Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway

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Enhancement of cerebral blood flow by hypoxia is critical for brain function, but signaling systems underlying its regulation have been unclear. We report a pathway mediating hypoxia-induced cerebral vasodilation in studies monitoring vascular disposition in cerebellar slices and in intact mouse brains using two-photon intravital laser scanning microscopy. In this cascade, hypoxia elicits cerebral vasodilation via the coordinate actions of H_2S formed by cystathionine β-synthase (CBS) and CO generated by heme oxygenase (HO)-2. Hypoxia diminishes CO generation by HO-2, an oxygen sensor. The constitutive CO physiologically inhibits CBS, and hypoxia leads to increased levels of H_2S that mediate the vasodilation of precapillary arterioles. Mice with targeted deletion of HO-2 or CBS display impaired vascular responses to hypoxia. Thus, in intact adult brain cerebral cortex of HO-2–null mice, imaging mass spectrometry reveals an impaired ability to maintain ATP levels on hypoxia.

gas biology | neurovascular unit | energy metabolism | gasotransmitter

The cerebral circulation is maintained by autoregulation, which prevents marked alterations in response to changes in blood pressure, whereas functional hyperemia links blood flow to neural activity (1). Blood flow regulation in the brain is modulated by O_2 (2), with increased cerebral blood flow in response to hypoxia critical for protecting the brain against diverse insults. Such regulation also participates in functional hyperemia, as demonstrated by functional MRI investigations indicating a transient decrease in O_2 levels preceding activation of blood flow in response to neuronal firing (3).

Alterations in cerebral blood flow in response to hypoxia and neural activity are mediated via several neurotransmitter systems, with prominent involvement of the gaseous mediator nitric oxide (NO) (1, 2). In response to glutamate acting on NMDA receptors, neuronal NO synthase (nNOS) is activated by increases in intracellular calcium, with the generated NO stimulating soluble guanylyl cyclase, thereby increasing cGMP levels to dilate blood vessels (4). Functional hyperemia is decreased by ∼50% in rats in response to inhibition of nNOS (5). Another gaseous mediator, CO (6–8), is also vasoactive. In some blood vessel systems (e.g., liver sinusoids), CO causes vasodilation, and inhibition of its biosynthetic enzyme HO-2 leads to vasoconstriction (9–13). However, in the cerebral circulation, CO elicits vasoconstriction. Thus, HO inhibitors cause cerebral vasodilation, an effect reversed by CO (14). This action of CO cannot be readily explained by previously identified CO receptors, such as soluble guanylyl cyclase (6–12, 15) or potassium channels (13, 16), both of which mediate vasodilation. The CO and NO systems interface; thus, the vasodilatory actions of HO inhibitors are partially reversed by inhibitors of NOS (14).

A third gaseous mediator, H_2S, is also vasoactive, eliciting vasodilation in both the peripheral and cerebral circulation (17–21). H_2S can be physiologically generated by two enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). In peripheral blood vessels, CSE is localized to the endothelium, and endothelial-derived relaxing factor activity in peripheral vessels is reduced by ∼75% in CSE-null mice (18). H_2S exerts its physiological actions by covalently modifying the sulfhydryl group of cysteines in target proteins in a process termed *S*-sulfhydration, which is analogous to *S*-nitrosylation by NO (22–25). The vasodilating actions of H_2S are associated with sulfhydration of a specific cysteine in ATP-sensitive potassium (K_{ATP}) channels of blood vessels, activating the channels and hyperpolarizing the vascular endothelial and smooth muscle cells (25).

The brain generates micromolar amounts of CO via HO-catalyzed reactions using O_2 as a substrate, with HO-2 accounting for ∼80% of the total rodent brain HO activity (14, 26). A recent metabolomic study in murine liver using capillary electrophoresis-mass spectrometry (CE-MS) identified CBS as a CO sensor regulating bicarbonate-dependent biliary choleresis (27). CBS is a hemethiolate enzyme (28) that catalyzes multiple H_2S-generating reactions (29, 30). The prosthetic heme of this enzyme (31–33) allows the reversible coordinate bonding of gaseous ligands, such as CO and NO (34). Interestingly, CO, but not NO, inhibits the activity of CBS in vitro (31, 32) and in vivo (27), making CBS a CO-specific sensor.

In the present study, we show that hypoxia-induced arteriolar vasodilation in brain slices and intact mice is mediated via a signaling system in which HO-2 is the O_2 sensor. HO-2 uses O_2 to generate CO, which tonically inhibits the ability of astrocytic CBS, a CO-specific sensor (27, 31, 32), to generate vasodilatory H_2S (17–21). During hypoxia, inhibition of HO-2–mediated CO production occurs, with a corresponding release of the tonic inhibition of CBS, allowing it to generate H_2S, which in turn elicits arteriolar vasodilation.

