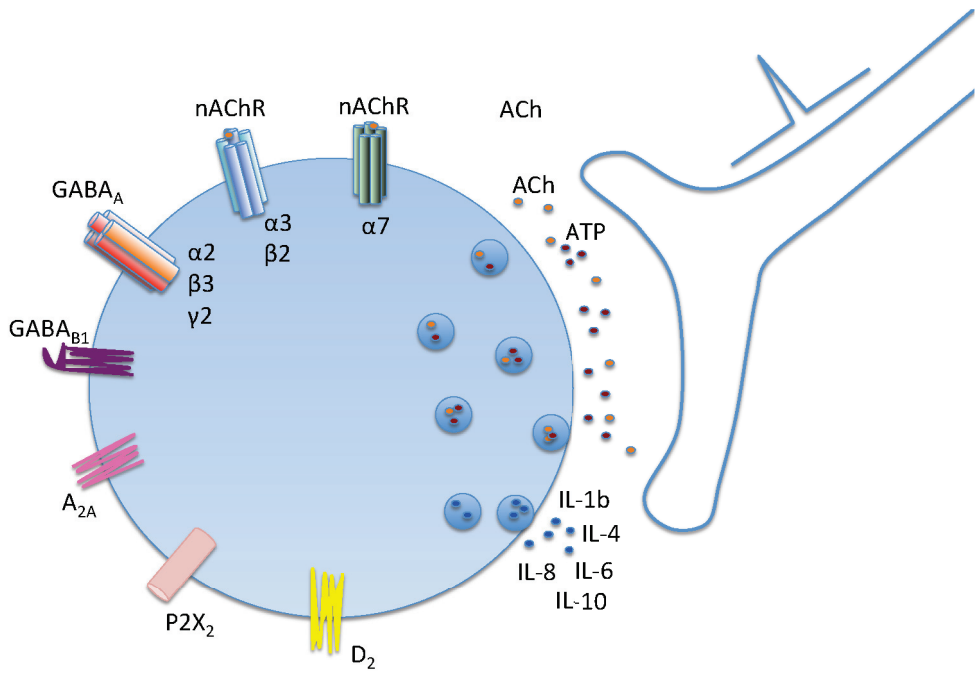


Fig. 16. Oxygen signaling components in the human CB type 1 cell demonstrated in this thesis.

INFLAMMATION SENSING AND SIGNALING IN THE HUMAN CB

There is a growing body of evidence for a role of the CB in immune-to-brain signaling. The CB possesses an ideal location for sensing and transferring information on blood-borne inflammatory molecules to the CNS and in consequence, an immunological input could initiate cardiorespiratory responses through CB activation.
In the human and mouse CB we show enrichment of inflammatory response genes in comparison with brain and adrenal gland tissues. Over-representation of the Gene Ontology groups *inflammatory response*, *response to cytokine stimuli*, *acute inflammatory response*, *adaptive immune response*, *response to LPS* and *chemokine receptor binding*, among others, was demonstrated. In the human CB we further point out specific gene expression of inflammatory mediators and receptors, the latter confirmed as proteins in the type 1 cell. Finally, in response to prolonged hypoxia there was an increased release of pro- and anti-inflammatory mediators. These components are summarized in figure 17.

Previous studies on animals have established several inflammatory factors in the CB, as reviewed by Porzionato et al.[148]. More specifically, mRNA and protein of IL-1β, IL-6 and their receptors IL1-R1 and IL-6 (the gp 130 subunit) have been demonstrated in rat CBs with the receptors located at the type 1 cell[143, 144]. Further, TNF-α, NFκB, TNF-R1 and TLR-4 were shown as mRNA and protein in cat and rat CBs, in both type 1 cells, endothelial cells (TNF-α and TNF-R1) and petrosal neurons (TLR-4)[137, 218]. We confirm these animal findings in the human CB, with the exception of TNF-α and IL-1α but with the addition of HMGB1 and the receptors IL-10R, TLR-1 and TGFβR1. The rationale for the undetected human CB expression of TNF-α, IL-1α and IL-10, who's corresponding receptors all were expressed, could be a low expression in normoxia, as was the milieu for the human CBs prior to extraction. Hence, the expression data are exclusively from CBs exposed to physiological normoxia. Under these

