A Critical Role for Astrocytes in Hypercapnic Vasodilation in Brain

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Cerebral blood flow (CBF) is controlled by arterial blood pressure, arterial CO₂, arterial O₂, and brain activity and is largely constant in the awake state. Although small changes in arterial CO₂ are particularly potent to change CBF (1 mmHg variation in arterial CO₂ changes CBF by 3%–4%), the coupling mechanism is incompletely understood. We tested the hypothesis that astrocytic prostaglandin E₂ (PgE₂) plays a key role for cerebrovascular CO₂ reactivity, and that preserved synthesis of glutathione is essential for the full development of this response. We combined two-photon imaging microscopy in brain slices with \textit{in vivo} work in rats and \textit{in vitro} mice to examine the hemodynamic responses to CO₂ and somatosensory stimulation before and after inhibition of astrocytic glutathione and PgE₂ synthesis. We demonstrate that hypercapnia (increased CO₂) evokes an increase in astrocyte [Ca²⁺], and stimulates COX-1 activity. The enzyme downstream of COX-1 that synthesizes PgE₂ (microsomal prostaglandin E synthase-1) depends critically for its vasodilator activity on the level of glutathione in the brain. We show that, when glutathione levels are reduced, astrocyte calcium-evoked release of PgE₂ is decreased and vasodilation triggered by increased astrocyte [Ca²⁺] \textit{in vitro} and by hypercapnia \textit{in vivo} is inhibited. Astrocyte synthetic pathways, dependent on glutathione, are involved in cerebrovascular reactivity to CO₂. Reductions in glutathione levels in aging, stroke, or schizophrenia could lead to dysfunctional regulation of CBF and subsequent neuronal damage.

Key words: astrocyte; calcium; cerebral blood flow; glutathione; hypercapnia

Significance Statement
Neuronal activity leads to the generation of CO₂, which has previously been shown to evoke cerebral blood flow (CBF) increases via the release of the vasodilator PgE₂. We demonstrate that hypercapnia (increased CO₂) evokes increases in astrocyte calcium signaling, which in turn stimulates COX-1 activity and generates downstream PgE₂ production. We demonstrate that astrocyte calcium-evoked production of the vasodilator PgE₂ is critically dependent on brain levels of the antioxidant glutathione. These data suggest a novel role for astrocytes in the regulation of CO₂-evoked CBF responses. Furthermore, these results suggest that depleted glutathione levels, which occur in aging and stroke, will give rise to dysfunctional CBF regulation and may result in subsequent neuronal damage.

Introduction
Astrocyte [Ca²⁺], transients have been shown to directly alter diameters of cerebral arterioles in experiments using either direct astrocyte stimulation or calcium uncaging in astrocytes of juvenile (Zonta et al., 2003; Mulligan and MacVicar, 2004; Gordon et

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GSH-dependent mechanism of CBF regulation, which involves astrocytes and the GSH-sensitive release of PgE2.

Materials and Methods
Slice preparation
Four hundred μm hippocampal-neocortical slices were prepared from male and female juvenile (postnatal age 16–21 d) Sprague Dawley rats. Treatment of animals was approved by the University of British Columbia Animal Care and Use Committee. As previously described (Gordon et al., 2008), rats were anesthetized with halothane, decapitated, and the brains removed into ice-cold slicing solution containing the following (in mM): 2.5 KCl, 26 NaHCO3, 0.5 CaCl2, 10 MgSO4, 1.25 NaH2PO4, 10 glucose, 230 sucrose, saturated with 95% O2/5% CO2. The 400 μm transverse semi-sections were incubated at 32°C–34°C in aCSF containing the following (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO3, 2.0 CaCl2, 2.0 MgCl2, 1.25 NaH2PO4, 10 glucose, saturated with 95% O2/5% CO2 for 60 min. For experiments, slices were at 22°C–24°C, aCSF was saturated with 20% O2/5% CO2, balanced Na2, and perfused at −2 ml/min. Healthy slices can be maintained in 20% O2, which provides a PO2 at the low end of the physiological range (Gordon et al., 2008). Astrocytes were loaded with the caged IP3 compound, NV-IP3/AM (5 μm/mL), and/or the Ca2+ indicator rhod-2/AM (10 μm, Invitrogen) as previously described (Mulligan and MacVicar, 2004; Gordon et al., 2008). Slices were loaded with mono- chlorobimane (MCB, Fluka) in the dark at room temperature for 30 min as previously described (Robillard et al., 2011).

Two-photon imaging and uncaging in acute brain slices
A two-photon laser-scanning microscope (Zeiss LSM510-Axioskop-2 fitted with a 40 X-W/1.0 numerical aperture objective lens) coupled to a Chameleon ultra II Ti:sapphire laser (~140 fs pulses 80 MHz, Coherent) provided excitation of rhod-2 and was used to uncage IP3. Images were acquired 50–100 μm below the slice surface. Rhod-2 fluorescence imaging and two-photon uncaging were performed using laser settings and emission filters as previously described (Gordon et al., 2008). MCB was excited at 780 nm and detected with a PMT at 512–562 nm as previously described (Robillard et al., 2011). Astrocytes (defined as vessels with diamter >10 μm, surrounded by a visible layer of smooth muscle cells) were imaged by acquiring the transmitted laser light and using IR-DIC optics.

