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Carbon dioxide influence on nitric oxide production in endothelial cells and astrocytes: Cellular mechanisms

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Abstract

Cerebral vessels may regulate cerebral blood flow by responding to changes in carbon dioxide (CO₂) through nitric oxide (NO) production. To better determine the role of NO production by human adult cerebral microvascular endothelial cells and human fetal astrocytes under different CO₂ conditions, we studied endothelial cell and astrocyte production of NO under hypo-, normo- and hypercapnic conditions. Human cerebral endothelial cell and fetal astrocyte cultures were exposed to hypocapnic (pCO₂ 21.7±6.7 mmHg), normocapnic (pCO₂ 40.1±0.9 mmHg) and hypercapnic (pCO₂ 56.3±8.7 mmHg) conditions. NO production was recorded continuously over 24 hours with stable pH. N-nitro-L-arginine [NLA; a nitric oxide synthase (NOS) inhibitor] and L-arginine (substrate for NO production via NOS) were used to further define the role of NOS in chemoregulation. NO levels in endothelial cells increased during hypercapnia by 36% in 8 hours and remained 25% above baseline. NO increase in astrocytes was 30% after 1 hour but returned to baseline at 8 hours. NLA blocked NO increase in endothelial cells under hypercapnia. During hypocapnia, NO levels in the endothelial cells decreased by 30% at 8 hours but were unchanged in astrocytes. L-arginine prevented NO decrease in endothelial cells under hypocapnia. NO changes in the endothelial cells correlated with changes in pCO₂ (R=0.99) and were independent of pH. This study suggests that cerebral endothelial cells and astrocytes release NO under normocapnic conditions and NO production is increased during hypercapnia and decreased during hypocapnia independent of pH. Further, this demonstrates that endothelial cells may play a pivotal role in chemoregulation by modulating NOS activity.

Keywords

nitric oxide; chemoregulation; endothelial cell; astrocyte; cerebral blood flow; neurovascular unit

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1. Introduction

Modulation of cerebral vascular tone in response to changes in the arterial partial pressure of carbon dioxide ($p\text{CO}_2$) is defined as chemoregulation. In humans hypocapnia produces vasoconstriction resulting in decreased cerebral blood flow (CBF), whereas hypercapnia produces vasodilation and increased CBF (Lavi et al., 2003). Chemoregulation is disturbed in various brain pathologies including cerebral vasospasm after subarachnoid hemorrhage, ischemic stroke and brain trauma in humans and primates (Dernbach et al., 1988; Jakubowski et al., 1982). Nitric oxide (NO), a powerful vasodilator also known as endothelium-derived relaxing factor (EDRF) (Palmer et al., 1987), plays a key role in regulating cerebral vascular tone (Thompson et al., 1996; Toda et al., 2009). Using nitric oxide synthase (NOS) inhibitors, several *in vivo* studies have suggested that vasodilation in response to increased $p\text{CO}_2$ may be mediated by NO (Lavi et al., 2006). However, the underlying cellular mechanism of chemoregulation has not been elucidated and the source of NO as a response to CO_2 changes in human cells is still unclear.

Animal experiments suggest that NO in the brain can be produced by endothelial cells, astrocytes (Toda et al., 2009) and neurons (Bhardwaj et al., 2000). These cells are the components of the neurovascular unit (Allan, 2006) that controls cerebrovascular chemoregulation. To elucidate the chemoregulatory role of NO in these cells, we studied NO production by adult human cerebral microvascular endothelial cells and human fetal astrocytes in response to changes in the $p\text{CO}_2$ under constant pH conditions.