Results

Neuronal and Endothelial Localization of HO-2 and Glial Localization of CBS. Because CBS accounts for the great bulk of H_2S generation in the brain (35), and because its heme group provides a CO-

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sensing mechanism (27, 31, 32), we compared the localizations of CBS in the brain with those of HO-2, the main CO-producing isozyme in the brain (14). Immunohistochemical analysis in the neonatal mouse cerebellum revealed expression of HO-2 in neurons and endothelial cells (Fig. 1 *A–D*). In contrast, CBS was expressed in Bergmann glia and astrocytes (Fig. 1 *E–H*). Consistent with these observed CBS localizations, generation of H_2S was abolished in primary astrocytic cultures from CBS-null mice (Fig. S1). Moreover, metabolomic analysis using CE-MS revealed that levels of cystathionine, another CBS product, were below the limit of detection in primary astrocytic cultures from CBS-null mice (Fig. S1*C*). In the cortex, CBS appears to be the main H_2S-producing enzyme, given that expression of CSE, another H_2S-producing enzyme, is limited to vascular smooth muscle cells surrounding large vessels in the subarachnoid space and in specific neurons in striatum and hippocampus (Fig. S2). Pericytes, key effector contractile cells (36) surrounded by HO-2–containing endothelia and CBS-containing astrocytic endfeet that physically link neurons to the vasculature, compose a neurovascular unit with optimal spatial localizations for multiple gaseous molecular signals to control microvascular resistance (Fig. 1*I*).

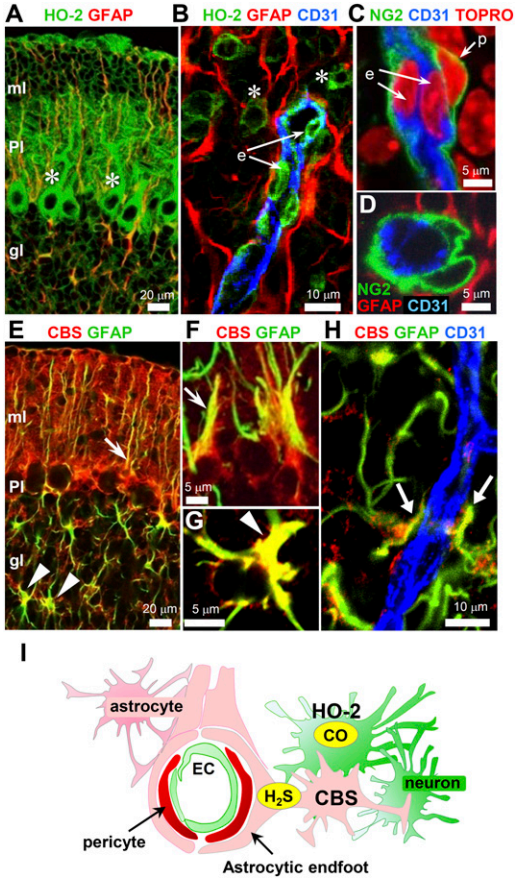

Fig. 1. Immunohistochemical localization of HO-2 and CBS in the neurovascular unit of neonatal mouse cerebellar cortex. (*A*) HO-2, a CO-producing enzyme, is abundantly expressed in neurons. Note the strong labeling of Purkinje cells (asterisks). (*B–D*) HO-2 surrounds microvessels. HO-2$^+$ cells along the vessel wall in *B* are endothelial, not pericytic, because nuclei of cells positive for NG2 (a pericytic marker) stained with TO-PRO-3 (TOPRO, a nucleic acid stain), are completely devoid of CD31 (endothelial marker) labeling in *C*. In *D*, the arteriolar wall is surrounded by NG2$^+$ pericytes, important contractile cells within the neurovascular unit. (*E–H*) CBS, an H_2S-producing enzyme, is concentrated at the ascending processes of Bergmann glia (arrow in *E* and *F*) and radial processes of astrocytes (arrowheads in *E* and *G*), as evidenced by the colocalization with GFAP, an established marker of glial cells. The astrocytic endfeet in contact with the vessel wall are CBS-positive in *H*. ml, molecular layer; Pl, Purkinje cell layer; gl, granular layer; e, endothelium; p, pericyte. (*I*) Schematic depiction of localization of HO-2 and CBS in the neurovascular unit.