Glutathione and PgE2 measurements
Protocols in suppliers’ instructions were followed for the PgE2 ELISA and glutathione assays. When measuring PgE2, release from acute brain slices, TTX (1 μM, Alamone Labs) was added to dampen neuronal activation. PgE2 release from acute brain slices was measured using a Specific Parameter PgE2, ELISA kit (R&D systems). Measurements of tissue glutathione levels were made using a specific total glutathione assay kit from either BioVision or Assay Designs.

Immunohistochemistry
Rats were anesthetized with halothane, given an intraperitoneal injection of urethane (0.5 ml of 30% urethane per 50 g body weight), and perfused with saline (0.9% NaCl in 0.1 M phosphate buffer) followed by 4% PFA (in 0.1 M PBS). The brain was extracted, postfixed (10% formalin in 4% PFA) overnight, and cryoprotected (30% sucrose in PBS) overnight. Using a cryostat, 40 μm serial sections in the horizontal plane were collected throughout the brain. Free-floating sections were blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories) and 0.4% Triton X-100 in PBS for 1 h and incubated in PBS containing 0.1% Triton X-100 and primary antibodies against PgE2 synthase (anti-mPGES-1) (Orajie et al., 2014; Tuure et al., 2015) (Agrisera, catalog #AS03 031, 1:200) as well as an astrocyte phenotypic marker (anti-GFAP (Lathia et al., 2008) Sigma, catalog #G8939, clone G-A-5, 1:500) overnight at 4°C. Tissue was rinsed and incubated in AlexaFlour-488 goat anti-mouse and AlexaFlour-546 goat anti-rabbit secondary antibodies (Invitrogen; diluted 1:500 in PBS, 2.5% normal goat serum and 0.4% Triton X-100) for 1.5 h at room temperature. The tissue was rinsed, mounted onto slides, and coverslipped using Fluorsave mounting medium
Table 1. Blood gases for BSO experiment (Nota Bene blood gases taken 24 h after drug but before hypercapnia and whisker stimulation experiments)

| Condition | Treatment | pH   | pCO₂ (mmHg) | pO₂ (mmHg) |
|-----------|-----------|------|--------------|-------------|
| Predrug   | Predrug   | 7.47 (0.01) | 34.5 (2.3) | 161 (4) |
| Postdrug  | Predrug   | 7.46 (0.01) | 35.8 (1.5) | 154 (7) |

Data are mean (SEM).

An inhibitor of γ-glutamylcysteine synthetase.

Table 2. Blood gases for SC560 intravenous experiment (Nota Bene blood gases taken before and after drug administration)

| Condition | Treatment | pH   | pCO₂ (mmHg) | pO₂ (mmHg) |
|-----------|-----------|------|--------------|-------------|
| Predrug   | Predrug   | 7.47 (0.01) | 33.5 (1.5) | 164 (5) |
| SC560     | Predrug   | 7.45 (0.01) | 36.8 (1.2) | 140 (5) |
| Postdrug  | Predrug   | 7.46 (0.01) | 34.1 (0.6) | 156 (6) |
| SC560     | Predrug   | 7.45 (0.03) | 37.1 (2.1) | 140 (5) |

Data are mean (SEM).

Animals: in vivo blood flow measurements in rats

All procedures were approved by the University of Oxford Ethical Review Committee and complied with the requirements of the Animals (Scientific Procedures) Act, 1986, United Kingdom. Animals were housed in an animal housing facility in a 12 h alternating light/dark cycle with ad libitum access to food and water. Male Wistar rats were used (256–367 g).

Intracerebral injection

For surgical procedures, rats were anesthetized with 4% isoflurane and maintained at 1.5%–2% isoflurane in 30% O₂ and 70% N₂. Anesthesia was induced with 4% isoflurane and maintained during surgical procedures, all procedures involving animals were approved by the Danish National Ethics Committee according to the guidelines set forth in the European Council’s Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The 8- to 10-week-old male C57BL/6J mice were used.

In vivo calcium imaging

For experiments involving mice, anesthesia was induced with bolus injections of the α2-adrenergic receptor agonist xylazine (10 mg/kg i.p.) and the NMDA-receptor antagonist ketamine (60 mg/kg i.p.). Anesthesia was maintained during surgery with supplemental doses of ketamine (30 mg/kg/20 min i.p.). Upon completion of all surgical procedures, anesthesia was switched to continuous infusion with α-cloralose (50 mg/kg/h i.v.).