2. Results

2.1 Cerebral Microvascular Endothelial Cells

Under normocapnic conditions ($p\text{CO}_2$ 40.1 ± 0.9 mmHg), mean NO concentration in the media overlying the cells was $7.5 \pm 1.1 \times 10^{-10}$ M (Figure 1A). Mean NO concentration increased minimally by 3-5% over 24 hours. Under hypercapnic conditions ($p\text{CO}_2$ 56.3 ± 8.7 mmHg), NO concentration increased from baseline levels to a mean of $10 \pm 0.6 \times 10^{-10}$ M during the first 4 hours (Figure 1A). NO concentration peaked at 36% ($10.2 \pm 0.5 \times 10^{-10}$ M) above baseline at 8 hours and stabilized 25% ($9.4 \pm 0.5 \times 10^{-10}$ M) above baseline until completion of the experiment. Within the first 4 hours of hypocapnia ($p\text{CO}_2$ 21.7 ± 6.7 mmHg), mean NO levels decreased to $5.9 \pm 0.5 \times 10^{-10}$ M and reached a nadir of $5.4 \pm 0.5 \times 10^{-10}$ M at 8 hours, a mean decrease of 30% (Figure 1A). This decrease remained stable for the remainder of the recording. In the presence of N-nitro-L-arginine (NLA; 10^{-3} M), increased NO production induced by hypercapnia was eliminated (Figure 1B). Furthermore, adding L-arginine (10^{-3} M) prevented decreased NO production induced by hypocapnia (Figure 1C). By plotting NO changes as a function of $p\text{CO}_2$, we could disregard time as a variable in NO production (Figure 3) to establish that changes in NO levels correlate with changes in $p\text{CO}_2$ ($R=0.99$).

2.2 Astrocytes

During normocapnia ($p\text{CO}_2$ 40.1 ± 0.9 mmHg), mean NO concentration was $1.9 \pm 0.8 \times 10^{-10}$ M (Figure 2). Except for a minimal 3-5% increase over the 24-hour experimental period, NO production remained relatively constant. Under hypercapnic conditions ($p\text{CO}_2$ 56.3 ± 8.7 mmHg), human fetal astrocytes increased NO production by 30% over baseline values to a mean level of $2.5 \pm 1.2 \times 10^{-10}$ M in the first hour of hypercapnia (Figure 2). NO production then gradually decreased to control levels after 8 hours and remained at control levels for the remainder of the experiments. During hypocapnia ($p\text{CO}_2$ 21.7 ± 6.7 mmHg), NO production decreased minimally (3%) to a mean level of $1.9 \pm 0.9 \times 10^{-10}$ M (Figure 2) during the 24-hour period. Changes in NO production correlated ($R=0.93$) with changes in $p\text{CO}_2$ levels (Figure

3). However, there was a significant difference in the angle of the regression lines (endothelial cells, $y=30+1.2x$; astrocytes, $y=18+0.06x$; $P<0.001$).

2.3 pH Measurements

To confirm that pH was maintained in a narrow range during the alteration of pCO_2 , a series of pH measurements was performed in which a pH probe was placed in the media above the cultured cells for a 24-hour period during normocapnia, hypercapnia, and hypocapnia. The pH values were kept stable within a neutral gap under normocapnic (7.39 ± 0.01), hypercapnic (7.36 ± 0.02) and hypocapnic (7.40 ± 0.01) conditions.

2.4 Western Blot and Immunofluorescence Analysis

Western blotting and immunofluorescence were performed to confirm the presence of NOS isoforms in both cell types. NOS was present in both cell lines as determined by Western blot analysis (Figure 4). Immunofluorescent staining revealed iNOS as the primary NOS isoform expressed in astrocytes, while eNOS was represented mainly in the cerebral microvascular endothelial cells (Figure 5).

3. Discussion

3.1 NO and Chemoregulation

These results support the hypothesis that NO production in response to hypercapnia is increased under stable pH values. NO levels were consistent with the values previously reported by Malinski et al. (Malinski and Taha, 1992). Stimulation of NOS in the endothelial cells is consistent with the NO-dependent vasodilation and increased CBF that occur in vivo during hypercapnia, as has been shown in rats (Iadecola, 1992) and in primates (Thompson et al., 1996). Decreased NO production by endothelial cells also correlates with the in vivo vasoconstrictive response to hypocapnia shown previously (Lavi et al., 2003; Thompson et al., 1996).

