HIGHLIGHTED TOPIC | Regulation of the Cerebral Circulation

Carbon monoxide and hydrogen sulfide: gaseous messengers in cerebrovascular circulation

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Leffler, Charles W., Helena Parfenova, Jonathan H. Jaggar, and Rui Wang. Carbon monoxide and hydrogen sulfide: gaseous messengers in cerebrovascular circulation. J Appl Physiol 100: 1065–1076, 2006; doi:10.1152/japplphysiol.00793.2005.—This review focuses on two gaseous cellular messenger molecules, CO and H2S, that are involved in cerebrovascular flow regulation. CO is a dilatory mediator in active hyperemia, autoregulation, hypoxic dilation, and counteracting vasoconstriction. It is produced from heme by a constitutively expressed enzyme [heme oxygenase (HO)-2] expressed highly in the brain and by an inducible enzyme (HO-1). CO production is regulated by controlling substrate availability, HO-2 catalytic activity, and HO-1 expression. CO dilates arterioles by binding to heme that is bound to large-conductance Ca2+-activated K+ channels. This binding elevates channel Ca2+ sensitivity, that increases coupling of Ca2+ sparks to large-conductance Ca2+-activated K+ channel openings and, thereby, hyperpolarizes the vascular smooth muscle. In addition to dilating blood vessels, CO can either inhibit or accentuate vascular cell proliferation and apoptosis, depending on conditions. H2S may also function as a cerebrovascular dilator. It is produced in vascular smooth muscle cells by hydrolysis of l-cysteine catalyzed by cystathionine γ-lyase (CSE). H2S dilates arterioles at physiologically relevant concentrations via activation of ATP-sensitive K+ channels. In addition to dilating blood vessels, H2S promotes apoptosis of vascular smooth muscle cells and inhibits proliferation-associated vascular remodeling. Thus both CO and H2S modulate the function and associated vascular remodeling. Both the HO-CO and CSE-H2S systems have potential to interact with NO and prostanoids in the cerebral circulation. Much of the physiology and biochemistry of HO-CO and CSE-H2S in the cerebral circulation remains open for exploration.
opposed to the destructive actions, is limited (for reviews, see Refs. 32, 44, 114).

**CARBON MONOXIDE**

Most information on contributions of endogenously produced CO to control of cerebrovascular circulation derives from studies on newborn pigs. Thus the review below is biased toward newborn pig cerebrovascular circulation. Far fewer data are available on CO in the cerebrovascular circulation from newborns of other species or, for the most part, adults of any species.

The following discussion is summarized pictorially in Fig. 1.

**Control of CO production.** The gas CO is produced physiologically by catabolism of heme to CO, iron, and biliverdin (94). This reaction is catalyzed by heme oxygenase (HO) with reduction of NADPH. HO is expressed as three known isoforms: HO-1, HO-2, and a third isoform (HO-3) with much lower heme-degrading activity (101) and low expression in the brain (129). Recent reports indicate that HO-3 genes are processed pseudogenes derived from HO-2 transcripts that have no known functional significance (47). In freshly isolated cerebral microvessels, as in the intact brain, in vivo, only HO-2 expression was detected on Western blots (117). The constitutive nature of HO-2 in the brain and brain vasculature requires mechanisms for regulation of CO production either by control of enzyme activity or substrate delivery. CO production by HO-2 could be controlled by delivery of electrons from NADPH via cytochrome P-450 reductase, by O2 availability, by delivery of heme to the enzyme, and by the catalytic activity of the enzyme.

Although evidence to indicate NADPH concentration or Po2 regulates CO production in the cerebral circulation has not been produced, data suggesting that O2 availability can affect CO production are available from other tissues. In microsomal fractions of human placenta chorionic villi, CO production is increased by ionotropic, but not metabotropic, glutamate receptor stimulation (116) (see **Fig. 1**). In cerebral microvessels and cortical neurons, calmidizolium increased HO-2 activity and calmidizolium, which inhibits calmodulin (CaM), decreased HO-2 catalytic activity, suggesting elevation of free heme as the rate-limiting step is production in mitochondria of δ-aminolevulinic acid from succinyl CoA and glycine catalyzed by δ-aminolevulinic acid synthase (66, 100). δ-Aminolevulinic acid synthase activity is tightly regulated, being inhibited in a negative feedback manner by heme and hemin (oxidized heme). Control conceivably could also occur by regulation of ferrochelatase or porphobilinogen deaminase, but such control mechanisms have not been demonstrated. In intact cerebral microvessels, increasing cytosolic Ca2+ with ionomycin or activation of protein kinases C (PKC) with phorbol ester increased CO production by increasing heme availability (80). However, in neither case is the cellular mechanisms involved known.