Calcium activity during hypercapnia was measured in vivo in eight C57BL/6J mice. A cranialotomy over the somatosensory cortex was covered with agar and parselyed with a glass coverslip. Oregon Green Bapta-1/AM (OGB; Invitrogen) was dissolved in DMSO and Pluronic F-127 (10%, BASF Global) and diluted in acSF to yield a final dye concentration of 0.8 mM. It was mixed with the astrocyte marker sulforhodamine 101 (SR101; Sigma-Aldrich, 100 μM) (Nimmerjahn et al., 2004) and was pressure injected (4–6 psi, 4 s) into the somatosensory cortex through a micropipette at a depth of 100–150 μm below the cortical surface. Ca²⁺ imaging was performed using a commercial two-photon microscope (SPS multiphoton/confocal Laser Scanning Microscope; Leica), and a Mai Tai HP Ti:sapphire laser (MillePro, Pro, Spectra Physics) with a 20× 1.0 NA-water-immersion objective (Leica). The excitation wavelength was 820 nm. The emitted light was filtered to retain both red and green light using a TRITC/FITC filter.

The hypercapnia challenge was presented as follows: Following 1 min baseline recording, 10% CO₂ in air was applied for 30 s and imaging continued for subsequent 4 min. Five trials were performed with 3 min between trials. For each animal, a second field of view was selected and the hypercapnia challenge repeated. Blood gases were taken after each experiment, and all mice had pCO₂ in the range 30–40 mmHg and pO₂ in the range 95–130 mmHg.

Data collection, analysis, and statistics

In vitro data. An image (512 × 512 pixels) was collected in 7.86–12.68 s, using 8-line averaging. Measurements of lumen diameter and Ca²⁺.
changes were performed offline with Zeiss LSM (version 3.2) software and ImageJ (National Institutes of Health). As previously described (Gordon et al., 2008), fluorescence signals were defined as $F/F_0$ (%) = $\frac{(F_1 - B_1)/(F_0 - B_0)}{100}$, where $F_1$ and $F_0$ are fluorescence at a given time and the mean fluorescence during the control period, respectively. $B_1$ and $B_0$ are the corresponding background fluorescence signals, taken from the neuropil. Pseudo-color images show absolute changes in fluorescence (ImageJ, 16-color linear Lut). Experimental values are mean ± SEM; $n$ is the number of experiments conducted or, for calcium changes, number of astrocytes analyzed. Either a two-tailed Student’s $t$ test or a one-way ANOVA with a Newman–Keuls post hoc test for comparison between multiple groups was used, and $p < 0.05$ was considered statistically significant. As these were novel experiments, the effect size was unknown before the experiment. Therefore, sample size estimates were based on our previous experience. Experiments were alternately performed under control or treatment conditions with slices chosen at random for each experiment. Data were excluded from analysis if any of the following occurred during imaging: unstable baseline vessel diameters or astrocyte calcium levels, or movement leading to significant focus changes during the experiment. To perform statistical analysis, data were assumed to be normally distributed.

In vivo data. All laser Doppler and LFP data were collected in Spike 2 software, whereas laser speckle data were collected using Moor FLPi software. Quantification of CBF changes and electrophysiology were performed in MATLAB (The MathWorks, version 7.12). To obtain the region of interest (ROI) for calculation of CBF changes using laser speckle imaging, a principal components analysis was used to identify the focal point of the change in response to stimulation. The same region of interest was used within each animal’s data. Experimental values are the mean ± SEM, and $n$ is the number of animals. To perform statistical analysis, data were assumed to be normally distributed. An $F$ test was used to compare variances of groups being statistically compared. For CBF data, a one-tailed $t$ test with Welch’s correction (as groups had significantly different variances) was used to compare means between groups. A two-tailed $t$ test was used to compare means of groups for both GSH analysis (see Fig. 5C) and electrophysiology data in response to whisker pad stimulation (Welch-corrected for SC560 experiment, see Fig. 6C). For electrophysiology data collected during hypercapnia challenge experiments, a two-way ANOVA with Bonferroni correction for multiple comparisons was used to compare means between groups. $p < 0.05$ was considered statistically significant. For experiments involving rats, due to effect sizes being unknown before experiment, sample size estimates were based on previously published sample sizes (e.g., Niwa et al., 2001). Assignment of animals was alternated between treatment and control groups, and neither experiments nor analysis were blinded. Three animals were excluded from all data analysis (1 for SC560 and 2 for B6 levels or the recording ended. Experimental values are expressed as mean ± SEM. A paired $t$ test was used for the calcium imaging data, each animal served as its own control. $p < 0.05$ was accepted as statistically significant. For experiments involving mice, as there have been no previous studies reporting astroglial calcium changes during hypercapnia, it was impossible to estimate an expected value for change in fluorescence or its SD. Hence, no sample size calculation could be performed. However, we expected similar calcium changes to those we observe for low frequency whisker stimulation, and so sample sizes were based on our previous experiments (6–8 mice). Calcium signals obtained during hypercapnia exceeded an SNR of 4:1, and hypercapnia-induced calcium responses were recorded in every animal tested. As all mice were subjected to hypercapnia, there was no randomization method used. Control measurements of calcium activity (i.e., activity without application of hypercapnia) were taken at random time points during the experiment. Analysis of calcium changes was not blinded, assessment of these changes was based on a MATLAB program, which analyzes the image sequences in an unbiased manner, rather than by visual inspection.

