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Citation for published version:

Digital Object Identifier (DOI):
10.1523/JNEUROSCI.0005-16.2016

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Journal of Neuroscience

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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 in vivo work in rats and C57BL/6j 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 

**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 release of 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+]i 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

Received Jan. 1, 2016; revised Nov. 21, 2016; accepted Dec. 14, 2016.


This work was supported by a Sir Henry Wellcome Post-Doctoral Fellowship to C.H., a Government of Canada Post-Doctoral Research Fellowship to C.H., a Michael Smith Foundation for Health Research Post-Doctoral Fellowship to C.H., a Natural Sciences and Engineering Research Council Post-Doctoral Fellowship to J.M.P.P., a Canadian Institutes of Health Research Doctoral Stipend to R.K., a Canada Research Chair in Neuroscience to B.A.M., Canadian Institutes of Health Research Operating Grants 146397, 8545, 115121, and T5E-117869 in the framework of the ERA-NET NEURON to B.A.M., Fondation Leducq to B.A.M., A.M.B., and M.L., Human Frontier Science Program to B.A.M. and G.E.-D., National Institutes of Health Grants GM03355 and NS067920 to G.E.-D., Heart and Stroke

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

**Cellular/Molecular**
et al., 2008), or adult animals (Takano et al., 2006). However, several laboratories have published contradictory evidence on whether, in adult animals, astrocyte \([Ca^{2+}]_{i}\) signaling is evoked by synaptic activity leading to neurovascular coupling (Zonta et al., 2003; Petzold et al., 2008; Schulz et al., 2012; Lind et al., 2013; Otsu et al., 2015) or not (Nizar et al., 2013; Takata et al., 2013; Bonder and McCarthy, 2014). More recently, astrocyte \([Ca^{2+}]_{i}\) was shown to modify basal arteriole tone in adult animals (Rosenegger et al., 2015). Therefore, it is still poorly understood when, how, and under what conditions, astrocyte \([Ca^{2+}]_{i}\) signaling contributes to the regulation of cerebral blood flow (CBF).

In this work, we investigated the mechanisms underlying CBF responses to increased blood CO2 concentrations (hypercapnia) and the potential contribution of astrocytes to those CBF responses. Arterial CO2 has a potent effect on CBF, with a 1 mmHg variation eliciting a 3%–4% CBF change (Hauge et al., 1980). However, the mechanism coupling a change in CO2, to a change in CBF is incompletely understood. There are parallels between the vasoactive signals generated by astrocytes and those underlying hypercapnia-evoked CBF responses. Astrocytes have been shown to directly modify arteriole diameter when their intracellular \([Ca^{2+}]_{i}\) increases, activating astrocytic phospholipase A2 (PLA2) (He et al., 2012) and thereby generating arachidonic acid (AA) and several vasoactive metabolites including PgE2, which causes vasodilation (Zonta et al., 2003; Takano et al., 2006; Gordon et al., 2008; Attwell et al., 2010). In addition to their roles in neurovascular coupling, both PgE2 (Wagerle and Mishra, 1988; Wagerle and Degiulio, 1994) and cyclooxygenase-1 (COX-1) activity (Niwa et al., 2001) are involved in increasing CBF during hypercapnia. We examined the potential link between astrocytes and increased CBF during hypercapnia because astrocytes express the enzymes that are involved in synthesizing PgE2, from AA during hypercapnia-induced CBF changes (Niwa et al., 2001). For example, mRNA for both COX-1 and microsomal prostaglandin E synthase-1 (mPGES-1) are reported in transcriptome studies to be highly expressed in astrocytes but not neurons (e.g., ptgs1, also known as COX-1, is 15-fold higher in astrocytes than in neurons) (Cahoy et al., 2008; Zhang et al., 2014). Astrocytes are immunoreactive for both the enzyme proteins COX-1 (Takano et al., 2006; Gordon et al., 2008) and mPGES-1 (see Fig. 3A and Tachikawa et al., 2012). mPGES-1, the form of prostaglandin E synthase expressed in astrocytes, requires the cofactor glutathione (GSH) (Jakobsson et al., 1999; Murakami et al., 2000) that is present in high levels in astrocytes (see Fig. 3B and Sun et al., 2006; Bragin et al., 2010; Robillard et al., 2011). We investigated whether hypercapnia can evoke an increase in astrocyte \([Ca^{2+}]_{i}\), \textit{in vivo} and, if so, whether this results in activation of a PgE2-mediated vasodilation. In doing so, we demonstrate a novel, 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 hemi-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 N2, 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 μg/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 monochlorobimane (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 TSaperture 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 diameter >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% sucrose in 4% Paraformaldehyde) overnight at 4°C, cryosectioned, and free-floating sections were blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories) and 0.4% Triton X-100 in PBS containing 20% normal goat serum and 1% bovine serum albumin, washed, and then exposed to primary antibodies overnight at 4°C. The primary antibodies used were: goat anti-glutathione (1:500) (Sigma, catalog #G3893, clone #G-A-5), goat anti-COX-1, antibody (1:200), rabbit anti-ST2 antibody (1:400) (Agrisera, catalog #AS03 031, 1:400), and rabbit anti-GFAP antibody (1:200) as well as an astrocyte phenotypic marker (anti-GFAP (Lathia et al., 2008) Sigma, catalog #G893, clone #G-A-5, 1:500) overnight at 4°C. Tissue was rinsed and incubated in AlexaFluro-488 goat anti-mouse and AlexaFluro-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.
Rats had their left femoral artery and vein cannulated, and were also tracheotomized and ventilated. A laser speckle camera (Moor Instruments) was used to monitor relative CBF over a thin skull window over the right whisker barrel cortex while an LFP electrode for neuronal activity was inserted through a burr hole. Bipolar stimulating electrodes were placed in the left whisker pad. Animals had a steady-state blood gas before and after drug administration (Table 2). An electrical stimulus (10 Hz, 16 s duration, 1.6 mA, 0.3 ms pulsewidth, 60 s interstimulation interval) to evoke a blood flow response in the right whisker barrel cortex was performed for 10 trials per animal. Following this, animals were exposed to 10% CO2 for 30 s at 3 min intervals repeated four times to induce a hypercapnic blood flow response. Animals were then administered 5 mg/kg SC560 or 10% DMSO (vehicle) intravenously. SC560 is a highly lipophilic COX-1 inhibitor and distributes widely into tissues (Teng et al., 2003), and this dose was chosen for maximal target efficiency (Zhang et al., 2003). After 20 min, the effect of COX-1 inhibition on the evoked CBF responses to whisker stimulation and hypercapnia was measured.