**HO-2 catalytic activity, i.e., CO production per amount of enzyme from exogenous heme, includes HO catalytic efficiency and activation by intracellular relocation. Negative feedback control of HO-2 catalytic activity by bilirubin has been described (93). Other control mechanisms of HO-2 catalytic activity may be cell type and tissue specific. For example, in neurons HO-2 activity can be stimulated by casein kinase II (CK2)-catalyzed phosphorylation of serine 79 (10). Glutamatergic activation of HO-2 results from metabolotropic glutamate receptor-induced Ca2+ release, activation of PKC, and CK2 phosphorylation (12). Conversely, in freshly isolated piglet cerebral microvessels and microvascular endothelial cells in culture, CO production is increased by ionotropic, but not metabotropic, glutamate receptor stimulation (116) (see **Fig. 1**). In addition, protein tyrosine kinase inhibition decreased and tyrosine phosphatase inhibition increased basal and glutamate-stimulated HO-2 catalytic activity and CO production (79). Furthermore, inhibition of neither PKC nor CK2 altered HO-2 catalytic activity (80, 81).

In cerebral microvessels and cortical neurons, calmidizolium, which inhibits calmodulin (CaM), decreased HO-2 catalytic activity and blocked glutatione stimulation of CO production (11, 81). Boehning et al. (11) demonstrated Ca2+-dependent CaM binding to HO-2 expressed in yeast. Ionomycin increased HO-2 activity and calmidizolium blocked the response to ionomycin in HEK-293 cells transfected with rat HO-2 gene. Interestingly, in cerebral microvessels we found that the Ca ionophore, ionomycin, in Ca2+-replete media increased CO production but did not detectably increase HO-2 catalytic activity, suggesting elevation of free heme as the major mechanism behind ionomycin-induced stimulation of CO production (81). Nevertheless, the results in both neurons and microvessels are consistent with HO-2 expressed in yeast showing calcium-calmodulin (Ca2+/CaM) regulation of HO-2.
catalytic activity. The reasons for the apparent discrepancy between effects of cytosolic Ca\textsuperscript{2+} manipulation and those of CaM inhibition are not known.

Another gaseous mediator, NO, can affect HO-2 catalytic activity. HO-2 expressed in *Escherichia coli* is inhibited by NO donors via binding of NO to a heme regulatory motif on HO-2 (27). Also, a direct inhibitory effect of NO has been reported in HO-1-rich aortic endothelial cell microsomes where nitrosylation of heme prevented catabolism by HO (62). Conversely, in cerebral microvessels we found that NO increases HO-2 catalytic activity and thus CO production via a cGMP-dependent mechanism (80). The mechanism by which cGMP increases HO-2 activity is unknown, but it appears reasonable to suspect protein kinase G activation of tyrosine kinases or phosphatase inhibition (79, 81). Also, in isolated heart (99) and porcine aortic endothelial cells (108), NO increased CO production. It is possible that NO can have a direct inhibitory effect on HO-2 that is masked in the intact system by cGMP-induced stimulation.

Extravascular sources of CO in vivo may contribute to pial arteriolar dilation to glutamate. Of particular note, data on isolated vessels and those from intact cerebrovascular circulation in vivo are not entirely consistent. Thus, in isolated microvessels NO synthase (NOS) inhibition totally abolished glutamate-induced CO production (80). However, in vivo, whereas NO inhibition blocked glutamate-induced dilation (71). If glutamate increases NO that increases CO, which is the final mediator of dilation, glutamate should not cause dilation if NO is held constant, but it does. In vivo, cerebral microvessels are accompanied by astrocytes and neurons that also have glutamate receptors (46, 54, 89), HO-2 (129), and neuronal NOS (nNOS) (157). In fact, involvement of nNOS in glutamate-induced cerebrovascular dilation in mouse cerebellum has been demonstrated (156). Furthermore, because both astrocytes and neurons have HO-2, glutamate could stimulate CO production in these cells. The dilator response to CO would still be endothelial dependent because the endothelium is necessary to provide the obligatory permissive signal of NO.

**Localization of HO isoforms and regulation of HO-1 and HO-2 expression.** HO-1 and HO-2 isoforms are products of distinct genes located on different chromosomes. The amino acid sequences of HO-1 and HO-2 have only 40% similarity (13). Human HO-1 is a single polypeptide of 288 residues and ~32 kDa, whereas human HO-2 is a 316-residue protein (36 kDa) due to an addition at the NH\textsubscript{2} terminus. A common 24-amino-acid domain that forms the heme catalytic pocket is evolutionarily conserved in HO-1 and HO-2 except for a single amino acid residue (95). Catalytic mechanisms of heme degradation by the isoforms are similar.

Both HO-1 and HO-2 are membrane-bound proteins anchored to the endoplasmic reticulum membrane via a COOH-terminal hydrophobic tail (130). In porcine cerebral vascular endothelial cells, HO-1 and HO-2 have similar intracellular localization with strong preference for the nuclear envelope, perinuclear area of the cytoplasm, and endoplasmic reticulum (117). Electron immunocytochemistry data in rat kidney epithelial cells also demonstrated association of HO-2 with the nuclear outer membrane and the endoplasmic reticulum (52). Intracellular localization of HO corresponds to localization of NOS and prostaglandin cyclooxygenase (COX) that may suggest functional cross talk among CO, NO, and prostanooids. In the carotid body, intracellular HO-2 colocalizes with large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK\textsubscript{Ca}) channels (150). Localization with BK\textsubscript{Ca} channels may be important for cerebral circulatory control because HO-2-derived CO activates BK\textsubscript{Ca} channels to produce vascular smooth muscle relaxation (see *Mechanism of CO-induced cerebrovascular dilation*, below).