Measurements of NO production in both cell types show a delayed response to changes in pCO_2 compared to the immediate in vivo response (Thompson et al., 1996). Thus, it is unlikely that eNOS is responsible for the early or fast phase response during chemoregulation in vivo. There are several explanations for this phenomenon. First, nitrite (NO_2^-), being a storage pool of NO, can be reduced to NO under acidic and hypoxic conditions in vivo (Cosby et al., 2003). Under these conditions nitrite releases NO in the presence of deoxygenated hemoglobin in blood (Cosby et al., 2003; Nagababu et al., 2003) or neuroglobin (Burmester et al., 2000) in neurons acting as a nitrite reductase (Petersen et al., 2008). Thus, nitrite in blood and the brain can be an endogenous, on-demand NO donor. This mechanism could be crucial for rapid vasodilation and CBF changes in response to hypercapnia (Peebles et al., 2008). In this study, we eliminated this mechanism since no deoxygenated hemoglobin or neuroglobin was present in the media. Furthermore, as the pH values and nitrite concentrations were stable during each experiment, nitrite as a source of NO was unlikely. Second, our experiment was performed in cultured cells bathed in a stable medium, which equilibrates slowly (about 5-10 minutes) with pCO_2 changes in the environment. This differs from the situation in vivo where endothelial cells are continuously exposed and adjusting NO production to pCO_2 levels in the circulating blood. The chemoregulatory response to CO_2 changes in vivo is rapid, occurring on the order of milliseconds; our results did not demonstrate this component of the chemoregulatory response. Nevertheless, this does not preclude NO involvement in early vasodilation as it can be released in the presence of deoxygenated hemoglobin in acidic conditions from ubiquitous nitrite as mentioned earlier. Finally, individual cell types in culture lack feedback

from other cell types, which may facilitate and/or accelerate the chemoregulatory response (Faraci et al., 1995; Iuliano et al., 2004).

3.2 Timing of Response

In humans and other mammals, the vasoconstrictive response to hypocapnia is lost after less than 6 hours (Hirata et al., 1999), perhaps due to changes in pH (Severinghaus, 1965). In our buffered system, this did not occur. These responses suggest the existence of homeostatic mechanisms in vivo that re-established the balance between vasoconstriction (Faraci et al., 1995) (e.g., endothelin-1) and vasorelaxation agents (NO). These observations suggest that pCO₂ regulation of CBF in vivo is achieved via redundant mechanisms and that at least one of them is NO-dependent (Iadecola and Zhang, 1994; McPherson et al., 1995). Other vasoactive substances, such as endothelium-derived hyperpolarizing factor (EDHF), vasodilator prostaglandins (Toda et al., 2009), carbon monoxide (CO) (Leffler et al., 2006), hydrogen sulfide (Yang et al., 2008), vasoconstricting prostanoids, and endothelin-1 (Toda et al., 2009) may play a role in chemoregulation.

3.3 Cellular Basis of Chemoregulation

A neurovascular unit consists of neurons, astrocytes, endothelial cells and smooth muscle cells. The neurovascular unit has been implicated in regulating CBF (Allan, 2006). Neuronal NOS-containing neurons are relatively rare in the brain (only 2% of neurons) (Bredt et al., 1991) and adventitial noxinergetic (NOS-containing) neurons are unlikely to be involved in chemoregulation because they are mostly present in the conductive rather than resistance vessels, and are regulated by the sphenopalatine ganglion (Nozaki et al., 1993). Thus, we restricted this study to human cerebral endothelial cells and astrocytes because they are elements of the neurovascular unit that should contribute to NO increase during chemoregulation (Koehler et al., 2009; Simard et al., 2003). Multiple signaling pathways (Koehler et al., 2009; Xu et al., 2008) have been proposed to regulate CBF in response to pCO₂ changes, but it is still unclear whether and how this signaling could be induced by pCO₂ changes.

