Reversal of drug-affected breathing

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Reversal of Drug-Affected Breathing

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1 Introduction

The maintenance of normal blood gas values is of major importance in the perioperative setting. Both anesthetics and analgesics (particularly opioids) may affect the ability of the human body to respond adequately to possible life-threatening decreases in oxygen tension (PO_2) and rises in carbon dioxide tension (PCO_2) in arterial blood.[1-3] In the control system regulating pulmonary ventilation, the peripheral chemoreceptors, located within the carotid bodies, and central chemoreceptors in the brain stem play a crucial role in correcting the arterial PO_2 and PCO_2 once they are compromised by depressant effects of pharmacological agents that are used in anesthetic practice before, during and after a surgical procedure.[1-3]

In extreme cases, for example in patients suffering from obstructive and/or central postoperative sleep apnea (often related to underlying disease such as the obstructive sleep apnea syndrome, the residual effects of anesthetics and the relative overdosing of opioid analgesics for postoperative pain relief), the availability of agents that are able to reverse severe respiratory depression is of life-saving importance. In the past decades, several studies in our laboratory have addressed the ability to reverse the respiratory depressant effects of opioids and anesthetics in animal and human studies.[4,5] An example of an agent that reverses the adverse effects of opioids on breathing is the opioid receptor antagonist naloxone. Naloxone is closely related to morphine (figure 1) and antagonizes the following three opioid receptors: μ-, κ- and δ-opioid receptors. Naloxone is able to reverse respiratory depression from commonly used perioperative opioids, such as morphine and fentanyl, at relative low intravenous doses ranging from 100 to 400 μg (dose for a 70–80 kg person).[6] Note, however, that with the disappearance of the opioid-induced side effect, also the intended effect (pain relief) is severely affected (*i.e.*, reduced) by naloxone.

Buprenorphine: respiratory behavior and reversal with naloxone

Surprisingly little is known about the ability of naloxone to reverse respiratory depression from one specific opioid, buprenorphine. Buprenorphine is a potent opioid analgesic used in the treatment of chronic and acute pain and used in the treatment of heroin addiction. The use of buprenorphine has gained in popularity due to the recent introduction of buprenorphine matrix patches for the treatment of chronic (malignant and non-malignant) pain and replacement of methadone by buprenorphine in the treatment of heroin addiction. It has several special and unique properties.[7] Buprenorphine is a semisynthetic opioid derived from thebaine, an alkaloid present in the poppy *Papaver somniferum*. The chemical structure of

Box 1: Physiology of the Control of Breathing

Breathing is the process in which air flows into (inspiration) and out of (expiration) the lungs. The lungs expand during inspiration due to contraction of the diaphragm and external intercostal muscles. Expiration is passive (at least during rest). Skeletal muscles of the mouth and pharynx (incl. tongue and glottis) and smooth muscles of the airways play an important role in flow formation due to their role in modulation of airway resistance.

Breathing results from rhythmic activity of the respiratory centers in the brain stem. Recent studies suggest the existence of two separate but coupled brainstem respiratory oscillators.[28] The dominant one, the preBötzinger complex (preBötC), contains μ-opioid receptors and is one of the sites involved in opioid-induced respiratory depression.[29] The other respiratory center is the retrotrapezoid nucleus (RTN). The preBötC and RTN are part of the ventral respiratory column.

Breathing is well adjusted to the metabolic and non-metabolic needs of the body. The respiratory centers themselves cannot account for adjustment of breathing in response to changes in metabolic requirements. They receive input from various sites in the body (hypothalamus, lung receptors, proprioreceptors in muscles and joints, peripheral and central chemoreceptors, cortex, subcortical regions) and send neuronal signals to the respiratory muscles. With respect to the chemical control of breathing, the chemical composition of arterial blood primarily regulates breathing through their effect on the peripheral and central chemoreceptors.[1,2] The peripheral chemoreceptors in the carotid bodies are sensitive to changes in arterial pH, PCO_2 and pO_2. The central chemoreceptors on the surface of the ventral medulla are sensitive to changes in brain tissue PCO_2 and pH. To maintain a chemical equilibrium in the body, the metabolic control system makes use of two reflex arcs. The peripheral chemoreflex loop consists of the peripheral chemoreceptors, the sinus nerve, sites in the brainstem that receive and process afferent input from the carotid bodies, the brainstem respiratory centers and the neuromechanical link between brainstem and ventilation (phrenic nerve, spinal motoneurons, diaphragm, intercostal nerves and muscles, lungs). The central chemoreflex loop involves the central chemoreceptors, and neuronal connections between these receptors and the brainstem respiratory centers and the above-mentioned link between respiratory centers and ventilation. Animal studies showed the presence of opioid receptors in the carotid bodies.[30]

Pure chemical control of breathing operates during non rapid-eye-movement (REM) sleep and during anesthesia (at least in spontaneous breathing patients). During wakefulness and REM-sleep another equally important system, the behavioral control system, will influence breathing and may even temporarily override the chemical system. Behavioral control of breathing allows for the adjustment of breathing to voluntary efforts such as speech, singing, eating, diving, *et cetera*. Pain may both stimulate and depress breathing. These effects are chemoreflex- and arousal-state-independent.

buprenorphine contains the general morphine skeleton (figure 1). In contrast to morphine, buprenorphine is considered a partial agonist for the μ-opioid receptor. This indicates that despite full receptor occupancy it shows a partial or limited effect. Some animal studies show ceiling (the occurrence of an apparent maximum in effect), a bell-shaped dose-response curve (or inverse U-shaped response curve) with respect to buprenorphine's antinociceptive and respiratory depressant effects (see also figure 2).[8-10] In this respect, no human data is available. Furthermore, buprenorphine displays high μ-opioid receptor affinity (*K*-values in the subnanomolar range) with slow receptor association/dissociation kinetics.[7,11] In humans, buprenorphine's μ-opioid receptor affinity is so high that reversal of buprenorphine's adverse effects is impossible with low doses of naloxone (up to 400 μg). And even with high naloxone bolus doses (up to 10 mg), reversal is at best partial and short-lived.[12,13]

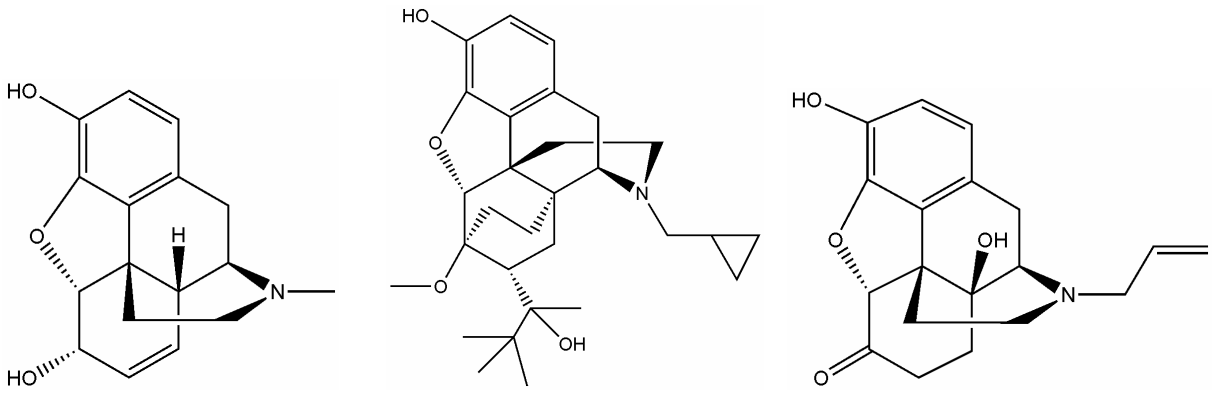

Figure 1. Chemical structures of morphine (left), buprenorphine (middle) and naloxone (right).

The first objective of this thesis is to describe the behavior of buprenorphine in humans with respect to its most important unwanted side effect, respiratory depression (chapter 2), and compare this with its intended effect, analgesia (chapter 3). The studies were designed to enable us to answer the question whether buprenorphine shows ceiling in its respiratory and/or analgesic behavior. Next, we studied the ability of naloxone to reverse buprenorphine-induced respiratory depression in an extensive set of experiments. The ability of naloxone to reverse buprenorphine-induced respiratory depression was tested in a dose range from 0.5 to 7 mg per 70 kg (using a continuous naloxone infusion rather than bolus infusions) and compared to naloxone's ability to reverse morphine- and alfentanil-induced respiratory depression (chapter 4).

Box 2: The Opioid System and Pain Inhibition

The opioid system is composed of a family of structurally related endogenous peptides that act at three receptor types known as μ-, κ- and δ-opioid receptors.[31] Recently, a fourth receptor has been discovered with a strong homology with the three classical opioid receptors. Because of its likeness with these receptors the new receptor was named ORL_1 or opioid receptor-like type 1.[32] The opioid system is involved in responses to pain, stress and emotion and has a modulatory influence on various physiological functions such as the control of breathing, thermoregulation, appetite, nociception and the immune response.[31] Various endogenous opioid peptides have been discovered (for example, β-endorphin, enkephalin, dynorphin, endomorphin, nociceptin/orphanin FQ) but there is still much discussion about their exact function and distribution within the central nervous system.

The opioid receptor is a seven-transmembrane G-protein-coupled receptor. Activation of the receptor will cause inhibition of neuronal activity due to:[33]

(i) Inhibition of adenylate cyclase causing a decrease in cAMP,
(ii) Opening of potassium channels (causing hyperpolarization of postsynaptic cells),
(iii) Closure of calcium channels (causing a presynaptic decrease in neurotransmitter –such as substance P and glutamate– release).

The G-proteins play a crucial role in these processes.

The majority of opioid receptors (70% μ-opioid receptors, 25% δ-opioid receptors) come to expression on the membranes of neurones in the lamina I and substantia gelatinosa of the spinal cord. Activation of the opioid system for pain relief (from endogenous and exogenous opioids) is due to:[33]

(i) Inhibition of neurones involved in pain perception (in afferent pathways in the spinal cord and supraspinal in pons and thalamus) and activation of descending pain inhibition pathways (locus coeruleus, raphe nuclei),
(ii) Inhibition of pain perception, pain realization and central modulation of pain (supraspinal in thalamus and cortex),
(iii) Suppression of the emotional component of pain (limbic system),
(iv) Reduction of the level of arousal and autonomic responses to pain (reticular formation and locus coeruleus),
(v) Furthermore, in case of inflammation at peripheral sites (for example, in case of arthritis), opioid receptors are expressed in the inflamed tissue and endogenous opioids are released from activated leucocytes.

Typical μ-opioid side effects include: nausea, vomiting, respiratory depression, miosis, itch, inhibition of gastric motility, euphoria/dysphoria, hallucinations, sedation, muscle rigidity, cardiovascular depression, excitation, tolerance and addiction. Furthermore, recent findings indicate that opioids, aside from producing analgesia, display hyperalgesic behavior in various pain tests and clinical situations.

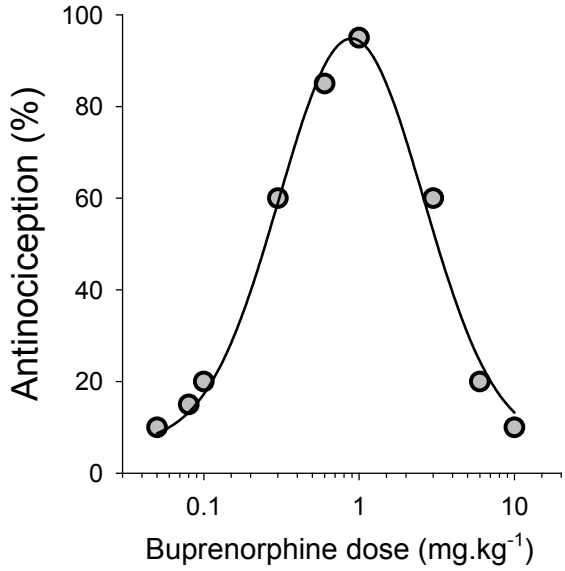

Figure 2. Buprenorphine dose-effect curve for antinociception in rats (electrical stimulation was used to assess antinociception). The curve is bell-shaped. Adapted from ref. 8.

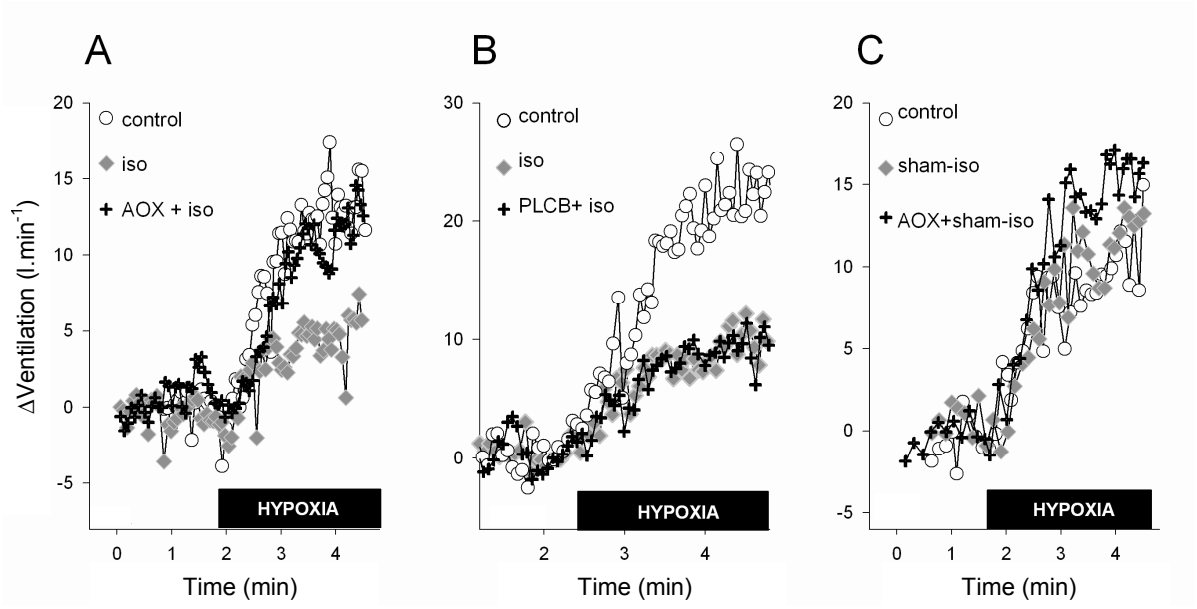

Figure 3. Examples from one subject of the influence of an antioxidant cocktail (ascorbic acid plus α-tocopherol) on depression of the ventilatory response to acute hypoxia by low-dose isoflurane.
A. Isoflurane causes > 50% depression of the ventilatory response to hypoxia; full reversal of effect is observed after pre-treatment with antioxidants. B. Isoflurane causes > 50% depression of the ventilatory response to hypoxia; no effect is seen after pre-treatment with placebo (NaCl 0.9%). C. No effect is observed after sham-isoflurane or antioxidants plus sham-isoflurane on the ventilatory response to acute hypoxia. AOX is the antioxidant cocktail; iso is isoflurane; PLCB is placebo. Data are from ref. 16.

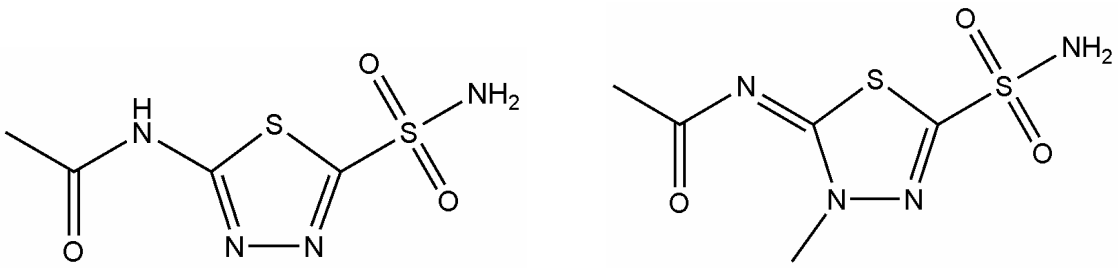

Figure 4. Chemical structures of acetazolamide (left) and methazolamide (right).

Antioxidant-reversal of respiratory effects of drugs

A major challenge in anesthesiology is to treat (*i.e.*, reverse) respiratory depression due to opioids without affecting pain relief. A more detailed knowledge about the mechanisms by which the peripheral and central chemoreceptors operate is a prerequisite to bring this important clinical aim closer to reality. Therefore, a second focus of our studies in the past decades was put on the mechanism of the hypoxic and hypercapnic ventilatory responses.[14,15] One important finding was the ability of antioxidants (we used a combination of oral α-tocopherol –vitamin E– and intravenous ascorbic acid –vitamin C) to completely reverse the depression of the acute hypoxic ventilatory response (AHR) by subanesthetic concentrations of halothane and isoflurane (see also figure 3).[5,16] Data from the literature strongly suggest that the AHR is mediated by the carotid bodies.[17] The stimulus-response cascade in the carotid bodies is not completely known but studies in the last two decades have shown that the hypoxic response may be initiated by (various classes of) potassium channels that upon closure by hypoxia are responsible for depolarization of oxygen-sensitive cells. This depolarization is followed by influx of Ca^{2+} ions and release of neurotransmitters (presumably acetylcholine and ATP) that depolarize nearby nerve-endings of the glossopharyngeal nerve.[18] Potassium channels are also sensitive to (*i.e.*, are opened by) halogenated volatile anesthetics. Interestingly, most potassium channels are also sensitive to reducing and oxidizing agents,[19,20] and this may explain the ability of antioxidants to reverse the inhibiting effects of halothane and isoflurane. These previous experiments raised the question whether the ability of antioxidants to reverse an inhibiting effect on the carotid bodies would also apply to inhibiting effects caused by other agents than anesthetics and thus might reflect a more general property of the carotid bodies when they are in a state of inhibition. Another pharmacological means to inhibit the carotid bodies is the carbonic anhydrase inhibitor acetazolamide (figure 4). In large intravenous doses, this agent completely abolishes the steady-state hypoxic response in animals,[19,21] but literature data indicate that already clinical oral doses have an inhibiting effect on the carotid bodies.[20,22] One question that we wished to answer in this thesis is whether antioxidants (the same mixture as described above) would also be able to reverse an inhibiting effect of a low intravenous dose of acetazolamide on the ventilatory response to acute isocapnic hypoxia in man (chapter 5).

The aforementioned inhibition of the AHR by acetazolamide is thought to result from inhibition of carbonic anhydrase that has been shown to be present in the carotid bodies.[21,23] However, methazolamide, a more lipid soluble carbonic anhydrase with similar affinity for carbonic anhydrase II and IV (the two major carbonic anhydrase isoforms that are blocked by acetazolamide and methazolamide), does not seem to affect the hypoxic response of an *in vitro* carotid body preparation,[21,24] and this raises the question as to whether inhibition of carbonic anhydrase by acetazolamide is the underlying mechanism of its inhibiting effect on the hypoxic response indeed. Furthermore, recent studies have shown that acetazolamide has a specific opening effect on large conductance voltage-sensitive potassium (BK) channels, and possibly also on calcium- and sodium channels.[22,25-27] These differences in physiological and pharmacological actions of acetazolamide and methazolamide raised the idea of comparing the effects of both agents on the steady-state hypercapnic and hypoxic response in the cat (chapters 6 and 7). The major question that we wished to answer was whether full activity of carbonic anhydrase is a necessary condition for an intact hypoxic response to occur, and whether the profound inhibiting effects of acetazolamide can be ascribed to inhibition of this enzyme or rather to another pharmacological action.

The specific aims of this thesis are

1. To describe the dose-respiratory response relationship of the opioid analgesic buprenorphine and compare this to the opioid fentanyl (**chapter 2**). Experiments were performed in healthy young volunteers. Respiration was measured using the computer-controlled 'dynamic end-tidal forcing technique'.

2. To compare the respiratory and analgesic behavior of buprenorphine in healthy volunteers (**chapter 3**).

3. To assess the ability of the opioid-antagonist naloxone to reverse buprenorphine-induced respiratory depression in humans (**chapter 4**). Studies focused on continuous infusions and doses. The buprenorphine data were compared to naloxone-reversal of morphine- and alfentanil-induced respiratory depression.

4. To assess the ability of antioxidants to reverse acetazolamide-induced depression of the ventilatory response to acute hypoxia in humans (**chapter 5**).

5. To describe and compare the effects of the carbonic anhydrase inhibitors acetazolamide and methazolamide on the ventilatory CO_2 sensitivity and the hypoxic ventilatory response in anesthetized cats (**chapters 6 and 7**).

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SECTION 1

Ceiling and Naloxone-Reversal of Buprenorphine-Induced Respiratory Depression

2 Comparison of the Respiratory Effects of Intravenous Buprenorphine and Fentanyl in Humans and Rats

Buprenorphine is a semi-synthetic opioid analgesic used in clinical practice since 1979.[1] In patients, buprenorphine is used for treatment of acute and chronic pain using various administration modes, such as intravenous, sublingual and spinal/epidural administration. Recently, interest in buprenorphine has been renewed due to the introduction of a new transdermal formulation for treatment of chronic malignant pain and due to the use of buprenorphine in the treatment of heroin addiction.[1-3] Buprenorphine is a potent analgesic with agonistic activity at the μ-opioid receptor and the opioid receptor-like 1 (ORL_1) receptor, and antagonistic properties at the κ-opioid receptor.[4,5] In humans, buprenorphine behaves as a typical μ-opioid receptor agonist showing analgesia, sedation, nausea, delayed gastric emptying and respiratory depression.[4,6] Animal studies suggest that buprenorphine, in contrast to other opioids, shows ceiling in its μ-agonist behavior such as analgesia and respiratory depression.[4,7-9] Ceiling is best defined as an apparent maximum effect regardless of drug dose. The occurrence of ceiling is an important observation that is poorly studied in humans. Ceiling in respiratory effect has evident clinical advantages as opioids that possess this property are considered to be safer, because in theory they can not cause excessive respiratory depression.

In this study we wished to assess whether there exists an apparent maximum in opioid-induced respiratory depression for buprenorphine. We measured the respiratory responses to buprenorphine and compared them to fentanyl, a μ-opioid receptor agonist for which no ceiling has been observed.[10] We performed experiments in two species, humans and rats, and obtained dose-response relationships using double-blind, placebo-controlled randomized designs. We studied both human and animal subjects, allowing us to expand the dose range beyond the rather limited dose range that is acceptable in awake humans. The dose range for buprenorphine was 0 to 3 $mg.kg^{-1}$, for fentanyl from 0 to 0.09 $mg.kg^{-1}$.

Methods

Human Studies

Forty-eight volunteers (24 men, 24 women) participated in the protocol after approval was obtained from the local Human Ethics Committee. All subjects were healthy and did not have a history of illicit substance abuse. They were asked to refrain from stimulants and depressant substances for at least 12 h before the study. Each subject participated once.

Respiration. To study ventilation we used the dynamic end-tidal forcing technique.[11] This technique enables us to force end-tidal PCO_2 ($P_{ET}CO_2$) and end-tidal PO_2 ($P_{ET}O_2$) to follow a specific pattern in time. In this study we clamped the $P_{ET}CO_2$ and $P_{ET}O_2$ to 7 kPa and 14.5 kPa, respectively, throughout the studies. The subjects were comfortably positioned in a hospital bed and breathed through a face mask (Vital Signs, Totowa, NJ, USA), positioned over nose and mouth (a nose clip was not used). The face mask received fresh gas (45 $l.min^{-1}$) from a gas mixing system consisting of three mass flow controllers (F202, Bronkhorst High-Tech BV, Veenendaal, The Netherlands) for the delivery of oxygen, carbon dioxide and nitrogen. A personal computer running ACQ software (Erik Kruyt, Leiden University Medical Center, Leiden, The Netherlands) provided control signals to the mass flow controllers allowing the adjustment of the inspired gas concentrations to obtain the desired end-tidal concentrations. The in- and expired gas flows were measured at the mouth using a pneumotachograph connected to a pressure transducer (Hans Rudolph, Wyandotte, MI, USA) and electronically integrated to yield a volume signal. The volume signal was calibrated with a motor-driven piston pump. The oxygen and carbon dioxide concentrations were measured using a gas monitor (Multicap, Datex, Helsinki, Finland); a pulse oximeter (Masimo, Irvine, CA, USA) continuously measured the oxygen saturation (SpO_2) of arterial hemoglobin with a finger probe. All relevant variables (minute ventilation, SpO_2, $P_{ET}CO_2$ and $P_{ET}O_2$) were available for on-line analysis (using RRDP software, Erik Olofsen, Leiden University Medical Center, Leiden, The Netherlands) and stored on a breath-to-breath basis for further analysis. The bispectral index (BIS) of the EEG was monitored with a BIS XP machine (Aspect Medical Systems Inc., Newton, MA, USA; release 2002) using a four leads electrode placed on the forehead as specified by the manufacturer. BIS values were collected at 1-min intervals.

The study was double-blind, randomized and placebo controlled. Prior to testing all subjects received 4 mg ondansetron i.v. (GlaxoSmithKline BV, Zeist, The Netherlands). The subjects were randomly assigned to receive placebo (0.9% NaCl), buprenorphine (Reckitt Benckiser Healthcare Ltd, Hull, UK) or fentanyl (Janssen-Cilag BV, Tilburg, The Netherlands). The following doses were given, placebo: 9 ml (n = 7); buprenorphine in 9 ml saline: 0.7 $\mu g.kg^{-1}$ (n = 5), 1.4 $\mu g.kg^{-1}$ (n = 5), 4.3 $\mu g.kg^{-1}$ (n = 5) and 8.6 $\mu g.kg^{-1}$ (n = 5); fentanyl in 9 ml saline: 1.1 $\mu g.kg^{-1}$ (n = 5), 2.1 $\mu g.kg^{-1}$ (n = 5), 2.9 $\mu g.kg^{-1}$ (n = 5), 4.3 $\mu g.kg^{-1}$ (n = 5) and 7.1 $\mu g.kg^{-1}$ (n = 1). The highest fentanyl dose (7.1 $\mu g.kg^{-1}$) was tested only once. After the first subject was dosed with this dose, the respiratory effects were so severe (apnea > 5 min and SpO_2 drop below 70%) that the blinding of this experiment was broken and a decision was made to no longer infuse the highest fentanyl dose. The data of the single subject receiving 7.1 $\mu g.kg^{-1}$ were used in the analysis.

The respiratory studies started after a period of acclimatization to the apparatus and ventilation (at a $P_{ET}CO_2$ of 7 kPa) had reached a steady state. Next the drug was infused slowly over 90 s. Subsequently continuous respiratory measurements were obtained for 80 to 90 min, followed by 5 to 10 min measurements at 30 min intervals (until 4 h after drug

infusion) and next at 60 min intervals. In case ventilation returned to baseline values (defined by at least 5 min at or above baseline) prior to the end of the measurement periods the study was ended. In case this did not happen or in case there was no systematic respiratory depressant effect, the study ended seven hours after drug infusion.

Data Analysis. We performed a 1-min average on the ventilation data of individual subjects. From these data we calculated peak ventilatory depression, time to peak effect and time to end of effect (*i.e.*, return to baseline). The dose–peak ventilatory depression data were analyzed using the following sigmoid E_{max} model (Hill equation):

$$\text{Effect(dose)} = E_{max} - (E_{max} - E_{min}) \cdot [(\text{dose}/ED_{50})^{\gamma} / (1 + (\text{dose}/ED_{50})^{\gamma})]$$

where dose is the drug dose applied, ED_{50} the estimated dose causing 50% effect, E_{max} and E_{min} the maximum and minimum of the sigmoid function, respectively, and γ a shape parameter. The model parameters were estimated using nonlinear regression analysis (NONMEM version V, level 1.1, a data analysis program for nonlinear mixed effects modeling). The likelihood ratio criterion was used to assess whether E_{min} differed significantly from 0 $l.min^{-1}$.

In order to get an impression of the average drug effect on respiration over the measurement time we assessed the area between the curves standardized by the length of the study.[12] The involved curves are measured ventilation normalized by predrug baseline (which per definition equals 1; see also figure 1). An average drug effect of 40 indicates an average 40% respiratory depression over the measured time period (*i.e.*, from time of drug infusion to time to end of effect or end of study if time to end of effect had not been reached within 420 min).

The time to peak effect and average drug effect data were analyzed using a one-way analysis of variance (factor: dose), with *post-hoc* Bonferroni correction for multiple comparisons. The buprenorphine and fentanyl groups were analyzed separately. *P*-values < 0.05 were considered significant. Values are given as mean (SD), unless otherwise stated.

Animal Studies

Male Wistar rats (weight 225–250 g, $n = 63$) were housed in groups before surgery and individually after surgery in plastic cages. The animals were housed under laboratory standard conditions at constant room temperature (21 ºC) and on a 12-h light/dark cycle (lights on at 07:00 AM; lights off at 07:00 PM). Food (RMH-TM; Hope Farms BV, Woerden, The Netherlands) and acidified water were allowed *ad libitum*. The animals were handled and allowed for acclimation to the experimental environment for ten days prior to the start of the experiment. The animal protocol was approved by the University Animal Ethics Committee.

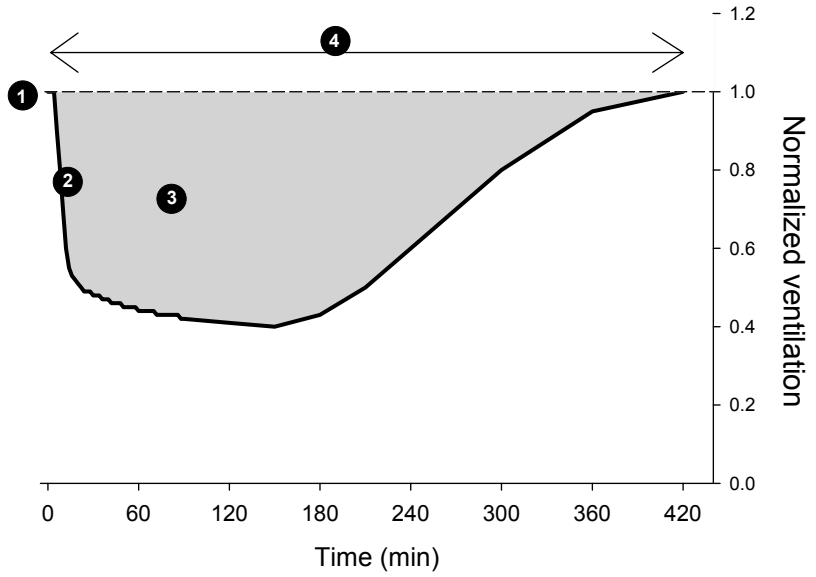

Figure 1. Calculation of the average drug effect. **1**: baseline ventilation (per definition = 1), **2**: ventilation normalized by the baseline value, **3**: the area between lines 1 and 2 equals the average drug effect, **4**: the time to end of effect. The average drug effect is divided by the time to end of effect or 420 min if time to end of effect had not been reached within 420 min.

