Low-dose acetazolamide reduces CO_2–O_2 stimulus interaction within the peripheral chemoreceptors in the anaesthetised cat

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1. Using the technique of end-tidal CO_2 forcing, we measured the effect of the carbonic anhydrase inhibitor acetazolamide (4 mg kg^{-1}, I.V.) on the CO_2 sensitivities of the peripheral and central chemoreflex loops both during hyperoxia and hypoxia in 10 cats anaesthetised with α-chloralose–urethane.

2. In the control situation, going from hyperoxia (arterial $P_{O_2}(P_{a,O_2})$ 47.40 $\pm$ 3.62 kPa, mean $\pm$ S.D.) into moderate hypoxia (P_{a,O_2} 8.02 $\pm$ 0.30 kPa) led to an almost doubling of the peripheral CO_2 sensitivity (S_P): a rise from 0.09 $\pm$ 0.07 to 0.16 $\pm$ 0.06 l min^{-1} kPa^{-1}. After acetazolamide, however, lowering the P_{a,O_2} from 46.95 $\pm$ 5.19 to 8.02 $\pm$ 0.66 kPa did not result in a rise in S_P, indicating the absence of a CO_2–O_2 stimulus interaction.

3. In hypoxia, acetazolamide reduced S_P from 0.16 $\pm$ 0.06 to 0.07 $\pm$ 0.05 l min^{-1} kPa^{-1}. In hyperoxia, however, the effect on S_P was much smaller (an insignificant reduction from 0.09 $\pm$ 0.07 to 0.06 $\pm$ 0.05 l min^{-1} kPa^{-1}).

4. Acetazolamide reduced both the hyperoxic and hypoxic sensitivities (S_C) of the central chemoreflex loop: from 0.45 $\pm$ 0.16 to 0.27 $\pm$ 0.13 l min^{-1} kPa^{-1} and from 0.40 $\pm$ 0.16 to 0.26 $\pm$ 0.13 l min^{-1} kPa^{-1}, respectively. In hyperoxia, the apnoeic threshold B (X-intercept of the ventilatory CO_2 response curve) decreased from 2.91 $\pm$ 0.57 to 0.78 $\pm$ 1.9 kPa ($P = 0.005$). In hypoxia, B decreased from 1.59 $\pm$ 1.22 to −0.70 $\pm$ 2.99 kPa ($P = 0.03$).

5. Because acetazolamide abolished the CO_2–O_2 interaction, i.e. the expected increase in S_P when going from hyperoxia into hypoxia, we conclude that the agent has a direct inhibitory effect on the carotid bodies. The exact mechanism by which the agent exerts this effect will remain unclear until more detailed information becomes available on the identity of the carbonic anhydrase iso-enzymes within the carotid bodies and their precise subcellular distribution.

The carbonic anhydrases are a family of zinc metallo-enzymes catalysing the reversible hydration of CO_2. In higher vertebrates including humans, at least 10 different carbonic anhydrase iso-enzymes have been discovered. These isoforms are characterised by different distributions within tissues, kinetic properties, subcellular locations and susceptibility to inhibitors (Sly & Hu, 1995). Hydration and dehydration of CO_2 are key events in physiological processes such as respiration, bicarbonate reabsorption in kidney (urinary acidification), the formation of aqueous humour and cerebrospinal fluid and the secretion of fluids in the gastro-intestinal system (Maren, 1967).

Several of our previous studies in the anaesthetised cat were focused on the role of carbonic anhydrase in the control of breathing. One significant finding was that large intravenous doses of the sulfonamides benzolamide and acetazolamide caused a large reduction in the slope of the ventilatory CO_2 response curve (Teppema *et al.* 1995; Wagenaar *et al.* 1996), and completely abolished the hypoxic response (Teppema *et al.* 1988, 1992). Using the technique of dynamic end-tidal forcing (DEF) we also found that both during normoxia and moderate hypoxia a low dose (i.e. 4 mg kg^{-1}) of acetazolamide reduced the CO_2 sensitivity of the central chemoreflex loop by about 45%; the sensitivity of the peripheral chemoreflex loop was reduced by 50 and 33% during moderate hypoxia and normoxia, respectively (Wagenaar *et al.* 1996, 1998). The effects with low-dose acetazolamide occurred at inhibitor concentrations insufficient to cause effective inhibition of red cell carbonic anhydrase, as indicated by the absence of significant arterial–end-tidal P_{CO_2} gradients. We attributed the effects of low-dose acetazolamide on the peripheral chemoreflex loop to a direct action of the agent on the carotid bodies. This is not unreasonable

because glomus cells are known to contain carbonic anhydrase (Lee & Mattenheimer, 1964; Ridderstråle & Hanson, 1984; Rigual *et al.* 1985; Nurse, 1990), while enzyme inhibitors have been shown to alter their *in vivo* and *in vitro* CO_2 responses (Hanson *et al.* 1981*b*; Iturriaga *et al.* 1991; Lahiri *et al.* 1996; Torrance, 1996). Although it is conceivable that within the carotid bodies the carbonic anhydrases play a role in the (de)hydration of CO_2, their precise function is unclear because both the identity and the subcellular location(s) of the isoforms are unknown.