In pulmonary artery endothelial cells, HO-1 was detectable in plasma membrane caveolae, where caveolin binds to HO-1 and regulates its enzymatic activity (68). Under certain conditions, HO-1, but not HO-2, is localized to the nucleus in differentiated astroglial cells (85) and pulmonary artery endothelial cells (68), indicating a possibility of nuclear functions of HO-1.

The highest HO-2 expression is found in the brain, cerebral vasculature, and testes (85, 93, 95). Steroid hormones are the only known regulators of HO-2 expression (4, 92, 94, 116, 147). The presence of glucocorticoid response element in the promoter region of the HO-2 gene accounts for upregulation of HO-2 expression (88). High expression in the brain, including selected neuronal populations (29, 33, 34, 57, 158), glia (129), and cerebral vasculature (84, 117), indicates an important role of HO-2 in brain physiological functions that include regulation of cerebral blood flow and mediation of neuronal activity.

Under basal conditions, HO-1 is expressed strongly only in reticuloendothelial cell-rich tissues, such as spleen and liver, where it functions to eliminate potentially toxic heme released from degraded red blood cells (94). In the brain and in cerebral vasculature, no HO-1 expression has been detected under basal conditions (34, 84, 117, 119). HO-1 is an early response gene (heat shock protein 32) that can be induced by a variety of stress factors, including heme, metalloporphyrins, heavy metals, cytokines, oxidative stress, and oxidized lipids (2, 18, 31, 38, 56, 65, 75, 77, 93, 95, 119, 125). Induction of HO-1 is regulated at the level of gene transcription via cell-specific multiple regulatory elements in the promoter region of HO-1 gene, including but not limited to stress response elements and antioxidant response elements in conjunction with the redox-sensitive transcription factor Nrf2 (98, 111), NF-κB (76), and cAMP-responsive element (75). Enhanced HO-1 gene transcription may also occur via binding of the basic helix-loop-helix-leucine zipper family of transcription factors upstream stimulating factor (USF)1 and USF2 to the class B E-box located in the proximal promoter of the human HO-1 gene (49). In addition, HO-1 protein induction via translation-independent mRNA stabilization has been described (13).

**Mechanism of CO-induced cerebrovascular dilation.** It has been proposed that CO-induced cell signaling may be via activation of GC (51, 97). Indeed, treatment of platelets (16) or aorta (41) with CO or a CO-releasing molecule (37), as well as overexpression of HO-1 in pulmonary artery or aorta (3, 107, 127), increase cGMP. CO is much less effective at stimulating GC than is NO (69–71), but involvement of an endogenous substance that increases GC sensitivity to CO has been suggested (24, 142). Nevertheless, cGMP as a direct mediator of CO dilation in cerebrovascular circulation under physiological conditions appears unlikely. An increase in cGMP production coincident with CO-induced dilation has not been demonstrated, and normal dose-dependent dilation to CO occurs with cGMP held constant (72, 82). Furthermore, the ability of GC inhibition to attenuate vasodilation to CO (37, 51, 79, 144), in
the cerebrovascular circulation at least, can be entirely explained by a role of cGMP as a necessary permissive enabling factor (see below).

In contrast to cGMP, extensive evidence suggests CO-induced dilation is mediated by BKCa channel activation. BKCa channel inhibition blocks CO-induced vasodilation (84, 110, 159). HO inhibitors reduce BKCa channel activity in renal and tail artery smooth muscle cells, suggesting that HO-derived products activate BKCa channels (63, 152). O2 can regulate BKCa channels indirectly via CO, because O2 is necessary for heme metabolism by HO (150). Conversely, inhibitors of soluble GC do not attenuate CO-induced BKCa channel activation in smooth muscle cells isolated from cerebral and renal arteries (63, 153). Furthermore, CO activates BKCa channels in excised arterial smooth muscle cell membrane patches that are removed from the intracellular milieu (150, 152, 153). Chemical modification of histidine residues blocks CO-induced BKCa channel activation, suggesting an important role for this amino acid (147). CO activates BKCa channel α-subunits expressed in the absence of auxiliary β-subunits in mammalian cells, suggesting that CO acts on the pore-forming α-subunit (152, 153). Antisense downregulation of the β-subunit abolished NO-induced BKCa channel activation but did not alter CO-induced BKCa channel activation in rat tail artery smooth muscle cells, indicating that CO and NO activate BKCa channels by different mechanisms (152). Taken together, these findings show that CO activates BKCa channels by interacting with the α-subunit or an associated regulatory element.