Surgical Procedure. Surgery was carried out under anesthesia with medetomidine hydrochloride (0.1 mg.kg^{-1} i.m.; Pfizer BV, Capelle a/d IJssel, The Netherlands) and ketamine base (1 mg.kg^{-1} i.m.; Parke-Davis BV, Hoofddorp, The Netherlands). Two days before the experiment two indwelling cannulae were implanted, one in the left femoral artery and one in the right jugular vein. The cannula in the right jugular vein was used for administration of the opioid or vehicle while the cannula in the left femoral artery was used for collection of arterial blood samples. The cannulae were made from pyrogen-free, non-sterile polyethylene tubing. The cannulae were tunnelled subcutaneously and fixed at the back of the neck with a rubber ring. In order to prevent clotting and cannula obstruction the cannulae were filled with a 25% (w/v) polyvinylpyrrolidone solution (PVP; Brocacef NV, Maarssen, The Netherlands) in pyrogen-free physiological saline (B. Braun Melsungen AG, Melsungen, Germany) containing 20 IU.ml^{-1} heparin.

Drugs and Dosages. Fentanyl was dissolved in saline; buprenorphine was dissolved in saline with aid of two drops of polysorbate 80 (Hospital Pharmacy, Leiden University Medical Center, Leiden, The Netherlands). Henceforth, the doses of buprenorphine and fentanyl are expressed as free base. Each animal was tested once. For buprenorphine the following dosages were tested: 0 (vehicle), 100, 300, 1000 and 3000 μg.kg^{-1}. For fentanyl the dosages were: 0 (vehicle), 50, 68 and 90 μg.kg^{-1}. Fentanyl and buprenorphine were infused i.v. over 20 min *via* a constant-rate infusion using an infusion pump (BAS Bioanalytical Systems, Inc.,

West Lafayette, IN, USA). Animals were randomly assigned to the treatment groups with seven animals in each treatment level.

Measurement of arterial PCO_2. Arterial blood samples were obtained at fixed times for measurement of arterial PCO_2 ($PaCO_2$) using a blood gas analyzer (278, Bayer BV, Mijdrecht, The Netherlands). For buprenorphine these times were: baseline (5 to 10 min prior to drug infusion), 0, 30, 60, 120, 270 and 390 min after the start of drug administration; and for fentanyl: baseline, 5, 10, 15 and 20 min after the start of drug administration. Each blood sample withdrawn was replaced by an equal volume of heparinized 0.9% saline (heparin 20 $IU.ml^{-1}$). The difference in sampling schedule is related to the difference in speed of onset of the two tested opioids, with immediate changes in $PaCO_2$ observed after fentanyl but not after buprenorphine infusion. During the experiments body temperature was maintained at 37.5 ºC by a CMA/150 Temperature Controller (BAS Bioanalytical Systems, Inc.)

Statistical Analysis. The buprenorphine and fentanyl studies were analyzed separately. A one-way analysis of variance was performed to assess the effect of drug dose per time, with *post-hoc* Bonferroni correction for multiple comparisons. *P*-values < 0.05 were considered significant. Values are given as mean (SD).

Results

Human studies

The average age of the subjects was 22 years (range 19–34 years). Mean weight and height of the subjects was 72 kg (range 53–93 kg) and 176 cm (range 160–192 cm), respectively.

Placebo. Placebo had no systematic effect on ventilation over the 420-min measurement period. Predrug ventilation was 22.7 (6.1) $l.min^{-1}$. The lowest ventilation after drug infusion was at 180 min: 19.6 (4.6) $l.min^{-1}$. The mean average drug effect was -0.1 (0.1).

Fentanyl and Buprenorphine Time Profiles. The individual ventilatory responses of the subjects are given in figures 2 and 3. For both drugs, predrug ventilation did not differ among the doses: fentanyl 24.1 (6.0) $l.min^{-1}$, buprenorphine 24.5 (4.1) $l.min^{-1}$. After fentanyl, four subjects developed a period of apnea shortly after the infusion, one after 2.9 $\mu g.kg^{-1}$ (duration < 3 min, lowest SpO_2 measured 92%), two after 4.3 $\mu g.kg^{-1}$ (duration < 3 min, lowest SpO_2 measured 91% and 93%) and one after 7.1 $\mu g.kg^{-1}$ (8 min). Time to fentanyl peak effect did not differ among the doses tested: 4.8 (2.2) min. None of the subjects receiving buprenorphine developed apnea. Time to buprenorphine peak effect was dose independent and averaged 117 (58) min.

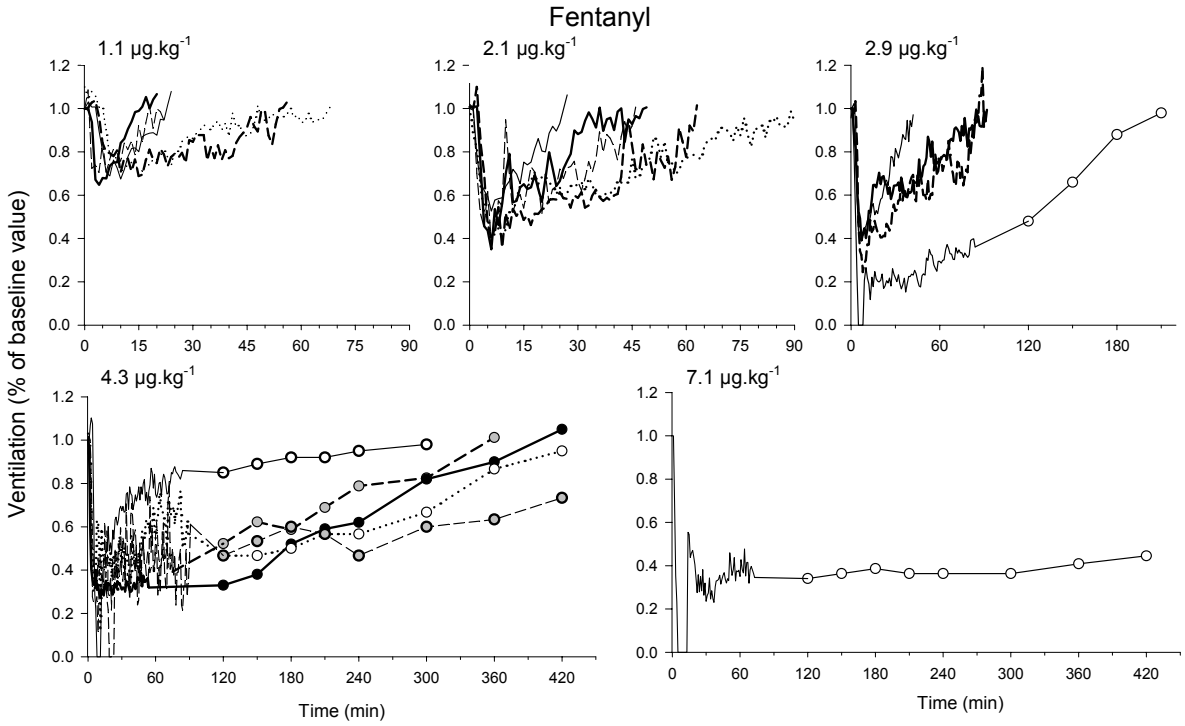

Figure 2. Individual human ventilatory responses after 1.1, 2.1, 2.9, 4.3 and 7.1 µg.kg^{-1} fentanyl infusions. Ventilation is normalized relative to baseline values. Different symbols and lines depict different subjects.

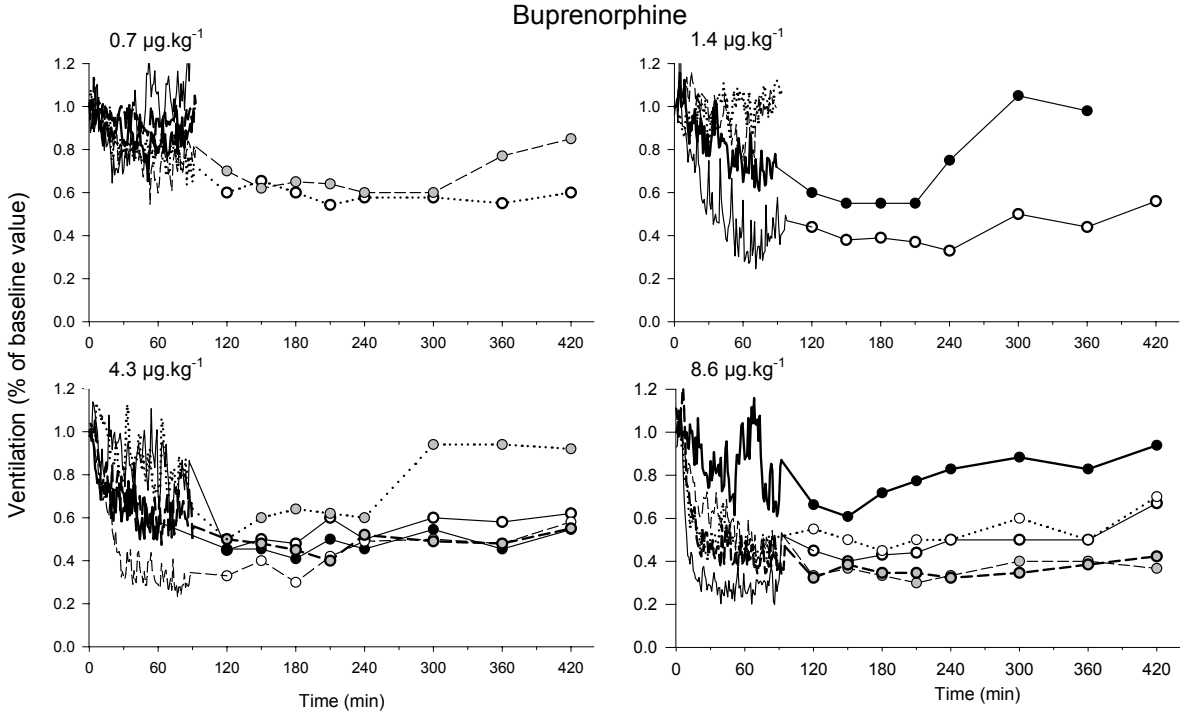

Figure 3. Individual human ventilatory responses after 0.7, 1.4, 4.3 and 8.6 µg.kg^{-1} buprenorphine infusions. Ventilation is normalized relative to baseline values. Different symbols and lines depict different subjects.

Peak Drug Effect and Average Drug Effect. The dose–peak effect relationships are given in figure 4. For fentanyl there was a steep dose–response relationship eventually reaching 0 l.min^{-1} (apnea). The data fit yielded the following parameter values (median (SE)): E_{max} 20.1 (2.9) l.min^{-1}, ED_{50} 1.5 (0.5) μg.kg^{-1} and γ 2.0 (1.4); E_{min} did not differ significantly from 0 l.min^{-1}. For buprenorphine the dose–peak effect relationship showed an initial steep decrease in ventilation which levelled off at doses > 2 μg.kg^{-1}. The data fit yielded the following parameter values: E_{max} 20.0 (0.8) l.min^{-1}, ED_{50} 0.9 (0.1) μg.kg^{-1} and γ 3.0 (0.9); E_{min} differed significantly from 0 l.min^{-1} and was estimated at 9.1 (0.6) l.min^{-1}. The E_{min} value indicates that at a background $P_{ET}CO_2$ of 7 kPa, the lowest value of minute ventilation after buprenorphine is 9 l.min^{-1}.

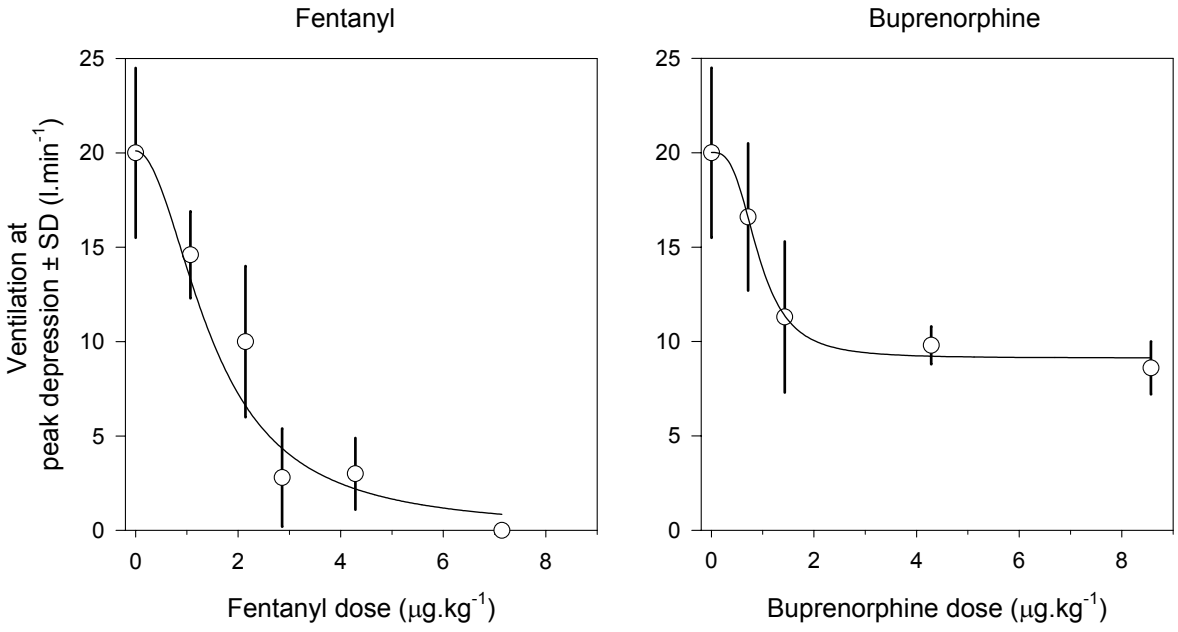

Figure 4. Dose–response relationships for fentanyl (left) and buprenorphine (right) in humans. The response is the peak ventilatory depression. The line through the data is the fit to the Hill equation. 0 μg.kg^{-1} is placebo.

The dose–average drug effect relationships are shown in figure 5. For fentanyl there was a steep dose–effect relationship, with increasing values at increasing fentanyl doses ($P < 0.001$). In contrast, for buprenorphine the average drug effect did not differ among doses.

Side Effects. The one subject dosed with 7.1 μg.kg^{-1} fentanyl developed apnea within 4 min of infusion with a drop in SpO_2 to 68%. She was instructed to take regular breaths and 100% inspired oxygen was given. This resulted in a quick return to SpO_2 values > 90%. The apneic episode lasted about 8 min after which breathing resumed with $P_{ET}CO_2$ values > 7 kPa. We

did not remove the ventilation data points at a higher $P_{ET}CO_2$ level than targeted in the study. One subject receiving 8.6 μg.kg^{-1} buprenorphine developed severe nausea about 60 min after the infusion of the drug. During this period of nausea (lasting about 30 min) he had a hyperventilatory response. Time to end of effect was set at 420 min. We decided to leave the data as they were as it did not affect the peak effect and time to peak effect data. It did cause underestimation of the average drug effect of this subject, however. All other side effects were mild ranging from mild nausea to sedation. Sedation developed in most subjects after having received an opioid but was marked after 8.6 μg.kg^{-1} buprenorphine. BIS values were > 90 during respiratory measurements in all subjects, irrespective of the opioid dose. Decreases in BIS did occur in between respiratory measurements and were always related to the occurrence of sleep.

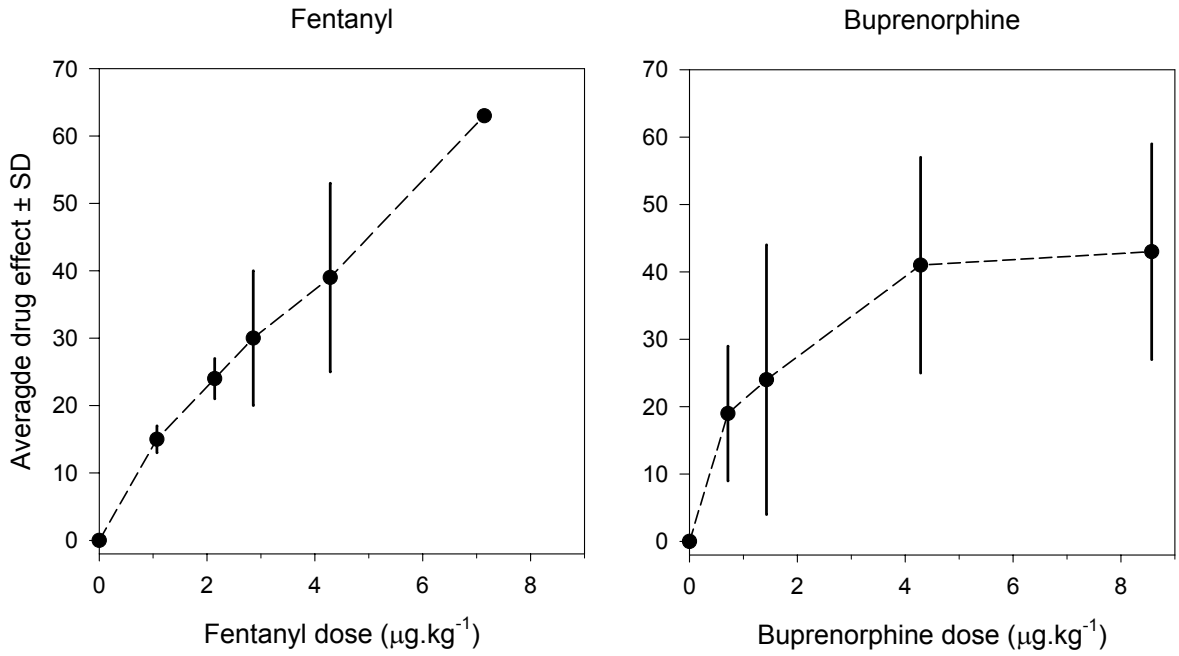

Figure 5. Average drug effect for fentanyl (left) and buprenorphine (right) in humans. Analysis of variance revealed a significant dose effect for fentanyl ($P < 0.001$) but not for buprenorphine. 0 μg.kg^{-1} is placebo.

Rat Studies

The effects of both opioids on $PaCO_2$ are shown in figures 6 and 7. As predicted the increase in $PaCO_2$ after the infusion of fentanyl was rapid with significant effects apparent just 5 min after the initiation of the fentanyl infusion.

After fentanyl, a significant dose–effect was observed at times t = 5, 10 and 15 min ($P = 0.01$) with an almost linear dose–response relationship (figure 6). At t = 20 min, a maximum in respiratory depression ($PaCO_2$ 7.5 kPa) was observed at all doses tested (fentanyl *versus*

vehicle, $P < 0.001$; no significant differences among the fentanyl doses). The changes in $PaCO_2$ dissipated rapidly after the termination of the fentanyl infusion.

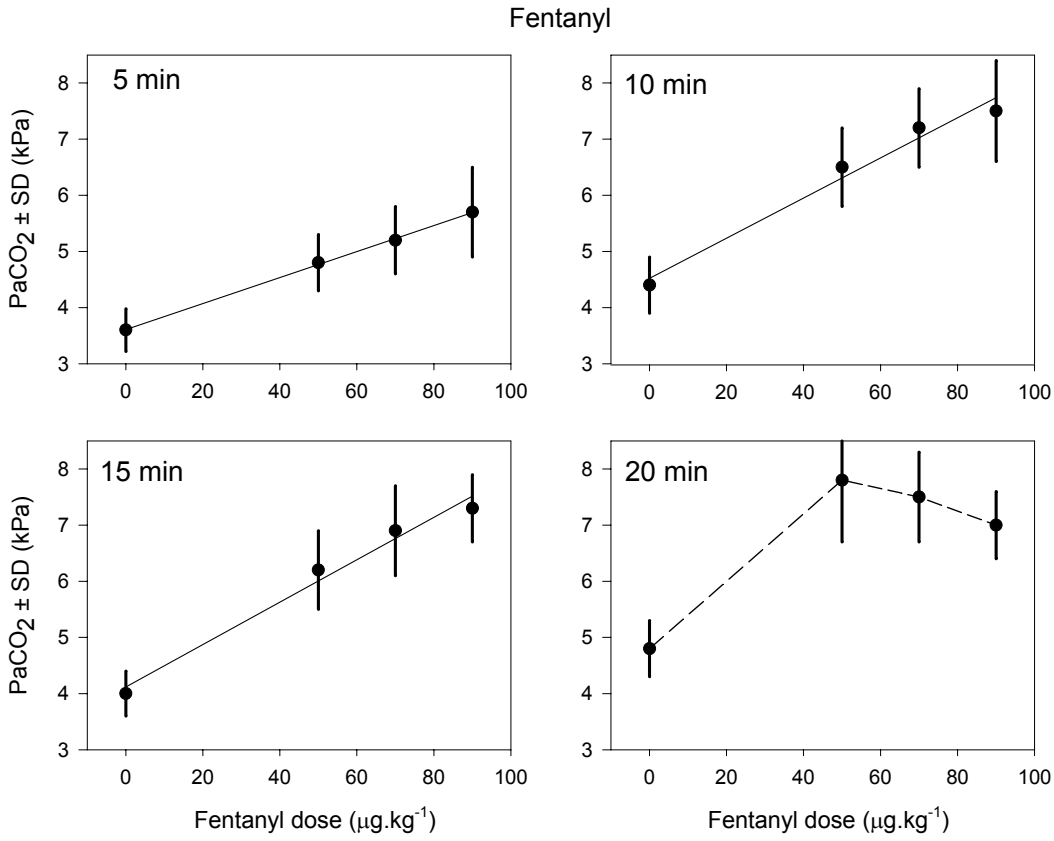

Figure 6. Fentanyl dose–response relationship obtained in rats at times t = 5, 10, 15 and 20 min after the initiation of the fentanyl infusions. A linear dose–response relationship was observed at times t = 5, 10 and 15 min ($P = 0.01$). After 20 min, maximum effect was reached at all fentanyl doses with no significant differences among the fentanyl doses. 0 $\mu g.kg^{-1}$ is vehicle. To guide the eye, linear regression curves are plotted through the data (continuous lines) at times t = 5, 10 and 15 min.

Irrespective of the time at which measurements were obtained and dose, buprenorphine showed a relatively small increase in $PaCO_2$ of 1–1.5 kPa (buprenorphine *versus* vehicle, $P < 0.05$; no significant differences among the buprenorphine doses). This indicates that a plateau in respiratory depression occurred at a dose of 0.1 $mg.kg^{-1}$ buprenorphine causing an increase in $PaCO_2$ of about 50% of the maximum increase in $PaCO_2$ observed after fentanyl.

Discussion

In humans and rats, we studied two potent opioids, buprenorphine and fentanyl, and observed distinct differences in their respiratory behavior. The data obtained in both species were in

good correspondence leading to similar conclusions. In contrast to fentanyl, buprenorphine showed a ceiling (or apparent maximum) effect in its ability to cause respiratory depression.

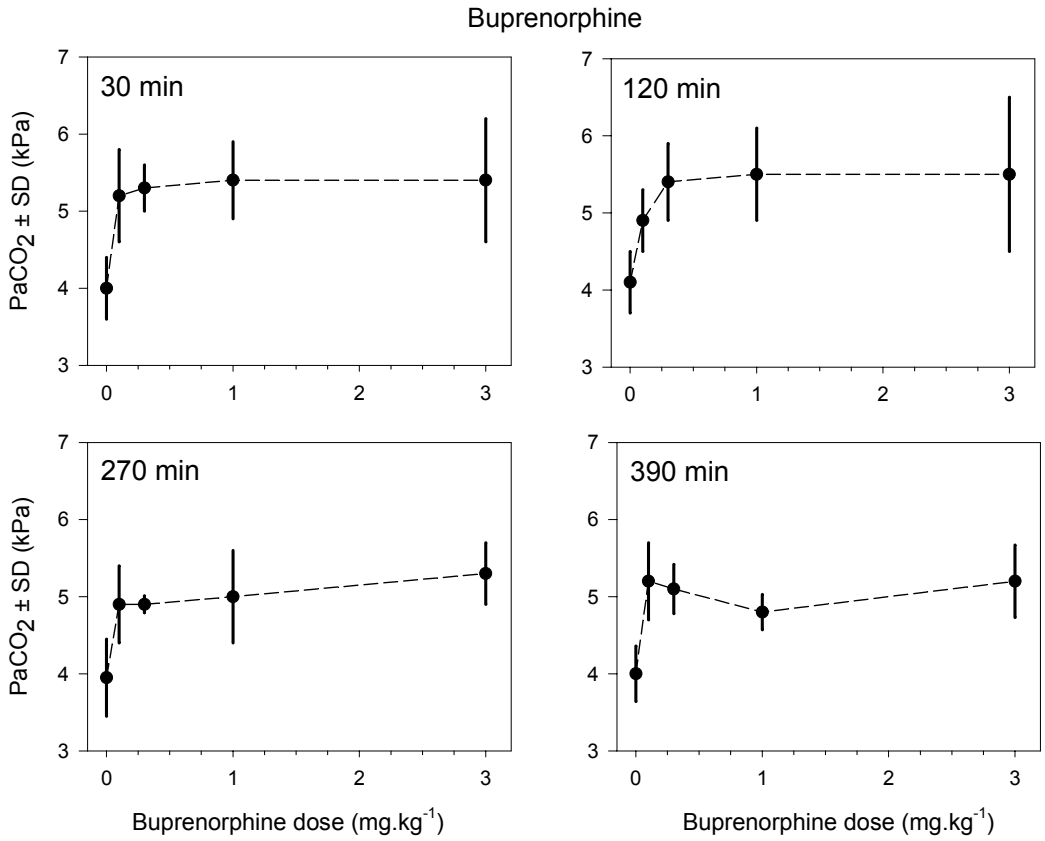

Figure 7. Buprenorphine dose–response relationship obtained in rats at times t = 30, 120, 270 and 390 min after the initation of the buprenorphine infusions. At all measured times a plateau in effect was observed at doses > 100 μg.kg^{-1} (100, 300, 1000 and 3000 μg.kg^{-1} buprenorphine *versus* vehicle, $P < 0.01$; no significant differences among the buprenorphine doses). 0 μg.kg^{-1} is vehicle.

The $P_{ET}CO_2$ was controlled within 0.1 kPa (mean SD of $P_{ET}CO_2$ fluctuations). In some cases deviations from target $P_{ET}CO_2$ greater than 0.4 kPa did occur related to the short periods of relative hyperventilation following apnea (see figure 2). While these deviations from target $P_{ET}CO_2$ may have influenced the time profile of individual curves and underestimated the average drug effect, we do not believe that the final conclusions of the study were influenced significantly. In those instances that apnea did occur we coached the subjects to take deep breaths. Coaching may have activated behavioral control of breathing and consequently may have influenced the study results (average drug effect).[13] However, the influence of 3–8 min of coaching during apnea on a 7-h experiment was minimal.

We were able to successfully coach the subjects through the episode of apnea after 2.9 and 4.3 $\mu g.kg^{-1}$ fentanyl. However we felt that the prolonged period of apnea with low SpO_2 observed in the first subject dosed with 7.1 $\mu g.kg^{-1}$ fentanyl was unacceptable and hence decided to restrict our study to a maximum fentanyl dose of 4.3 $\mu g.kg^{-1}$. Similarly, in rats, we had observed in a pilot study that short-term infusions of fentanyl but not buprenorphine caused the death of several of our animals. To overcome this problem we infused both tested drugs over 20 min in the rats. The different method of drug administration between humans and rats may have resulted in differences in plasma drug time profiles.

In the human study we focused our data analysis on two measures of respiratory outcome: peak effect and the average drug effect. Average drug effect divided by the duration of effect is considered a weighted average of a response,[12] and allows comparisons among drug doses when no pharmacokinetic data are available. Although both measures (peak effect and average drug effect) are related to the pharmacokinetics and pharmacodynamics of the infused drug, they represent two distinct features of the drug that complement each other. Peak effect is related to the rise of the opioid concentration in the brain compartment, subsequent attachment to the opioid receptor and neuronal dynamics. The average drug effect is related to the accumulation of the drug within the brain compartment, receptor kinetics (association and more important dissociation) and neuronal dynamics and gives an indication of the opioid's respiratory efficacy. Both indices showed great variability (see SD's in figures 4 and 5). The early effects were especially variable, which may be due to variability in the central volume of distribution, transit and uptake of the drug in the lungs (fentanyl)[14] and passage across the blood brain barrier.

In humans, the relationships between buprenorphine dose and peak effect and average drug effect were non-linear. These findings contrast with the observations that the relationships of peak effect and average drug effect and dose of fentanyl were linear over the dose range from 0 to 4.3 $\mu g.kg^{-1}$. We remain uninformed on the effect of fentanyl at doses > 4.3 $\mu g.kg^{-1}$ with only data from one subject at 7.1 $\mu g.kg^{-1}$. However, when we take into account the data from this one subject together with the animal data it is evident that also at the higher fentanyl doses, the human dose–response curve has linear characteristics. Only few studies systematically addressed the issue of buprenorphine-induced respiratory depression in humans. Comparison of our data with these studies is difficult. In our studies we used isocapnia (constant end-tidal PCO_2) and measured ventilation. Other studies used either no control for carbon dioxide, a constant inspired CO_2 concentration or less informative measures such as respiratory rate. In two studies, Walsh *et al.* assessed the effect of 0.5–32 mg sublingual buprenorphine on respiratory rate and oxygen saturation.[15,16] They observed a non-linear dose–response curve with a plateau arising between 0.4 and 0.8 mg. Despite the fact that neither respiratory rate nor oxygen saturation are valid markers of respiration, the results of the studies of Walsh *et al.* do give a qualitative suggestion of buprenorphine's behavior in humans. Our data, obtained at isocapnia, give a quantitative

indication of the occurrence of ceiling in buprenorphine-induced respiratory depression at intravenous doses of ≥ 2 μg.kg^{-1}.