circumstances the human CB expresses early and late mediators of inflammation and inflammatory receptors. It would, however, be interesting to investigate the change in expression pattern of inflammatory response genes in the human CB exposed to a longer period of hypoxia.
A recent study showed that cytokines contribute to an increased CB response to prolonged hypoxia in rats[219]. Whole rabbit CBs exposed to hypoxia release prostaglandin E_2[220], but hypoxia-evoked CB release of cytokines has not been investigated further. We hereby present an increased release of IL-1β, IL-4, IL-6, IL-8 and IL-10 in comparison to hyperoxia. The cytokine levels in study IV were measured in arbitrary units and the relative change in cytokine levels during hypoxia 1h was compared to the relative change in cytokine levels during hyperoxia 1h (control). These arbitrary levels cannot be correlated to clinical levels of circulating cytokines. Instead, a paracrine or autocrine mechanism for the released cytokines is proposed. Although an increased release in hypoxia could be detected, the levels of IL-4 and IL-10 were low, and further data is needed to investigate the cytokine release in depth. Based on the demonstrated cytokine release in response to hypoxia, immune signaling in response to prolonged hypoxia is possible in the human CB. However, since only one time point was studied (i.e. 1 hour of hypoxia) conclusions regarding the inflammatory release pattern of early and late mediators must be made with great caution.

Systemic inflammation may be transmitted to the CNS through either blood-borne humoral and cellular signaling, where circulating inflammatory mediators may pass the blood-brain barrier and activate immune competent cells within the CNS to produce second messengers ultimately affecting the brain, or via neural signaling where e.g. vagus afferents are activated by immune-cell generated cytokines. In a similar way, the CB has been proposed to participate in neural signaling (complimentary to the vagal-dependent inflammatory reflex pathway) of peripheral inflammation by relaying the information to the CNS through nerve afferent signal modulation[142]. Ackland et al. showed that CBs of mice exposed to the pathogen zymosan exhibited an increased chemoafference at rest and in response to hypoxia in the absence of acidemia or hypercapnia[140]. Moreover, IL-1β and IL-6 increases the release of Ca^{2+} from clusters of CB type 1 cells[138, 139], and both IL-1β and LPS administered intraperitoneally induces an increased expression of cytokines and cytokine receptors in the rat CB[218, 221].The tachypnea, tachycardia and hypotension instigated in rats with LPS administered intravenously was prevented with a previous neurotomy of the CSN and aortic nerve[137]. Although contradicting findings exist, such as the demonstration of a decreased CSN-frequency with TNF-α stimuli on the *in vitro* rat CB[137], the main body of evidence points towards a CB function in communication between the immune system and the CNS. This could be important in early stages of inflammatory activity by inducing appropriate respiratory and cardiovascular responses. In fact, rats with carotid sinus denervation exhibit a shorter survival time in endotoxin-induced sepsis[222].
Fundamental components for the immune-to-brain communication link are now established in the human CB. However, the inflammasome immunoreactivities NLRP 1 and 3 were not expressed in our human CB samples, as in the CBs of the previously described rats exposed to zymosan[140].
Another component shown in the human CB and essential for immune-to-brain signaling is the α7 nAChR subunit. This subunit is necessary for the cholinergic anti-inflammatory reflex pathway, a vagus-nerve circuit that ultimately inhibits TNF-α production in spleen macrophages expressing the α7 nAChR subunit[142]. It was recently discovered that vagus nerve efference results in adrenergic activation of spleen T-cells relaying the cholinergic ACh signal

required for regulation of the macrophage TNF-α release[223]. In the same way, it may be speculated that the human CB could be activated by circulating early inflammatory mediators and transforming this stimuli into an afferent signal to the brainstem, from where a cardiovascular and possibly an immunomodulating response is evoked.