One way to study possible direct effects of the agents on the peripheral chemoreceptors in a whole animal preparation is to focus on the interaction between CO_2 and O_2, which is known to originate in the carotid bodies (Lahiri & Delaney, 1975; Fitzgerald & Deghani, 1982). At the level of minute ventilation, this interaction is translated into steeper CO_2 ventilatory response curves at lower P_{O_2} levels. An effect of carbonic anhydrase inhibitors on the CO_2–O_2 interaction within the carotid bodies has not been systematically investigated. Our previous experiments with low-dose acetazolamide were performed in separate animals, and thus did not allow a study of the CO_2–O_2 interaction within animals. Therefore, the aim of this study in the anaesthetised cat was to determine the effect of 4 mg kg^{-1} acetazolamide on the CO_2 sensitivities of the peripheral and central chemoreflex loops both during hyperoxia and moderate hypoxia within the animals. In this way we should be able to see if the inhibitor affects the expected increase in peripheral chemoreflex gain on going from hyperoxia into moderate hypoxia. We used the DEF technique to separate the CO_2 sensitivities of the peripheral and central chemoreflex loops from each other (DeGoede *et al.* 1985).

METHODS

Animals and measurements

Experiments were performed in 10 female adult cats (weight 2.8–4.2 kg). The Ethical Committee for Animal Experiments of the University of Leiden approved the use of animals and the experimental protocols. The animals were sedated with 10 mg kg^{-1} ketamine hydrochloride (I.M.). Anaesthesia was induced with 2% sevoflurane in 30% O_2 in N_2. The right femoral artery and vein were cannulated, 20 mg kg^{-1} α-chloralose and 100 mg kg^{-1} urethane were slowly administered intravenously, and the volatile anaesthetic was gradually withdrawn. About 1 h later, an infusion of a α-chloralose–urethane solution was started at a rate 1.0–1.5 mg kg^{-1} h^{-1} α-chloralose and 5.0–7.5 mg kg^{-1} h^{-1} urethane. This regimen is sufficient to suppress the pain-withdrawal reflex but light enough to preserve the corneal reflex. In previous studies we found that the maintenance of this condition has little effect on the ventilatory response to changes in end-tidal P_{CO_2} (P_{ET,CO_2}; Teppema *et al.* 1997). The animals were monitored throughout the experiment to ensure an adequate level of anaesthesia.

The trachea was cannulated at the 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 (Statham PM 197, Los Angeles, CA, USA). The respiratory fractions of O_2 and CO_2 were continuously measured with a Datex gas monitor (Multicap, Helsinki, Finland), which was calibrated with gas mixtures of known composition. The inspiratory gas concentrations were made with computer-steered mass flow controllers (type AFC 260, Advanced Semiconductor Materials, DeBilt, Netherlands). The animals were connected to an extra-corporeal circuit (ECC) for continuous blood gas measurement. Using the ECC, blood from the cannulated left femoral artery was pumped back via the right femoral vein with a flow of 6 ml min^{-1}. Arterial pH, P_{CO_2} and P_{O_2} in the blood passing the extra corporeal circuit were measured continuously with a pH electrode (Radiometer E-5037-0, Copenhagen, Denmark) calibrated with phosphate buffers, a CO_2 electrode (General Electric A312AB, Milwaukee, WI, USA) and a home-made Clark-type O_2 electrode, respectively, which were calibrated with water equilibrated with CO_2–O_2–N_2 gas mixtures delivered by a gas mixing pump (Wösthoff, Bochum, Germany). The transport delay from the lungs to the CO_2 electrode was approximately 50 s. The CO_2 electrode was recalibrated every 2 h and corrections for drift were made when necessary. Arterial blood pressure was measured using a Statham pressure transducer (Statham P23AC).

Rectal temperature was measured and controlled within 1 °C. All signals were recorded on polygraphs, converted to digital values (sample frequency 100 Hz) and processed by a PC. All signals were stored on a breath-by-breath basis.

Experimental design

Using the technique of end-tidal CO_2 forcing, we performed step changes in end-tidal P_{CO_2} before and after intravenous infusion of 4 mg kg^{-1} acetazolamide (Diamox, AHP Pharma, Hoofddorp, The Netherlands; 2 mg ml^{-1} in saline) against two different background conditions, namely hyperoxia (mean P_{a,O_2} 47.40 ± 3.62 and 46.95 ± 5.19 kPa before and after administration of the inhibitor, respectively) and moderate hypoxia. Because the CO_2 sensitivity of the peripheral chemoreflex loop increases with decreasing levels of P_{a,O_2}, the hypoxic DEF runs in particular were performed at equal levels of arterial P_{O_2} before and after acetazolamide (mean P_{a,O_2} 8.02 ± 0.30 and 8.02 ± 0.66 kPa, respectively). In each condition (control–hyperoxia, control–hypoxia, acetazolamide–hyperoxia, acetazolamide–hypoxia) two to four dynamic end-tidal forcing (DEF) runs were performed and the dynamic ventilatory responses analysed (see below). The P_{ET,CO_2} pattern during a DEF run was as follows. After a 10–15 min period of steady-state ventilation at constant P_{ET,CO_2} (about 0.5 kPa above the apnoeic threshold), the P_{ET,CO_2} was increased by 1–1.5 kPa in a stepwise 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.