Despite the overt differences in methodology between the human and animal studies, the results of the studies were similar. In the animal studies we used arterial PCO_2 as a surrogate measure of minute ventilation. The measured $PaCO_2$ in our studies is not only determined by the respiratory depression *per se*, but also by its complex interaction with ventilation. The increase in $PaCO_2$ has a drug-dependent stimulatory effect on breathing causing the (drug-dependent) elimination of CO_2 from the lung. We therefore may have underestimated any respiratory depression observed in the animals. An example of the underestimation of respiratory depression in human studies performed under poikilocapnic conditions (*i.e.,* end-tidal PCO_2 was not kept constant) is the observation by Mildh *et al.* of a very high EC_{50} value (drug concentration causing 50% effect) for fentanyl-induced respiratory depression (6.1 ng.ml^{-1}).[10] In that study ventilation was measured and fentanyl was infused slowly allowing the accumulation of CO_2 which prevented the occurrence of severe respiratory depression and apnea. Bouillon *et al.* addressed this issue by using indirect–response models to calculate the EC_{50} and taking into account both drug and CO_2 effects.[17] Although calculation of the EC_{50} is not directly possible from our study, using pharmacokinetic data from the literature, we were able to estimate a value of 1.5 ng.ml^{-1} which is a factor of 4 smaller than the value of Mildh *et al.* Taken into account all of the above, we do not believe that our animal data lack importance. Like the studies of Walsh *et al.*, these data give qualitative proof of the behavior of both tested opioids.

In humans, the occurrence of apnea shortly after the 90-s infusion of high dose fentanyl (≥ 200 μg) is related to the rapid increases in blood fentanyl concentration, its rapid passage across the blood–brain barrier (the fentanyl blood–effect site equilibration half-life is about 5 min),[18] with consequently high brain concentrations and almost immediate attachment to the μ-opioid receptor. This caused rapid depression of respiratory neurons expressing the μ-opioid receptor (peak respiratory effect after fentanyl occurred at 4.8 min). Buprenorphine, like fentanyl, is highly lipophilic with a relatively rapid passage across the blood–brain barrier. However, in contrast to fentanyl, buprenorphine displays slow opioid receptor association and dissociation kinetics.[19] This may have prevented rapid changes in ventilation in our population despite relatively high brain concentrations (peak respiratory effect after buprenorphine occurred at 117 min).

It is generally believed that both fentanyl and buprenorphine produce their intended effect (analgesia) *via* an action at the μ-opioid receptor gene (*OPRM1*) product. Using exon 2 *Oprm* knockout mice, we observed that the μ-opioid receptor is the source of morphine-induced antinociception *and* respiratory depression.[20,21] Involvement of other opioid receptors (κ-, δ- or ORL_1 receptors) in morphine-induced respiratory depression seems unlikely. We believe this also holds true for other opioids such as fentanyl. Hence, we postulate that the effect of fentanyl at the μ-opioid receptor is exclusively responsible for the (almost-linear) dose–

response relationship found in our volunteers and animals (figures 4–6). In rats, the finding of a maximum effect of fentanyl 20 min after the start of the infusion shows the maximum effect on respiration that is possible in animals (fentanyl doses ≥ 90 μg.kg^{-1} are fatal). Our human (average drug effect, figure 5) and animal data indicate that fentanyl is a full agonist at the μ-opioid receptor with high intrinsic activity. The non-linear buprenorphine dose–response relationship we observed is in agreement with earlier human and animal studies on its respiratory effect.[7-9] Especially rat data systematically show ceiling in buprenorphine-induced respiratory depression at doses above 0.1 mg.kg^{-1}.[7,8] In rhesus monkeys a similar observation was made for doses greater than 1.0 mg.kg^{-1}.[9] This latter study is of interest since it measured steady-state minute ventilation at a fixed inspired CO_2 concentration of 5%. Partial agonism of buprenorphine at the μ-opioid receptor is generally held responsible for the ceiling phenomenon.[4,7] Partial agonism indicates a partial (respiratory) effect despite full μ-opioid receptor occupancy. Recently, Lutfy *et al.* proposed a different mechanism for the non-linear dose-response.[5] They showed that buprenorphine (but not morphine) given to mice activates ORL_1 receptors compromising antinociception mediated *via* μ-opioid receptors. Extrapolation of these animal data on antinociception to our respiratory studies would suggest that buprenorphine's action at the ORL_1 receptor would cause the reduction of respiratory depression from buprenorphine's action at the μ-opioid receptor. In this respect buprenorphine would act as a respiratory stimulant at the ORL_1 receptor. Just one study addressed the influence of the ORL_1 receptor on respiration.[22] In an *in vitro* preparation of the newborn rat brainstem activation of the ORL_1 receptor produced depression of respiratory rhythm generation. This observation does not support the hypothesis of involvement of the ORL_1 receptor in the development of ceiling in buprenorphine's respiratory effect. The current data are very scanty and further studies are required to elucidate the involvement of the ORL_1 receptor in opioid induced respiratory depression.

The observation of ceiling in buprenorphine-induced respiratory depression has contributed to the notion that buprenorphine's respiratory effects are limited.[1] (Significant or fatal respiratory depression has only been reported when buprenorphine was combined with sedative drugs such as benzodiazepines.[23]) However, buprenorphine's safety profile should be considered against the background of its analgesic profile. For example, if a ceiling in respiratory depression coincided with ceiling in analgesia, then the value of buprenorphine would be limited in clinical practice. While there is evidence from animal data of the occurrence of a ceiling or even a bell-shaped response curve in the analgesic effect of buprenorphine (at doses > 1.0 mg.kg^{-1}),[5,7,24] there are no good (placebo-controlled, randomized) human studies available. Our data and those of others obtained in rodents indicate that ceiling in respiratory effect occurs at a much lower dose (0.1 mg.kg^{-1}) than ceiling in analgesic effect (1.0 mg.kg^{-1}) which indicates the relative safety of buprenorphine combined with its ability to produce effective analgesia in these animals.[5,7-9,24] Before we can extrapolate these claims to humans we need good clinical studies to determine whether there is a ceiling for analgesia and assess the dose at which it occurs. Our study can not address the issue of buprenorphine's efficacy and safety in light of its analgesic properties.

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3 Buprenorphine Induces Ceiling in Respiratory Depression but Not in Analgesia

Buprenorphine is a semi-synthetic opioid in clinical use for treatment of acute and chronic pain since 1979.[1] Buprenorphine is a potent analgesic with agonistic activity at the μ-opioid receptor and antagonistic properties at the κ-opioid receptor.[2] Human studies show that buprenorphine behavior is typical of μ-opioid receptor agonists, with respect to its intended effect (potent and long-lasting analgesia) and side effects (for example sedation, nausea, delayed gastric emptying and respiratory depression).[2,3] In a previous study, in a group of healthy volunteers buprenorphine-induced respiratory depression demonstrated ceiling (or an apparent maximum effect) at doses > 0.1 mg (per 70 kg).[4] Ventilation at a fixed end-tidal PCO_2 reached maximum peak depression of about 50% of baseline. Buprenorphine's behavior contrasts that of fentanyl, which showed irregular breathing and apnea at high dose (> 200 μg per 70 kg). These findings suggest a greater margin of safety for buprenorphine relative to other potent opioids frequently used to treat severe acute and chronic pain. However, buprenorphine's safety profile must be considered against the background of its analgesic profile. For example, would ceiling in respiratory depression coincide with ceiling in analgesia then the value of buprenorphine would be limited in clinical practice. Recently we observed in rats the occurrence of ceiling in buprenorphine's respiratory effect while no ceiling was observed in buprenorphine's antinociceptive behavior.[4,5] There are no good experimental human studies available on buprenorphine's analgesic behavior at doses causing ceiling in respiratory effect. To address this important issue we assessed the effect of two doses of intravenous buprenorphine (0.2 and 0.4 mg/70 kg) on respiration and analgesia in a group of young and healthy volunteers. Previous studies indicated that the respiratory effect of 0.2 and 0.4 mg buprenorphine have similar and limited respiratory effects.[4]

Methods

Twenty volunteers (10 men, 10 women; aged 22–35 years, weight 62–92 kg) participated in the protocol after approval was obtained from the local Human Ethics Committee. All subjects were healthy and did not have a history of illicit substance abuse or smoking. They were asked to refrain from stimulants and depressant substances for at least 12 h before the study. Each subject participated once. After arrival in the laboratory the subjects were familiarized with the pain test and breathing apparatus for about 60 min.

Half of the subject group ($n = 10$, 5 men, 5 women) received 0.2 mg (per 70 kg) buprenorphine i.v., the other half 0.4 mg (per 70 kg) buprenorphine i.v. Buprenorphine (Reckitt Benckiser Healthcare Ltd, Hull, UK) was infused slowly over 90 s.

Respiration

To study ventilation we used the dynamic end-tidal forcing technique.[6] This technique enables us to force end-tidal PCO_2 ($P_{ET}CO_2$) and end-tidal PO_2 ($P_{ET}O_2$) to follow a specific pattern in time. In this study we clamped the $P_{ET}CO_2$ and $P_{ET}O_2$ to 7 kPa and 14.5 kPa, respectively, throughout the studies. The subjects were comfortably positioned in a hospital bed and breathed through a face mask, positioned over nose and mouth (a nose clip was not used). The face mask received fresh gas (45 $l.min^{-1}$) from a gas mixing system consisting of three mass flow controllers (Bronkhorst High-Tech BV, Veenendaal, The Netherlands) for the delivery of oxygen, carbon dioxide and nitrogen. A personal computer provided control signals to the mass flow controllers allowing the adjustment of the inspired gas concentrations to obtain the desired end-tidal concentrations. The in- and expired gas flows were measured at the mouth using a pneumotachograph connected to a pressure transducer (Hans Rudolph, Wyandotte, MI, USA) and electronically integrated to yield a volume signal. The volume signal was calibrated with a motor-driven piston pump. The oxygen and carbon dioxide concentrations were measured using a gas monitor (Multicap, Datex, Helsinki, Finland); a pulse oximeter (Masimo, Irvine, CA, USA) continuously measured the oxygen saturation (SpO_2) of arterial hemoglobin with a finger probe. All relevant variables (minute ventilation, SpO_2, $P_{ET}CO_2$ and $P_{ET}O_2$) were available for on-line analysis and stored on a breath-to-breath basis for further analysis.

At several times respiration was measured: t = -30 min (30 min before the drug was infused) and at times t = 15, 75, 140, 180, 240, 300, 360, 420 and 480 min after the infusion of buprenorphine. The respiratory studies started after ventilation (at a fixed $P_{ET}CO_2$ of 7 kPa) had reached a steady state. Next the mean value of 10 consecutive breaths was calculated and used in the data analysis. Generally no more than 7 minutes were needed before a measurement at steady state was obtained. In between respiratory measurements the subjects were taken off the mask and analgesia testing was performed.

Analgesia

Acute pain was induced by an electrical current through two surface electrodes (Red Dot, 3M, London, Ontario, Canada) placed on the skin overlaying the tibial bone (shin bone) of the left leg.[7] The electrodes were attached to a computer interfaced current stimulator (CICS, Leiden University Medical Center, Leiden, The Netherlands). The intensity of the noxious stimulation was increased from 0 mA in steps of 0.5 mA per 1 s. The stimulus train consisted of a square-wave pulse of 0.2 ms duration applied at 10 Hz and had a cutoff at 128 mA. The subjects were instructed to press a button on a control panel when no further increase in stimulus intensity was acceptable (pain tolerance). Upon pressing the pain tolerance button, the stimulus train ended. This procedure was performed three times prior to drug infusion (the

mean of which was used as baseline value) and at fixed times after drug infusion (at times t = 5, 10, 45, 80, 110, 130, 165, 210, 270, 330, 390, 450 and 490 min after the drug infusion). The current at which pain tolerance occurred was automatically collected and stored on the hard disc of a computer for further analysis. The involvement of the observers in the pain assessments was restricted to training the subjects and to the initiation of the stimulus train during the studies.

Data Analysis

Data analysis was performed on the absolute respiration and pain tolerance values and on the values relative to baseline (*i.e.,* Δventilation and Δpain tolerance). Data are reported as mean (SD). Statistical analysis was performed using SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA, USA). A two-way repeated measures analysis of variance was applied to detect a significant difference of buprenorphine dose on respiration or analgesia and to detect whether sex differences were present. *Post-hoc* analysis was by *t*-test. *P*-values < 0.05 were considered significant.

Results

All subjects completed the study without major side effects. Most prominent side effects were nausea and vomiting that occurred in 80% and 40% of subjects, respectively. Nausea/vomiting remained untreated throughout the study period.

Respiration

The two buprenorphine doses had a similar effect on ventilation. Baseline ventilation at a $P_{ET}CO_2$ of 7 kPa did not differ between the two dose groups: 24.2 (2.3) $l.min^{-1}$ in the 0.2 mg *versus* 23.5 (1.9) $l.min^{-1}$ in the 0.4 mg buprenorphine group. After infusion of the drug ventilation showed a rapid decline and reached peak depression between t = 150 and 180 min (see figure 1). This effect was dose-independent with respect to timing and magnitude: at peak respiratory depression ventilation was 13.1 (1.8) $l.min^{-1}$ (0.2 mg) *versus* 12.0 (1.3) $l.min^{-1}$ (0.4 mg, n.s.). The overall effect of buprenorphine on ventilation was dose-independent over the 8 hours of the study: 0.2 *versus* 0.4 mg, $P > 0.05$. No sex difference was observed (factors sex and the interaction term between sex and dose, $P > 0.05$).

Analgesia

A significant increase in analgesia was observed going from 0.2 to 0.4 mg buprenorphine. Baseline pain tolerance currents did not differ between the two groups: 16.3 (3.9) mA (0.2 mg) *versus* 15.0 (2.6) mA (0.4 mg) (n.s.). At 0.2 mg buprenorphine a small short-lived analgesic effect was observed with a maximum increase in pain tolerance current of 6.7 (2.8) mA occurring at t = 75 min (figure 2). Peak analgesic effect was 41% above baseline current. In contrast, 0.4 mg buprenorphine caused a large and long-lived analgesic effect with a maximum increase in pain tolerance current of 23.8 (7.4) mA occurring at t = 130 min.

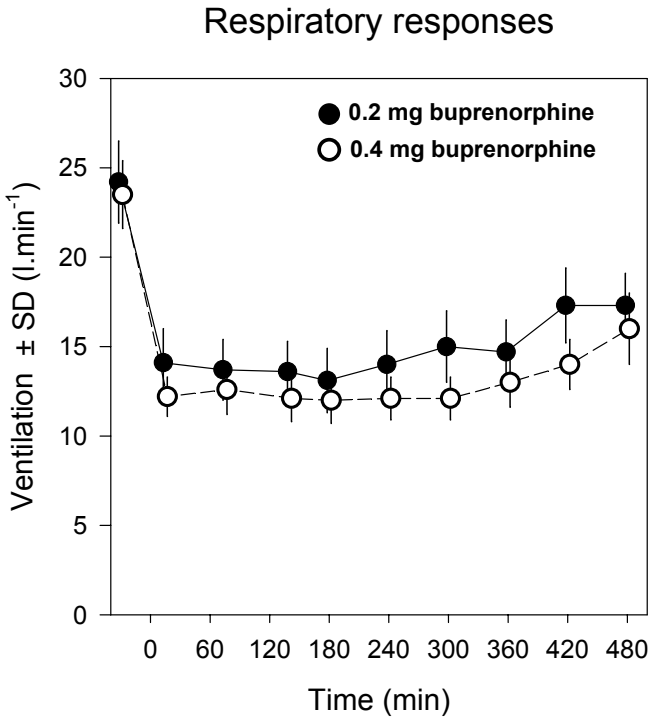

Figure 1. Influence of intravenous buprenorphine, 0.2 and 0.4 mg (per 70 kg), on inspired minute ventilation at a fixed $P_{ET}CO_2$ of 7 kPa in healthy volunteers (each dose: n = 8). The influence of the two buprenorphine doses is similar with respect to peak respiratory depression and duration of effect.

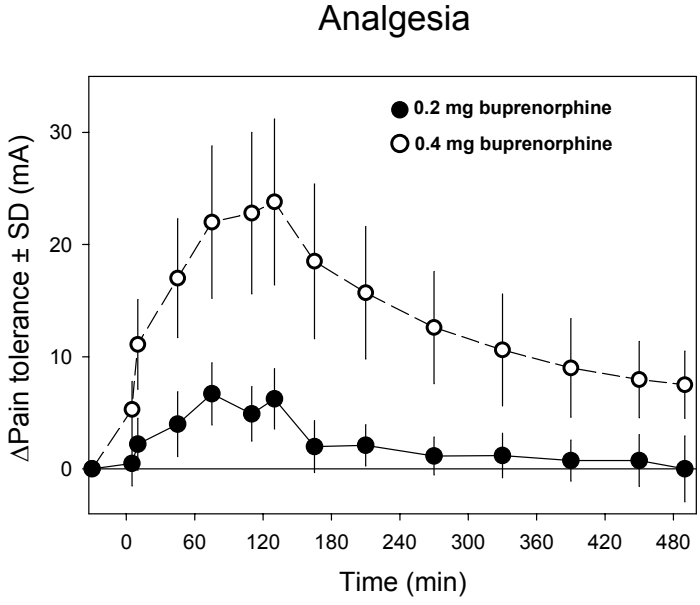

Figure 2. Influence of intravenous buprenorphine, 0.2 and 0.4 mg (per 70 kg), on pain tolerance in healthy volunteers (each dose: n = 8). Values are the increase in currents to achieve pain tolerance relative to baseline pain tolerance currents. A significant increase in analgesia is observed going from 0.2 to 0.4 mg buprenorphine.

Peak analgesic effect was 159% above baseline current ($P < 0.01$ *versus* 0.2 mg). The overall effect of buprenorphine on analgesia was dose-dependent over the 8 hours of the study (0.2 *versus* 0.4 mg, $P < 0.01$). No sex difference was observed (factors sex, sex × dose, $P > 0.05$).

Discussion

In the current study we examined the effect of 0.2 and 0.4 mg intravenous buprenorphine (dosed per 70 kg) on ventilation and on analgesia in healthy volunteers. We observed that doubling the dose of buprenorphine increased its peak analgesic effect by a factor of 3.5 (from 6.7 mA to 23.8 mA). In contrast, the timing and magnitude of respiratory depression remained unchanged by doubling the buprenorphine dose (compare figures 1 and 2). These data suggest that buprenorphine displays a plateau for respiratory depression over a dose range where no plateau in analgesic effect is observed. However, before definite conclusions can be drawn our data needs to be viewed in context, that is against the background of a more extensive dose–response relationship. This is important taken into account the observation in some animal studies of a bell-shaped (inverse U-shaped) dose-response relationship for buprenorphine's analgesic effects.[8,9] In a previous study we assessed the effect of 0.05, 0.1, 0.3 and 0.6 mg buprenorphine on breathing in a similar study population.[4] We observed a ceiling effect in peak respiratory depression of the drug at doses > 0.1 mg. Although the design of the previous and current studies differs with respect to the duration of measurements (in contrast to the current study, we previously measured breathing continuously for 90 min), we were able to combine these two data sets on peak respiratory depression (figure 3). The continuous line in figure 3 is the data fit using a sigmoid E_{max} model to all data presented (including the data from the current study). The broken line is the data fit using a decaying exponential model to the complete data set. Both models give similar results, that is, that the 0.3 and 0.6 mg buprenorphine data is on the flat part of the dose–response relationship, about 50% of baseline ventilation. To the best of our knowledge, an extensive dose–response relationship of the analgesic properties of buprenorphine in humans is not available in the literature. We recently assessed the analgesic effect of 0.05, 0.1 and 0.3 mg i.v. buprenorphine over time in 15 healthy young volunteers using our electrical pain model (Dahan, unpublished observation). In figure 4 we plotted the mean increase in current to achieve pain tolerance (relative to baseline pain tolerance current) *versus* dose and added the data from the current study. A dose-dependent increase in analgesic effect is observed without any sign of ceiling. We believe that this is sufficient proof to state that buprenorphine displays ceiling in respiratory depression over a dose range (0.05–0.4 mg) without causing any ceiling in analgesic effect. Evidently, this is true for the applied acute pain model and the subset of subjects that we used (young and healthy volunteers using no co-medication). Finally, we cannot exclude that ceiling in analgesic effect occurs at greater doses than tested by us.

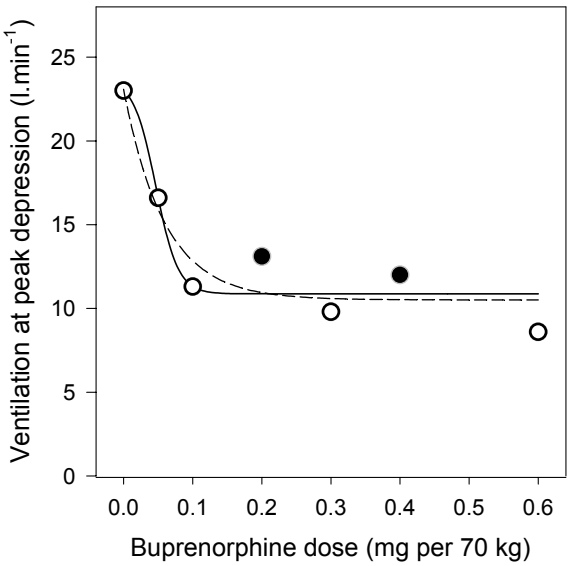

Figure 3. Human buprenorphine dose–respiratory response (peak respiratory depression) relationship. Values are mean. Open symbols: data from ref. 4; closed symbols: data from the current study. Both a sigmoid E_{max} model (continuous line) and a decaying exponential model (broken line) were fitted to the data. Both model fits indicate that the 0.3 and 0.6 mg buprenorphine data are on the flat part of the dose–response relationship.

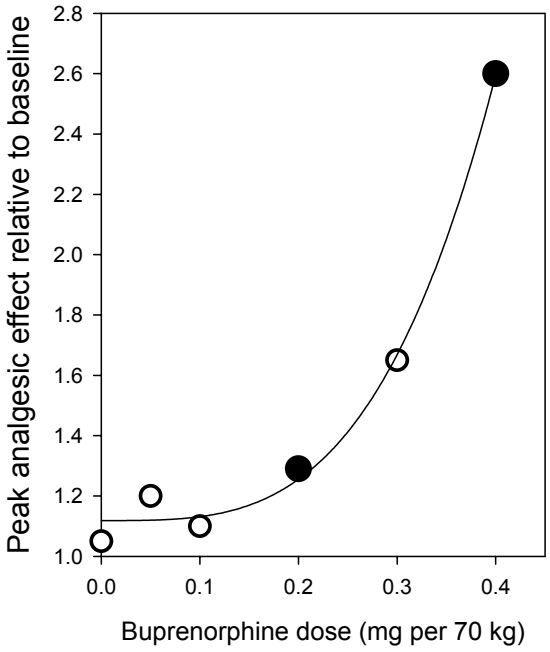

Figure 4. Human buprenorphine dose–analgesic response (peak analgesic effect) relationship. Data are mean. Values are relative to baseline: a value of 1.5 indicates a 50% increase in current to achieve pain tolerance. 0 mg per 70 kg is placebo (0.9% NaCl). Open symbols: data from unpublished observations; closed symbols: data from the current study. To guide the eye a power model was fitted to the data.[24]

Differential Effect of Buprenorphine on Analgesia and Respiration

Our data suggest that buprenorphine is a full agonist at μ-opioid receptors (MOR's) involved in pain processing but a partial agonist at MOR's involved in respiratory depression. Partial agonism indicates a partial effect despite full MOR occupancy. These findings are in agreement with rat data from our laboratories and with some clinical studies which show the absence of ceiling effect for analgesia (tested at much greater buprenorphine doses than tested by us) and the ability to produce 100% pain relief despite the observation of ceiling for properties other than analgesia (such as sedation and the decrease in respiratory frequency).[2,4] Buprenorphine behaves very differently from other opioids (full agonists with respect to respiratory effect and analgesia) such as morphine and fentanyl. For example, we previously measured the respiratory depressant effect of morphine simultaneously with morphine's antinociceptive effects in humans.[7] We observed that over the concentration range that caused a systematic increase in analgesia, morphine caused concentration-dependent respiratory depression without any plateau or ceiling.

It is possible that differences in receptor density may be the cause of the differential buprenorphine effect at the two typical μ-opioid end points studied by us. For example, Garrido *et al*. showed in rats that progressive MOR knockdown (*i.e.*, the reduction in MOR binding sites) with the irreversible MOR antagonist β-funaltrexamine, caused a marked decrease in alfentanil efficacy.[10] Alfentanil transformed from a full MOR agonist into a partial agonist at reduced MOR availability. It may then be argued that MOR density is greater at pathways in the central nervous system concerned with processing pain than at the respiratory centers in the brainstem.

Another explanation for buprenorphine's behavior may be found in a difference in the agonist/MOR/G-protein/β-arrestin complex in pain and respiratory neurons. Opioid receptors belong to the superfamily of seven-transmembrane G-protein-coupled receptors which bind to G-proteins and the regulatory protein β-arrestin upon activation.[11] Raehal *et al*.[12] showed that genetic disruption of the β-arrestin type 2 (βarr2) gene (βarr2 knockout mice) attenuated the respiratory depression (and acute constipation) caused by morphine. In contrast, morphine-induced antinociception was augmented in the βarr2 knockout mice.[13] The authors hypothesized that β-arrestin may play an important G-protein independent role in signal transduction *via* MOR's that lead to respiratory depression (and gastrointestinal transit inhibition) but not *via* MOR's that lead to analgesia. G-protein independent but β-arrestin dependent activation has been observed for other receptors of the seven-transmembrane receptor superfamily, such as the β_2-adrenergic receptor.[14] Following the reasoning of Raehal *et al.*[12] and taking into account our data, this suggests that MOR activation of a G-protein independent signal transduction pathway is ligand specific: some ligands (such as fentanyl and morphine) cause changes in neuronal physiology fully dependent on G-protein activation causing full MOR responses, while others (such as buprenorphine) activate the β-arrestin protein with diminished responses. We hypothesize that signal transduction due to buprenorphine-activation of MOR's expressed on respiratory neurons is *via* β-arrestin

mediation and not *via* G-protein activation. Interestingly, morphine's active metabolite morphine-6-glucuronide (M6G) displays significantly less respiratory depression than morphine (*i.e.*, a rightward shift of the dose–response relationship).[15] A shared difference in the structure of M6G and buprenorphine with morphine is modification of the hydroxyl group at position C6 of the morphine molecule (morphine: C6–OH, buprenorphine: C6–O–CH_3 and M6G: C6–glucuronide). Possibly this modification at C6 may be the cause for the reduced effect at MOR's expressed on respiratory neurons (*cf.* reference 16). Further studies are needed to clarify this important issue.

In contrast to our previous study,[4] we now can address the issue of buprenorphine's respiratory safety in light of its analgesic properties. Opioid-induced respiratory depression is related to overdosing, concurrent sedation/sleep, co-medication, the periodic nature of pain and underlying disease. The frequency of serious respiratory events related to opioid use remains poorly reported and probably poorly studied. In chronic cancer and non-cancer pain patients, respiratory complications are often erroneously taken for progression of disease and sometimes accepted –and hence unreported– in the light of the poor prognosis of the patient. However, a series of recent case-reports on fentanyl-induced severe respiratory depression and death in old and relatively healthy young patients has lead to several warnings related to the use of fentanyl patches for treatment of chronic pain.[17-20] The question is whether buprenorphine can make a difference, or –in other words– whether the use of buprenorphine in pain patients will cause less respiratory events than commonly used potent opioids such as morphine and fentanyl. Our data support the notion that since buprenorphine's respiratory effects are limited, buprenorphine has an advantage over other opioids such as fentanyl and morphine which do not show ceiling at high dose but eventually cause breathing instability and apnea. However, whether this advantage persists under specific conditions such as old age, (lung) disease and use of co-medication needs further study. In opioid-addicts acute co-administration of buprenorphine and benzodiazepines is sometimes associated with fatal respiratory depression.[21]

Critique of Methods

We used our pain model as a pharmacological tool and did not intend to simulate clinical (acute or chronic) pain. We previously used this acute pain model (electrical transcutaneous stimulation of the skin) successfully to study the antinociceptive effects of morphine and M6G.[7,22] The results of these studies were comparable with clinical observations on morphine and M6G pain relief in acute and chronic pain patients with respect to analgesia and drug potency. Since the healthy volunteers tested in our studies were without pain or inflammation, the term antinociception seems a more appropriate description of the opioid behavior in the acute pain test. The choice of the term analgesia throughout this paper was taken –somewhat arbitrarily– to make a distinction from animal studies. In the current study we combined respiratory and analgesia measurements.

It may be argued that pain measurement may interfere with respiratory measurements and *vice versa*. This is true, pain testing may have a significant effect on breathing, often causing hyper- and/or hypoventilatory responses due to activation of behavioral respiratory drives;[23] and hypercapnia has been shown to influence pain testing.[24] We tried to minimize the complex interactive effects of pain testing and respiratory measurements by allowing ample time between measurements, but we can not exclude some mutual disturbing effects on both systems.

We were unable to detect significant sex differences in buprenorphine's respiratory and analgesic responses, although there was a clear trend in the data ($P = 0.09$) with greater responses in women. Our current study was not designed to examine sex differences, such as observed earlier for morphine.[25,26] *Post-hoc* power analysis indicated that 20 subjects (10 men/10 women) were needed to reveal a sex difference in analgesia after 0.4 mg buprenorphine at the $P = 0.05$ level.

