Sustained hypercapnia induces cerebral microvascular degeneration in the immature brain through induction of nitrative stress

Jean-Claude Honoré,1,2 Amna Kooli,3,1 Xin Hou,1 David Hamel,1,2 José Carlos Rivera,1,2 Émilie Picard,1,2 Pierre Hardy,1,2 Sophie Tremblay,1 Daya R. Varma,3 Robert P. Jankov,4 Joseph A. Mancini,1 Michael Balazy,5 and Sylvain Chemtob1,2,3

1Department of Pediatrics, Research Center-Centre Hospitalier Universitaire Ste-Justine, Montréal, Quebec, Canada; 2Department of Pharmacology, Université de Montréal, Quebec, Canada; 3Department of Pharmacology and Therapeutics, McGill University, Montréal, Quebec, Canada; 4Department of Pediatrics, University of Toronto, Toronto, Ontario, Canada; and 5Department of Pathology, New York Medical College, New York, New York

Submitted 4 December 2009; accepted in final form 26 March 2010

Hyperoxia has long been proposed to exert detrimental effects to immature neural tissue, notably retina but also brain (8, 34). Hypoxia has also been associated with long-term neurological disorders (8, 32), albeit a cause-effect relationship has never been demonstrated. Additionally, newborn infants are regularly exposed to hypercapnia especially as a result of inhaled chronic lung disease, such as bronchopulmonary dysplasia (BPD) (25). BPD is itself associated with adverse long-term neurological and neurodevelopmental prognosis (33). Although chronic hypercapnia has been suggested to exert lung protection during mechanical ventilation (5, 23, 36), brain injury and neurodevelopmental disability may be complications (9, 37). Increased tissue oxygenation in response to clinically relevant hypercapnia (PaCO2 ∼ 60–65 mmHg) may, in part, explain adverse consequences of hypercapnia (37). On the other hand, direct cytotoxicity of hypercapnia to the retinal vasculature and possibly to the brain has been reported (13, 16). Interestingly, in vitro CO2 catalyzes the generation of nitrating products (3, 14). Whether hypercapnia affects brain integrity in vivo by inducing nitration remains unknown. This aspect is especially relevant for the newborn neural vasculature, which is particularly susceptible to nitro-oxidative stress (34), as exemplified by the retinovascular endothelial cell susceptibility to cytotoxic effects of the trans- arachidonic acids (TAA)—a family of nitrative stress-derived lipids, including the equipotent 5E, 8E, 11E-, and 14E-AA isomers—in a model of retinopathy of prematurity (17, 20). We, therefore, investigated the effects of clinically relevant prolonged hypercapnia on the newborn brain, by focusing on its neural vasculature, and explored mechanisms implicated.

We, hereby, report that chronic exposure of newborn rat pups to hypercapnia (∼65 mmHg) leads to cerebrovascular nitrative stress associated with increased generation of TAA, which exert cerebrovascular endothelial cytotoxicity and curtail brain growth; these hypercapnia-induced adverse effects were prevented by decreasing the nitrative stress.

MATERIALS AND METHODS

Animals. Sprague-Dawley rat pups (n = 200; Charles River Laboratories) were used following approval of the research protocol by the Animal Care Committee of the Centre Hospitalier Universitaire Ste-Justine, in accordance with guidelines of the Canadian Council on Animal Care.

Within 24 h of birth, rat pups and their mothers were placed in a 10% CO2, 21% O2, and 69% N2 environment (Oxycycler; Biospherix) up to postnatal day 7 (P7). Some animals were treated from P1 to P7 with intraperitoneal injections of a nitric oxide synthase inhibitor (l-Nω-nitroarginine methyl ester, l-NAME; 20 mg · kg−1 · day−1 twice a day; Sigma-Aldrich), a peroxynitrite decomposition catalyst (FeTPPS; 10 mg · kg−1 · day−1 twice a day; Calbiochem) or vehicle (0.9% NaCl).