Results

Increased CO2 evokes [Ca2+]i responses in astrocytes in vivo

Elevation of tissue CO2 concentration, which can be caused by neuronal metabolism, is known to dilate cerebral blood vessels in a process dependent on PGF2 (Wagerle and Mishra, 1988; Wagerle and Degiulio, 1994) formation via COX-1 activity (Niwa et al., 2001). However, the cells that both are responsible for sensing CO2 and that also express the enzymes for synthesizing PGF2 (COX-1 and PGES) have not been resolved. Astrocytes can produce PGF2, but it is unknown whether astrocytes generate [Ca2+]i signals in response to CO2. Therefore, we tested whether an increase in inspired CO2 (hypercapnia) in vivo evokes astrocyte [Ca2+]i, when it also triggers CBF increases.

Two-photon laser scanning microscopy (2PLSM) in vivo was used to examine the simultaneous responses of both neurons and astrocytes to hypercapnia in the intact brain as a first step to investigate which cell type might be the primary sensor of CO2 (Fig. 1). Remarkably, we found consistent and significant increases in [Ca2+]i in the soma and endfeet of astrocytes in cortical layers II/III of mouse (Fig. 1) during the period of hypercapnia. The dramatic increases that we observed in astrocytes were of significantly higher amplitude (Fig. 1A–C; $p < 0.01$) than increases in [Ca2+]i, observed in neuronal soma during the period of hypercapnia. The number of astrocytes with [Ca2+]i responses was also much greater in hypercapnia compared with the number showing spontaneous calcium activity (control time period: Fig. 1D; $p < 0.01$). Although neurons could display increased [Ca2+]i during hypercapnia, with onset times within seconds (Fig. 1B, C, E), there was no significant difference in the number of neurons with [Ca2+]i responses during hypercapnia compared with the number showing spontaneous calcium activity (control time period: Fig. 1D). Measurements taken in the neuropil where there were no defined cell bodies, and it is difficult to separate signals in fine astrocyte processes from neuronal processes did not show correlated changes in [Ca2+]i signals during hypercapnia (Fig. 1D). The astrocyte [Ca2+]i responses (Fig. 1B, E, F) appear to occur within a similar timescale as the increased CBF evoked by hypercapnia (as measured by laser speckle contrast imaging and laser Doppler flowmetry in rat; see Fig. 5A, D, respectively). During hypercapnia, an increased number of astrocyte soma (Fig. 1D) displayed increased [Ca2+]i, with onsets within seconds (Fig. 1B, E) and variable durations of tens of seconds (Fig. 1B, F). While there were no differences between the three groups (astrocyte soma, neuronal soma, and neuropil) with regards to the delay of the hypercapnia-induced Ca2+ responses (average Ca2+ response delay [Fig. 1E]: neuron soma = 12.14 ± 1.19 s ($n = 33$), neuropil = 12.83 ± 4.18 s ($n = 3$), and astrocyte soma = 14.57 ± 1.55 s ($n = 47$)), the average Ca2+ response
Figure 1. Astrocyte [Ca$^{2+}$]i transients are evoked by CO$_2$ in vivo. A, Example still images of mouse cortical layer II/III from 2PLSM. OGB is used as a calcium indicator (Ai–Aiii) and sulforhodamine 101 (SR101, Aiv, average image for whole recording) is used to stain astrocytes. Color scale refers to images Ai–Aiii. White arrows indicate astrocytes that show a Ca$^{2+}$ response to CO$_2$ of at least twice its baseline Ca$^{2+}$ fluctuation. In this case, CO$_2$ stimulus begins at $t = 0$ s and is applied for 36 s. Aiii, Recovery of immediate CO$_2$ induced Ca$^{2+}$ transient. Scale bars, 40 μm. B, Bi, Biii, Further example images of mouse cortical layer II/III from 2PLSM showing example ROI placement. Merge images showing OGB and SR101 (Bi, Bii). Red ROI1 indicates astrocyte endfoot. Red ROI2 indicates astrocyte soma (layer II: n = 181, 8 mice). Green ROI indicates neuron soma (layer II: n = 153, 8 mice). Blue ROI indicates neuropil (layer II: n = 104, 8 mice). Scale bar, 20 μm. Example time series (Bi, Biv) of [Ca$^{2+}$]i response in astrocyte and neuron soma ROIs (as indicated in Bi, Bii). Blue box represents time during which expired CO$_2$ level is increased. C, Mean Ca$^{2+}$ response in ROIs. Colors represent description in Bi. D, Percentage of ROIs for each cell type that showed a Ca$^{2+}$ response with and without a hypercapnia stimulus. For no hypercapnia (control), n = 170 astrocyte somas, n = 148 neuronal soma, and n = 96 neuropil ROIs, n = 8 mice. Colors represent description in Bi. E, Delay from hypercapnia start time to start of Ca$^{2+}$ response in ROI. F, Duration of Ca$^{2+}$ response in each ROI in response to CO$_2$ stimulus. E, F, Box plots represent the mean (small square). Edges of the box represent 25% and 75% of data. End lines indicate maximum and minimum values. Data are mean ± SEM. ***p < 0.001.
duration (Fig. 1F) was found to be significantly longer in astrocytes than in neurons: neuron soma = 119.41 ± 8.82 s (n = 33), astrocyte soma = 155.47 ± 8.32 s (n = 47) (p < 0.05, ANOVA).