HO-2/CO Cerebral Vasoconstrictor Tone Is CBS-Dependent. It was previously shown that HO inhibitors cause vasodilation in the pial microcirculation, suggesting that CO is responsible for a cerebral vasoconstrictor tone (14). We confirm these findings, showing that the HO inhibitors zinc protoporphyrin-IX (ZnPP; 1 μM) and chromium (III) mesoporphyrin-IX (CrMP; 1 μM) dilated arterioles in cerebellar slices by ~50% at 60 min (Fig. 2). In contrast, copper protoporphyrin-IX, an analog that does not inhibit HO, had no effect. Tricarbonyldichlororuthenium(II) dimer, $[Ru(CO)_3Cl_2]_2$, a CO-releasing molecule (CORM-2; 100 μM) (37), reversed the effect of ZnPP (Fig. 2*C*), confirming that CO is a tonic vasoconstrictor under basal conditions.

This action of CO cannot be readily explained by previously identified CO receptors, such as soluble guanylyl cyclase (6–12, 15) or potassium channels (13, 16), both of which mediate vasodilation. Based on previous studies indicating that physiological concentrations of CO can inhibit the ability of CBS to generate vasodilatory H_2S (17–21, 26, 27, 38), we hypothesize that endogenous CO serves as a tonic vasoconstrictor by inhibiting CBS, thus preventing the H_2S-mediated vasodilatory response. The H_2S donor sodium hydrosulfide (NaHS; 30 μM) elicits comparable vasodilation to that provided by ZnPP. In CBS-null mice, whose astrocytes lose the ability to produce H_2S (Fig. S1*B*), inhibition of endogenous CO production by ZnPP did not cause vasodilation (Fig. 2*C*). On the other hand, NaHS induced substantially greater vasodilation in CBS-null mice than in WT mice (Fig. 2*C*), indicating that the vasodilatory machinery in CBS-null mice is intact

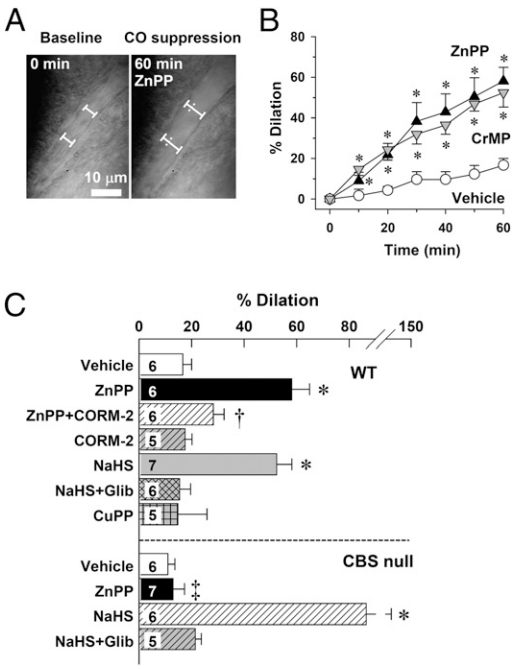

Fig. 2. Pharmacologic inhibition of endogenous CO production evokes a CBS-dependent arteriolar vasodilation in neonatal mouse cerebellar slices. (*A*) A typical change in arteriolar diameter of cerebellar slices in response to ZnPP (1 μM), an HO inhibitor. (*B*) Time course of arteriolar dilation during superfusion of the potent HO inhibitors ZnPP (1 μM) and CrMP (1 μM). (*C*) Summary of changes in arteriolar diameter at 60 min after superfusion of various reagents. Vasodilation induced by HO inhibition is reversed by tricarbonyldichlororuthenium(II) dimer, $[Ru(CO)_3Cl_2]_2$, a CO-releasing molecule (CORM-2; 100 μM), indicating that CO acts as a tonic vasoconstrictor. The vasodilatory response of ZnPP does not occur in CBS-null slices. Glib, glibenclimide, an inhibitor of K_{ATP} channels. *$P < 0.05$ compared with the vehicle-treated control; †$P < 0.05$ compared with the ZnPP-treated WT mice; ‡$P < 0.05$ compared with the ZnPP-treated WT mice. Values are mean ± SEM.

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and appears to display supersensitivity of H_2S-responsive elements after depletion of endogenous H_2S. Physiological vasodilation elicited by H_2S is mediated by K_{ATP} channels (21), the blockade of which by glibenclamide (100 μM) abolishes the effects of NaHS. Glibenclamide was seen to abolish CrMP-induced arteriolar vasodilation (Fig. S3). These findings suggest that cerebral vessels are regulated by a signaling cascade in which CO formed by HO-2 residing in the endothelia and/or neurons basally inhibits H_2S production from glial CBS.