Address for reprint requests and other correspondence: S. Chemtob, Research Center-CHU Ste-Justine, Dept.s of Pediatrics, Ophthalmology, and Pharmacology, 3175, Chemin Côte Ste-Catherine, Montréal, Québec, Canada, H3T 1C5 (e-mail: sylvain.chemtob@umontreal.ca).
Fig. 1. Effects of hypercapnia on cerebral cortex microvascular density (A), brain cell apoptosis (B), brain weight (C), and body weight (D). A: representative lectin-stained cerebral sections. Rat pups were exposed from 24 h of birth to normocapnia (control) or hypercapnia (CO₂) until 3 (P3) or 7 (P7) days postpartum. Scale bar = 100 μm. Values in histogram are expressed as means ± SE of vessel density in cortical regions. n = 6 per group. *P < 0.05 vs. respective age-matched control. B: brain cell DNA fragmentation from P3 rat pups assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Scale bar = 100 μm. Values in histogram are expressed as means ± SE; n = 5 per group. *P < 0.05 compared with control. C: time-dependent effect of hypercapnia on brain weight; n = 6–20 per group. *P < 0.05 vs. respective aged-control. D: time-dependent effect of hypercapnia on body weight; n = 6–20 per group.
Intracerebroventricular injections of TAAs. In separate sets of experiments, P7 rat pups were anesthetized with isoflurane (2%) and injected (stereotaxic coordinates of the left lateral ventricle: PA-1.0 mm, lateral-1.0 mm from bregma, and ventral-2.0 mm relative to dura) with vehicle (0.5% ethanol in saline) or 14E-AA (10 μM) using a 10-μl Hamilton syringe; 14E-AA was synthesized from corresponding epoxides, as previously described (22). Animals were killed 24 h postinjection, and brains were processed for immunohistochemistry.

Endothelial cell viability assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed to assess cell viability as described previously (1). Briefly, endothelial cells previously isolated from newborn pig brain (1, 20) were starved and cultured for 24 h in endothelial growth medium-2 (Clonetics) in 5% (control) or 10% (hypercapnic) CO2 levels, by maintaining normal pH with NaHCO3; endothelial cells of ≤5 passages were used. MTT (0.5 mg/ml) was then added and following a 3-h incubation, cells were solubilized in acidified isopropanol (40 mM HCl). Optical density was measured at 560 nm with 690 nm as a reference readout. Cell viability was expressed as a percentage of optical density relative to control.

Aortic ring explants. The microvascular sprouting assay was performed as reported previously (20). Aortic rings were cultured for 3 days in endothelial growth medium-2 (Clonetics) in 5% (control) or 10% (hypercapnic) CO2 levels; pH was controlled with NaHCO3. Photomicrographs of individual explants were taken before and after completion of the treatment.

Brain explants. Brain cortex explants from rat pups (P3–P6) were cultured in vitro based on modifications from retinal explant protocols (6, 28). Brains were sectioned in ice-cold culture medium using a
vibratome. Sections obtained (150 μm thick) were delicately placed in six-well dishes on top of a free-floating membrane (Nucleopore polycarbonate Track Etch, pore size 0.03 μm; Whatman). Brain explants were cultured without FBS for 3 days in an endothelial basal medium-2 (Clonetics) containing vehicle (control) or 14E-AA (5 μM). Control explants did not show signs of vascular degeneration for up to 5 days of culture.

Measurement of TAAs. Lipids were extracted from the brain cortex, separated by HPLC, and analyzed by isotopic dilution followed by GC/MS techniques, as previously described (39). Since TAA seem to elicit comparable effects on neural vasculature (1, 20), 14E-AA was measured as an indication of TAA levels.

Determination of nitrite/nitrate levels. Nitrite plus nitrate concentrations in the brain cortex were determined by means of a colorimetry-based assay using Griess reagents, as previously reported (24).

Immunohistochemistry. Brain cryossections (20 μm) were labeled overnight at 4°C with TRITC-conjugated endothelial cell marker lectin Griffonia simplicifolia (1:100; Sigma-Aldrich), alone or in combination with a monoclonal antibody against endothelial nitric oxide synthase (eNOS; 1:200; BD Biosciences) or a polyclonal antibody against 3-nitrotyrosine (3-NT; 1:200; Upstate/Millipore). Alexa-488-conjugated secondary antibody (1:200, Molecular Probes/Invitrogen) was applied for 60 min and DAPI (300 nM) for 2 min. Labeled sections were examined under an epifluorescent microscope (Eclipse E800; Nikon) equipped with a digital image analysis system (ACT-1 software; Nikon).

Measurement of cerebral cortex vascular density. Vascular density was quantified using the Image J 1.41 software (National Institutes of Health, Bethesda, MD) from pictures of brain sections labeled with the TRITC-conjugated lectin. Vascular density in the cortical area was expressed as a percentage of the cortical area. The use of this technical approach to measure vascular density has already been reported in the retina (2, 20) and the brain (34).

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on paraffin-embeded brain sections, as indicated by the manufacturer (Chemicon/Millipore).

