Published in final edited form as:
J Appl Physiol. 2006 March ; 100(3): 1065–1076.

Carbon monoxide and hydrogen sulfide: gaseous messengers in cerebrovascular circulation

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Abstract

This review focuses on two gaseous cellular messenger molecules, CO and H_2S, 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 Ca^{2+} activated K^+ channels (BK_{Ca} channels), which elevates channel Ca^{2+} sensitivity, increases coupling of Ca^{2+} sparks to BK_{Ca} 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. H_2S may also function as a cerebrovascular dilator. It is produced in vascular smooth muscle cells by hydrolysis of L-cysteine catalyzed by cystathione gamma-lyase (CSE). H_2S dilates arterioles at physiologically relevant concentrations via activation of K_{ATP} channels. In addition to dilating blood vessels, H_2S promotes apoptosis of vascular smooth muscle cells and inhibits proliferation-associated vascular remodeling. Thus, both CO and H_2S modulate the function and the structure of circulatory system. Both the HO/CO and CSE/H_2S systems have potential to interact with NO and prostanoids in the cerebral circulation. Much of the physiology and biochemistry of HO/CO and CSE/H_2S in the cerebral circulation remains open for exploration.

Introduction

The group of gaseous autocrine/paracrine messengers continues to expand and now includes nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S), and several molecules from the broad category of reactive oxygen species (ROS). Diverse functions of the messengers are being detected at an astounding rate, demonstrating that the gaseous autocrine/paracrine messengers are critical to vertebrate, and possibly all living organism, physiology; and extend far beyond just NO. Gaseous messengers, or gasotransmitters (145), are employed for intra- and intercellular communication with high specificity in many, if not all, organ systems. While gaseous messengers function analogously to hormones, neurotransmitters, and lipid mediators, they have distinct attributes. Because they are lipid soluble gases, gasotransmitters are not constrained by cellular membranes. Thus, storage in vesicles for later release is not possible. Although it has been suggested that CO and NO could be bound to heme containing proteins that may be induced to release the messenger (27,53,141), clearly the predominant determination of the strength of signal of gasotransmitters is de novo synthesis. Because gasotransmitters diffuse down partial pressure gradients and are unconstrained by cell

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membranes, specific uptake or metabolizing processes to terminate the signals need not exist. Signals are terminated by falling concentrations upon reduction of production that are caused by reacting with cellular components (particularly ROS and NO), binding to cellular components (e.g. CO to ferrous heme), or diffusing away. The natures of the interactions of the gasotransmitters with their receptors are, with the exception of NO and guanylyl cyclase, only beginning to be uncovered.

While the overall category of gasotransmitters encompasses NO, CO, H_2S, various ROS, and certainly others not yet realized, the present mini-review will be limited to CO and H_2S in regulation of cerebrovascular circulation, because roles of NO have been extensively discussed and reviewed before (35,78,121) and the understanding of signaling roles of ROS, as opposed to the destructive actions, is limited (see reviews 32,44,114).

Carbon monoxide (CO)

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 below 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 P450 reductase, by O_2 availability, by delivery of heme to the enzyme, and by the catalytic activity of the enzyme.

While evidence to indicate NADPH concentration or P_{O2} regulates CO production in the cerebral circulation has not been produced, data suggesting O_2 availability can affect CO production are available from other tissues. In microsomal fractions of of human placenta chorionic villi, CO production is directly related to P_{O2} from 18–166 mmHg (5). Also, electrophysiological results from membrane patches of carotid body glomus cells suggest hypoxia may decrease CO production (77). However, hypoxia dilates cerebral arteries, as does CO, so the functional significance of hypoxic inhibition of CO production in the cerebral circulation is unknown.

