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Factors of lowered respiratory CO_2 sensitivity by acetazolamide in anaesthetized rabbits

Heidrun F. Kiwull-Schöne[1*], Luc J. Teppema[2], Peter J. Kiwull[1]

[1] *Department of Physiology, Faculty of Medicine, Ruhr-University, 44780 Bochum, Germany*

[2] *Department of Physiology and Anesthesiology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands*

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Abstract: The carbonic anhydrase (CA) inhibitor acetazolamide is a classic drug to treat patients with breathing disorders. Recent studies in rabbits showed that low-dose acetazolamide (not causing appreciable inhibition of red cell CA) significantly weakened respiratory muscle performance, accompanied by diminished ventilatory CO_2-sensitivity, which implies stabilizing loop-gain properties. Now is aimed to explore the interaction of these factors under conditions of complete CA-inhibition by acetazolamide in a higher dose-range.

In anesthetized rabbits (N=7), acetazolamide (up to 75 mg·kg^{-1}) distinctly lowered the base excess (to −7.6 ±0.9mM, mean ± SEM) without respiratory compensation of arterial pH. Ventilatory CO_2-sensitivity was nearly abolished to 15.1 ±5.2% of control, but the transmission of a CO_2-mediated rise in tidal phrenic activity into respiratory work was only reduced by 51.6 ±6.4%, P<0.001, not very much more than (∼38%) already observed at low-doses.

Thus, the large reduction of ventilatory CO_2-sensitivity in the high-dose range cannot be ascribed to respiratory muscle weakening, but rather may relate to complete inhibition of red cell CA. Conversely, CA-inhibition may not be the only cause for the weakening effect of acetazolamide on (respiratory) muscles. Adverse effects on respiratory muscles, impaired CO_2-transport and acid-base imbalance may limit to make use of stabilizing effects on breathing control functions by high-dose acetazolamide.

Keywords: Acetazolamide, CO_2 sensitivity, metabolic acidosis, respiratory muscle fatigue, rabbits

* E-mail: Heidrun.Kiwull-Schoene@ruhr-uni-bochum.de

1 Introduction

The ubiquitous presence of carbonic anhydrase (CA) in erythrocytes, lung endothelial cells, kidney tubules, carotid bodies, striated and smooth muscles as well as neurons, glial cells and brain capillary endothelium, implies multifactorial effects of CA inhibition on the control of breathing [1]. Several studies in men [1–4] and animals [5–7] indicate that acetazolamide, as the most widely used CA inhibitor, can have both stimulatory and inhibiting effects on breathing, depending on the dose and route of administration.

The known beneficial effects on blood gases that are seen in (some but not all) COPD patients are usually ascribed to a rise in ventilation secondary to renal metabolic acidosis [1, 3, 4, 8]. Another known application of acetazolamide in respiratory medicine is to prevent and/or reduce the symptoms of acute mountain sickness [8] or sleep disordered breathing [2, 9, 10].

Previously, we have shown that dose-dependent mechanisms may underlie the effects of CA inhibition on the control of breathing in the cat. Low-dose acetazolamide (∼4.5 $mg{\cdot}kg^{-1}$) decreased the apnoeic threshold and reduced peripheral and central chemosensitivity by about 35%, most likely through actions on the carotid bodies and cerebral blood vessels, respectively [6, 7]. However, benzolamide at high doses that cause >99.99% inhibition of red cell CA led to very large reductions in central CO_2 sensitivity, most likely caused by a decreased slope of the in vivo CO_2 dissociation curve, so that a given change in arterial PCO_2 would be followed by a much smaller change in brainstem tissue PCO_2 than in the uninhibited state [5].

In rabbit, we recently reported an additional and surprising effect of low-dose acetazolamide consisting of a substantial impairment of respiratory muscle function during hypercapnia by about 40% [11], accompanied by ∼30% decrease in ventilatory CO_2 sensitivity, implying protective loop-gain properties against the incidence of periodic breathing [12]. In line with the observation that acetazolamide causes respiratory muscle weakening in rabbits is the reduction of skeletal muscle performance and exercise endurance reported also for healthy humans after oral intake of acetazolamide [13, 14].

Because the effects on ventilatory CO_2 sensitivity were found to be largely different at different levels of red cell CA inhibition [5–7], we now tried to separate possible dose-dependent factors on respiratory muscle function from those on central chemosensitivity. To this end, phrenic nerve activity, intrapleural pressure and ventilation were determined simultaneously. Studying the dose-dependent role of acetazolamide under this aspect may be of clinical relevance to distinguish between desired (stabilizing) and undesired (muscle weakening) factors.

