Acetazolamide protects against posthypoxic unstable breathing in the C57BL/6J mouse

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Yamauchi M, Dostal J, Strohl KP. Acetazolamide protects against posthypoxic unstable breathing in the C57BL/6J mouse. J Appl Physiol 103: 1263–1268, 2007. First published August 2, 2007; doi:10.1152/japplphysiol.01287.2006.—Acetazolamide (Acz), a carbonic anhydrase inhibitor, is used to manage periodic breathing associated with altitude and with heart failure. We examined whether Acz would alter posthypoxic ventilatory behavior in the C57BL/6J (B6) mouse model of recurrent central apnea. Experiments were performed with unanesthetized, awake adult male B6 mice (n = 9), ventilatory behavior was measured using flow-through whole body plethysmography. Mice were given an intraperitoneal injection of either vehicle or Acz (40 mg/kg), and 1 h later they were exposed to 1 min of 8% O2-balance N2 (poikilocapnic hypoxia) or 12% O2-3% CO2-balance N2 (isocapnic hypoxia) followed by rapid reoxygenation (100% O2). Hypercapnic response (8% CO2-balance O2) was examined in six mice. With Acz, ventilation, including respiratory frequency, tidal volume, and minute ventilation, in room air was significantly higher and hyperoxic hypercapnic ventilatory responsiveness was generally lower compared with vehicle. Poikilocapnic and isocapnic hypoxic ventilatory responsiveness were similar among treatments. One minute after reoxygenation, animals given Acz exhibited posthypoxic frequency decline, a lower coefficient of variability for frequency, and no tendency toward periodic breathing, compared with vehicle treatment. We conclude that Acz improves unstable breathing in the B6 model, without altering hypoxic response or producing short-term potentiation, but with some blunting of hypercapnic responsiveness.

ventilation; periodic breathing; carbonic anhydrase inhibitor

PERIODIC BREATHING (PB) reflects an instability in the respiratory control system (4, 13). The prevalence of PB is increased in heart failure patients or those exposed to altitude. The presence of PB during the night worsens the symptoms and prognosis of patients with heart failure (16). One mechanism thought responsible for initiation and propagation of apneas is increased chemosensitivity (16). Another is posthypoxic response. Patients without short-term potentiation (STP) of ventilation, i.e., those with posthypoxic frequency decline (PHFD), will show PB (1). Thus one might consider modification of posthypoxic ventilatory behavior as well as chemosensitivity as targets in attempts to modify recurrent central apneas.

The carbonic anhydrase has at least 14 different carbonic anhydrase isoenzymes identified genetically in mice and humans, located in different tissues and subcellular locations, each exhibiting different levels of activity (15, 22). In addition, there are proteins with “carbonic anhydrase-like” domains. Thus there are many sites and mechanisms by which a carbonic anhydrase inhibitor might act. Acetazolamide (Acz) is one carbonic anhydrase inhibitor well recognized as producing a systemic, metabolic acidosis by a decreased renal absorption of bicarbonate (5), and it increases CO2 levels by inhibiting bicarbonate formation in red blood cells and tissues (3, 23). In addition, Acz may act directly on the central nervous system (CNS) neurons and/or carotid body cells to influence breathing (14, 33). Hence the effect of Acz on ventilation can be the result of many physiological factors, but a consistent result is that it stimulates breathing and increases ventilation. Previous animal studies have shown that systemic administration of Acz increased resting ventilation in mice (21) and that it stimulated ventilation and modulated hypercapnic ventilatory responsiveness in the cat (27–29). In two human studies, Acz stimulated breathing at rest in patients with chronic obstructive lung disease and heart failure (2, 30). In a third randomized double-blind human study, Acz decreased by half the number of central apneas during sleep in patients with heart failure (12).

The hypothesis of this study was that Acz would improve posthypoxic unstable breathing in the C57BL/6J (B6) mouse, which exhibits posthypoxic breathing instability including PB (9). The subhypotheses were that PB in this model would correlate with decreased hypoxic, and/or decreased hypercapnic sensitivity, and/or posthypoxic ventilatory behavior.