Real-time PCR analysis. RNA from brain cortex samples was extracted using TRIzol (Invitrogen) and cDNA synthesized from 2 μg of RNA. Quantitative real-time PCR was performed in a sequence detection system (MxPro 3000 QPCR Systems, Stratagene). All PCR reactions were performed in duplicate. Rat endothelial and inducible NOS primers were selected according to Kaur et al. (19) and beta-

Fig. 3. Effects of hypercapnia on cerebral cortex nitrite/nitrate concentrations (A), 14E-arachidonic acid (14E-AA) levels (B), and cerebral nitrative stress (C). A: brain cortex nitrite/nitrate levels; n = 8 per group. *P < 0.05 compared with control. B: brain cortex 14E-AA levels extracted 6, 18, or 24 h after hypercapnia; n = 6 or 7 per group. *P < 0.05 vs. normocapnia. C: representative brain sections from rat pups exposed for 18 h to normocapnia (control) or hypercapnia (CO2) and stained with lectin (red) and 3-nitrotyrosine antibody (green). Arrows indicate lectin and 3-nitrotyrosine colocalization. Scale bar = 100 μm.

AJP-Regul Integr Comp Physiol • VOL 298 • JUNE 2010 • www.ajpregu.org
actin primers according to Sullivan et al. (35). Neuronal NOS (nNOS) primers were designed using the DNAMAN software (Lynnon) and had the following forward and reverse sequences: 5’-AAGCCGTCGATCTGTCTCA-3’ and 5’-CCACACCATGCTTTGGA-3’, respectively.

Western blot analysis. Standard SDS-PAGE techniques were conducted on 60 μg of proteins. eNOS primary antibody (BD Biosciences) was used at a 1:1,000 dilution. Equal protein loading was ensured by probing with a beta-actin antibody (1:10,000; Santa Cruz Biotechnology). Densitometry was measured using the ImageJ 1.41 software (National Institutes of Health, Bethesda, MD).

Statistical analysis. Values are presented as means ± SE. Statistical analysis were performed by one-way ANOVA, followed by Tukey’s post hoc test for multiple comparisons, except for the data in Fig. 1, C and D, where a two-way ANOVA followed by Bonferroni’s post hoc test was performed. For comparisons simply between two groups, Student’s t-test was used. $P < 0.05$ was considered to denote significant differences.

RESULTS

Effects of hypercapnia on cerebral cortex vascular density and brain weight. Exposure of neonatal rat pups to hypercapnia (PaCO₂ = 60–70 mmHg) from P1 to P7 resulted in a 20% decrease in cerebral cortex microvasculature density by P3 (Fig. 1A), at which time (apoptotic) cell death (TUNEL positivity) was detected mostly in microvasculature and also to

Fig. 4. Effects of pharmacological treatments on hypercapnia-induced brain cortex 14E-AA levels (A), nitric stress (B), brain cortex microvascular density (C, D), and brain weight (E). A: brain cortex 14E-AA levels determined 18 h after hypercapnia. Pups were treated or not with a nitric oxide synthase inhibitor (l-NAME; 20 mg/kg) and exposed for 18 h to hypercapnia (CO₂); $n = 6$ or 7 per group. $*P < 0.05$ vs. normocapnia. B: representative brain sections stained with lectin (red) and 3-nitrotyrosine antibody (green). Pups were treated or not with l-NAME (20 mg/kg) or a peroxynitrite decomposi- tion catalyst (FeTPPS; 10 mg/kg) and exposed to hypercapnia (CO₂). B: arrows indicate lectin and 3-nitrotyrosine colocalization. Scale bar = 100 μm. C: representative lectin-stained cerebral sections from P3 rat pups treated as in B. Scale bar = 100 μm. D: quantification of cerebrovascular density from rat pups treated as indicated in C. Values in histogram are expressed as means ± SE of vessel density in cortical regions; $n = 5$ or 6 per group. Scale bar = 100 μm. $*P < 0.05$ vs. normocapnia. E: brain weight assessed in P7 rat pups treated or not with l-NAME (20 mg/kg) or FeTPPS (10 mg/kg) and exposed to hypercapnia (CO₂); $n = 10$–20 per group. $*P < 0.05$ vs. normocapnia.
some extent on parenchyma (Fig. 1B). By P7, microvascular density was equivalent to that in normocapnia-exposed animals, indicative of accrued revascularization following the early vasoobliteration, as reported in other conditions associated with vessel loss (34). Nonetheless, brain mass growth remained curtailed into adolescence (P30), despite resuming normocapnia since P7 (Fig. 1C). It is noteworthy that body weight did not change in any time points investigated (Fig. 1D).