HO-2/CO and CBS/H_2S Pathways Mediate Hypoxia-Induced Arteriolar Vasodilation. The coordinate actions of HO-2 and CBS might form a signaling system that mediates hypoxia-induced arteriolar vasodilation. Hypoxia inhibits oxidative phosphorylation, as well as the activities of various enzymes that use molecular O_2 as a substrate. HO-2 is such an enzyme, which functions as an O_2 sensor in the carotid body (39, 40). We wondered whether the vasodilation observed on hypoxia reflects a decrease in HO-2–derived CO, diminishing the inhibition of CBS and leading to an increase in CBS-derived H_2S with resulting vasodilation. In this model, HO-2 serves as the O_2 sensor, modulating its generation of CO in proportion to O_2 levels. To test this hypothesis, we examined how the deletion of HO-2 or CBS influences the vasodilatory responses to hypoxia in neonatal cerebellar slices from HO-2–null (7) and CBS-null (41) mice (Fig. 3*A* and *B*). Decreasing the O_2 concentration of the superfusate elicited a time-dependent arteriolar dilation, with a maximal dilation of 64% ± 8% within 10 min in WT mice (Fig. 3*B*). The extent of the vasodilation was reduced by >50% in slices prepared from both HO-2–null mice (20% ± 3%) and CBS-null mice (31% ± 8%) (Fig. 3*B*). The decreased vasodilation seen in CBS-null mice supports the notion that H_2S is a physiological vasodilator that mediates responses to hypoxia. It might seem paradoxical that the loss of the vasoconstrictor HO-2/CO leads to decreased arteriolar vasodilation; however, note that in our model, hypoxia acts by inhibiting HO-2–derived CO generation, that is, by relieving the HO-2/CO vasoconstrictor tone. Thus, in HO-2–null mice, there is no longer any HO-2, and hence no HO-2/CO vasoconstrictor tone to be affected by hypoxia.

We substantiated the regulation of HO-2 by O_2 by measuring the endogenous CO concentration in cerebellar slices exposed to various O_2 concentrations (Fig. 3*C*). In slices from WT mice, lowering O_2 from 10% to 1% decreased the endogenous CO concentration by 60% (Fig. 3*D*). HO-2 deletion abolished the O_2-dependent reduction in CO generation (Fig. 3*D*). Moreover, quantitative measurements of H_2S by bimane-assisted MS analyses (Fig. S4) revealed that hypoxia caused a significant elevation of endogenous H_2S in WT mice, but not in HO-2–null mice (Fig. 3*E*). Under normoxia, H_2S levels were similar in WT and HO-2–null mice, presumably due to compensation from other sources of H_2S in the transsulfuration pathway (27). In addition, CORM-2 inhibited H_2S production in WT whole-brain lysates in a concentration-dependent fashion (Fig. S5*A*), and CO gas similarly inhibited H_2S production in WT whole-brain homogenates (Fig. S5*B*). These data indicate a role for the HO-2/CO system in the hypoxia-induced elevation of H_2S in the brain.

Further evidence that HO-2 functions as an O_2 sensor comes from our finding of a K_m value of ~15 μM (~11 mm Hg) of recombinant mouse HO-2 for O_2 in vitro (Fig. S5*C*)—a suitable K_m value for an O_2 sensor to detect and respond to changes in the brain tissue O_2 concentration (42). Although isoenzyme HO-1 is expressed in nucleolar-like structures in nuclei, no immunoreactivity for bilirubin-IXα (43), a byproduct of HO-catalyzed reactions, was detected, suggesting that CO is not produced in the nucleus by HO-1 (Fig. S6 *A* and *B*). Thus, it is unlikely that HO-1 participates in this system. These data indicate that HO-2 is the O_2 sensor in the brain that mediates the vascular responses to hypoxia.

Hypoxia-Induced Vasodilation of Precapillary Arterioles in Vivo Is Attenuated in HO-2–Null Mice and Abolished in CBS-Null Mice. Although the cerebellar slice preparation provides refined experimental conditions ex vivo, it cannot adequately mimic the complex O_2 gradient geometry in a living organ, that is, the O_2 gradient

