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Review

Gas biology: Tiny molecules controlling metabolic systems☆

Mayumi Kajimura[a,b,*], Tsuyoshi Nakanishi[a,c], Toshiki Takenouchi[a,d], Takayuki Morikawa[a], Takako Hishiki[a], Yoshinori Yukutake[a,b], Makoto Suematsu[a,b,*]

[a] Department of Biochemistry, School of Medicine, Keio University, Tokyo 160-8582, Japan
[b] JST, ERATO, Suematsu Gas Biology Project, Tokyo 160-8582, Japan
[c] MS Business Unit, Shimadzu Corporation, Kyoto 604-8511, Japan
[d] Department of Pediatrics, School of Medicine, Keio University, Tokyo 160-8582, Japan

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ABSTRACT

It has been recognized that gaseous molecules and their signaling cascades play a vital role in alterations of metabolic systems in physiologic and pathologic conditions. Contrary to this awareness, detailed mechanisms whereby gases exert their actions, in particular *in vivo*, have been unclear because of several reasons. Gaseous signaling involves diverse reactions with metal centers of metalloproteins and thiol modification of cysteine residues of proteins. Both the multiplicity of gas targets and the technical limitations in accessing local gas concentrations make dissection of exact actions of any gas mediator a challenge. However, a series of advanced technologies now offer ways to explore gas-responsive regulatory processes *in vivo*. Imaging mass spectrometry combined with quantitative metabolomics by capillary-electrophoresis/mass spectrometry reveals spatio-temporal profiles of many metabolites. Comparing the metabolic footprinting of murine samples with a targeted deletion of a specific gas-producing enzyme makes it possible to determine sites of actions of the gas. In this review, we intend to elaborate on the ideas how small gaseous molecules interact with metabolic systems to control organ functions such as cerebral vascular tone and energy metabolism *in vivo*.

1. Gas signaling *in vivo*: why difficult to study?

Gas-transducing signaling involves many regulatory roles including neurotransduction, transcription, vascular resistance, and metabolism, and has attracted much attention. However, investigation of gas-transducing signaling is a challenge. Criteria that must be fulfilled for a standard signaling such as hormonal signaling include: (i) specific receptor triggering the change of functions of target molecules; (ii) transducing the initial change to downstream effectors; and (iii) reversibility allowing the cascade to be controlled. Unlike hormonal signaling where specific targets are identified, mechanisms that mediate gas signaling are relatively unsolved. There are reasons why it is difficult to characterize the molecular nature involving each of the three steps above. First, gas has an ability to coordinate with metal centers of prosthetic groups of proteins (*e.g.* heme, and transition-metal center other than iron), but also some gases (*e.g.* NO and H_2S) exert their actions by covalently modifying the sulfhydryl group of cysteines in target proteins, processes designated as *S*-nitrosylation by NO and *S*-sulfhydration by H_2S (Mustafa et al., 2009). Thus, gas actions are pleiotropic in nature (Fig. 1). Second, their small-size and neutral-charge provide gases with the ability to permeate through cell membrane and inside the macromolecular structure, allowing gases to contact rapidly with various functional groups of different molecules. Third, the redox state of a metal center modulates the affinity of the binding of a gas ligand to a metal atom. Since the alteration of redox states is a hallmark of disease conditions such as ischemia and metabolic disorders, it needs to be taken into account. However, it adds a further challenge to elucidation of gas-signaling mechanisms *in vivo*. See review (Hishiki et al., 2012; Kajimura et al., 2010) for more comprehensive account on this subject.

Recent biochemical investigations of purified enzymes to correlate molecular structure of a heme binding pocket with functional relation (*e.g.* catalytic reaction) have found many answers for gas-sensing and gas-transduction mechanisms on the specific protein *in vitro*. How can we make a bridge between findings *in vitro* and solving problems *in vivo*? One approach could be to examine not only expressions of enzymes but also the abundance of substrates and cofactors of a gas-producing enzyme that is more likely to determine the rate of gas formation in the tissues with

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* Corresponding authors at: Department of Biochemistry, School of Medicine, Keio University, JST, ERATO, Suematsu Gas Biology Project, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel.: +81 3 5363 3461; fax: +81 3 5363 3466.
E-mail addresses: myk30@z5.keio.jp (M. Kajimura), gasbiology@z6.keio.jp (M. Suematsu).