In conclusion, we tested two incremental intravenous doses of buprenorphine (0.2 and 0.4 mg) on pain relief and respiratory depression in a group of healthy young volunteers. We observed that while buprenorphine's analgesic effect increased significantly, respiratory depression showed a similar magnitude and timing for the two doses tested. Taking into account additional data from our laboratory we conclude that buprenorphine displays ceiling in respiratory effect but not in analgesic effect over a dose range from 0.05 to 0.4 mg.

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4 Naloxone-Reversal of Opioid-Induced Respiratory Depression with Special Emphasis on Buprenorphine

Long-acting opioids are important tools in the treatment of postoperative acute pain and chronic cancer and non-cancer pain. When selecting one of the available compounds, not only the analgesic properties but also the safety profile of the drug needs to be considered. In general, opioids are well tolerated. Among the opioid-typical side effects, however, respiratory depression is of special importance because of the risk of fatal outcome for the patient.

Buprenorphine is a potent analgesic (100 times more potent than morphine) with μ-agonistic, ORL_1-agonistic and κ-antagonistic opioid properties.[1] In patients, buprenorphine is used for treatment of acute and chronic pain *via* various administration modes, such as intravenous (i.v.), transdermal, sublingual, epidural or spinal administration. In humans, buprenorphine behaves as a typical μ-opioid receptor agonist showing analgesia, euphoria, sedation, respiratory depression and pupillary constriction.[1,2] Buprenorphine has high affinity for opioid receptors and slow receptor association and dissociation kinetics as compared to other opioids.[3] After an intravenous infusion of 0.2–0.4 mg/70 kg the duration of action of buprenorphine is about 6 to 8 hours. Data obtained in opioid-naïve volunteers indicate that buprenorphine causes dose-dependent respiratory depression which levels off at greater buprenorphine doses (*i.e.,* plateau or ceiling in respiratory effect).[4]

Surprisingly few studies have addressed the ability to reverse the respiratory effects of opioids in general and buprenorphine in specific. Just two studies from the literature published in the 1980's as well as some anecdotal data suggest that the respiratory depression from buprenorphine is resistant to antagonism by naloxone.[5-7] While relatively low bolus doses of intravenous naloxone had no effect, high doses (2.5 to 10 mg) caused only partial reversal of buprenorphine's respiratory effects. These results may be explained by the short duration of action of a bolus dose of naloxone resulting from a rapid elimination combined with the high affinity of buprenorphine for μ-opioid receptors. Consequently, a bolus dose of naloxone may be unable to displace buprenorphine from the opioid receptors. The buprenorphine/naloxone data contrast data on the ability to reverse fentanyl-induced respiratory depression, which is considered relatively easy. Short naloxone infusions up to 0.4 mg cause full reversal of fentanyl-induced respiratory depression in patients during halothane/nitrous oxide anesthesia.[8]

We performed a series of experiments to study the influence of naloxone on buprenorphine-induced respiratory depression. Our aim was to obtain a naloxone-dosing regimen that would

cause full reversal of buprenorphine-induced respiratory depression. Initially (Study 1) we assessed the effect of 0.8 mg naloxone (or placebo) on 0.2 mg i.v. buprenorphine-induced respiratory depression in healthy volunteers. In a subsequent study (Study 2) we explored which naloxone dose causes full reversal of 0.2 mg i.v. buprenorphine-induced respiratory depression. In order to do so we tested various naloxone doses in the range from 0.5 to 7 mg in separate subjects. In another study (Study 3) we assessed the effect of a continuous naloxone (or placebo) infusion on 0.2 and 0.4 mg i.v. buprenorphine-induced respiratory depression. Finally, in order to compare the results of studies 1–3 we assessed the effect of just 400 μg naloxone (or placebo) on 0.15 $mg.kg^{-1}$ i.v. morphine-induced respiratory depression (Study 4) and 4 $\mu g.kg^{-1}$ naloxone on alfentanil-induced depression of the ventilatory response to hypoxia (Study 5). We hypothesized that while 300–400 μg naloxone was sufficient to fully reverse morphine- and alfentanil-induced respiratory depression, much greater doses and a continuous infusion are needed to reverse buprenorphine-induced respiratory depression.

Methods

A total of ninety-nine male and female subjects (age range 20–30 years, weight 54–93 kg) participated in the studies after approval of the protocols was obtained from the local Human Ethics Committee. We obtained oral and written consent. All subjects were healthy and did not have a history of illicit drug use or mental disease. All women were taking oral contraceptives. Subjects were asked to have a normal night of sleep and not to eat or drink for at least 6 h prior to the study. They were comfortably seated in a hospital bed for the duration of the study.

Respiration

In- and expired gas flows were measured with a pneumotachograph (Hans Rudolph, Wyandotte, MI, USA) connected to a pressure transducer and electronically integrated to yield a volume signal. The volume signal was calibrated with a motor-driven piston pump (stroke volume 1000 ml, at a frequency of $20.min^{-1}$). The pneumotachograph was connected to a T-piece. One arm of the T-piece received a gas mixture with a flow of 45 $l.min^{-1}$ from a gas mixing system, consisting of three mass flow controllers (Bronkhorst High-Tech BV, Veenendaal, The Netherlands) with which the flow of O_2, CO_2 and N_2 could be set individually at a desired level. A personal computer provided control signals to the mass flow controllers so that the composition of the inspired gas mixtures could be adjusted to force end-tidal oxygen and carbon dioxide concentrations ($P_{ET}O_2$ and $P_{ET}CO_2$) to follow a specified pattern in time, independent of the ventilatory response (*i.e.*, dynamic end-tidal forcing).[9] In the studies 1–4, $P_{ET}CO_2$ was clamped at 7.0 kPa throughout the measurements (about 1 kPa above resting values), while $P_{ET}O_2$ was maintained at a normoxic value of 14.5 kPa. In study 5, $P_{ET}CO_2$ was clamped at 7.0 kPa and $P_{ET}O_2$ lowered to 6.0 kPa for 3 min. The O_2 and CO_2 concentrations and the arterial Hb-O_2 saturation (SpO_2) were measured with a gas

monitor near the mouth (Multicap, Datex, Helsinki, Finland) and a pulse oximeter (Masimo, Irvine, CA, USA) using a finger probe, respectively. The gas monitor was calibrated with gas mixtures of known composition delivered by a gas-mixing pump (Wösthoff, Bochum, Germany). $P_{ET}CO_2$, $P_{ET}O_2$, inspired minute ventilation and SpO_2 were collected on a breath-to-breath basis and stored on disc for further analysis.

Study Design and Data Analysis

The studies were placebo-controlled and had a double blind design (except study 5, see below). The local pharmacy delivered the buprenorphine hydrochloride (Reckitt Benckiser Healthcare Ltd, Hull, UK), alfentanil hydrochloride (Janssen-Cilag BV, Tilburg, The Netherlands), morphine hydrochloride and naloxone hydrochloride (both manufactured by the local pharmacy), and placebo (NaCl 0.9%). Randomization and preparation of the syringes was performed by a physician not involved in the study using randomization lists obtained from www.randomization.com. All buprenorphine and naloxone doses are per 70 kg. All bolus infusions were given over 90 s. Each subject participated once in any of the studies. Values are reported as mean ± SEM, unless otherwise stated.

Study 1

Sixteen subjects participated in this study. All received 0.2 mg buprenorphine i.v. (at time t = 0) followed by 800 μg naloxone i.v. (n = 8) or placebo (n = 8) at t = 120 min. At the following times steady-state ventilation was measured (measurement period 7 min): -10 min (ten min prior to drug infusion), 15, 75, 140, 180, 240, 300, 360, 420 and 480 min after drug infusion. *t*-Tests were performed to detect a significant effect of naloxone on ventilation at the $P < 0.05$ level.

Study 2

Twenty-four subjects participated in this study. All received 0.1 mg buprenorphine i.v. at t = 2 min, followed by a continuous infusion for 1 h of 0.1 $mg.h^{-1}$ (total dose = 0.2 mg in 60 min). At t = 32 min, x mg naloxone i.v. was given followed by a continuous infusion for 30-min of $2{\cdot}x.h^{-1}$ (total dose = $2{\cdot}x$ mg in 30 min). The following values of $2{\cdot}x$ were tested: 0 (placebo), 0.5, 1, 2, 3, 4, 5, 6 and 7 mg. Each dose was tested in two subjects, except for placebo that was tested in eight subjects. Breathing was measured continuously from 2 min prior to buprenorphine infusion until 90 min after the start of infusion.

The breath-to-breath data were averaged over 1-min periods. An ensemble average was performed on the data of the eight subjects receiving the buprenorphine/placebo combination, allowing the calculation of buprenorphine/placebo-induced respiratory effect at various times. To quantify the respiratory effect of naloxone relative to placebo we used the following formula on the data of each subject that had received the buprenorphine–naloxone combination:

$$\text{Reversal}(z) = [V_{i,\,\text{naloxone}}(z) - V_{i,\,\text{placebo}}(z)] \,/\, [V_{i,\,\text{baseline}} - V_{i,\,\text{placebo}}(z)]$$

where period *z* ranges from t = 61 to t = 63 min (*i.e.,* 1 min before to 1 min after the end of the continuous naloxone infusion); $V_{i,\,placebo}(z)$ is mean minute ventilation in the placebo during period *z*, $V_{i,\,naloxone}(z)$ mean ventilation of the equivalent period after naloxone, and $V_{i,\,baseline}$ mean ventilation of the 2 min prior to the buprenorphine infusion. This analysis will yield a quantitative measure of reversal with 0 indicating no reversal (naloxone no better than placebo) and 1 full reversal (response returned to pre-buprenorphine level).

In order to get an impression of the naloxone dose-effect relationship on this 0.2 mg buprenorphine-induced respiratory depression, we fitted the dose-reversal data using the following sigmoid E_{max} function incorporating an inhibitory component (using NONMEM):

$$\text{Reversal} = (\text{dose}/D_1)^{\gamma} / (1 + (\text{dose}/D_1)^{\gamma}) \cdot [1/(1 + (\text{dose}/D_2)^{\gamma})]$$

where D_1 and D_2 are the naloxone doses causing 50% reversal and return to 50% depression, respectively, and γ a shape parameter.

Study 3

Thirty-two subjects participated in this study.

STUDY 3.1: Sixteen received 0.1 mg buprenorphine i.v. at t = 2 min, followed by a continuous infusion for 1 h of 0.1 $mg.h^{-1}$ (total dose = 0.2 mg in 60 min). At t = 32 min, 2 mg naloxone (*n* = 8) or placebo (*n* = 8) was infused, followed by an infusion of 4 $mg.h^{-1}$ for 2 h.

STUDY 3.2: Sixteen other subjects received 0.2 mg buprenorphine i.v. at t = 2 min, followed by a continuous infusion for 1 h of 0.2 $mg.h^{-1}$ (total dose = 0.4 mg in 60 min). At t = 32 min, 3 mg naloxone (*n* = 8) or placebo (*n* = 8) was infused, followed by a continuous infusion of 4 $mg.h^{-1}$ for 2 h. The bolus naloxone dose was 50% greater than that of Study 3.1. This was based on a pilot study in 3 subjects that showed the need for a greater initial dose of naloxone after 0.4 mg buprenorphine compared to 0.2 mg buprenorphine.

Ventilation was initially measured continuously from 2 min prior to buprenorphine infusion until 120 min after the start of infusion. Subsequently measurements were made at 30-min intervals until t = 240 min, after which hourly measurements were performed until t = 420 min.

The breath-to-breath data were averaged over 1-min periods. An ensemble average was performed on the naloxone and placebo data groups. The values were compared to baseline ventilation (± its 95% confidence interval).[10] When the mean ventilation value equalled or crossed [baseline ventilation – 1×95% confidence interval], we somewhat arbitrarily assumed that ventilation had returned to pre-drug baseline.

Study 4
Sixteen subjects participated in this study. All received 0.15 $mg.kg^{-1}$ morphine i.v. at t = 0. Eight subjects received 400 µg naloxone i.v. at t = 30 min; eight others received placebo at t = 30 min. Ventilation was initially measured continuously from t = 0 until t = 90 min. Subsequently measurements were obtained at 30-min intervals until t = 240 min. The breath-to-breath data were averaged over 1-min periods. Next ensemble averages were obtained and the data was compared to baseline ventilation in a similar fashion as in Study 3.

Study 5
Eight subjects received alfentanil i.v. by Target Controlled Infusion (TCI) using a palm-top computer (Psion 3C, London, UK) programmed with the population pharmacokinetic data set of Maitre *et al.* In this study we used a target plasma alfentanil concentration of 30 $ng.ml^{-1}$. This concentration was used previously to reduce the hypoxic ventilatory response by 50%. Before the alfentanil infusion and 15 min after a 'steady-state' concentration was obtained, we obtained a hypoxic ventilatory response, after which naloxone, 4 $\mu g.kg^{-1}$ i.v. (280 µg in a 70 kg person), was administered and 15 min later the third and last hypoxic response was obtained. Ten-breath averages were used in the data analysis. The hypoxic sensitivity was calculated by dividing the change in ventilation from normoxia to hypoxia by the change in arterial hemoglobin-oxygen saturation:[11]

$$\text{Hypoxic sensitivity} = [V_{i,\,\text{hypoxia}} - V_{i,\,\text{normoxia}}] \,/\, [SpO_{2,\,\text{normoxia}} - SpO_{2,\,\text{hypoxia}}]$$

(data points derived from the last ten breaths prior to hypoxia and the last ten breaths of hypoxia). Statistical analysis was by one-way analysis of variance with *post-hoc t*-test. *P*-values < 0.05 were considered significant.

Results

All subjects completed the studies without major side effects. The most frequent side effect was nausea and vomiting which occurred in 70% after buprenorphine, 50% after morphine and 0% after alfentanil.

Study 1
The averaged responses of the naloxone (800 µg) and placebo administration following 0.2 mg buprenorphine are shown in figure 1. At none of the measurement times did these two groups differ in the change in ventilation (or absolute ventilation values, data not shown). In order to detect a small effect of naloxone on ventilation unobserved in the averaged data, we calculated the difference in ventilation from t = 75 to t = 180 min. In the placebo group the change in ventilation was 0.2 ± 0.5 $l.min^{-1}$ against 2.2 ± 0.7 $l.min^{-1}$ in the naloxone group. This difference did not reach the level of significance (*P* = 0.08, one-tailed t-test, assuming a

larger response in the naloxone group). Our data indicate that the buprenorphine effect on ventilation was similar at all times irrespective of 800 μg naloxone infusion at t = 120 min.

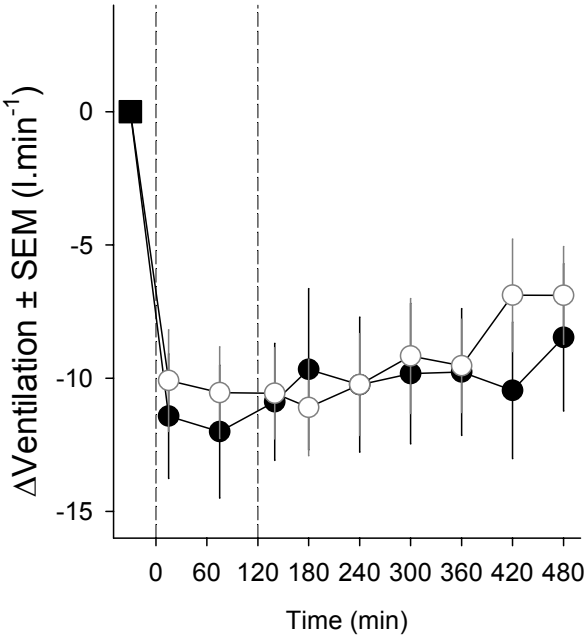

Figure 1. Influence of 800 μg naloxone on 0.2 mg buprenorphine-induced change in ventilation (Δventilation). Buprenorphine was given at time t = 0 min; naloxone or placebo at time t = 120 min. Square is predrug baseline value (0 $l.min^{-1}$). Closed circles: naloxone (*n* = 8); open circles: placebo (*n* = 8).

Study 2

The mean effect of buprenorphine/placebo on minute ventilation is given in figure 2 (grey area). Baseline ventilation was 24.0 ± 3.3 $l.min^{-1}$ at a fixed $P_{ET}CO_2$ of 7.0 ± 0.1 kPa. Peak depression of ventilation occurred at t = 71 min after the start of the buprenorphine infusion reaching a value of 13.5 ± 1.5 $l.min^{-1}$. Relative to baseline, peak depression was 62 ± 11%, indicating a reduction of baseline ventilation by 38%. In order to get an impression of the naloxone data, we plotted the data of two subjects given 2 and 6 mg naloxone in figure 2. The subject receiving 2 mg showed full reversal back to baseline (reversal = 1). In contrast, the subject given the higher naloxone dose showed little reversal (reversal = 0.1). In figure 3 we plotted the individual dose–reversal data for the time frame 61–63 min. The data show that full reversal ± 20% was obtained at doses between 2 and 4 mg naloxone, but that at higher doses reversal gradually declined. We calculated the naloxone dose causing 50% reversal of 0.95 ± 0.09 mg and the naloxone dose causing the return to 50% depression of 5.2 ± 0.22 mg (data fitted to a sigmoid E_{max} model with inhibitory component, see methods; values are median ± SE).

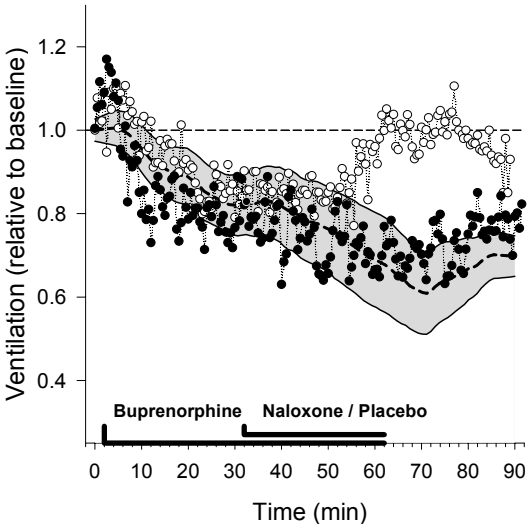

Figure 2. Buprenorphine (0.2 mg given over 60 min) induced respiratory depression.
Grey area is the mean ± SEM of the combination of buprenorphine and placebo (*n* = 8). The individual data (1-min averages) of two subjects receiving the combination of buprenorphine and naloxone are superimposed. Open circles: subject receiving 2 mg naloxone, closed circles: subject receiving 6 mg naloxone. Naloxone or placebo was given over 30 min.

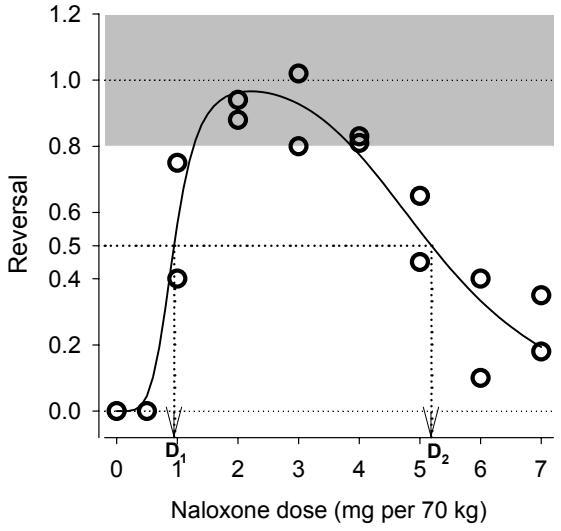

Figure 3. Influence of placebo and 0.5 to 7 mg naloxone reversal on 0.2 mg buprenorphine-induced respiratory depression. Reversal ranges from 0 (no reversal) to 1 (complete reversal). Circles are values of individual subjects (two subjects received 0.5 mg naloxone; 8 subjects placebo). Grey field indicates the area of full reversal ± 20%. The line through the data is the fit according to the sigmoid E_{max} function.

Study 3

Baseline ventilation averaged to 21.9 ± 2.5 l.min^{-1} (data from studies 3.1 and 3.2 combined). The effect of both doses of buprenorphine (0.2 and 0.4 mg) were successfully reversed by a continuous infusion of naloxone at the dose chosen by us, which was, at least partly, based on the data from Study 2.

STUDY 3.1. See figure 4. Buprenorphine, 0.2 mg, caused a rapid decrease in ventilation. Prior to naloxone or placebo infusion (t = 32 min), ventilation was 84 ± 3% and 79 ± 5% of baseline, respectively. In the placebo group, ventilation declined further to a nadir of 57 ± 6% of baseline at t = 120 min. In the naloxone group, the nadir was 78 ± 4% of baseline at t = 48 min (at the same time ventilation was 61 ± 5% of baseline in the placebo group). From that point on ventilation increased to reach baseline values (that is baseline ventilation – 1×95% confidence interval) at t = 70 min. Ventilation remained not different from baseline during the remainder of the naloxone infusion. After termination of the naloxone infusion (at t = 152 min) ventilation decreased but it never reached the level observed in the placebo group.

STUDY 3.2. See figure 4. A rapid decrease in ventilation occurred after the initiation of the 0.4 mg buprenorphine infusion. Prior to naloxone or placebo infusion (t = 32 min), ventilation

was 62 ± 5% and 64 ± 5% of baseline, respectively. In the placebo group, ventilation declined further to a nadir of 40 ± 3% of baseline at t = 150 min. In the naloxone group, the ventilation nadir was 61 ± 5% of baseline at t = 34 min (ventilation of the placebo group was 66 ± 7% at t = 34 min). From that point on ventilation increased to reach baseline values at t = 93 min. Ventilation remained not different from baseline during the remainder of the naloxone infusion. After termination of the naloxone or placebo infusion (at t = 152 min) the changes in ventilation were similar to those observed in Study 3.1.

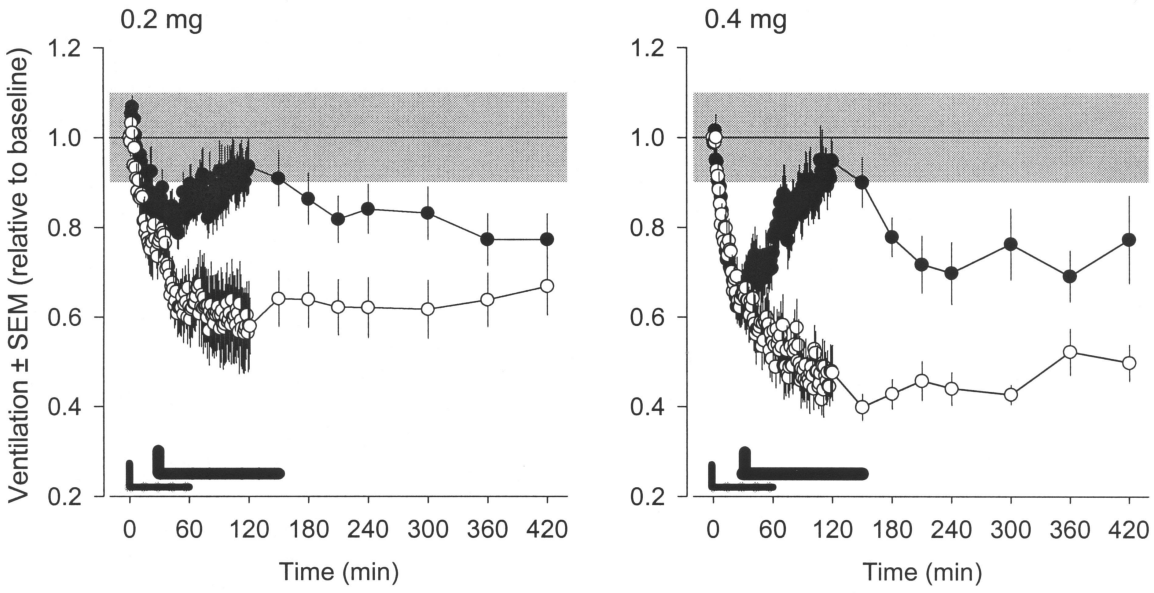

Figure 4. Influence of a continuous infusion of naloxone and placebo on 0.2 mg buprenorphine- (left) and 0.4 mg buprenorphine-induced respiratory depression (right). The buprenorphine dose was given over 60 min from t = 2 to t = 62 min. Naloxone or placebo was given for 2 h, from t = 32 to t = 152 min. Closed circles: naloxone (n = 8); open circles: placebo (n = 8). The grey area represents the 95% confidence interval of predrug baseline ventilation.

Study 4

See figure 5. Morphine, 0.15 mg.kg^{-1}, caused a rapid decrease in ventilation (baseline = 20.4 ± 1.0 l.min^{-1}). Prior to infusion of naloxone and placebo, ventilation was reduced to 14.1 ± 0.6 and 12.4 ± 1.5 l.min^{-1} in the naloxone and placebo groups, respectively. While placebo had little effect on ventilation, 400 µg naloxone caused a rapid return of ventilation to baseline. Ventilation was not different from baseline from t = 37 to t = 66 min, after which it gradually returned towards placebo-levels.

Study 5

The predrug hypoxic sensitivity was 1.22 ± 0.21 l.min^{-1}.%$^{-1}$. Alfentanil caused the severe impairment of the hypoxic sensitivity (reduction > 50% to 0.48 ± 0.35 l.min^{-1}.%$^{-1}$; $P < 0.05$

versus predrug control and naloxone) which was fully restored by 4 μg.kg^{-1} naloxone (1.15 ± 0.29 l.min^{-1}.%$^{-1}$; n.s. *versus* control).

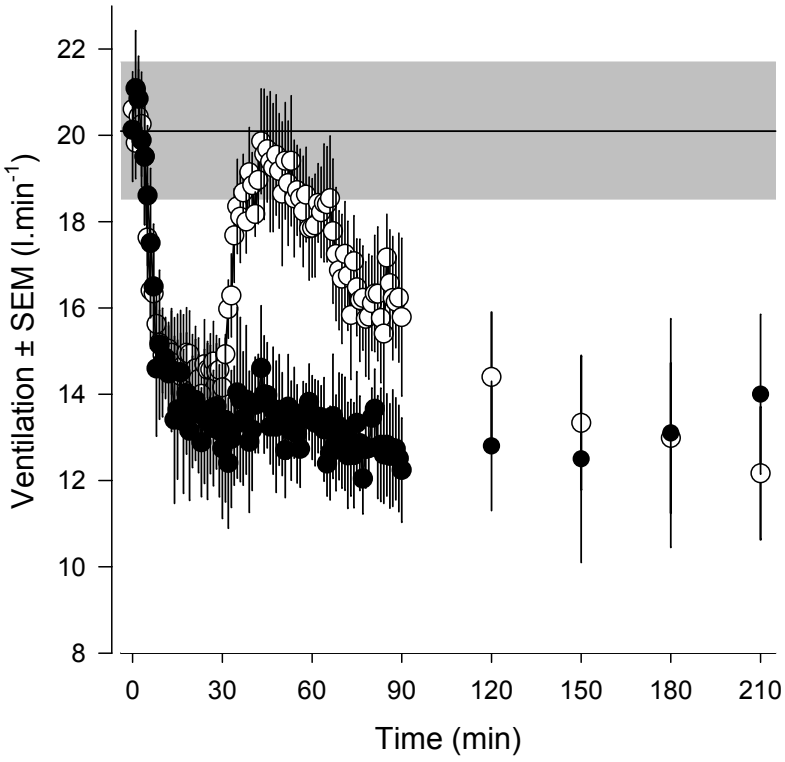

Figure 5. Influence of 400 μg naloxone or placebo (given at t = 30 min) on 0.15 mg.kg^{-1} morphine-induced respiratory depression. Open circles are naloxone data (n = 8); closed circles placebo (n = 8). Grey area is the mean predrug ventilation ± 95% confidence interval.

Discussion

In our studies, we observed that, while 0.4 mg i.v. naloxone (given in 90 s) caused full reversal of morphine- and alfentanil-induced respiratory depression (both are full μ-opioid receptor agonists), a double dose of naloxone (0.8 mg) had no effect on respiratory depression induced by the opioid analgesic buprenorphine. We next explored the naloxone dose–response relationship and observed that increasing doses of naloxone caused full reversal of 0.2 mg buprenorphine-induced respiratory depression (2–4 mg naloxone given in 30 min). Further increasing the naloxone dose (5–7 mg), however, caused a decline in reversal activity. The form of the dose–response relationship is best described by a bell-shape or inverse U. Taken into account these data, we designed a naloxone infusion scheme intended to cause full reversal of the respiratory depression from 0.2 and 0.4 mg buprenorphine. A naloxone bolus dose of 2 to 3 mg, followed by a continuous infusion of 4 mg.h^{-1}, caused full reversal within

40 to 60 min. Renarcotization did occur upon the termination of the naloxone infusion. These data indicate that reversal of buprenorphine-induced respiratory depression is only possible (i) with a continuous infusion of naloxone and (ii) with a specific naloxone dose chosen based on the buprenorphine dose and the form of the naloxone dose–response curve.

All opioids that interact with the μ-opioid receptor system depress respiration.[12] Buprenorphine is no exception to that rule. The extent of respiratory effect is highly variable and is related to the specific opioid, the opioid dose, the administration mode, concurrent medication, underlying disease, pain and the state of arousal (these two factors vary over time), genetics and exogenous stimulatory factors. Since the occurrence of overt and sometimes life-threatening respiratory depression is often unpredictable, the ability to induce rapid opioid reversal is of evident importance. In contemporary medicine, naloxone has become the drug of choice for treatment of opioid-induced respiratory depression. Naloxone is a non-specific opioid receptor antagonist (*i.e.*, it antagonizes the μ-, κ- and δ-opioid receptor) with a relatively short duration of action resulting from rapid elimination; its half-life in plasma is about 30–45 min.[13]

Although there is ample evidence that buprenorphine, just like other μ-opioid receptor agonists, produces significant respiratory depression at clinical doses (see figures 1, 2 and 4), sparse data from the literature addressed the issue of reversal of buprenorphine-induced respiratory depression.[5-7] The picture that emerges from these few studies is that at relatively large bolus naloxone doses little (that is, just partial) reversal of buprenorphine's respiratory effects is observed. For example a recent short report indicated that an incremental naloxone dose of 2.4 mg has an effect on 0.4 mg buprenorphine-induced respiratory depression no greater than placebo in patients during sevoflurane/nitrous oxide anesthesia.[7] An older study by Gal showed only partial reversal of 0.3 mg buprenorphine-induced respiratory depression with 5 and 10 mg i.v. naloxone (given as single bolus).[5] The inability to obtain full reversal in these two studies may be related to various factors, such as: anesthesia (anesthesia must be considered a serious complication when studying opioid-induced respiratory depression due to the complex opioid–anesthetic interaction on breathing),[14,15] the lack of sensitivity of the respiratory model applied to assess naloxone–buprenorphine interaction, the use of single naloxone doses and finally the use of a too high dose of naloxone (see figure 3).