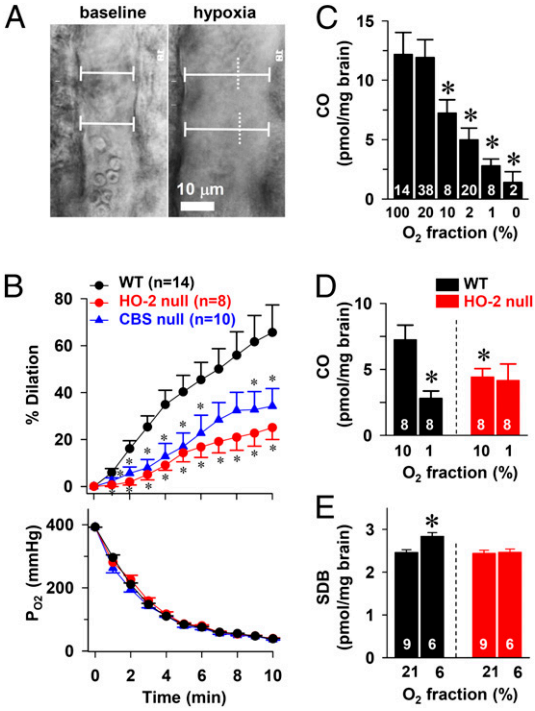

Fig. 3. The HO-2/CO and CBS/H_2S pathways mediate hypoxia-induced arteriolar vasodilation. (*A*) Hypoxia-induced arteriolar vasodilation of an arteriole in a cerebellar slice. Dashed lines indicate the previous position of the vessel wall. (*B*) Lowering the partial pressure of O_2 (P_{O_2}) in the superfusates is followed by robust arteriolar dilation in WT mice. Deletion of either HO-2 or CBS causes a significant reduction in the extent of hypoxia-induced arteriolar vasodilation. *$P < 0.05$ compared with WT. (*C*) Lowering the O_2 concentration gradually decreases CO production from cerebellar slices. *$P < 0.05$ compared with 100% O_2. (*D*) The O_2-dependent reduction in CO production is abolished in HO-2–null mice. The CO concentration in HO-2–null mice at 10% O_2 is approximately half that in WT mice. *$P < 0.05$ compared with WT at 10% O_2. (*E*) Endogenous H_2S concentration in neonatal brain (P12) measured by the reaction of monobromobimane, an electrophilic reagent, with HS^- to form sulfide dibimane (SDB). Lowering the O_2 concentration elevates the endogenous H_2S concentration. Hypoxia-induced elevation of H_2S concentrations is abolished in HO-2–null mice. *$P < 0.05$ compared with WT at 21% O_2.

profile along various segments of the microvascular tree and in the radial direction in tissues. To circumvent such ambiguity inherent in ex vivo systems, we used an intact live-mouse model in which cerebral blood vessels are visualized by two-photon imaging of the cerebral cortex in adult mice through a closed thinned-skull window (44, 45). This approach allowed us to maintain the physiological local concentrations of various gases. The neuro-glia-vascular unit in the adult cerebral cortex, similar to the neonatal cerebellum (Fig. 1), displays HO-2 in neurons and endothelium and CBS in glia (Fig. S6 *C* and *D*). To ascertain whether HO-2 and CBS contribute to the hypoxia-induced microvascular responses in vivo, we ventilated anesthetized mice first with normal air (21% O_2) and then with 10% O_2 (Fig. 4). We examined hypoxic responses of small arterioles at an ~100-μm depth in the cortex under controlled conditions (Fig. S7). Cerebral arteries penetrate from the surface into deeper layers, becoming diving (penetrating) arterioles, which lead to the smallest precapillary arterioles. In WT mice, reduction in inhaled O_2 caused an immediate and robust dilation of both diving and precapillary arterioles (Fig. 4*C*). The magnitude of the hypoxia-induced precapillary arteriolar vasodilation was severely attenuated in HO-2–null mice (Fig. 4*C*). These results suggest that different mechanisms mediate the hypoxic vasodilatory responses at different points in the hierarchy of the microvasculature, even within consecutive arteriolar segments. In proximal arterioles, close to arteries, tissue O_2 concentrations are higher and decrease more slowly during hypoxia compared with

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peripheral arterioles (46). Tissue O_2 concentrations in the vicinity of diving arterioles during hypoxia could be well above the K_m for O_2 of HO-2, whereas the tissue O_2 concentration outside precapillary arterioles would fall to the required threshold for initiating the gaseous signaling to bring about vasodilation. Although CBS-null mice on a C57BL/6J background rarely survive past the weaning age, CBS-null mice on a C3H/HeJ background survive (47), allowing us to examine the microvascular responses to hypoxia in adult CBS-null mice in vivo. For experiments using neonatal cerebellar slices (Figs. 2 and 3 *A* and *B*), we used CBS-null mice on a C57BL/6J background, given that survival past weaning was not critical. We presumed that the difference in background of the two mouse strains does not influence the CO/H_2S dynamics. We found that the hypoxia-induced precapillary arteriolar vasodilatory response was abolished in CBS-null mice, but not in CSE-null mice (48) (Fig. 4*F*), indicating that CBS-derived H_2S, but not CSE-derived H_2S, mediates this vasodilatory response. This finding is in agreement with the lack of CSE's endothelial localization in the brain (Fig. S2), in contrast to its localization to endothelium in peripheral blood vessels (18).

