UMEÅ UNIVERSITY MEDICAL DISSERTATIONS
NEW SERIES NO. 1134 ISSN 0346-6612 ISBN 978-91-7264-431-1

From the Department of Surgical and Perioperative Sciences
Anesthesiology and Intensive Care
Umeå University, Umeå, Sweden

Carbon Monoxide in biological systems

An experimental and clinical study

Anna-Maja Åberg

Fakultetsopponent:
Docent Dick Delbro
Avdelningen för kirurgi
Institutionen för kliniska vetenskaper
Sahlgrenska universitetssjukhuset

Umeå 2007

Copyright © 2007 Anna-Maja Åberg
ISBN 978-91-7264-431-1

Printed in Sweden by
Print Media, Umeå, 2007, 2003780

Om du tror att du vet allt är du dåligt underrättad.
Kinesiskt ordspråk

TABLE OF CONTENTS

ABSTRACT	v
SAMMANFATTNING	vi
LIST OF PAPERS	viii
INTRODUCTION.....	1
Chemical and physical properties of carbon monoxide	1
History of carbon monoxide	1
Haemoglobin.....	2
Haem oxygenase	3
Carbon monoxide in the body	5
Interactions of carbon monoxide in the body	6
Effects.....	9
Kinetics of carbon monoxide	12
Analytical methods for the analysis of carbon monoxide	12
Levels of carbon monoxide in the environment	13
AIMS OF THIS THESIS	14
MATERIAL AND METHODS	15
Gas chromatography	15
Animal model.....	15
Blood donors	17
Methodological considerations	17
Statistics and pharmacokinetic calculations	19
RESULTS	20
Development of an analytical method for carbon monoxide in blood	20
Kinetics of carbon monoxide	21
Possible circulatory effects after carbon monoxide administration.....	23
Investigation of anti-inflammatory properties for carbon monoxide.....	23
Carbon monoxide concentrations in blood from blood donors	25
Summary of results.....	29
DISCUSSION	30
Interpretation of the results	30
Carbon monoxide research today	33
Future considerations	34
ACKNOWLEDGEMENTS.....	35
REFERENCES	37

ABSTRACT

Background: Carbon monoxide (CO) is a toxic gas, but it is also produced endogenously when haem is degraded. When produced in vivo, CO is believed to have positive biological effects. For example it activates the production of cyclic guanosine mono-phosphate and causes vasodilatation. CO is also believed to have anti-inflammatory properties by binding to Mitogen activated protein (MAP) kinase. Several studies in cells, mice and rats support this opinion regarding both the circulatory as well as the anti-inflammatory properties. However, studies in larger animals regarding circulatory effects have demonstrated contradictory results. The only study in humans regarding anti-inflammatory properties of CO could not demonstrate such effects.

Methods: This thesis consists of four different models. In paper I a method for analysis of CO in blood was developed using gas chromatography. In paper II a porcine model was used to investigate the elimination time for CO. The pigs in paper II had a high concentration of CO administered via blood, and CO concentrations were followed over time and kinetically parameters calculated. Circulatory parameters were also measured to evaluate if there were any circulatory changes after CO administration. In paper III CO's anti-inflammatory properties were investigated in an endotoxin-induced systemic inflammatory model in pigs. Paper III was a randomized study where one group inhaled CO and the other group served as controls. Plasma cytokine concentrations were measured and followed over time as an indication of the inflammatory state. In paper IV, CO concentrations in blood from blood donors at the Blood Centre in Umeå were investigated. The blood donors also completed a questionnaire about age, smoking history and other possible sources for exogenous contamination of CO in the blood.

Results and conclusions: In paper I we developed a method suitable for analysis of low concentrations of CO in blood. The half-life of CO at levels of 250 μ M in pigs was found to be 60 minutes. CO did not show anti-inflammatory effects after an endotoxin-induced systemic inflammation in pigs. In banked blood CO was present at concentrations up to six times higher than normal concentrations. This could be a risk when transfusing such blood to susceptible patients.

SAMMANFATTNING

(Abstract in Swedish)

Bakgrund: Kolmonoxid (CO) är en gas som är mest känd för sina toxiska egenskaper. Den bildas vid ofullständig förbränning, exempelvis i cigarettrök, bilavgaser och vid bränder. CO binder till hemoglobin i de röda blodkropparna och hindrar därmed syre från att binda. Höga CO nivåer i blodet leder till kvävning. Även ett enzym, cytokrom c oxidas, påverkas av CO och därmed påverkas också cellens energiförbrukning negativt. CO bildas också endogent, när hemoglobin bryts ner. De senaste åren har mycket forskning berört CO's funktion i kroppen. I olika studier har man sett att CO har flera positiva effekter. CO fungerar som en signalsubstans, den påverkar cirkulationen genom att agera som en kärldilator och tros ha anti-inflammatoriska egenskaper.

Syfte: Syftet med den här avhandlingen var att studera CO och dess effekter i kroppen. Avhandlingen består av fyra delarbeten. I det första arbetet var syftet att ta fram en analytisk metod för att bestämma låga nivåer av CO i blod och i utandningsluft. I arbete II var syftet att studera CO's kinetik vid relativt låga CO nivåer i blod samt att se ifall det blev några cirkulatoriska förändringar vid tillförsel av CO. CO's anti-inflammatoriska egenskaper skulle undersökas i det tredje arbetet. Eftersom CO även är toxiskt var syftet i det fjärde arbetet att undersöka CO's nivåer i blodbankens blod.

Material och metoder: För att ta fram en analysmetod för CO så har gaskromatografi använts. Blod har pipetterats till gastäta rör, ett reagens (saponin och svavelsyra) har tillsatts för att frigöra CO från hemoglobin. Proverna har skakats kraftigt innan gasfasen injicerats på kolonnen. Den detektionsmetod som använts har varit en flammjonisations detektor med en nickelkatalysator placerad före flammen. Den utvecklade metoden har sedan används vid CO analyser i arbete II-IV. Vid kinetikstudien har försök på grisar (n=6) gjorts. Blod har tappats från sövda grisar, bubblats med CO för att sedan administreras tillbaka till djuren. Koncentrationen av CO i blod har följts genom att ta blodprov vid olika tidpunkter. Även i arbete III har studier på sövda grisar (n=20) gjorts. Grisarna har delats upp i två grupper där en grupp har andats in CO och den andra gruppen varit kontroldjur. Alla djur har fått endotoxin vilket har gett dem en svår systemisk inflammation. Blodprover har tagits kontinuerligt och cytokiner (TNF-alfa, IL-6, IL-10 och IL-1beta) har analyserats, som ett mått på inflammationen. Grupperna har jämförts för att se

om CO påverkar cytokinkoncentrationerna. I det sista arbetet har blodgivare (n=410) lämnat ett rör med extra blod vid blodgivning samtidigt som de fyllt i en enkät om exempelvis rökvanor. CO har analyserats i blodet och normalnivåer samt förhöjda nivåer bestämts. Korrelationer mellan CO koncentration och olika faktorer som tagits upp i enkäten har också genomförts.

Resultat och slutsatser: Metoden för att analysera CO fungerar bra. Halveringstiden för CO är 60 minuter vid nivåer som startar på 250 μM CO. Eliminationen följer första ordningens kinetik och all elimination sker via lungan. Inga cirkulatoriska effekter kunde ses vid tillförsel av CO. CO visar inga anti-inflammatoriska egenskaper i den tredje studien. Den studien kännetecknas av en svår systemisk inflammation, relativt låg CO nivå (runt 350 μM) för att undvika toxiska effekter samt att studien är gjord på grisar. I litteraturen finns studier på celler, möss och råttor som visar att CO har anti-inflammatoriska egenskaper medan en studie på människa inte visar på några effekter. Vår slutsats blir att CO kan ha en anti-inflammatorisk effekt i små djur men inte hos gris och människa. I det sista arbetet ligger 6 % av proverna över 130 μM . Prover under 130 μM verkar vara den fysiologiska nivån med ett medel på 84.5 μM . Den hösta nivån ligger nästan sex gånger högre än normalnivåerna. Det finns en stark korrelation mellan förhöjda CO nivåer och rökning. Tiden som gått mellan rökning och blodgivning verkar vara den faktor som påverkar CO koncentrationen mest. Det kan vara en risk att ge blod med förhöjda CO nivåer till vissa patientgrupper, exempelvis barn och speciellt för tidigt födda barn med outvecklade lungor.

LIST OF PAPERS

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

- I. Sundin AM, Larsson JE.
Rapid and sensitive method for the analysis of carbon monoxide in blood using gas chromatography with flame ionisation detection.
J Chromatogr B Analyt Technol Biomed Life Sci. 2002 Jan 5;766(1):115-21.
- II. Åberg AM, Hultin M, Abrahamsson P, Larsson JE.
Circulatory effects and kinetics following acute administration of carbon monoxide in a porcine model.
Life Sci. 2004 Jul 16;75(9):1029-39.
- III. Åberg AM, Abrahamsson P, Johansson , Haney M, Winsö O and Larsson JE.
Does carbon monoxide treatment alter cytokine levels after endotoxin-infusion in pigs?
Submitted
- IV. Åberg AM, Nilsson-Sojka M, Winsö O, Abrahamsson P, Johansson G and Larsson JE.
Carbon monoxide concentration in donated blood - relation to cigarette smoking and other sources.
Manuscript

INTRODUCTION

Chemical and physical properties of carbon monoxide

The CO molecule consists of one carbon and one oxygen molecule. The molecule has a small dipole moment and a partial triple bond. This can be described with three resonance structures, where there is a major contribution from the form with the triple bond (Pauling, 1960). The bond between carbon and oxygen in CO is the strongest bond in chemistry, 1076.4 kJ/mol (Christian, Stranger, Petrie, Yates, & Cummins, 2007).

Figure 1 The three different resonance structures for CO.

CO is a colourless and odourless gas, with boiling point at minus 191.5°C and melting point at minus 205°C. The density is 1.25 g/l at 0°C and 1 atm. CO dissolves poorly in water, 23 ml/l at 20°C (*The Merck Index*, 1996). CO is a stable molecule, but can be reduced by transition metals. Coordinated CO is more reactive than the free gas. Proteins that contain transition metals at the active site form coordinated complexes with CO which interferes with their function. For example in red blood cells CO bind with the iron centre in the haemoglobin molecule (Piantadosi, 2002).

