3 Antioxidants prevent depression of the acute hypoxic ventilatory response by subanesthetic halothane

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[80] During the past decade considerable progress has been made in unraveling the cascade of events within carotid body type I cells upon exposure to a hypoxic environment, although there are still many areas of controversy.[80,118]

The general pictures emerging from most studies is that low oxygen decreases the open probability of potassium channels which causes membrane depolarisation and influx of Ca^{2+} ions. In several species, various types of potassium channels are described that may serve as oxygen sensing element that initiates the transduction cascade in hypoxia, for example K_V channels in rabbit, [146,148] and Maxi-K and TASK channels in rat.[25,158] Although it is known that potassium channels confer redox sensitivity and are sensitive to changes in the concentration of reactive oxygen species (ROS) it is unclear by what mechanism low oxygen is able to decrease the conductance of these channels.[102,105,118]

Volatile anaesthetics such as halothane can open potassium channels in various cell types such as TASK channels in rat carotid body.[25,143,184,140,141] At the same time, volatile anaesthetics, particularly halothane, are known to depress the acute hypoxic response, an effect that may be mediated through a preferential and potent action on the carotid bodies.[100,47] It is unknown if opening of potassium channels by halothane might occur through changes in the cell redox state and/or changes in ROS. It is known, however, that during hypoxia halothane undergoes a reductive metabolism in the liver by which radical species are produced and lipid peroxidation is initiated; this reductive metabolism of halothane is thought to be responsible for it's mild hepatotoxic effect.[56,57,95,187] In guinea pig liver, peroxidation of lipids following halothane administration can be inhibited by antioxidant treatment with vitamin E.[177]

The above findings on the sensitivity of potassium channels to ROS, the ability of halothane to produce radical species and to open potassium channels and finally the role of potassium channels in the hypoxic response raise the question if halothane may reduce the hypoxic response by producing ROS or by influencing the redox state of the carotid body. The aim therefore of the present studies in humans was to examine the influence of the potent antioxidants a-tocopherol and ascorbic acid on the acute hypoxic ventilatory response.

METHODS

Subjects and Apparatus

Thirty-two healthy, non-smoking, male subjects (age 20 to 35 yr) were recruited after protocol approval by the Leiden University Medical Center Committee on Medical Ethics. None of the volunteers was taking any medication or ever had surgery under general anaesthesia. All subjects performed 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. They were not instructed about respiratory physiology, anesthesia and the intensions of the study. All gave oral and written informed consent before their participation.

After arrival at the laboratory, an intravenous catheter was inserted in the left or right antecubital vein for drug infusion. Subsequently electrodes for EEG monitoring (BisSensor, Aspect Medical Systems, Newton, MA) were placed on the head at AT_1-FP_1 as specified by the manufacturer, and the subjects rested for 20 to 30 min. Next a facemask was applied over the mouth and nose.

The EEG was recorded using an Aspect A-2000 EEG monitor (software version 3.3). The monitor computed the bispectral index (BIS), an objective measure of hypnosis,[164] over 2-s epochs. We averaged the BIS values over 1 min-intervals and used data points obtained at 3-min intervals for further analysis.

See METHODS section *Apparatus* of *Chapter 2* for a description of the procedure and apparatus. Part of the nitrogen (5 L/min) passed through a halothane vaporizer (Dräger 19·2, Lubeck, Germany). During the initial part of the study (control experiments), the vaporizer was kept in the "off"-position. Dräger Nederland BV calibrated the vaporizer prior to its use in this study.

Study Design

In the first set of studies, which was designed to test the effect of antioxidant pre-treatment on the depression by halothane of the acute hypoxic response (AHR), two separate groups of 8 subjects underwent a control hypoxic study, followed by a halothane hypoxic study, and finally by a halothane hypoxic study after pre-treatment with a cocktail of antioxidants (study 1) or placebo (study 2). In a second set of studies, which was designed to study the effect of antioxidant pre-treatment on the hypoxic ventilatory response in the absence of halothane, two separate groups of 8 subjects underwent a control hypoxic study, followed by a sham halothane hypoxic study, and next followed by a sham halothane study after pre-treatment with a cocktail of antioxidants (study 3) or placebo (study 4). While the design of the halothane administration was randomised and blinded to the subjects only, both subjects and researchers were blinded to the pre-treatment with anti-oxidants or placebo.

