Effect of chronic elevated carbon dioxide on the expression of acid-base transporters in the neonatal and adult mouse

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Effect of chronically elevated carbon dioxide on the expression of acid-base transporters in the neonatal and adult mouse. Am J Physiol Regul Integr Comp Physiol 293: R1294–R1302, 2007. First published July 25, 2007; doi:10.1152/ajpregu.00261.2007.—Several pulmonary and neurological conditions, both in the newborn and adult, result in hypercapnia. This leads to disturbances in normal pH homeostasis. Most mammalian cells maintain tight control of intracellular pH (pHi) using a group of transmembrane proteins that specialize in acid-base transport. These acid-base transporters are important in adjusting pH, during acidosis arising from hypoventilation. We hypothesized that exposure to chronic hypercapnia induces changes in the expression of acid-base transporters. Neonatal and adult CD-1 mice were exposed to either 8% or 12% CO2 for 2 wk. We used Western blot analysis of membrane protein fractions from heart, kidney, and various brain regions to study the response of specific acid-base transporters to CO2. Chronic CO2 increased the expression of the sodium hydrogen exchanger 1 (NHE1) and electroneutral sodium bicarbonate cotransporter (NBCn1) in the cerebral cortex, heart, and kidney of neonatal but not adult mice. CO2 increased the expression of electrogenic NBC (NBCe1) in the neonatal but not the adult mouse heart and kidney. Hypercapnia decreased the expression of anion exchanger 3 (AE3) in both the neonatal and adult brain but increased AE3 expression in the neonatal heart. We conclude that: 1) chronic hypercapnia increases the expression of the acid extruders NHE1, NBCe1 and NBCn1 and decreases the expression of the acid loader AE3, possibly improving the capacity of the cell to maintain pHi in the face of acidosis; and 2) the heterogeneous response of tissues to hypercapnia depends on the level of CO2 and development.

anion exchanger; sodium bicarbonate cotransporter; sodium hydrogen exchanger; acidosis; hypercapnia

HYPERCAPNIA AND THE ENSUING respiratory acidosis are consequences of many disease conditions affecting both the newborn and adult. Emphysema, severe asthma, obstructive sleep apnea syndrome, central nervous system (CNS) depression, and neuromuscular disease (e.g., amyotrophic lateral sclerosis) can all lead to chronic or intermittent hypercapnia. The elevation in alveolar carbon dioxide (CO2), which leads to respiratory acidosis, also induces intracellular acidification. This drop in intracellular pH (pHi) will affect a great number of biological processes, cellular metabolic activity, and abundance and/or activity of cellular proteins (15, 18, 24, 55). Left unadjusted, these changes may lead to irreversible cellular damage.

Most mammalian cells have evolved several mechanisms for maintaining a tight pH control through intracellular buffering and a group of transmembrane proteins that specialize in acid-base exchange (14, 51, 56). Two major families of acid-base transporter proteins play a crucial role in pH homeostasis: 1) the sodium hydrogen exchangers 1-9 (NHE1-9) SLC9A1-SLCA9 (21, 30, 43, 45, 57, 60), and 2) the bicarbonate (HCO3)−-dependent acid-base transporters (53), which include: the electronegative Na/HCO3 co-transporters (NBCe1; SLC4A4) (9, 29, 54), as well as three electroneutral members, the electroneutral Na/HCO3 co-transporter (NBCn1; SLC4A7) (19, 20, 46), the Na+−driven Cl-HCO3 exchanger (NDCBE; SLC4A8) (32, 59), and the sodium chloride bicarbonate exchanger (NCBE; SLC4A10) (13, 47, 62) whose chloride requirement and/or transport is still unresolved (53). On the basis of their function in standard physiological conditions, these transporters can be classified as acid extruders or acid loaders (55). The acid extruders such as NHEs, NDCBES, and NBCs participate in pH recovery from acid loads. The acid loaders, such as chloride-bicarbonate exchange (AE) and some NBC’s (through altering their Na+/HCO3− stoichiometry), mediate pH recovery from alkaline loads.