Astrocytic [Ca2+]i signals evoke subsequent GSH-dependent PgE2 release

Having demonstrated in vivo that hypercapnia evokes an increase in astrocyte [Ca2+]i, we then used a combination of 2PLSM and PgE2 measurements using ELISA in acute brain slices to determine the mechanistic links between astrocyte [Ca2+]i responses and CBF regulation. Using a biochemical model, we investigated the role of GSH in the generation of PgE2.

Unlike in the in vivo situation, it is difficult to reliably evoke astrocyte [Ca2+]i signals and vasodilations by applying CO2 to acute brain slices. Thus, we needed an alternative method of elevating astrocyte [Ca2+]i. Previously, we have shown that bath application of the mGluR agonist tACPD is known to increase astrocyte [Ca2+]i in younger animals (Mulligan and MacVicar, 2004). Therefore, tACPD was used to evoke reliable, reproducible astrocyte [Ca2+]i elevations in acute brain slices from juvenile rats. To evoke widespread increases in astrocyte [Ca2+]i, hippocampal-neocortical slices were perfused with tACPD, an mGluR agonist. Application of tACPD (100 μM) to brain slices (from juvenile rats) caused a generalized increase in astrocyte [Ca2+]i, observed

Figure 2. Astrocyte [Ca2+]i signals evoke COX-1- and GSH-dependent vasodilations in vitro. A, 2PLSM imaging: example Ca2+ and arteriole diameter changes in response to tACPD with and without BSO. Images represent overlay of pseudo-colored Ca2+ changes and transmitted light images. Dotted line indicates initial vessel diameter. Scale bar, 10 μm. B, Mean time course of increase in astrocyte [Ca2+]i in response to tACPD. Colored box represents time of tACPD application. Control, n = 56 from 26 rats; BSO, n = 39 from 18 rats. C, Mean tACPD-evoked increase in astrocyte [Ca2+]i. tACPD, n = 56 from 26 rats; tACPD + SC560, n = 12 from 7 rats; tACPD + BSO, n = 39 from 18 rats. D, Mean tACPD-evoked PgE2 release, measured by ELISA. Within a group, each experiment (n) uses tissue from a different rat (i.e., control, n = 8 from 8 rats). E, Mean tissue GSH concentration; data from 4 rats for each group. F, Mean time course of tACPD-evoked change in lumen diameter. Colored box represents time of tACPD application. Control, n = 31 slices from 26 rats; BSO, n = 21 slices from 18 rats. G, Mean changes in lumen diameter evoked by tACPD and clonidine. tACPD, n = 31 slices from 26 rats; SC560 + tACPD, n = 7 slices from 7 rats; BSO + tACPD, n = 21 slices from 18 rats; clonidine, n = 8 slices from 8 rats; BSO + clonidine, n = 8 slices from 8 rats. H, Mean changes in lumen diameter evoked by PgE2 and NE. PgE2, n = 5 slices from 4 rats; BSO + PgE2, n = 3 slices from 3 rats; NE, n = 14 slices from 11 rats; BSO + NE, n = 8 slices from 8 rats. Data are mean ± SEM. **p < 0.01. ***p < 0.001. n, number of experiments conducted or, for calcium measurements, number of astrocyte ROIs analyzed.
using 2PLSM (Fig. 2A–C), that provided us with the ability to measure subsequent synthesis of PGE$_2$. Applying tACPD resulted in the formation and efflux of PGE$_2$, as measured by ELISA (Fig. 2D). The first step in the conversion of AA to PGE$_2$ in astrocytes is via COX-1 (Fig. 7) (Takano et al., 2006; Gordon et al., 2008; Font-Nieves et al., 2012). Neurons, in contrast, express COX-2 but not COX-1 (Nogawa et al., 1997). In support of a central role for COX-1, we found that, although the tACPD-evoked increase in astrocyte [Ca$^{2+}$]$_i$, was unaltered (Fig. 2C) in the presence of the COX-1 inhibitor SC560 (Smith et al., 1998; 100 nm: Blanco et al., 2008), the resulting formation and efflux of PGE$_2$, as measured by ELISA, was abolished ($p < 0.001$; Fig. 2D). Thus, astrocyte COX-1 activity is required for the subsequent PGE$_2$ release in acute brain slices, which is triggered by astrocyte [Ca$^{2+}$]$_i$ signals.