Effects of hypercapnia on eNOS expression and on products of nitration. Hypercapnia has been shown to increase eNOS expression in brain cortex (24). Concordantly, eNOS (but not inducible NOS or nNOS) mRNA expression and immunoreactivity were augmented by 18 h of exposure to high CO₂ (Fig. 2, A–C), and this was associated with increased generation of nitrite/nitrate (oxidation products of NO) and lipid nitration product 14E-AA in the brain cortex (Fig. 3, A and B). Immunohistochemistry also revealed that early nitration (of proteins) was foremost localized on brain microvessels (Fig. 3C), consistent with increased susceptibility of microvessels to nitro-oxidative stress (7, 34).

Nitric oxide synthase inhibition and enhanced peroxynitrite decomposition prevent vascular degeneration and loss of brain mass. Nitric oxide synthase inhibitor L-NAME (20 mg/kg ip) decreased generation of nitration products (14E-AA and 3-nitrotyrosine) (Fig. 4, A and B) as expected, and preserved cortical vascular density, as well as brain mass (Fig. 4, C–E); similar benefits were observed with the peroxynitrite decomposition catalyst FeTPPS (10 mg/kg ip). It should be noted that drug treatments in normocapnia-exposed animals did not affect vascular density or brain weight (Fig. 4, D and E).

Endothelial cytotoxicity to high CO₂ and TAA ex vivo. Chronic hypercapnia increases oxygen delivery to the brain of the newborn (24), which, in turn, can exacerbate peroxidation and tissue injury in immature subjects. We, therefore, tested whether high CO₂ can cause endothelial cytotoxicity independent of changes in oxygen concentration. Exposure of cultured cerebral microvascular endothelial cells to 10% CO₂ diminished cell viability and vascular sprouting of aortic explants; these effects were prevented by L-NAME or FeTPPS (Fig. 5, A and B); observations are consistent with increased nitration in response to high CO₂, as previously shown ex vivo in other tissues (31). Finally, in line with cytotoxicity of TAA (20), 14E-AA caused within 24 h microvascular degeneration in brain explants (Fig. 5C); in vivo intraventricular injection of 14E-AA at physiological concentration also markedly decreased periventricular brain vascular density 24 h later (Fig. 5D).

DISCUSSION

Hypercapnia may be beneficial for lungs of newborns (23), but neurodevelopment may be adversely affected by extremes of hypercapnia (>65 mmHg) (11, 37). Marked and prolonged increases in cerebral circulation may explain observed brain hemorrhages (24). However, hemorrhages do not seem to account for the majority of neurodevelopmental disorder asso-
Fig. 5. Effects of hypercapnia on vasculature are mimicked by TAA ex vivo and in vivo. 

A: endothelial cell viability quantified by MTT assay. Cells were cultured for 24 h in 5% (control) or 10% CO₂ with or without L-NAME (5 μM) or FeTPPS (5 μM); n = 4 experiments each performed in triplicate; *P < 0.05. 

B: representative microvascular sprouting from Matrigel-embedded aortic rings cultured in 5% (control) or 10% CO₂, with or without FeTPPS (5 μM). Scale bar = 1 mm; n = 6–9 explants per group; *P < 0.05 vs. normocapnia. Drug treatment in control explants did not affect vascular sprouting when compared with untreated explants (not shown). 

C: ex vivo effects of 14E-AA (5 μM) on cerebral cortex vascular density of rat brain explants. Scale bar = 100 μm; n = 4 separate experiments. *P ≤ 0.05 compared with control explants. 

D: representative lectin-stained cerebral sections 24 h after intracerebroventricular injections of 0.5% ethanol in saline (control) or 14E-AA (10 μM). Scale bar = 100 μm; n = 4 or 5 per group. *P < 0.05 compared with control.
associated with hypercapnia (37). In line with this inference, hypercapnia depletes the vascular bed of retina by causing microvascular dropout (7). The current study expands on this notion by showing that clinically observed chronic hypercapnia (P_{\text{ACO}_2} \approx 65 \text{ mmHg}) causes cerebral vasooobliteraton in the developing subject, by inducing nitrative stress; inhibition of the nitrative stress preserves brain microvasculature and mass.