3.4 The Role of Endothelial Cells and Astrocytes in Chemoregulation

Baseline production of NO during normocapnia is responsible for maintaining basal cerebrovascular tone in healthy humans (Lavi et al., 2003) and is important in regulating CBF (Toda et al., 2009). Cerebrovascular reactivity in response to CO₂ is impaired in diabetic or hypertensive patients with endothelial dysfunction (Lavi et al., 2006), suggesting an important role for endothelial cells in modulating CBF response to CO₂. Furthermore, in our experiments, only endothelial cells decreased NO production in response to hypocapnia. Although not strong at three points, the correlation between pCO₂ and NO production by endothelial cells and astrocytes indicates that production of NO by endothelial cells has a more efficient and rapid response during chemoregulation.

Direct in vitro measurements of NO production in human cerebral endothelial cells and astrocytes suggest that these two cell types can adjust NO production in a manner that is consistent with the in vivo chemoregulatory response in animals and humans (Iadecola, 1992; Lavi et al., 2003; Thompson et al., 1996). The difference in the quantity of NO produced by human endothelial cells (3.5 times more) versus astrocytes under normocapnic conditions suggests that endothelial cells play a critical role in adjusting basal vascular tone in response to changes in pCO₂. These findings highlight endothelial cells as the primary targets to modulate CBF changes in patients with impaired cerebral CO₂ vasoreactivity (Lavi et al., 2006).

3.5 NOS Involvement in Chemoregulation

Najarian et al. (Najarian et al., 2000) demonstrated that both increased CBF and eNOS mRNA expression induced by hypercapnia could both be blunted by nonselective NOS inhibitors but not a selective neuronal NOS (nNOS) inhibitor. In our endothelial cells, the addition of NLA, an inhibitor of NOS, prevented the increase of NO production induced by hypercapnia, and the supplementation of L-arginine, the substrate for NO, prevented decreased NO production in endothelial cells induced by hypocapnia. These findings indicate that changes of NO in response to pCO₂ were specific for NOS regulation. Thus, exogenous NO administration could affect the CO₂-dependent mechanism of chemoregulation by a feedback mechanism (Lavi et al., 2003). Western blotting results reveal a high concentration of eNOS in the endothelial cells and a primary expression of iNOS in astrocytes confirming the presence of the respective enzymes in our cell lines.

3.6 Role of pH

In these experiments, changes in NO production occurred in response to changes in pCO₂ under constant extracellular pH values. Our media was heavily buffered and the pH (7.36 to 7.40) did not change during normo-, hypo- and hypercapnic conditions. This finding questions an earlier report that hypercapnic vasodilation mainly occurs as a result of low pH (Nakahata et al., 2003). While we have shown that NO is produced in the absence of changes in extracellular pH, we cannot exclude the existence of redundant mechanisms, including changes in pH that alter NO production by modulating NOS (Niwa et al., 1993). In addition, we measured the extracellular pH values and since CO₂ is freely permeable, whereas hydrogen is not, the intracellular pH could be different and thus have additional influence on NOS activity. Future studies will be necessary to elucidate the effect of intra- versus extracellular pH on NO production.

It has been reported that the ATP-sensitive K⁺ channels play a pivotal role in microvessel vasodilation of the cerebral cortex in response to decreased pH corresponding to mild hypercapnia and that a NOS inhibitor could not alter this vasodilation (Nakahata et al., 2003). In addition, the influence of glucose and oxygen levels on NO production has to be considered. Since the oxygen and glucose concentrations were not altered we have no data about the influence of hyperoxic or hyperglycemic conditions on NO production. This is an important issue worth analyzing in future studies.

3.7 Conclusion

This study indicates that cerebral endothelial cells and astrocytes release NO under normocapnic conditions and the NO production is changed by hypercapnia and hypocapnia. Endothelial cells play a pivotal role in chemoregulation by modulating NOS and their response is independent of pH. Nevertheless, the interactions and close network of different cell types comprise redundant mechanisms and signaling pathways involved in chemoregulation in vivo.