In vivo calcium imaging

For in vivo experiments, 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-chloralose (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 partly sealed 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 μM. 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 (MILLIPORE 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 \( F/F_0 \) = \( [(F_1 - B_1)/(F_0 - B_0)] \times 100 \), 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 using Moor FLP1 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-correction 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 BSO), as these experiments did not correspond with experimental equipment.

For in vivo calcium imaging, frame size was \( 256 \times 256 \) pixels (189–207 ms/frame) during recordings. The \( \text{Ca}^{2+} \) changes were evaluated as the average change in fluorescence relative to baseline levels in ROIs. The ROIs were placed based on morphology over neuronal or astrocytic soma, or neuropil. Because of movement of astrocytes during hypercapnia, an increased number of astrocytes to hypercapnia in the intact brain as a first step to investigate which cell type might be the primary sensor of \( \text{CO}_2 \) (Fig. 1). Remarkably, we found consistent and significant increases in \( [\text{Ca}^{2+}]_i \) in the soma and endfeet of astrocytes in cortical layers II/III of mouse (Fig. 1) during the period of hypercapnia. The number of astrocytes with \( [\text{Ca}^{2+}]_i \) in vivo evokes astrocyte \( [\text{Ca}^{2+}]_i \) when it also triggers CBF increases. By using laser Doppler microscopy, we observed two distinct changes in astrocyte \( [\text{Ca}^{2+}]_i \) during hypercapnia: an increase in \( [\text{Ca}^{2+}]_i \) observed in the neuropil during the period of hypercapnia. The number of astrocytes with \( [\text{Ca}^{2+}]_i \) response 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 \( [\text{Ca}^{2+}]_i \) during hypercapnia, with onset times within seconds (Fig. 1B,C,E), there was no significant difference in the number of neurons with \( [\text{Ca}^{2+}]_i \) 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 \( [\text{Ca}^{2+}]_i \) signals during hypercapnia (Fig. 1D). The astrocyte \( [\text{Ca}^{2+}]_i \) 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 \( [\text{Ca}^{2+}]_i \), 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 \( \text{Ca}^{2+} \) responses (average \( \text{Ca}^{2+} \) response delay [Fig. 1E]: neuron soma = 12.14 ± 1.19 s \( (n = 33) \), neuropil = 12.85 ± 4.18 s \( (n = 3) \), and astrocyte soma = 14.57 ± 1.55 s \( (n = 47) \)), the average \( \text{Ca}^{2+} \) 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. Bi, Biii. Further example images of mouse cortical layer II/III from 2PLSM showing example ROI placement. Merge images showing OGB and SR101 (Bi, Biii). 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 (Bii, Biv) of [Ca$^{2+}$]$_i$ response in astrocyte and neuron soma ROIs (as indicated in Bi, Biii). Blue box represents time during which expired CO$_2$ level is increased. C. Mean Ca$^{2+}$ response in ROIs. Colors represent description in B. 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 B. 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 < 0.01. ***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 in acute brain slices. Although the adult mouse (Sun et al., 2013) and rat (Duffy and MacVicar, 1995) have been shown not to express functional mGluR5, 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 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 high levels of GSH (as indicated by MCB staining, green) than neurons (white arrows). Merge (yellow). Scale bar, 20 μm.