In rodents, the protein abundance of most of the above-mentioned acid-base transporters exhibits a developmental time course in which expression increases steadily until around 2 wk postnatally (11, 25, 49), after which expression plateaus into adulthood. Newborns and adults differ in their intracellular buffer capacity (64, 65), pH regulation (8, 22, 35), as well as their adaptive responses to pH altering stresses, such as hypoxia (37, 64, 66), and acute hypercapnia. For example, pH recovery responses to hypercapnia in medullary neurons in general has been shown to deteriorate as they age (44, 50, 51). Still, little is known about the effect of chronic hypercapnia on acid-base regulation in the CNS and other excitable tissues and organs. We hypothesized that, to maintain pH homeostasis during chronic CO2 exposure, there will be an increase in the expression of acid extruders and/or a decrease in the expression of acid loaders and that the extent of change will most likely be greater in the neonate compared with the adult. Previously, our laboratory and others have shown that varying the level of CO2 to which the animal is exposed, strongly influences the functional response as well as pattern of gene and/or protein expression in many organs including the brain, lung, and heart (33, 38, 42). Therefore, we exposed mice in this study to both a moderate (8% CO2) and a severe (12% CO2) hypercapnia and...
examinined by immunoblot analysis the effect of chronically elevated CO2 exposure on the membrane protein expression of the major acid-base transporter proteins in the brain, heart, and kidneys of neonatal and adult mice.

MATERIALS AND METHODS

CO2 Exposure

A computer-controlled system (OxyCycler; Reming Bioinstruments, Redfield, NY) was utilized to introduce and maintain constant levels of CO2 (either 8% or 12%) and oxygen levels of 21%, as described previously (34). CO2 concentrations of 8% and 12% were chosen to provide moderate and severe levels of hypercapnia. CD-1 mice, both neonates and adults, were utilized for all experiments. For neonates, litters were culled to eight pups each and placed at postnatal day 2 with their dams in Plexiglas chambers with regular 12:12-h light-dark cycles. For adults: 3-mo-old male CD-1 mice were placed in Plexiglas chambers with regular 12:12-h light-dark cycles. Mice were exposed to either 8% or 12% CO2, for 2 wk. Control litters were housed in identical chambers and exposed to room air. All experimental protocols in this study were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, CA, which is accredited by the American Association of Laboratory Animal Care.

Antibodies

The mouse monoclonal antibody to NHE1 (anti-NHE1; mAb 4E9) was derived from an MBP fusion protein containing the amino acids 514–818 of porcine NHE1 (Chemicon, Temecula, CA). We used rabbit polyclonal antibody 666 with specificity for mammalian NHE3 constructed by a GST transferase fusion protein to NHE3 cDNA (amino acids 528–648; Bookstein et al., 1994 (11a); gift of M. Musch, University of Chicago). In our study, we have utilized two rabbit polyclonal anti-NBC antibodies that detect all reported variants of NBCe1: 1) 1B1-B-NBC (9) recognizes the cytoplasmic COOH terminus unique to the brain-specific splice variant NBCe1-C, and 2) K1A-NBC (9) recognizes the cytoplasmic COOH terminus common to both the kidney splice variant NBCe1-A and the ubiquitous splice variant NBCe1-B (i.e., NBCe1-A/B). For NBCn1, the polyclonal antibody against NBCn1 is a new antibody that was generated by immunizing a rabbit with an MBP-fusion protein including an 87-residue peptide that corresponds to most of the predicted cytoplasmic COOH terminus of rat (r) NBCn1-B (19). For NDCBE, we used a rabbit polyclonal antibody directed against the first 18 amino acids at the NH2 terminus of NDCBE (32) that was developed and characterized in Dr. Walter Boron’s laboratory, Yale University School of Medicine (Boron W, personal communication). For NCBE, we used a rabbit polyclonal antibody directed against the first 135 amino acids at the NH2 terminus of human NCBE (Chen LMKM, Rojas J, Parker MD, Gill HS, Davis BA, and Boron WF, unpublished observation). The anti-anion exchanger 3 (anti-AE3) affinity-purified rabbit polyclonal antibody to the COOH-terminal 12 residues of AE3 detects a protein of ~180 kDa and does not cross-react with the abundant AE2 of choroid plexus (68). We also used the anti-AE2 affinity purified rabbit polyclonal antibody directed against the COOH terminus of mouse AE2 (28, 58).