The α-subunit of the BKCa channel contains a heme-binding pocket, and binding of heme to the BKCa channel inhibits BKCa channel activity (140). CO, by binding to channel-bound ferrous heme, changes the association of the heme with the channel leading to channel activation (60). Therefore, the BKCa channel isfunctionally a heme-protein. BKCa channel-bound heme is the receptor for CO, and CO binding increases BKCa channel Ca2+ sensitivity (see next paragraph).

In smooth muscle cells, BKCa channels are activated by local intracellular Ca2+ transients termed “Ca2+ sparks” that elevate the local Ca2+ concentration into the micromolar range (61). A single Ca2+ spark activates several BKCa channels, leading to an outward BKCa transient. In the arterial wall, summation of transient Ca2+-activated K+ currents induces a membrane hyperpolarization that reduces voltage-dependent Ca2+ channel (VDCC) activity and, thus, intracellular Ca2+ concentration. In cerebral artery smooth muscle cells, CO elevates BKCa channel Ca2+ sensitivity, particularly within the micromolar Ca2+ concentration range (153). Accordingly, endogenous HO-derived CO and exogenous CO elevate BKCa channel-transient frequency and amplitude by enhancing the effective coupling of Ca2+ sparks to BKCa channels (59). CO also elevates Ca2+ spark frequency that contributes to the CO-induced transient Ca2+-activated K+ current frequency and amplitude elevation. Because inhibitors of Ca2+ sparks and BKCa channels block CO-induced cerebrovascular dilation in vivo, Ca2+ spark to BKCa channel coupling is essential for vasodilatory actions of CO (59, 84).

In the piglet cerebrovascular circulation, CO interacts with two other prominent endothelium produced vasodilators. Inhibition of either NOS or COX blocks dilation to CO (83). However, these blockades are not the consequence of CO increasing NO and prostacyclin that cause the dilation. The contributions of NO and prostacyclin are permissive in that only sufficient background levels are necessary before CO will produce dilation of piglet cerebral arterioles. Thus, if the concentration of NO and/or prostacyclin is held constant by blocking synthesis, but providing exogenous NO and/or prostacyclin, CO produces dose-dependent pial arteriolar dilation that is indistinguishable from the responses when NOS and COX are not inhibited (83). Furthermore, the final mechanism behind the permissive actions of NO and prostacyclin appears to be the same because either the prostacyclin mimics iloprost or the NO-generating molecule sodium nitroprusside (SNP) can return dilation to CO when both COX and NOS are inhibited (82). The source of the permissive mediators may be endothelial because endothelial denudation blocks CO-induced dilation and SNP or 8-br-cGMP restores dilation to CO (8). The actions of both NO and prostacyclin are mediated by protein kinase G, but only the NO action is via cGMP (82). Whether the action of protein kinase G is on the BKCa channel, ryanodine receptor, both, or another mechanism has not as yet been determined.

Functional significance of CO in control of cerebrovascular circulation. Functional significance of purported messengers and processes is usually suggested by actions of pharmacological inhibitors. Pharmacological inhibition of HO with substituted metalloporphyrins is a major source of data on the functional significance of endogenous CO, in addition to measurements of CO production, overexpression of endogenous HO, and application of exogenous CO and CO-releasing molecules.

Available data suggest that the predominant effects of HO inhibitory metalloporphyrins on cerebrovascular circulation in vivo result from HO inhibition. For example, photooxidized porphyrins do not block HO (42), and photooxidized chromium mesoporphyrin (CrMP) does not alter cerebrovascular responses (123). Furthermore, copper mesoporphyrin, a metal porphyrin that does not inhibit HO, does not affect dilatory responses to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, (RS)-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-y1) propionic acid, or 1-amino-1,3-cyclo-pentanedicarboxylic acid (123).

Actions of metalloporphyrins unrelated to HO inhibition have been reported. Micromolar concentrations of metal porphyrins can both inhibit (55, 131) and activate GC (131). However, tin protoporphyrin (SnPP) does not affect dilation to SNP, a cGMP-dependent dilator (12). In hippocampal slices, effects of CrMP and zinc protoporphyrin (ZnP) on NOS activity were greater than those of tin mesoporphyrin and zinc deuterooporphyrin, even though all inhibited HO (102). These data suggest CrMP and ZnP might inhibit NOS independently of HO inhibition. Conversely, ZnPP stimulated NO production by rabbit internal anal sphincter, an action apparently related to HO inhibition (19). ZnPP, but not other porphyrins, may inhibit VDCC in pituitary cells (87). However, the consequence of inhibition of VDCC would be dilation, not constriction, so involvement of inhibition of VDCC in metalloporphyrin blockade of putative CO-dependent responses in cerebrovascular circulation is unlikely. Metalloporphyrin inhibition of G protein coupling to second-messenger systems has been reported (112, 143). However, in piglet cerebral circulation metalloporphyrins do not inhibit dilation to isoproterenol, suggesting no interference with G protein function (84). Finally,
inhibitory activity toward another necessary component of heme metabolism, NADPH-cytochrome P-450 reductase, and possible effects on the expression as well as activity of HO-2 in rat brain have been reported for SnPP, but not for ZnPP (96).