2 Statistical methods and Experimental Procedures

The experiments were officially approved according to the "German Law on the Protection of Animals". They were performed in 7 male rabbits (average weight 3.5 kg), anaesthetized by sodium pentobarbital (average initial dose: 55 $mg{\cdot}kg^{-1}$, continuous in-

fusion: 7.7 mg·kg^{-1}·h^{-1}), as described previously in detail [11]. Briefly, the tracheal cannula was connected to a Fleisch-tube (size 0) for pneumotachography (Gould-Godart pneumotachograph type 17212, SensorMedics, Bilthoven, The Netherlands). Transpulmonary pressure was determined in the oesophagus (Manometer/Amplifier, Validyne, Northridge, USA), phrenic nerve activity was measured by bipolar silver electrode, preamplifier (DAM 50, WPI, Sarasota, FL) and (self-constructed) leakage integrator, airway CO_2 by infrared-absorption (Binos 1, Leybold-Heraeus, Hanau, Germany) and arterial blood pressure by pressure transducer (Statham P 23Gb) and bridge amplifier (AWP4 DC, Astro-Med, Warwick, RI). All these variables were continuously recorded (Chart recorder MT95K2, Astro-Med, Warwick, RI).

Arterial blood gases and pH were measured at (the controlled body temperature of) 38° by conventional equipment (Clark type electrode E5046, BMS2 Mk2 blood microsystem and pH meter PHM 84, Radiometer, Copenhagen, Denmark). Standard bicarbonate and base excess (BE) were estimated directly from the pH of samples equilibrated with precision CO_2/O_2 mixtures (Precision gas-mixing pump, Wösthoff, Bochum, Germany). Concentrations of hemoglobin and lactate were determined photometrically (Spectrophotometer: Hitachi-100-10, Tokyo, Japan; Test solutions: Merckotest, Merck, Darmstadt, Germany and LACT MPR 3, Boehringer, Mannheim, Germany).

2.1 Experimental protocol

Control CO_2-responses of respiratory variables were assessed for three repeated baseline levels and four steady state levels of elevated arterial P_{CO2} between 4.41 ±0.25 and 7.98 ±0.40 kPa, by adding about 3, 4, 6 and 8% CO_2 to the inspiratory gas mixture. This was repeated after injections of acetazolamide at 15 minute intervals to reach high average (±SEM) cumulative doses of 9.3 ±0.5, 18.8 ±0.9, 37.7 ±1.8 up to 73.8 ±3.5 mg·kg^{-1}. The 8% CO_2-step was included to cover the range reached by high-dose acetazolamide, despite thereby having to accept non-linearity of control ventilatory CO_2 response curves.

2.2 Evaluation and data processing

Mean steady state values of tidal volume (V_T), inspiratory and expiratory durations (T_I,T_E), of tidal transpulmonary pressure changes (ΔP_{TP}), integrated tidal phrenic nerve activity (IPNA) and end-tidal PCO_2 (Pet_{CO2}), as well as of blood pressure and heart rate were obtained during five breaths immediately before blood sampling. Respiratory rate (f_R= 60/T_I+T_E), pulmonary ventilation ($\dot{V}$=V_T·f_R), tidal respiratory work ($V_T \cdot \Delta P_{TP}$) and phrenic minute activity (IPNA·f_R) were calculated. The maximum IPNA value reached in each animal was normalized to 100 units.

2.3 Statistical Analysis

Variables were tested for normal distribution (One-sample Kolmogorov-Smirnov test). Group mean values and standard errors (SEM) under control conditions and after application of acetazolamide were compared by paired-samples t-tests. Linear regression analysis was performed for various relationships between V_T and ΔP_{TP} and IPNA during CO_2-inhalation in each animal. The effect of acetazolamide was explored by paired comparisons of the resulting individual slopes (and intercepts). Accordingly, the slopes of the ventilatory CO_2 responses were assessed at base-line level from individual best fitting curves. Differences were regarded as significant with $P_D \leq 0.05$. Statistical analysis was performed by SPSS 11.0 for Windows (SPSS, Chicago, IL).

3 Results

3.1 Base-line conditions

The effects of high-dose acetazolamide on resting acid-base status and ventilatory variables are summarized in Table 1. The agent increased ventilation and tidal volume by up to about 125% and 90%, respectively, with smaller rise in respiratory rate (+16%). Mean end-tidal P_{CO2} decreased by up to 2.7 kPa, while the $P(a\text{-}et)_{CO2}$ difference rose considerably in a dose-dependent manner, indicating that the infused high dose of acetazolamide did inhibit erythrocytic carbonic anhydrase (CA) effectively (Fig. 1). Likewise, there was a progressive decrease in base excess by up to an average of -7.9 $mmol \cdot l^{-1}$ with no respiratory compensation of blood pH (Table 1). The mean arterial blood pressure was lowered by about 20%.