Endogenous CO production by brain and brain vessels is substrate limited because provision of exogenous heme increases CO production (80). Therefore, mechanisms that regulate cellular heme production will also regulate CO production. Cellular heme manufacture is a multi-step process that includes both mitochondrial and cytoplasmic elements, but the rate-limiting step is production in mitochondria of delta-aminolevulinic acid from succinyl CoA and glycine catalyzed by delta-aminolevulinic acid synthase (ALAS) (66,100). ALAS 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 Ca^{2+} with ionomycin or activation of protein kinases C (PKC) with phorbol ester increased CO production by increasing heme availability (80). However, in neither case are the cellular mechanisms involved known.

HO-2 catalytic activity, 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 CK2 catalyzed phosphorylation of serine 79 (10). Glutamatergic activation of HO-2 results from metabotropic glutamate receptor-induced Ca^{2+} 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 below in section on seizures for more extensive discussion of glutamate receptors and CO). 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, that inhibits calmodulin (CaM), decreased HO-2 catalytic activity and blocked glutamate stimulation of CO production (11, 81). Boehning et al (11) demonstrated Ca^{2+} - 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 Ca^{2+}-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 Ca^{2+}/CaM regulation of HO-2 catalytic activity. The reasons for the apparent discrepancy between effects of cytosolic Ca^{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 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 NOS inhibition totally abolished glutamate –induced CO production (80). However, in vivo, while NOS inhibition blocked glutamate-induced dilation, a constant background amount of NO completely restored dilation to glutamate (71). If glutamate increases NO that increases CO, that 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 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 approximately 32 kDa, while human HO-2 is a 316-residue protein (36 kDa) due to an addition at the N-terminus. A common 24 amino acid domain that forms the heme catalytic pocket is evolutionally 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 C-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 nitric oxide synthase (NOS) and prostaglandin cyclooxygenase (COX) that may suggest functional cross-talk among CO, NO, and prostanoids. In the carotid body, intracellular HO-2 co-localizes with BK_{Ca} channels (150). Localization with BK_{Ca} channels may be important for cerebral circulatory control because HO-2-derived CO activates BK_{Ca} channels to produce vascular smooth muscle relaxation (see 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 astrogial 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 (GRE) 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 were 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 Hsp 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 (StREs) and antioxidant response elements (AREs) in conjunction with the redox-sensitive transcription factor Nrf2 (98,111), nuclear factor kappa B (NFκB) (76), and cAMP responsive element CRE (75). Enhanced HO-1 gene transcription may also occur via binding of the basic helix-loop-helix-leucine zipper family of transcription factors USF1 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 guanylyl cyclase (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 guanylyl cyclase than is NO (69,70,71), 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 guanylyl cyclase 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 BK_{Ca} channel activation. BK_{Ca} channel inhibition blocks CO-induced vasodilation (84,110,159). HO inhibitors reduce BK_{Ca} channel activity in renal and tail artery smooth muscle cells, suggesting that HO-derived products activate BK_{Ca} channels (63,152). O_2 can regulate BK_{Ca} channels indirectly via CO, because O_2 is necessary for heme metabolism by HO (150). Conversely, inhibitors of soluble guanylyl cyclase do not attenuate CO-induced BK_{Ca} channel activation in smooth muscle cells isolated from cerebral and renal arteries (63,153). Further, CO activates BK_{Ca} 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 BK_{Ca} channel activation, suggesting an important role for this amino acid (147). CO activates BK_{Ca} channel α-subunits expressed in the absence of auxiliary β-subunits in mammalian cells, suggesting CO acts on the pore forming α-subunit (152,153). Antisense downregulation of the β-subunit abolished NO-induced BK_{Ca} channel activation, but did not alter CO-induced BK_{Ca} channel activation in rat-tail artery smooth muscle cells, indicating that CO and NO activate BK_{Ca} channels by different mechanisms (152). Taken together, these findings show CO activates BK_{Ca} channels by interacting with the α-subunit or an associated regulatory element.

The α-subunit of the BK_{Ca} channel contains a heme-binding pocket and binding of heme to the BK_{Ca} channel inhibits BK_{Ca} 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 BK_{Ca} channel is functionally a heme-protein. BK_{Ca} channel-bound heme is the receptor for CO, and CO binding increases BK_{Ca} channel Ca^{2+} sensitivity (see below).