Immunoblotting

Tissue preparation. Mice were deeply anesthetized with isoflurane (AErrane, Baxter; Deerfield, IL), weighed, and quickly decapitated with scissors. The brains were rapidly removed from the cranium and segregated into four components, i.e., the cerebral cortex, the hippocampus, the cerebellum, and the brain stem/diencephalon. Heart and kidney were also removed. The same tissues were pooled from 3–4 different animals and placed in ice-cold protein isolation buffer [200 mM mannitol, 80 mM HEPES, 41 mM KOH, 1 tablet/50 ml of complete protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany), and 230 μM PMSF, pH 7.5] for extraction.

Membrane preparation. Crude microsomes (31) were prepared from each of the four CNS regions, heart, and kidney. Pooled tissues were homogenized by 10–20 strokes with a Teflon-glass homogenizer operating at 2,000 rpm (Thomas Scientific, Swedesboro, NJ). For heart and kidney, the tissue was first minced with a razor and then homogenized (20–30 strokes). The homogenate was then centrifuged at 1,000 g for 10 min to remove cellular debris. The supernatant was withdrawn and centrifuged at 100,000 g in a Beckman SW41 rotor for 1 h. The resulting pellet was resuspended in 200–1,000 μl of protein isolation buffer and stored at ~80°C until used.

Western blot analysis. Sample protein concentration was determined with the use of the Bio-Rad DC Protein Assay Kit. Membrane protein (30 μg) was resolved on 4–12% precast NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were incubated in 5% nonfat dry milk (Carnation, Nestle Food, Glendale, CA)
in PBS (in mM: 148.9 NaCl, 2.8 NaH2PO4, 7.2 Na2HPO4, pH 7.4) with 0.1% Tween 20 (MPBST, Sigma) for 1–2 h to block nonspecific proteins. The membranes were then incubated overnight at 4°C in MPBST containing one of the following primary antibodies: mouse monoclonal anti-NHE1 (1:1,000), affinity-purified rabbit polyclonal anti-NBCe1 (K1A-NBC 1:3,000; B1B-NBC 1:5,000), anti-NBCn1 (1:1,000), anti-NDCBE (1:1,000), anti-NCBE (1:1,000), and affinity-purified rabbit polyclonal antibody to AE3 (1:1,000) and AE2 (1:2,000). Protein signal detection was achieved with the ECL chemiluminescence system (Amersham, Little Chalfont, UK). For normalization, membranes were stripped and reprobed with affinity-purified goat polyclonal antibody to actin at 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). Scanning densitometry of immunoblot films was performed on a Personal Densitometer SI scanner (Molecular Dynamics, Sunnyvale, CA) and analyzed with the aid of ImageQuant image analysis software (Molecular Dynamics, Sunnyvale, CA). Densitometry measurements of Western blots from each experimental group were obtained, and absolute values were normalized to actin. Results were reported in arbitrary units as percent change from age-matched normocapnic controls. Results were reported in arbitrary units as percent change from age-matched normocapnic controls. For neonates (n = 4–6) and for adults (n = 3). The n values represent the number of immunoblot lanes examined for each antibody and tissue sample (brain regions, heart, or kidney), each of which represents tissues pooled from three to four animals.

### Statistical Analysis

An unpaired two-tailed Student’s t-test was performed to examine the difference in body weight between the normocapnic and corresponding hypercapnic mice. A two-tailed, one-sample Student’s t-test was performed to examine the differences between the control-normalized 8% and 12% CO2 Western blot density data and a hypothetical value of 1.00. Data are presented as means ± SE and P values of ≤0.05 were considered statistically significant.