Alternative strategies to pharmacological inhibitors have limitations as well. Antisense oligonucleotide approaches suffer from cell toxicity and ineffectiveness (25, 26, 128), and siRNA, which can be more efficient than antisense oligonucleotides, also can have unintended actions (48, 126, 128). Loss of specific functions in knockout mice without genes of interest can be very convincing, but no in vivo data are available for HO in cerebrovascular circulation. Also, this approach can be limited by biological compensation for missing proteins and questions of removal of processes and pathways associated with the intended target during development.

Overall, pharmacological inhibition of enzymes and receptors remains the most rapid and effective approach for removal of messages and processes of interest. Although there are potential non-HO-mediated actions of metalloporphyrin HO inhibitors, none appear to account for the actions of these compounds on cerebrovascular circulation.

CO is a gasotransmitter related to neural function in the brain (10). Neurotically released CO could be a regulator of cerebral blood flow, but no data supporting this concept have been collected to date. In vivo, topical CO (nM) dilates pial arterioles (84). Brain production of CO results in accumulation in cerebrospinal fluid (CSF) placed under cranial windows that is contiguous with brain extracellular fluid. Dilator concentrations of CO in this CSF (88 ± 20 nM) have been measured under control conditions, and 10-fold increases have been measured with strong stimulation (12). Thus the level of CO production by brain and vessels is sufficient to provide dilator effects on the cerebral circulation.

Indications of functional significance of CO are further supported by alterations of responses to important physiological cerebrovascular regulatory stimuli upon inhibition of HO with substituted metalloporphyrins. Pial arteriolar dilations to both hypoxia and topical glutamate are selectively inhibited after topical treatment with CrMP that blocks CO production (84, 123). Furthermore, CO appears to be involved in pial arteriolar dilation to hypotension (64). Mediation of autoregulatory vasodilation could contribute to the protective effects of HO after brain ischemia, but most evidence suggests that the antioxidant properties of bilirubin are largely responsible (71). CO can also attenuate vascular responses to constrictor stimuli (151). For example, inhibition of HO accentuates the constriction of pial arterioles produced by hypertension but has minimal effects when piglets are normotensive. In addition, vasoconstriction to topical application of platelet activating factor is accentuated after inhibition of HO. In rat hypothalamus, CO has been shown to contribute to regulation of vascular tone that is particularly evident in the absence of NO (50). This finding in adult rats appears to be different from results of newborn pigs where NO plays an obligatory permissive role in CO-induced dilation and NOS inhibition blocks dilation to CO (see Mechanism of CO-induced cerebrovascular dilation, above).

Although reported actions of CO on cerebrovascular tone are uniformly dilating, it should be mentioned that in skeletal muscle vasculature CO has been shown to be capable of producing vasoconstriction, apparently by inhibiting NOS. Thus, in isolated, pressurized, phenylephrine-treated rat gracilis muscle arterioles, either exogenous CO or stimulation of endogenous CO production produced constriction that was prevented by endothelium removal (40). That this constriction involves reduction of endothelial-derived NO is indicated by the ability of L-arginine to prevent the constriction and of NOS inhibition to convert the CO-induced constriction to dilation. Of interest, results from rat gracilis muscle arterioles are opposite of those from small rat renal arteries where CO increased NO production that contributed to CO-induced dilation (140).

Protective effects of HO-CO and regulation of CBF during seizures. CO produced by the brain contributes to regulation of cerebral blood flow during seizures (17, 105, 122). In bicuculline-induced epileptic seizures in newborn pigs, pial arteriolar dilation that occurs simultaneously with neuronal activation correlated with a massive increase in CO concentration in cortical CSF that was sustained for the duration of seizures (17, 115). HO inhibitors CrMP and SnPP inhibited CO production by the brain and reduced cerebral dilation in response to seizures (17, 115, 122). The rapid increase in CO production by the brain during epileptic seizures is attributable exclusively to constitutive HO-2, because no induction of HO-1 or HO-2 protein was observed (17, 119). The rapid increase in HO-2 enzymatic activity in the cerebral vasculature occurs via a glutamate receptor-mediated mechanism.