In smooth muscle cells, BK_{Ca} channels are activated by local intracellular Ca^{2+} transients termed "Ca^{2+} sparks" that elevate the local Ca^{2+} concentration into the micromolar range (61). A single Ca^{2+} spark activates several BK_{Ca} channels, leading to an outward BK_{Ca} transient. In the arterial wall, summation of transient K_{Ca} currents induces a membrane hyperpolarization that reduces voltage-dependent Ca^{2+} channel (VDCC) activity, and thus, intracellular Ca^{2+} concentration. In cerebral artery smooth muscle cells, CO elevates BK_{Ca} channel Ca^{2+} sensitivity, particularly within the micromolar Ca^{2+} concentration range (153). Accordingly, endogenous HO-derived CO and exogenous CO elevate BK_{Ca} channel-transient frequency and amplitude by enhancing the effective coupling of Ca^{2+} sparks to BK_{Ca} channels (59). CO also elevates Ca^{2+} spark frequency that contributes to the CO-induced transient K_{Ca} current frequency and amplitude elevation. Since inhibitors of Ca^{2+} sparks and BK_{Ca}

channels block CO-induced cerebrovascular dilation *in vivo*, Ca^{2+} spark to BK_{Ca} channel coupling is essential for vasodilatory actions of CO (59,84).

In the piglet cerebrovascular circulation, CO interacts with two other prominent endothelial derived 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 appear to be the same because either the prostacyclin mimic, 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 BK_{Ca} 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, over expression of endogenous HO, and application of exogenous CO and CO releasing molecules (see below).

Available data suggest the predominant effects of HO inhibitory metalloporphyrins on cerebrovascular circulation *in vivo* result from HO inhibition. For example, photooxidized protoporphyrins do not block HO (42) and photooxidized CrMP does not alter cerebrovascular responses (123). Furthermore, CuMP, a metal porphyrin that does not inhibit HO, does not affect dilatory responses to AMPA, ATPA or ACPD (123).

Actions of metalloporphyrins unrelated to HO inhibition have been reported. Micromolar concentrations of metal porphyrins can both inhibit (55,131) and activate guanylyl cyclase (131). However, SnPP does not affect dilation to SNP, a cGMP dependent dilator (12). In hippocampal slices, effects of CrMP and ZnPP on NOS activity were greater than those of SnMP and ZnDP, even though all inhibited HO (102). These data suggest CrMP and ZnPP 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 protoporphrins 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 metalloporphryrin blockade of putative CO dependent responses in cerebrovascular circulation is unlikely. Metalloporphryrin 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, that can be more efficient than antisense oligonucleotides, also can have unintended

actions (48,126,128). Loss of specific functions in KO 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. While 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). Neurally 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 (<1nM) dilates pial arterioles (84). Brain production of CO results in accumulation in CSF placed under cranial windows that is contiguous with brain extracellular fluid. Dilator concentrations of CO in this CSF (88±20nM) 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 following 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 following 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 following 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 above).

While reported actions of CO on cerebrovascular tone are uniformly dilator, 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 NMDA- and AMPA/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). While 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 versus pentobarbital, is different but appears to be an unlikely contributor in an isolated vessel experiment. While the diameters of the vessels in the two studies are similar, the arteries selected may not be the same, since Simandle et al (135) could pressurize to 100 mmHg which was not tolerated by arteries used by Fiumana et al (unpublished observation). Heterogeneity of responses even in pial arteries and arterioles within a 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 (17,119). 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 a long-term protection of the cerebral vasculature from detrimental effects of epileptic seizures. However, up-regulation 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 pro-oxidant heme, and results in production of biliverdin/ bilirubin, which have powerful antioxidant properties as a redox cycling pair that scavenges ROS (7,29,95,104). Also, CO itself may have anti-apoptotic effects in vascular endothelial and smooth muscle cells (see below).