After the termination of the experiments, the animals were humanely killed without recovery by way of an intravenous infusion of 10 ml pentobarbital.

Dynamic end-tidal forcing

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

$$\dot{V}_I = (S_P + S_C)(P_{ET,CO_2} - B),$$

where S_P is the carbon dioxide sensitivity of the peripheral chemoreflex loop, S_C is the carbon dioxide sensitivity of the central chemoreflex loop, and B is the apnoeic threshold or extrapolated P_{ET,CO_2} at zero $\dot{V}_I$. The sum of S_P and S_C is the overall carbon dioxide sensitivity.

Table 1. Effects of low-dose acetazolamide on respiratory parameters during hyperoxia

	Control	Acetazolamide	P
B (kPa)	2.91 ± 0.57	0.78 ± 1.9	0.005
S_C (l min^{-1} kPa^{-1})	0.45 ± 0.16	0.27 ± 0.13	0.001
S_P (l min^{-1} kPa^{-1})	0.09 ± 0.07	0.06 ± 0.05	0.123
τ_P (s)	5.91 ± 4.28	7.27 ± 5.76	0.741
τ_{on} (s)	83.60 ± 47.60	88.56 ± 25.28	0.756
τ_{off} (s)	108.65 ± 45.04	128.98 ± 46.86	0.362
$P_{(a-ET),CO_2}$ (kPa)	0.05 ± 0.57	0.43 ± 0.81	0.029
P_{a,CO_2} (kPa)	47.40 ± 3.62	46.95 ± 5.19	0.633

Parameter values obtained from the optimal fits in the analysis of DEF runs performed during hyperoxia (32 control runs and 27 runs after acetazolamide). B, the apnoeic threshold or X-intercept of the ventilatory CO_2 response curve. S_C and S_P, the CO_2 sensitivities of the central and peripheral chemoreflex loops, respectively. τ_p, time constant of the peripheral chemoreflex loops. τ_{on} and τ_{off}, central time constants of responses to CO_2 on- and off transients, respectively. $P_{(a-ET),CO_2}$, arterial–end-tidal P_{CO_2} difference.

Table 2. Effects of low-dose acetazolamide on respiratory parameters during hypoxia

	Control	Acetazolamide	P
B (kPa)	1.59 ± 1.22	-0.70 ± 2.99	0.030
S_C (l min^{-1} kPa^{-1})	0.40 ± 0.16	0.26 ± 0.13	0.019
S_P (l min^{-1} kPa^{-1})	0.16 ± 0.06	0.07 ± 0.05	0.002
τ_P (s)	4.26 ± 4.92	5.12 ± 4.04	0.693
τ_{on} (s)	73.68 ± 37.63	60.37 ± 22.79	0.269
τ_{off} (s)	104.53 ± 50.35	126.82 ± 65.84	0.469
$P_{(a-ET),CO_2}$ (kPa)	-0.10 ± 0.57	0.39 ± 0.75	0.009
P_{a,CO_2} (kPa)	8.02 ± 0.30	8.02 ± 0.66	0.986

Parameter values obtained from the optimal fits in the analysis of DEF runs performed during hypoxia (32 control runs and 29 runs after acetazolamide). B, the apnoeic threshold or X-intercept of the ventilatory CO_2 response curve. S_C and S_p, the CO_2 sensitivities of the central and peripheral chemoreflex loops, respectively. τ_p, time constant of the peripheral chemoreflex loop. τ_{on} and τ_{off}, central time constants of responses to CO_2 on- and off transients, respectively. $P_{(a-ET),CO_2}$, arterial–end-tidal P_{CO_2} difference.

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 (DeGoede *et al.* 1985):

$$\dot{V}_P(t) + \tau_P \, \mathrm{d}/\mathrm{d}t \dot{V}_P(t) = S_P(P_{ET,CO_2}[t - T_P] - B),$$

$$\dot{V}_C(t) + \tau_C \, \mathrm{d}/\mathrm{d}t \dot{V}_C(t) = S_C(P_{ET,CO_2}[t - T_C] - B),$$

where τ_P and τ_C are the time constants of the peripheral and central chemoreflex loops, respectively, $\dot{V}_P(t)$ and $\dot{V}_C(t)$ 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 $P_{ET,CO_2}[t - T_C] =$ 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 (Ct) in our model. The total ventilatory response $\dot{V}_I(t)$ is made up of the contributions of the central and peripheral chemoreflex loops and Ct:

$$\dot{V}_I(t) = \dot{V}_P(t) + \dot{V}_C(t) + Ct.$$

The parameters of the model were estimated by fitting the model to the breath-by-breath data using 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 $T_P \leqslant T_C$, were tried until a minimum in the residual sum of squares was obtained. The minimum time delay was chosen, arbitrarily, to be 1 s; the 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 acetazolamide infusion, analysis of variance was performed on individual data. The level of significance was set at $P = 0.05$ or lowered in the case of multiple comparisons. Results are given as means of the mean per cat $\pm$ S.D.

RESULTS

The mean parameter values obtained from the optimal fits during hyperoxia, before (32 DEF runs) and after (27 DEF runs) administration of 4 mg kg^{-1} acetazolamide are summarised in Table 1.