After each hypoxic study blood was drawn from the capillary bed of a hyperaemic finger for the determination of blood acidity (Åstrup equilibration technique, Radiometer, Copenhagen, Denmark).

The Hypoxic Study. Hypoxia was induced with a dynamic end-tidal forcing system:[39,45] 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. Since peak hypoxic responses occur within three min,[39] hypoxia was maintained for three min, after which hyperoxia was introduced for 5 min ($F_iO_2 > 0{\cdot}5$). The $P_{ET}CO_2$ was maintained just above individual resting values.

Halothane. During the appropriate studies, the subjects inhaled halothane (Fluothane, Zeneca

Ltd, Macclesfield, UK). By manipulating the settings of the vaporizer, the subjects inhaled 0·11% end-expiratory halothane for 10 min before the hypoxic study started. Inhaling 0·11% halothane for 10 min results in a MAC equivalent of 0·13 (assuming an age adjusted MAC of 0·84% in our young subjects).[83] Note that because of the short (10 min) exposure time to this end-tidal level of halothane, the brain concentration is less than 0·11%, preventing the occurrence of significant central effects (i.e., within the central nervous system) of halothane. The subjects were under the impression that halothane was given during the sham halothane studies by manipulating an empty vaporizer.

The Antioxidant Cocktail (AOX). The antioxidant cocktail consisted of 200 mg of oral α-tocopherol (Organon, Oss, The Netherlands) given 1-h prior to the start of the appropriate hypoxic study, which was ingested with a cup of yoghurt and two 1 gram intravenous doses of ascorbic acid (Ascorbinezuur CF, 5 ml, Centrafarm, The Netherlands) given 10 and 4 min before the appropriate hypoxic study. Placebos consisted of cellulose tablets and 0·9% NaCl manufactured by the local pharmacy). The oral placebo was also ingested with yoghurt.

Data and Statistical Analysis

Analysis was performed on a blinded data set. The breath-to-breath data of the last 10 breaths of normoxia and the last 10 breaths of hypoxia were averaged. Since the relationship between ventilation and arterial oxygen saturation is found to be linear,[45] we calculated the difference between the mean $\dot{V}_i$- and the S_PO_2-data points and expressed the acute hypoxic ventilatory response (AHR) or sensitivity as follows:[45]

$$\text{AHR} = [\dot{V}_i(\text{hypoxia}) - \dot{V}_i(\text{normoxia})] \,/\, [S_PO_2(\text{normoxia}) - S_PO_2(\text{hypoxia})]$$

(units L/min per % desaturation). The statistical analysis was performed using SPSS v10.0 for Windows. To detect the significance of differences among the three treatment groups of each study, a two-way analysis of variance was performed. *Post-hoc* analysis was by least-significant differences and Bonferroni tests. To assess the effect of antioxidant-versus placebo-pre-treatment, Student *t*-tests were performed on the appropriate treatment levels of studies 1 and 2 and studies 3 and 4. Values reported are mean ± SD. *P*-values $< 0{\cdot}05$ were considered significant.

RESULTS

All subjects completed the protocols without side effects. During all studies $P_{ET}CO_2$ values were kept constant 0·1 to 0·2 kPa above individual resting values, with no differences between baseline (pre-hypoxia) and hypoxic $P_{ET}CO_2$ values and pH. In all hypoxic studies S_PO_2 values were 82 ± 2%.

The values of baseline ventilatory parameters and the control ventilatory responses to hypoxia are in agreement with earlier observations (table 1; refs. 48,45). We observed no effect from low dose halothane on baseline ventilation. Similarly, antioxidant and placebo pre-treatment had no significant effect on baseline parameters (table 1). Halothane (0·11% end-tidal) decreased the ventilatory response to hypoxia by more than 50%. As shown in figure 1, this effect was completely prevented by pre-treatment

Table 1. Influence of antioxidant and placebo pretreatment on halothane- and sham-halothane-induced depression of the ventilatory response to hypoxia