The resistance to naloxone reversal is related to the very high affinity of buprenorphine for the μ-opioid receptor.[13] This very high affinity explains why relatively high doses of naloxone (2–4 mg) are needed before complete reversal is observed. The need for a continuous infusion in this process (upon termination of the naloxone infusion there was a rapid return of respiratory depression, see figure 4) implies the need for continuous supply of naloxone to the opioid receptor sites in the brain involved in respiratory depression. Otherwise the naloxone bolus is rapidly washed out from the brain compartment and eliminated from the body. We believe that the use of a single dose naloxone infusion to reverse opioid-related overdose has several disadvantages which are unrelated to the opioid involved: renarcotization due to

naloxone's short duration of action (in case of morphine see figure 5), the inability to titrate to effect causing the return of pain and sympathico-excitation. An infusion regimen aimed at a prolonged and steady-state naloxone plasma concentration may overcome these shortcomings. For example, continuous (11 h) naloxone infusion after high-dose fentanyl anesthesia caused reversal of respiratory depression without causing renarcotization, pain, or sympathico-excitation.[16]

An interesting observation in Studies 3.1 and 3.2 is the higher ventilation levels after naloxone treatment than after placebo treatment at times when naloxone is washed out from the brain and possibly also from the body (see figure 4 at t > 240 min). This is probably due to washout from the brain compartment of buprenorphine that was replaced by naloxone at the μ-opioid receptor or at non-specific binding sites (*i.e.*, some buprenorphine was lost without replacement). A similar observation was not made for morphine (figure 5). This may suggest that the morphine, which accumulates at intracellular sites in the brain,[17] fully replaces the morphine which was washed-out after naloxone-replacement at the receptor.

Naloxone doses exceeding the maximal effective dose (> 4 mg) lead to a decrease in (0.2 mg) buprenorphine reversal efficacy (figure 3). Since the number of subjects was limited (just 2 subjects per naloxone dose over the dose range from 0.5–7 mg) we consider this observation preliminary. Evidently, further studies are needed. In a first attempt, we performed a set of experiments after 0.4 mg buprenorphine and applied various naloxone doses (1 dose per subject; duration of naloxone infusion 30 min) and observed a similar bell-shaped dose–response relationship albeit full reversal was not reached (Dahan, unpublished observation). Our unexpected observation may be specific to the naloxone molecule or to buprenorphine and its interaction with naloxone. If it is just a naloxone effect, our data suggest that naloxone turns from a full antagonist to an agonist at high dose. We are unaware of any studies on increasing doses of naloxone on opioid-analgesia or opioid-side effects. Buprenorphine *per se* has a long history of showing bell-shaped dose–response curves with respect to its analgesic and side effect profile.[1,18] Most of these observations were made in animals. For example, rodents display a bell-shape buprenorphine dose–response relationship in various antinociceptive assays (electrical pain, heat pain, visceral pain and spinal nerve ligation) and on respiratory depression (using arterial PCO_2 as end point).[19-21] Also in humans there are indications of the existence of a bell-shaped dose–response curve with respect to analgesia. For example, two patients treated with buprenorphine (30–40 $\mu g.kg^{-1}$) for postoperative pain showed improved pain relief after 0.4 mg naloxone infusion, probably due to shifting of the bell-shaped dose–analgesia curve to the right.[22] A rightward-shift of the bell-shaped buprenorphine dose–response curve after the infusion of an opioid-antagonist (naltrexone) has been observed in rats using an electrical pain test.[19] Note that a bell-shaped curve was actually never observed in experimental human studies and clinically complete analgesia is reached with buprenorphine. The mechanism of the bell-shape curve remains unknown. Some argued that the form of the curve is related to the type and intensity of (experimental) pain administered.[21] Others suggested non-competitive autoinhibition, in which there are two

receptor subpopulations, one mediating the agonistic properties at low dose, the other mediating the antagonistic properties at high dose.[3,19,21,23] Finally, Lutfy *et al.*[24] suggest the contribution of the ORL_1 receptor. They showed that buprenorphine, but not morphine, given to mice activates ORL_1 receptors, compromising (antagonizing) analgesia from μ-opioid receptors. The latter theory seems not plausible taken into account that the scanty literature which exists on the respiratory effects of stimulation of ORL_1 receptors shows respiratory depression rather than stimulation.[25] The existence of two μ-opioid receptor subpopulations as described above could also theoretically explain our findings with high-dose naloxone causing the antagonism of the receptors mediating the antagonistic effects of buprenorphine. We are not aware, however, of any observation of these two receptor subpopulations in *in vitro* or *in vivo* animal studies.

We studied respiration in a group of healthy young volunteers and not in patients of various ages with pain. Hence, we remain uninformed what the effect of the naloxone–buprenorphine interaction is on pain and respiration in acute and chronic pain patients. Clinical studies in patients on buprenorphine treatment will be necessary to elucidate these important matters.

Finally, studying the ability to reverse opioid-induced side effects is not only relevant to compounds with high affinity for the μ-opioid receptor, such as buprenorphine. Since only few studies addressed the issue of long-term opioid reversal, we believe that more studies are required to assess –in case of respiratory depression– the specific dose and administration mode of naloxone needed to restore breathing and maintain it at an adequate level. This is relevant to opioids given at high dose, long acting opioids and opioids given as a continuous infusion (*e.g.*, using the transdermal patch formulation).

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SECTION 2

Respiratory Effects of Carbonic Anhydrase Inhibitors and Antioxidants

5 Antioxidants Reverse Depression of the Hypoxic Ventilatory Response by Acetazolamide in Man

A major defense of the mammalian body to acute hypoxia is a rapid increase in pulmonary ventilation called the acute hypoxic response (AHR). This vital chemoreflex is primarily mediated by the carotid bodies located at the bifurcations of the common carotid arteries.[1] A decrease in oxygen tension in the blood leads to a complex cascade of events in carotid body type I cells, in which closure of one or more classes of potassium channels results in membrane depolarization.[2-4] The mechanistic link between hypoxia and potassium channel closure is unknown; however, potassium channels are sensitive to oxidizing and reducing agents, and reactive oxygen species (ROS).[5,6] Whether ROS indeed play a mediating role in oxygen sensing is controversial.[7] Recently, Hildebrandt *et al.* have shown that after a 5-day oral application, N-acetylcysteine increased the hypoxic response in young adults indicating a possible specific involvement of gluthathione in oxygen sensing.[8] In older women with an age-related reduction of hypoxic sensitivity, ascorbic acid supplementation appeared to increase their hypoxic response.[9] Recently, we showed that an antioxidant mixture, consisting of oral α-tocopherol and intravenous ascorbic acid was able to reverse the reduction of the hypoxic response in humans by subanesthetic concentrations of inhalational anesthetics.[10,11] These studies indicate that antioxidants may be able to increase carotid body sensitivity in a situation wherein their output is reduced. If this is a general property of antioxidants remains to be seen, and in this context it would be of interest to investigate whether they would also be able to reverse carotid body inhibition that is induced by other means than anesthetics or age-related phenomena. One other, pharmacological, means to reduce carotid body sensitivity is acetazolamide, a well-known carbonic anhydrase inhibitor. The mechanism by which acetazolamide reduces the hypoxic response in man[12] and animals[13,14] is unclear, but could involve opening of potassium channels[15] and/or inhibition of one or more carbonic anhydrase iso-enzymes that have been shown to be present in the carotid bodies,[16] and at least one of which appears to act as a scavenger of reactive oxygen species in cultured cells.[17] Previous studies have shown that the effect of acetazolamide on the hypoxic response strongly depends on the dose and route of administration.[12-14] The aims of the present study in healthy volunteers were twofold. First, we wished to examine if an intravenous acetazolamide dose as low as 4 $mg.kg^{-1}$ would reduce the carotid body-mediated *acute* hypoxic response in man. Second, we wished to examine if a reduction of the acute hypoxic response by acetazolamide could be reversed by antioxidants (1 g ascorbic acid i.v. and 200 mg α-tocopherol p.o.), suggesting a modulating role of the redox status of blood in oxygen sensing or a potential role of a carbonic anhydrase isoenzyme *via* an influence of the (extra) cellular redox state in the carotid bodies.

Methods

Sixteen volunteers (8 men, 8 women, equally distributed over two subject groups, age 19–24 years; all of them gave informed consent) participated in the study after approval from the local Human Ethics Committee. All experiments conformed to the Declaration of Helsinki. All subjects were healthy, did not smoke or use any illicit drugs. They underwent a series of test experiments to familiarize them with the apparatus and experimental procedures. The subjects were instructed not to eat or drink for at least 8 hours prior to the study. After arrival in the laboratory, a catheter was inserted into the right antecubital vein for drug infusion and into the left radial artery for arterial blood gas analysis (ABL 700 Radiometer Copenhagen, Brønshøj, Denmark).

Respiration

The subjects breathed through a face mask, which was connected to a gas mixing system which received oxygen, carbon dioxide and nitrogen from three mass flow controllers. Gas flow was measured with a pneumotachograph (Fleisch, Lausanne, Switzerland) connected to a pressure transducer and electronically integrated to yield a volume signal. This signal was calibrated with a motor-driven piston pump (stroke volume 1000 ml, frequency 20 min^{-1}). Corrections were made for the changes in gas viscosity due to changes in oxygen concentration of the inhaled gas mixtures. The pneumotachograph was connected to a T-piece. One arm of the T-piece received a gas mixture (with a flow of 50 $l.min^{-1}$) from a gas mixing system consisting of three mass flow controllers (Bronkhorst High-Tech BV, Veenendaal, The Netherlands). A personal computer provided control signals to the mass flow controllers so that the composition of the inspired gas mixtures could be adjusted to force end-tidal oxygen concentration ($P_{ET}O_2$) to follow a specified pattern in time while the end-tidal carbon dioxide concentration ($P_{ET}CO_2$) was kept constant.[18,19] The oxygen and carbon dioxide concentrations of inspired and expired gases were measured with a gas monitor (Multicap, Datex-Engstrom, Helsinki, Finland) by paramagnetic and infrared analysis. The gas monitor was calibrated with gas mixtures of known composition delivered by a gas-mixing pump (Wösthoff, Bochum, Germany). The arterial hemoglobin-O_2 saturation obtained *via* a finger probe (SpO_2) was measured by pulse oximetry (Sattelite Plus, Datex-Engstrom). Respiratory parameters were collected on a breath-to-breath basis and stored on disc for further analysis.

Study Design and Data Analysis

Subjects were randomly divided into two groups and exposed to three treatments: control (CON) followed by acetazolamide (AZ) and antioxidants for the antioxidants (AOX) group and control (CON) followed by acetazolamide (AZ) and placebo for the placebo (PLCB) group. In both groups, two hypoxic exposures were applied during each of the three treatments, one at a constant normocapnic end-tidal PCO_2 ($P_{ET}CO_2$, 'normocapnic experiments' in table 2, resulting in normocapnic clamped levels of ventilation), and one at a constant hypercapnic $P_{ET}CO_2$ ('hypercapnic experiments' in table 2, resulting in hypercapnic

clamped levels of ventilation). The order of these CO_2 levels was random. Hypoxia was induced with dynamic end-tidal forcing by which steps from normoxia ($P_{ET}O_2$ 15 kPa) into hypoxia ($P_{ET}O_2$ 6.2 kPa, obtained within 4 to 6 breaths) were applied.[11,14,18,19] Since peak hypoxic ventilatory responses occur within three min,[19] hypoxia was maintained for three min, after which hyperoxia was introduced for 5 min ($F_iO_2 > 0.5$). Subsequently, the $P_{ET}O_2$ sequence was repeated at the alternate $P_{ET}CO_2$ level.

In all subjects control hypoxic studies at both CO_2 levels (no drugs given) were followed by the intravenous administration of 4 $mg.kg^{-1}$ acetazolamide (AHP Pharma SA, France). Twenty minutes after the AZ infusion, a second set of hypoxic studies was performed at both $P_{ET}CO_2$ levels. Next, eight subjects received the antioxidant cocktail (AOX group), eight others placebo (PLCB group). The antioxidant mixture consisted of 200 mg α-tocopherol (dl-α-tocoferolacetaat, OPG Farma, Utrecht, The Netherlands) ingested with a cup of yoghurt and 1 gram ascorbic acid (Ascorbinezuur CF, Centrafarm, Etten-Leur, The Netherlands), infused intravenously 35 min after the α-tocopherol application. The placebo mixture (manufactured by the local pharmacy) consisted of cellulose tablets (ingested with yoghurt), and saline, infused 35 min after the ingestion of cellulose. The final sets of hypoxic studies were performed 10 min after the infusions of AOX and saline in the AOX and PLCB groups, respectively. With respect to the administration of the antioxidants and placebo, the study had a double blind, randomized design.

The breath-to-breath data of the last 10 breaths of normoxia (prior to hypoxia) and of hypoxia (prior to hyperoxia) were averaged. Hypoxic ventilatory sensitivity (or acute hypoxic ventilatory response, AHR; units $l.min^{-1}. \%^{-1}$) was calculated as:[18]

$$AHR = [V_{i,\,hypoxia} - V_{i,\,normoxia}] / [SpO_{2,\,normoxia} - SpO_{2,\,hypoxia}]$$

The statistical analysis was performed using SPSS v11.0 for Windows (SPSS Inc., Chicago, IL, USA, 2003). A two-way repeated measures analysis of variance with *post-hoc* Bonferroni correction was performed to detect significant differences among the three treatment levels within the AOX group (CON – AZ – AOX) and the PLCB group (CON – AZ – PCLB). *P*-values < 0.05 were considered significant. Unless otherwise indicated, values are reported as mean ± SEM.

Results

All subjects completed the sessions without side effects, except one subject belonging to the PLCB group who withdrew after one session for unknown reasons.

The effects of acetazolamide on resting arterial acid–base and $[K^+]$ are shown in table 1. In both groups of volunteers, the agent induced small reductions in mean potassium and

bicarbonate concentrations and base excess. No significant differences in mean resting minute ventilation and $P_{ET}CO_2$ were observed between control and AZ or AZ + AOX in the AOX group and control and AZ or AZ + PLCB in the PLCB group (table 2). After acetazolamide administration, there was no detectable increase in the arterial-to-end-tidal PCO_2 ($P_{(a–ET)}CO_2$) gradient indicating incomplete erythrocytic carbonic anhydrase inhibition. We observed no effect of low intravenous dose acetazolamide on arterial blood pressure (data not shown).

Table 1. Effects of acetazolamide on blood acid–base status.

Antioxidant group (*n* = 8)	**Control**	**Acetazolamide**
$PaCO_2$ (kPa)	5.6 ± 0.3	5.4 ± 0.4
$P_{(a–ET)}CO_2$ (kPa)	0.00 ± 0.15	-0.08 ± 0.24
Arterial pH	7.39 ± 0.01	7.37 ± 0.01 †
Arterial [bicarbonate] (mM)	24.8 ± 1.4	22.7 ± 1.6 †
Base excess (mM)	-0.3 ± 1.1	-2.5 ± 1.2 †
Arterial [K^+] (mM)	4.1 ± 0.3	3.9 ± 0.3 §
SpO_2 (%)	97.6 ± 0.4	96.8 ± 1.1 *
Placebo group (*n* = 7)		
$PaCO_2$ (kPa)	5.4 ± 0.5	5.2 ± 0.4
$P_{(a–ET)}CO_2$ (kPa)	0.04 ± 0.26	-0.09 ± 0.33
Arterial pH	7.38 ± 0.03	7.37 ± 0.02 *
Arterial [bicarbonate] (mM)	24.1 ± 1.8	21.9 ± 1.6 ‡
Base excess (mM)	-0.9 ± 1.6	-3.0 ± 1.2 †
Arterial [K^+] (mM)	4.1 ± 0.2	3.7 ± 0.3 †
SpO_2 (%)	97.6 ± 0.2	97.7 ± 0.4

Effect of acetazolamide on resting blood gas and electrolyte parameters in eupneic resting conditions. Data are given as mean ± SD. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.02$; § $P < 0.03$. Blood gas analysis was performed to check for possible $P_{(a–ET)}CO_2$ differences following acetazolamide infusion.

Normocapnic clamped $P_{ET}CO_2$ values were 5.9 ± 0.1 and 5.7 ± 0.2 kPa in the AOX and PLCB group, respectively. In both subject groups, the clamped $P_{ET}CO_2$ was increased by 0.5 kPa to perform a hypercapnic AHR. When going from the normocapnic to the hypercapnic $P_{ET}CO_2$, both subject groups showed an about equal increase in AHR (O_2–CO_2 interaction, table 2, see also figure 1).

Table 2. Effects of acetazolamide (AZ), antioxidants (AOX) and placebo (PLCB) on resting ventilatory parameters and acute normocapnic and hypercapnic hypoxic responses.

	Antioxidant group			Placebo group		
	Control	**AZ**	**AZ + AOX**	**Control**	**AZ**	**AZ + PLCB**
Resting ventilation ($l.min^{-1}$)	8.9 ± 0.4	9.3 ± 0.5	9.4 ± 0.4	9.1 ± 0.8	9.4 ± 0.5	9.2 ± 0.3
Resting $P_{ET}CO_2$ (kPa)	5.6 ± 0.1	5.5 ± 0.1	5.3 ± 0.2	5.4 ± 0.2	5.3 ± 0.2	5.2 ± 0.2
Normocapnic experiments						
Clamped $P_{ET}CO_2$ (kPa)	5.9 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	5.7 ± 0.2	5.7 ± 0.2	5.7 ± 0.2
Normoxic ventilation ($l.min^{-1}$)	11.1 ± 0.5	12.1 ± 0.8	14.0 ± 0.7 [c]	10.6 ± 0.8	11.7 ± 0.9	13.7 ± 0.6 [e]
Hypoxic ventilation ($l.min^{-1}$)	20.4 ± 0.7	17.0 ± 1.0	22.8 ± 1.0	23.7 ± 0.9	16.8 ± 0.9	19.5 ± 0.9
AHR ($l.min^{-1}.\%^{-1}$)	0.65 ± 0.1	0.41 ± 0.0 [*]	0.67 ± 0.1	0.61 ± 0.1	0.41 ± 0.1 [*]	0.46 ± 0.01 [*]
Hypoxic SpO_2 (%)	85.0 ± 0.8	84.0 ± 0.7	84.8 ± 0.6	85.1 ± 0.8	83.1 ± 0.6	83.0 ± 1.0
Hypercapnic experiments						
Clamped $P_{ET}CO_2$ (kPa)	6.4 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	6.2 ± 0.2	6.2 ± 0.2	6.2 ± 0.2
Normoxic ventilation ($l.min^{-1}$)	16.0 ± 0.8	18.1 ± 1.0	20.0 ± 0.8 [d]	15.9 ± 1.0	17.3 ± 1.5	18.2 ± 0.7 [d]
Hypoxic ventilation ($l.min^{-1}$)	27.3 ± 0.8	23.1 ± 1.2	29.8 ± 1.1	27.6 ± 1.1	23.8 ± 1.6	24.3 ± 0.8
AHR ($l.min^{-1}.\%^{-1}$)	0.86 ± 0.1 [†]	0.39 ± 0.0 [*‡]	0.74 ± 0.1 [§]	0.82 ± 0.1 [†]	0.44 ± 0.1 [*a]	0.53 ± 0.1 [*b]
Hypoxic SpO_2 (%)	85.1 ± 0.8	85.1 ± 0.6	84.8 ± 1.0	84.6 ± 0.5	82.9 ± 0.8	82.9 ± 0.8

Data are given as mean ± SEM. Note that in the AOX group AZ reduces the AHR both in normocapnia and hypercapnia and that these effects are reversed by AOX. In the PLCB group, no reversal is seen. * $P < 0.01$ *versus* control; † $P = 0.0002$ *versus* normocapnia; ‡ $P = 0.52$ *versus* normocapnia; § $P = 0.02$ *versus* normocapnia; a $P = 0.24$ *versus* normocapnia; b $P = 0.19$ *versus* normocapnia; c $P = 0.004$ *versus* control; d $P = 0.015$ *versus* control; e $P = 0.016$ *versus* control.

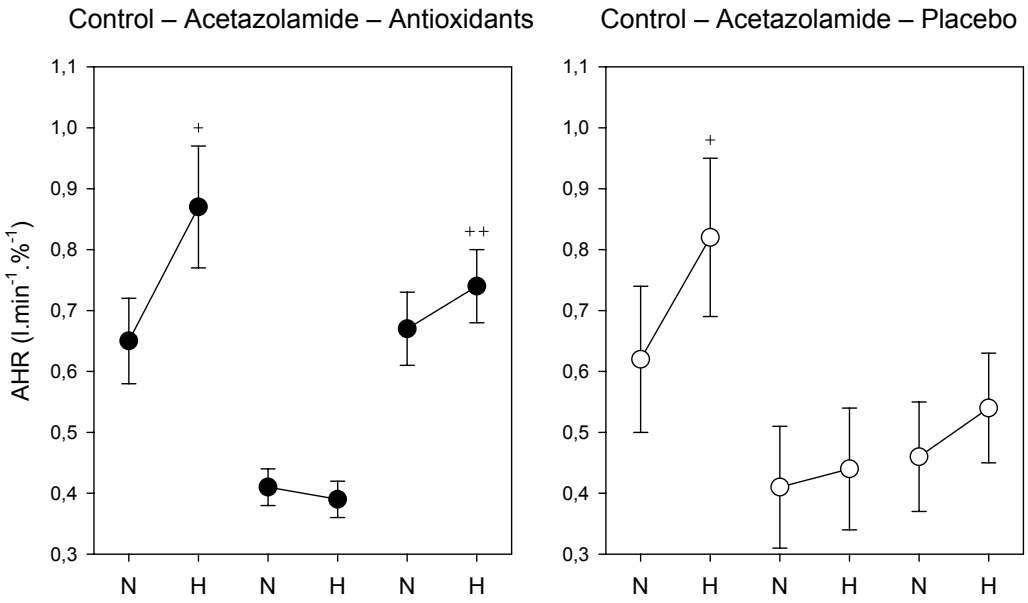

Figure 1. Effects of antioxidants (left panel) and placebo (right panel) on acetazolamide-induced inhibition of normocapnic (N) and hypercapnic (H) hypoxic responses. Control data are shown at left in both panels. Note the increase in hypoxic sensitivity when going from normocapnia to hypercapnia (O_2–CO_2 interaction) in control studies that is abolished after acetazolamide (shown in the middle of both panels). The left panel shows that after antioxidant administration, the normocapnic hypoxic response is fully restored, while the O_2–CO_2 interaction reappears. After placebo (right panel), the normocapnic and hypercapnic hypoxic responses clearly remain below control and no O_2–CO_2 interaction reappears. Paired *t*-test: + $P < 0.001$; ++ $P = 0.02$.

The effects of AZ, AOX and PLCB on the mean AHR in both subject groups are summarized in table 2 and graphically presented in figure 1. The effects of AZ, AOX and PLCB on the normocapnic AHR of one individual is presented in figure 2. The mean normocapnic AHR's were reduced by 37% and 33% in the AOX and PLCB groups, respectively (not significantly different between the two groups, table 2). This inhibiting effect on the *hypercapnic* AHR was even larger (52% and 47% reduction in the AOX and PLCB groups, respectively, not significantly different between the two groups): in fact, AZ abolished any O_2–CO_2 interaction (figure 1, table 2). In the subjects of the AOX group that showed a reduced normocapnic and hypercapnic AHR upon AZ, this effect was reversed by AOX. In normocapnia, this reversal was total, while in hypercapnia a complete or almost complete reversal occurred in all individuals except one in which it was partial (for mean data see table 2). After AOX, all these individuals showed a larger hypercapnic than normocapnic AHR indicating a reversal of the O_2–CO_2 interaction. Note that both the normocapnic and hypercapnic AHR's after AZ + AOX did not significantly differ from those during control.

In contrast to the subjects receiving AOX, those receiving placebo did not show reversal of the acetazolamide-induced inhibition of the normocapnic and hypercapnic AHR. At both $P_{ET}CO_2$ levels, the AHR after AZ + PLCB was not different from that after AZ alone. After

AZ + PLCB, some subjects in this group showed a somewhat larger, and others an equal or somewhat smaller hypercapnic than normocapnic AHR so that in contrast to those treated with AOX, the subjects of this group showed on average no signs of a re-appearing O_2–CO_2 interaction.

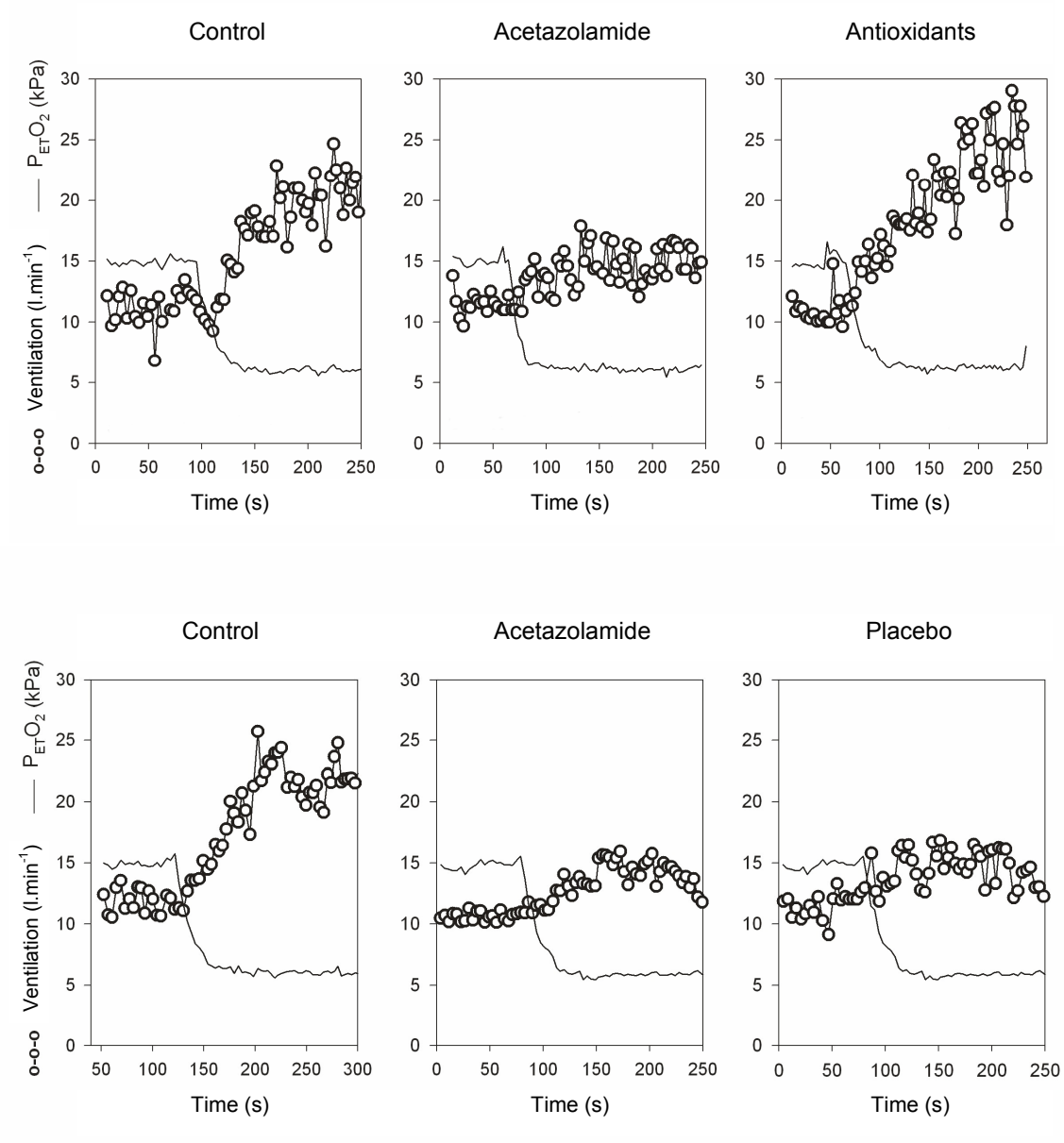

Figure 2. Effects of antioxidants (top) and placebo (bottom) on the acetazolamide-induced depression of the normocapnic acute hypoxic ventilatory response of one individual. Antioxidants reverse the depression but placebo fails to do so. Ventilatory data are show breath-by-breath.

Discussion

In this study in healthy volunteers, acetazolamide (4 $mg.kg^{-1}$ i.v.) reduced the normocapnic and hypercapnic hypoxic response by about 35% and 50%, respectively, and prevented the increase in hypoxic response that normally occurs with a rise in arterial PCO_2 (O_2–CO_2 interaction; note also that the inhibiting effect of acetazolamide occurred despite a small significant decrease in arterial pH that normally would tend to increase the AHR). A mixture of antioxidants (200 mg α-tocopherol p.o. and 1 g ascorbic acid i.v.) reversed these inhibiting effects of acetazolamide on the hypoxic response.