Before infusion of the inhibitor, the mean arterial P_{O_2} tension was maintained at 47.40 ± 3.62 kPa. After acetazolamide, the DEF runs were performed at a mean P_{a,O_2} of 46.95 ± 5.19 kPa.

Acetazolamide caused a small increase in the mean arterial-to-end-tidal P_{CO_2} difference from 0.05 to 0.43 kPa. The mean value of the apnoeic threshold B decreased from 2.91 to 0.78 kPa. The CO_2 sensitivity S_C of the central chemoreflex loop decreased significantly from a mean value of 0.45 to 0.27 l min^{-1} kPa^{-1}. The sensitivity S_P of the peripheral chemoreflex loop, however, was less clearly affected by the inhibitor: a decrease from 0.09 to 0.06 l min^{-1} kPa^{-1} which did not reach significance. Figure 1 displays the effects of acetazolamide on S_C and S_P in all individual animals in scatter diagrams. No significant effects of acetazolamide on the peripheral and central time constants were observed (see Table 1). Also, no acetazolamide-induced differences in peripheral and central transport delay times were found.

The effects of acetazolamide on the parameter values obtained during moderate hypoxia are summarised in Table 2 (32 DEF runs before and 29 runs after infusion). To prevent an effect of changes in P_{a,O_2} on the sensitivity of the peripheral chemoreflex loop, we maintained the level of the arterial oxygen tension at equal levels before
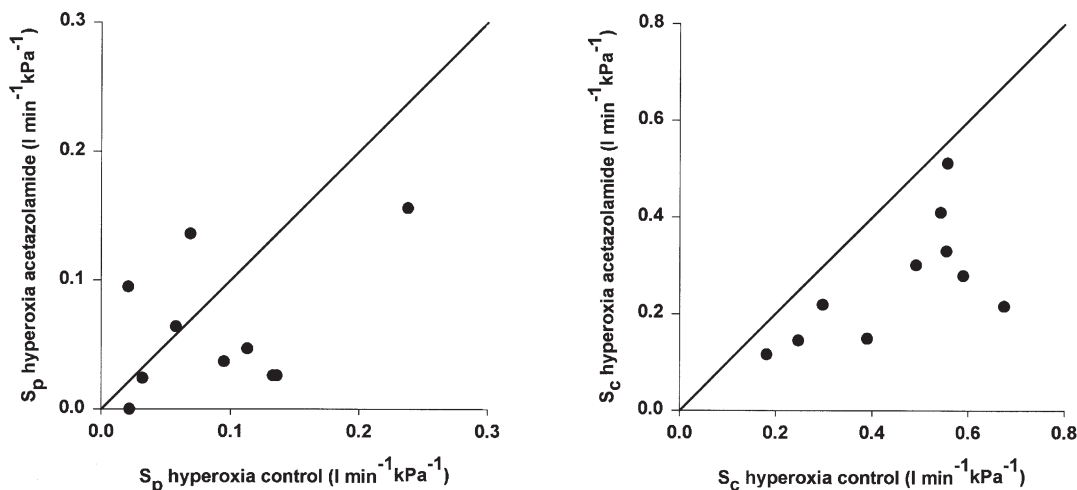
Figure 1. Effect of 4 mg kg⁻¹ acetazolamide (I.V.) on the hyperoxic CO_2 sensitivities of the peripheral and central chemoreflex loops

Effect of acetazolamide (4 mg kg⁻¹, I.V.) on the CO_2 sensitivities of the peripheral (S_P, left panel) and central (S_C, right panel) chemoreflex loops during hyperoxia. Each animal is represented by one symbol. Diagonal line represents line of identity.

and after acetazolamide administration. In the hypoxic situation, the inhibitor caused a decrease of about 2 kPa in mean value of B, similar to that found during hyperoxia. During hypoxia, the mean CO_2 sensitivities of both the peripheral and central chemoreflex loops were considerably reduced by acetazolamide (from 0.16 to 0.07, and from 0.40 to 0.26 l min⁻¹ kPa⁻¹, respectively), and this is illustrated by the individual data in all animals shown in Fig. 2. Peripheral and central time constants and transport delay times were not altered by acetazolamide. Similar to the hyperoxic condition, a small but significant increase in the arterial–end-tidal P_{CO_2} difference ($P_{(a-ET),CO_2}$) was seen (see Table 2).