History of carbon monoxide

Arnold of Villanova
(ca 1238 – ca 1310)

In the late 1200s, the Spanish alchemist Arnold of Villanova described a poisonous gas produced by the incomplete combustion of wood that was almost certainly CO ("Carbon monoxide - history, sources, physiological effects, uses."). Between 1772 and 1799 an English chemist, Joseph Priestley recognized and characterized CO. In America Priestley is considered to have discovered oxygen as well. In Europe however, the discovery of oxygen is dedicated to a Swedish apothecary, Carl Wilhelm Scheele (Severinghaus, 2002). In 1857, CO was discovered by Claude Bernard to have an

asphyxiating effect. This effect was due to a reversible binding of CO to haemoglobin (Piantadosi, 2002). Studies by Haldane in 1895 showed that a high delivery of oxygen to the blood prevented this effect (Haldane, 1895). During 1920-1960, Roughton did several kinetic studies on CO and haemoglobin. In 1944 he discovered that CO bound to haemoglobin changed the oxyhaemoglobin dissociation curve. (Roughton & Darling, 1944). Sjostrand showed 1949 that CO was produced in the body during the metabolism of the haemoglobin molecule (T Sjostrand, 1949; T. Sjostrand, 1952). Some years later, it was discovered that the enzyme haem oxygenase (HO) was responsible for the degradation of the haem group from the haemoglobin protein (Tenhunen, Marver, & Schmid, 1968).

Haemoglobin

Haemoglobin is a metalloprotein in red blood cells responsible for oxygen transportation. The most common type of haemoglobin in mammals contains four globular subunits, each with a haem group containing an iron atom. Oxyhaemoglobin is formed during breathing when oxygen binds to iron in the haem group of the haemoglobin molecule. Deoxyhaemoglobin is the reversed, when no oxygen or other molecule is bound. Methaemoglobin is the protein species when iron in the haem centre is reduced from Fe^{2+} to Fe^{3+} . When reduced, the molecule can no longer bind oxygen (*The Merck Index*, 1996). Metabolism of haemoglobin produces biliverdin, iron and CO. The enzyme HO catalyzes the first and rate-limiting step in the degradation of haem to form biliverdin. Biliverdin is subsequently converted to bilirubin by an NADPH- or NADP -dependent reductase. HO releases iron (Fe^{2+}) and CO by cleaving the haem molecule. The HO enzymatic activity requires three molecules of oxygen per molecule oxidized haem. For electron donation the enzyme uses NADPH or NADH (Ryter, Alam, & Choi, 2006).

Figure 2 Chemical degradation of haem to biliverdin by the enzyme HO, resulting in the formation of iron and CO.

Haem oxygenase

There are two genetically distinct isoenzymes of HO: HO-1 and HO-2 (Maines, Trakshel, & Kutty, 1986). In 1997 cDNA from a third isoform of HO was isolated. When expressed in e-coli, the protein HO-3 did not show any activity for haem degradation. (McCoubrey, Huang, & Maines, 1997). A recent study concluded that the HO-3 gene represents pseudogenes originating from HO-2 transcripts since the gene have stop codons within the coding regions as well as the lack of detectable mRNA or protein product (Hayashi et al., 2004). Examinations in rats show that both HO-1 and HO-2 is active in the conversion of haem to biliverdin, but they have different kinetics (Trakshel, Kutty, & Maines, 1986).

HO-1 is present in higher animals with structural and functional similarities. A HO-1-like protein is also present in flies, bacteria, fungi, plants and algae with similar function, which is to metabolise haem and produce CO and biliverdin (Ryter et al., 2006).

Tissue distribution of HO has been investigated, and in rats HO occurs with the highest concentration in the spleen followed by the testes, brain and liver. The enzyme is also present at lower concentrations in the thymus, adrenals, kidneys, bone marrow, heart, lungs, epididymis, prostate, seminal vesicles and small intestine (Maines, 1988; Tenhunen, Marver, & Schmid, 1969,

1970). One study in rats found higher concentrations in bone marrow than in liver and brain (Tenhunen et al., 1970). The abundance of HO in different tissues in humans is not well established but it is probably present in all tissues that degrade red blood cells. The two iso-forms of HO have different tissue distribution. A study in rats showed that HO-1 is most abundant in the spleen. This is probably the only organ where the HO-1 concentration is higher than the HO-2 concentration. In rat brain and testes, only the isoform HO-2 is expressed (Ewing & Maines, 1992). In rat liver, the ratio between HO-1 and HO-2 is 1:2 (Trakshel et al., 1986). In rabbits and rats, the distribution of the two isoforms of HO is similar (Trakshel & Maines, 1989). The total capacity of spleen to produce CO is increased after haemolysis in rats (Tenhunen et al., 1970).

In the cell, HO enzymes are found largely in the endoplasmic reticulum. Recent studies have detected the enzymes also in nucleus, plasma membranes, caveolae in endothelial cells and possibly in the mitochondria. In different cell lines it has been shown that HO-1 can be induced by several stimuli, like hypoxia, hyperoxia, cytokines, NO donors, UVA radiation, peroxide, Cadmium-, Cobalt- and Stannous Chloride etc. HO-2 on the other hand is constitutively expressed and does not respond to environmental stress (Ryter et al., 2006). Metalloproteins like SnPPIX and ZnPPIX can induce HO-1 transcription and also inhibit HO-1 activation (Sardana & Kappas, 1987).

Figure 3 Subcellular localisation of haem metabolic enzymes.

Carbon monoxide in the body

There is an exogenous uptake of CO by the lungs, and endogenous production of CO by the cells. CO diffuses rapidly between the alveolar and capillary membranes. The elimination of CO from the body is mainly through the lungs, a small amount is also oxidized to CO₂. Most of the CO found in the body is bound to haemoglobin in blood. Approximately 10-15 % of the total amount of CO is distributed in tissues, bound to different haemoproteins like myoglobin and different cytochromes. Only about 1 % of the total amount of CO is represented as the free molecule (Coburn, 1970).

Endogenous production of CO gives blood carboxyhaemoglobin (COHb) concentrations of 0.4-1 % (Von Burg, 1999). In women during the progesterone phase, CO production is higher, and during pregnancy CO production is almost doubled. At 24 hours post partum, CO production is increased by 4.5 times. Newborns have a CO production of approximately twice the production in women during their estrogen phase or the production in men (Longo, 1977).

Interactions of carbon monoxide in the body

CO binds to cellular haem proteins and thereby alters their effects. After binding, CO often inhibits electron transfer and/or catalytic activity.

Haemoglobin

Apart from the fact that CO is produced during the degradation of haemoglobin, CO also interacts with the molecule by binding to the iron centre of the haem group. CO competes with oxygen for the same binding site on the haemoglobin molecule. Each haemoglobin molecule has four haem centres and can thereby bind four CO or oxygen molecules (*The Merck Index*, 1996). CO's affinity for haemoglobin is approximately 240 times greater than oxygen's affinity for the same molecule (Ryter et al., 2006). Iron in haemoglobin is normally in the Fe^{2+} form; therefore it binds CO. Fe^{2+} is present both in deoxyhaemoglobin and oxyhaemoglobin. In methaemoglobin, however, iron is present as Fe^{3+} , and neither CO nor O_2 binds to the ferrous molecule in this state (*The Merck Index*, 1996). When CO is bound to haemoglobin the oxyhaemoglobin dissociation curve is changed. If CO is bound to two haem sites in the haemoglobin molecule, the affinity for oxygen increases, making it harder for oxygen to be released in the tissues (Roughton & Darling, 1944). The ratio of oxyhaemoglobin to COHb is always proportional to the relative partial pressure of CO and oxygen. The ratio varies between species and individuals (Douglas, Haldane, & Haldane, 1912). The binding between CO and haemoglobin is reversible and CO can be replaced with oxygen. To increase elimination oxygen therapy under hyperbaric conditions may be applied (Gorman, Drewry, Huang, & Sames, 2003).

Myoglobin

Myoglobin, like haemoglobin, is a globular protein with an iron-centred haem group that can bind both oxygen and CO. Myoglobin is smaller than haemoglobin and each molecule binds only to one oxygen- or CO-molecule. Myoglobin is present in cardiac and skeletal muscle cells and is important for maintenance of both oxygen consumption and tension generation in exercising muscle (Cole, 1982). The ratio between haemoglobin-CO in blood cells and myoglobin-CO in muscle cells is constant when oxygen pressure is between 40-700 mmHg. However, when oxygen pressure decreased below 40 mmHg the CO concentration shifted from blood to muscle (Coburn & Mayers, 1971). This phenomenon has been studied during different conditions. During muscle hypoperfusion or hypoxemia, the CO uptake by myoglobin increased (Coburn, Ploegmakers, Gondrie, & Abboud, 1973).

Cytochrome P₄₅₀

This is a group of enzymes with an iron haem centre with a sulphur atom ligated. These enzymes occur in most animal tissues and organelles and are involved in several reactions. The enzymes catalyse aliphatic and aromatic hydroxylation, N-oxidation, sulfoxidation, epoxidation, N-, S-, and O-dealkylation, peroxidation, deamination, desulfuration and dehalogenation. They are also active in reductions which involve electron transfer between several molecules. Two examples of important reactions catalysed by cytochrome P₄₅₀ enzymes are metabolism of lipids and xenobiotics. The metabolism of xenobiotics usually lead to detoxification but it can also yield products with greater cytotoxic properties (White & Coon, 1980). Tissue pCO is normally too low to inhibit P₄₅₀ enzymes significantly. When COHb increases to 15-20 %, CO binds to these enzymes and is thought to cause some biological effects, including changes in drug metabolism and vascular tone (Piantadosi, 2002).

Cytochrome c oxidase

Cytochrome c oxidase is a large transmembrane protein in the mitochondria which consists of two haem and two copper centres. It is the last enzyme in the respiratory chain in mitochondria, and it catalyses the reaction with O₂ to form H₂O. This reaction accounts for approximately 90 % of all oxygen utilized by tissues. When O₂ is reduced four electrons is released, these electrons are responsible for most of the ATP produced in the cell. Cytochrome c oxidase can bind to CO in its reduced form. The enzyme is under normal conditions oxidised, but when hypoxia is present the enzyme gets reduced (Piantadosi, 2002). When CO binds to cytochrome c oxidase, one would expect the oxygen utilization by mitochondria to be reduced.

CO is also oxidised to carbon dioxide by mitochondria and by purified cytochrome c oxidase (Young & Caughey, 1986).