In acclimatization to chronic hypoxia, inflammatory changes in the CB are induced, such as infiltration of immune cells and an increased expression of cytokines and cytokine receptors together with an increased chemosensitivity and augmented HVR, as reviewed by Porzionato et al[148]. In the human CB we demonstrate that genes involved in angiogenesis were enriched, in line with the CB ability to undergo hyperplastic and immunomodulatory changes in response to chronic hypoxia[12, 144, 175]. Interestingly, in animals these changes are blocked by treatment with anti-inflammatory agents[145]. Accordingly, we show that the only patient in study IV that received an anti-inflammatory drug prior to CB extraction displayed a diminished release of cytokines in response to hypoxia.
Notably, in rats exposed to chronic *intermittent* hypoxia, ibuprofen prevents the augmentation of the HVR and the hypertension but not the potentiation of CB chemoreactivity. This suggests a different mechanism behind the increased CB chemoresponse in chronic intermittent hypoxia compared to chronic hypoxia, supposedly through altered HIF-balance[95].
Surgery is known to trigger an inflammatory response involving local and systemic upregulation of early cytokines through activation of peripheral immune cells[224, 225]. The fact that the CB samples were taken during surgery with ongoing trauma-induced inflammatory response combined with lack of perfusion before removal may have contributed to residual immune cells with the CB capillaries. However, we believe that the impact of this is minimal since the same CB was used to provide slices for both the hypoxic and control experiments, i.e., slices used for comparison in the control and hypoxic challenges came from the same CB and hence the same conditions.
Lymphocytes have previously been reported in the human CB and mast cells in vicinity of the vessels in the human CB[18]. However, the degree of RNA contribution is most likely low because the limited amount of circulating macrophages or monocytes in the blood in early phases of the aseptic trauma caused by surgery. Furthermore, the tissues that the CB was compared to in study III (brain and adrenal gland) had not been perfused before analysis and the comparison would be influenced equally with regards to contribution of inflammatory cells.

Despite the limited number of patients studied, we show increased release of both pro- and anti-inflammatory cytokines from the human CB in response to 1-hour prolonged hypoxia, indicating that the CB orchestrates a cascade of inflammatory mediators with the ability to modulate the inflammatory response to hypoxia. An alternate interpretation would be that simultaneous pro- and anti-inflammatory effects resulting from activation of corresponding receptors are part of auto- or paracrine regulation of both neurotransmitter and further cytokine release. Finally, it may also be argued that the human CB gene expression of early and late inflammatory mediators and receptors is associated with the cholinergic anti-inflammatory reflex pathway. In this way, systemic inflammation may activate the CB to further evoke early signs of the inflammatory response such as tachycardia and hyperventilation.