Comparison of the data in Tables 1 and 2 shows that before acetazolamide infusion during hypoxia the CO_2 sensitivity of the peripheral chemoreflex loop was considerably larger than during hyperoxia (mean sensitivities 0.16 *vs.* 0.09 l min⁻¹ kPa⁻¹, $P = 0.006$). We illustrate this with two DEF runs from one animal in Fig. 3. Figure 4, obtained from the same animal, shows the absence of this CO_2–O_2 interaction after acetazolamide administration. In all 10 animals studied, a difference between the hypoxic and hyperoxic peripheral CO_2 sensitivity was no longer present after acetazolamide administration (0.07 *vs.* 0.06 l min⁻¹ kPa⁻¹, $P = 0.558$), indicating the absence of a CO_2–O_2 stimulus interaction.
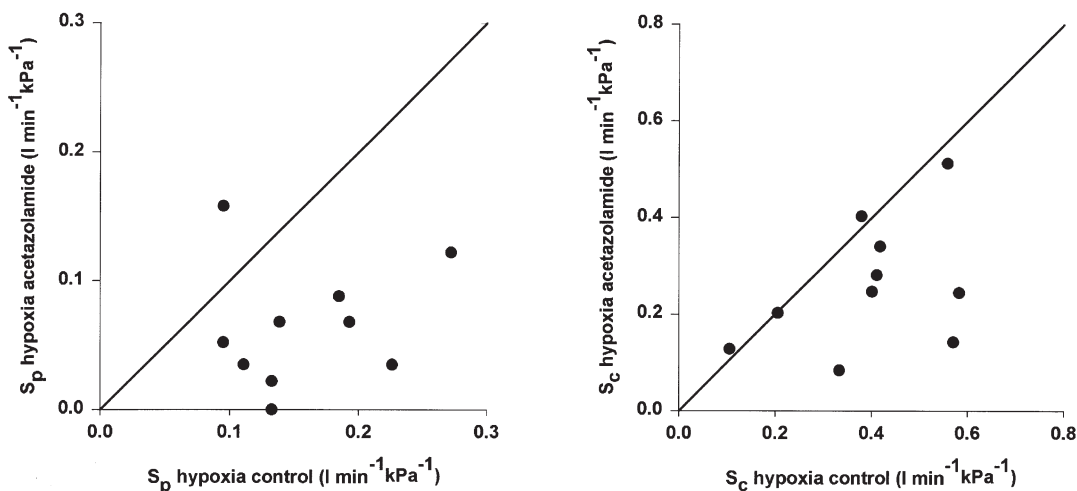

Figure 2. Effect of 4 mg kg⁻¹ acetazolamide (I.V.) on the hypoxic CO_2 sensitivities of the peripheral and central chemoreflex loops

Effect of acetazolamide (4 mg kg⁻¹, I.V.) on the CO_2 sensitivities of the peripheral (S_P, left panel) and central (S_C, right panel) chemoreflex loops during hypoxia. Each animal is represented by one symbol. Diagonal line represents line of identity.
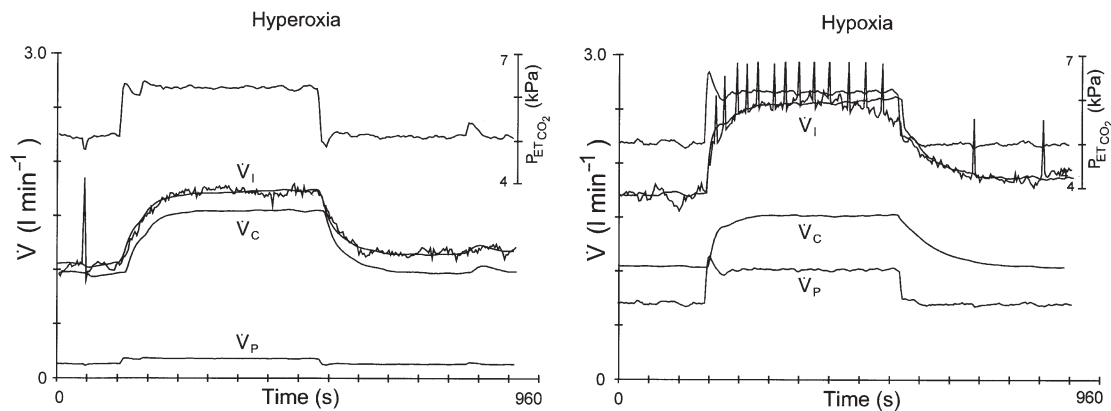
Figure 3. CO$_2$–O$_2$ stimulus interaction in the control situation

Two DEF runs obtained in the control situation during hyperoxia (left panel) and hypoxia (right panel). Upper traces are end-tidal P_{CO_2}. Breath-by-breath data points of inspiratory ventilation ($\dot{V}_I$) are connected; the smooth line represents the optimal model fit. $\dot{V}_C$ and $\dot{V}_P$ are the calculated contributions of the central and peripheral chemoreflex loops, respectively, to the change in ventilation. The sensitivity of the peripheral chemoreflex loop is considerably larger during hypoxia.

Standard bicarbonate (determined for the hypoxic condition only) was not significantly changed by acetazolamide: 20.1 ± 1.5 mmol l^{-1} (control) *vs.* 18.5 ± 1.9 mmol l^{-1} (acetazolamide).

DISCUSSION

The main results obtained in this study can be summarised as follows.

(1) Going from hyperoxia to moderate hypoxia in the control situation (mean P_{a,O_2} from 47.60 to 8.02 kPa) led to an almost doubling of CO$_2$ sensitivity of the peripheral chemoreflex loop. After administration of 4 mg kg^{-1} acetazolamide, this CO$_2$–O$_2$ interaction was no longer present.

(2) Both during hyperoxia and during moderate hypoxia, acetazolamide reduced the CO$_2$ sensitivity of the peripheral chemoreflex loop, but during hyperoxia this reduction was much smaller and statistically insignificant.

(3) Acetazolamide caused similar decreases in the CO$_2$ sensitivities of the central chemoreflex loop during hyperoxia and moderate hypoxia of 35 and 40 %, respectively, and a decrease in the value of the apnoeic threshold of approximately 2 kPa.