3.2 Respiratory CO_2-responses

The effect of high-dose acetazolamide on the CO_2 responses of ventilation and tidal volume, as well as their neuronal equivalents, are shown in Fig. 2 A-D. In spontaneously breathing rabbits, starting from a more than doubled base-line ventilation, high-dose acetazolamide considerably reduced ventilatory CO_2 sensitivity, from a mean control value of 334 ±67 to 53 ±25 $ml \cdot min^{-1} \cdot kg^{-1} \cdot kPa^{-1}$, ($P < 0.001$). Concomitantly, the hypercapnic maximum of ventilation was significantly lower than under control conditions (by 24.2 ±6.1%, $P < 0.01$, Fig. 2A).

As shown by Fig. 5, the relative average reduction in ventilatory CO_2-sensitivity was 84.9 ±5.2%, whereas a mean reduction of only 33.4 ±9.7% resulted from our previous data for low doses [11].

Table 1 Eucapnic base-line values before and after high-dose acetazolamide application.

Control	Acetazolamide	
$\dot{V}$[ml·min^{-1}·kg^{-1}]	258.5 ± 15.3	581.2 ± 68.2**
V_T [ml·kg^{-1}]	6.1 ± 0.5	11.6 ± 1.1**
f_R [min^{-1}]	43.1 ± 2.5	49.8 ± 1.9*
T_I [s]	0.51 ± 0.04	0.47 ± 0.02
T_E [s]	0.91 ± 0.06	0.75 ± 0.04*
Pa_{CO2} [kPa]	4.41 ± 0.25	6.08 ± 0.71*
Pet_{CO2} [kPa]	4.18 ± 0.23	1.46 ± 0.17***
pHa	7.456 ± 0.007	7.271 ± 0.037**
BE [mmol·l^{-1}]	0.3 ± 1.2	-7.6 ± 0.9**
Lac [mmol·l^{-1}]	2.4 ± 0.4	4.1 ± 0.9
Pa_{O2} [kPa]	20.21 ± 0.44	23.30 ± 1.00*
MAP [kPa]	12.48 ± 0.58	9.68 ± 1.03**

Means ±SEM (N=7) of minute ventilation ($\dot{V}$), tidal volume (V_T), respiratory rate (f_R), inspiratory and expiratory times (T_I, T_E), end-tidal P_{CO2} (Pet_{CO2}), arterial P_{CO2} (Pa_{CO2}), pH (pHa), base excess (BE), lactate concentration (Lac), arterial P_{O2} (Pa_{O2}) and mean arterial blood pressure (MAP) each obtained before CO_2 elevation under control conditions and about 20 min after cumulative application of acetazolamide (73.8 ± 3.5 mg·kg^{-1}).
Significant difference of means by paired samples t-test: *P ≤0.05, ** P ≤0.01 and *** P ≤0.001.

3.3 Transmission of tidal phrenic nerve activity into tidal volume, transpulmonary pressure and respiratory work

Comparison of the V_T-Pa_{CO2} and IPNA-Pa_{CO2} response curves in Fig. 2B and 2D shows that during hypercapnia only tidal IPNA but not V_T is significantly enhanced by complete CA inhibition. At any level of P_{CO2}, IPNA was distinctly higher with acetazolamide than under control conditions (at maximum by 38.4 ±5.8%, P<0.001), which indicates an impaired transmission between neuronal drive and tidal volume response, as is also visualized by the original recording in Fig. 3.

Individual regression analysis revealed that acetazolamide reduced the mean slope of the V_T-IPNA relationship for different levels of $Pa_{CO2}$by 53.5%±6.6%, and the slope of the relationship between the transpulmonary pressure changes ΔP_{TP} and IPNA by 47.9±7.9% (not shown). This implies also reduced tidal respiratory work ($\Delta P_{TP} \cdot V_T$) for a given central respiratory drive (Fig. 4A), whereby dynamic lung compliance remained unaffected, as acetazolamide did not change the mean $V_T - \Delta P_{TP}$ relationship (Fig. 4B). The average relative reduction of respiratory work performance by high-dose