	STUDY 1			STUDY 2		
	control	**halothane**	**AOX + halothane**	**control**	**halothane**	**placebo + halothane**
Baseline $\dot{V}_I$ (L/min)	12·1 ± 1·5	12·5 ± 3·3	14·0 ± 2·1	12·5 ± 1·6	12·7 ± 3·2	13·7 ± 4·1
$P_{ET}CO_2$ (kPa)	6·1 ± 0·4	6·1 ± 0·3	6·2 ± 0·2	6·0 ± 0·02	6·1 ± 0·2	6·0 ± 0·2
pH	7·41 ± 0·02	7·41 ± 0·02	7·42 ± 0·02	7·40 ± 0·02	7·40 ± 0·03	7·41 ± 0·02
Halothane ET vol.%	–	0·11 ± 0·01*	0·11 ± 0·01*	–	0·11 ± 0·01*	0·11 ± 0·01*
AHR (L min^{-1} %$^{-1}$)	0·79 ± 0·31	0·36 ± 0·14*	0·77 ± 0·32	0·79 ± 0·40	0·36 ± 0·19*	0·36 ± 0·27* †
AHR (% of control)	100	46 ± 11*	96 ± 20	100	47 ± 14*	40 ± 15* †
BIS	96 ± 2	96 ± 2	96 ± 2	97 ± 1	97 ± 2	97 ± 1

	STUDY 3			STUDY 4		
	control	**sham-halothane**	**AOX + sham halothane**	**control**	**sham-halothane**	**placebo + sham halothane**
Baseline $\dot{V}_I$ (L/min)	13·9 ± 1·9	14·5 ± 3·6	14·5 ± 2·8	14·6 ± 3·3	16·9 ± 3·8	16·1 ± 2·3
$P_{ET}CO_2$ (kPa)	5·8 ± 0·3	5·9 ± 0·2	5·8 ± 0·3	5·9 ± 0·4	5·9 ± 0·4	5·9 ± 0·4
pH	7·43 ± 0·03	7·43 ± 0·02	7·43 ± 0·03	7·42 ± 0·03	7·42 ± 0·02	7·41 ± 0·02
Halothane ET vol.%	–	–	–	–	–	–
AHR (L min^{-1} %$^{-1}$)	0·89 ± 0·42	0·90 ± 0·44	1·00 ± 0·54	0·83 ± 0·42	0·88 ± 0·45	0·88 ± 0·45
AHR (% of control)	100	102 ± 14	116 ± 22	100	104 ± 15	110 ± 10
BIS	97 ± 2	97 ± 2	97 ± 1	96 ± 2	96 ± 3	96 ± 3

Values are mean ± SD; pH values are obtained during air breathing; BIS is bispectral index of the EEG; AHR is Acute Hypoxic Response;
* = P < 0·01 *vs.* control of identical study (two-way analysis of variance);
† = P < 0·01 *vs.* AOX-pretreated halothane run on study 1 (Student-*t*-test).

with the antioxidant cocktail (study 1) but not by placebo pre-treatment (study 2). Sham halothane did not affect any of the ventilatory baseline and hypoxic parameters, neither did antioxidant (study 3) or placebo (study 4) pre-treatment (table 1 and fig. 2)). The 95% confidence intervals of antioxidant effect relative to halothane or sham-halothane (ratio AOX+halothane/halothane in study 1, and ratio AOX+sham halothane/sham halothane in study 3) did not overlap: 1·7, 3·1 and 0·6, 1·1 in studies 1 and 3, respectively (figure 3). This indicates that the effect of AOX to abolish halothane's depressant effect cannot be explained by an increase of the AHR by the antioxidants *per se*.

Bispectral index values did not differ among control, halothane, sham-halothane, antioxidant pre-treatment and placebo pre-treatment studies (table 1), indicating that there were no differences in the subjects' level of arousal across the various runs of all four studies.

DISCUSSION

We have found that while an antioxidant cocktail had only a small, statistically not significant, effect on the acute hypoxic response (fig. 3), it did reverse the large depression in the hypoxic response caused by low dose halothane. To place this result into context, we need to discuss methodological considerations; the modulating role of reactive oxygen species (ROS) in the chemoreception process; and the mechanism by which halothane depresses the hypoxic ventilatory response and how this effect might depend on the redox state in (the membrane of) chemoreceptors cells. The measurement of the hypoxic ventilatory response requires isocapnia both across drug treatments as well as during the hypoxic test. As seen in table 1 the mean differences in $P_{ET}CO_2$ for the different treatment conditions in the four studies were closely matched and did not contribute to the changes in the measured AHR.