Guanylate cyclase

CO can diffuse from endothelial cells into neighbouring vascular smooth muscle cells which contain soluble guanylate cyclase (sGC). sGC contains a haem regulatory subunit which binds to CO in the ferrous (Fe³⁺) state, and CO thereby activates the molecule. When sGC is activated, the production of cyclic guanosine monophosphate (cGMP) is increased. cGMP is an important regulator of vascular tone. In rat tail artery, CO induces vasorelaxation since it acts on the cGMP signal transduction. In the same study, when the cGMP pathway was blocked, vasorelaxation was attenuated but not eliminated, indicating that there are other mechanisms for CO to act as a vasorelaxant

then converted into bilirubin by the enzyme biliverdin reductase. In terms of the interactions between NO and CO, CO can bind to the haem centre of NOS and inhibit its activity. Moreover, Fe²⁺ liberated from the degradation of haem can result in a decrease in NOS expression, which occurs at the level of transcription. On the other hand NO can induce the expression of HO-1, which subsequently results in CO synthesis. NO that is bound to haem centres can be displaced by CO, and the liberated NO can then bind Fe released from the degradation of haem via HO (Watts, Ponka, & Richardson, 2003).

Mitogen activated protein kinase signalling pathway

Studies *in vitro* and *in vivo* in mice show that CO interacts with the mitogen activated protein (MAP) kinase signalling pathway, in particular the MKK3/p38 MAP kinase pathway, bringing about anti-inflammatory effects (Otterbein et al., 2000).

Effects

Toxicity

The most widely known effect of CO in the body is that it is toxic. It binds to haemoglobin and thereby prevents oxygen from binding. Results of this are dizziness, shortness of breath and headache. Lethality after CO exposure results from tissue hypoxia. In order to achieve the amount of CO needed to produce these effects, one has to be exposed to CO from external sources such as cigarette smoke, internal combustion engines or fire. All toxic symptoms can not be explained by the formation of COHb in the blood. CO is also toxic for mitochondria in man, where it binds to cytochrome c oxidase and alters function of the respiratory electron transport chain. This effect may explain persistent symptoms after CO poisoning, when CO has been eliminated from the bloodstream (Alonso, Cardellach, Lopez, Casademont, & Miro, 2003). Toxicity of different concentrations of CO in man has been investigated, though the clinical response to different CO levels of exposure may vary between individuals. Symptoms of CO poisoning begin at 20 % COHb while death occurs at 50-80 % COHb (Ryter & Otterbein, 2004). In a retrospective study it was stated that concentration of 23 % COHb can lead to loss of consciousness (Mannaioni, Vannacci, & Masini, 2006). In children a COHb concentration of 20 % in the blood can lead to unconsciousness (Kondo et al., 2007). Effects of CO in healthy men during exercise can be seen at low CO levels (Eklblom & Huot, 1972). For example, there is an inverse correlation between COHb levels and maximal oxygen uptake at levels between 4.8 and 21.2 % COHb. Studies in patients with angina pectoris show that CO at levels from 2.0 % to 4.5 % COHb shortens the time to pain after

exercise and it also gives a longer duration of the pain (Anderson, Andelman, Strauch, Fortuin, & Knelson, 1973; Aronow, 1981; Aronow & Isbell, 1973; Aronow, Stemmer, & Isbell, 1974). One study showed that relevant environmental concentrations of CO (10-100 ppm) liberate oxidants as well as causes cell death (Thom, Xu, & Ischiropoulos, 1997). There is no easy way to determine at what concentration CO is toxic since its toxicity probably differs with different circumstances. Healthy persons can probably survive higher doses of CO than persons with severe pre-existing diseases. A study investigating the mechanisms of CO toxicity stated that CO was not toxic when already bound to haemoglobin, only toxic when administrated as the free molecule (Goldbaum, Orellano, & Dergal, 1976).

Circulation

CO and the whole HO system seem to have a role in circulatory control. In rats there is evidence for CO's positive circulatory effects. For example, in systemic hypertensive rats HO-1, CO and cGMP are increased. When inhibiting HO-1, CO as well as cGMP levels decreased (Motterlini et al., 1998). In perfused rat liver, addition of CO decreased vascular resistance (Suematsu et al., 1995). HO inhibition led to increased portal vascular resistance in rat liver *in vivo* (Pannen & Bauer, 1998). During endotoxic shock, HO-1 mRNA levels were increased in aortic tissue from rats, and this was associated with an increase of HO-1 activity *in vivo* which contributes to a reduction in vascular tone (Yet et al., 1997).

Several *in vivo* studies on pulmonary circulatory effects of CO in large animals have provided diverging results. Two studies, one in dogs during hypoxemia and another in late-gestation fetal lambs under normal conditions, showed no change in pulmonary vascular resistance (PVR) after inhaling CO or inhibiting HO-1 (Grover, Rairigh, Zenge, Abman, & Kinsella, 2000; Vassalli et al., 2001). In sheep during hypoxemia, a decrease in PVR was shown after inhalation of CO (Nachar et al., 2001). Investigation of the coronary microcirculation in dog hearts *in vivo* showed that during ischemia HO activation caused dilatation, but only when other systems such as NO, prostaglandins and K⁺ channels were impaired (Nishikawa, Stepp, Merkus, Jones, & Chilian, 2004).

Inflammation

The anti-inflammatory effects of CO have been investigated in cell-lines, mice and rats with convincing results. In a study on mice exposed to endotoxin-induced systemic inflammation CO was associated with decreased TNF-alfa. An anti-inflammatory cytokine, IL-10, was increased in the presence

of CO (Otterbein et al., 2000). In that study, the mechanism for these findings was investigated and found to be through p38 MAP kinase. In a mouse-to-rat cardiac transplant, inhibition of HO-1 caused graft-rejection which did not occur in control animals. When the HO-1 inhibited rats were exposed to CO, this effect was suppressed. The graft rejection was associated with widespread platelet sequestration, thrombosis of coronary arterioles, myocardial infarction and apoptosis (Sato et al., 2001). In another study where small intestinal transplantation was performed in rats, one group was exposed to CO and the other was not. The rats that inhaled CO had improved micro-vascular blood flow, decreased m-RNA for inflammatory mediators such as IL-6, COX-2, iNOS and ICAM-1, and almost no histopathological changes. The CO exposed animals also had improved gastrointestinal transit and no animal deaths after the operation, compared to a survival of 58 % for the non-exposed rats (Nakao et al., 2003). In a model of acute lung injury in mice, CO showed cytoprotective effects and an attenuation of the lung injury, along with prolonged survival during exposure to lethal hyperoxia (Otterbein et al., 2003). All of these studies indicate that CO has anti-inflammatory effects. However, in humans there has been only one study published which showed no anti-inflammatory effects of CO after endotoxin-induced systemic inflammation (Mayr et al., 2005).

Studies in patients during systemic inflammation have shown significant elevated COHb and end tidal CO levels in traumatized or septic patients (Moncure, Brathwaite, Samaha, Marburger, & Ross, 1999; Zegdi, Perrin, Burdin, Boiteau, & Tenaillon, 2002).

Nervous system

NO is known to act as a neurotransmitter by inducing sGC and increasing cGMP levels. Investigations of different regions of the brain show that HO-2 is present in the same locations as sGC and that NO is missing in some of these. When inhibiting HO-2, cGMP is decreased indicating that CO may act as a neurotransmitter in the brain (Verma, Hirsch, Glatt, Ronnett, & Snyder, 1993). Experiments concerning CO as a neurotransmitter in the brain of rats have shown that CO participates in regulation of circadian rhythms (Artinian, Ding, & Gillette, 2001). The mechanisms underlying learning and memory may also be affected by CO: it could act as a retrograde messenger which links post and presynaptic sites (Wu & Wang, 2005). In salamander olfactory receptor neurons it has been shown that CO regulates long-term adaptation to odorant stimulation via its effect on cGMP levels (Zufall & Leinders-Zufall, 1997). Regarding studies on signal substances involved in pain, CO has been demonstrated to be involved in the regulation of neuropathic

pain in mice (Li & Clark, 2003). Another area of interest for CO is in the human ear where local production of CO has been found in the middle ear epithelium (Andersson, Uddman, Tajti, & Cardell, 2002).

Asthma

Individuals with asthma have an increased level of CO in their exhaled air (Horvath et al., 1998). In a culture of human airway smooth muscle cells, addition of CO inhibited cell proliferation. This inhibition was cGMP independent, instead mediated through the MAP kinase pathway (Song et al., 2002).

Kinetics of carbon monoxide

The pharmacokinetics of endogenous CO produced under normal conditions has not been fully investigated. Previous studies have focused on patients exposed to CO in high concentrations, including fire victims (Weaver, Howe, Hopkins, & Chan, 2000) or after inhaling CO (Kreck, Shade, Lamm, McKinney, & Hlastala, 2001; Sasaki, 1975; Shimazu et al., 2000; Wagner, Horvath, & Dahms, 1975; Wazawa, Yamamoto, Yamamoto, Matsumoto, & Fukui, 1996). These studies often had a high start dose of CO and they examined best means of elimination of CO. The large range of half-lives (5-190 minutes) among these studies is probably a result of differences in protocol design, in particular oxygen delivery and different species. The elimination pattern for CO has not been fully elucidated either. Some have claimed that CO follows first orders kinetics with one compartmental elimination (Kreck et al., 2001; Sasaki, 1975; Weaver et al., 2000), while others speculate that it has second or multi-compartmental elimination (Shimazu et al., 2000; Wagner et al., 1975); (Wazawa et al., 1996). In a population pharmacokinetic study investigating the elimination time for CO in smokers, the results showed two-compartmental kinetics with a half-life of 1.6 h for the first phase and 30.9 h for the second phase (Cronenberger, Mould, Roethig, & Sarkar, 2007). In that study 190 smokers participated and a mathematic model to predict COHb fractions in adult smokers was developed. The differences in kinetics between studies can be a result of differences in sampling times where long sampling times more often result in multi-compartment models with a second slow elimination phase.

Analytical methods for the analysis of carbon monoxide

Previously described methods for CO measurement include microdiffusion, colorimetric, infrared absorption, volumetric or spectrophotometric methods (Feldstein, 1967). Other investigators have use

manometric oxidation, microcalorimetric or indirect methods (Commins, 1975). However, gas chromatography (GC) has been suggested as a reference method (Johansson & Wollmer, 1989), since it often has much lower detection limits and higher accuracy. Detection of CO is possible using thermal conductivity detection (TCD) (Goldbaum, Chace, & Lappas, 1986) or flame ionisation detection (FID) after methane conversion by a nickel catalyst (Cardeal et al., 1993). The TCD often needs larger volumes than the FID with a nickel catalyst system. When analysing CO in blood, some preparation is necessary. CO has to be released from haemoglobin, and this can be done by various methods. One method is by using sulphuric acid together with saponin.

Levels of carbon monoxide in the environment

In the environment CO is produced naturally during bush fires and volcanic eruptions. CO also originates from car exhaust, cigarette smoke and other sources of incomplete combustion. Levels of CO in the environment have been measured in different areas as can be seen in table 1 (Longo, 1977). The levels can be 1 million times higher when exposed to external sources like cigarette smoke or car exhaust compared to fresh air. The limit for exposure of CO at workplaces is 35 ppm (Arbetsmiljöverket, 2000).