Methodology and Study Design: General Considerations

Two issues that should be discussed concern the acetazolamide dose and the timing of the ventilatory measurements. The acetazolamide dose that is needed to obtain an acute maximal physiological effect from carotid body carbonic anhydrase inhibition has not been systematically explored. For most organs it is 5–10 $mg.kg^{-1}$, but for kidneys, containing an enzyme concentration similar to carotid bodies, it is smaller.[20,21] From available human data,[22] we estimate a free unbound (diffusible) drug concentration in our subjects of about 8 μM at the beginning of the elimination phase which is insufficient for impairment of red cell function,[20,21] but well within a concentration range necessary for lowering intraocular pressure (EC_{50} ~7 μM).[23] The concentration needed for maximal inhibition of carotid body function probably lies between 8 and 15 μM, because already at 12–15 μM the hypoxic response is abolished.[12] In order to reach this concentration of ~15 μM, we considered initially to administer 500 mg to our subjects, because this could have told us whether the hypoxic response after AOX would reappear after its abolishment by acetazolamide. However, this could have the disadvantage of studying an effect that could be confounded by sufficient erythrocytic CA inhibition to result in tissue acidosis[21] and the inability to perform real isocapnic measurements (note the absence of a $P_{(a–ET)}CO_2$ gradient after acetazolamide infusion, see table 1).[24] Nevertheless, together with those of Swenson and Hughes[12], our data indicate a strong dose-dependent effect of acetazolamide on carotid body function in a narrow concentration range.

The time course of the effect of intravenous acetazolamide on the control of breathing is not well described. In the anesthetized cat, an intravenous dose of 4 $mg.kg^{-1}$ abolishes the O_2–CO_2 interaction and reduces hypoxic sensitivity by half, effects that are still demonstrable about 100 min after infusion.[14,24] Similar to Swenson and Hughes,[12] we performed the ventilatory measurements within one elimination half time of acetazolamide which after an intravenous bolus is ~1.7 h.[22,25] The estimated prevailing plasma concentration (~5 μM) at the time of the last ventilatory measurements (AHR after AZ + AOX/PLCB) is well within the range reported to cause physiological effects in various peripheral tissues except erythrocytes.[20-23] A long-lasting inhibition of carotid body function by acetazolamide was

confirmed in our subjects receiving AZ + PLCB because on average they still showed a hypoxic response that was significantly lower than control.

Reduction of the AHR by Acetazolamide and Reversal by AOX

Our observations are from intact organisms and this compels us to be cautious with extrapolating them back to processes occurring at the cellular level. However, available data obtained from reduced preparations may help us to place our observations into a proper context. For the sake of brevity, we will only discuss two scenarios that may be helpful to explain our findings.

Scenario 1. Inhibition of the AHR by acetazolamide is due to inhibition of a carbonic anhydrase (CA) isoform. It is assumed that the antioxidants that we administered did not influence acetazolamide's binding to the isoenzyme but rather exerted an independent reducing effect, for example by scavenging ROS.[26,27] Because AOX reversed an inhibitory effect of a carbonic anhydrase inhibitor, we consider a possible role of some CA isoforms as a ROS scavenger. Two candidates for such an antioxidant function could be CA III and IV, the latter because it contains cysteine groups that may participate in redox reactions,[28] and the former because it behaves as an important ROS scavenger[17] and is present in type I carotid body cells from rat.[16] CA III is a cytoplasmic enzyme with a relatively low affinity for sulfonamide inhibitors,[29] while at the same time acetazolamide is only moderately permeable.[30] These two factors, acetazolamide's limited permeability and its low affinity for CA III could then explain why a relatively high dose is needed to completely abolish the hypoxic ventilatory response.[13] With regard to CA IV, we are not aware of data showing a role of this protein in regulating the (extracellular) redox state, for example by S-glutathiolation of one or more of its cysteine residues.[28] It should also be mentioned that as yet the presence of CA IV in the carotid bodies has not been demonstrated.

Because AOX reversed the acetazolamide-induced depression of the AHR, scenario 1 could involve a role for CA as a ROS scavenger in hypoxia, which then could contribute to (*i.e.*, increase) the magnitude of the ventilatory response. However, this remains a matter of speculation since experimental evidence is lacking. Also, note that it is unsettled if hypoxia is associated with either an increase or decrease in [ROS] and that ROS do not seem to be directly involved in oxygen sensing,[7] and our results do not shed more light on this. It is interesting to note that AOX are also able to reverse cutaneous photosensitivity by acetazolamide and other sulfonamide-derived diuretics and antidiabetics,[31,32] and that AOX such as vitamin E may affect CA activity.[33]

Scenario 2. Acetazolamide causes intracellular alkalosis in type I carotid body cells, which results in a decrease in carotid body output and sensitivity. AOX's reverse this by an independent effect on potassium channels. Acetazolamide causes alkalosis in type I carotid body cells from neonatal rat[34] and in guinea pig mesenteric smooth muscle cells, it induces

alkalosis followed by hyperpolarization, opening of maxi-K (BK) channels and a decreased Ca^{2+} influx.[35] Apart from TASK-1 channels,[36] maxi-K channels have been shown to play a role in oxygen sensing by type I cells from rat carotid body (note that in rabbit and mouse K_v channels have been shown to be involved).[37-40] A reversal by AOX of the inhibitory effects of acetazolamide on the hypoxic response could be explained by the known redox sensitivity of potassium channels.[5,6] In rabbit type I glomus cells, reducing agents decrease the open probability of potassium channels.[2]

In rat muscle cells, acetazolamide has a BK channel-opening effect which may be mediated by a direct action and is not shared by the more lipophilic carbonic anhydrase inhibitor methazolamide.[15] In cats, 4 $mg.kg^{-1}$ acetazolamide has a similar inhibitory effect on the hypoxic response as we report here in humans.[14] Recently, we have compared the effect of low-dose acetazolamide and methazolamide on the hypoxic response in this species and the surprising finding was that even high-dose methazolamide did not affect it (unpublished observations). This suggests that at least in the cat, but possibly also in humans the inhibition of the hypoxic response by acetazolamide is not related to inhibition of carbonic anhydrase (thereby making the above scenario 1 less likely) but rather to some other pharmacological effect of the agent. It remains a matter of speculation whether the inhibiting effect of acetazolamide on the hypoxic response may be mediated *via* a specific effect on one or more potassium channels, or, alternatively, to a possible action on various types of L-type calcium and/or sodium channels.[41,42]

Clinical Implications?

Because at this stage the possible clinical implications of our observations are a matter of speculation we only briefly address two issues, the first of which concerns a possible effect of AOX on the hypoxic response *per se.* Because in previous studies[10,11] we were unable to demonstrate an effect of AOX on normoxic ventilation and the AHR, we decided not to include a separate protocol with AOX alone. Putting our present and previous findings together, it seems that in resting conditions, in the absence of inhibiting factors, AOX may not alter carotid body output but may increase it when such factors are present. This could be important in elderly people (> 70 years): they have been reported to possess a considerably lower O_2 sensitivity than healthy young adults,[43,44] while in women 60–80 years of age ascorbic acid supplementation appeared to increase their hypoxic response.[9] The ability of antioxidants to alter the hypoxic response may vary between different reducing agents. For example, Hildebrandt *et al.* showed that after a 5-day oral application, N-acetylcysteine increased the hypoxic response in young adults indicating a possible specific involvement of glutathione in oxygen sensing.[8]

A second issue concerns the possibility that AOX may also alter physiological effects of acetazolamide other than the depression of the hypoxic response. For example, the agent has well-known beneficial effects in the prevention and treatment of acute mountain sickness

(AMS).[45] Ascent to high altitude is associated with increased production of ROS that may have both advantageous and adverse roles in the adaptation to low environmental PO_2.[46] Acetazolamide may prevent or ameliorate symptoms of AMS by an acidosis-induced rise in ventilation and/or by reducing pulmonary vasoconstriction, an oxygen-sensitive process that, similar to O_2 sensing in the carotid bodies, involves potassium channels.[47] It remains to be seen whether supplemental antioxidants at altitude might interfere with the beneficial effect of acetazolamide or influence adaptive processes in a hypobaric environment.

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6 Effects of Low-Dose Methazolamide on the Control of Breathing in Cats

Inhibitors of carbonic anhydrase (CA) have complex effects on respiration. Many cells and tissues that are involved in the control of breathing contain various isoforms of CA, *e.g.*, red cells, carotid bodies, lung and brain capillary endothelial cells, muscle and neurons closely associated with central chemoreceptors.[1-9] In human and cats, low intravenous doses of acetazolamide have both stimulatory and inhibitory effects on the control of breathing.[10,11] One of the inhibitory effects applies to the peripheral chemoreceptors because acetazolamide has been shown to reduce the hypoxic response and also the O_2–CO_2 interaction that is known to reside in the carotid bodies.[10,12,13] The mechanism by which this occurs is unclear: however, due to its physical-chemical properties acetazolamide does not easily cross biological membranes,[1,2] so that at low dose this inhibiting effect is unlikely due to inhibition of an intracellular isoform of CA in the carotid bodies. Methazolamide, another CA inhibitor with an about equal affinity for sulfonamide-sensitive CA isoforms, is much more lipophilic and rapidly permeates into cells.[1,2] Therefore, this agent would be a suitable tool to study the effect of intracellular CA inhibition on carotid body-mediated responses. Another difference between acetazolamide and methazolamide refers to their effects on large-conductance Ca^{2+}-dependent potassium (BK) channels: while acetazolamide specifically opens these channels, methazolamide is without any stimulating effect on them.[14] Because BK channels may play a crucial role in the hypoxic response of type I carotid body cells,[5] it is therefore interesting to compare the effects of both agents on the carotid body responses to both hypoxia and hypercapnia.

Dynamic end-tidal CO_2 forcing (DEF) is a suitable means to study the separate effects of pharmacological agents on the CO_2 sensitivity of the peripheral and central chemoreflex loops.[16] In this study we have applied this technique to study the effects of low-dose methazolamide on the control of breathing in the cat.

Methods

Experiments were performed in nine female adult cats (weight 2.5–4.1 kg). The Ethical Committee for Animal Experiments of the University of Leiden approved the use of animals. The animals were sedated with 10 $mg.kg^{-1}$ ketamine hydrochloride i.m. Anesthesia was induced with 2% sevoflurane in 30% O_2 in N_2. Both femoral arteries and the right femoral vein were cannulated, 20 $mg.kg^{-1}$ α-chloralose and 100 $mg.kg^{-1}$ urethane were slowly

administered intravenously, and the volatile anesthetic was gradually withdrawn. About 1 h later, an infusion of an α-chloralose-urethane solution was started at a rate of 1.0–1.5 $mg.kg^{-1}.h^{-1}$ α-chloralose and 5.0–7.5 $mg.kg^{-1}.h^{-1}$ urethane.

Respiration

The trachea was cannulated at midcervical level and connected to a respiratory circuit. Tidal volume was measured electronically by integrating airway gas flow obtained from a pneumotachograph (number 0 flow transducer, Fleisch, Lausanne, Switzerland) connected to a differential pressure transducer (PM 197, Statham, Los Angeles, CA, USA). The respiratory fractions of O_2 and CO_2 were continuously measured with a gas monitor (Multicap, Datex, Helsinki, Finland), which was calibrated with gas mixtures of known composition. The inspiratory gas concentrations were made with computer-steered mass flow controllers (AFC 260, Bronkhorst High-tech BV, Veenendaal, The Netherlands). The end-tidal PCO_2 ($P_{ET}CO_2$) and end-tidal PO_2 ($P_{ET}O_2$) were controlled independently by a PC by adjusting the inspiratory gas fractions. Arterial blood pressure was measured using a pressure transducer (P23ac, Statham). Arterial blood samples were taken for blood gas analysis (ABL 700, Radiometer Copenhagen, Brønshøj, Denmark).

Experimental Design

Using the DEF technique, we performed step changes in $P_{ET}CO_2$ before and after intravenous infusion of 3 $mg.kg^{-1}$ methazolamide (Sigma, Zwijndrecht, The Netherlands), dissolved in 0.1 N NaOH and 0.1 N HCl (pH was adjusted to 7.3–7.4). $P_{ET}O_2$ was kept constant throughout the experiments at a normoxic level of 14 kPa. Both before and after methazolamide administration, 2 to 4 DEF runs were performed and the dynamic ventilatory responses were analyzed (see below). The $P_{ET}CO_2$ pattern during a DEF run was as follows. After a 10 to 15 min period of steady-state ventilation at constant $P_{ET}CO_2$ (about 0.5 kPa above the apneic threshold), the $P_{ET}CO_2$ was increased by 1–1.5 kPa in a step-wise fashion and kept constant for 7 min. Thereafter, the $P_{ET}CO_2$ was returned to its previous value and maintained for another 7 min.

Dynamic End-Tidal Forcing

The steady-state relation of inspiratory ventilation (V_i) to $P_{ET}CO_2$ at constant $P_{ET}O_2$ can be described by:

$$V_i = (S_p + S_c)(P_{ET}CO_2 - B)$$

where S_p and S_c are the carbon dioxide sensitivities of the peripheral and central chemoreflex loops, respectively, and B is the apneic threshold or extrapolated $P_{ET}CO_2$ at zero V_i. The sum of S_p and S_c is the overall carbon dioxide sensitivity.

For the analysis of the dynamic response of ventilation to a step-wise change in $P_{ET}CO_2$ we used a two-compartment model:[16]

$$V_p(t) + \tau_p \, d/dt \, V_p(t) = S_p \, (P_{ET}CO_2[t - T_p] - B)$$

$$V_c(t) + \tau_c \, d/dt \, V_c(t) = S_c \, (P_{ET}CO_2[t - T_c] - B)$$

where τ_p and τ_c are the time constants of the peripheral and central chemoreflex loops, respectively, $V_p(t)$ and $V_c(t)$ are the outputs of the peripheral and central chemoreflex loops, respectively, $P_{ET}CO_2[t - T_p]$ is the stimulus to the peripheral chemoreflex loop delayed by the peripheral transport delay time (T_p), and $P_{ET}CO_2[t - T_c]$ is the stimulus to the central chemoreflex loop delayed by the central transport delay time (T_c).

To allow the time constant of the ventilatory on transient to be different from that of the off transient, τ_c is written as:

$$\tau_c = x.\tau_{on} + (1 - x) \, \tau_{off}$$

where τ_{on} is the time constant of the ventilatory on transient, τ_{off} is the time constant of the off transient, and x = 1 when $P_{ET}CO_2$ is high, while x = 0 when $P_{ET}CO_2$ is low.

In most experiments a small drift in ventilation was present. We therefore included a drift term (C·t) in our model. The total ventilatory response $V_i(t)$ is made up of the contributions of the central and peripheral chemoreflex loops and C·t:

$$V_i(t) = V_p(t) + V_c(t) + C \cdot t$$

The parameters of the model were estimated by fitting the model to the breath-by-breath data with a least-squares method. To obtain optimal time delays, a grid search was applied, and all combinations of T_p and T_c, with increments of 1 s and with T_p smaller than or equal to T_c, were tried until a minimum in the residual sum of squares was obtained. The minimum time delay was chosen, arbitrarily, to be 1 s, and τ_p was constrained to be at least 0.3 s.

Statistical Analysis

To compare the means of the values obtained from the analysis of the DEF runs in the control situation with those obtained after methazolamide infusion, analysis of variance was performed on individual data. The level of significance was set at $P = 0.05$. Results are given as mean of the mean per cat ± SD.

Results

The dose of 3 $mg.kg^{-1}$ methazolamide did not cause an appreciable arterial-to-end-tidal PCO_2 ($P_{(a-ET)}CO_2$) gradient indicating the absence of effective erythrocytic CA inhibition ($P_{(a-ET)}CO_2$ differences were -0.14 ± 0.27 kPa in control and 0.18 ± 0.27 kPa after

methazolamide, $P = 0.11$). Altogether 57 DEF runs (32 before and 25 after methazolamide) were analyzed and the results are summarized in table 1.

Table 1. Effects of methazolamide on respiratory parameters. Values are given as mean of the mean per cat ± SD ($n = 9$).

	Control	**Methazolamide**	*P*
B (kPa)	3.60 ± 0.72	1.77 ± 1.41	0.00006
S_c (l.min.kPa^{-1})	0.68 ± 0.27	0.44 ± 0.22	0.013
S_p (l.min.kPa^{-1})	0.08 ± 0.04	0.06 ± 0.03	0.13
Base excess (mM)	-6.65 ± 1.75	-7.84 ± 1.90	0.009

Methazolamide reduced the apneic threshold and the CO_2 sensitivity of the central chemoreflex loop. The CO_2 sensitivity of the peripheral chemoreflex loop was not significantly reduced. The individual data are shown in figure 1. Time constants, delays and drift were not significantly influenced by methazolamide (data not shown).

Discussion

Our data show a clear decrease in apneic threshold and central CO_2 sensitivity by low-dose methazolamide. However, we could not demonstrate a significant decrease in peripheral CO_2 sensitivity in our animals. The agent caused a small but significant decrease in base excess.

Previously, we have attributed the decrease in apneic threshold by low-dose acetazolamide to a possible effect on the relationship between cerebral blood flow (CBF) and brain tissue PCO_2 ($PtCO_2$).[11] Similar to our previous experiments with low-dose acetazolamide, a significant $P_{(a-ET)}CO_2$ gradient was also absent after methazolamide, albeit at a somewhat lower dose. Methazolamide, however, is a much more permeable inhibitor with an about equal affinity for carbonic anhydrase II,[1,2] so that compared to acetazolamide (4 mg.kg^{-1}) after 3 mg.kg^{-1} methazolamide the fractional inhibition of intracellular CA can be expected to be at least equal if not larger. Avoiding complete inhibition is important to prevent large tissue acidosis, which then could have explained the large decrease in apneic threshold. Our data show that the decrease in mean apneic threshold was considerably larger than after low-dose acetazolamide, and this may be caused by additional inhibition of intracellular CA in brain capillary cells.

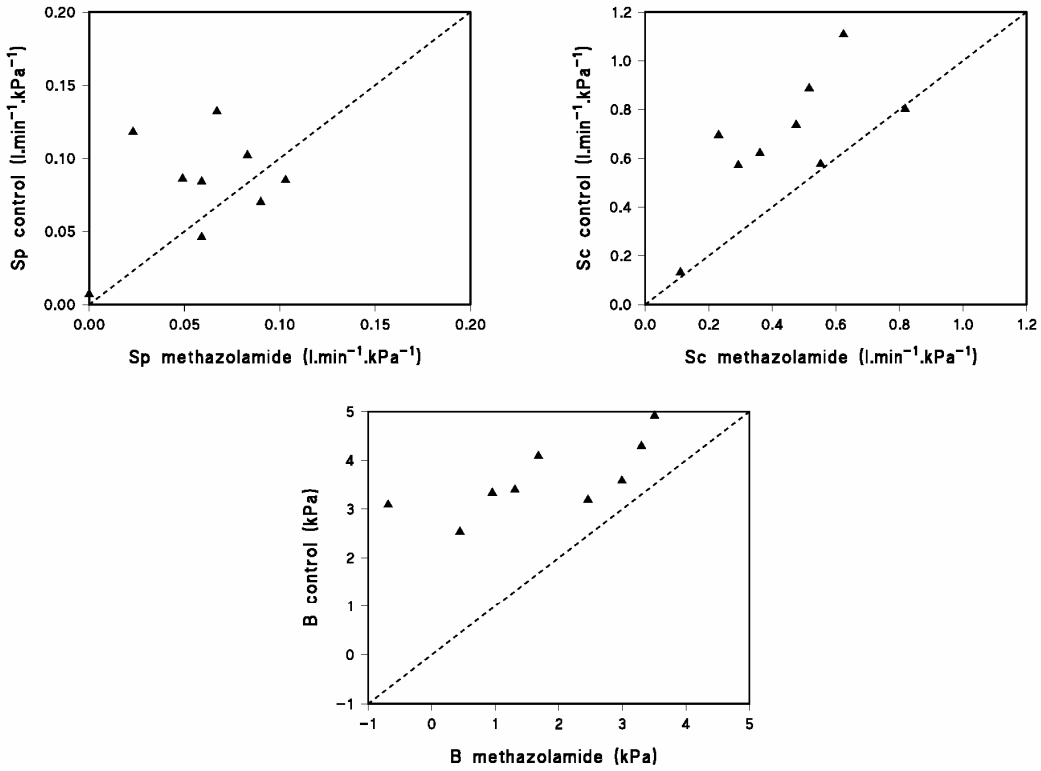

Figure 1. Scatter diagrams of the respiratory parameters before (control) and after methazolamide.

A change in the CBF–$PtCO_2$ relationship may also result in a change in CO_2 sensitivity of the central chemoreflex loop. Compared to acetazolamide, we now find a smaller decrease in central CO_2 sensitivity. After low-dose methazolamide, however, inhibition of brain carbonic anhydrase could have altered central chemoreceptors CO_2 sensitivity for at least two reasons. First, carbonic anhydrase has been shown to be present in rostroventrolateral medullary structures associated with central chemoreceptors,[8,9] and second, in a previous study we showed that a high-dose methazolamide caused *an increase* in CO_2 sensitivity independently of extracellular and erythrocytic CA inhibition.[17] Inhibition of brain carbonic anhydrase is followed by decrease in intracellular buffer capacity against CO_2.[18] Intracellular pH changes play a crucial role in central CO_2 chemoreception (references see 19). In conclusion, the lower decrease in central CO_2 sensitivity by methazolamide compared to that by acetazolamide may be caused by a combined effect of the former on the CBF–$PtCO_2$ relationship (tending to decrease the CO_2 sensitivity of the central chemoreflex loop) and on the buffer capacity of central chemoreceptors (tending to increase their sensitivity to changes in PCO_2).

An interesting finding was that, in contrast to acetazolamide,[11] methazolamide did not significantly reduce the CO_2 sensitivity of the peripheral chemoreflex loop. The carotid bodies contain several CA isoforms.[4-6,20] Because 3 $mg.kg^{-1}$ methazolamide will be sufficient for complete inhibition of sulfonamide-sensitive carbonic anhydrases within the carotid bodies (but not in erythrocytes due to their very large CA content),[1,2] its failure to reduce peripheral CO_2 sensitivity may seem surprising. Causing extracellular rather than extracellular *and* intracellular CA inhibition, low-dose acetazolamide induces a clear reduction in peripheral CO_2 sensitivity, while the steady-state hypoxic response is reduced by 50%.[11,12] Our findings are reminiscent of data obtained from *in vitro* carotid body preparations in which complete CA inhibition appeared to reduce the fast initial rather than the steady-state CO_2 response.[21] One possible explanation of the different effects of methazolamide and acetazolamide on the peripheral chemoreflex loop may be related to a specific effect of acetazolamide on Ca^{2+}-dependent large-conductance potassium (BK) channels that is not shared by methazolamide.[14] While acetazolamide has a specific, powerful stimulating effect on these channels (*i.e.*, BK channels from skeletal muscles of K^+-deficient rat), methazolamide entirely lacks such an opening effect.[14] As recently shown by Williams *et al.,*[15] BK channels may play a crucial role in the response of type I carotid body cells to hypoxia. Unpublished data from our lab indicate that in contrast to acetazolamide, low-dose methazolamide does *not* reduce the steady-state hypoxic response in the cat indicating that BK channels may indeed be involved in the inhibiting effect of acetazolamide and that CA inhibition in the carotid bodies not necessarily reduces their steady-state response to changes in PO_2 and PCO_2.

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7 The Carbonic Anhydrase Inhibitors Methazolamide and Acetazolamide Have Different Effects on the Hypoxic Ventilatory Response in the Anesthetized Cat

Carbonic anhydrase (CA) catalyzes the interconversion of CO_2 and bicarbonate and plays a crucial role in respiration. To date, fourteen iso-enzymes have been characterized in higher vertebrates including humans.[1] A specific role of CA in respiration is indicated by the presence of various isoforms in tissues and cells that are directly or indirectly involved in ventilatory control such as lung and brain capillary endothelium, kidneys, muscles, carotid bodies and central chemosensitive areas in the rostroventrolateral medulla oblongata.[1,2]

Inhibitors of CA have a profound influence on the control of respiration. Due to different physical-chemical properties, various sulfonamide CA inhibitors have distinct dose-dependent effects. Acetazolamide (AZ), a moderately permeable sulfonamide, is the most frequently used inhibitor to study the role of CA in the hypercapnic response of the carotid body and has been shown to diminish its steady-state CO_2 sensitivity,[3,4] although other studies reported only a reduced response speed and/or elimination of over- and undershoots in carotid sinus nerve activity upon (removal of) sudden hypercapnic stimuli.[5,6] AZ reduces the CO_2-induced initial fast depolarization and nerve activity in type I carotid body cells and co-cultured petrosal ganglion neurons, respectively, without changing steady-state responses.[7] This is consistent with a role of CA in regulating the speed and magnitude of the initial CO_2-induced fall in intracellular pH in type I cells.[8] In a superfused carotid body preparation from cat, AZ reduces both the release of dopamine and the increase in carotid sinus nerve discharge upon acidic stimuli.[9] In the cat, AZ exerts inhibitory effects on carotid body-mediated reflexes. For example, low intravenous doses reduce both steady-state hypoxic sensitivity and the O_2–CO_2 interaction.[10,11] In a dose that completely inhibits erythrocytic CA, it causes a much greater rise in ventilation in carotid body denervated than in intact cats,[12] while in the latter the hypoxic ventilatory response is virtually abolished, an observation that also has been reported in man after an intravenous infusion of 500 mg.[12-14] To our knowledge, effects of AZ on the hypoxic response of the carotid sinus nerve or type I cells have not been reported, but the above findings in intact organisms clearly suggest that it may have inhibitory effects.

Methazolamide (MTZ) is a more lipophilic sulfonamide with an about equal K_I as AZ for CA II and IV.[15] MTZ does not seem to affect the magnitudes of the cat carotid sinus nerve responses to hypercapnia and hypoxia.[5,16,17] Whether MTZ affects the hypoxic response in intact organisms is unknown.

It would be interesting to compare the effects of AZ and MTZ on the hypoxic ventilatory response for two reasons. First, both agents may have different pharmacological effects. Recently, Tricarico *et al.* showed a direct, stimulating, action of AZ on voltage-sensitive large-conductance Ca^{2+}-dependent potassium (BK) channels in muscle cells from K^+ depleted rats,[18] an effect that is not shared by MTZ. At least in the rat, BK channels play a role in oxygen sensing by the carotid bodies,[19-21] but this could not be confirmed in type I cells from adult cats.[22] Despite the paucity of data on oxygen sensitive potassium channels in type I cells from cat, it would be interesting to compare the effects of MTZ and AZ in this species, given the different pharmacological properties of these agents. Second, if the elimination of the hypoxic response by high-dose AZ is due to inhibition of one or more CA isoenzymes, a high dose of the more permeable CA inhibitor MTZ should also abolish it. The purpose of the present study in anesthetized cats was to examine if inhibition of carbonic anhydrase in all body tissues by an agent other than AZ would reduce or abolish the hypoxic response and, second, if AZ could reduce it by a mechanism other than by inhibition of CA isoforms. To study the effect of CA inhibition on the hypoxic response, we administered MTZ in a dose (33 $mg.kg^{-1}$) that would inhibit CA in all body tissues. To compare the effects of low doses of both sulfonamides, we infused low doses (3 $mg.kg^{-1}$) of MTZ and AZ (in this order) in a separate group of animals.

Methods

Experiments were performed in fifteen adult cats of either sex (mean body weight 3.55 ± 0.98 kg) after approval by the Ethical Committee for Animal Experiments of the University of Leiden. The animals were sedated with 10 $mg.kg^{-1}$ ketamine hydrochloride i.m. Anesthesia was induced with 2% sevoflurane in 30% O_2 in N_2. Both femoral arteries and the right femoral vein were cannulated, 20 $mg.kg^{-1}$ α-chloralose and 100 $mg.kg^{-1}$ urethane were slowly administered intravenously, and the volatile anesthetic was gradually withdrawn. Then an infusion of a α-chloralose-urethane solution was started at a rate of 1.0–1.5 $mg.kg^{-1}.h^{-1}$ α-chloralose and 5.0–7.5 $mg.kg^{-1}.h^{-1}$ urethane to obtain a stable but light anesthesia.[23]

Respiration

The trachea was cannulated at midcervical level and connected to a respiratory circuit. Tidal volume was measured electronically by integrating airway flow obtained from a pneumotachograph (number 0 flow transducer, Fleisch, Lausanne, Switzerland) connected to a differential pressure transducer (PM 197, Statham, Los Angeles, CA, USA). The respiratory fractions of O_2 and CO_2 were continuously measured with a gas monitor (Multicap, Datex, Helsinki, Finland), which was calibrated with gas mixtures of known composition. The inspiratory gas concentrations were made with computer-steered mass flow controllers (AFC 260, Bronkhorst High-Tech, Veenendaal, The Netherlands). The end-tidal PCO_2 ($P_{ET}CO_2$) and end-tidal PO_2 ($P_{ET}O_2$) were controlled independently by a PC by adjusting the inspiratory gas fractions. Arterial blood samples were taken from the right femoral artery for

blood gas analysis (ABL 700, Radiometer Copenhagen, Brønshøj, Denmark). Arterial blood pressure was measured using a pressure transducer (P23ac, Statham). Rectal temperature was maintained within 1 ºC. All signals were converted to digital values (sample frequency 100 Hz), processed by a PC and stored breath-by-breath.

Study Design

In a first group of ten animals (group I), we measured the steady-state ventilatory O_2 response at constant $P_{ET}CO_2$ in the control situation. Then, 3 $mg.kg^{-1}$ methazolamide (Sigma, Zwijndrecht, The Netherlands; dissolved in NaOH, adjusting the pH to about 7.4 with HCl) was infused in a volume of about 5 ml (~1 $ml.min^{-1}$). About 60 min thereafter, a second isocapnic steady-state hypoxic response curve was determined. After finishing these measurements, 3 $mg.kg^{-1}$ acetazolamide (Diamox, AHP Pharma, Hoofddorp, The Netherlands; 2 $mg.ml^{-1}$ in saline) was infused and after another 60 min a third steady-state isocapnic hypoxic response was measured.

After control measurements in a second group of five animals (group II), an isocapnic hypoxic response curve was determined after 3 $mg.kg^{-1}$ MTZ as described above. Then, to obtain complete inhibition of CA in all tissues, these animals were given an additional dose of 30 $mg.kg^{-1}$ MTZ, whereafter another isocapnic hypoxic reponse curve was determined.