METHODS

Animals. Experiments were performed on unanesthetized adult male B6 mice (n = 9, 2.5 mo old, 22.0 ± 1.7 g, means ± SD). Animals were obtained from Jackson Laboratory (Bar Harbor, ME) and were housed in the Louis Stokes Department of Veterans Affairs Medical Center (LSDVAMC) Animal Research Facility for at least 3 wk before investigation (food and water ad libitum; with a 7 AM to 7 PM and 7 PM to 7 AM light-dark cycle). Experimental protocols used were approved by the LSDVAMC Institutional Animal Care and Use Committee and were in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental protocols. Measurements were made between 10:00 AM and 2:00 PM. All experiments were carried out when the animals appeared awake, as determined by behavioral observation. On days 1 and 3, the mice were put into the chambers at the usual start time, but they did not undergo any testing. After testing hours, they were returned to the LSDVAMC Animal Research Facility. On day 2 or 4, the animals received one of two experimental protocols involving either injection with Acz or vehicle. Except for the injection received, the protocols for days 2 and 4 were identical. Mice received Acz or vehicle on day 2 and a crossover on day 4. The presentation of Acz or vehicle was randomized in each group.

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Animals were first weighed and then placed in the test apparatus at 10:00 AM and given 60 min to acclimate to their surroundings. At 11:00 AM, the mice received an intraperitoneal injection of either saline adjusted to a pH of 9.4 with sodium hydroxide (vehicle) or Acz dissolved in saline with sodium hydroxide at a dose of 40 mg/kg, resulting in a pH of 9.4. The volume of the injection was 0.02 ml/g of body weight. One hour later, the animals underwent testing.

During resting breathing, using room air, O2 consumption (V\textsubscript{O2}) and CO\textsubscript{2} production (V\textsubscript{CO2}) were measured by the open-circuit method (8). After a 5-min recording of baseline breathing, mice were then given a 1-min poikilocapnic hypoxia challenge (8% O\textsubscript{2}-balance N\textsubscript{2}). The hypoxic gas was then flushed out of the chamber and replaced with 100% O\textsubscript{2} for 5 min. After a 20-min interval, the mice were given an isocapnic hypoxia challenge (3% CO\textsubscript{2}-12% O\textsubscript{2}-balance N\textsubscript{2}). Body temperature, measured with a thermocouple inserted rectally to a depth of 1 cm, was assessed immediately after the last hypoxic challenge.

Measurements of ventilatory behavior. Animals were placed in a round Lucite chamber (600-ml volume) containing an inlet port for the administration of test gases (see Experimental protocols). An outlet port was connected to a vacuum sufficient to create a bias flow of 300 ml/min through the chamber, as measured by a flow rotameter. Because respiration immediately following hypoxia was the focus of this experiment, test gases were flushed out of the chamber at a flow rate of 15 l/min, and then the flow rate through the chamber was returned to baseline. The chamber was connected to one side of a pressure transducer (Validyne DP45, Validyne Engineering, Northridge, CA) with a sensitivity of ±2 cmH\textsubscript{2}O, referenced to a chamber of equal volume. As the animal breathed, swings in chamber pressure were recorded and then processed as a voltage signal. Comparison of this voltage signal to calibration volumes permitted an estimation of values that would represent tidal volume (VT). For each animal, the calibration volumes before and after each testing period and the voltage signals were recorded on a strip-chart recorder (Linerecorder WR3320, Graphtec, Irvine, CA) and stored in a computer with custom-written respiratory acquisition software (LabView programming by I.C.E., Cleveland, OH). With the chamber empty, a calibration volume of 0.22 ml of air was repeatedly introduced into the chamber before and after data collection. Other calibration volumes above and below this volume were also routinely performed as a quality control for the linearity of the voltage signal.

Method for assessment of hypercapnic response. In six mice on separate days, hypercapnic response was examined, similar to the procedure for hypoxic gas challenge; after a 5-min recording of baseline breathing, mice were then given a 1-min hyperoxic hypercapnic gas challenge (8% CO\textsubscript{2}-balance O\textsubscript{2}). 1 h after intraperitoneal injection of either Acz or vehicle.