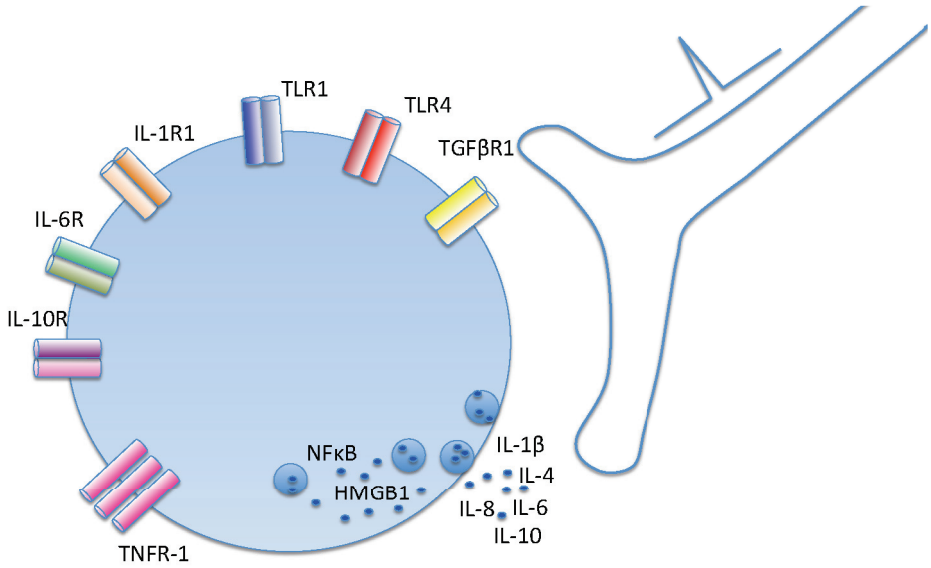


Fig. 17. Inflammatory components demonstrated in the human CB

THE HUMAN CB GENE EXPRESSION PROFILE

Neither the human nor the mouse CB gene expression closely resembled any cell or tissue type available in the Barcode database. The tissue closest to the CB in developmental origin from the neural crest, the trigeminal ganglion, only displayed weak gene expression profile similarities. Rather, the comparison with functionally related tissues accessible in public databases, brain and adrenal gland, provided with interesting information on the differences between the gene expression profiles of these tissues. In addition to the up-regulation of genes related to the inflammatory response discussed previously, the human CB exhibits a distinct neurological profile in the comparison with adrenal gland. In relation to a recent publication where the human CB contains nestin-expressing stem cells with TH-expressing "blebs" growing on the neurosphere[48] it is notable that the human CB over–expresses genes involved in neuron development, axonogenesis and neuron projection in comparison to adrenal gland. Furthermore, angiogenesis and vasculature development was over-expressed in the human CB compared to both brain and adrenal gland. Comparison of the mouse CB with mouse brain and adrenal gland show similar patterns to the comparison in corresponding human tissues. Taken together, these results indicate a dynamic organ with cells able to proliferate and differentiate in response to for example chronic hypoxia, as demonstrated previously in mouse and rat CBs[16]. Hence, the CB is proposed to be a neurogenic center containing stem cells that can differentiate into functional type 1 cells and this demonstration of neurogenesis in the CB by Pardal et al. is notably the first neurogenesis shown in the peripheral nervous system[16]. It is so far unclear as to whether the human type 2 cell can differentiate into a functional type 1 cell.

METHODOLOGICAL CONSIDERATIONS

Human CB tissue for investigation may be obtained with several different approaches. The CBs can be harvested during early autopsy when the tissue might still be intact enough to perform morphological investigations. Further, during carotid endarterectomy due to carotid artery

stenosis the CB is accessed, however, due to the arterial occlusion the blood flow to the CB is proposed to be severely impaired and the typical patient going through such surgery per definition displays severe cardiovascular disease that might affect CB structure and function. Another option is to extract CBs from organ donors during or after the actual organ donation surgery. Yet, these patients are confirmed brain dead before surgery and are typically subjected to potent pharmacological treatment in the preceding time period, besides the fact that the cerebral circulation per definition is abolished with following, plausible effects on CB perfusion. Our approach has been to obtain fresh CB tissue directly after surgical removal from patients without interference from co-morbidities, pharmacological influence or radiation with an assured denervation of the CB prior to extraction. This was achieved by utilizing CBs extracted unilaterally from ENT patients going through elective modified radical neck surgery. With this approach, we could investigate the human CB in terms of morphology, gene expression and function with carefully selected methods.

During the course of the studies we faced a number of challenges. With the narrow inclusion criteria of our protocol, patient availability became limited. In each included patient, there was a limited amount of tissue related to the small size of the organ and furthermore a low RNA yields. The heterogeneity of the human CB has been a further constraint. Methods were selected according to these prerequisites and after establishing a platform based on experiences and acquired methods from study I on the mouse CB.
We chose to first characterize human CB oxygen signaling components with immunohistochemistry, microarray and PCR in study II, oxygen sensing and inflammation gene expression with microarray, PCR and gene-expression comparison software tools in study III and finally human CB function using an hypoxia experimental setup as well as immunohistochemistry to confirm the expression of selected proteins.
As immunohistochemistry is known to provide a risk for false positive results due to non-specific binding and conversely, low sensitivity due to fixation errors, steric obstacles in binding of antibodies and low affinity of the antibody, the method is preferred in combination with other, overlapping methods. In study II and III we also used microarray and PCR to demonstrate mRNA expression.
The PCR technique has a lower sensitivity threshold compared to the microarray technique, which could explain discrepancies in gene expression, as noted in study II. This is one reason why PCR is often used as a complement or confirmation technique to microarray. Since the translation of mRNA to protein is not a linear process, demonstration of proteins with immunohistochemistry is of added value, besides the profit of gaining information on the cellular localization of proteins in different cells. The use of Western blot would have added quantitative information but would have provided no indication of protein localization. Due to the limitation in tissue amount this method was not incorporated in the protocol.

An obvious matter when exposing CB tissue to hypoxia is the level of hypoxia actually attained in the CB tissue in relation to the level of hypoxia in the perfusate or medium. *In vivo* CB tissue PO_2 is usually lower than PaO_2 and this difference between surface and tissue PO_2 is even larger *in vitro* using a perfusate or medium. The measured *in vivo* CB tissue PO_2 diverges between studies but a normal to high value compared to other tissues is considered a present consensus[13]. Studies on perfused CBs have demonstrated a difference in the PO_2 of the perfusate and tissue PO_2, with tissue PO_2 values being less than half of the perfusate PO_2[36, 226]. In our experiments the perfusate PO_2 was approximately 14kPa during the hypoxic challenges and the levels of achieved hypoxia were sufficient to result in increased neurotransmitter and cytokine release.