Isocapnic Hypoxic Response and Data Analysis

Near step-wise changes in $P_{ET}O_2$ were achieved by adjusting the O_2 fraction of the inspired air; the $P_{ET}CO_2$ was kept constant by adjusting the inspired CO_2 concentration.[24] In this way, a new steady-state level of ventilation has established after 5–6 min.[24] The last 20 breaths of this period were averaged to yield steady-state ventilation at a given $P_{ET}O_2$. Blood samples were taken at the end of the steady-state periods to analyze blood gases. Using a least square method, inspiratory ventilation (V_i) was fitted to arterial PO_2 (PaO_2) according to the exponential function:[24,25]

$$V_i = G \cdot \exp(-D \cdot PO_2) + A$$

in which G is the overall hypoxic sensitivity ($l.min^{-1}$), D is a shape parameter (kPa^{-1}) and A is the ventilation during hyperoxia ($l.min^{-1}$). None of these three parameters was fixed but all were estimated with the aid of an iteration method.

The statistical analysis was performed using SPSS v11.0 for windows (SPSS Inc., Chicago, IL, USA). To detect significant differences between the three treatments (control, MTZ and AZ), we performed a one-way repeated measures analysis of variance with *post-hoc* Bonferroni correction. *P*-values < 0.05 were considered significant. Unless otherwise indicated, data are presented as mean ± SD.

Results

In the animals of group I (n = 10), the hypoxic responses were measured by forcing the $P_{ET}O_2$ level from hyperoxia to hypoxia resulting in PaO_2 levels that covered the range from 60.5 ± 4.1 to 5.6 ± 1.4 kPa in control, 57.1 ± 5.7 to 5.7 ± 1.0 kPa after MTZ and 57.6 ± 7.4 to 5.9 ± 1.2 kPa after AZ. The dose of 3 $mg.kg^{-1}$ MTZ did not cause a rise in the mean arterial-to-end-tidal PCO_2 ($P_{(a–ET)}CO_2$) gradient indicating the absence of effective erythrocytic carbonic anhydrase inhibition (see table 1). The additional dose of 3 $mg.kg^{-1}$ AZ caused a significant increase in the gradient by about 0.3 kPa, probably too small to cause appreciable tissue acidosis and a further rise in hyperoxic ventilation (see table 1). Table 1 also shows that MTZ caused a mild acidosis that was more pronounced after the subsequent infusion of AZ.

Table 1. Effects of methazolamide (MTZ) and acetazolamide (AZ) on the steady-state hypoxic response in ten cats.

	Control	**MTZ**	**AZ**
G ($l.min^{-1}$)	1.93 ± 1.32	1.89 ± 0.90	1.09 ± 0.92 *
D (kPa^{-1})	0.20 ± 0.07	0.22 ± 0.06	0.14 ± 0.06
A ($l.min^{-1}$)	0.86 ± 0.33	1.30 ± 0.40 †	1.32 ± 0.43 ‡
$PaCO_2$ (kPa)	4.63 ± 0.75	4.55 ± 0.78	4.78 ± 0.81
$P_{(a–ET)}CO_2$ (kPa)	-0.01 ± 0.30	-0.08 ± 0.38	0.29 ± 0.61 §
Arterial pH	7.338 ± 0.04	7.307 ± 0.05 §	7.253 ± 0.05 a
Base excess (mM)	-6.75 ± 1.26	-8.56 ± 1.83 b	-10.53 ± 1.97 a

* P = 0.003 *versus* control and P = 0.01 *versus* MTZ; † P = 0.003 *versus* control; ‡ P = 0.002 *versus* control; § P = 0.007 *versus* control; a P = 0.000 *versus* control; b P = 0.006 *versus* control

An example of the effect of both agents on the hypoxic response in one animal is shown in figure 1. The overall results, summarized in table 1, show that the mean control hypoxic sensitivity in the ten animals studied was not different from that after MTZ (P = 0.88 *versus* control) while AZ reduced it by 44%. Compared to the control situation, all animals except one had a lower hypoxic sensitivity after AZ administration. The shape parameter D was unaffected by MTZ, while AZ tended to reduce it (P = 0.023 *versus* control). The hyperoxic ventilation A increased after MTZ but did not rise further after AZ.

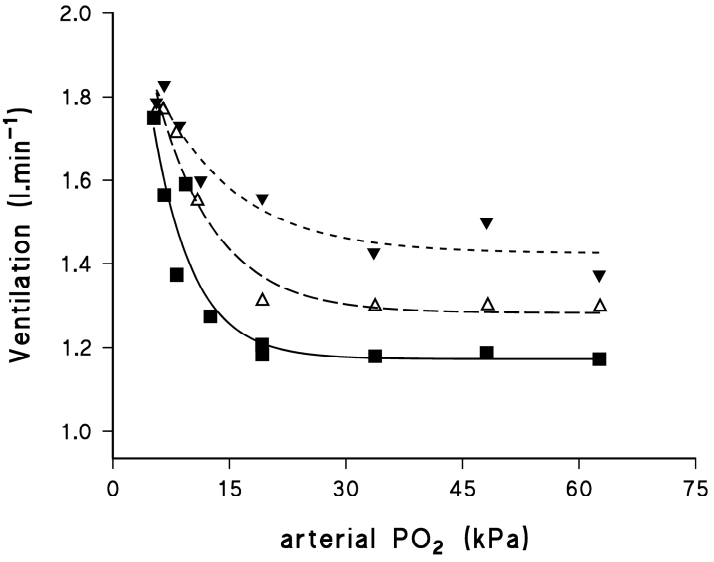

Figure 1. Steady-state hypoxic response curves in one animal before (squares) and after infusions of, respectively, 3 mg.kg^{-1} MTZ (open triangles) and 3 mg.kg^{-1} AZ (closed triangles). Curves are optimal fits (least square method) to a monoexponential equation with residual. Parameter values for G (l.min^{-1}), D (kPa^{-1}) and A (l.min^{-1}) were 1.52, 0.19 and 1.17 in control, 1.1, 0.13 and 1.28 after MTZ and 0.68, 0.10 and 1.43 after AZ, respectively.

The animals of group II (n = 5) were given 3 mg.kg^{-1} MTZ, followed by 30 mg.kg^{-1}. Hypoxic responses in these animals were measured by forcing the $P_{ET}O_2$ level from hyperoxia to hypoxia resulting in PaO_2 levels that covered the range from 58.1 ± 1.1 to 4.9 ± 0.8 kPa in control, 54.4 ± 4.3 to 4.9 ± 0.9 kPa after 3 mg.kg^{-1} MTZ and 56.6 ± 1.5 to 5.0 ± 0.9 kPa after 30 mg.kg^{-1} of the agent. In table 2, note the appearance of a large $P_{(a-ET)}CO_2$ gradient after 33 mg.kg^{-1}, indicating effective inhibition of erythrocytic carbonic anhydrase. An example of the effect of low- and high-dose MTZ in one animal is shown in figure 2. The scatter diagram of figure 3 shows the individual effect of low-dose MTZ in all fifteen animals (group I and II) studied. Note the absence of a systematic increase or decrease of parameter G by MTZ. All five animals of group II showed a substantial response to hypoxia after 33 mg.kg^{-1} MTZ (in three animals parameter G was even larger than in control), which is in sharp contrast after high-dose AZ, when the hypoxic response is abolished.[12,13] Overall, high-dose MTZ did not induce significant changes in hypoxic sensitivity, shape parameter and hyperoxic ventilation (table 2).

Table 2. Effects of methazolamide (MTZ) (3 and 33 mg.kg^{-1}, respectively) on the steady-state hypoxic response in five cats.

	Control	**MTZ 3 mg.kg^{-1}**	**MTZ 33 mg.kg^{-1}**
G (l.min^{-1})	3.26 ± 1.44	3.23 ± 1.16	3.00 ± 1.23
D (kPa^{-1})	0.27 ± 0.07	0.29 ± 0.10	0.27 ± 0.09
A (l.min^{-1})	0.79 ± 0.16	0.59 ± 0.21	0.65 ± 0.21
$PaCO_2$ (kPa)	5.75 ± 0.39	5.76 ± 0.31	6.14 ± 0.21
$P_{(a-ET)}CO_2$ (kPa)	0.40 ± 0.45	0.66 ± 0.72	2.40 ± 0.54 †
Arterial pH	7.262 ± 0.03	7.239 ± 0.04	7.217 ± 0.02 *
Base excess (mM)	-7.03 ± 0.88	-8.23 ± 1.27	-8.38 ± 1.09 *

Note that after 33 mg.kg^{-1} MTZ, the $PaCO_2$, pH and base excess represent equilibrium values *in vitro*; *in vivo* $PaCO_2$ in the blood perfusing the carotid bodies must have been considerably lower and pH considerably higher than the *in vitro* values shown here. * $P = 0.012$ *versus* control; † $P = 0.000$ *versus* control.

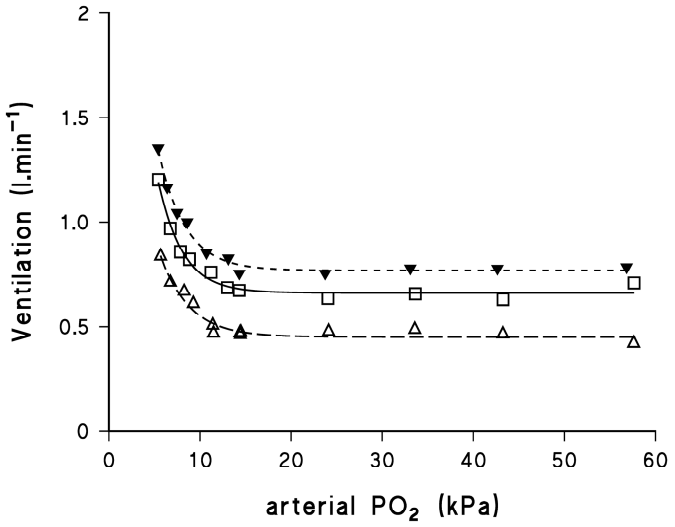

Figure 2. Example of steady-state response curves in one animal in control (squares), 3 mg.kg^{-1} MTZ (open triangles) and 33 mg.kg^{-1} MTZ (closed triangles), respectively. Curves are optimal fits (least square method) to a monoexponential equation with residual. Parameter values for G (l.min^{-1}), D (kPa^{-1}) and A (l.min^{-1}) were 3.99, 0.37 and 0.66 in control, 2.31, 0.31 and 0.45 after low-dose MTZ and 3.70, 0.34 and 0.77 after high-dose MTZ, respectively.

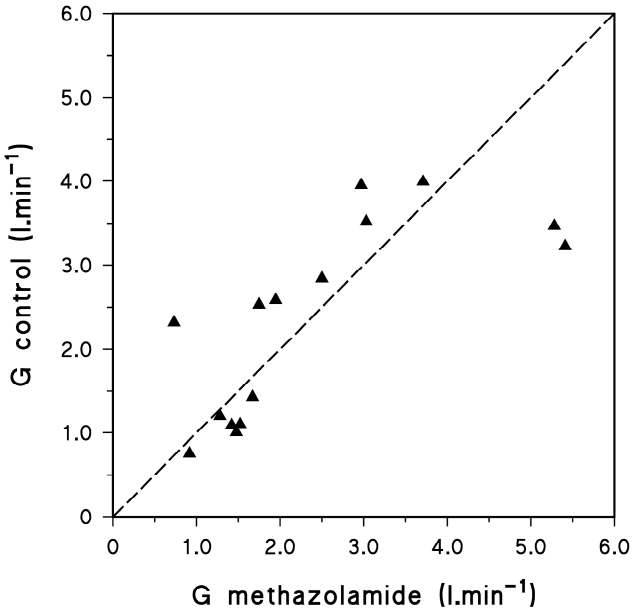

Figure 3. Scatter diagram of the effect of 3 mg.kg^{-1} MTZ on hypoxic sensitivity G in fifteen animals. Low-dose MTZ did not cause a systematic change in G.

Discussion

The main findings of this sudy can be summarized as follows. First, in a dose (33 mg.kg^{-1}) that completely inhibits carbonic anhydrase in all body tissues, methazolamide did not alter the steady-state hypoxic ventilatory response in the cat. Second, in a dose of 3 mg.kg^{-1}, the less lipophilic sulfonamide acetazolamide reduced hypoxic sensitivity by 44%, while an equal low dose of MTZ lacked this effect. These results indicate that full inhibition of CA does not reduce the steady-state hypoxic response in the cat and that the depressing effect of AZ may be caused by a pharmacological action other than on CA.

Study Design

Full physiological inhibition of CA is reached when 99.99% of the enzyme is inhibited.[26] The concentration of CA II in cat erythrocytes is very high providing a possible explanation why 3 mg.kg^{-1} MTZ did not widen the $P_{(a-ET)}CO_2$ gradient.[27] Because red cells contain much more CA than carotid bodies,[2] the fractional inhibition of the enzyme after 3 mg.kg^{-1} MTZ (which is evenly distributed) will be larger in the latter. In cat choroid plexus, also an organ with a relatively high CA concentration, this dose has shown to result in 99.58% inhibition.[28] We thus speculate that in the carotid bodies the inhibition will then be close to 99.99% if not more, so that a subsequent AZ dose would not exert a physiological effect *via* inhibition of local CA. Although in the absence of concrete data we can not exclude that the additional AZ

dose (group I) could have made the difference between incomplete and full carotid body CA inhibition, our observation of an entirely intact hypoxic response after a MTZ dose that completely inhibits CA in all tissues,[2,28] lends support to the view that the inhibiting effect of AZ in the animals of group I must be due to an effect unrelated to CA inhibition. Note that the AZ dose of 3 $mg.kg^{-1}$ was somewhat smaller than that was previously shown to reduce the hypoxic response.[11]

Tables 1 and 2 show that 3 $mg.kg^{-1}$ MTZ caused a decrease in mean pH of 0.031 in group I and 0.023 in group II. This mild acidosis could have counteracted an inhibitory effect of the agent on the hypoxic response. Low-dose AZ, however, causing a similar degree of mild acidosis, clearly reduces hypoxic sensitivity,[11,29] indicating that MTZ and AZ have different effects on the hypoxic response indeed. In the present study, AZ enlarged the MTZ-induced acidosis, but this did not prevent a clear reduction in hypoxic sensitivity. Consequently, AZ must have a potent inhibitory effect on the hypoxic response that is not shared by MTZ.

Effects of MTZ and AZ on the Hypoxic Ventilatory Response: Parameters A, D and G

In both men and animals, the hypoxic response is biphasic, starting with an initial rise in ventilation mediated by the carotid bodies, followed by a secondary decrease called hypoxic ventilatory depression (HVD). HVD is a poorly understood phenomenon: it is related to the magnitude of the initial carotid body stimulation, but possibly also to an increased washout of CO_2 from brain due to a rise in brain blood flow, and/or a release of inhibitory neurotransmitters.[30-32]

We examined the effects of MTZ and AZ on the steady-state hypoxic response curve, described by the exponential function $V_i = G \cdot \exp(-D \cdot PO_2) + A$, with hypoxic sensitivity G (comprising both the stimulation by the carotid bodies and HVD), shape factor D and hyperoxic ventilation A. Neither low- nor high-dose MTZ changed mean hypoxic sensitivity, while AZ, given after an equal initial MTZ dose (3 $mg.kg^{-1}$), reduced it by 44%. The hyperoxic ventilation A increased after low- but not high-dose MTZ. AZ tended to reduce parameter D.

Parameter A, the ventilation during hyperoxia is a complex variable and is influenced by the CO_2 sensitivity of the central chemoreflex loop, the X-intercept of the CO_2 response curve (apneic threshold), the prevailing (arterial and brain stem tissue) PCO_2/pH and a small, PCO_2-dependent, contribution of the carotid bodies to total ventilation. Similar to AZ,[29] low-dose MTZ causes a decrease in sensitivity of the central chemoreflex loop resulting in a less steep CO_2 response curve (unpublished observations in nine cats) and this would tend to reduce the value of parameter A. At the same time, however, also similar to AZ,[29] it causes a large decrease in the apneic threshold (so CO_2 response curves before and after MTZ intersect). The influence of the latter effect on parameter A will thus depend on the prevailing PCO_2: the lower the PCO_2 the higher the tendency for low-dose MTZ to increase parameter A.

Therefore, because the experiments in the animals of group II were performed at a background PCO_2 considerably higher than in group I, our finding of an unchanged value of A in this group by low-dose MTZ is not necessarily conflicting with the increase in group I.

The physiological significance of the shape parameter D remains unknown and we cannot explain the tendency for AZ to decrease it. In our previous study, we did not find an influence of AZ on D.[11]

The most important and surprising findings of this study are that neither low- nor high-dose MTZ changed the hypoxic sensitivity G. Previously, we ascribed the elimination of the hypoxic response by high-dose AZ solely to a total inhibition of CA isoenzymes in the carotid bodies.[12,13] Now, however, we may have to reconsider this view because the MTZ dose administered in group II (33 $mg.kg^{-1}$) will inhibit all extracellular membrane-bound CA IV as well as intracellular CA I and II.[2,26,28] An action of MTZ other than inhibition of CA is not known to us, so we can not speculate about a scenario in which complete carotid body CA inhibition by MTZ would abolish the O_2 response while at the same time an additional pharmacological action, not shared by AZ, would reverse this. The alternative, however, an action of AZ other than CA inhibition alone that is not shared by MTZ, may open a way to discuss our results against a background of recent studies showing unexpected actions of this agent.

Although there is evidence indicating that AZ has inhibiting effects on the carotid bodies (see introduction), we will discuss possible different effects of MTZ and AZ on *both* components of the hypoxic response namely the initial carotid body-mediated increase in ventilation and the secondary decrease (HVD).

Different effects of MTZ and AZ on HVD?

A hypoxia-induced release of inhibitory neurotransmitters may be one of the mechanisms that contributes to HVD.[30,32] AZ is known to reduce the excitability of neurons that are involved in seizures and as such it is used as an anticonvulsant.[2,33] Changes in extra- and intracellular pH of neurons will influence their excitability, and it is possible that compared to AZ, MTZ, by its larger permeability, may have different effects particularly on intracellular pH (pH_i) of carbonic anhydrase-containing neurons. In hippocampal CA3 neurons, CA inhibitors cause intracellular acidosis, and this effect, which is at least partly responsible for their anticonvulsant action, is larger with membrane permeant inhibitors.[34] Compared to AZ, MTZ has a superior inhibiting effect on seizures,[2,35] and if this would reflect a general decrease in activity in CA-containing neurons, the agent might be expected to promote rather than reduce HVD (compared to AZ), assumed that these neurons (or some of them) play a role in it.

Possible different effects of MTZ and AZ on the hypoxia-induced increase in cerebral blood flow (CBF) may also result in distinct effects on HVD. AZ has a reputation as a dilator of

cerebral vessels, but this effect is clearly dose-dependent and does not seem to be operative with intravenous doses lower than 5 $mg.kg^{-1}$.[36] Also, it remains to been seen whether low-dose AZ would alter the hypoxia-induced rise in CBF. In humans, after a usual oral clinical dose this does not seem to be the case.[37] We are not aware of studies showing a dilating effect of low-dose MTZ on cerebral vessels. At high dose, CBF will rise by the increase in tissue PCO_2 due to inhibition of erythrocytic CA.

Different effects of MTZ and AZ on the Carotid Bodies?

The most likely explanation for our results is a different effect of MTZ and AZ on the carotid bodies. One possibility is that AZ, with its vasodilatory reputation, increases carotid body blood flow, while MTZ lacks this effect. Recently, Pickkers *et al.* ascribed the vasodilatory effects of AZ on the peripheral circulation (forearm) in humans to a stimulating effect on large-conductance calcium-dependent potassium (BK) channels,[38] which they thought might be mediated by intracellular alkalosis that they had previously demonstrated in pig mesenteric arterial smooth muscle cells.[39] Because sulfonamides lacking CA inhibitory effects had a much smaller vasorelaxant effect, the authors argued that the inhibition of a CA isoenzyme results in intracellular alkalosis, followed by opening of BK channels.[38] In an *in vitro* carotid body preparation from cat, the specific BK channel inhibitor charybdotoxin has been reported to induce vasodilation indicating that this channel type may be involved in the regulation of carotid blood flow in this species.[40] For reasons explained above, we believe that if AZ would have increased carotid blood flow in our animals, this would most likely be due to an effect unrelated to CA inhibition (direct opening of BK channels? –see also below). Unfortunately, data on possible effects of MTZ on the peripheral circulation are lacking. From the available data in the literature we would certainly expect high-dose AZ to cause a large increase in carotid body blood flow. Whether this could explain the elimination of the hypoxic response that we showed previously remains to be seen.[12,13] Would high-dose MTZ fail to induce changes in carotid body blood flow? Studies examining the effects of low- and high-dose MTZ on the peripheral circulation and/or carotid body blood flow are warranted.

Finally, we discuss possible different effects of MTZ and AZ on type I carotid body cells. Type I cells contain carbonic anhydrase isoenzymes of which the precise subcellular locations remain to be elucidated except for the cytosolic isoforms CA II and III (possibly a membrane-bound isoform is also involved, see references 41–44). There is ample data to indicate that in the carotid bodies CA regulates the speed and magnitude of changes in pH_i of type I cells upon (removal of) sudden hypercapnic stimuli (reference see above). Hypoxia, however, does not cause a fall in pH_i,[45] and in this respect the absence of an effect of MTZ on the hypoxic response may not be as surprising as it may seem (also, see references 16 and 17). In this context then, we could ascribe the inhibiting effect of AZ to an action that is not related to inhibition of CA. The next issue is whether such an effect of AZ, unrelated to CA inhibition, could be mediated *via* BK channels. While there is ample data on the existence of specific oxygen-sensitive potassium channels in the carotid bodies of other species, *e.g.*, K_v channels

in rabbit and mouse, and TASK-1 and BK channels in rat, the information from cat is scarce.[20,21,46-48] In one report, it was found that a voltage-sensitive potassium current (inhibited by hypoxia) recorded in type I cells from adult cats was insensitive to charybdotoxin.[22] Another study in an *in vitro* perfused carotid body reported a decrease in carotid sinus nerve activity by this agent during hypoxia.[40] Clearly more studies are needed, particularly in type I cells from neonatal cat, to identify the oxygen-sensitive potassium channels in this species. It would also be interesting to compare the effects of MTZ and AZ in rat, because it is now well established that in this species BK channels play a role in oxygen sensing by the carotid bodies.[21] Finally, because AZ also appears to inhibit (directly or indirectly) various types of voltage-sensitive Ca^{2+} channels,[49] we can not exclude that this may also have contributed to the difference in effect of MTZ and AZ on the hypoxic response.

In conclusion, we have shown different effects of MTZ and AZ on the steady-state hypoxic response in the cat that in our opinion are best explained by an action of AZ on carotid body blood flow or type I cells that is not related to inhibition of carbonic anhydrase. Our data indicate that normal CA activity in the carotid bodies is not a prerequisite for a normal steady-state hypoxic response to occur. The abolishment of the hypoxic response by high-dose AZ that we previously reported is probably mediated by an action other than inhibition of carbonic anhydrase.

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8 Summary and Conclusions

The frequent intake and administration of substances for treatment and prevention of disease and/or pain has become an inherent part of daily life in the western world. Apart from their intended effect, most of these agents (especially those used in anesthetic practice) produce side effects. While some side effects are mild and have limited relevance others are potentially harmful to the patient. For example, respiratory depression from opioid analgesics may cause severe hypoxia, hypercapnia and acidosis and eventually the death of the patient (Br J Anaesth 2003; 91: 40-9). It is therefore of clinical importance to study the effects of such drugs on the ventilatory control system and understand the underlying mechanisms. This may be done by studying the drugs involved (such as opioids) or by studying other agents which unearth complex underlying mechanisms and increase our knowledge on ventilatory control. Furthermore, in order to avert (that is, prevent or treat) serious complications from these drugs it is of imminent importance to develop regimens which reverse drug-affected respiratory depression.

The first part of this thesis describes the respiratory effects of the opioid analgesic buprenorphine and the ability to reverse buprenorphine-induced respiratory depression with the non-specific opioid receptor antagonist naloxone. In the second part of the thesis the focus is on the mechanisms of the hypoxic and hypercapnic ventilatory responses. In these studies carbonic anhydrase inhibitors are infused in healthy volunteers and anesthetized cats and ventilatory responses to isocapnic hypoxia and hypercapnia are measured. Furthermore, the interaction of antioxidant treatment and inhibition of the enzyme carbonic anhydrase by acetazolamide on the ventilatory response to hypoxia is assessed in healthy volunteers.

In **chapter 2,** the analgesics buprenorphine and fentanyl were compared with respect to their respiratory effects. Data were obtained in humans (healthy volunteers) and awake adult rats. Like fentanyl, buprenorphine is a μ-opioid receptor agonist (J Neurosci 2003; 23: 10331-7), and causes respiratory depression. For both agents, dose–response relationships were obtained (human studies: fentanyl 0–7 $\mu g.kg^{-1}$, buprenorphine 0–9 $\mu g.kg^{-1}$; these are equianalgesic doses). It was observed that buprenorphine displayed a ceiling in respiratory effect: in humans respiration declined by about 50% of baseline ventilation at doses > 2 $\mu g.kg^{-1}$; in rats, arterial PCO_2 increased by just 1–1.5 kPa irrespective of dose. In contrast, fentanyl caused a dose-dependent respiratory effect with periodic breathing and apnea in humans at doses greater than 2 $\mu g.kg^{-1}$, and a linear dose-dependent increase in arterial PCO_2 in rats with death of the animals at doses > 90 $\mu g.kg^{-1}$. Although these findings suggest that buprenorphine is relatively safe (in contrast to fentanyl), one needs to be aware that data were obtained in

healthy volunteers (and animals) free of underlying disease, pain and co-medication. How buprenorphine behaves in (elderly) patients with pain, co-medication and underlying (pulmonary) disease remains unclear and needs further study.

It is clear from the data that buprenorphine performs like a partial agonist over the dose range tested with respect to respiratory depression (partial agonism indicates a partial effect despite full receptor occupancy). Evidently the data on buprenorphine may be considered clinically advantageous only when a similar ceiling effect is *not* observed for its analgesic (antinociceptive) effects. Buprenorphine's analgesic behavior was examined in **chapter 3.** In humans, the influence of two doses of buprenorphine (0.2 and 0.4 mg per 70 kg) on respiration and analgesia was assessed. As expected (**chapter 2**), buprenorphine's respiratory effects did not differ among the two doses tested. In contrast, a sharp increase in analgesic effect was observed going from 0.2 to 0.4 mg buprenorphine. These data (**chapters 2 and 3**) indicate a distinct pharmacological effect of buprenorphine on μ-opioid receptors expressed on neurons in the central nervous system involved in analgesia/antinociception (absence of ceiling effect, at least over the dose range tested) and μ-opioid receptors expressed on neurons involved in respiration (ceiling effect). Although there are various explanations possible for this distinct behavior (**chapter 3**), the most plausible and elegant explanation may be related to differences in μ-opioid receptor density in brain areas involved in analgesia/antinociception and areas involved in the control of breathing (Pharmaceut Res 2000; 17: 653-9). A relatively reduced μ-opioid receptor density in the latter areas may cause the partial agonist buprenorphine to display ceiling in respiratory depression, while the abundance of μ-opioid receptors in areas involved in analgesia/antinociception may cause the partial agonist buprenorphine to behave like a full agonist on analgesia (in contrast, the full μ-opioid agonist fentanyl shows an unlimited effect at both end points).

In **chapter 4,** the ability of naloxone to reverse opioid-induced respiratory depression is explored in an extensive set of experiments (data were obtained in 99 subjects). The data showed that naloxone reversed morphine- and alfentanil-induced respiratory depression at relatively low doses (naloxone doses up to 6 $\mu g.kg^{-1}$). In contrast, much greater doses were needed to reverse 0.2 and 0.4 mg buprenorphine-induced respiratory depression (doses $> 30\ \mu g.kg^{-1}$). Furthermore, full reversal was only possible when naloxone was given as a continuous infusion. The need for the high naloxone dose is related to the high buprenorphine μ-opioid receptor affinity (*K*-values are in the nanomolar range (Eur J Pharmacol 2005; 507: 87-98)); the need for a continuous infusion to the very short elimination half-life of naloxone. Finally, a remarkable and important observation was that naloxone displays a bell-shaped dose–response curve (or inverse U-shaped response curve). At doses greater than 5 mg, reversal of respiratory depression decreased dose-dependently. These data do remind of the bell-shaped dose–response curve which is often observed in animal studies (Br J Pharmacol 1981; 74: 627-33 (data on analgesia); Br J Pharmacol 1982; 75: 118 (data on respiration); see also **chapter 1**). The best explanation for these findings is the existence of non-competitive autoinhibition, in which there are two μ-opioid receptor subpopulations, one mediating the

agonistic properties at low dose, the other mediating the antagonistic properties at high dose (Life Sci 1981; 29: 2699-708). Irrespective of the mechanism, the data indicate that buprenorphine-induced respiratory depression is relatively resistant to reversal by naloxone needing a high dose and a continuous infusion. Furthermore, one needs to take into account the shape of the naloxone dose–response curve, as too high naloxone doses will be inactive in causing reversal of buprenorphine-affected breathing.

The reduction in CO_2 sensitivity of the central chemoreflex loop by low-dose methazolamide (**chapter 6**) confirms earlier findings with acetazolamide from the same animal preparation (J Physiol Lond 1996; 495: 227-37), and could be explained by assuming a role of carbonic anhydrase (CA) in the regulation of cerebral blood flow. The rise in brain blood flow is one of the factors that determine the magnitude of the change in brain stem PCO_2 –the assumed stimulus to the central chemoreceptors– upon (step) changes in end-tidal PCO_2. The lower central CO_2 sensitivity after low-dose acetazolamide and methazolamide might be due to a larger brain blood flow response after inhibition of membrane-bound carbonic anhydrase (CA IV) in capillary endothelium or at the extracellular face of astrocytes and/or oligodendrocytes (J Neurocytol 2000; 29: 263-9; Proc Natl Acad Sci USA 1992; 89: 6823-7; Cerebrovasc Dis 2000; 10: 65-9). Acetazolamide is known to cause cerebral vasodilation but this effect is dose-dependent and seems to occur only at intravenous doses > 5 $mg.kg^{-1}$ (Cerebrovasc Dis 2000; 10: 65-9). Further studies are warranted to examine the influence of low-dose acetazolamide and methazolamide on the steady-state relationship between arterial PCO_2 and brain blood flow.