Although we attempted to achieve blinding, the subjects were probably aware of when the halothane was being inhaled. The depression, however, of the AHR by halothane is large and consistent across subjects (fig. 1) while the changes in the AHR with the sham-halothane are variable and similar to the variation expected with repeated hypoxic tests. In testing the effects of inhalational anaesthetics, the experimental conditions are very important. We have previously shown that arousing the subject with audio visual stimulation can reverse the depression of the AHR by isoflurane.[200]

In the present experiments, the subjects were awake but left undisturbed. Because we did not see any influence of either halothane or the antioxidants on the bispectral index (table 1), we have no observational evidence of an influence of the subject's level of arousal on our results. While we believe that the antioxidant cocktail that we utilized was effective in altering the intracellular or extracellular redox state, we have no direct measurement of their efficacy in our subjects. We rationalize the use of an antioxidant cocktail as follows. We had to take into account that the effects of halothane could be located at several sites: at the outer face of the membrane, within the membrane or in the cytosol or possibly at the mitochondrial level. For this reason we used the water-

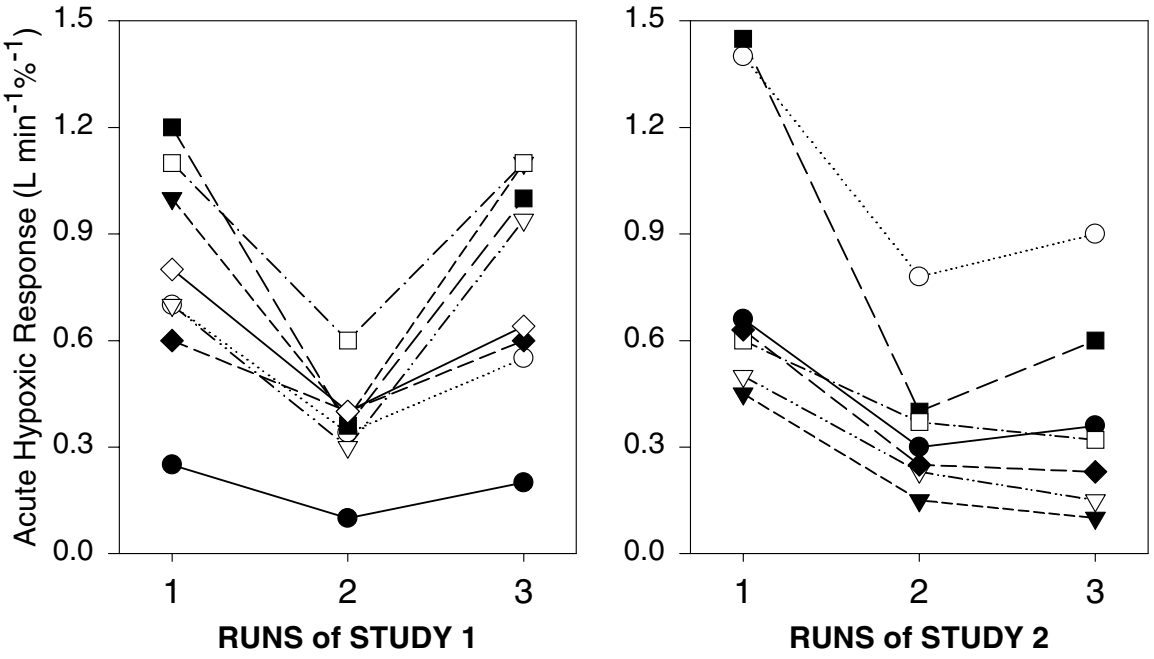

Figure 1. Hypoxic ventilatory responses of individual subjects of studies 1 and 2. Study 1: Control, run 1, and halothane hypoxic ventilatory responses, run 2, and influence of antioxidant, run 3 pretreatment on halothane-induced impairment of the hypoxic drive. Study 2: Control, run 1, and halothane hypoxic responses, run 2, and influence of placebo pre-treatment on halothane-induced impairment of the hypoxic drive, run 3. Note the ability of antioxidant but not placebo pre-treatment to prevent depression of the hypoxic response by halothane.