The present studies were performed in anaesthetised animals and we are cautious about applying our results to conscious animals. In cats, inhalational anaesthetics depress respiration due to an effect on the peripheral and central chemoreflex loops (Dahan *et al.* 1999). For
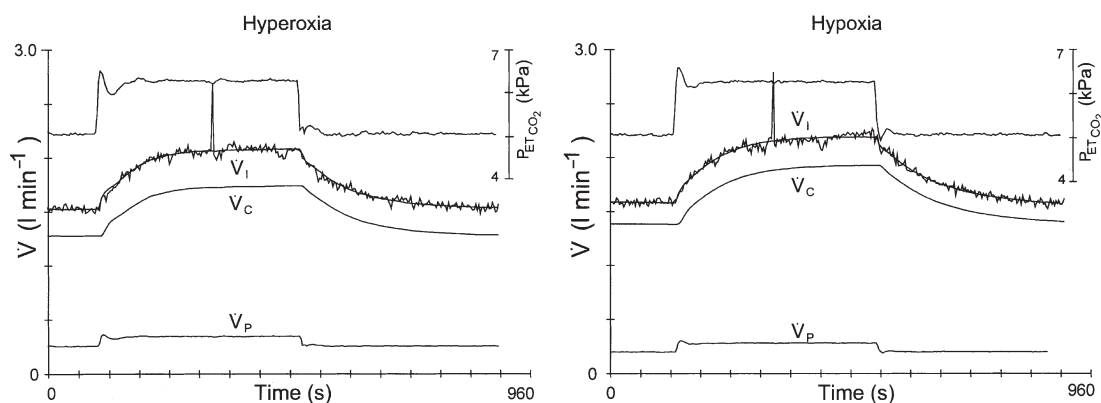

Figure 4. Absence of CO$_2$–O$_2$ stimulus interaction after administration of acetazolamide

Two DEF runs obtained during hyperoxia (left panel) and hypoxia (right panel) after 4 mg^{-1} kg^{-1} acetazolamide. Upper traces are end-tidal P_{CO_2}. Breath-by-breath data points of inspiratory ventilation ($\dot{V}_I$) are connected; the smooth line represents the optimal model fit. $\dot{V}_C$ and $\dot{V}_P$ are the calculated contributions of the central and peripheral chemoreflex loops, respectively, to the change in ventilation. Going from hyperoxia into hypoxia does not result in an increased contribution of the peripheral chemoreflex loop to the change in ventilation.

sevoflurane, the effect-site half-life of respiratory changes is about 3.5 min (Dahan *et al.* 1999). In this study, sevoflurane was discontinued for at least 1 h (> 15 half-lives) before the respiratory studies started. This may also be relevant given the known effect of the inhalational anaesthetic halothane on background potassium channels in carotid body cells from neonatal rats (Buckler *et al.* 2000). The infusion of α-chloralose–urethane has little effect on respiration and the ventilatory responses to hypercapnia compared with the awake state and does not yield systematic changes in CO_2 sensitivity over time (Gautier & Bonora, 1979; Teppema *et al.* 1997).

Essential features of the present study included the continuous measurement of arterial blood gases. This is crucial for two reasons. First, with regard to the P_{a,O_2}, we determined the CO_2 sensitivity of the peripheral chemoreflex loop, which in the (moderate) hypoxic range may be very sensitive for small variations in P_{a,O_2}. Because end-tidal P_{O_2} does not reflect P_{a,O_2}, and, among other factors, depends on the level of ventilation, the experimental design required continuous measurement of the arterial P_{O_2} tension. By varying the inspired oxygen concentration when necessary, we were able to perform DEF runs at equal arterial oxygen tensions before and after acetazolamide infusion. Second, regarding the P_{a,CO_2}, we wished to use $P_{(a-ET),CO_2}$ as a measure of erythrocytic carbonic anhydrase inhibition (Maren, 1967). Not only would effective inhibition of the erythrocytic enzyme severely compromise the blood CO_2 transport system (resulting in $P_{(a-ET),CO_2}$ differences as large as 2.5–3.5 kPa – Teppema *et al.* 1988, 1992, 1995; Wagenaar *et al.* 1996), it would also reduce ventilatory CO_2 sensitivity by mechanisms other than inhibition of the endothelial and carotid body enzyme alone (Teppema *et al.* 1995; Wagenaar *et al.* 1996). After infusion of 4 mg kg^{-1} acetazolamide, the animals in this study developed small increases in the arterial–end-tidal P_{CO_2} gradients (see Tables 1 and 2) only. Our data do not allow us to estimate the extent of the resulting inhibition of the erythrocytic enzyme, but it must have been less than that needed to cause the physiological effect of complete (i.e. $> 99.9\%$) inhibition (Maren, 1967, 1977). In the cat, maximal widening of the arterial–end-tidal P_{CO_2} gradient occurs after about 30 mg kg^{-1}, a dose substantially higher than is needed in dogs (see Wagenaar *et al.* 1996, 1998, 2000). Altogether, we have no reasons to assume that the applied dose of acetazolamide caused substantial slowing of the CO_2 hydration reaction kinetics in the blood, which in that case could have slowed or even masked a fast (peripheral) component in the ventilatory response upon stepwise changes in end-tidal P_{CO_2}.