Table 1

Measured concentrations of carbon monoxide in different areas.

	CO (ppm)
Fresh sea air	0.06-0.5
Urban air	1-30
Street corner	5-50
Major interchange	50-100
Automobile exhaust	30000-80000
Cigarette smoke	20000-60000
Alveolar conc. in smokers	300-400

(Longo, 1977)

Human levels of CO also differ depending on to which external sources they have been exposed. COHb levels in blood should be under 1.5 % according to air quality standards (Aronow, O'Donohue, Freygang, & Sketch, 1984). In smokers, levels of 2.1-9.3 % COHb have been reported (Aker, 1987).

AIMS OF THIS THESIS

- To develop and validate a sensitive method for determination of CO in blood.
- To investigate the kinetics of CO in pigs at low levels of CO and to observe possible circulatory effects after CO administration.
- To study possible anti-inflammatory effects of CO after an endotoxin-induced systemic inflammation.
- To examine concentrations and origins of CO in banked blood.

MATERIAL AND METHODS

Gas chromatography (paper I-IV)

In paper I, a method for determination of CO was developed. This method was then used in paper II-IV.

To establish an analytical method for the small amounts of CO that are produced in the body, gas chromatography (HP 5790) with flame ionization detection (FID) was used. A nickel catalyst system was placed before the FID detector. The column (6 ft Heysept 80/100 Mesh) was packed, which is an advantage when gas samples are to be analyzed, since larger amounts can be injected on the column.

The method was developed for utilisation with both blood and exhaled gas. Blood, 400 µl, was put in gas-tight tubes, together with a glass pearl. A liberating solution (LS), 800 µl, containing sulphuric acid (1 M) and saponin (15 g/l) was added through a needle. The samples were mixed for 40 minutes, and 200 µl of the gas phase injected on the column. Before the final preparation procedure was settled a lot of experiments were made. The relationship between blood volume and volume of LS was investigated, with the goal of complete release of CO but without over exceeding the gas phase. Mixing time, acid strength, and the effect of saponin were other examined factors. The procedures were tested both with human blood as well as with porcine blood. Exhaled gas was collected in gas-tight bags and then directly injected on the column.

Standard curves were made from samples containing 400 µl water and 800 µl LS, CO was added to the tubes through a needle, and the preparation proceeded in the same way as for the blood samples. Standards used to calculate concentrations in exhaled gas were made in gas-tight bags.

A cross validation experiment was performed where human blood samples from intensive care patients were collected and analyzed both with a spectrophotometer (OSM3) and with the developed GC method. In total, 42 samples were analysed.

Animal model (paper II-III)

In paper II and III an animal model was used. Female pigs weighing 23-40 kg were premedicated (ketamine 10 mg/kg, azaperone 4 mg/kg and atropine sulfate 0.05 mg/kg), then anesthetised (sodium pentobarbital 10-30 mg/kg/h), tracheotomized and mechanically ventilated with air containing 30 % oxygen. The ventilation was adjusted to obtain normoventilation using arterial blood gas analysis. Three intravascular catheters were inserted in

external jugular veins and in a branch of the external carotid artery. One litre of Ringer's acetate was given to the animals directly after preparation, in order to prevent hypovolemia. Infusion of ringer's acetate (15-20 ml/kg/h) was maintained during the whole protocol. The body temperature was held at 37-39°C. Measurements included mean arterial pressure (MAP), central venous pressure (CVP), mean pulmonary arterial pressure (MPAP), heart rate and cardiac output. Blood samples were collected and analysed for CO, haemoglobin, COHb, pH, pCO₂, pO₂, SBC and BE.

In paper II 150 ml blood was drained from the pigs, mixed with sodium citrate and filtered through leukocyte removal filters. The blood was exposed to CO bubbles to obtain 100 % COHb before injected back to the blood stream. The volume of blood injected back was determined based on a goal to achieve approximately 3 % COHb in the pigs. In addition to determination of CO in blood, CO was analysed in exhaled air. Some of the blood was radio labelled with ⁵¹Cr and divided into two equal volumes: one part was injected back immediately whereas the other part was mixed with the rest of the blood and bubbled with CO before re-injected into the blood stream. The radio activity was measured in blood samples before and after CO administration, to make sure there were no changes in the blood cells during this procedure.

Figure 5 This illustration stylistically shows the procedure when blood is withdrawn from the animals, bubbled with CO and administrated back. The figure also shows how some of the blood is radio-labelled before administrated back or bubbled with CO before administrated back together with the rest of the blood.

In paper III, after the preparation and one hour of equilibrium time, animals were divided into two groups. One group (CO-group) received CO through the airways. The animals were first given a bolus with the goal to

obtain 5 % COHb in the blood, as determined by haemoximetry. Then, CO was delivered (4-50 ml/h) throughout the protocol to match clearance and maintain a stable CO level both by predicted clearance (Aberg, Hultin, Abrahamsson, & Larsson, 2004) and observed COHb levels. The other group were controls and did not receive any CO. Two hours after preparation, endotoxin (lipopolysaccharide, 0111:B4, Sigma, USA) was infused intravenously, beginning at 0.05 µg/kg/h and reaching 0.25 µg/kg/h after 30 minutes. That infusion rate was then maintained for the rest of the protocol. This infusion rate aimed at a total dose of 1.175 µg/kg for each animal. The endotoxin dose was not adjusted when the animals demonstrated respiratory or circulatory dysfunction. Blood samples were taken every 30 minutes. The total protocol time was six hours, including five hours of endotoxin infusion. Arterial blood samples were analysed for blood gas parameters (pCO₂, pO₂, haemoglobin and haemoglobin saturation), CO and cytokines (TNF-α, IL-1β, IL-6 and IL-10). Mixed venous blood was analysed for blood gas parameters.

Blood donors (paper IV)

In this study 410 blood donors participated after being asked for approval when they arrived at the Blood Centre in Umeå for their original blood donation. After giving informed consent they filled out a questionnaire and an additional blood sample was taken during the blood donation process. These blood samples were analysed for haemoglobin and CO concentrations. In addition, a small study regarding smokers and their elimination time for CO was performed. In that study six smoking volunteers participated. Blood samples were taken before, directly after as well as one, three and five hours after the last smoked cigarette. This blood was also analysed for haemoglobin and CO concentrations.

Methodological considerations

In paper I, an analysis method for determination of CO was developed with gas chromatography since, according to the literature, this was considered to be the best method. It was important to develop a method with excellent sensitivity in order to analyse low concentrations of CO and to be able to detect small differences. For practical reasons a FID detector with a nickel catalyst was used instead of TCD. Regarding the method for cleaving the bond between CO and haemoglobin, the literature suggests several methods of which saponin and sulphuric acid is one.

In paper II, the kinetics of CO at relatively low concentrations was determined in pigs. Most of our research is performed on pigs and therefore it

was important to investigate the kinetics in these animals. The pig is an animal with cardiac and circulatory functions similar to humans, and is therefore widely used in research. Since CO is bound to haemoglobin in the body, the administration of CO was as COHb, using the animal's own blood. If given through the airways it would have been harder to control the start dose of CO. Even with the same ventilation and the same amount of CO administered it could result in different COHb concentrations. Also, administering CO through the airways could influence the lungs in a non desirable way. The start dose of 3 % COHb is approximately five times higher than the pig's normal COHb concentration and was considered high enough for the study questions.

The work by Otterbein and co-workers (Otterbein et al., 2000) performed in mice, showed anti-inflammatory properties of CO after an endotoxin-induced systemic inflammation. There are a number of current review articles that state that CO has anti-inflammatory effects (Mannaioni et al., 2006; Ryter et al., 2006; Ryter & Otterbein, 2004; Wu & Wang, 2005). But these generalisations are all based on studies on cells, mice and rats. One study performed on humans did not show any effect of CO after an endotoxin-induced systemic inflammation. This study had, obvious for ethical reasons, a low inflammatory state. The body could perhaps cope with the inflammation itself without the effect of CO. Our study was designed to test for possible anti-inflammatory effects of CO in an endotoxin-induced systemic inflammatory situation. There has been one study before ours that performed a similar intervention in pigs though without definitively answering this question (Mazzola et al., 2005). However, the conclusions in that study can be questioned. In our present study we chose the same cytokines as in the study by Otterbein and co-workers (TNF-alpha, IL-10) as well as in the other porcine study (TNF-alpha, IL-10, IL-1beta). In addition we measured IL-6 concentrations. The size of the study group in paper III was determined after power analysis based on the results by Otterbein and co-workers. With only TNF-alpha, the power was almost 100 % with eight animals in each group. However, after analysing six animals in each group without finding any difference between the groups, we increased the group size to ten animals in each group in order to avoid type II errors.

In paper IV, blood from donors was analysed instead of analysing blood bags. For practical reasons it was easier to match the blood with the right questionnaire. The blood from the donor is filtered, separated and the bags kept in a refrigerator. Since there is a large consumption of blood at the hospital it would be hard to get blood from the right bag before it is consumed. That type of study was considered to be insufficient. Regarding the

study on smokers, the time intervals between blood samples was based on the results from the 410 blood donor's.

Statistics and pharmacokinetic calculations

In paper I a cross validation experiment was performed and the correlation coefficient calculated.

For kinetic calculations in paper II, the CO concentration in blood was plotted *vs* time using the pharmacokinetics software WinNonlin. The basal concentration was first withdrawn from all values.

For comparison between the groups in paper III, multivariate statistics was used. ANOVA for repeated measures (SPSS Inc., Chicago, USA) was used to calculate differences between the two groups based on all measures made during the protocol time.

The Kolmogorov-Smirnov test was used to test for normality, and a p value <0.05 was used to reject a null hypothesis of normal distribution. Since blood CO concentrations in paper IV in general did not follow normal distribution (Kolmogorov-Smirnov test, $p < 0.001$), nonparametric tests have been used in all of the statistical calculations. Differences between groups were analyzed using Mann-Whitney U-test (SPSS Inc., Chicago, USA). Receiver operating characteristic (ROC) analysis on the CO concentration was performed using Microsoft® Excel (Microsoft Corporation, Washington, USA) after grouping the data in smokers *vs* non-smokers (van Erkel & Pattynama, 1998). ROC-analysis on CO concentrations was also made with regard to recent smoking (≤ 3 hours since the last cigarette) *vs* late smoking (> 6 hours since the last cigarette). Non-smokers were also included in the latter group. The group with 3 to 6 hours between smoking and blood donation was discarded since it only consisted of one subject.