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 anti-apoptotic 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 NFkB-mediated apoptosis signaling also has been proposed (15,90,136). Pro-apoptotic effects of CO have been also 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. Following vascular injury as well as hypoxia, CO suppresses vascular smooth muscle proliferation by increasing cellular cGMP that activates p38 mitogen-activated protein kinase, up-regulating caveolin-1 that prevents proliferation (69). Conversely, CO has been shown to promote proliferation of microvascular endothelial cells (64). Thus, depending upon the conditions, background stimuli, and specific cell type CO can either increase or decrease apoptosis and cell proliferation.

Hydrogen sulfide (H_2S)

The below discussion is summarized pictorially in Fig. 2.

Control of H_2S production

Production and utilization of H_2S have been demonstrated in tissues from different life forms, including bacteria and archae (113), non-mammalian 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 beta-synthase (CBS) and cystathionine gamma-lyase (CSE), are responsible for the endogenous production of H_2S in mammalian tissues (1,145,146,163). While expression of CBS is more abundant in liver and neuronal tissues, CSE is the dominant H_2S-generating enzyme in cardiovascular system. CSE and CBS catalyze the hydrolysis of cysteine via beta-elimination to generate H_2S. A beta-replacement reaction in which cysteine is condensed in the presence of CSE with homocysteine to form cystathionine and H_2S 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 H_2S production (21).

The synthetic pathway for H_2S production is intermingled with the synthetic pathway for NO. The expression and activity of CSE are up-regulated 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 H_2S production (70,132). Kimura reported that CBS-mediated hydrolysis of cysteine was regulated by Ca^{2+} and CaM in mouse brain (70). This observation, however, could not be repeated by Chen et al. in mouse brain, mouse liver, or purified recombinant human CBS expressed in *E. coli* or yeast (21).

Mechanism of H_2S vascular actions

Compelling evidence indicates that K_{ATP} channels are major targets of H_2S. Opening of K_{ATP} 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 H_2S increases whole-cell K_{ATP} channel currents in single smooth muscle cells isolated from both aorta and mesenteric arteries (22,163). This stimulatory effect of H_2S is concentration dependent and reversible. The K_{ATP} inhibitor, glibenclamide blocks and the activator, pinacidil, mimics the effect of H_2S on K_{ATP} channels. H_2S hyperpolarizes smooth muscle cells and the action is antagonized by glibenclamide (163). The molecular mechanisms underlying the effect of H_2S on K_{ATP} channels are still largely unknown. Alteration of intracellular ATP concentration cannot explain the interaction between H_2S and K_{ATP} channels since clamping intracellular ATP at different levels did not alter the relative stimulatory effect of H_2S on the whole-cell K_{ATP} channels (163). Because H_2S is a reductant (68), it is possible that H_2S directly interacts with K_{ATP} channel proteins by reducing the cysteine residues. A mutagenesis approach that replaces cysteine residues with structurally similar serine residues would support this hypothesis if H_2S fails to stimulate K_{ATP} channels after selective point mutation of cysteine residues.

The pro-apoptotic effect of H_2S (see below) is related to increased activity of extracellular signal-regulated kinase (ERK), but not p38 MAPK or c-Jun N-terminal kinase activity. Activation of caspase-3 by ERK could be one downstream target of H_2S-ERK interaction (154).

Functional significance of H_2S in circulatory regulation

Vascular contractility is regulated by endogenous and exogenous H_2S at physiologically relevant concentrations. Intravenous injection of H_2S 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 weeks elevates of systolic blood pressure (162). Since this PPG treatment suppresses H_2S production in vascular and other tissues, PPG-induced hypertension may result from reduced endogenous H_2S production in vascular tissues (162). H_2S 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 H_2S (16). Although rat aortic and mesenteric artery tissues generate comparable levels of H_2S (15,161,162,163), rat mesenteric arteries are much more sensitive to H_2S (EC_{50} of 25.2±3.6 μM) than rat aorta (EC_{50} of 125±14 μM). L-cysteine, a substrate of CSE and CBS, increases endogenous H_2S production and decreases contractility of mesenteric arteries. In contrast, PPG abolishes the L-cysteine-dependent increase in H_2S production and relaxation of mesenteric arteries. These findings indicate the importance of endogenous H_2S in regulating vascular contractility (22).