Because, due to its physicochemical properties, acetazolamide crosses biological membranes rather slowly (Holder & Hayes, 1965), the fact that a dose as low as that applied in the present study has such large ventilatory effects is remarkable. The effects on the carbon dioxide sensitivity of the central chemoreflex loop and on the apnoeic threshold B confirm and extend earlier findings obtained in normoxic and moderate hypoxic conditions, respectively (Wagenaar *et al.* 1996, 1998). Briefly, we attributed the decreases in S_C and B to the inhibition by acetazolamide of a membrane-bound carbonic anhydrase on the luminal side of brain capillaries (cf. Hanson *et al.* 1981*a*; Ridderstråle & Hanson, 1984). This may influence the relationship between brain tissue P_{CO_2} and arterial P_{CO_2} by altering the cerebral blood flow response to changes in arterial P_{CO_2} (Wagenaar *et al.* 1996, 1998). In the same studies we ascribed the effect on the peripheral chemoreflex loop to a possible direct action on the peripheral chemoreceptors, and because the interaction between O_2 and CO_2 is known to occur at the level of the carotid bodies (Lahiri & Delaney, 1975; Fitzgerald & Deghani, 1982), we focused in the present study on the effect of acetazolamide on this interaction.

From our finding that acetazolamide abolished the increase in S_P, on going from hyperoxia to moderate hypoxia, we conclude that indeed the low dose must affect the peripheral chemoreflex by a direct action on the carotid bodies.

It is known that chemosensitive (type I) carotid body cells posses carbonic anhydrase but the exact subcellular location(s) and the identity of the isoform(s) are unknown (Ridderstråle & Hanson, 1984; Rigual *et al.* 1985; Nurse, 1990). Histochemical studies by Ridderstråle & Hanson (1984) and Rigual *et al.* (1985) showed the existence of both cytosolic and membrane-bound carbonic anhydrase in type I cells from cat. Biochemical assays of rabbit carotid bodies suggested the presence of at least two isoforms (one cytosolic and one membrane-bound) of the enzyme (Botrè *et al.* 1994). Because we observed such pronounced effects after a low dose of a moderately slow-permeating inhibitor, we should consider the possibility that its effect may be mediated by inhibition of an easily accessible isoform of the enzyme, possibly CA IV, at the extracellular face of the membrane of type I cells. In this context we refer to the finding of Hanson *et al.* (1981*b*) that the non-permeant inhibitor benzolamide, at a dose as low as 2 mg kg^{-1}, reduced the overshoot in the carotid body response to fast local applications of CO_2-saturated saline. Their *in vivo* finding in cats, that acetazolamide (50–100 mg kg^{-1}, I.V.) quickly (i.e. within a few minutes) reduced the speed of the carotid body response to a fast hypercapnic stimulus and prevented adaptation, led Torrance and co-workers initially to suggest that an extracellular carbonic anhydrase was mediating the fast hypercapnic response (Black *et al.* 1971). Because a large dose (25 mg kg^{-1}) of the more lipid-soluble and less ionised agent acetazolamide had more pronounced effects than benzolamide, they suggested later that carotid body responses are triggered by an intracellular pH change rather than by an extracellular one (acidic hypothesis; see Hanson *et al.* 1981*b*).

At present, it is generally accepted that intracellular acidosis is a crucial factor in the cascade of events from a rise in P_{CO_2} to an increase in carotid body discharge. The activity of local carbonic anhydrase is necessary to obtain the normal biphasic response of the afferent nerve upon a fast change in P_{CO_2}, i.e. an over- or undershoot followed by a slower adaptation to the new steady-state level: apart from a decrease in resting activity, inhibitors of the enzyme cause a reduction in the peak and steady-state carotid sinus nerve responses (Hanson *et al.* 1981*b*; Iturriaga *et al.* 1991; Iturriaga, 1993; Lahiri *et al.* 1996). *In vitro*, the predominant effect of *complete* carbonic anhydrase inhibition is to reduce the fast initial rather than the steady-state response (Itturiaga *et al.* 1993; Lahiri *et al.* 1996). This, however, may depend on the degree of inhibition, since during *partial* inhibition the *in vitro* steady-state response may be reduced (Itturiaga *et al.* 1991). The effects of carbonic anhydrase inhibitors may be due to a rise in intracellular pH. Not only has intracellular alkalosis by acetazolamide been shown to occur in type I carotid body cells from neonatal rats (Buckler *et al.* 1991), it has also been observed in other tissues such as smooth muscle cells from guinea-pig mesentery arteries (Pickkers *et al.* 1999). So if the effects of acetazolamide on S_P and on the CO_2–O_2 interaction that we report are also due to intracellular alkalosis in type I cells, normal activity of the uninhibited enzyme will have an acidifying effect. How then could a carbonic anhydrase iso-enzyme, possibly located at the extra-cellular face of the membrane (see above), acidify the cell interior? One possibility is that normal enzyme activity is necessary for the operation of a bicarbonate–chloride (bicarbonate out, chloride in) exchanger in the membrane of type I cells, which indeed appears to exist (Buckler *et al.* 1991). Inhibition of the exchanger results in intracellular alkalosis (Buckler *et al.* 1991; Wilding *et al.* 1992), and in a reduced steady-state response of the carotid body to hypoxia and hypercapnia (Itturiaga *et al.* 1998). In several tissues, intracellular alkalosis by inhibition of a membrane-bound carbonic anhydrase may be due to inhibition of a chloride–bicarbonate exchanger (Pickkers *et al.* 1999).