Figure 4. Astrocyte [Ca\(^{2+}\)] transient-evoked vasodilations are GSH dependent in vitro. A. Mean IP\(_3\) evoked increases in astrocyte [Ca\(^{2+}\)]. Control, n = 21 from 6 rats; + BSO, n = 11 from 4 rats. B. Mean time course of increase in astrocyte [Ca\(^{2+}\)]. Dotted line indicates time of photolysis of caged IP\(_3\), n as described in A. C, Mean 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.

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 the COX-1 inhibitor SC560 (Smith et al., 1998; Niwa et al., 2001), which reduced the tissue GSH concentration by 64% (p < 0.001; Fig. 2D). 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 either tACPD application or IP\(_3\) uncaging 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\(^{2+}\)] 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\(^{2+}\)] 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\(^{2+}\)]-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\(^{2+}\)] signals were unaltered (Fig. 2A–C). However, the vasodilations triggered by these [Ca\(^{2+}\)] signals were abolished (Fig. 2A,F,G; p < 0.01). Vasoconstrictions evoked 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 vasodilations 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\(^{2+}\)] increases can be triggered by two-photon uncaging of IP\(_3\) within the cell body of an astrocyte. Using this
technique, we directly examined the effect of decreasing GSH levels on astrocyte \([\text{Ca}^{2+}]_{\text{i}}\)-evoked arteriole dilations. Astrocytes in hippocampal slices from juvenile rats were bulk-loaded with the caged IP3 compound, NV-IP3/AM. Two-photon photolysis was used to uncage IP3 within an astrocyte soma specifically, generating a \([\text{Ca}^{2+}]_{\text{i}}\), increase within the soma, processes, and endfeet. This local increase in \([\text{Ca}^{2+}]_{\text{i}}\), could evoke an increase in \([\text{Ca}^{2+}]_{\text{i}}\), in nearby astrocytes (Fig. 4A, B represents local and propagated responses) and elicited vasodilation of the neighboring arteriole (Fig. 4C). Although astrocyte \([\text{Ca}^{2+}]_{\text{i}}\), signals were unaltered following BSO treatment to reduce GSH levels \((p = 0.1; \text{Fig. 4A,B}), \) dilations were not observed and vasocostrictions were now evoked \((p = 0.008; \text{Fig. 4C})\). Thus, when GSH levels are reduced, astrocyte \([\text{Ca}^{2+}]_{\text{i}}\), signals can no longer evoke vasodilations normally triggered by the release of \(\text{PgE}_2\).

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

Having determined in acute brain slices the vasodilatory molecules underlying astrocyte \([\text{Ca}^{2+}]_{\text{i}}\)-evoked vasodilations, we examined whether these same enzymes and molecules were involved in the CBF response, which occurs downstream of \(\text{CO}_2\)-evoked astrocyte \([\text{Ca}^{2+}]_{\text{i}}\), responses *in vivo*. Hypercapnia *in vivo* evoked a CBF increase in the barrel cortex of adult rat (Fig. 5A, B, D, E), whereas neural activity was unchanged (Fig. 5F). The calculated area under the curve (AUC) of the CBF response was significantly attenuated by SC560 \((p = 0.032; \text{Fig. 5A,B}), \) confirming that COX-1 plays a critical role in hypercapnia-evoked CBF increases *in vivo* (Niwa et al., 2001).

We examined the impact of decreased tissue GSH levels on \(\text{CO}_2\)-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. 5D, E; 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 \([\text{Ca}^{2+}]_{\text{i}}\)-related, CBF increases require \(\text{PgE}_2\) 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. 6A, B; \(p = 0.10\)) 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.