Downstream of COX-1, the synthesis of PGE$_2$ involves the astrocyte-expressed, GSH-dependent, enzyme mPGES-1 (Tachikawa et al., 2012). Therefore, a role for astrocytes in the regulation of arteriolar diameter would be supported if [Ca$^{2+}$]$_i$-evoked vasodilations were attenuated when GSH levels were depressed. We examined whether there is a reduction in subsequent vasodilations in hippocampal slices after treatment with BSO. When GSH levels were decreased, tACPD-evoked astrocyte [Ca$^{2+}$]$_i$ signals were unaltered (Fig. 2A–C). However, the vasodilations triggered by these [Ca$^{2+}$]$_i$ signals were abolished (Fig. 2A,F,G; $p < 0.01$). Vasocostrictionvoked by NE (100 µM) or the α$_1$ agonist clonidine (10 µM), which act directly on arteriolar smooth muscle cells (Busija and Leffler, 1987), were unchanged in the presence of BSO (Fig. 2A,G,H), indicating that arterioles were not damaged by the BSO treatment. Furthermore, BSO treatment did not alter the vasodilation evoked by either 1 µM PGE$_2$ (Fig. 2H) or high [K$^+$] (10 mM), which causes vasodilation by hyperpolarizing arteriolar smooth muscle cells (Filosa et al., 2006) (K$^+$: 8.6 ± 2.3%, n = 5 slices from 5 rats; BSO + K$^+$: 6.5 ± 0.8%, n = 6 slices from 3 rats, $p = 0.37$).

Astrocyte [Ca$^{2+}$]$_i$ increases can be triggered by two-photon uncaging of IP$_3$ within the cell body of an astrocyte. Using this

**Figure 3.** Astrocytes express mPGES-1 and contain high levels of GSH. **A.** Immunohistochemistry showing astrocytic expression of GSH-dependent mPGES-1 in the CA3 of the hippocampus. Astrocyte marker, GFAP (red), mPGES-1 (green), and merge (yellow). Scale bar, 20 µm. **B.** MCB-loaded hippocampal-neocortical slices. Astrocytes (identified by SR101, red, white arrowheads) contain higher levels of GSH (as indicated by MCB staining, green) than neurons (white arrows). Merge (yellow). Scale bar, 20 µm.

**Figure 4.** Astrocyte [Ca$^{2+}$]$_i$ transient-evoked vasodilations are GSH dependent in vitro. **A.** Mean IP$_3$-evoked increases in astrocyte [Ca$^{2+}$]$_i$, Control, n = 21 from 6 rats; + BSO, n = 11 from 4 rats. **B.** Mean time course of increase in astrocyte [Ca$^{2+}$]$_i$. Dotted line indicates time of photolysis of caged IP$_3$, as described in A. **C.** Mean lumen diameter change in response to uncaging of IP$_3$. Uncage IP$_3$, n = 11 slices from 6 rats; + BSO, n = 6 slices from 4 rats. Data are mean ± SEM. **p < 0.01. n, number of experiments conducted or, for calcium measurements, number of astrocyte ROIs analyzed.

**Figure 5.** Astrocyte [Ca$^{2+}$]$_i$ signals evoked COX-1 and GSH-dependent vasodilations in brain slices

As COX-1 activity (Niwa et al., 2001) and PGE$_2$ release (Wagerle and Mishra, 1988; Wagerle and Degiulio, 1994) have been shown to lead to increased CBF in response to hypercapnia, we examined whether COX-1-dependent PGE$_2$ release evoked by astrocyte [Ca$^{2+}$]$_i$ signals triggered by either tACPD application or IP$_3$ uncaging resulted in vasodilations.

Bath perfusion of tACPD induced arteriolar dilation in acute brain slices (Fig. 2A,F,G), which was abolished in the presence of SC560 ($p < 0.01$; Fig. 2G), whereas the amplitude of evoked astrocyte [Ca$^{2+}$]$_i$ signals was unchanged ($p > 0.05$; Fig. 2C). Thus, combined with the results discussed above, these data confirm that astrocyte COX-1 activity and subsequent PGE$_2$ release are required for vasodilations in acute brain slices that are triggered by astrocyte [Ca$^{2+}$]$_i$ signals.

As previously discussed, downstream of COX-1, the synthesis of PGE$_2$ involves the astrocyte-expressed, GSH-dependent, enzyme mPGES-1 (Tachikawa et al., 2012). Therefore, a role for astrocytes in the regulation of arteriolar diameter would be supported if [Ca$^{2+}$]$_i$-evoked vasodilations were attenuated when GSH levels were depressed. We examined whether there is a reduction in subsequent vasodilations in hippocampal slices after treatment with BSO. When GSH levels were decreased, tACPD-evoked astrocyte [Ca$^{2+}$]$_i$ signals were unaltered (Fig. 2A–C). However, the vasodilations triggered by these [Ca$^{2+}$]$_i$ signals were abolished (Fig. 2A,F,G; $p < 0.01$). Vasocostrictionvoked by NE (100 µM) or the α$_1$ agonist clonidine (10 µM), which act directly on arteriolar smooth muscle cells (Busija and Leffler, 1987), were unchanged in the presence of BSO (Fig. 2A,G,H), indicating that arterioles were not damaged by the BSO treatment. Furthermore, BSO treatment did not alter the vasodilation evoked by either 1 µM PGE$_2$ (Fig. 2H) or high [K$^+$] (10 mM), which causes vasodilation by hyperpolarizing arteriolar smooth muscle cells (Filosa et al., 2006) (K$^+$: 8.6 ± 2.3%, n = 5 slices from 5 rats; BSO + K$^+$: 6.5 ± 0.8%, n = 6 slices from 3 rats, $p = 0.37$).
We examined the impact of decreased tissue GSH levels on CO₂-evoked CBF increases in vivo. To lower GSH levels in vivo, BSO was injected into rat barrel cortex. After 24 h, tissue GSH levels in the ipsilateral cortex were reduced by 45% (Fig. 5C, p = 0.018). Treatment with BSO reduced the hypercapnia-evoked CBF response (Fig. 5DE; AUC reduced by 65%, p = 0.048). Neural activity was no different in BSO-treated rats compared with saline-treated rats (Fig. 5G). Combining all the data described so far suggests that hypercapnia-evoked, astrocyte [Ca²⁺]-related, CBF increases require PG₂ release and, thus, are compromised when brain GSH levels are reduced.