We carefully sought to avoid a CB PO_2 below the limit of its own metabolism in this precious tissue. The control slices were exposed to hyperoxia since we cannot exclude that exposure to a "normoxic" medium would rather be a milder form of hypoxia in the center of the tissue. This is based on experiences with perfusion of rabbit CBs that were distinctly activated by a PO_2 reduction to "hyperoxic-normoxic" levels[131].
Finally, the size of the CBs in study II is very large compared to what is reported in the literature[12]. Since none of the patients had a hypoxemic disease leading to CB hyperplasia, the relatively large size of the human CBs in this study is most likely due to insufficient cleaning of the tissue piece. It is also evident that the tissue that we study contains many cell types besides type 1 cells, for example type 2 cells, nerve cells, endothelial cells, fibroblasts and circulating blood cells. The microarray results must therefore be interpreted with caution and complementary methods therefore provide guiding in the distinguishing between the contributions of the different cell types to the microarray results.

CLINICAL IMPLICATIONS

When the individual is exposed to acute or chronic hypoxia the CBs evoke the HVR to ultimately increase O_2 delivery to the tissues. As stated, many anesthetic agents blunt this reflexive chemoresponse, not only during the course of anesthesia but also in the postoperative period. During the postoperative period, the patient is usually less monitored compared to the intraoperative period with regards to vital signs, which may expose the patient to a higher risk for adverse respiratory events. In this sense, the clinical relevance of anesthesia-induced respiratory depression has the most important impact on patient care and safety in the early postoperative period (< 24 h), where residual effects of anesthetic agents are common[227]. In the later postoperative phase (> 24 h), respiratory adverse events may also occur during sleep, where the pharmacological effects on the control of breathing could be even more pronounced since behavioral control of breathing is less during sleep. Depressed CB activity acts in synergy with atelectasis, decreased arousal response and a reduced upper airway tone, increasing the risk of hypoxia in the vulnerable, postoperative period. It is possible that hypoxic adverse events further disturb several postoperative vital processes such as cognitive recovery, wound healing and immunological function.
In this thesis we have demonstrated receptor targets in the human CB for inhaled and intravenous anesthetics as well as NMBAs. Consequently, with residual effects of one or more of these agents it may be argued that the first line of defense to hypoxia might be reduced or even absent in the postoperative period. Therefore, adequate reversal of NMBAs and other anesthetic agents known to interfere with regulation of breathing during hypoxia is warranted. In addition, we believe it is desirable to consider alternative anesthetic management in at-risk patients prone to hypoxia and postoperative pulmonary complications such as patients with chronic pulmonary disease and sleep-related breathing disorders. As an example, patients with OSA are at risk of postoperative complications due to hypoxemia, leading to unplanned ICU-transfer and longer hospital stay[228]. In animal models of OSA, transcriptional remodeling in the CB type 1 cells related to altered HIF-balance and immunoactivation, results in increased oxidative stress and augmentation of CB sensory output. The cardiorespiratory pathology in OSA including hypertension and elevated catecholamine levels has been shown directly related to the enhanced CB activity[179]. We have showed that the human CB responds to prolonged hypoxia with release of cytokines, indicating recruitment of the inflammatory mechanism that is part of the pathogenesis in OSA. Moreover, both HIF-1α and HIF-2α are demonstrated as

mRNA and proteins in the human CB, although the levels in hypoxia and hyperoxia have not been explored.

The human CB thus exhibits properties of an immunological organ. Triggering of the innate immune system aims to ultimately restore homeostasis and provide healing but may become excessive and damaging or suppressed and insufficient. In this context, the cholinergic anti-inflammatory response is important in regulating the magnitude of the inflammatory response in several conditions, such as endotoxin induced shock, pancreatitis and ileus[142]. We speculate whether the human CB can participate in sensing of immunological reagents and relay this information to the CNS to ultimately contribute to adjusting the level of inflammatory response through vagus efference as well as eliciting appropriate cardiorespiratory responses. It is demonstrated that CB chemoafferent activity is strikingly increased in early systemic inflammatory exposure in the absence of hypoxia, hypercapnia and acidemia, and that inflammasome-induced cytokine increase activates the CB[140]. This activation results in increased ventilation, tachycardia, vasodilation and airway constriction, all common features of critical illness. Notably, clinical studies have showed a correlation between a reduced chemoreflex function (as a part of the autonomic dysfunction in sepsis) and outcome from critical illness[229].