soluble ascorbic acid which is a particularly potent anti-oxidant in plasma and in the cytosol[27,74] and α-tocopherol which, due to its lipid solubility, may be the most important free radical and lipid peroxide scavenger in membranes.[26] Furthermore, it is known that the combined effectiveness of ascorbate and α-tocopherol is synergistic, with the net result that radicals originating from the membrane are removed using two different antioxidants.[134,138] Combined administration of α-tocopherol (2000 I.U. i.m.) and ascorbic acid (2 g i.v.) has been shown to reduce lipid peroxidation in patients undergoing cardiac bypass operation.[9] The oxygen transduction cascade in the carotid body (as in the similarly oxygen sensitive pulmonary artery smooth muscle and the pulmonary neural epithelial cell bodies) has been subject to considerable research over the past decade and while a much clearer picture of the process has emerged, there are many areas of considerable controversy.[80,118] The most generally accepted model is that low oxygen decreases the open probability of potassium channels in the membrane of carotid body type I cells which results in depolarisation. This membrane depolarisation opens voltage gated calcium channels with the resulting influx of Ca^{2+} causing neurotransmitter release, which activates the synaptically adjacent carotid sinus nerve. Currently, much interest has focused on the oxygen sensitive potassium channels in the carotid bodies

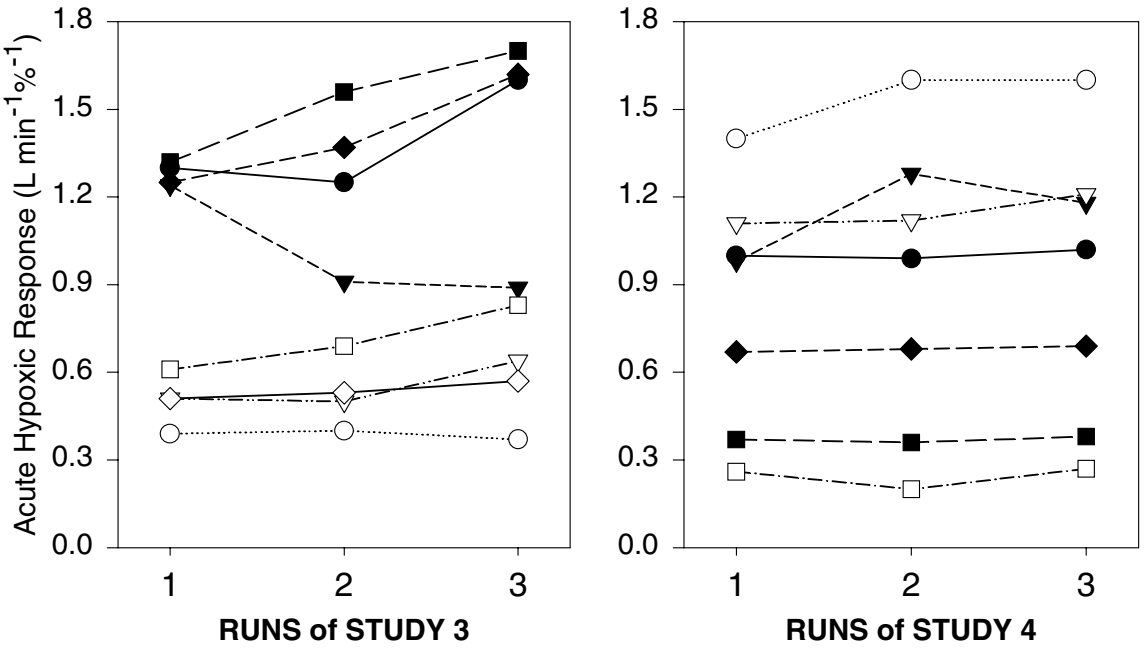

Figure 2. Hypoxic ventilatory responses of individual subjects of studies 3 and 4. Study 3: Control, run 1, and sham halothane hypoxic ventilatory responses, run 2, and influence of antioxidant pretreatment on the hypoxic drive during inhalation of sham halothane, run 3. Study 4: Control, run 1, and sham halothane, run 2, and the influence of placebo pre-treatment on the hypoxic drive during inhalation of sham halothane, run 3.