H_2S can be involved in the control of both proliferation and apoptosis in vascular smooth muscle cells. H_2S at physiologically relevant concentrations did not induce necrosis of human aortic smooth muscle cells (154). However, H_2S (200–500 μM) increased the numbers of condensed apoptotic nuclei, TUNEL-positive cells, and oligonucleosomal DNA fragmentation. The cells showed the morphological changes typical of apoptosis within 2 h of H_2S application. Interestingly, baseline endogenous H_2S modulates exogenous H_2S-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 pro-apoptotic effect of exogenously applied H_2S. The threshold concentration of H_2S to induce apoptosis was reduced from 200 μM to 100 μM under this condition. It is hypothesized that basal endogenous H_2S may be high enough to desensitize apoptotic signaling pathways in vascular smooth muscle cells. As such, the basal H_2S level may serve as a set point for the basal apoptotic status of smooth muscle cells. Increases or decreases in endogenous H_2S levels may consequently alter homeostatic control of smooth muscle cell apoptosis.

H_2S also can inhibit cell proliferation. Stable over-expression of CSE in HEK-293 cells increased endogenous H_2S production and inhibited cell proliferation and DNA synthesis (154). These effects were significantly reversed in the presence of the H_2S scavenger, methemoglobin. Exogenous H_2S at the physiologically relevant concentration of 100 μM also inhibited cell proliferation. However, neither over-expression of CSE nor application of exogenous H_2S induced apoptosis of HEK-293 cells. The difference between the endogenous H_2S basal levels in vascular smooth muscle cells and HEK-293 cells may underlie the lack of pro-apoptotic response of HEK-293 cells to H_2S. 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 H_2S in cerebral circulation remains poorly understood. H_2S 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 H_2S metabolism (70). Environmental exposure to H_2S at low concentrations for 2 weeks 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 H_2S 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 H_2S and cerebral vascular smooth muscle cells possess the K_{ATP} channels (144), the target of H_2S 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 K_{ATP} channels (121). In glutamatergic neurons, endogenous H_2S has been shown to enhance NMDA receptor-mediated transmembrane currents (70).

Conclusions

CO and H_2S 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 H_2S, 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 counteracting vasoconstriction. 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 up-regulated 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 BK_{Ca} channel Ca^{2+} sensitivity, leading to increased coupling to Ca^{2+} 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 prostanoids. While 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 where 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 unknown. Knowledge of the mechanisms regulating CO production is only beginning to become available, and those data appear to contrast at times. While mechanisms by which CO can affect the activity of BK_{Ca} 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 upon 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.

H_2S 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, H_2S modulates both the function and structure of the circulatory system. The mechanisms behind H_2S-induced activations of K_{ATP} 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 H_2S may help to decisively establish physiological and pathophysiological importance of H_2S in regulation of cerebrovascular tone in particular and cardiovascular function in general. Future studies should examine vasorelaxant and pro-apoptotic, antiproliferative effects of H_2S on cerebral vascular tissues. Advance in this understanding also has the potential to provide a novel therapeutic avenue for treatment of many vascular diseases linked to H_2S-related abnormal cellular contractility and proliferation.

Acknowledgements

C.W. Leffler, J. H. Jaggar, and H Parfenova are supported by NHLBI/NIH. H. Parfenova is also supported by NINDS/NIH. R. Wang is supported by CIHR. We thank G. Short for construction of the figures and M. Lester for clerical assistance.