Our observations point to an important role for the NOS pathway and reactive NO products in cerebral cortical microvascular degeneration upon exposure to hypercapnia. The expression particularly of eNOS was found to augment by 18 h in response to hypercapnia (Fig. 2, A–C), as previously demonstrated (24). The ensued rise in NO can exert either protective or cytotoxic effects, depending on local tissue redox potential (2); accordingly, excessive NO production in the presence of incompletely developed antioxidant systems, as seen in the immature subject, leads to generation of reactive nitrogen species, which can cause endothelial cell injury (2), as evidenced herein (Figs. 3 and 4). Along these lines, CO₂ enhances nitration by interacting with peroxynitrite to form a more unstable and efficient nitrating adduct nitrosoperoxocarbonate (14), which shifts peroxynitrite from forming reactive oxygen species to reactive nitrogen species (3). This nitrative capacity is clearly exemplified in vitro by the fact that peroxynitrite in combination with increased CO₂ levels enhances protein nitration (31). We, hereby, demonstrate in vivo in brain that hypercapnia not only increases generation of nitrites but also of protein and lipid nitration products (Fig. 3).

Neurovascular endothelial cells are particularly susceptible to nitrative stress (1, 15, 20, 34), more so than glia (4). In line with this claim, early detection (18 h) of nitrotyrosine was largely confined to the microvasculature (Fig. 3C) and preceded vasooobliteraton (Fig. 1A). More relevantly, products of nitration (3-nitrotyrosine and TAA), as well as vessel loss, were decreased by NOS inhibition and catalyst of peroxynitrite decomposition (Fig. 4). In addition, microvascular endothelial cytotoxicity to the nitration product TAA (20) was reproduced on brain explants and in vivo (Fig. 5, C and D). Vulnerability of the endothelium to nitration product TAA has been attributed to the formation of the proapoptotic antiangiogenic thrombospondin-1 (20). Correspondingly, we detected an increased expression of thrombospondin-1 in the brain of hypercapnia-exposed rats, which was prevented by pretreatment with FeTPPS (data not shown).

Rat pups provide an interesting model to study brain development occurring in preterm neonates since neuronal generation is complete and axonal and dendritic branching is robust, corresponding to the development observed in preterm infants at the second trimester of gestation (10, 12). Interestingly, neuronal metabolism and the peak of angiogenic activity during vascular development coincides (29). In cortical areas of rat pups, vascular development continuously occurs from P3 to P24, then stabilizing between P24 and P33 (26). Upon prolonged hypercapnic exposure, alteration of the cortical cerebrovascular density as evidenced in the current study (Fig. 1A) impairs neurovascular metabolism and neuronal development, leading to the brain growth retardation observed at P7 (Fig. 1C). In line with this hypothesis, formation and sprouting of blood vessels are very important for brain maturation (27). Importantly, and despite the observed revascularization phase (Fig. 1A), brain retardation remains in P30 rat pups (Fig. 1C); this suggests that early alteration of angiogenesis induced by sustained hypercapnia, as encountered in uncured BPD patients, may curtail growth of the rapidly developing cortex of fetus and premature infants (30).

In conclusion, we, hereby, show that clinically relevant prolonged hypercapnia causes brain microvascular degeneration by generating a nitrative stress triggered likely by eNOS. Despite an accelerated revascularization and resulting normalization of the vascular density following the cerebral vasooobliteraton, brain mass growth remained curtailed well after the insult.

**Perspectives and Significance**

The cerebral endothelial cytotoxicity to sustained high blood CO₂ observed in this study may have broader implications than simply those presented. Ischemia per se results in local elevation in CO₂ (18, 38). Despite the fact that a transient increase in CO₂ may preserve cerebral blood flow after hypoxia/ischemia (21), one can surmise that the sustained accumulation of CO₂ in ischemic brain tissue may partake in intensifying neuromicrovascular degeneration. As such, achievable preventative measures are foreseeable. Modulation of NO formation may prove difficult because of the benefits incurred to the vasculature by this mediator. However, inhibition of nitrative stress carries greater potential, as it could specifically improve revascularization and assist in preserving organ integrity following detrimental factors commonly encountered by the preterm infant, specifically not only the well-described hyperoxia (34), ischemia, and possibly hypocapnia (8, 32), but also hypercapnia as described herein.

**GRANTS**

This study was supported by grants from the Canadian Institutes of Health Research, the Heart and Stroke Foundation of Quebec, and the Fonds de la Recherche en Santé du Québec. J.-C.H. holds a “Focus on Stroke” postdoctoral fellowship, and S. Chemtob holds a Canada Research Chair in Perinatology and the Leopoldine Wolfe Chair in Clinical/Translational Research for the Prevention of Age-related Macular Degeneration.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**


HYPERCAPNIA AND CEREBROVASCULAR DEGENERATION