### RESULTS

#### Body Weight

Neither neonate nor adult mice exhibited a difference in total body weight after a 2-wk exposure to 8% CO2 compared with normocapnic controls. However, mice exposed to 12% CO2 weighed significantly less than control (P < 0.05) for both neonates and adults compared with their normocapnic controls (Fig. 1). Neonatal CO2-exposed mice showed a 15% decrease in body weight (6.96 ± 0.07 g) compared with control (8.16 ± 0.12 g), whereas the CO2-exposed adults showed a 7% decrease in body weight (35.3 ± 0.61 g), compared with control (37.70 ± 0.43 g). To document increased blood CO2 in the CO2-exposed mice, we have, in the past, measured their serum bicarbonate and found it elevated compared with normocapnic controls (42). Other studies have also reported increased arterial HCO3 and CO2 (38, 39), and/or decreased pH (16, 38) when exposing animals to CO2 levels similar to those in our study.

#### Acid-Base Transporters

NHE3, NDCBE, NCBE, NBCe1-C, and AE2 membrane protein expression showed no significant change with elevated CO2.
exposure in any of the various tissues and organs examined. In contrast, several acid-base transporter subtypes showed significant changes in membrane protein abundance with chronic hypercapnia, namely NHE1, NBCn1, NBCe1-A/B, and AE3. Therefore, we detail below our results on these particular acid-base transporters in neonatal and adult mouse brain, heart, and kidney exposed to either 8% or 12% CO2.

Hypercapnia increases the expression of NHE1 in the brain, heart, and kidney of neonatal but not adult mice. As has been previously reported (25, 26), expression of NHE1 protein was robust in mouse CNS, heart, and kidney (Fig. 2, A and C and Fig. 3, A and C). In neonatal mice, chronic hypercapnia (8% CO2) significantly increased NHE1 protein expression in the cerebral cortex (22%) (Fig. 2, A and B) and heart (54%) (Fig. 3, A and B), compared with control (P < 0.05). Similarly, 12% CO2 significantly increased NHE1 expression in cerebral cortex (29%) (Fig. 2, A and B), heart (66%), and kidney (64%) (Fig. 3, A and B) compared with normocapnic control (P < 0.05). Chronic hypercapnia did not change NHE1 expression in adult mouse brain, heart, or kidney (Fig. 2, C and D and Fig. 3, C and D).

Hypercapnia increases expression of NBCn1 in the brain, heart, and kidney of neonatal but not adult mice. The response of the NBCn1 to hypercapnia followed the same pattern as that of NHE1. In neonates, 8% CO2 exposure led to an increase in NBCn1 expression in cerebral cortex (29%) (Fig. 4, A and B) and heart (30%) (Fig. 5, A and B) (P < 0.05). When P2 mice were exposed to 12% CO2, the expression of NBCn1 increased in cortex (57%) (Fig. 4, A and B), heart (70%), and kidney (50%) (Fig. 5, A and B) (P < 0.05). Adult mice exposed to chronic hypercapnia displayed no change in expression of NBCn1 neither in brain (Fig. 4, C and D) nor kidney (Fig. 5, C and D), but 12% CO2 significantly increased the expression of NBCn1 in the adult heart (Fig. 5, C and D).

Hypercapnia increases the expression of NBCe1-A/B in heart and kidney of neonatal but not adult mice. Hypercapnia produced no change in abundance of the NBCe1-A/B in any region of neonatal or adult brain (data not shown). In contrast, 12% CO2 (but not 8% CO2) significantly increased NBCe1-A/B abundance in neonatal heart and kidney by ~40% (P = 0.05) and 22% (P < 0.05), respectively (Fig. 6, A and B), but produced no statistically significant change in NBCe1-A/B levels in adult heart or kidney (Fig. 6, C and D).