The major excitatory neurotransmitter, glutamate, is massively released from neurons during seizures (103). Cerebral microvascular endothelial cells from rat, human, and pig, in addition to neurons and glia, have receptors for glutamate (14, 23, 74, 116, 133, 134). Both ionotropic and metabotropic subtypes have been identified in cerebral vascular endothelial cells (23, 116, 133, 134). Conversely, other investigators were unable to detect glutamate receptors on cerebral microvascular endothelial cells from sheep, rat, or human (9, 108). The reason for these opposing results is not apparent. Consistent with the presence of receptors, glutamate receptor agonists cause functional responses in cerebral vascular endothelial cells that include increases in HO activity (116), changes in endothelial monolayer permeability (134), and increases in formation of ROS (14, 134). Of significance to the present review, piglet cerebral microvessels respond directly to glutamate and selective N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate receptor agonists by increasing CO production (79, 116). We found pressurized pial arteries respond to glutamate by endothelium-dependent vasodilation (11% at 10⁻⁵ M) (36), although less strongly than in vivo (20% dilation at 10⁻⁵ M) (123). Others have failed to detect any glutamate-receptor-mediated responses in pressurized bovine middle cerebral arteries (149) or middle cerebral arteries from newborn pigs (135). Although the difference in species, age, and artery examined could account for divergent results in the first instance, the reason for the inconsistent results in piglets is not evident. The choice of anesthetic, ketamine vs. pentobarbital, is different but appears to be an unlikely contributor in an isolated vessel experiment. Although the diameters of the vessels in the two studies are similar, the arteries selected may not be the same, because Simandle et al. (135) could pressurize to 100 mmHg, which was not tolerated by arteries used by Fiumana et al. (36). Heterogeneity of responses even in pial arteries and arterioles within a 1-cm-diameter circle of surface parietal cortex has
been described (118). Regardless, it is clear that cerebral arterioles respond more strongly to glutamate in vivo than in vitro, suggesting extravascular cells contribute to glutamatergic dilation.

Epileptic seizures result in prolonged postictal cerebral vascular dysfunction characterized by reduced vasoreactivity to physiologically relevant dilators, including hypercapnia and bradykinin (119). When HO-2 activity is inhibited before seizures, cerebral vascular dysfunction is observed immediately after the ictal episode and is extended for at least 2 days of the postictal period (117, 119). In contrast, in animals with intact HO-2 activity, no immediate reduction of cerebral vascular reactivity is detected, but cerebral vascular reactivity is greatly reduced 2 days later (12, 86). Therefore, HO-2 is necessary for a short-term protection but not sufficient for long-term protection of the cerebral vasculature from detrimental effects of epileptic seizures. However, upregulation of cerebral HO-1 expression can completely protect the cerebral vasculature during the delayed postictal period (119). Thus it appears that HO-1 can provide long-term protection against postictal cerebral vascular dysfunction. HO-derived CO is important in increasing blood flow to the brain to match excessive neuronal activity during the ictal episode, thus protecting neurons and preventing cerebral vascular injury. HO activity also reduces the amount of prooxidant heme and results in production of biliverdin and bilirubin, which have powerful antioxidant properties as a redox cycling pair that scavenges ROS (7, 29, 95, 104). Also, CO itself may have antiapoptotic effects in vascular endothelial and smooth muscle cells.

**CO, apoptosis, and cell proliferation.** In addition to its role as a neuronal and vascular messenger, CO can suppress apoptosis (15, 136, 160). The molecular mechanism of antiapoptotic protection by CO is not clear. CO may inhibit generation of free radicals by mitochondria via a direct interaction with the heme protein of the mitochondrial electron transport chain, or interact with the p38 MAPK- or cGMP-signaling pathways that can modulate apoptosis in a cell- and signal-specific manner (136). In addition, CO inhibits the activity of caspases that play a major role in executing apoptosis (138, 160). A possible interaction of CO with NF-κB-mediated apoptosis signaling also has been proposed (15, 90, 136). Proapoptotic effects of CO have also been reported (139). It appears that the ability of CO to suppress or promote apoptosis depends on the specific apoptotic signal and cell type.

In addition to effects on apoptosis, CO may affect cerebrovascular circulation via regulation of vascular cell proliferation. In rat aortic smooth muscle cells, increasing CO inhibited and scavenging CO increased cell proliferation, actions apparently mediated by increasing cGMP that increases E2F-1 expression (106). Furthermore, CO can reduce endothelial cell proliferation caused by hypoxia by inhibiting VEGF production by adjacent vascular smooth muscle (91). This action as well appears to be mediated via cGMP, by decreasing binding of a hypoxic enhancer to hypoxia-inducible factor-1. After vascular injury as well as hypoxia, CO suppresses vascular smooth muscle proliferation by increasing cellular cGMP that activates p38 mitogen-activated protein kinase, upregulating caveolin-1 that prevents proliferation (69). Conversely, CO has been shown to promote proliferation of microvascular endothelial cells (86). Thus, depending on the conditions, background stimuli, and specific cell type, CO can either increase or decrease apoptosis and cell proliferation.

**HYDROGEN SULFIDE**

The following discussion is summarized pictorially in Fig. 2. Control of H2S production. Production and utilization of H2S have been demonstrated in tissues from different life forms, including bacteria and archa (113), nonmammalian vertebrates (28), and mammals (145, 146) with physiological concentrations in the range of 20–160 μM (1, 163).

Two pyridoxal-5’-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), are responsible for the endogenous production of H2S in mammalian tissues (1, 145, 146, 163). Although expression of CBS is more abundant in liver and neuronal tissues, CSE is the dominant H2S-generating enzyme in the cardiovascular system. CSE and CBS catalyze the hydrolysis of cysteine via β-elimination to generate H2S. A beta-replacement reaction in which cysteine is condensed in the presence of CSE with homocysteine to form cystathionine and H2S has been recently reported in vitro (21). The condensation of cysteine is believed to be 50 times more efficient than hydrolysis of cysteine in terms of H2S production (21).