This finding was specific to hypercapnia-evoked CBF increases. We examined the impact of decreased tissue GSH levels in vivo on functional hyperemia in the somatosensory cortex. Whisker pad stimulation (10 Hz) evoked a blood flow increase in the barrel cortex (Fig. 6A). In agreement with previous findings (Niwa et al., 2000), inhibiting COX-1 with SC560 had no effect on either the CBF response to whisker pad stimulation (Fig. 6AB; p = 0.10) or evoked neural activity (LFP) (Fig. 6C, p = 0.91). Furthermore, the AUC of the stimulation-evoked CBF response was not significantly different in BSO-treated animals (Fig. 6D; p = 0.14) compared with saline-treated animals, demonstrating that the CBF response is not GSH-sensitive. The magnitude of the neural response to whisker pad stimulation was unaffected by BSO (Fig. 6E; p = 0.68). These results indicate that, under these experimental conditions, COX-1 and GSH play little, if any, role in the CBF response to somatosensory stimulation. These findings confirm that several different pathways exist that account for CBF regulation under differing conditions and in response to different stimuli.

**In vivo hypercapnia-evoked CBF responses are GSH dependent**

Having determined in acute brain slices the vasodilatory molecules underlying astrocyte [Ca²⁺]-evoked vasodilations, we examined whether these same enzymes and molecules were involved in the CBF response, which occurs downstream of CO₂-evoked astrocyte [Ca²⁺] responses in vivo. Hypercapnia in vivo evoked a CBF increase in the barrel cortex of adult rat (Fig. 5ABDE), whereas neural activity was unchanged (Fig. 5F). The calculated area under the curve (AUC) of the CBF response was significantly attenuated by SC560 (p = 0.032; Fig. 5AB), confirming that COX-1 plays a critical role in hypercapnia-evoked CBF increases in vivo (Niwa et al., 2001).
Discussion

We demonstrate a novel mechanism of CBF regulation involving astrocytes, which is GSH dependent. Previously, Niwa et al. (2001) demonstrated that hypercapnia-evoked CBF increases are principally COX-1 dependent. In this study, we examined the mechanism of such CBF regulation, both upstream and downstream of hypercapnia-evoked increases in COX-1 activity (Fig. 7). We demonstrate in vivo that, upstream of evoked COX-1 activity, CO₂ increases [Ca²⁺], in astrocytes. These data demonstrate a new signal (hypercapnia) that activates astrocyte COX-1 and specifically identifies the involvement of astrocytes in the regulation of CBF in response to changes in arterial CO₂.

In vitro, using brain slices from juvenile animals in which it is possible to examine calcium signals by bulk loading a calcium indicator dye, we confirm that increased astrocyte [Ca²⁺], results in the subsequent release of PgE₂ and vasodilation which are COX-1 activity-dependent (Fig. 7). Our assumption that the evoked response in juvenile rat slices is the same as in adult rat with respect to COX-1 dependence is supported by the fact that the same COX-1 dependence has been shown in adult mice (Takano et al., 2006). We demonstrate that these findings hold in vivo, confirming previous findings in adult mice (Niwa et al., 2001). Astrocytic endfeet, which are apposed to cerebral vascular smooth muscle, express all the machinery necessary for PgE₂ synthesis (COX-1) (Takano et al., 2006; Gordon et al., 2008), mPgES-1 (Fig. 3A) (Tachikawa et al., 2012), and GSH: (Fig. 3B) (Sun et al., 2006; Bragin et al., 2010; Robillard et al., 2011), providing further evidence for the involvement of astrocytes in the regulation of CBF responses to hypercapnia. mPgES, an enzyme selectively expressed in astrocytes compared with neurons (Tachikawa et al., 2012), is the enzyme responsible for producing PgE₂ downstream of COX-1 activity. Intriguingly, the formation of PgE₂ is regulated by the availability of GSH in astrocytes, as PgES requires GSH as a cofactor (Jakobsson et al., 1999; Murakami et al., 2000). In vitro, we demonstrate that astrocyte [Ca²⁺]i-evoked vasodilations are attenuated when GSH levels are depleted, whereas in vivo, we demonstrate that CO₂-evoked CBF increases occur via a GSH-dependent mechanism. As astrocytes contain high levels of GSH (Fig. 3B) (Sun et al., 2006; Bragin et al., 2010; Robillard et al., 2011), the dependence of the CO₂-evoked CBF response on GSH is further evidence of astrocytic involvement. Together, our findings suggest a novel mechanism of astrocyte-evoked CBF regulation, which is GSH dependent. We propose that increased CO₂ levels evoke [Ca²⁺]i responses in astrocytes, subsequently activating a signaling pathway, involving COX-1 and the GSH-dependent PgES, which results in the release of the vasodilator PgE₂. Thus, an increase in CO₂ results in an astrocyte-driven, GSH-dependent vasodilation (Fig. 7).