Data analysis. Ventilatory parameters were measured continuously throughout the testing period and scored by computer using a respiratory-based software program (LabView programming by I.C.E.). The following variables were calculated and analyzed: inspiratory VT (µl), breath frequency (f; breaths/min), minute ventilation (V\textsubscript{E}; ml/min; VT × f), V\textsubscript{O2} (ml/min), V\textsubscript{CO2} (ml/min), and respiratory quotient (RQ: V\textsubscript{CO2}/V\textsubscript{O2}). VT, V\textsubscript{E}, V\textsubscript{CO2}, and V\textsubscript{O2} were adjusted by body weight (g). For each animal, f and VT were obtained from the mean of consecutive breaths for 30 s during baseline and hypoxic gas challenge and for 240 s after reoxygenation. Values for steady-state V\textsubscript{E} and its components (VT × f) were determined during 5-min baseline breathing; the first 30 s and latter 30 s of the hypoxic gas challenge; and 0–60 s, 60–120 s, 120–180 s, and 180–240 s after rapid reoxygenation with 100% O\textsubscript{2}. Sighs and sniffs and erratic breathing during grooming were excluded from the analysis. Sighing was defined as a nonartifactural volume signal at least a 50% above the average amplitude compared with the preceding 10 breaths, and in general, sniffing was identified as rapid (>1.5 × baseline), shallow oscillations. To quantify breathing variability after reoxygenation, we calculated the coefficient of variation (CV) for f. We defined the presence of PB, as defined as cyclic fluctuations in f of respiration interrupted by periods of apnea or near apnea, defined as an end-expiratory pause of ≥2 average breath durations that was not preceded by a sigh. A minimum of three instances of apnea were needed to classify a given set of breaths as PB (9). Figure 1 illustrates the definitions above.

Significant differences in ventilation at all points in the protocol between drug treatments were examined by a two-way ANOVA (time and treatment as factors). If an ANOVA was significant, probing for differences within the model was performed using t-tests of estimated marginal means (simple main effects) with adjustments for multiple comparisons made via Sidak’s correction. Paired Student’s t-tests were performed to examine the difference in hypoxic and hypercapnic ventilatory responsiveness, which were expressed as percent change from baseline breathing, and the differences in V\textsubscript{E} after reoxygenation were expressed as percent change compared with resting breathing to detect STP and PHFD. To detect a difference in breathing stability, CVs were compared between treatments using paired Student’s t-test. Significant differences in the presence of PB between groups were detected using x\textsuperscript{2} statistics. Differences of P < 0.05 were considered significant. All results are expressed as means ± SD. Statistical analysis was performed with SPSS version 10.0 for Windows software (SPSS, Chicago, IL).

RESULTS

Ventilatory behavior during resting air breathing, poikilocapnic hypoxia, isocapnic hypoxia, and hyperoxia with Acz. During resting room air breathing, Acz significantly increased f (P < 0.01), body weight-adjusted VT (P < 0.01), V\textsubscript{E} (P < 0.01), and V\textsubscript{CO2} compared with vehicle treatment; however, body temperature (in °C), RQ, and body weight-adjusted V\textsubscript{O2} were not significantly affected by Acz treatment. The increase
in $V_E$ for Acz was proportionally higher for any given $V_{CO_2}$ or $V_{O_2}$ ($V_{E}/V_{CO_2}$; $P < 0.01$, $V_{E}/V_{O_2}$; $P < 0.01$, respectively) than that of vehicle-treated animals, indicating the presence of hyperventilation (Table 1). For the poikilocapnic hypoxic challenge, f during all segments, including the hypoxic and hyperoxic states, was significantly increased with Acz compared with vehicle. $V_E$ during the hypoxic state and the first to second minute of reoxygenation was increased with Acz compared with vehicle (Fig. 2, A and B).

For the isocapnic hypoxic challenge, Acz significantly increased f as well as $V_E$ for all segments of the hypoxic and hyperoxic challenges except the second minute of reoxygenation compared with vehicle (Fig. 2, C and D). In summary, Acz increased f and $V_E$ at almost every point in the protocol compared with vehicle-treated animals.

**Hypoxic and hypercapnic responses.** In both the poikilocapnic and isocapnic hypoxic challenges, an increase in f for mice treated with Acz was significantly less during the first 30 s of exposure to hypoxia compared with that of vehicle ($P < 0.05$ and $P < 0.01$, respectively). However, there was no statistically significant difference in $V_E$ between treatments (Fig. 3).

During the hypercapnic gas challenge, an increase in f as well as $V_E$ for animals treated with Acz was significantly less in the first 30 s of exposure to hypercapnia compared with that of vehicle ($P < 0.01$ and $P < 0.05$ respectively; Fig. 3). Figure 4 shows responses expressed as absolute increases in value. Although hypercapnic response in $V_E$ tended to decrease in Acz-treated animals, it did not reach statistical significance ($P = 0.067$).

**Posthypoxic frequency changes.** When comparing ventilatory behavior with reoxygenation with prehypoxic baseline, B6 mice treated with Acz showed a posthypoxic frequency and $V_E$ decline with reoxygenation after both poikilocapnic ($P < 0.01$ and $P < 0.05$, respectively) and isocapnic hypoxic challenges ($P < 0.05$, for both) (Fig. 5).