The synthetic pathway for H2S production is intermingled with the synthetic pathway for NO. The expression and activity of CSE are upregulated by NO, but hydroxylamine (a precursor of NO) inhibits the activity of CBS (114). S-adenosylmethionine and pyridoxal-5’-phosphate stimulate CSE activity to increase H2S production (70, 132). Kimura (70) reported that CBS-mediated hydrolysis of cysteine was regulated by Ca2+ and CaM in mouse brain. This observation, however, could not be repeated by Chen et al. (21) in mouse brain, mouse liver, or purified recombinant human CBS expressed in E. coli or yeast.

**Mechanism of H2S vascular actions.** Compelling evidence indicates that ATP-sensitive K+ (KATP) channels are major

![Fig. 2. Flow chart summarizing the section of this review on hydrogen sulfide](https://jap.physiology.org/)

**H2S**

**CSE**

**H2S**

**L-cysteine**

**L-arginine**

**Hydroxylamine**

**NO**

**p-ERK 1/2 activation**

**KATP channel open**

**Caspase 3 activation**

**Hyperpolarization**

**Apoptosis**

**Vasorelaxation**

**Proliferation**

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targets of H2S. Opening of KATP channels hyperpolarizes cells and closes VDCC. This provides an important mechanism linking cellular metabolism to excitability and contractility of vascular smooth muscle cells. Patch-clamp studies show that H2S increases whole cell KATP channel currents in single smooth muscle cells isolated from both aorta and mesenteric arteries (22, 163). This stimulatory effect of H2S is concentration dependent and reversible. The KATP inhibitor glibenclamide blocks and the activator pinacidil mimics the effect of H2S on KATP channels. H2S hyperpolarizes smooth muscle cells, and the action is antagonized by glibenclamide (163). The molecular mechanisms underlying the effect of H2S on KATP channels are still largely unknown. Alteration of intracellular ATP concentration cannot explain the interaction between H2S and KATP channels because clamping intracellular ATP at different levels did not alter the relative stimulatory effect of H2S on the whole cell KATP channels (163). Because H2S is a reductant (68), it is possible that H2S directly interacts with KATP channel proteins by reducing the cysteine residues. A mutagenesis approach that replaces cysteine residues with structurally similar serine residues would support this hypothesis if H2S fails to stimulate KATP channels after selective point mutation of cysteine residues.

The proapoptotic effect of H2S is related to increased activity of extracellular signal-regulated kinase (ERK), but not p38 MAPK or c-Jun NH4-terminal kinase activity. Activation of caspase-3 by ERK could be one downstream target of H2S-ERK interaction (154).

Functional significance of H2S in circulatory regulation. Vascular contractility is regulated by endogenous and exogenous H2S at physiologically relevant concentrations. Intravenous injection of H2S decreases mean arterial blood pressure of anesthetized rats by decreasing vascular resistance (163). Daily intraperitoneal injections of d,l-propargylglycine (PPG), a specific blocker of CSE, for 2–3 wk elevates systolic blood pressure (162). Because this PPG treatment suppresses H2S production in vascular and other tissues, PPG-induced hypertension may result from reduced endogenous H2S production in vascular tissues (162). H2S concentration-dependently relaxes phenylephrine-precontracted rat aorta, a conduit artery (161). The isolated and perfused rat mesenteric vascular bed, a model of peripheral resistance arteries, is also relaxed by H2S (16). Although rat aortic and mesenteric artery tissues generate comparable levels of H2S (15, 161–163), rat mesenteric arteries are much more sensitive to H2S (EC50 of 25.2 ± 3.6 μM) than rat aorta (EC50 of 125 ± 14 μM). l-Cysteine, a substrate of CSE and CBS, increases endogenous H2S production and decreases contractility of mesenteric arteries. In contrast, PPG abolishes the l-cysteine-dependent increase in H2S production and relaxation of mesenteric arteries. These findings indicate the importance of endogenous H2S in regulating vascular contractility (22).

H2S can be involved in the control of both proliferation and apoptosis in vascular smooth muscle cells. H2S at physiologically relevant concentrations did not induce necrosis of human aortic smooth muscle cells (154). However, H2S (200–500 μM) increased the numbers of condensed apoptotic nuclei, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells, and oligonucleosomal DNA fragmentation. The cells showed the morphological changes typical of apoptosis within 2 h of H2S application. Interestingly, baseline endogenous H2S modulates exogenous H2S-induced apoptosis of human aortic smooth muscle cells. Treatment of these cells with the CSE inhibitor PPG alone for 1 h did not induce any apoptotic changes. This preconditioning treatment, however, significantly enhanced the proapoptotic effect of exogenously applied H2S. The threshold concentration of H2S to induce apoptosis was reduced from 200 to 100 μM under this condition. It is hypothesized that basal endogenous H2S may be high enough to desensitize apoptotic signaling pathways in vascular smooth muscle cells. As such, the basal H2S level may serve as a set point for the basal apoptotic status of smooth muscle cells. Increases or decreases in endogenous H2S levels may consequently alter homeostatic control of smooth muscle cell apoptosis.