In guinea-pig mesenteric smooth muscle cells, benzolamide and acetazolamide, apart from causing intracellular alkalosis, stimulate large-conductance (Ca^{2+}- dependent) potassium channels, resulting in hyperpolarisation, reduced Ca^{2+} influx and relaxation (Pickkers *et al.* 1999). Conversely, Peers & Green (1991) showed that weak-acid-induced intracellular acidosis in type I cells from neonatal rats inhibits large-conductance potassium channels.

A reduced influx of calcium ions secondary to acetazolamide-induced hyperpolarisation of glomus cells could well result in a substantial reduction in CO_2–O_2 interaction: Dasso *et al.* (2000) recently showed that within individual type I cells from neonatal rats a combined hypoxic–hypercapnic stimulus invariably led to a greater rise in intracellular rise [Ca^{2+}] than either stimulus alone.

In summary, considering the data available in the literature we suggest that the reduction in S_p and the loss of CO_2–O_2 interaction induced by low-dose acetazolamide may be due to an alkalising effect on carotid body cells, followed by an increased opening probability of large conductance potassium channels, hyperpolarisation and reduced calcium influx. Obviously, such a scenario warrants electrophysiological verification. In addition, the mechanism(s) by which carbonic anhydrase influences the pH in glomus cells will remain incompletely understood until more detailed information on both the identity and subcellular location(s) of the isoforms becomes available.

An inhibiting effect of acetazolamide has also been shown in human subjects. After an intravenous infusion of 500 mg, Swenson & Hughes (1993) reported that the eucapnic hypoxic ventilatory response was abolished. Scheuermann *et al.* (1999*a*,*b*) showed that an intravenous dose of 10 mg kg^{-1} slowed the $\dot{V}_E$ and $\dot{V}_{CO_2}$ kinetics and prevented a decrease in ventilation upon the transition from normoxia to hyperoxia during steady-state exercise, and they attributed this to a decrease in carotid body gain. However, after a standard clinical oral dosage of 250 mg every 8 h (for 3 days), we did not find any change in the CO_2 sensitivity of the peripheral chemoreflex loop in healthy volunteers (Teppema & Dahan, 1999). It seems therefore that the effect of the inhibitor on the carotid bodies depends critically on the dose and route of administration. In most clinical applications of acetazolamide, an oral dose is given. Usually this results in metabolic acidosis, frequently (but not always) followed by a rise in ventilation (Teppema & Dahan, 1999). Acute intravenous administration, however, does not lead to metabolic acidosis but may be followed by inhibiting effects on the control of breathing (reviewed by Swenson, 1998).

In this and previous studies in cat and rabbit, we have shown that an intravenous dose as low as 4 mg kg^{-1} may have both stimulating (decrease in apnoeic threshold) and inhibiting (decrease in CO_2 sensitivity) effects (Wagenaar *et al.* 1995, 1996, 2000). Although we cannot exclude effects at the (neuro)muscular level as shown in rabbit (see Kiwull-Schöne *et al.* 2001), the fact that in the present study the agent abolished or at least reduced the CO_2–O_2 interaction suggests a direct action on the carotid bodies. Preliminary data from our laboratory show that in the cat acetazolamide, at the same dose, also reduces the isocapnic hypoxic response; larger doses, sufficient to cause effective inhibition of intracellular (e.g. red cell) carbonic anhydrase, completely abolish it (Teppema *et al.* 1988, 1992). Again this indicates that the effect is dose dependent. If carbonic anhydrase inhibitors cause physiological effects only when inhibition exceeds 99 %

(Maren, 1967, 1977), this dose dependency could result from enzyme activity in different compartments with variable accessibility. Thus a relatively high dose of a moderately slow-permeating agent would be needed to inhibit the enzyme at intracellular locations.

Our data do not provide a direct clue as to the specific site of action of acetazolamide within the carotid bodies. As mentioned above, data obtained from cat and rabbit indicate the existence of at least two iso-enzymes in type-I cells. Biochemical analysis of rabbit carotid bodies showed that during hyperoxia enzyme activity was lower than during hypoxia (Botrè *et al.* 1994). This may explain our present finding that during hyperoxia the effect of acetazolamide on S_P was smaller than during hypoxia (see also previous data from hypoxic animals; Wagenaar *et al.* 1998). Carbonic anhydrase may thus be an oxygen-sensitive element within the carotid bodies.

In conclusion, the data presented here show that low-dose acetazolamide lowers the CO_2 sensitivity of the peripheral chemoreflex loop (S_P) particularly during hypoxia. Furthermore, the agent prevented the increase in S_P on going from hyperoxia to moderate hypoxia. Because the interaction between O_2 and CO_2 resides within the carotid bodies, it was concluded that the effect of acetazolamide is probably mediated by a direct action on the carotid bodies. The precise role of carbonic anhydrase within the carotid bodies is not completely known at this stage, but our data show that normal activity of the enzyme is crucial for the carotid bodies to function normally, even in steady-state conditions.

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