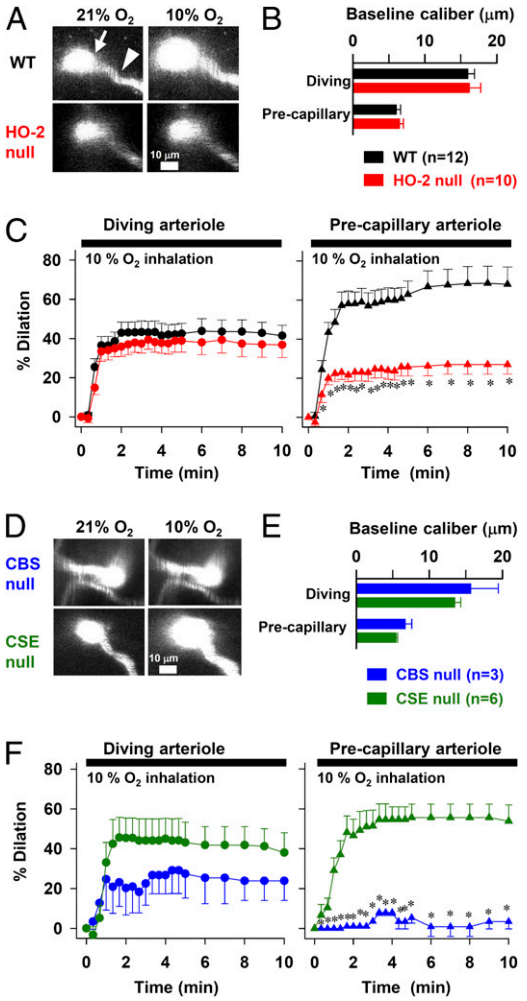

Fig. 4. Hypoxia-induced vasodilation of precapillary arterioles in vivo is attenuated in HO-2-null mice and abolished in CBS-null mice. (*A*) Vasodilatory responses of diving (*arrow*) and precapillary arterioles (*arrowhead*) imaged in vivo in live mouse cerebral cortex through the thinned skull at a depth of 50–90 μm using two-photon laser scanning microscopy. Qdot655 was injected i.v. to outline the vasculature. (*B*) Baseline diameter of arterioles. (*C*) Inhalation of 10% O_2 evokes rapid vasodilation of both diving and precapillary arterioles. HO-2 deletion causes a marked attenuation in the vasodilatory response of precapillary arterioles, but not of diving arterioles. *$P < 0.05$ compared with WT mice. (*D–F*) Hypoxia-induced vasodilation of precapillary arterioles is abolished in CBS-null mice, but not in CSE-null mice.

Impaired Ability of HO-2–Null Mice to Maintain ATP Levels on Hypoxia. We found that hypoxia-induced precapillary arteriolar dilation was impaired by 50% in HO-2–null mice (Fig. 4*C*). To examine whether such a compromised microvascular response to hypoxia might lead to impaired energy metabolism, we monitored ATP and its degradation metabolites, ADP and AMP, using CE-MS in the whole brain of WT and HO-2–null mice after 1 min of hypoxia (10% O_2) (Fig. 5*A*). Hypoxia resulted in elevated concentrations of AMP, and to a lesser extent of ADP, in both WT and HO-2–null mice. However, with hypoxia, ATP levels declined by 50% in the HO-2–null mice, but remained unchanged in the WT mice. As a result, energy charge values on hypoxia were unchanged in the WT mice, but dropped to <0.5 in the HO-2–null mice. These data indicate that impaired energy metabolism ensues from impairment of the microvascular responses to hypoxia in HO-2–null mice.

Glucose consumption varies regionally in the brain, with higher levels in the cerebral cortex compared with the hippocampus (49). Because these two regions comparably express HO-2 (6, 14), we examined regional variation in hypoxic responses using semiquantitative imaging mass spectrometry (IMS) (Fig. 5 *B* and *C*). Under normoxia, cortical ATP levels were higher in HO-2–null mice than in WT mice. After exposure to hypoxia (10% O_2) for 1 min, cortical and hippocampal ATP levels remained unchanged in the WT mice but decreased by 50% in the HO-2–null mice. These findings indicate that the HO-2/CO system is a determinant of basal energy metabolism and its tolerance to hypoxia in these regions.

Discussion

In the present study, we have demonstrated major roles for HO-2 and CBS, forming CO and H_2S, respectively, in regulating the cerebral microvascular response to hypoxia. We have substantiated these findings both in cerebellar slices and in the cerebral cortex of live mice. We have shown that H_2S, formed by CBS, is critical to the hypoxic responses of the cerebral microcirculation, which are abolished in intact CBS-null mice.