Hypercapnia decreases AE3 expression in brain and increases AE3 expression in heart. As previously seen in other studies (36), the AE3 band appears as a doublet, likely reflecting heterogeneous glycosylation (Fig. 7A). Control mice exhibited substantial expression of AE3 protein in the cortex, hippocampus, and brain stem/diencephalon, but less in the cerebellum. In the neonatal brain, exposure to 12% (but not 8%) CO2 led to statistically significant decreases in AE3 (Fig. 7, A and B) abundance in hippocampus (33%), cerebellum (38%), and brain stem/diencephalon (45%), but not in cortex (P < 0.05). Adult mice showed a similar pattern in which only 12% CO2 significantly decreased AE3 expression in all brain regions, whether in cortex (70%), hippocampus (55%), cerebellum (35%), or brain stem/diencephalon (50%) (P < 0.05) (Fig. 7, C and D). In the heart, neonates showed a 50% increase in AE3 (P < 0.05) (Fig. 8, A and B) with exposure to 8% CO2. Curiously, this increase in AE3 in heart was absent after exposure to 12% CO2. Hypercapnia did not change AE3 expression in the adult heart (Fig. 8, C and D).

DISCUSSION

In this study, we have demonstrated that: 1) exposure of mice to chronically elevated CO2 increases the abundance of acid extruder protein levels and decreases acid loader protein levels in the brain, heart, and kidney of mice; 2) chronic hypercapnia produces greater changes in acid-base transporter protein expression in neonatal mice than in adult mice; 3) 12% CO2 produces a broader range of effects than does 8% CO2 on
acid-base transporter protein expression; and 4) brain, heart, and kidney respond differently to hypercapnia; in the brain, hypercapnia alters AE3 levels to the greatest degree, whereas alteration in NHE1, NBCn1 and NBCe1 levels predominate in heart and kidney.

Chronic hypercapnia elicits disturbances in acid-base homeostasis, and indeed, prolonged hypercapnia of levels comparable to those used in our experiments induces chronic acidosis in neonatal and adult mammals (16, 38). Although the extent of renal compensation and altered metabolism may contribute to the compensatory changes in response to respiratory acidosis, the major factor in the readjustment of steady-state pH homeostasis is the activity and level of expression of plasma membrane acid-base transporters (12). The contribution of the latter becomes more important with the prolongation of hypercapnia. In our study, we have focused on the response of the major known acid-base transporters to prolonged, elevated, inspired CO2 in vivo in brain, heart, and kidney. We have found that mice exposed to chronic hypercapnia significantly increased expression of acid extruder proteins and decreased the expression of acid loader proteins. This pattern of changes in transporter expression is highly suggestive of an adaptive response to attenuate intracellular acidosis induced by hypercapnia. This may prove to be protective, as severe intracellular acidosis adversely affects, among multiple cellular functions, second messenger systems, neurotransmitter release, and myocontractility (10, 61).

The response of acid-base transporters to hypercapnia differed markedly between neonates and adults. The more widespread response to elevated CO2 in newborn mice compared with adults may reflect several developmental differences in pH homeostasis: 1) maturation of mouse kidney occurs during the first 2 wk postnatally, where there is a coordinated increase in proteins that are involved in bicarbonate reabsorption and acid excretion both in the proximal tubule and the intercalated cells of the collecting duct (11). This results in lower levels of neonatal plasma bicarbonate and decreased ability to excrete acid loads compared with adults; and 2) hypercapnia in adults decreases production of metabolic acid equivalents (reflected by lowered arterial lactate levels), by induction of hypothermia and reduction in metabolism, oxygen consumption, and protein synthesis (16, 41). Adult mice may rely on these mechanisms to counteract the hypercapnia-induced acidosis, thus diminishing the need to alter expression of acid-base transporter proteins.

The acid-base transporter protein in mice exposed to 8% CO2 showed only a few changes in neonates and none in adults. In contrast, exposure to 12% CO2 altered expression of a larger number of transporters in a wider range of organs and decreased body weight in both neonatal and adult mice. This finding supports the idea of a response threshold for hypercapnia. We caution the reader that 8% CO2 should not be considered a mild or permissive level of hypercapnia that does not elicit major responses in mice. Lung (42) and brain microarray

Fig. 4. A and C: typical Western blots of the electroneutral sodium bicarbonate cotransporter (NBCn1) and the corresponding actin in the central nervous system (CNS) during chronic hypercapnia (8% and 12% CO2) in neonatal (A) and adult (C) mice. B and D: graphical representation of NBCn1 protein expression as a ratio of NBCn1 to actin normalized to control. Data are for neonates (n = 4–6) and for adults (n = 3), where each n represents pooled tissues from 3–4 animals. Values are means ± SE. *P < 0.05.
data from mice exposed to 8% CO₂ showed significant changes in gene expression, in contrast to much fewer changes after exposure to 12% CO₂. Along the same line, we have seen in this study that 8% CO₂, but not 12% CO₂, induces an increase in heart AE3 membrane protein expression.