of several species.[149,113] The rat and the rabbit have been most commonly studied and they appear to have different types of oxygen sensitive potassium channels. The rat appears to have both TASK,[25] and Maxi-K channels,[158] that are oxygen sensitive, while in the rabbit K_V channels seem to serve this role.[146,148] However, within this general model, it is not determined how low oxygen closes the potassium channel that seems to initiate the cascade. Several studies have shown that potassium channels show redox sensitivity and considerable sensitivity to levels of ROS.[102,105,142,117] It is unsettled whether potassium channels possess intrinsic oxygen sensitivity, or, alternatively, are influenced or modulated by other O_2 sensing elements in the cascade, for example by (membrane associated) cytosolic redox couples . Intrinsic oxygen sensitivity could exist in the form of reduction/oxidation of thiol containing free cysteine residues in b subunits that are required for hypoxic sensitivity.[147] One proposed redox model associated with enzymatic production of ROS that may influence potassium channel conductance is the cytochrome P-450 system that utilizes NAD(P)H as an electron donor. Inhibition of this enzyme system has been shown to prevent the hypoxic inhibition of potassium channels[87] but this has not been found in all model systems.[165]

It is clear that within this general framework of hypoxic chemoreception there is considerable variety in specific sensor elements and couplings. Particularly when channels

are expressed in heterologous systems, all the elements for the in vivo cascade may not be present. In addition, there may be substantive differences between sensing elements of the cascade between the different oxygen sensitive tissues. Thus, it has been difficult to verify the role for ROS in carotid body chemotransduction in more physiologically intact preparations. In fact, there is considerable controversy as to whether ROS increases (pulmonary arterial smooth muscle)[111,214] or decreases (carotid body)[108] with hypoxia in oxygen sensitive cells. Experiments in which the redox state of carotid body cells was altered would seem to indicate that ROS may not be a direct link between hypoxia and the membrane depolarisation initiated by the closure of the K^+ channel.[165,167] Exogenous reductants, on the other hand, have been shown to mimic the effect of hypoxia on O_2 sensitive potassium channels in carotid body cells.[11] Thus, whatever the precise mechanism, there is likely to be at least a modulating role for the redox state of the type-I cell in O_2 sensing. The depressant effect of subanesthetic halothane in humans on ventilation during hypoxia may occur via a preferential and potent action on the carotid bodies.[47,100] The mechanism for this depression is unknown but inhalational anaesthetics can directly open two-pore domain potassium (TASK) channels in various cell types,[140,142,143,184] and in particular in the rat carotid body.[25] The action ion of inhalational anaesthetics on TASK channels may be located at a specific region at the junction between the final transmembrane domain and the cytoplasmic C-terminus.[143,192] This site is also involved in neurotransmitter inhibition of the channel but does not contain a motif that is known to be involved in cell signalling mechanisms.[192] How changes in ROS or redox state could alter the properties of this binding site is unknown. In the lung carcinoma cell line H146, a representative model for pulmonary oxygen-sensitive neuroepithelial body cells, halothane transiently reverses hypoxic inhibition of potassium currents, similar to the reversal caused by the reactive species H_2O_2.[86] The metabolism of halothane itself may also change the redox status of cells. In hypoxia, halothane undergoes a reductive metabolism that in the liver is catalysed by isoforms of cytochrome P450 but in other tissues possibly also by other heme-containing proteins.[57,95,187] Reduction of halothane yields CF_3CHCl radicals able to inactivate cytochrome P450 by covalent binding, or, alternatively, to remove hydrogen from polyunsaturated lipids thus initiating lipid peroxidation.[56,95] In guinea pig, the hepatotoxic effect caused by this reductive metabolism of halothane can be prevented by antioxidant treatment.[176] In humans, induction with hemin of heme oxygenase-1, which has an antioxidant role in oxidative stress, has been shown to be effective against halothane-induced liver damage.[136] The susceptibility of halothane's depressant effect to antioxidant treatment that we found in this study indicates that the cellular redox state influences the effect of halothane on the oxgyen sensing mechanism. This could be explained by a modulation by ROS of the coupling of halothane to the potassium channel (or other channels). Whether or not the ROS was generated from halothane's metabolism or from other intracellular processes,[214] the reduction in ROS with antioxidant treatment could reduce the coupling of halothane to the channel and prevent it from opening it. An alternative way to explain our findings would be to suggest that an increase in the concentration of ROS has an inhibitory effect on the mechanism involved in the acute hypoxic response. In