The existence of a CO-dependent vasoconstrictor tone is established by the substantial arteriolar vasodilation elicited by HO inhibitors, as well as by the elevated basal ATP levels in the brains of HO-2–null mice. Despite the loss of vasoconstrictor tone with HO-2 deletion, the absence of the enzyme eliminates the proposed mechanism for hypoxia-induced vasodilation, namely inhibition of HO-2 with consequent activation of CBS to generate vasodilatory H_2S (17–21). Accordingly, in HO-2–null mice, hypoxia fails to induce arteriolar dilation. Moreover, our sensitive analytic methodology establishes that endogenous CO levels (several of which are micromolar) are sufficient to inhibit CBS by binding its ferrous heme in vivo (27). The direct inhibition of CBS activity by CO donor and CO gas (Fig. S5 *A* and *B*) supports the physiological interplay of CO and H_2S. However, caution must be used in extrapolating these in vitro data to in vivo physiology. In vivo relevance for the postulated CO–H_2S link is afforded by our experiments showing that the hypoxia-induced increase in brain H_2S is abolished in HO-2–null mice (Fig. 3*E*).

The localization of HO-2 and CBS supports these physiological interactions. CBS is localized exclusively to astrocytes, including the Bergmann glia of the cerebellum. Astrocytes are well disposed to influence blood vessels, given their prominent endfeet that contact the vessels directly. Astrocytes also can transduce information from neurons to blood vessels, because they typically ensheath synapses. HO-2 is localized to both neurons and blood vessel endothelium, confirming previously reported findings in the brain (6, 14) and peripheral tissues (7, 9). Because hypoxia affects all cells in the brain, ascertaining whether the physiological influence of CO on CBS involves neuronal or endothelial HO-2 is difficult. In either instance, we presume that CO diffuses into the astrocytes containing CBS.

Our measurements of vasodilation have focused on arterioles, which are classically considered the primary regulators of the cerebral circulation, given that capillaries do not contain smooth

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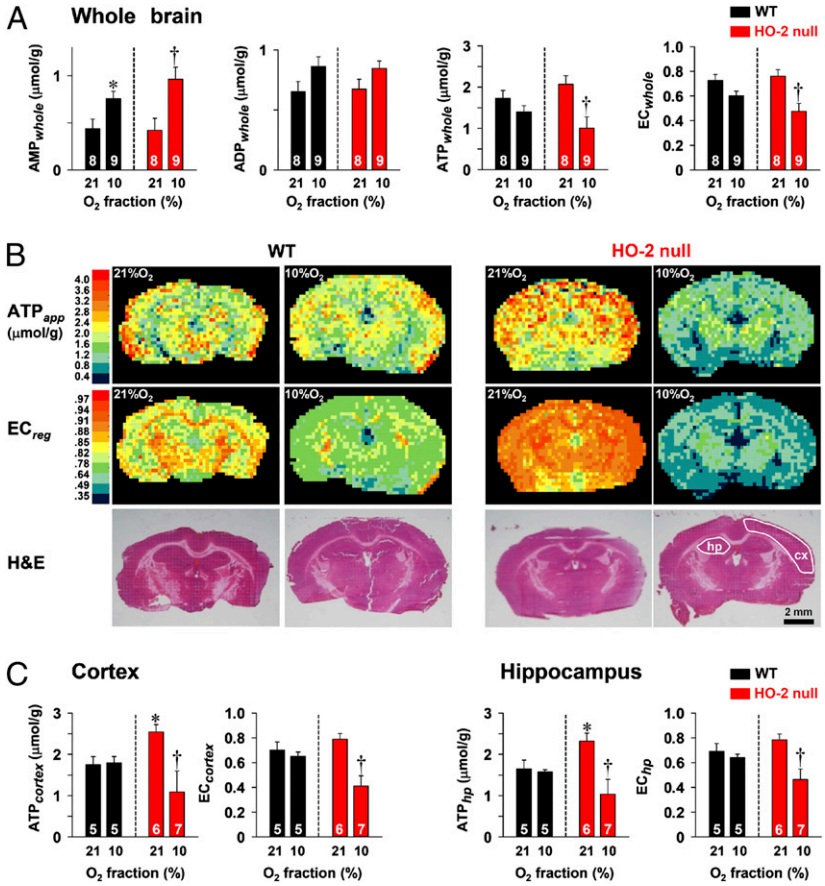

Fig. 5. Impaired ability of HO-2-null mice to maintain ATP levels on exposure to 10% O_2 for 1 min. (*A*) Alterations in AMP (AMP_{whole}), ADP (ADP_{whole}), ATP (ATP_{whole}), and energy charge (EC_{whole}) in the whole brain. The concentrations of adenylates were determined by CE-MS. $^{*}P < 0.05$ compared with WT normoxia; $^{\dagger}P < 0.05$ compared with HO-2-null normoxia. (*B*) Representative IMS showing spatial distribution of apparent ATP concentration (ATP_{app}) and energy charge (EC_{reg}). Note the basal increase in ATP in HO-2-null mice. (*Bottom*) H&E staining after IMS. cx, cortex; hp, hippocampus. (*C*) Quantitative analysis of regional ATP concentration and energy charge in WT and HO-2-null mice. $^{*}P < 0.05$ compared with WT normoxia; $^{\dagger}P < 0.05$ compared with HO-2-null normoxia.

muscle. However, capillaries are surrounded by pericytes, cells with contractile properties that can regulate capillary blood flow (36). Astrocytes form intimate connections with pericytes (1, 2). Thus, it is possible that astrocytic H_2S diffuses to pericytes to alter their contractile activity, thereby influencing capillary blood flow.