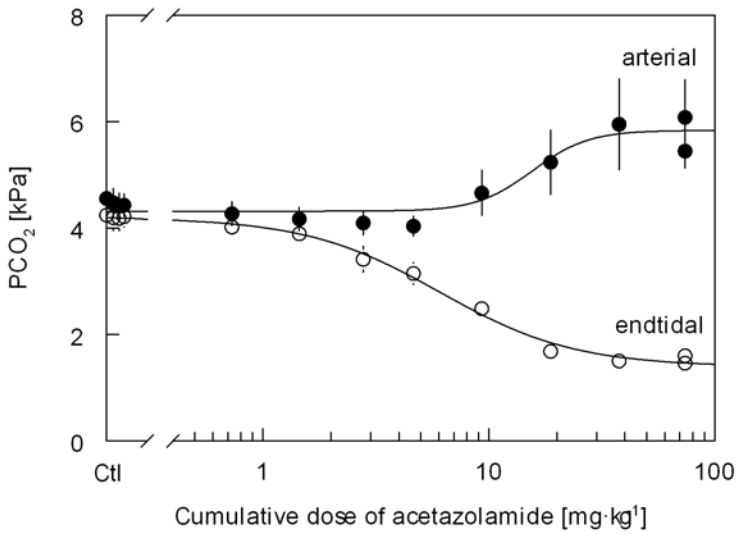

Fig. 1 Dose-dependent effect of acetazolamide on the arterio-endtidal P_{CO2} gradient. Values are means ±SEM (N=7) of arterial (•) and endtidal (○) P_{CO2} in response to cumulative injection of acetazolamide (logarithmic scale).
Note the considerable rise in the P_{CO2} gradient between blood and alveolar space at cumulative doses above ∼5 mg·kg^{-1}. Data for the lower dose-range were taken from [11].

acetazolamide was 51.6 ± 6.4%, P <0.001 (Fig. 5). Compared to that, already low doses of acetazolamide reduced the transmission of a CO_2-induced phrenic neuronal drive into respiratory work by as much as 37.6 ±8.0%, P <0.01 (Fig. 5), recalculated from previous data [11].

4 Discussion

The main findings of this study are that compared to low doses, high-dose acetazolamide up to ∼75 mg·kg^{-1} has only little additional weakening effects on respiratory muscles, but causes a much larger decrease in ventilatory CO_2 sensitivity.

4.1 Base-line conditions

In rabbits, high intravenous doses of acetazolamide elicit a large rise in baseline ventilation, in much the same way as in cats [15]. This hyperventilation may be due to brainstem tissue CO_2 retention, since the ability of the circulating blood to remove CO_2 from the tissues is reduced upon >99.99% inhibition of erythrocytic CA [16]. Under these conditions, brainstem extracellular-fluid P_{CO2} reached rather high values between 7 and 13 kPa in cats [17, 18] and rabbits [19, and unpublished own data], which also explains large augmentations of central neuronal drive (Fig. 2C).

Additional driving forces on ventilation may arise from the considerable metabolic acidosis. Since no lactic acid accumulation was found, the observed metabolic acidosis