This GSH-dependent mechanism of CBF regulation exists alongside other COX-1 and GSH-insensitive mechanisms. For example, we found no effect of blocking COX-1 activity or of lowering GSH levels on CBF responses following 10 Hz whisker pad stimulation. Although it is possible that an astrocyte calcium response (and, thus, a GSH-sensitive mechanism of CBF regulation) may be evoked by an intense sensory stimulus (Schulz et al., 2012; Sekiguchi et al., 2016), our results are in agreement with previous work suggesting that COX-1 is involved in CBF responses to hypercapnia (Niwa et al., 2001) but not sensory stimulation (Niwa et al., 2000). Although we saw no evidence that this pathway was important for functional (neuronal activity-evoked) increases in CBF under our experimental conditions, astrocytes appear to be an important intermediary for physiological (hypercapnia-evoked) increases in CBF. Our findings suggest that CBF regulation may involve astrocytes, and their [Ca²⁺]i signals, under certain conditions and not under others.

Previous studies have provided evidence for several mechanisms linking astrocyte [Ca²⁺]i increases and changes in CO₂ concentration. For example, within the respiratory center, increased astrocyte [Ca²⁺]i and astrocytic release of ATP can be triggered by CO₂-evoked decreases in pH (Gourine et al., 2010). This [Ca²⁺]i increase may be the result of increased Na⁺/HCO₃⁻ cotransport and reversal of Na⁺/Ca²⁺ transport (Turovsky et al., 2016). It is unknown whether this mechanism also occurs within the cortex. Alternatively, increased CO₂ can evoke hemichannel-mediated release of ATP (Huckstepp et al., 2010), which may act
on astrocytic purinergic receptors to elicit an increase in [Ca\textsuperscript{2+}]. (Pelligrino et al., 2011). Depending on the mechanism linking increases in CO\textsubscript{2} to astrocyte [Ca\textsuperscript{2+}], responses, therefore, astrocytes could act as either a pH or CO\textsubscript{2} sensor. Although it is beyond the scope of this paper to determine the link between an increase in CO\textsubscript{2} and the increase in astrocyte [Ca\textsuperscript{2+}], we have demonstrated that the depletion of GSH levels leads to a reduction in the ability of astrocytes to release PgE\textsubscript{2}, following such a rise in [Ca\textsuperscript{2+}], and so reduces their ability to evoke vasodilation in response to hypercapnia. This occurs because astrocytes express GSH-dependent mPgES-1.

Our finding that CBF responses to increased CO\textsubscript{2} are GSH-sensitive suggests that global CBF regulation, which is sensitive to the partial pressure of arterial CO\textsubscript{2} (Ainslie and Duffin, 2009), will be affected in conditions where GSH levels are depleted. Alterations in the redox status of brain tissue that are ultimately linked to cellular GSH levels have been observed in numerous neurological and psychiatric disorders (Slivka and Cohen, 1993; Tohgi et al., 1999; Ansari et al., 2010). Alterations in GSH levels may lead to metabolic changes in astrocytes, which are apposed to the smooth muscle layer surrounding arterioles, resulting in activation of K\textsuperscript{+} channels, a decrease in Ca\textsuperscript{2+} entry into the smooth muscle cell and vasodilation.

![Diagram of astrocyte with CO2 entering and leading to Ca2+ increase, and then PLA2, GSH, PgE2, PGES, COX enzymes, and CO2 exit.] Figure 7. Increases in astrocytic [Ca\textsuperscript{2+}], may lead to GSH-dependent, PgE\textsubscript{2}-mediated vasodilation. Schematic diagram depicting how CO\textsubscript{2}-evoked increases in astrocytic [Ca\textsuperscript{2+}], may lead to PgE\textsubscript{2}-mediated vasodilation. As a result of elevated [Ca\textsuperscript{2+}], PLA\textsubscript{2} is activated. PLA\textsubscript{2} generates AA from the plasma membrane. AA can be processed locally by COX enzymes to produce AA derivatives, such as prostaglandin H\textsubscript{2} (PgH\textsubscript{2}). PgE\textsubscript{2} is produced from PgH\textsubscript{2} by the enzyme PGES, which requires GSH as a cofactor (Jakobsson et al., 1999; Murakami et al., 2000; Tanioka et al., 2000). PgE\textsubscript{2} is released from astrocyte endfeet, which are apposed to the smooth muscle layer surrounding arterioles, resulting in activation of K\textsuperscript{+} channels, a decrease in Ca\textsuperscript{2+} entry into the smooth muscle cell and vasodilation.

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