How does regulation of the cerebral circulation by H_2S and CO interact with the effects of other neurotransmitters and neuromodulators? H_2S appears to be a physiological mediator of hypoxia-induced precapillary arteriolar vasodilation, which is virtually abolished in CBS-null mice. Functional hyperemia is largely determined by nNOS, with a 50% reduction seen in rats treated with nNOS inhibitors (5). However, NO does not appear to play a significant role in the cerebral vasodilation elicited by hypoxia; in most studies, NOS inhibitors were found to not attenuate this vasodilation, although reductions were seen in some studies (50). Functional hyperemia also involves the arachidonic acid pathway (5), which might be modulated by endogenous CO (34, 51). Thus, glutamate acting on metabotropic receptors on astrocytes stimulates the formation of arachidonic acid, which gives rise to a variety of eicosanoids, some vasoconstricting and some vasodilating (2). Accordingly, agents that affect the eicosanoids elicit variable effects on the cerebral circulation.

Although numerous mediators appear to be involved in the regulation of vascular tone at different points in the hierarchy of the cerebral microvasculature, our findings in the present study provide evidence suggesting that HO-2 generates CO in an O_2-dependent manner and reserves the capacity to dilate precapillary arterioles during hypoxia through a mechanism involving the ability of the gas to inhibit the CBS/H_2S system. On hypoxia, the mechanism is unlocked through a fall in CO that triggers the microvascular dilation. Lack of such an adaptive vascular response in HO-2–null mice compromises the brain's ability to maintain ATP levels and the energy charge. In these mice, the failure to maintain the local ATP concentration on hypoxia appears to result from impaired hypoxia-induced arteriolar vasodilation; however, our investigations using semiquantitative IMS combined with CE-MS suggest that targeted deletion of HO-2 in the brain leads to the lack of a "respiratory lock" by the constitutive CO, resulting in the surprising elevation of the basal ATP concentration observed in the cerebral cortex and hippocampus. That is, CO modestly suppresses ATP production under normoxia, but once this tonic inhibition is eliminated on hypoxia, it gives way to the rise in dynamic strength of compensatory ATP maintenance.

In HO-2–null mice, neurovascular units lacking the lock are unable to immediately enhance further respiration to salvage ATP. This notion is consistent with previous studies indicating that pharmacologic inhibition of HO increases basal O_2 consumption in the liver (52) and that an increase in endogenous CO, by enzyme induction inhibits cellular respiration through its inhibitory effect on cytochrome *c* oxidase (53). H_2S also might modulate mitochondrial respiration through reversible inhibition of cytochrome *c* oxidase (54); however, at least in the present study, basal H_2S levels in the brain are unchanged in HO-2–null mice compared with WT mice (Fig. 3*E*), possibly due to compensatory changes in endogenous H_2S production from other enzymes in the transsulfuration pathway (27). Although our findings provide evidence for a unique protective mechanism in the neurovascular units against hypoxia through the coordinate actions of two gaseous mediators, further investigation is needed to reveal functional links between neuronal and microvascular coupling through gas-responsive metabolic systems.

Materials and Methods

Animals. See Table S1 for primers used in genotyping.

Immunohistochemistry. Table S2 summarizes the antibodies used.

Cerebellar Slice Preparation and Arteriolar Diameter Measurements. Transverse slices (400 μm thick) of the cerebellar vermis from 9- to 15 d-old male mice were prepared using a vibratome (Leica VT1000S), as described previously (55, 56).

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Blood vessels were visualized with an upright microscope equipped with a differential interference contrast. The vessels were pretreated with thromboxane A_2 receptor agonist 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619; 0.3 nM). See *SI Materials and Methods* for details.

Visualization of the Cerebral Microvasculature of Adult Mice in Vivo Using Two-Photon Intravital Laser Scanning Microscopy. The cortical microvasculature was visualized through a closed thinned-skull window as described previously (44, 45, 57) with a few modifications, as detailed in *SI Materials and Methods*.

Semiquantitative IMS. Semiquantitative IMS was performed as described previously (58) and described in detail in *SI Materials and Methods*.

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