H2S also can inhibit cell proliferation. Stable overexpression of CSE in HEK-293 cells increased endogenous H2S production and inhibited cell proliferation and DNA synthesis (154). These effects were significantly reversed in the presence of the H2S scavenger, methemoglobin. Exogenous H2S at the physiologically relevant concentration of 100 μM also inhibited cell proliferation. However, neither overexpression of CSE nor application of exogenous H2S induced apoptosis of HEK-293 cells. The difference between the endogenous H2S basal levels in vascular smooth muscle cells and HEK-293 cells may underlie the lack of proapoptotic response of HEK-293 cells to H2S. It has been reported that CSE expression level and its enzymatic activity were higher in kidney (HEK) than in smooth muscle (58).

The function of H2S in cerebral circulation remains poorly understood. H2S is produced in brain tissues, reaching an endogenous level of 50–160 μM (1). The release of corticotropin-releasing hormone from hypothalamus and facilitation of hippocampal long-term potentiation appear to be influenced by H2S metabolism (70). Environmental exposure to H2S at low concentrations for 2 wk activates protein synthesis in nerve cells and myelinated fibers in the cerebral cortex (137), but the physiological meaning of this study is unclear. It appears likely that endogenously produced H2S exerts similar vasorelaxant effects on the cerebral circulation as in the systemic circulation. Cerebral vascular smooth muscle cells would be exposed to significant amounts of H2S, and cerebral vascular smooth muscle cells possess the KATP channels (144), the target of H2S on other vascular smooth muscle cells (145). A recent study showed that NMDA-induced dilation of pial arteries in newborn pigs was partially due to activation of KATP channels (121). In glutamatergic neurons, endogenous H2S has been shown to enhance NMDA receptor-mediated transmembrane currents (70).

CONCLUSIONS

CO and H2S are gaseous cellular messenger molecules that are involved in cerebrovascular flow regulation. Thus they join the group with the most studied gaseous mediator, NO. The collective differences between the gasotransmitters and the classical neurotransmitters, hormones, and lipid and peptide autocrine/paracrine messengers in intercellular movement, cellular action mechanisms, and signal termination are already revolutionizing our concepts of cellular communication. This review has focused on two of these molecules, CO and H2S,
the physiological significance of which have begun to be appreciated in the last decade or less.

CO exerts a dilator influence on the cerebral circulation and is involved in active hyperemia, autoregulation, hypoxic dilation, and countering vasocostricton. CO is produced by metabolism of cellular heme by a constitutive enzyme expressed highly in the cerebral microcirculation and by an inducible enzyme that is readily upregulated in brain by potentially injurious conditions. CO production is regulated by controlling substrate, HO-2 catalytic activity, and HO-1 expression. CO causes cerebrovascular dilation by elevating smooth muscle BKCa channel Ca2+ sensitivity, leading to increased coupling to Ca2+ sparks, thereby hyperpolarizing the cell. The HO-CO vasodilatory system interacts at both the level of messenger production and action with other important mediators of cerebral circulatory control, including NO and prostanooids. Although much is becoming known, much more remains unknown about CO and cerebrovascular circulatory control. Even though CO is involved in several cerebral circulatory control mechanisms, many other situations in which CO may be a key mediator remain to be explored, and very little is known about pathological alterations of HO-CO system in the cerebral circulation. The cellular origins of CO mediating distinct responses in the intact cerebral circulation are largely unknown. Knowledge of the mechanisms regulating CO production is only beginning to become available, and those data appear to contrast at times. Although mechanisms by which CO can affect the activity of BKCa channels are being revealed, these mechanisms are still not completely understood. In addition, CO can contribute to vascular remodeling by enhancing or depressing both apoptosis and cellular proliferation, depending on cell type and particularly other impinging signals. Thus much of the physiology and biochemistry of HO-CO in the cerebral circulation remains open for exploration.

H2S is a very recently identified gasotransmitter that is produced by many mammalian cells, including vascular smooth muscle cells. By relaxing vascular smooth muscle cells to dilate blood vessels, promoting apoptosis of vascular smooth muscle cells, and inhibiting proliferation-associated vascular remodeling, H2S modulates both the function and structure of the circulatory system. The mechanisms behind H2S-induced activations of KATP channels on the cell membrane and the ERK signaling pathway inside cells are not yet understood. Genetic approaches to manipulate CSE expression and endogenous production of H2S may help to decisively establish physiological and pathophysiological importance of H2S in regulation of cerebrovascular tone in particular and cardiovascular function in general. Future studies should examine vasorelaxant and proapoptotic, antiproliferative effects of H2S on cerebral vascular tissues. Advancing in this understanding also has the potential to provide novel therapeutic avenues for treatment of many vascular diseases linked to H2S-related abnormal cellular contractility and proliferation.

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