Tissue type and the particular type of acid-base transporter demonstrated important specificities that are not unprecedented. For example, acidosis was previously shown to increase NBCn1 protein abundance in the thick ascending limb but not in the inner medullary collecting duct where NBCn1 is also present (46). Moreover, previous studies from our laboratory have shown that pH₁-altering stimuli, such as the NHE1 null mutation and hypoxia will differentially alter the expression of different acid-base transporter isoforms in various brain regions (26, 67). Of particular interest is hypoxia, because it usually accompanies hypercapnia in the clinical setting. We have reported that in the heart, hypercapnia increases NHE1 expression. Similarly, hypoxia/reperfusion increases NHE1 expression in heart, where it is associated with cardiac hypertrophy and injury (5, 6). In contrast, whereas chronic intermittent hypoxia in the brain decreases expression of the acid extruders NHE1 and NBCe1-A/B (26), our present work has revealed that hypercapnia increases the expression of the acid extruder NHE1 and decreases the acid loader AE3 in the brain; a response that may potentially render neurons less acidotic and more resistant to subsequent acute acidosis.

In addition to their role in pH₁ homeostasis, acid-base transporters regulate cell electrolytes, cell volume, cell growth, and cell signaling (2, 43, 52, 53). NHE1 and NBCn1 affect sodium homeostasis, which is important for nerve conductance, cell

![Fig. 5. A and C: typical Western blots of NBCn1 and the corresponding actin in the heart and kidney during chronic hypercapnia (8% and 12% CO₂) in neonatal (A) and adult (C) mice. B and D: graphical representation of NHE1 protein expression as a ratio of NHE1 to actin normalized to control. Data are for neonates (n = 4–6) and for adults (n = 3), where each n represents pooled tissues from 3–4 animals. Values are means ± SE. *P < 0.05.](http://ajpregu.physiology.org/)

![Fig. 6. A and C: typical Western blots of the electrogenic NBC (NBCe1) and the corresponding actin in the heart and kidney during chronic hypercapnia (8% and 12% CO₂) in neonatal (A) and adult (C) mice. B and D: graphical representation of NHE1 protein expression as a ratio of NHE1 to actin normalized to control. Data are for neonates (n = 4–6) and for adults (n = 3), where each n represents pooled tissues from 3–4 animals. Values are means ± SE. *P < 0.05.](http://ajpregu.physiology.org/)
volume, and apoptotic signaling. AE3 influences chloride ion concentration (2), which plays a major role in neurotransmission and cardiac contractility. Therefore, the changes in acid-base transporters seen with hypercapnia potentially impact many cellular functions other than pH homeostasis.

We have described changes in body weight and transporter protein abundance in mouse brain, heart, and kidney after exposure to chronically elevated CO2, but the underlying mechanisms that lead to these changes still need to be elucidated. Although it could be argued that some of the changes
CO2 enhances sodium channel maturation and excitability in sure to chronically elevated CO2 alters acid-base transporter be similarly modulated by acidosis (3), we believe that, at least these transporters have been shown in tissue culture models to stress response. Inhalation of 35% CO2 leads to an increase in HCO3
whether the causative agent in our study is CO2, acidosis, expression (1, 7). At the moment, we are unable to pinpoint transporters or may even have a direct effect on transporter metabolism and pH homeostasis and, in turn, the expression of the acid-base transporter expression and/or activity have been shown in the ischemic hypertrophied hyperthyroid rat heart. Am J Physiol Heart Circ Physiol 281: H2398–H2409, 2001.

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CO2 ALTERS ACID-BASE TRANSPORTER EXPRESSION