For the comparisons in all of the papers a p value <0.05 was considered statistically significant.

RESULTS

Development of an analytical method for carbon monoxide in blood (Paper I)

After preparation of blood samples and analysis on GC, a chromatogram was recorded where the response from the detector was plotted *vs* time. In figure 6 a typical chromatogram after CO analysis from a porcine blood sample is shown. The chromatography showed very good performance with no disturbing peaks. Detection limit was determined to 0.01 μM gas. The calibration curve ranged between 12-515 μM liquid, so that the limit of quantification (LOQ) was 12 μM . Within-day variation and between-day variation was calculated and are presented as coefficient of variation in table 2.

Figure 6 A representative example of a chromatogram from a porcine blood sample.

Table 2

Variations for porcine blood, prepared and analyzed with the developed method

	CV (%)	n
Within-day variation	1.4	10
Between-day variation	2.6	13

When analyzing a mixture (CH_4 , CO, CO_2 , 10 ppm of each gas in He) with this method it was shown that, if present, methane easily separated from the other peaks. Neither N_2 nor O_2 give any peaks in this analytic system.

Results

Blood samples analysed in this assay had concentrations between 30-50 μM . The equation for the calibration curve was $y = 561x + 2558$ with a R^2 -value of 0.9995 (x are CO concentration in μM liquid and y the response in mV).

The stability of the blood samples was investigated and the samples were found to be stable at least 35 days if stored in a refrigerator, and, after preparation the samples were stable eight days in the refrigerator. When stored in a freezer (-20°C) the blood samples showed larger variations.

Results from a cross validation experiment between the developed GC method and a spectrophotometric method (OSM3) are shown in figure 7.

Figure 7 Cross validation plots between the developed GC method and a spectrophotometric method (OSM3) with human blood. CO expressed in M in the GC method was transformed to % COHb using the formula; $\text{COHb (\%)} = [\text{CO (M)} * 64400 \text{ (g/mol)}] / [4 * \text{Hb (g/l)}] * 100$ (Aberg et al., 2004).

Kinetics of carbon monoxide (Paper II)

CO was administered to pigs and the concentration of CO in blood was then analyzed and plotted *vs* time, as depicted figure 8. The equation of the curve was calculated after correction for basal CO concentrations. Correlation between of observed CO values and the curve equation was high. The elimination of CO followed first orders kinetics. Pharmacokinetic data are presented in table 3.

Results

Figure 8 Concentrations of CO (mean $\pm$ SEM, $n=6$) in porcine blood *vs* time. At time 0, CO saturated blood was injected intravenously. Reprinted with the permission from Elsevier B.V.

Table 3

Pharmacokinetic data in arterial blood from 6 pigs, injected with a known amount of carbon monoxide in blood.

	Min	Max	Mean	SEM
Weight (kg)	24	30	28	0.9
Dose (μ mol) X	302	455	393	21
Correlation coeff.	0.985	0.995		
Half life (min)	40.4	75.4	60.5	4.7
Clearance (ml/min)	20.0	28.6	24.5	1.5

Along with blood analysis for CO, CO was also analyzed in exhaled air. The results showed that after 160 minutes all exogenously administered CO had been cleared.

To study the effect of retransfusion and CO saturation of the blood on red blood cells kinetics, ^{51}Cr was incorporated in red blood cells and the radio activity in blood was followed. First, a small bolus of control red blood cells was given and then the CO containing bolus of red blood cells. The apparent distribution volumes were 72 ± 4.0 ml/kg for the first injected fraction and 72 ± 3.3 ml/kg for the second injected fraction. There was no significant change in distribution volume, or in decay, indicating that no major changes in red blood cell kinetics were induced by this step.

Possible circulatory effects after carbon monoxide administration (Paper II)

SVR and PVR did not change after CO administration.

Investigation of anti-inflammatory properties for carbon monoxide (Paper III)

The results showed that we had a stable model of systemic inflammation in pigs and there were no physiological differences between the groups (one control group and one group that inhaled CO). The pigs in paper III suffered from a severe systemic inflammation as can be seen from the MPAP increase depicted in figure 9 and the increase in cytokine levels depicted in figure 10.

Figure 9 Mean pulmonary artery pressure in pigs after endotoxin-induced systemic inflammation. The values are presented as means $\pm$ SEM, for CO treated animals (open circles, $n=10$ unless at 270 and 300 min where $n=9$) and controls (closed circles, $n=10$ unless at 150, 180 and 210 min where $n=9$ and at 240, 270 and 300 min where $n=8$). Immediately after -60 min CO was administered. At time 0, endotoxin was administered ($0.05 \mu\text{g/kg/h}$) reaching maximum infusion rate after 30 min ($0.25 \mu\text{g/kg/h}$). No significant difference between the groups ($p=0.697$, ANOVA repeated measures).

CO concentrations in blood were measured in both groups. The mean CO level for all measurements after CO administration was $321 \mu\text{M}$ with a SEM of 8.6 ($n=127$) compared with the control group with a mean of $46.3 \mu\text{M}$ and a SEM of 0.8 ($n=121$). This difference was statistically significant ($p<0.001$, ANOVA repeated measures).

Results

In figure 10 cytokine levels in plasma from the animals are shown. There was a massive response. The largest increase was found in TNF-alpha, with levels up to 50 000 pg/ml. TNF-alpha showed a peak concentration after one hour of endotoxin infusion. There was also a release of IL-6 after endotoxin infusion, with a peak after 2.5 hour. Regarding IL-10 the increase was of a smaller magnitude but still significant. IL-1 beta increased with the highest concentration at the end of the protocol. However, there were no differences in cytokine concentrations for any of the measured cytokines between the two groups (CO and Controls) using mixed between-within subject's analysis of variance for repeated measures (ANOVA).

Figure 10 Plasma cytokine concentrations in pigs with endotoxin-induced systemic inflammation with or without CO treatment. The values are presented as individual

Results

measurements and means for CO treated animals (open circles, $n=10$ unless at 270 and 300 min where $n=9$) and controls (closed circles, $n=10$ unless at 150, 180 and 210 min where $n=9$ and at 240, 270 and 300 min where $n=8$). At time 0, endotoxin was administered ($0.05 \mu\text{g/kg/h}$) reaching maximum infusion rate at time 30 ($0.25 \mu\text{g/kg/h}$). No significant difference between the groups for any of the cytokines ($p=0.317$ for TNF, $p=0.360$ for IL-6, $p=0.264$ for IL-10 and $p=0.210$ for IL-1 β , ANOVA).

Carbon monoxide concentrations in blood from blood donors (Paper IV)

Figure 11 shows individual CO concentrations in 410 blood donors after donating blood at the Blood Centre in Umeå. The majority of the studied group (94 %) had concentrations below $130 \mu\text{M}$. The CO concentrations in this group were normally distributed ($p=0.072$, Kolmogorov-Smirnov) with a mean of $84.5 \mu\text{M}$ and a SEM of 0.8. In 22 or 5.4 % of the blood samples the concentrations were above $150 \mu\text{M}$. Of these 22 donors, 20 smoked cigarettes and two were non-smokers. The highest detected CO concentration was $561 \mu\text{M}$.

Figure 11 Individual ($n=410$) blood CO concentrations from blood donors after donating blood at the Blood Centre in Umeå.

There was a significant difference in CO concentration between smokers (cigarette, cigar and pipe) and non-smokers ($p<0.01$, Mann-Whitney U). The median concentration was $142 \mu\text{M}$ or 1.5 % COHb in smokers and $83 \mu\text{M}$ or 0.9 % COHb in non-smokers. The group of cigarette smokers had a median CO concentration of $229 \mu\text{M}$ or 2.4 % COHb whereas smokers of cigar and

pipe had concentration with in the same range as non-smokers. In figure 12 the blood donors are grouped after cigarette smoking habits, e.g. how many cigarettes a person smokes per day (figure 12A), and time intervals between cigarette smoking and blood donation (figure 12B). The median CO concentrations with quartiles for each group are presented. There was a clear correlation between number of cigarettes and CO concentration in their blood, with a significant difference in all the groups compared to non-smokers. When comparing CO concentrations based on time interval between cigarette smoking and blood donation there was a significant difference between groups. The highest concentration was found in the two groups with up to three hours between smoking and blood donation.

Figure 12 Median CO concentrations with quartiles grouped by number of consumed cigarettes per day for the blood donors (figure A) and grouped by the elapsed time after the last smoked cigarette before blood donation (figure B). Significant difference at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ *vs* the non-smoking group, ††† $p < 0.001$, †† $p < 0.01$ *vs* the group with more than six hours since last cigarette (Mann-Whitney U-test), as indicated.

In figure 13 ROC curves are shown, and a cut-off between non-smokers *vs* smokers at 100 µM CO resulted in a sensitivity of 67.6 % and a specificity of 84.4 %. In the same figure a ROC curve is shown for CO concentrations grouped by elapsed time after a smoked cigarette and blood donation. At 135 µM the sensitivity was 100 % and the specificity 98.9 % for recent (less than three hours ago) *vs* late (more than six hours ago together with non smokers) elapsed time since a smoked cigarette. Taking the smoking history (e. g. elapsed time after smoking a cigarette) from a blood donor can predict the CO concentration quite accurately.

Figure 13 ROC curves. The dotted line represents non-smokers *vs* smokers and the solid line represents persons with recent smoking (less than three hours since last cigarette) *vs* late smoking (more than six hours since last cigarette as well as non-smokers).

Elimination time for CO after a smoked cigarette was followed in six volunteers. The results can be seen in figure 14. Calculations show that the median elimination half-life was 5.3 hours with a range between 4.7 and 8.4 hours. One individual had higher CO concentrations than the others; this person had a more frequent smoking habit (21-40 cigarettes per day) than the others (less than 21 cigarettes per day).

Results

Figure 14 Individual CO concentrations for smokers, before and after smoking one cigarette. Baseline CO concentrations are depicted ten minutes before they smoked the cigarette. The open symbols (○ □ △) in the figure represent persons who had smoked two - three hours prior to the baseline sample and the closed symbols (● ■ ▲) represent persons who had smoked 12-13 hours before baseline measurement. Open circles (○) represent an individual smoking 21 to 40 cigarettes every day, whereas the others smoked less than this. A dotted line representing non-smokers is also given in the figure, and denotes the median CO concentration in 366 non-smokers analyzed on one occasion.