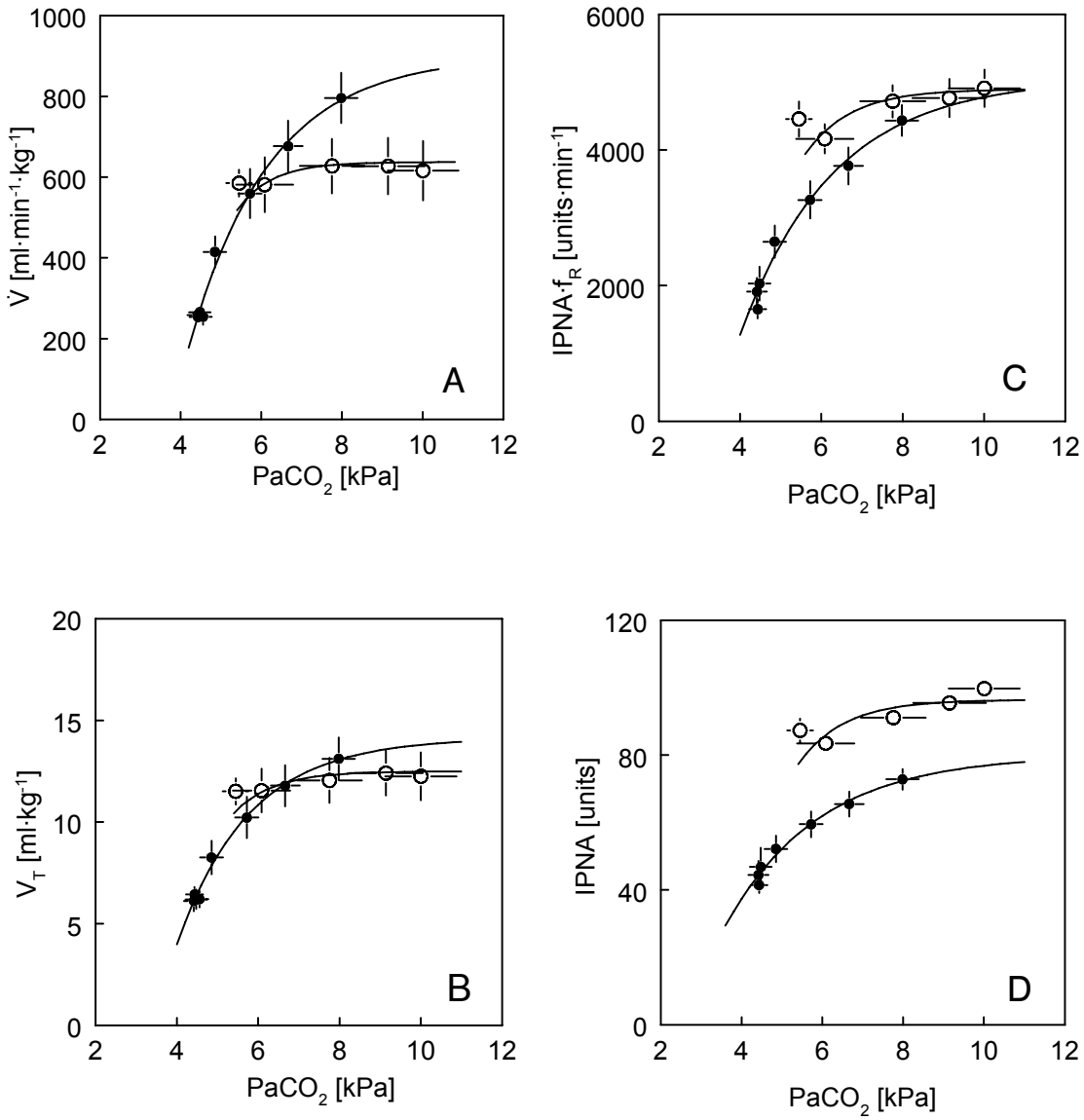

Fig. 2 Different effects of high-dose acetazolamide on CO_2 responses of mechanical compared to neuronal respiratory variables.
As functions of arterial PCO_2 (Pa_{CO2}) are shown in *diagram A*: Pulmonary ventilation ($\dot{V}$), in *diagram B:* Tidal volume (V_T), in *diagram C*: Phrenic minute activity ($IPNA \cdot f_R$) and in *diagram D*: Integrated tidal phrenic nerve amplitude (IPNA). Values are means ±SEM of seven rabbits under control conditions (•) and after acetazolamide (○).
Note stronger attenuating effect of acetazolamide on ventilation and tidal volume compared to corresponding neuronal respiratory drives.

may be due to inhibition of renal CA [1]. In rabbits, however, on alkali-rich herbivore alimentation, the role of renal CA inhibition for the development of metabolic acidosis remains to be explored, since daily oral treatment with 20mg/kg acetazolamide - other than in humans - does not change fractional renal base re-absorption [20]. Metabolic acidosis normally leads to an increase in ventilation mediated by the peripheral chemore-