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**Figure 5.** \(\text{CO}_2\)-evoked CBF responses *in vivo* are GSH dependent. **A,** Mean traces of local CBF response to hypercapnia, measured by laser speckle contrast imaging, in vehicle (DMSO)- (blue) and SC560- (red) injected animals \(n = 7\) rats for each group. Colored box represents time of \(\text{CO}_2\) application. Data shown as fractional change with baseline of 0 (baseline taken during 60 s prechallenge) and a pretreatment peak of 1 (black dotted line on graph). **B,** Mean AUC of CBF response to hypercapnia in the presence of vehicle (DMSO) or SC560 (normalized to pretreatment maxima for each animal). \(n = 7\) rats for each group. **C,** Tissue GSH levels 24 h after injection of BSO or saline into the barrel cortex \((n = 7\) rats). **D,** Mean trace of local CBF response to hypercapnia, measured by laser Doppler flowmetry, in saline- (blue) and BSO- (red) injected rats. \(n = 6\) rats in each group. **E,** Mean values of AUC of CBF response to hypercapnia. \(n = 6\) rats in each group. **F, G,** Neural activity. Power in frequency bands. **F,** During baseline (Base) and in response to hypercapnia (HCN) for saline- (blue) and BSO- (red) treated animals. \(n = 3\) rats. **G,** Hypercapnia (HCN)/baseline (Base). Treatment with BSO does not change the effect of hypercapnia on neural activity. \(n = 3\) rats. Data are mean ± SEM. *\(p < 0.05\).
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 [Ca^{2+}]_i 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 [Ca^{2+}]_i 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 (Jacobsson et al., 1999; Murakami et al., 2000). In vitro, we demonstrate that astrocyte [Ca^{2+}]_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 [Ca^{2+}]_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 [Ca^{2+}]_i signals, under certain conditions and not under others.

Previous studies have provided evidence for several mechanisms linking astrocyte [Ca^{2+}]_i increases and changes in CO₂ concentration. For example, within the respiratory center, increased astrocyte [Ca^{2+}]_i, and astrocytic release of ATP can be triggered by CO₂-evoked decreases in pH (Gourine et al., 2010). This [Ca^{2+}]_i increase may be the result of increased Na⁺/HCO₃⁻ cotransport and reversal of Na⁺/[Ca^{2+}]_i 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 $[Ca^{2+}]_i$, (Pelligrino et al., 2011). Depending on the mechanism linking increases in CO$_2$ to astrocyte $[Ca^{2+}]_i$ responses, therefore, astrocytes could act as either a pH or CO$_2$ sensor. Although it is beyond the scope of this paper to determine the link between an increase in CO$_2$ and the increase in astrocyte $[Ca^{2+}]_i$, we have demonstrated that the depletion of GSH levels leads to a reduction in the ability of astrocytes to release PgE$_2$, following such a rise in $[Ca^{2+}]_i$, 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$_2$ are GSH-sensitive suggests that global CBF regulation, which is sensitive to the partial pressure of arterial CO$_2$ (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., 1995, 1999; Ansari and Scheff, 2010; Zhang et al., 2012; Kulak et al., 2013). Therefore, the impact of changes in GSH levels on the sensitivity of astrocyte regulation of vasodilation could contribute to several CNS pathologies. Thus, it is critical to understand the signaling pathways underly- ing changes in CBF, both in health and disease.

It has previously been shown that, in addition to astrocytic production of PgE$_2$ via COX-1/mPgES activity, neurons (which express COX-2 but not COX-1) (Nogawa et al., 1997; Lecrux et al., 2011), are capable of producing COX-2-derived PgE$_2$ (which contributes to neurovascular coupling) (Lecrux et al., 2011; Lacroix et al., 2015). In this study, we used a pharmacological approach to increase astrocyte $[Ca^{2+}]_i$, and to inhibit either the $de$ $novo$ synthesis of glutathione or the activity of COX-1, specifically, to demonstrate that, down-stream of an increase in astrocyte $[Ca^{2+}]_i$, COX-1 activity and glutathione are required for vasodilation to occur. However, as this pharmacological approach lacks cellular specificity, a contribution of neurona$lly$ produced PgE$_2$ to the hypercapnia-evoked CBF response cannot be completely excluded. Nevertheless, our conclusion that astrocyte COX-1-derived PgE$_2$, rather than neuronal COX-2-derived PgE$_2$, is involved in the CBF response to hypercapnia is in agreement with previous findings (Niwa et al., 2001). Future studies could use an astrocyte-specific genetic strategy (such as cell-specific knock-out) (Casper et al., 2007) to confirm that hypercapnia-evoked vasodilations, occurring downstream of astrocyte $[Ca^{2+}]_i$, responses, are dependent on astrocyte glutathione levels and COX-1 activity.

In conclusion, we demonstrate a novel mechanism by which astrocytes detect hypercapnia and, via $[Ca^{2+}]_i$ signals, increase CBF in response to CO$_2$. Astrocytes are therefore poised to detect the metabolic activity of neurons and to mod- ify vascular tone appropriately to deliver glucose and O$_2$. This important pathway may be impaired in conditions in which oxidative stress reduces GSH levels in astrocytes, leading to impaired CBF responses and altered vascular readouts of neu- ral activity.

References