Inflammatory modulations in the CB are also a key mechanism in the process of acclimatization to chronic hypoxia. Ibuprofen is reported to block these alterations and it could be speculated that NSAID treatment of acute mountain sickness delays the physiological acclimatization rather than resolving the problem. In this sense, it can be further speculated whether NSAID may block or postpone the necessary acclimatization to chronic hypoxia in patients with chronic hypoxemia due to for example chronic obstructive pulmonary disease (COPD).

Finally, patients with COPD rely on hypoxic pulmonary vasoconstriction to optimize ventilation-perfusion in the lung and thereby improve gas exchange. It is a common misconception that supplemental oxygen to this group of patients in exacerbation provokes CO_2 retention due to inhibition of ventilatory drive, i. e. depression of the peripheral chemoresponse. However, excessive oxygen administration has a negligible effect on minute ventilation and respiratory drive in patients with COPD exacerbation[230]. Instead, the increase in P_aCO_2 occurs as a result of opposed hypoxic pulmonary vasoconstriction by oxygen in the alveoli leading to ventilation-perfusion mismatch and oxygen induced Haldane effect (rightward shift of the CO_2 dissociation curve), increasing the P_aCO_2 for these patients that are unable to further elevate their minute ventilation[231]. Nevertheless, one should not deny these patients O_2 treatment when hypoxemic, but rather titrate the administration with caution, aiming for arterial oxygen saturation between 88-92%[232].

FUTURE PERSPECTIVES

In this thesis we have provided the basis for a map of human CB expression of oxygen sensing and signaling genes as well as inflammatory response genes. Besides the gene expression data, we further demonstrate CB release of neurotransmitters and cytokines in response to hypoxia. But the map is far from complete and further investigations to elucidate human CB molecular structure and function are necessary.

Over the past five years, massive RNA-sequencing has added new dimensions to RNA biology, from characterization of different non-coding RNA species and detection of alternative splicing to quantification of very small amounts of RNA. The advantage over conventional microarray is precisely the accuracy over a larger range of expression level. The overall aim and challenge of both DNA and RNA-sequencing is to interpret the functional consequences of genomic or expressional variability[233]. To our knowledge, RNA-sequencing of the human CB has not been performed. This could provide with important information on further splice variants in the human CB as well as non coding RNA's such as transfer RNA and micro RNA, all affecting the protein product and thereby the CB function. Comparing CB samples exposed to either hypoxia or normoxia/hyperoxia would give important information on hypoxia effects on transcription of key genes in the CB and hence clues on CB function. It would further be interesting to correlate the RNA profile with the phenotype of the individual patient, i. e. the HVR. Single-nucleotide polymorphism (SNP) in the human genome is currently heavily investigated in relation to development of diseases and in relation to the response to various pathogens and drugs. One possible explanation to the diversity in HVR seen between individuals is SNP in key genes in oxygen sensing and signaling. Identification of SNPs with functional consequences for the HVR could further provide with important information on the oxygen sensing and signaling mechanism.

The HVR in humans is studied under influence of many pharmacological agents. However, in the field of anesthesia and intensive care, we are lacking knowledge on the effect on hypoxic regulation of breathing for many of the agents used on a daily basis. Dexmedetomidine, an α2-adrenoreceptor agonist used for sedation in the ICU has recently been introduced in Sweden as a beneficial sedation alternative due to its minor effects on respiration. It is shown that dexmedetomidine does not depress resting ventilation and only has a slightly depressant effect on the HCVR[234, 235]. Yet, the effects of dexmedetomidine on the HVR and CB function are unknown. Since the use of this sedating drug is now expanding, further insights into its consequences on the human regulation of breathing in hypoxia. Furthermore, the effects of