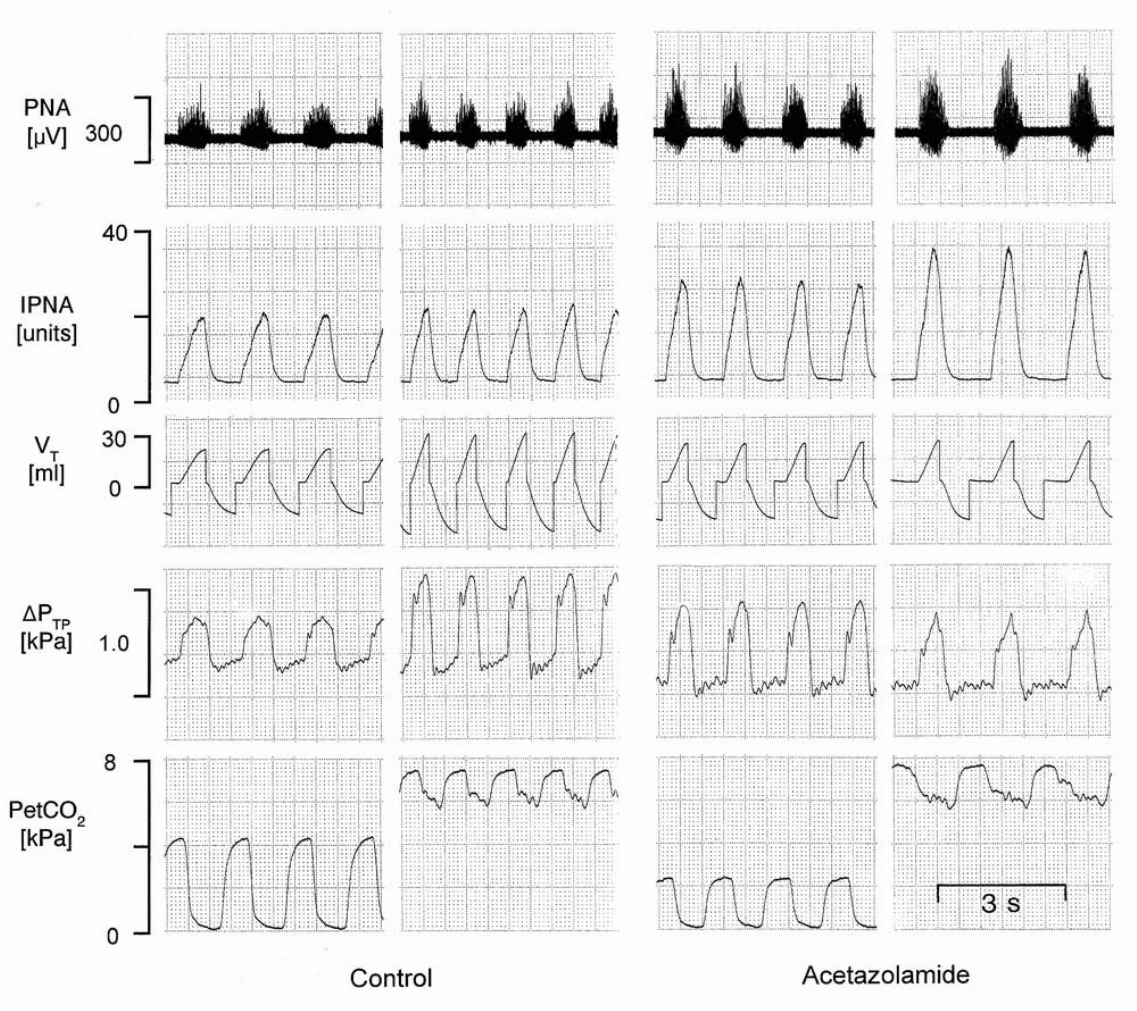

Fig. 3 Effect of high-dose acetazolamide on the relationship between tidal phrenic nerve activity and tidal volume or transpulmonary pressure in response to elevated P_{CO2}.
Recordings from top to bottom: Phrenic nerve compound potential (PNA), integrated phrenic nerve activity (IPNA), tidal volume (V_T), tidal transpulmonary pressure change (ΔP_{TP}.) and endtidal P_{CO2} (Pet_{CO2}), recorded in a male rabbit (3.55 kg).
Note that upon acetazolamide higher neuronal respiratory drive is needed to achieve a given tidal volume or transpulmonary pressure change. Thereby, the neuronal CO_2 response shows a rather deep and slow type of breathing pattern.

ceptors [1, 3, 4, 8]. This factor may be negligible in our experiments first, because of the sustained arterial hyperoxia [11], and second, because of the directly inhibiting effect of acetazolamide on peripheral chemoreceptors and/or ventilatory chemoreflex responses [7, 17, 21].

For the significant decrease in mean blood pressure (by about 20%) that we observed, vasodilatation at various locations in the systemic circulation [22] and a maximal vasodilatation in the brain shown for high doses [23] may be responsible.