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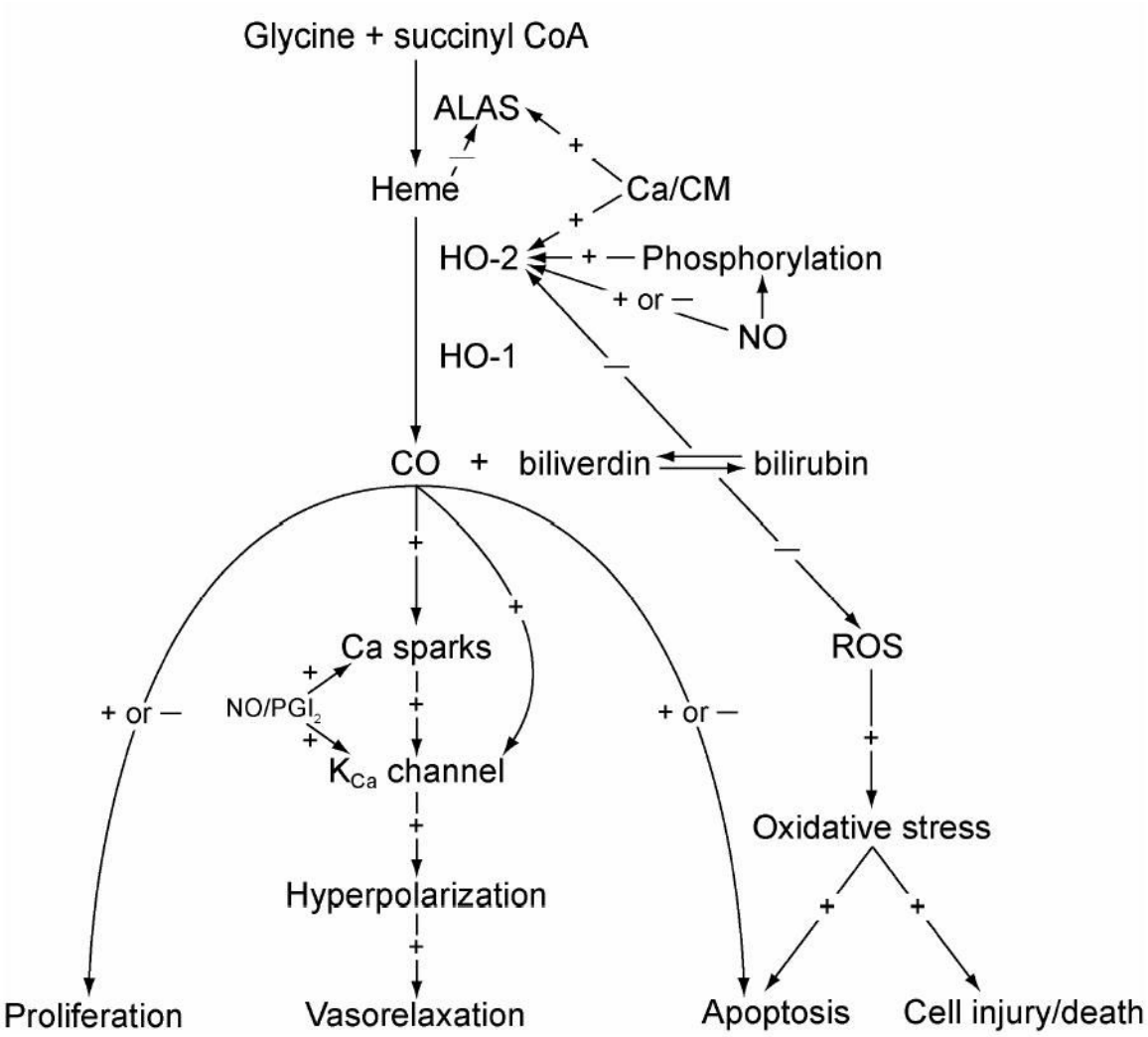

Figure 1.
Flow chart summarizing the section of this review on CO.