4. Experimental Procedure

4.1 Endothelial Cell Culture

Adult human cerebral endothelial cells were obtained from surgically resected temporal lobe specimens of epilepsy patients under an Institutional Review Board approved protocol at the National Institutes of Health (#03-N-0164). Endothelial cells were isolated from microvessels and cell lines were established as described by Manconi et al. (Manconi et al., 2000). Cells were grown in Dulbecco Modified Eagle's Medium (DMEM; GIBCO Invitrogen, Carlsbad, CA) and Media 199 (GIBCO Invitrogen, Carlsbad, CA) in equal parts, supplemented with 10% (vol/vol) fetal bovine serum (GIBCO Invitrogen, Carlsbad, CA),

5% heat-inactivated human serum (Scantibodies Laboratory Inc., Santee, CA) and penicillin-streptomycin (GIBCO Invitrogen, Carlsbad, CA). The baseline concentration of L-arginine was 84 mg/L in DMEM and 70 mg/L in Media 199. Endothelial cells were confirmed by Factor VIII-related antigen staining (1:500, Abcam, Cambridge, MA) (Manconi et al., 2000). Confluent cells ($1.3 \pm 0.6 \times 10^6$ cells/well) between passages 4 to 7 were used for experimentation in 6-well plates.

4.2 Astrocyte Cell Culture

Human fetal astrocytes were obtained from Dr. Eugene Major (Laboratory of Viral and Molecular Pathogenesis, NINDS, NIH). The cells were grown in DC 10 media consisting of DMEM (GIBCO Invitrogen, Carlsbad, CA) with 10% (vol/vol) fetal bovine serum and 0.2% of each L-glutamine and penicillin-streptomycin (GIBCO Invitrogen, Carlsbad, CA). Astrocytes were confirmed by staining for glial fibrillary acidic protein (GFAP; 1:1000, Abcam, Cambridge, MA) using standard immunofluorescence techniques (DAKO, Carpinteria, CA) (Sabri et al., 2008). Confluent cells ($3.4 \pm 0.8 \times 10^6$ cells/well) between passages 4 to 8 were studied in 6-well plates.

4.3 Chemoregulation Chamber

pCO₂ was adjusted to achieve stable conditions in the media of normocapnic (pCO₂ 40.1 ± 0.9 mmHg), hypercapnic (pCO₂ 56.3 ± 8.7 mmHg), and hypocapnic (pCO₂ 21.7 ± 6.7 mmHg) cells at 37°C in a modified cell culture incubator. As it took 5 minutes to achieve steady-state CO₂ levels in media, each experiment was initiated 20 minutes after such equilibrium was established. Oxygen (pO₂) levels in the cell media were maintained at a constant value of 156.0 ± 13.0 mmHg. We achieved a stable, narrow pH range (7.36 to 7.40) by using buffered media.

4.4 NO, pCO₂ and pH Measurements

NO was measured directly using an electrochemical probe (World Precision Instruments, Sarasota, FL) with the tip positioned in the media just above the cell layer. An electrode (World Precision Instruments, Sarasota, FL) was used to measure CO₂ levels in the cell media. A pH electrode (World Precision Instruments, Sarasota, FL) was used to measure the dynamic pH in the media throughout the experiments. Each electrode signal was recorded every 3 seconds for 24 hours by a computerized data acquisition system (MacLab/8, ADInstruments, Colorado Springs, CO). For subsequent analysis, raw voltage signals were converted to concentration values using previously recorded individual calibration curves for NO, pCO₂ and pH.

4.5 Experimental Design

Confluent primary human adult cerebral endothelial cells and human fetal astrocytes were examined under the following conditions: normocapnia, hypercapnia, and hypocapnia. To confirm the specificity of the responses, two control experiments were performed in the endothelial cells: hypercapnia with addition of 10^{-3} M NLA (a NOS inhibitor; Sigma-Aldrich, St. Louis, MO) and hypocapnia with addition of 10^{-3} M L-arginine (substrate for NO; Sigma-Aldrich, St. Louis, MO) to the media. Before each experiment, confluent cells were washed with phosphate buffered saline and fresh media was added. The cells were placed in the modified cell culture incubator, probes were positioned and the system was allowed to equilibrate for 15 to 30 minutes before data acquisition. Each experiment was performed in triplicate.