Summary of results

- A method suitable for analysis of low concentrations of CO in blood was developed and validated.
- The half-life of CO at a start concentration of 250 μM CO in pigs was found to be 60 minutes. No circulatory effects were detected after CO administration.
- CO did not show anti-inflammatory effects after an endotoxin-induced systemic inflammation in pigs.
- In 6 % of the blood samples CO was present at concentrations above 130 μM , which we consider to be the upper physiological level. The highest detected concentration was 561 μM . Cigarette smoking is the main source for elevated CO concentrations. Elapsed time between smoking a cigarette and blood donation seems to be a particularly important factor.

DISCUSSION

Interpretation of the results

Paper I

The goal to develop an analytical method able to measure small amounts of CO was successfully achieved. Background levels of CO in our study were approximately 50 μM in blood which is four times higher than the LOQ. This shows that background concentrations of CO can be determined with great certainty. Both blood and exhaled air can be analysed with the developed method. It is a reliable and stable method and it is possible to store samples in a refrigerator for several weeks. Since the interactions and effects of CO in the body is very complex and not fully understood, it is important that the analytical method is reliable and exact. This method could make it possible to detect small changes in CO concentration in the body and thereby evaluate possible relationships between physiological effects and endogenously produced CO.

Paper II

When investigating CO and its effects in the body, it is important to know the half-life of CO. In the literature the elimination of CO has only been studied at high start doses of CO, including after intoxication. It is hard to draw any conclusions regarding the elimination of endogenously produced CO from these data since endogenously produced CO probably results in much lower concentrations. Also, the goal of the studies in the literature is generally to find the best means of elimination and therefore they have administered high oxygen concentrations. Results from such studies show half-lives between 5-190 minutes (Kreck et al., 2001; Sasaki, 1975; Shimazu et al., 2000; Wagner et al., 1975). In our study, under normal conditions, we found the elimination time to be 60 minutes. We also found the elimination to follow first order kinetics, after correction for basal CO concentrations. This is not in agreement with other publications (Shimazu et al., 2000; Wagner et al., 1975; Wazawa et al., 1996), where observed differences in elimination kinetics may be explained in large part by different concentration levels. When high concentrations of CO are present, the distribution to tissues may be more significant, resulting in a biphasic concentration decrease. The elimination seemed to occur only through the lungs since the cumulative fraction was 100 % after 160 minutes in the protocol.

In paper II we also attempted to determine if there were any circulatory changes after administering CO. We measured SVR and PVR and did not observe any changes in these parameters. Some investigators have found *in vivo* effects and some have not (Grover et al., 2000; Nachar et al., 2001; Vassalli et al., 2001). Studies *in vitro*, however, show that CO has vascular effects (Suematsu et al., 1995; Wang, 1998). In our study CO was administered as COHb in the blood stream. It is possible that the vascular effect is local and involves free CO. When administering CO as COHb, the amount of free CO is also increased but the local concentrations may still be too low to induce vascular responses. The interactions between CO and NO are complex (Watts et al., 2003). Perhaps when CO is administered, less NO is produced resulting in unaltered vascular tone. Accordingly, one finding *in vivo* showed an effect of CO only when the NO system was inhibited (Nishikawa et al., 2004).

Paper III

We were unable to show that treatment with CO gave any effects on cytokine release after an endotoxin challenge resulting in severely increased MPAP. The hypothesis that the pro-inflammatory cytokine increase (TNF-alpha, IL-6 and IL-1beta) would be attenuated in animals treated with CO was rejected as well as the hypothesis that the anti-inflammatory cytokine increase (IL-10) would be augmented in the same group. These results were unexpected and contrasted to findings in an endotoxin-injected mouse model where a CO treated group showed lower TNF-alpha and IL-1beta and higher IL-10 levels compared to a control group (Otterbein et al., 2000). A power analysis based on this mouse model was performed prior to our porcine experiment. The power analysis showed that eight animals in each group would give a power of 100 % based on only TNF-alpha concentrations. Our design with ten animals in each group together with analysis of four different cytokines measured on ten occasions would give a minimal risk of type II errors. Published data on inflammatory effects of CO in pigs is limited to one other study. In that study, higher levels of TNF-alpha in the CO pre-treated group were observed compared with the control group (Mazzola et al., 2005). The authors of that porcine study (Mazzola et al., 2005), based on only four animals, concluded that even though the TNF-alpha levels were higher in the CO treated group, CO ameliorated several of the acute pathological changes. In the study by Mazzola and co-workers, IL-1beta was suppressed in the CO-infused group but not in the control group. This is in contrast to our findings which showed no difference in IL-1beta concentration between the control group and the CO infused group. In a study in man who were treated with CO before a bolus of endotoxin was injected, there were no differences in plasma cytokines (TNF-

alpha, IL-6, IL-8, IL-10), cytokine mRNA (IL-1alpha, IL-1beta), heart rate, MAP or SpO₂ between the pre-treated CO group and the control group (Mayr et al., 2005). Our interpretation of previously available information together with data from our study is that CO may have anti-inflammatory effects in mice but not in humans and pigs.

The administration of CO given to the animals in this study was chosen based on the aim to quickly achieve constant blood CO levels and to avoid toxic effects. Instead of giving a fixed CO dose, the rate of delivery was modulated in order to provide constant blood CO concentrations. The uptake of CO decreased throughout the experiments as a result of the severe inflammatory situation, resulting in a need to increase the CO delivery. The constant CO levels was achieved which is a strength in this study, compared to other studies where the CO level in blood have decreased throughout the experiments (Mayr et al., 2005; Mazzola et al., 2005) or never been measured (Otterbein et al., 2000). The level of CO chosen (5 % COHb) in this study was determined to be a clinically relevant dose since higher doses may induce toxic symptoms.

Paper IV

The results showed that blood samples from 94 % of the donors had CO concentrations below 130 µM, with a mean of 84.5 µM which seem to represent the upper physiological level. Another author claims that a level over 1.5 % COHb exceeds Air Quality Standard (Aronow et al., 1984), transformation of 1.5 %COHb results in 130 µM at a haemoglobin value of 140 g/l. However, concentrations over 130 µM CO could be seen in 6 % of the studied population. The highest detected concentration was 561 µM. From our findings it is difficult to speculate what effect transfused blood with CO concentrations over the physiological level may have in transfused recipients. In normal sized adults, the volume from one bag or unit of erythrocytes is small (perhaps 5 %) in comparison with the total blood volume. Thus, the influence from one bag on the CO concentration in the whole body is therefore probably minimal. However, if a newborn child requires a significant blood transfusion or blood exchange, one bag containing elevated CO concentrations might produce significantly elevated COHb fractions (Kandall, Landaw, & Thaler, 1973). With poorly developed lungs, negatively influencing the capacity for gas exchange, it could be a risk with transfusion of such blood.

At what concentration CO is toxic is hard to determine. Thus, in various patient groups, the negative effects of CO are probably seen at lower CO concentrations than in healthy persons. For example the effect on CNS and in the mitochondria can be the main effect in healthy persons whereas the

influence of CO on oxygen delivery probably is more important in persons with an impaired oxygen delivery capacity. A study investigating the mechanisms of CO toxicity stated that CO is toxic only when administered as the free molecule, and not toxic already bound to haemoglobin (Goldbaum, Ramirez, & Absalon, 1975). Applying this latter observation to our present results might allow one to conclude that these CO contaminated erythrocyte bags may not be toxic at all when transfused. Before relying on the results from the study in dogs from 1975 it would be advisable to investigate this further and if possible in humans.

Smoking appears to be the main factor causing increased blood concentrations of CO. The time interval between smoking and blood donation seems to be a particularly important factor. In our study, we investigated kinetics for CO after smoking a cigarette in six volunteers. Since all six volunteers did not have the same elimination half-life (range, 4.7 – 8.4 h), there are probably other factors involved that need to be further investigated. In another study, kinetics for CO elimination in smokers has been investigated. The result showed two-compartmental kinetics with half-life of 1.6 h for the first phase and 30.9 h for the second phase (Cronenberger et al., 2007). One study suggests that blood donors avoid cigarettes for 24 h before blood donation. (Aronow et al., 1984).

Carbon monoxide research today

Research about CO and HO has increased markedly these last years. Several reviews have been published recently about CO and its positive biological effects (Mannaioni et al., 2006; Ryter et al., 2006; Ryter & Otterbein, 2004; Wu & Wang, 2005). CO has anti-inflammatory, anti-apoptotic and anti-proliferative effects. CO inhibits platelet aggregation and acts as a vessel relaxant. In many of the recent reviews, this is stated as a fact without taken into consideration in what species the original discovery has taken place. CO has been shown to have positive effects in mice, rats and in different cell-lines. But experimental research in larger animals and human data are sparse and has so far been inconclusive. The published conclusions or generalisations that CO has positive biological effects are potentially dangerous and can lead to a public impression that CO has positive biological effects in humans as well when it might be in some settings and mounts predominantly toxic. One article published in 2007 raised the question if CO and HO is ready for the clinic (Scott, Chin, Bilban, & Otterbein, 2007). In that article the effect of CO and HO is considered clear, the consideration is mainly to avoid toxic effects of CO. In my opinion it is not proved that CO has any positive effect in humans. Even though effects have been shown in different human cell types in isolated

cell experiments, it is not proved that this effect can be extrapolated to the whole complex human body. In cell studies it could be hard to get the whole picture since often only one isolated biological effect is studied and measured. The same could apply for small animals where it might be difficult to observe and measure several aspects.

There are studies showing increased levels of CO during systemic inflammation in traumatized or septic patients (Moncure et al., 1999; Zegdi et al., 2002). What this increase is related to is not clear. It could be dangerous to interpret that evaluated CO levels is due to anti-inflammatory properties of the molecule. Anti-inflammatory properties of CO are one possible explanation, but there could be other mechanisms for these observations, as for instance increased red blood cell degradation due to the concurrent disease.

Since CO also is a toxic molecule, this area of research is interesting and should be reviewed with care even if the application is to study positive effects of CO. Toxicological researchers do not often discuss the positive effects of CO and researchers looking for therapeutic effects usually do not study toxicological effects. The difference between toxic and pharmacological effects of a substance is often related to the dose. In toxicological articles, negative effects of CO have been described at levels from 2 % COHb (Aronow, 1981). In physiological articles, positive effects are shown at levels of 10 % COHb (Mazzola et al., 2005).

Future considerations

There is convincing evidence that CO has positive biological effects in mice and rats. It would be valuable if there could be a discussion about CO and the fact that it is not yet proved that CO has positive biological effects in larger animals and humans. Perhaps there are many negative studies in mice and rats that never have been published. It is not easy to perform similar investigations in humans as those which have shown positive effects in animals. More studies in larger animals could increase the understanding of CO and its effect in biological systems more similar to humans. Another advantage with experiments in larger animals compared to small animals is the possibility to monitor and measure in a more invasive and serial fashion. Nevertheless, studies in humans have to be performed, concurrently evaluating both toxicity as well as possible positive biological effects. The only study performed in humans with an endotoxin-induced systemic inflammation showed no effect of CO. This circumstance is strong evidence that CO is not yet ready, and may never be ready, for use in clinical anti-inflammatory treatment.