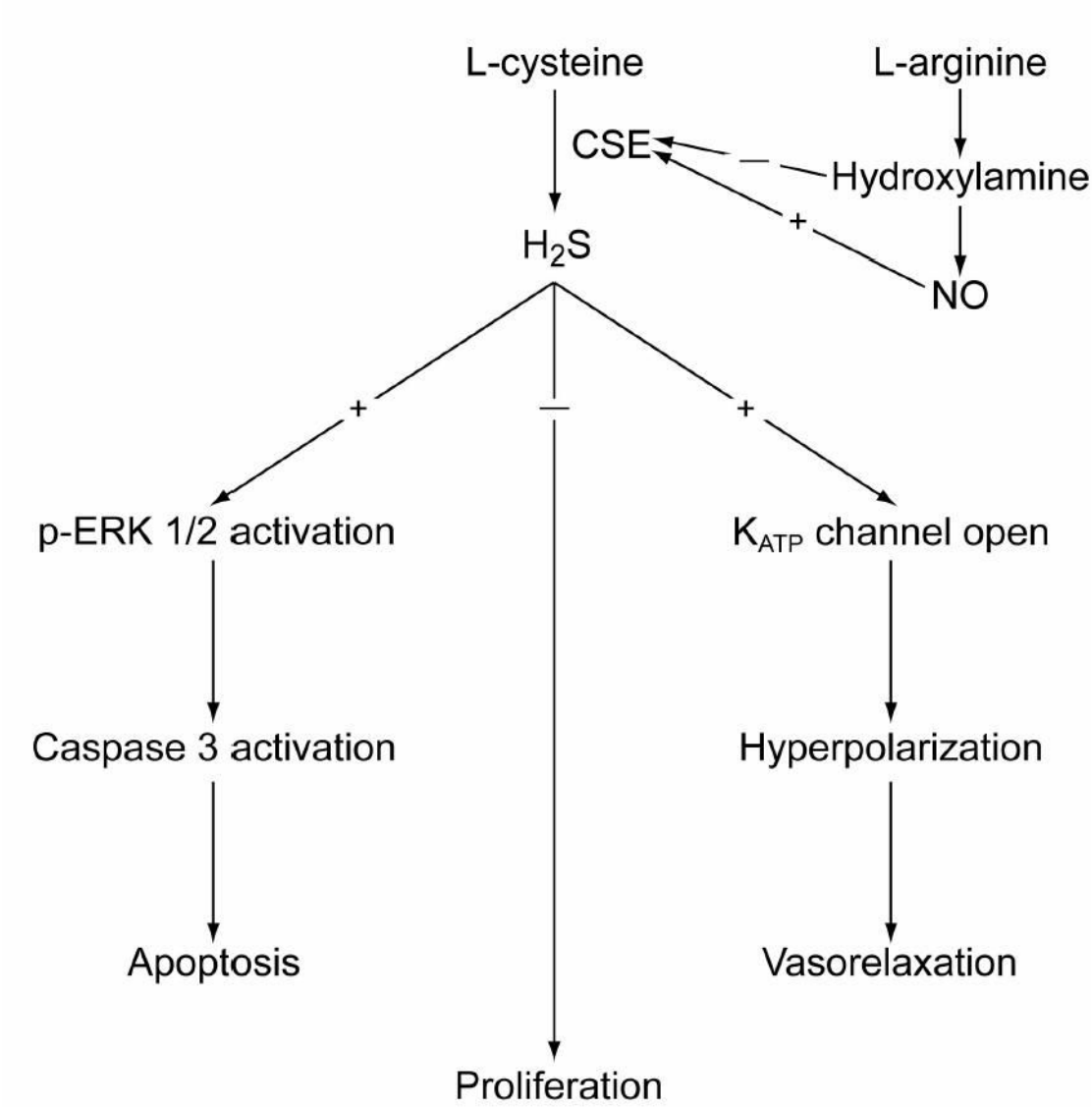

Figure 2.
Flow chart summarizing the section of this review on H_2S.