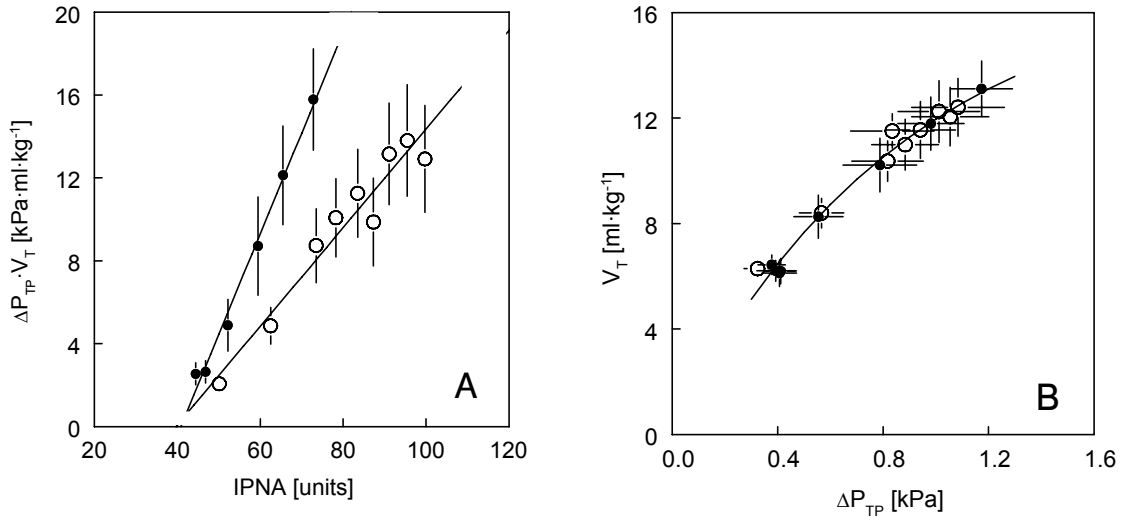

Fig. 4 The effect of high-dose acetazolamide on respiratory work performance and dynamic lung compliance.
Diagram A: Tidal respiratory work ($\Delta P_{TP} \cdot V_T$) as a function of integrated tidal phrenic nerve amplitude (IPNA). *Diagram B:* Tidal volume (V_T) as a function of tidal transpulmonary pressure changes (ΔP_{TP}). Values are means ±SEM of seven rabbits obtained at different levels of inhaled CO_2 under control conditions (•) and after acetazolamide (○). Note reduced respiratory work performance with acetazolamide, but no change in dynamic lung compliance.

4.2 CO_2 -responsiveness and respiratory muscle function

Cumulative application of acetazolamide was followed by a substantial rise in the arterial-to-endtidal P_{CO2} difference (Fig. 1), supporting previous reports for cats and rabbits [15, 24] and indicating progressive inhibition of erythrocytic CA towards the high-dose range. During complete and selective inhibition of red cell CA by benzolamide in cats, the observed large decrease in ventilatory sensitivity to inhaled CO_2 has been proven to be caused by a reduced CO_2 transport capacity to the central chemoreceptors due to a decreased slope of the *in vivo* CO_2 dissociation curves in blood and brain tissue [5].

High-dose acetazolamide also in rabbits largely reduced the ventilatory CO_2-sensitivities by ∼85%, whereas a mean reduction of only ∼33% resulted for low doses. Concomitantly, we found that low doses of acetazolamide reduced the transmission of a CO_2-induced phrenic neuronal drive into respiratory work by approximately the same order of magnitude. A crucial finding of our present study is that a high dose of the agent did not very much add to this inhibiting effect on respiratory muscle performance.

Therefore, we suggest that the reduced ventilatory CO_2 sensitivity by low-dose acetazolamide may quite be caused through respiratory muscle weakening [11], but not the considerable further reduction in ventilatory CO_2 sensitivity at high doses. Since respiratory muscle function was not very much more confined than by low doses, the large reduc-