ACKNOWLEDGEMENTS

Jan-Erik Larsson - Min handledare som har hjälpt mej genom hela doktorandtiden. Vi har haft ett bra samarbete och även om vi inte haft så mycket tid tillsammans så har det var kvalitetstid. Du är duktig och jag har förtroende för dej samtidigt som du låter mej hitta mitt eget sätt att jobba. Du har tvingat mej att bli självständig och att ställa högre krav på mej själv vilket jag är glad för.

Magnus Hultin - Min bihandledare som har funnits som ett stöd i bakgrunden hela tiden. Vi har inte diskuterat mina forskningsprojekt så mycket däremot är jag glad att du funnits där och engagerat dej i andra frågor som arbetssituation, konflikter, karriärmöjligheter mm.

Pernilla Abrahamsson - Min dagliga kollega, tack för att du har lyssnat när jag ventilerat alla aspekter av mina forskningsprojekt. Jag är också glad att ha en kollega att prata med när det gäller barn, hus, hem, träning, semestrar mm.

Göran Johansson - Min andra kollega som fixar och ser till att allt fungerar. Att jag alltid har en fungerade dator med uppdaterade program och backup. Sedan är jag glad att det är så lätt att komma till dej och be om hjälp, när det gäller Excel, statistiska beräkningar eller egentligen vad som helst.

Ola Winsö - Min chef som har trott på mej och mina projekt och därmed också finansierat dem. Du har också hjälpt till med en del skrivarbete och planering.

Michael Haney – Du är en kollega som alltid hjälper till om du kan, du engagerar dej och tycker aldrig någonting känns jobbigt tror jag. Du har hjälpt mej både på lab, med språkgranskning och funnits som stöd.

Björn Biber - Min förra chef som drog igång hela CO forskningen och såg till att jag blev doktorand från första början, Tack.

Fikarummet på UKBF - Tack för att du finns och att det finns så många trevliga personer som fikar och äter där. Det är skönt att ha någonstans att gå och tänka på något annat ibland.

Acknowledgements

Mamma, pappa, Gudrun och Olle - Tack för att ni alltid ställer upp och hjälper oss. Ni tar hand om sjuka barn, klipper gräset när vi inte hinner och är barnvakt när vi behöver en stund för oss själva. Utan er skulle det varit tufft att doktorera.

Katarina - Tack för att du alltid kommer att finnas där.

Mina vänner - Tack för att vi har roligt ihop. Tack för att jag får vara en vän och inte bara mamma och fru.

Alma - Min dotter som är en fantastisk liten människa, så klok och duktig, med en vilja av stål och ett hiskeligt temperament. Du är också väldigt omtänksam.

Svante - Min bebis som har blivit stor. Du skrattar och skojar jämt och slutar aldrig att prata. Du är så fysisk, en kram från dej kan jag leva på en hel dag.

Mathias - Min man som alltid stöttar och ställer upp och som helt enkelt är en underbar människa.

Dictum Factum

REFERENCES

- Aberg, A. M., Hultin, M., Abrahamsson, P., & Larsson, J. E. (2004). Circulatory effects and kinetics following acute administration of carbon monoxide in a porcine model. *Life Sci*, 75(9), 1029-1039.
- Aker, J. (1987). Carboxyhemoglobin levels in banked blood: a comparison of cigarette smokers and non-smokers. *Aana J*, 55(5), 421-426.
- Alonso, J. R., Cardellach, F., Lopez, S., Casademont, J., & Miro, O. (2003). Carbon monoxide specifically inhibits cytochrome c oxidase of human mitochondrial respiratory chain. *Pharmacol Toxicol*, 93(3), 142-146.
- Anderson, E. W., Andelman, R. J., Strauch, J. M., Fortuin, N. J., & Knelson, J. H. (1973). Effect of low-level carbon monoxide exposure on onset and duration of angina pectoris. A study in ten patients with ischemic heart disease. *Ann Intern Med*, 79(1), 46-50.
- Andersson, J. A., Uddman, R., Tajti, J., & Cardell, L. O. (2002). Heme oxygenase and nitric oxide synthase in human middle ear epithelium indicates local carbon monoxide and nitric oxide production. *Acta Otolaryngol*, 122(6), 634-637.
- Arbetskyddsstyrelsen. (2000). Hygieniska gränsvärden. In A. 2000:3 (Ed.) (pp. 46): Arbetsmiljöverket.
- Aronow, W. S. (1981). Aggravation of angina pectoris by two percent carboxyhemoglobin. *Am Heart J*, 101(2), 154-157.
- Aronow, W. S., & Isbell, M. W. (1973). Carbon monoxide effect on exercise-induced angina pectoris. *Ann Intern Med*, 79(3), 392-395.
- Aronow, W. S., O'Donohue, W. J., Jr., Freygang, J., & Sketch, M. H. (1984). Carboxyhemoglobin levels in banked blood. *Chest*, 85(5), 694-695.
- Aronow, W. S., Stemmer, E. A., & Isbell, M. W. (1974). Effect of carbon monoxide exposure on intermittent claudication. *Circulation*, 49(3), 415-417.
- Artinian, L. R., Ding, J. M., & Gillette, M. U. (2001). Carbon monoxide and nitric oxide: interacting messengers in muscarinic signaling to the brain's circadian clock. *Exp Neurol*, 171(2), 293-300.
- Brune, B., & Ullrich, V. (1987). Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol Pharmacol*, 32(4), 497-504.
- Carbon monoxide - history, sources, physiological effects, uses., *Science encyclopedia vol 1* (Vol. 1): JRank.
- Cardeal, Z. L., Pradeau, D., Hamon, M., Abdoulaye, I., Pailler, F. M., & Lejeune, B. (1993). New calibration method for gas chromatographic assay of carbon monoxide in blood. *J Anal Toxicol*, 17(4), 193-195.
- Christian, G., Stranger, R., Petrie, S., Yates, B. F., & Cummins, C. C. (2007). Breaking Chemistry's Strongest Bond: Can Three-Coordinate [M{N(R)Ar}(3)] Complexes Cleave Carbon Monoxide? *Chemistry*.
- Coburn, R. F. (1970). The carbon monoxide body stores. *Ann N Y Acad Sci*, 174(1), 11-22.

References

- Coburn, R. F., & Mayers, L. B. (1971). Myoglobin O₂ tension determined from measurement of carboxymyoglobin in skeletal muscle. *Am J Physiol*, 220(1), 66-74.
- Coburn, R. F., Ploegmakers, F., Gondrie, P., & Abboud, R. (1973). Myocardial myoglobin oxygen tension. *Am J Physiol*, 224(4), 870-876.
- Cole, R. P. (1982). Myoglobin function in exercising skeletal muscle. *Science*, 216(4545), 523-525.
- Commins, B. T. (1975). Measurement of carbon monoxide in the blood: review of available methods. *Ann Occup Hyg*, 18(1), 69-77.
- Cronenberger, C., Mould, D. R., Roethig, H. J., & Sarkar, M. (2007). Population pharmacokinetic analysis of carboxyhaemoglobin concentrations in adult cigarette smokers. *Br J Clin Pharmacol*.
- Douglas, C. G., Haldane, J. S., & Haldane, J. B. (1912). The laws of combination of haemoglobin with carbon monoxide and oxygen. *J Physiol*, 44(4), 275-304.
- Ekblom, B., & Huot, R. (1972). Response to submaximal and maximal exercise at different levels of carboxyhemoglobin. *Acta Physiol Scand*, 86(4), 474-482.
- Ewing, J., & Maines, M. (1992). In situ hybridisation and immunohistochemical localization of heme oxygenase-2 mRNA protein in normal rat brain: differential distribution of isozyme 1 and 2. *Molecular and cellular neurosciences*, 3, 559-570.
- Feldstein, M. (1967). Methods for the determination of carbon monoxide. *Prog Chem Toxicol*, 3, 99-119.
- Goldbaum, L. R., Chace, D. H., & Lappas, N. T. (1986). Determination of carbon monoxide in blood by gas chromatography using a thermal conductivity detector. *J Forensic Sci*, 31(1), 133-142.
- Goldbaum, L. R., Orellano, T., & Dergal, E. (1976). Mechanism of the toxic action of carbon monoxide. *Ann Clin Lab Sci*, 6(4), 372-376.
- Goldbaum, L. R., Ramirez, R. G., & Absalon, K. B. (1975). What is the mechanism of carbon monoxide toxicity? *Aviat Space Environ Med*, 46(10), 1289-1291.
- Gorman, D., Drewry, A., Huang, Y. L., & Sames, C. (2003). The clinical toxicology of carbon monoxide. *Toxicology*, 187(1), 25-38.
- Grover, T. R., Rairigh, R. L., Zenge, J. P., Abman, S. H., & Kinsella, J. P. (2000). Inhaled carbon monoxide does not cause pulmonary vasodilation in the late-gestation fetal lamb. *Am J Physiol Lung Cell Mol Physiol*, 278(4), L779-784.
- Haldane, J. (1895). The Relation of the Action of Carbonic Oxide to Oxygen Tension. *J Physiol*, 18(3), 201-217.
- Hayashi, S., Omata, Y., Sakamoto, H., Higashimoto, Y., Hara, T., Sagara, Y., et al. (2004). Characterization of rat heme oxygenase-3 gene. Implication of processed pseudogenes derived from heme oxygenase-2 gene. *Gene*, 336(2), 241-250.
- Horvath, I., Donnelly, L. E., Kiss, A., Paredi, P., Kharitonov, S. A., & Barnes, P. J. (1998). Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress. *Thorax*, 53(8), 668-672.