**Effects of Acz on unstable breathing.** To assess the stability of f after rapid reoxygenation, we calculated the CV for f at 1-min durations during reoxygenation. The f CVs for mice treated with Acz were significantly lower than the f CVs for vehicle for all phases of both the poikilocapnic and isocapnic gas challenges ($P < 0.01$). Furthermore, during the first minute after reoxygenation, PB, by the definitions above, was present in all of the vehicle-treated mice with poikilocapnic hypoxic gas challenge and in five of nine vehicle treated mice following isocapnic hypoxic gas challenge. Acz eliminated PB during reoxygenation from both the poikilocapnic and isocapnic gas challenges. A statistically significant difference from the vehicle-treated group was seen only in the first minute of reoxygenation (Table 2).

**DISCUSSION**

The present study shows that Acz (40 mg/kg) did improve the stability of posthypoxic ventilatory behavior as evaluated by a reduction in the CV for f and by elimination of PB. In addition, Acz significantly decreased hypercapnic ventilatory responsiveness as measured by percent change without producing a significant difference in hypoxic responsive-

![Table 1. Ventilatory behavior during resting breathing with administration of the vehicle and Acz](#)
ness. Furthermore, Acz eliminated PB despite the presence of PHFD.

There was a general ventilatory stimulant effect associated with Acz in regard to both an absolute increase in $V\dot{E}$ as well as an increase in $V\dot{E}$ in regard to $V\dot{O}_2$ or $V\dot{CO}_2$. The mechanisms for this ventilatory stimulation have been attributed to an elevation of cerebral blood flow (24), metabolic acidosis due to the decreased renal absorption of bicarbonate (5), and CO$_2$ elevations due to inhibition of carbonic anhydrases in red blood cells.

### Table 2. Coefficient of variation for posthypoxic breath frequency and presence of periodic breathing

<table>
<thead>
<tr>
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<th>Vehicle</th>
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<td><strong>Poikilocapnic hypoxic gas challenge</strong></td>
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<tr>
<td>Reoxy 1</td>
<td>CV</td>
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<td>3/9</td>
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<tr>
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<td>CV</td>
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<td></td>
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<td>0/9</td>
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Values are means ± SD; CV, coefficient of variation; PB, periodic breathing; Reoxy 1, the first minute after reoxygenation; Reoxy 2, the second minute of reoxygenation; Reoxy 3, the third minute of reoxygenation; Reoxy 4, the forth minute of reoxygenation. Significant difference from vehicle-treated animals: *$P < 0.05$; †$P < 0.01$.
cells and tissues (3, 23). Although Acz passes the blood-brain barrier very slowly (10, 17), another possible mechanism for the ventilatory stimulation is a direct effect of Acz on the CNS neurons. Carbonic anhydrase is present in the glial cells of the CNS, and Acz can cause intracellular alkalinization by increasing the accumulation of HCO₃⁻ levels, which may influence the function of various channels and neurotransmitters (14).

A few former studies support the action of Acz within the brain. Inhibition of carbonic anhydrase by microinjection of Acz into the fastigial nucleus of the cerebellum of anesthetized and spontaneously breathing rats increased VE and the minute phrenic nerve activity (31). When the retrotropeizoidal nucleus or the nucleus tractus solitarius was focally perfused with Acz in awake rats, VE was increased in room air (11, 18). We suggest that one of the effects of Acz might be by a central effect in this model, although this assumption was not supported by direct measures of Acz penetration or effect.

We found that Acz significantly decreased the response to hypercapnia expressed as a percent change from baseline, i.e., ventilatory responsiveness. Described as absolute values, there was a strong trend toward reduced responsiveness. Although the mechanism for this effect could include a direct effect on cerebral vessels on cardiac output, consequent effects on cerebral blood flow or its regulation, a change in the relationship between brain tissue PCO₂ and PO₂ (25), and/or a smaller cerebral blood flow or its regulation, a change in the relationship between brain tissue PCO₂ and PO₂, would act to stabilize hypercapnic responses. However, Acz did not alter hypoxic responsive-ness, nor did it produce STP. Our theory is that in this model the resultant depression of ventilation is caused by pathways to or within the central respiratory controller. Furthermore, we believe that this aberration encourages reentry into apnea and that it is independent of PHFD. Acz acts directly or indirectly on this pathway to reduce this form of instability.

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