ketamine and barbiturates on the human HVR are not determined, and there is still controversy regarding the effect of volatile anesthetics in different concentrations on the HVR.
The molecular effects of anesthetic agents, blockers of various K^+ channels and modulators of ROS-metabolism are studied in animal CBs, but once again the map is far from complete.
It has been demonstrated that exposure of rat neonates to intermittent hypoxia results in CB epigenetic changes, most prominently DNA hypermethylation that induce irregular breathing and hypertension in adulthood[236]. Considering this, it would be of interest to study the pattern of epigenetic changes (DNA methylation as well as small interfering RNAs and histone modifications) with a concurrent exposure to anesthetic agents, in order to investigate the modulatory role of repeated anesthetic exposures on the inflammatory part in acclimatization to chronic or chronic intermittent hypoxia.
In addition, in the continued quest for the CB oxygen sensor/s it is desirable to expose CB slices or type 1-cell cultures to hypoxia in the presence of blockers of for instance the enzymes producing the various gaseous messengers.
The secondary cardiovascular pathology in OSA is evoked by increased CB activity as described before, and ROS is proposed to have a key function in developing the pathology. It is reported that the augmentation of HVR is reversed with continuous positive airway pressure (CPAP) treatment for merely one month. Whether OSA patients exhibit a specific blood biomarker profile that is reversed simultaneously as the HVR is not known but would be of interest, especially as a tool to evaluate the efficacy of the CPAP-treatment.

CONCLUSIONS

With this thesis we characterized the human carotid body gene expression with focus on molecules in sensing and signaling of oxygen and inflammation. The global carotid body transcriptome profile was described in relation to other species and tissues and we also identified the human carotid body neurotransmitter and cytokine release in response to hypoxia. In detail:

- Nicotinic, purinergic and dopaminergic receptors and receptor subunits and the K^+ channel TASK-1 are present in the mouse carotid body type 1-cell

- The human carotid body type 1 cell expresses the proteins HO-2, Maxi-K TASK-1 in oxygen sensing, nACh and $GABA_A$ receptor subunits critical for anesthesia, purine and dopamine receptors as well as HIF-1α and HIF-2α.

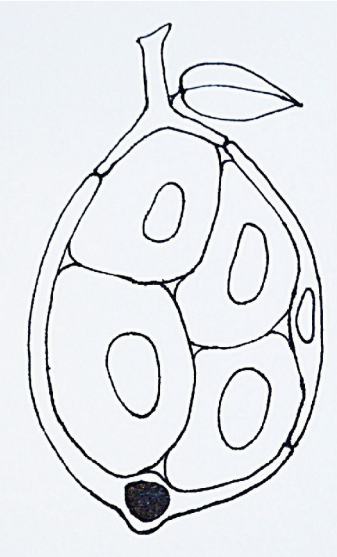
- The human carotid body transcriptome shows a specific tissue profile and overexpresses genes in relation to the inflammatory response and angiogenesis compared to brain and adrenal gland and genes in neurological processes in comparison with adrenal gland.

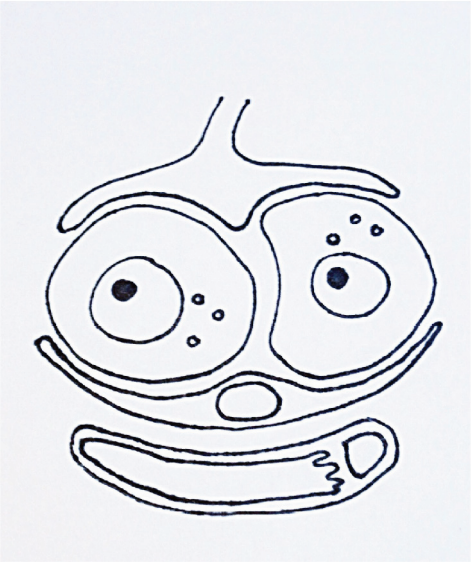
- The human carotid body shares expression of genes related to oxygen sensing with the mouse carotid body, yet differs in expression of chemosensory genes such as TASK-1 and CSE that are expressed in the human carotid body but were absent in one or two mouse strains compared to. The human carotid body type 1 cell lacks the α4 nAChR subunit and the $P2X_3$ receptor that were demonstrated in the mouse carotid body type 1 cell.

- Acute hypoxia increases the release of the neurotransmitters ACh and ATP from the human carotid body and prolonged hypoxia induces release of pro- and anti-inflammatory cytokines.

- Apart from transcriptome enrichment of inflammatory response gene groups, the human carotid body specifically expresses early and late inflammatory mediators and cytokine receptors of which IL-1R1, IL-6R and IL-10R were demonstrated both as mRNA and protein.

CB ART





INTERPRETATIONS OF THE CAROTID BODY BY FEDRA AMORIM AND ANNA PARKE

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