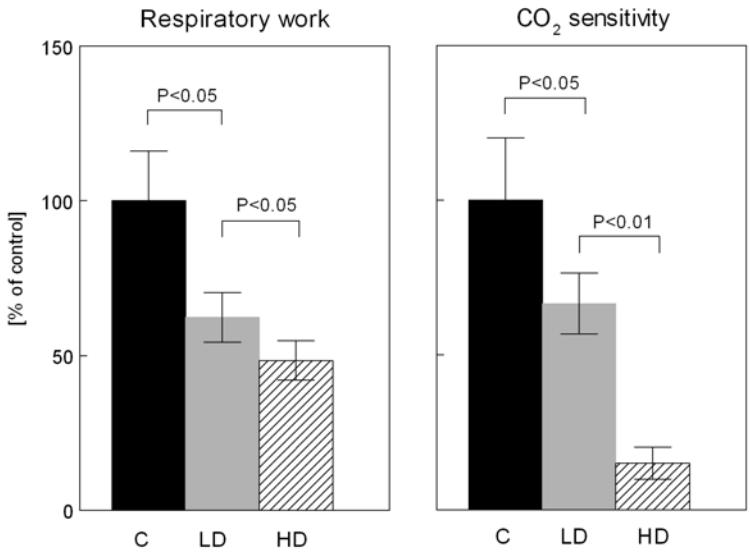

Fig. 5 Dose-dependent relative changes of respiratory work performance and ventilatory CO_2 sensitivity upon treatment with acetazolamide.
Tidal respiratory work for a given neuronal drive was calculated from the individual slopes underlying the data of Fig. 4A. Correspondingly, individual slopes of the ventilatory CO_2 response curves underlying Fig. 2A were estimated at base-line level. Results for the low-dose condition were recalculated from data published by Kiwull-Schöne et al. [11].
Bars represent mean values ± SEM of these slopes. Control values normalized to 100% (C, black bars). Relative changes by acetazolamide: Low dose (LD, grey bars), high dose (HD, hatched bars). Significance of differences was estimated by paired samples t-test.
Note that major reduction of respiratory work performance by acetazolamide is already accomplished by low-doses, whereas the most substantial reduction of CO_2 sensitivity occurs by high-doses.

tion of ventilatory CO_2 sensitivity in the high-dose range may rather relate to the role of complete red cell CA inhibition to attenuate CO_2 signals between blood and central chemoreceptor sites [5]. Indeed, Fig. 2B demonstrates that high-dose acetazolamide does attenuate the central neuronal CO_2 sensitivity in a way theoretically to be expected [5]. Vice versa, since only marginal inhibition of red cell carbonic anhydrase prevails upon low-doses of acetazolamide, an effect on muscle cells different from CA inhibition cannot be excluded.

4.3 Acetazolamide's possible mode of action

Since acetazolamide diffuses relatively slowly through biological membranes [16], the effect of low doses on respiratory muscles was assumed to be mediated by inhibition of the easily accessible extracellular iso-form of carbonic anhydrase CA IV in skeletal muscles [11, 25]. Disturbed CO_2 excretion and interstitial buffering capacity in the working muscle would explain why acetazolamide in low doses is able to counteract the efficacy

of anticholinesterase therapy in myasthenic patients [26] or to inhibit isometric muscular force [13] and to limit exercise endurance in healthy subjects [14]. In high doses, acetazolamide may reach intracellular targets, e.g. the cytosolic isoforms CA II and III [25], thereby potentially disturbing the regulation of intracellular pH, $[Ca^{2+}]$ or energy metabolism [27].

On the other hand, there is growing evidence that acetazolamide specifically opens large conductance Ca^{2+} dependent potassium (BK) channels in muscle cells from the rat [28]. Since the applied high dose of the agent, despite effective CA inhibition, did not lead to much additional respiratory muscle weakening, we cannot exclude that it may occur independently from carbonic anhydrase inhibition. Further studies are necessary to decide whether the weakening effect of acetazolamide on (respiratory) muscles in the rabbit is due to a specific influence e.g. on BK channels [28].

4.4 Clinical application of acetazolamide

Controlled clinical studies revealed that patients with chronic obstructive pulmonary disease (COPD) could benefit from mild carbonic anhydrase inhibition by improvement of blood gases [9], usually ascribed to a rise in ventilation secondary to renal metabolic acidosis [1, 3, 4, 8]. Metabolic acidosis primarily appears to be useful for COPD patients on steroids to compensate the consecutive metabolic alkalosis (and CO_2 retention). However, steroid-induced myopathy [29] could eventually be worsened by acetazolamide.

Our findings in anaesthetized rabbits indicate that the respiratory effects of acetazolamide are strongly dose-dependent, because low doses augmented ventilation, improved blood gases and effectively compensated metabolic acidosis, but had adverse effects on muscular performance [11], while high doses greatly reduced central CO_2 sensitivity without significant additional effects on muscular level.

Based on control theory of breathing, the overall gain of the feedback loop includes both the controller gain (e.g. CO_2 sensitivity) and the gain of the controlled system (depending on base-line PCO_2 and metabolic CO_2 production). A reduced closed-loop gain is theoretically expected to have stabilizing effect on ventilation [30, 31], which view is supported by clinical experience showing the severity of the obstructive sleep apnoea syndrome to be linked with the loop gain for chemical breathing control [32]. One reason, why in some patients with sleep apnoea acetazolamide has proven to be useful [10], could be a decrease in base-line PCO_2, whereby the greater difference between the prevailing PCO_2 and the apneic threshold may increase and the propensity to develop apneas decrease [33]. At least in our animal model, we found a significant reduction in loop gain upon treatment with low-dose, due to decreased CO_2 sensitivity and diminished base-line PCO_2 at constant metabolic rate [12].

As far as application of high-dose acetazolamide is concerned, adverse effects on respiratory muscles, impaired CO_2-transport and acid-base imbalance may limit to make use of stabilizing effects on breathing control functions.

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