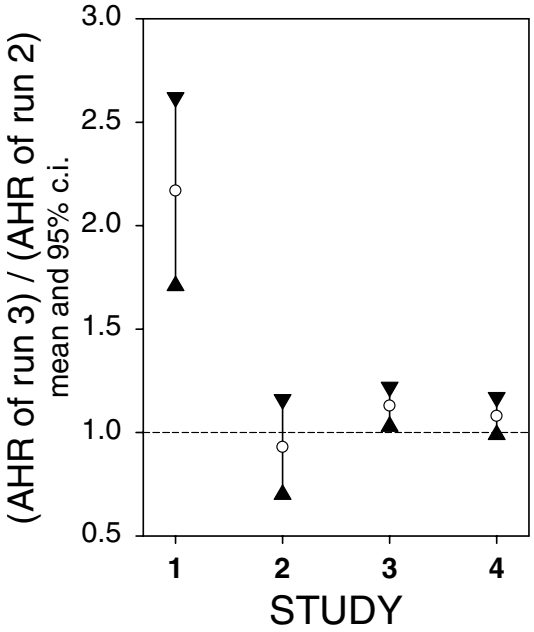

Figure 3. The effect of the antioxidant cocktail or placebo on halothane or sham-halothane induced depression of the acute hypoxic response. Values are the ratio of the third hypoxic run (antioxidants or placebo) over the second hypoxic run (halothane or sham halothane) of studies 1 to 4. ∘ is mean, the triangles depict the 95% confidence intervals (c.i.). A value of 1 indicates no effect of the AOX or placebo pre-treatement on the acute hypoxic ventilatory response. Note that the 95% c.i.'s of studies 1 (AOX + halothane) and 3 (AOX + sham-halothane) do not overlap.

this scenario, the cellular redox state or the signalling from a particular ROS would be the coupling from low oxygen to potassium channel closure. In this model , an NAD(P)H oxidase has been proposed as the membrane bound source of oxygen sensitive ROS implying a decrease in ROS in hypoxia.[94,96,106,181] The increase in local ROS caused by the reductive metabolism of halothane in hypoxia would thus counter the hypoxia- induced decrease in ROS and prevent the hypoxic closure of the K^+ channel. This effect would be most noticeable in hypoxia since halothane's reductive metabolism is increased in hypoxia.

In animal species, the effect of halothane on the hypoxic ventilatory response is variable. In the goat, for example, an end-tidal concentration of 0·5% does not significantly depress it.[104] In the rabbit and cat, 0·5-1% halothane reduces the hypoxic response, the effect in the latter species being larger.[52,150] As shown in this and previous studies, the effect of 0·1 MAC in man is to reduce hypoxic sensitivity by more than 50%. These species differences could originate from the differences in the type of oxygen sensitive potassium channel that initiates the transduction cascade (e.g., TASK versus K_V) and their differences in anaesthetic sensitivity or in splice variants of the expressed channel. An alternative explanation could also lie in species differences in the defence against ROS. Goats produce large quantities of ascorbic acid,[31] and may thus be better protected against the adverse effects of free radicals produced by halothane. To a

lesser degree this may also be the case for rabbits. Cats produce low quantities of ascorbic acid,[31] and this might explain their higher susceptibility to halothane than rabbits. Humans have lost the ability to synthesize ascorbic acid and may therefore be more vulnerable to the adverse effects of reactive species that are produced by halothane. It is worth mentioning that in a previous study we were not able to demonstrate a clear depression of the normocapnic AHR by desflurane.[45] This volatile anaesthetic has a low metabolism, with little production of free radicals.[103] In *Chapters 4 and 5* we show that low dose propofol, which is known to have antioxidant properties,[58] neither depressed the CO_2 sensitivity of the peripheral chemoreflex loop nor the fast - carotid body mediated - component of the acute hypoxic response. Together with the present findings, these previous data suggest that the (lack of) depressant effects of anaesthetics on the hypoxic response may be related to their pro-oxidant (antioxidant) properties, but further studies are needed to support this hypothesis. From the data that we presented in this study we conclude that changing the cellular redox state can modulate the depressant effect of halothane on the acute hypoxic response. Further, although our results do not supply direct evidence for an inhibitory role of ROS in the acute hypoxic response, they could be explained by ascribing at least a modulating role to radical species in the AHR. Our observation that anti-oxidant pre-treatment markedly reduces the depressant effect of halothane on the AHR demonstrates a specific pharmacological reversal of an anaesthetic effect. Further work will be needed in both humans and animal preparations to clarify the interaction of cellular redox status, inhalational anaesthetics and oxygen sensitive potassium channels in the carotid body.

SECTION 2

Pharmacology