References

- Johansson, M. B., & Wollmer, P. (1989). Measurement of carboxyhaemoglobin by spectrophotometry and gas chromatography. *Clin Physiol*, 9(6), 581-586.
- Kandall, S. R., Landaw, S. A., & Thaler, M. M. (1973). Carboxyhemoglobin exchange between donors and recipients of blood transfusions. *Pediatrics*, 52(5), 716-718.
- Kondo, A., Saito, Y., Seki, A., Sugiura, C., Maegaki, Y., Nakayama, Y., et al. (2007). Delayed neuropsychiatric syndrome in a child following carbon monoxide poisoning. *Brain Dev*, 29(3), 174-177.
- Kreck, T. C., Shade, E. D., Lamm, W. J., McKinney, S. E., & Hlastala, M. P. (2001). Isocapnic hyperventilation increases carbon monoxide elimination and oxygen delivery. *Am J Respir Crit Care Med*, 163(2), 458-462.
- Li, X., & Clark, J. D. (2003). Heme oxygenase type 2 participates in the development of chronic inflammatory and neuropathic pain. *J Pain*, 4(2), 101-107.
- Longo, L. D. (1977). The biological effects of carbon monoxide on the pregnant woman, fetus, and newborn infant. *Am J Obstet Gynecol*, 129(1), 69-103.
- Maines, M. D. (1988). Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *Faseb J*, 2(10), 2557-2568.
- Maines, M. D., Trakshel, G. M., & Kutty, R. K. (1986). Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *J Biol Chem*, 261(1), 411-419.
- Mannaioni, P. F., Vannacci, A., & Masini, E. (2006). Carbon monoxide: the bad and the good side of the coin, from neuronal death to anti-inflammatory activity. *Inflamm Res*, 55(7), 261-273.
- Mayr, F. B., Spiel, A., Leitner, J., Marsik, C., Germann, P., Ullrich, R., et al. (2005). Effects of carbon monoxide inhalation during experimental endotoxemia in humans. *Am J Respir Crit Care Med*, 171(4), 354-360.
- Mazzola, S., Forni, M., Albertini, M., Bacci, M. L., Zannoni, A., Gentilini, F., et al. (2005). Carbon monoxide pretreatment prevents respiratory derangement and ameliorates hyperacute endotoxic shock in pigs. *Faseb J*, 19(14), 2045-2047.
- McCoubrey, W. K., Jr., Huang, T. J., & Maines, M. D. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem*, 247(2), 725-732.
- The Merck Index*. (twelfth ed.)(1996). twelfth ed.). WhitehouseStation, NJ: Merck Research Laboratories, division of Merck & CO., Inc.
- Moncure, M., Brathwaite, C. E., Samaha, E., Marburger, R., & Ross, S. E. (1999). Carboxyhemoglobin elevation in trauma victims. *J Trauma*, 46(3), 424-427.
- Motterlini, R., Gonzales, A., Foresti, R., Clark, J. E., Green, C. J., & Winslow, R. M. (1998). Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. *Circ Res*, 83(5), 568-577.
- Nachar, R. A., Pastene, C. M., Herrera, E. A., Riquelme, R. A., Sanhueza, E. M., Troncoso, S., et al. (2001). Low-dose inhaled carbon monoxide reduces pulmonary vascular resistance during acute hypoxemia in adult sheep. *High Alt Med Biol*, 2(3), 377-385.

References

- Nakao, A., Kimizuka, K., Stolz, D. B., Seda Neto, J., Kaizu, T., Choi, A. M., et al. (2003). Protective effect of carbon monoxide inhalation for cold-preserved small intestinal grafts. *Surgery*, 134(2), 285-292.
- Nishikawa, Y., Stepp, D. W., Merkus, D., Jones, D., & Chilian, W. M. (2004). In vivo role of heme oxygenase in ischemic coronary vasodilation. *Am J Physiol Heart Circ Physiol*, 286(6), H2296-2304.
- Otterbein, L. E., Bach, F. H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., et al. (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med*, 6(4), 422-428.
- Otterbein, L. E., Otterbein, S. L., Ifedigbo, E., Liu, F., Morse, D. E., Fearn, C., et al. (2003). MKK3 mitogen-activated protein kinase pathway mediates carbon monoxide-induced protection against oxidant-induced lung injury. *Am J Pathol*, 163(6), 2555-2563.
- Pannen, B. H., & Bauer, M. (1998). Differential regulation of hepatic arterial and portal venous vascular resistance by nitric oxide and carbon monoxide in rats. *Life Sci*, 62(22), 2025-2033.
- Pauling, L. (1960). Types of resonance in molecules. In *The nature of the chemical bond* (pp. 265-266): Cornell University Press.
- Piantadosi, C. A. (2002). Biological chemistry of carbon monoxide. *Antioxid Redox Signal*, 4(2), 259-270.
- Roughton, F., & Darling, R. (1944). The effect of carbon monoxide on the oxyhemoglobin dissociation curve. *Am J Physiol*, 141, 17-31.
- Ryter, S. W., Alam, J., & Choi, A. M. (2006). Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev*, 86(2), 583-650.
- Ryter, S. W., & Otterbein, L. E. (2004). Carbon monoxide in biology and medicine. *Bioessays*, 26(3), 270-280.
- Sardana, M. K., & Kappas, A. (1987). Dual control mechanism for heme oxygenase: tin(IV)-protoporphyrin potently inhibits enzyme activity while markedly increasing content of enzyme protein in liver. *Proc Natl Acad Sci U S A*, 84(8), 2464-2468.
- Sasaki, T. (1975). On half-clearance time of carbon monoxide hemoglobin in blood during hyperbaric oxygen therapy (OHP). *Bull Tokyo Med Dent Univ*, 22(1), 63-77.
- Sato, K., Balla, J., Otterbein, L., Smith, R. N., Brouard, S., Lin, Y., et al. (2001). Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J Immunol*, 166(6), 4185-4194.
- Scott, J. R., Chin, B. Y., Bilban, M. H., & Otterbein, L. E. (2007). Restoring HOmeostasis: is heme oxygenase-1 ready for the clinic? *Trends Pharmacol Sci*, 28(5), 200-205.
- Severinghaus, J. W. (2002). Priestley, the furious free thinker of the enlightenment, and Scheele, the taciturn apothecary of Uppsala. *Acta Anaesthesiol Scand*, 46(1), 2-9.

References

- Shimazu, T., Ikeuchi, H., Sugimoto, H., Goodwin, C. W., Mason, A. D., Jr., & Pruitt, B. A., Jr. (2000). Half-life of blood carboxyhemoglobin after short-term and long-term exposure to carbon monoxide. *J Trauma*, 49(1), 126-131.
- Sjostrand, T. (1949). Endogenous formation of carbon monoxide in man. *Nature*, 164, 580-581.
- Sjostrand, T. (1952). The formation of carbon monoxide by the decomposition of haemoglobin in vivo. *Acta Physiol Scand*, 26(4), 338-344.
- Song, R., Mahidhara, R. S., Liu, F., Ning, W., Otterbein, L. E., & Choi, A. M. (2002). Carbon monoxide inhibits human airway smooth muscle cell proliferation via mitogen-activated protein kinase pathway. *Am J Respir Cell Mol Biol*, 27(5), 603-610.
- Suematsu, M., Goda, N., Sano, T., Kashiwagi, S., Egawa, T., Shinoda, Y., et al. (1995). Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J Clin Invest*, 96(5), 2431-2437.
- Tenhunen, R., Marver, H. S., & Schmid, R. (1968). The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci U S A*, 61(2), 748-755.
- Tenhunen, R., Marver, H. S., & Schmid, R. (1969). Microsomal heme oxygenase. Characterization of the enzyme. *J Biol Chem*, 244(23), 6388-6394.
- Tenhunen, R., Marver, H. S., & Schmid, R. (1970). The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med*, 75(3), 410-421.
- Thom, S. R., Xu, Y. A., & Ischiropoulos, H. (1997). Vascular endothelial cells generate peroxynitrite in response to carbon monoxide exposure. *Chem Res Toxicol*, 10(9), 1023-1031.
- Trakshel, G. M., Kutty, R. K., & Maines, M. D. (1986). Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform. *J Biol Chem*, 261(24), 11131-11137.
- Trakshel, G. M., & Maines, M. D. (1989). Multiplicity of heme oxygenase isozymes. HO-1 and HO-2 are different molecular species in rat and rabbit. *J Biol Chem*, 264(2), 1323-1328.
- Wagner, J. A., Horvath, S. M., & Dahms, T. E. (1975). Carbon monoxide elimination. *Respir Physiol*, 23(1), 41-47.
- van Erkel, A. R., & Pattynama, P. M. (1998). Receiver operating characteristic (ROC) analysis: basic principles and applications in radiology. *Eur J Radiol*, 27(2), 88-94.
- Wang, R. (1998). Resurgence of carbon monoxide: an endogenous gaseous vasorelaxing factor. *Can J Physiol Pharmacol*, 76(1), 1-15.
- Wang, R., Wang, Z., & Wu, L. (1997). Carbon monoxide-induced vasorelaxation and the underlying mechanisms. *Br J Pharmacol*, 121(5), 927-934.
- Vassalli, F., Pierre, S., Julien, V., Bouckaert, Y., Brimiouille, S., & Naeije, R. (2001). Inhibition of hypoxic pulmonary vasoconstriction by carbon monoxide in dogs. *Crit Care Med*, 29(2), 359-366.

References

- Watts, R. N., Ponka, P., & Richardson, D. R. (2003). Effects of nitrogen monoxide and carbon monoxide on molecular and cellular iron metabolism: mirror-image effector molecules that target iron. *Biochem J*, 369(Pt 3), 429-440.
- Wazawa, H., Yamamoto, K., Yamamoto, Y., Matsumoto, H., & Fukui, Y. (1996). Elimination of CO from the body: an experimental study on the rabbit. *Nihon Hoigaku Zasshi*, 50(4), 258-262.
- Weaver, L. K., Howe, S., Hopkins, R., & Chan, K. J. (2000). Carboxyhemoglobin half-life in carbon monoxide-poisoned patients treated with 100% oxygen at atmospheric pressure. *Chest*, 117(3), 801-808.
- Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V., & Snyder, S. H. (1993). Carbon monoxide: a putative neural messenger. *Science*, 259(5093), 381-384.
- White, R. E., & Coon, M. J. (1980). Oxygen activation by cytochrome P-450. *Annu Rev Biochem*, 49, 315-356.
- Von Burg, R. (1999). Carbon monoxide. *J Appl Toxicol*, 19(5), 379-386.
- Wu, L., & Wang, R. (2005). Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev*, 57(4), 585-630.
- Yet, S. F., Pellacani, A., Patterson, C., Tan, L., Folta, S. C., Foster, L., et al. (1997). Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *J Biol Chem*, 272(7), 4295-4301.
- Young, L. J., & Caughey, W. S. (1986). Mitochondrial oxygenation of carbon monoxide. *Biochem J*, 239(1), 225-227.
- Zegdi, R., Perrin, D., Burdin, M., Boiteau, R., & Tenaillon, A. (2002). Increased endogenous carbon monoxide production in severe sepsis. *Intensive Care Med*, 28(6), 793-796.
- Zufall, F., & Leinders-Zufall, T. (1997). Identification of a long-lasting form of odor adaptation that depends on the carbon Monoxide/cGMP second-messenger system. *J Neurosci*, 17(8), 2703-2712.