4.6 Western Blot

Primary culture of fetal astrocyte and human brain microvascular endothelial cells were maintained in growth medium according to manufacturer's protocol (ScienCell Research Laboratories, Carlsbad, CA). Total protein of cells was extracted using RIPA lysis buffer (Thermo Scientific, Waltham, MA). The quantity of protein was determined in the supernatant solution using a Bio-Rad Protein Assay Kit. Proteins were separated by NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). Membranes were blocked in 5% skim dried milk and probed with primary antibody. Antibodies used for this study were eNOS (1:1,000, BD Bioscience, San Diego, CA), iNOS (1:1,000, Millipore, Billerica, MA) and β -actin (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA).

4.7 Immunofluorescence Analysis

Primary cultures of fetal astrocyte and human brain microvascular endothelial cells were fixed in Histochoice and in PBS with 0.3% Triton X-100 for 30 minutes. Cells were labeled with primary antibodies against eNOS (1:100) or iNOS (1:100) overnight and then probed with Alexa Fluor 488 conjugated secondary antibody (1:200, Invitrogen, Carlsbad, CA). Cell nuclei were counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA). The specimens were visualized using a Zeiss LSM 510 confocal microscope at 40x magnification.

4.8 Statistical Methods

Mean values are expressed as mean $\pm$ standard deviation (mean $\pm$ SD) throughout. Linear regression analysis was utilized to explain the correlation of pCO₂ and NO production.

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Figure 1.

(A) Nitric oxide (NO) levels during normocapnia, hypercapnia and hypocapnia in human adult cerebral endothelial cells. This graph depicts levels of NO measured during normocapnia ($p\text{CO}_2$ 40.1 ± 0.9 mmHg), hypercapnia ($p\text{CO}_2$ 56.3 ± 8.7 mmHg), and hypocapnia ($p\text{CO}_2$ 21.7 ± 6.7 mmHg) in cultured human cerebral endothelial cells. (normocapnic [$n=17,280$], hypercapnic [$n=17,280$] and hypocapnic measures [$n=17,280$]). (B) NO levels during hypercapnia with and without NLA in endothelial cells. This graph depicts levels of NO measured during hypercapnia ($p\text{CO}_2$ 40.1 ± 0.9 mmHg) in human cerebral endothelial cells with and without the addition of 10^{-3} M N-nitro-L-arginine (NLA). [$n=17,280$] (C) NO levels during hypocapnia with and without L-arginine in endothelial cells. This graph depicts levels of NO measured during hypocapnia ($p\text{CO}_2$ 21.7 ± 6.7 mmHg) in human cerebral endothelial cells with and without the addition of 10^{-3} M L-arginine [$n=17,280$].

*Values are expressed as mean $\pm$ standard deviation.

Figure 2.

Nitric oxide (NO) levels during normocapnia, hypercapnia and hypocapnia in astrocytes. This graph depicts levels of NO measured during normocapnia ($p\text{CO}_2$ 40.1 ± 0.9 mmHg), hypercapnia ($p\text{CO}_2$ 56.3 ± 8.7 mmHg), and hypocapnia ($p\text{CO}_2$ 21.7 ± 6.7 mmHg) in human fetal astrocytes. (normocapnic [n=17,280], hypercapnic [n=17,280] and hypocapnic measures [n=17,280]).

*Values are expressed as mean $\pm$ standard deviation.

Figure 3.

Correlation of $p\text{CO}_2$ and nitric oxide (NO) levels in endothelial cells and astrocytes. This graph depicts the correlation between mean $p\text{CO}_2$ levels and mean NO concentrations for human cerebral endothelial cells and fetal astrocytes. Linear regression correlation coefficient was 0.99 for endothelial cells and 0.93 for astrocytes. Error bars represent standard deviations.

Figure 4. Direct Western blot detection of eNOS and iNOS in human astrocytes and brain endothelial cell lysates. The primary NOS isotype expression in astrocytes is iNOS whereas eNOS is expressed in higher concentrations in endothelial cells.

Figure 5. iNOS and eNOS detection in fetal astrocytes and human brain cerebral microvascular endothelial cells by immunofluorescence microscopy confirms results of Western blot with robust staining of eNOS in endothelial cell types and relatively high levels of iNOS signal in astrocytes. Magnification 40x.