From the perspective of enzyme inhibition alone, the reduction in the acute (**chapter 5**) and steady-state (**chapter 7**) ventilatory sensitivity to hypoxia by low-dose acetazolamide is difficult to understand. The carotid body type I cells contain several carbonic anhydrase iso-enzymes of which CA II and CA III are identified in the rat (J Anat 2003; 202: 573-7), while also at least one membrane-bound isoform seems to be present (Neurosci Lett 1994; 166: 126-30). From animals studies it is clear that inhibition of carbonic anhydrase in the carotid bodies by methazolamide slows down its dynamic response upon sudden acidic (hypercapnic) stimuli without altering its steady-state sensitivity (Am J Physiol 1991; 265: C565-73). Note that, in contrast to low-dose acetazolamide (J Physiol Lond 1996; 495: 227-37), low-dose methazolamide did *not* appear to reduce the CO_2 sensitivity of the peripheral chemoreflex loop in the cat (**chapter 6**). A further interesting finding in the *in vitro* carotid body from cat was that methazolamide does *not* reduce the response to hypoxia. The failure of low-dose methazolamide to reduce isocapnic hypoxic sensitivity in the intact cat (**chapter 7**) may fit very well in this scenario. Even more interesting was the finding (**chapter 7**) that, in sharp contrast to acetazolamide (J Appl Physiol 1992; 72: 1717-23), even high-dose methazolamide does not reduce hypoxic sensitivity indicating that normal functioning of carbonic anhydrase is not a prerequisite for a normal hypoxic response to occur.

The above findings clearly indicate that acetazolamide and methazolamide have different effects on carotid body-mediated responses to changes in PCO_2 and PO_2. Because on the one hand methazolamide, causing full inhibition of carbonic anhydrase in all body tissues, did not reduce the hypoxic response, while on the other hand acetazolamide, a less lipophilic inhibitor with an even somewhat less affinity to CA II and IV (Mol Pharmacol 1993; 44: 901-5) had profound inhibitory effects, suggest that the latter agent may exert its effect by a distinct pharmacological action. One interesting possibility is an influence on voltage-sensitive large conductance Ca^{2+}-dependent potassium (BK) channels. BK channels belong to the candidates of potassium channels that play a role in oxygen sensing by the carotid bodies (Science 2004; 306: 2093-7). In muscular cells from potassium depleted rats, acetazolamide has shown to behave as a specific BK channel opener, while methazolamide entirely lacks this effect (FASEB J 2004; 18: 760-1). It is tempting to speculate about the possibility of acetazolamide acting *via* BK channels in the carotid bodies although we have no experimental evidence to show this. However, it is of interest to note that the vasorelaxant effect of low-dose acetazolamide on the human peripheral circulation has been supposed to be mediated *via* BK channels (Br J Pharmacol 2001; 132: 443-50). Thus low-dose acetazolamide may well have measurable physiological effects that are mediated *via* BK channels. Effects of acetazolamide on other channel types such as calcium and sodium channels can also not be excluded (**chapter 7**).

Another interesting feature of potassium channels is their redox sensitivity (Am J Physiol 1998; 275: C1-24; Clin Exp Pharmacol Physiol 2002; 9: 305-11). **Chapter 5** described the reduction of the isocapnic ventilatory response to acute (< 5 min) hypoxia and the abolishment of the O_2–CO_2 interaction (*i.e.*, the increase in hypoxic sensitivity with increasing PCO_2 levels) by acetazolamide and the reversal of these effects by antioxidants in humans. The most plausible, although entirely speculative, explanation for these interesting observations is that acetazolamide and antioxidants may exert independent and different effects on potassium channels (opening by acetazolamide, closure by antioxidants, both responses are reported from reduced preparations). The O_2–CO_2 interaction seems related to the intracellular Ca^{2+} concentration in type I carotid body cells (Am J Physiol 2000; 279: L36-42). A hyperpolarizing action of acetazolamide (no matter this is caused by alkalizing the cell interior or by directly opening BK channels) could reduce the influx of calcium ions into oxygen sensing cells, thus explaining the abolishment of the O_2–CO_2 interaction by acetazolamide that we already had reported previously in the cat (J Physiol 2001; 537: 221-9).

Combining the data from **chapter 5** with those of previous studies which showed a reversal by antioxidants of carotid body inhibition by subanesthetic concentrations of halogenated volatile anesthetics (J Physiol Lond 2002; 544: 931-8 (data on halothane); Anesthesiology 2005; 102: 47-53 (data on isoflurane); see also **chapter 1**), the question arises whether antioxidants could be a general, easy and relatively harmless tool to relieve the carotid bodies from pharmacological inhibition and whether such property of antioxidants could be of clinical significance in anesthetic care. More specifically, would antioxidants also be able to

reverse depressant effects from opioids (*e.g.*, buprenorphine) more easily than naloxone and without affecting their analgesic effect? Another, as yet unexplored, issue is whether (membrane permeable) antioxidants would also be able to reverse depressant effects of pharmacological agents on central chemoreceptors and respiratory neurons. Both issues will be subject of future investigations in our laboratories.

In conclusion, at the end it is now possible to address the aims of this thesis as outlined on page 15:

1. **Description of the dose–respiratory response relationship of buprenorphine and fentanyl.** The opioid analgesic buprenorphine displays a dose-dependent depression of ventilation which levels off at a relatively low dose of 2 $\mu g.kg^{-1}$. This phenomenon is well described by the term ceiling. In this respect, buprenorphine behaves very differently from fentanyl which shows a dose-dependent respiratory effect causing cyclic breathing and apnea at doses greater than 2 $\mu g.kg^{-1}$. These data indicate that buprenorphine is a partial agonist of the μ-opioid receptor.

2. **Comparison of the respiratory and analgesic behavior of buprenorphine.** In contrast to its respiratory behavior (ceiling), buprenorphine's analgesic behavior is different. Over the dose range tested (0 to 6 $\mu g.kg^{-1}$) no ceiling is observed. A possible mechanism may be found in differences in μ-opioid receptor density in brain areas involved in pain and respiration with a reduced density of opioid receptors in areas involved in the control of breathing.

3. **Reversal of opioid-induced respiratory depression with special interest on buprenorphine.** In contrast to morphine and alfentanil, buprenorphine is relatively resistant to reversal by the aspecific opioid antagonist naloxone. High naloxone doses are needed at the correct naloxone dose-window (2 to 4 mg per 70 kg, given in 30 min). Greater doses of naloxone produce a decrease in reversal activity. The results of these 5 studies demonstrate that the specific dose and mode of administration of naloxone is very important to obtain a full and long-lasting reversal of buprenorphine-induced respiratory depression. The data show that even after administration of large boluses of naloxone or boluses plus brief infusions, respiratory depression induced by buprenorphine recurs and persists for the duration of the study. Additional studies are required to define the dose and the mode of administration of naloxone to restore breathing and maintain it at an adequate level in the clinical setting, which is complicated by acute and chronic pain, high doses of opioids, long-acting opioids, and various sustained release preparations of opioids.

4. **Ability of antioxidants to reverse acetazolamide-induced depression of the ventilatory response to acute hypoxia.** In a complex set of experiments in healthy volunteers, it is observed that acetazolamide reduces the isocapnic ventilatory response to acute hypoxia (< 3 min) as well as the O_2–CO_2 interaction at the carotid bodies. A mix of ascorbic acid and α-tocopherol (vitamin C and E) is able to reverse the acetazolamide-induced effects at the carotid bodies. It is argued that these observations are due to independent effects of acetazolamide and antioxidants on potassium channels of type I carotid body cells. The results of this study not only shed important light on the mechanism of oxygen sensing at the carotid bodies but also on the mechanism of acetazolamide and its beneficial effect in high altitude disease (mountain sickness). Further studies are needed to explore this issue and to address the question whether similar mechanisms play a role in oxygen sensing at other sites, such as the pulmonary vasculature.

5. **Effects of the carbonic anhydrase inhibitors acetazolamide and methazolamide on ventilatory responses to hypoxia and hypercapnia.** In anesthetized cats, low-dose methazolamide lacks a depressant effect on peripheral CO_2 sensitivity and the ventilatory response to steady-state hypoxia. Even at high dose the ventilatory response to steady-state hypoxia remains unaffected. In contrast, even at low dose, acetazolamide reduces the ventilatory response to acute and steady-state hypoxia. These data strongly suggest that carbonic anhydrase is not involved in oxygen sensing at the carotid bodies. They further indicate that acetazolamide but not methazolamide interacts with a system (such as a protein) which plays a crucial role in oxygen sensing and the ventilatory response to hypoxia. A possible candidate is the Ca^{2+}-dependent potassium (BK) channel.

8 Samenvatting en Conclusies

Het gebruik van geneesmiddelen in de behandeling en preventie van ziekte en pijn is niet meer weg te denken uit onze samenleving. De meeste geneesmiddelen hebben behalve hun beoogd effect ook bijwerkingen (vooral die middelen die door de anesthesioloog worden gebruikt). Het merendeel van deze bijwerkingen is mild en heeft geen consequenties voor de patiënt. Sommige bijwerkingen daarentegen zijn potentieel bedreigend. Bijvoorbeeld, ademdepressie veroorzaakt door opioïden (morfine en aanverwante stoffen) kan aanleiding geven tot het ontstaan van hypoxie, hypercapnie en acidose, en kan soms zelfs de dood van de patiënt tot gevolg hebben (Br J Anaesth 2003; 91: 40-9). Het is daarom van belang het effect van geneesmiddelen op de ademhaling te bestuderen en de onderliggende mechanismen te doorgronden. Dit kan men doen door deze geneesmiddelen zelf te onderzoeken (bijvoorbeeld opioïden) of door de bestudering van andere agentia die ons inzicht verschaffen in het complexe mechanisme van de ademregulatie. Verder is het van evident belang om behandelingen te ontwikkelen die toegepast kunnen worden indien zich levensbedreigende bijwerkingen en situaties voordoen zoals bijvoorbeeld ademdepressie door opioïden.

Het eerste deel van dit proefschrift beschrijft de ademhalingseffecten van de opioïde pijnstiller buprenorfine en de mogelijkheden om buprenorfine-geïnduceerde ademdepressie om te keren met behulp van de niet-specifieke opioïd receptor antagonist naloxon. In het tweede deel ligt de nadruk op het mechanisme van de reflexmatige ademhalingsreactie op hypoxie en hypercapnie. Remmers van het enzym carbo-anhydrase worden toegediend aan vrijwilligers en genarcotiseerde katten en de ademhalingsreacties op isocapnische hypoxie en hypercapnie worden gemeten. Tenslotte wordt de interactie tussen antioxidanten en de carbo-anhydrase remmer acetazolamide op de ademhalingsrespons op hypoxie bestudeerd in gezonde vrijwilligers.

In **hoofdstuk 2** werden de ademhalingseffecten van twee analgetica vergeleken: fentanyl en buprenorfine. Experimenten werden uitgevoerd in de mens (gezonde vrijwilligers) en de (wakkere) rat. Buprenorfine veroorzaakt zijn effect, net als fentanyl, door activatie van de μ-opioïd receptor (J Neurosci 2003; 23: 10331-7) en veroorzaakt (net als fentanyl) ademdepressie. De doses die werden getest varieerden in de mens voor fentanyl van 0 tot 7 $\mu g.kg^{-1}$ en voor buprenorfine van 0 tot 9 $\mu g.kg^{-1}$ (het betreft equi-analgetische doses). Buprenorfine toonde een duidelijk plafond in het ademhalingseffect: in een dosering groter dan 2 $\mu g.kg^{-1}$ daalde de ademhaling met maximaal 50% in de mens; in de rat steeg de arteriële PCO_2 met maximaal 1–1,5 kPa onafhankelijk van de dosis. In tegenstelling tot buprenorfine veroorzaakte fentanyl een dosisafhankelijk effect op de ademhaling in de mens met cyclisch

ademen en apneu (ademhalingsarrest) bij doses groter dan 2 $\mu g.kg^{-1}$, en een lineaire dosisafhankelijke stijging van de arteriële PCO_2 in de rat met de dood tot gevolg hebbend bij doses groter dan 90 $\mu g.kg^{-1}$. Hoewel deze bevindingen suggereren dat buprenorfine een relatief veilig analgeticum is (in tegenstelling tot fentanyl), moet men in ogenschouw nemen dat de hier besproken studies zijn uitgevoerd in gezonde vrijwilligers (en dieren) zonder pijn, medicatie en onderliggende ziekten. Hoe buprenorfine zich gedraagt in (oudere) patiënten met pijn, medicatie en onderliggende (pulmonale) problematiek blijft onduidelijk en verdient verdere studie.

Het is duidelijk dat de data aangeven dat buprenorfine zich in het geteste dosisbereik gedraagt als een partiële agonist voor de ademdepressie die het teweegbrengt (een partiële agonist veroorzaakt een partieel effect ondanks de volledige bezetting van de betrokken receptoren). Deze bevindingen zijn echter dan pas klinisch relevant indien zich een soortgelijk plafondeffect *niet* voordoet voor de beoogde pijnstillende effecten van buprenorfine. Om dit te onderzoeken werden in **hoofdstuk 3** de ademhalingseffecten en pijnstillende effecten van twee doses buprenorfine (0,2 en 0,4 mg per 70 kg) vergeleken in de mens. Zoals eerder voorspeld (**hoofdstuk 2**) was er geen verschil in de ademhalingseffecten van deze twee doses buprenorfine. Het pijnstillend effect nam daarentegen significant toe gaande van 0,2 naar 0,4 mg buprenorfine. De data die zijn verzameld in **hoofdstukken 2 en 3** geven aan dat buprenorfine zich anders gedraagt voor de effecten die worden veroorzaakt door activatie van μ-opioïd receptoren betrokken bij pijnstilling (geen plafondeffect) *versus* μ-opioïd receptoren betrokken bij de ademhaling (wel plafondeffect). Hoewel er voor dit verschijnsel verschillende verklaringen mogelijk zijn (**hoofdstuk 3**), is de meest waarschijnlijke en elegante verklaring een mogelijk verschil in receptordichtheid in de hersendelen betrokken bij de twee gemeten eindpunten (Pharmaceut Res 2000; 17: 653-9). Een relatief lage dichtheid van μ-opioïd receptoren in die hersendelen betrokken bij ademhaling en een relatief hoge receptordichtheid in die delen betrokken bij pijnstilling kan verklaren dat de partiële opioïd agonist buprenorfine wel een plafondeffect vertoont voor ademhaling maar niet voor pijnstilling (terwijl de volle μ-opioïd agonist fentanyl een ongelimiteerd effect voor beide eindpunten laat zien).

In **hoofdstuk 4** werd onderzocht of de niet-selectieve opioïd antagonist naloxon in staat is de ademdepressie door opioïden om te keren. Het betreft een uitgebreide set experimenten in 99 proefpersonen. De buprenorfine data moeten worden bezien tegen de achtergrond van de waarneming dat naloxon al in een relatief lage dosering (< 6 $\mu g.kg^{-1}$) in staat is de ademdepressie veroorzaakt door morfine en alfentanil volledig om te keren. Veel hogere doses naloxon waren nodig om ademdepressie door buprenorfine (0,2 en 0,4 mg) om te keren (doses groter dan 30 $\mu g.kg^{-1}$). Ook bleek dat volledige omkering dan slechts mogelijk is indien naloxon als continue infusie wordt toegediend. De noodzaak van een hoge dosis naloxon is gerelateerd aan de hoge affiniteit van buprenorfine voor de μ-opioïd receptor (Eur J Pharmacol 2005; 507: 87-98); de noodzaak van een continue infusie aan de korte eliminatiehalfwaardetijd van naloxon. Een opmerkelijke bevinding was dat de naloxon dosis–

responscurve een omgekeerde U-vorm laat zien. Doses groter dan 5 mg veroorzaakten een dosis-afhankelijke afname van de omkering van de ademdepressie. Deze data zijn vergelijkbaar met de omgekeerde U-vorm van de buprenorfine dosis–responscurven voor antinociceptie en ademdepressie in dierexperimentele studies (Br J Pharmacol 1981; 74: 627-33 (analgesiedata); Br J Pharmacol 1982; 75: 118 (ademhalingsdata); zie ook **hoofdstuk 1**). Een mogelijke verklaring van deze resultaten is het optreden van zogenaamde non-competitieve autoinhibitie. Hierbij is er sprake van twee μ-opioïd receptorpopulaties: één populatie die de agonistische effecten van lage doses buprenorfine medieert, en één populatie die de antagonistische effecten medieert na toediening van hoge doses buprenorfine (Life Sci 1981; 29: 2699-2708). Deze data zijn van klinisch belang. Immers, voor omkering van de ademdepressie door buprenorfine zijn relatief hoge doses naloxon en een continue infusie noodzakelijk, maar een te hoge dosis zal de omkering bemoeilijken of zelfs tegenwerken.

De afname van de koolzuurgevoeligheid van de centrale chemoreceptoren door een lage dosis methazolamide (**hoofdstuk 6**) is een bevestiging van eerdere bevindingen met acetazolamide in hetzelfde dierpreparaat (J Physiol 1996; 495: 227-37). Beide resultaten kunnen verklaard worden indien men uitgaat van de rol van het enzym carbo-anhydrase (CA) in de regulatie van de hersendoorbloeding. Een toename van de hersendoorbloeding is één van de factoren die een rol spelen in de verandering in de hersenstam PCO_2 (de vermoedelijke stimulus van de centrale chemoreceptoren) na een verandering in eind-expiratoire PCO_2. De lagere centrale koolzuurgevoeligheid na lage doses acetazolamide en methazolamide kan dan verklaard worden door een toename van de hersendoorbloedingsgevoeligheid (voor CO_2) na remming van de membraangebonden CA isovorm IV. Deze isovorm bevindt zich in het endotheel van de bloedvaten in het brein en/of aan de extracellulaire membraanzijde van astrocyten en/of oligodendrocyten (J Neurocytol 2000; 29: 263-9; Proc Natl Acad Sci USA 1992; 89: 6823-7; Cerebrovasc Dis 2000; 10: 65-9). Acetazolamide veroorzaakt cerebrale vasodilatatie maar dit effect is dosisafhankelijk en treedt alleen op indien de dosis groter is dan 5 $mg.kg^{-1}$ (Cerebrovasc Dis 2000; 10: 65-9). Verder onderzoek is nodig om de invloed van lage doses acetazolamide en methazolamide op de relatie tussen de arteriële PCO_2 en de hersendoorbloeding vast te stellen.

Vanuit het gezichtspunt van een enzymremming alleen is de afname van de acute (**hoofdstuk 5**) en subacute of 'steady-state' (**hoofdstuk 7**) hypoxische ventilatoire gevoeligheid door een lage dosis acetazolamide moeilijk te begrijpen. De type I cellen van de glomuslichaampjes bevatten verschillende carbo-anhydrase iso-enzymen waarvan CA II en CA III zijn aangetoond in de rat (J Anat 2003; 202: 573-7), terwijl ook tenminste één membraangebonden isovorm aanwezig is (Neurosci Lett 1994; 166: 126-30). Dierexperimentele studies toonden aan dat remming van het carbo-anhydrase in de glomuslichaampjes door methazolamide de dynamische reactie op plotselinge zure (hypercapnische) stimuli vertraagt terwijl de steady-state hypoxische ventilatoire gevoeligheid niet verandert (Am J Physiol 1991; 265: C565-73). Opmerkelijk is dat, in tegenstelling tot lage doses acetazolamide (J Physiol Lond 1996; 495: 227-37), een lage dosis methazolamide de koolzuurgevoeligheid van de perifere

chemoreceptoren in de kat *niet* verlaagde (**hoofdstuk 6**). Een interessante bevinding in het *in vitro* glomuslichaampje van de kat was dat methazolamide de respons op hypoxie *niet* reduceert. Het resultaat dat een lage dosis methazolamide de isocapnische hypoxische gevoeligheid in de intacte kat *niet* remt (**hoofdstuk 7**) past hierbij. Nog interessanter is de bevinding (**hoofdstuk 7**) dat, in tegenstelling tot acetazolamide (J Appl Physiol 1992; 72: 1717-23), zelfs een hoge dosis methazolamide geen depressie van deze respons veroorzaakt. Dit alles geeft aan dat een normale activiteit van carbo-anhydrase niet noodzakelijk is voor een normale hypoxische ventilatoire reactie.

De bovengenoemde bevindingen wijzen erop dat acetazolamide en methazolamide een verschillend effect hebben op de door de glomuslichaampjes gemedieerde reacties op veranderingen in arteriële PCO_2 en PO_2. Terwijl methazolamide, in staat om een volledige remming van het enzym carbo-anhydrase in alle weefsels te veroorzaken, de hypoxische ventilatoire respons niet deprimeerde, veroorzaakte acetazolamide, een minder lipofiele CA-remmer met een zelfs geringere affiniteit voor CA II en CA IV (Mol Pharmacol 1993; 44: 901-5) juist wel depressie van de hypoxische respons. Dit suggereert dat beide middelen hun effect sorteren langs verschillende farmacologische wegen. Acetazolamide heeft invloed op de spanningsgevoelige Ca^{2+}-afhankelijke kalium (BK) kanalen. BK kanalen behoren tot die kaliumkanalen die een rol spelen bij de zuurstofmeting in het glomuslichaampje (Science 2004; 306: 2093-7). In spiercellen van kalium-gedepleerde ratten blijkt acetazolamide BK kanalen te openen terwijl methazolamide dit effect mist (FASEB J 2004; 18: 760-1). Het is verleidelijk te speculeren dat acetazolamide op BK kanalen in de glomuslichaampjes werkt hoewel er geen direct bewijs voor is. Indirect bewijs wordt gevormd door het feit dat een lage dosering acetazolamide een vaatverwijdend effect heeft door een vermoedelijk effect op BK kanalen (Br J Pharmacol 2001; 132: 443-50). Een effect van acetazolamide op andere ionkanalen (calcium- of natriumkanalen) kan echter ook niet worden uitgesloten (**hoofdstuk 7**).

Een belangrijke eigenschap van kaliumkanalen is hun redoxgevoeligheid (Am J Physiol 1998; 275: C1-24; Clin Exp Pharmacol Physiol 2002; 9: 305-11). In **hoofdstuk 5** werd beschreven dat acetazolamide in de mens de isocapnische acute (< 5 min) hypoxische ventilatoire respons deprimeert en dat de O_2–CO_2 interactie (de toename in hypoxische ventilatoire gevoeligheid bij een stijgende PCO_2) verdwijnt. Tevens werd aangetoond dat een cocktail van antioxidanten het effect van acetazolamide omkeert. De meest plausibele maar vooralsnog speculatieve verklaring is dat acetazolamide en antioxidanten onafhankelijke en verschillende effecten sorteren op kaliumkanalen (opening door acetazolamide, sluiting door antioxidanten). De O_2–CO_2 interactie blijkt te worden veroorzaakt door een toename in de intracellulaire Ca^{2+}-concentratie in de type I glomuscellen (Am J Physiol 2000; 279: L36-42). Hyperpolarisatie door acetazolamide (ofwel door een intracellulair alkaliserend effect ofwel door de opening van BK kanalen) zou de influx van calciumionen in de zuurstofmetende cellen verminderen, hetgeen het verdwijnen van de O_2–CO_2 interactie, zoals al eerder door ons in de kat aangetoond (J Physiol 2001; 537: 221-9), verklaard.

Als we alle data beschouwen betreffende de omkering van (acetazolamide en inhalatieanesthesie geïnduceerde) ademdepressie door de toediening van antioxidanten (**hoofdstuk 5**; J Physiol Lond 2002; 544: 931-8 (halothaandata); Anesthesiology 2005; 102: 47-53 (isofluraandata); zie ook **hoofdstuk 1**), dan rijst de vraag of antioxidanten beschouwd mogen worden als een algemene, eenvoudige en relatief onschadelijke manier om farmacologische inhibitie van de perifere chemoreceptoren (ter plekke van het glomuslichaampje) tegen te gaan. Een tweede vraag is dan of antioxidanten een belangrijke rol zouden kunnen spelen in de anesthesiologische praktijkvoering. Zouden antioxidanten, makkelijker dan naloxon, de ademdepressieve effecten van opioïden (zoals buprenorfine) kunnen omkeren, zonder dat de pijnstillende effecten van deze middelen worden beïnvloed? En verder nog, zouden (membraandoorgankelijke) antioxidanten ook de deprimerende effecten van farmaca op de centrale chemoreceptoren en ademhalingsneuronen kunnen opheffen? Deze vragen zijn onderwerp van verder onderzoek in onze laboratoria.

Concluderend, aan het einde van dit proefschrift, is het nu mogelijk de doelstellingen beschreven op pagina 15 te bespreken:

1. **Beschrijving van de dosis–ademhalingsrespons relatie van buprenorfine en fentanyl.** De pijnstiller buprenorfine vertoont een dosis-afhankelijke ademdepressie dat een plateau bereikt bij 2 $\mu g.kg^{-1}$. Dit fenomeen wordt ook wel plafondeffect genoemd. Buprenorfine gedraagt zich in dit opzicht anders dan fentanyl dat een dosis-afhankelijke ademdepressie veroorzaakt met periodiek ademen en apneu's na toediening van doses groter dan 2 $\mu g.kg^{-1}$. Deze data geven aan dat buprenorfine een partiele agonist is van de μ-opioïd receptor.

2. **Vergelijking van de pijnstillende- en ademhalingseffecten van buprenorfine.** In tegenstelling tot de ademdepressie (waarbij er sprake is van een plafondeffect) laat buprenorfine geen plateau zien in de pijnstilling (dosisbereik 0 tot 6 $\mu g.kg^{-1}$). Een mogelijk mechanisme voor dit verschil zou kunnen liggen in een verschil in μ-opioïd receptordichtheid in die delen van het brein die betrokken zijn bij de regeling van de ademhaling en de pijnstilling, met een relatieve afgenomen μ-opioïd receptordichtheid in de gebieden betrokken bij de ademhaling.

3. **Omkering van opioïd-geinduceerde ademdepressie met nadruk op buprenorfine.** In tegenstelling tot morfine en alfentanil is buprenorfine relatief ongevoelig voor omkering door de aspecifieke opioïd antagonist naloxon. Hoge doses naloxon zijn nodig om volledige omkering te verkrijgen (2 tot 4 mg per 70 kg, gegeven in 30 min). Hogere doses veroorzaken daarentegen een afname van de omkering. Deze 5 separate studies tonen aan dat de specifieke naloxon dosis als ook de manier van toediening van groot belang is om een volledige en langdurige omkering te krijgen van de ademdepressie die buprenorfine veroorzaakt. Nadat de toediening van naloxon (als bolus met of zonder kortdurende

infusie) wordt gestopt keert de ademdepressie weer snel terug. Verder onderzoek is nodig om de specifieke naloxon dosis en toedieningswijze te bepalen die gebruikt kan worden in de kliniek. In de patiënt spelen factoren een rol die niet aanwezig zijn in gezonde vrijwilligers, zoals bijvoorbeeld acute en chronische pijn en de toediening van hoog gedoseerde opioïden en langwerkende opioïden.

4. **Vermogen van antioxidanten om acetazolamide-geïnduceerde depressie van de ventilatoire respons op acute hypoxie om te keren.** In een serie complexe experimenten in gezonde vrijwilligers wordt waargenomen dat acetazolamide de isocapnische ventilatoire respons op acute hypoxie (< 3 min) als ook de O_2–CO_2 interactie in de glomuslichaampjes deprimeert. Een cocktail van ascorbinezuur en α-tocopherol (vitamine C en E) is in staat het door acetazolamide veroorzaakte effect op de ademhaling volledig teniet te doen. Het is waarschijnlijk dat hier sprake is van twee onafhankelijke effecten van acetazolamide en antioxidanten op kaliumkanalen in de type I cel van het glomuslichaampje. De resultaten van deze studie geven ons niet alleen inzicht in het mechanisme van de zuurstofmeting in de cel, maar ook in het mechanisme van acetazolamide en in het gunstig effect dat dit middel heeft bij hooggebergteziekte. Verdere studies zijn nodig om dit nader te bestuderen en om in te gaan op de vraag of dit zelfde mechanisme een rol speelt in de zuurstofmeting in andere cellen dan de type I glomuscel (bijvoorbeeld in het longvaatbed).

5. **Effecten van de carbo-anhydrase remmers acetazolamide en methazolamide op de ventilatoire hypoxische en hypercapnische respons.** In de genarcotiseerde kat deprimeert een lage dosis methazolamide noch de perifere koolzuurgevoeligheid, noch de ventilatoire respons op steady-state hypoxie. Zelfs bij hoge dosis blijft de ventilatoire respons op steady-state hypoxie onveranderd. In tegenstelling tot methazolamide deprimeert een lage dosis acetazolamide wel de ventilatoire respons op acute en steady-state hypoxie. Deze data suggereren dat carbo-anhydrase geen bepalende rol speelt in het mechanisme van zuurstofmeting in de type I glomuscel. Het geeft verder aan dat acetazolamide maar niet methazolamide inwerkt op een systeem (bijvoorbeeld een eiwit) dat een cruciale rol speelt in de zuurstofmeting en de hypoxische ventilatoire respons. Een mogelijke kandidaat is het Ca^{2+}-afhankelijke kalium (BK) kanaal.

Curriculum Vitae

Johan Haiko Leonard Bijl was born on November 28, 1973 in Roelofarendsveen, The Netherlands. He obtained his Atheneum diploma at the Bonaventura College in Leiden in June 1992. From September 1992 to August 1993 he studied Biology at the University of Leiden. In September 1993 he started studying Biomedical Sciences at the University of Leiden, and in September 1994 he also started Medical School at the same university. As part of the former study, he participated in research projects at the departments of Surgery (Dr. G.N. Jukema), Physiology (Prof. Dr. D.L. Ypey) and Anesthesiology (Prof. Dr. A. Dahan) at the Leiden University Medical Center. In 2003, he obtained his Medical Degree and later that year he graduated in Biomedical Sciences. During the same year, he was appointed research fellow at the department of Anesthesiology (Leiden University Medical Center), and started the investigations described in this thesis.

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