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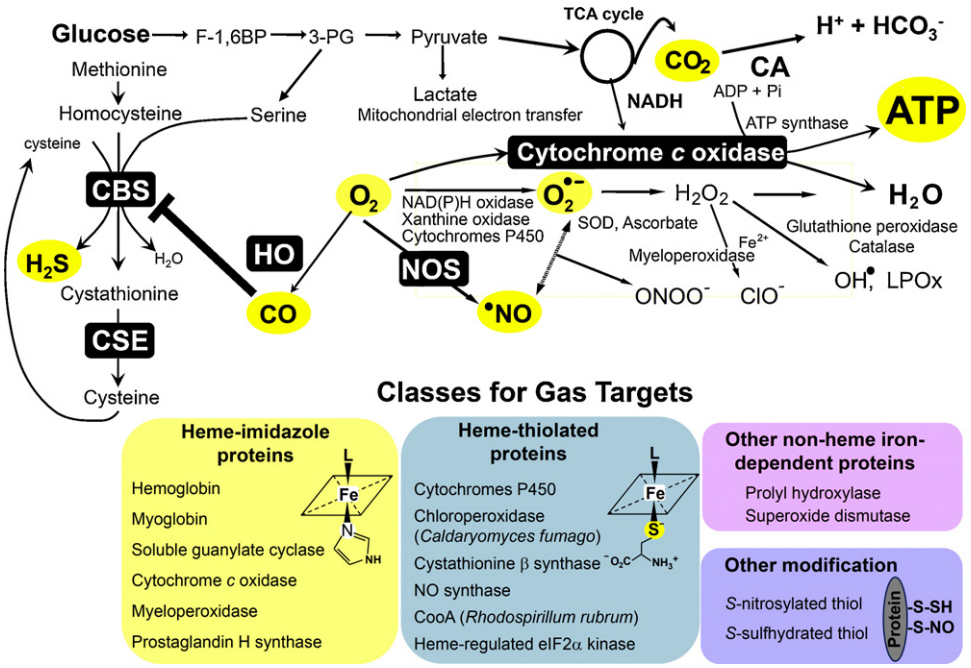

Fig. 1. Simplified metabolic links among cellular energetics, gas synthesis and gas targets. Molecular oxygen functions primarily as a terminal acceptor of electrons of the electron transport chain in the mitochondria. Most of the oxygen is reduced to generate water through the reaction of cytochrome *c* oxidase. However, a small fraction of oxygen is used to generate compounds including CO, NO, and reactive oxygen species. Gases exert biologic activity through interactions with macromolecules. These interactions involve; (i) the coordinate bonding of a gas ligand to prosthetic metal complexes in their receptor proteins leading to a wide range of interactions, depending on the strength of the bond formed, and (ii) their binding to the critical region for the protein function such as the cysteine thiol group. The interactions are further modulated by the redox state of the metal before ligand binding which is not shown in this scheme. Here gas targets are classified by four groups depending on the nature of the bond. L, ligand; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; HO, heme oxygenase; NOS, nitric oxide synthase; CA, carbonic anhydrase.
Adapted by permission from Suematsu et al. (2003).

spatial and temporal resolution. Imaging mass spectrometry combined with quantitative metabolomics can satisfy these criteria as it provides spatio-temporal profiles of many metabolites simultaneously. Comparing the metabolic footprinting from an animal model with a targeted deletion of a specific gas-producing enzyme induces logic to identify the sites of actions of the gas. This article aims to outline how these technical advances can help solve critical issues laid out above, with focus on physiological significance of coordinate actions of CO and H_2S and their relation to O_2 metabolism *in vivo* proposed in the recent literature.

2. Interaction of CO and H_2S: cystathionine β-synthase as a specific CO sensor

Recent literature indicates that heme oxygenase (HO)/CO and cystathionine β-synthase (CBS)/H_2S systems interface (Morikawa et al., 2012). What is a molecular mechanism of this interaction? CO derived from HO can regulate the activity of CBS, an H_2S-producing enzyme, which has been known as a CO-specific sensor *in vitro* (Taoka et al., 2002, 1999). However, it is only within several years that CO was found to control the function of CBS *in vivo* (Shintani et al., 2009). We start this section by providing a brief summary of structural characteristics of purified CBS *in vitro*. Then we describe how metabolomic approaches can be used to examine altered functions of this enzyme by CO.

2.1. Fundamental mechanisms of CO sensing by CBS in vitro

Many heme enzymes including cytochromes P450 were once considered putative CO-sensitive signal transducers. However, the ferrous heme of these enzymes has been found sensitive to both CO and NO, ruling them out as CO-specific sensors. By contrast, CBS remained a strong candidate for a CO-specific sensor. CBS was discovered as an interesting soluble heme protein that showed an absorption peak at 448-nm on its reduction without addition of CO (Kim and Deal, 1976). Since the 450-nm absorption peak of the CO-ligated P450 in the reduced state is the hallmark of cytochrome P450, it was named H450 as a 'pseudo-cytochrome P450′ (Omura, 2005). Subsequently, Omura et al. (1984) identified that the axial ligand at the 5th coordinate position is a thiolated anion, and the 6th position is occupied by histidine, confirming the heme-thiolated nature of this protein (Fig. 2A and B). Authors showed that adding CO causes the spectral shift of the absorption peak from 448 to ~420 nm, indicating that the thiolate-anion ligand of the heme is replaced with CO to produce a spectrum similar to the CO-ligated heme–imidazole protein (Omura et al., 1984). This is the first study suggesting the gas-sensing function of this enzyme.

Why is the heme-thiolated form useful to function as a sensor? This effect might derive from a weak, reversible binding of CO to the heme. Coordination of thiolate anion to heme is weaker than that of the imidazol group, particularly when the iron atom of the heme is in the ferrous state. This labile nature of the thiolate-anion ligand in the heme–thiolate proteins explains the functions of the protein as a sensor for detecting CO. In such a case, binding of CO to the heme results in the displacement of the thiolate-anion ligand and induces a conformational change of the protein moiety, which is transduced to a change in its enzyme activity (Fig. 2B). See review by Omura (2005) for more comprehensive account on gas-sensing mechanisms by heme-thiolated proteins.

2.2. CBS inhibition by CO in vitro

CBS is unique in that it is the only known pyridoxal phosphate (PLP)-dependent enzyme that possesses prosthetic heme

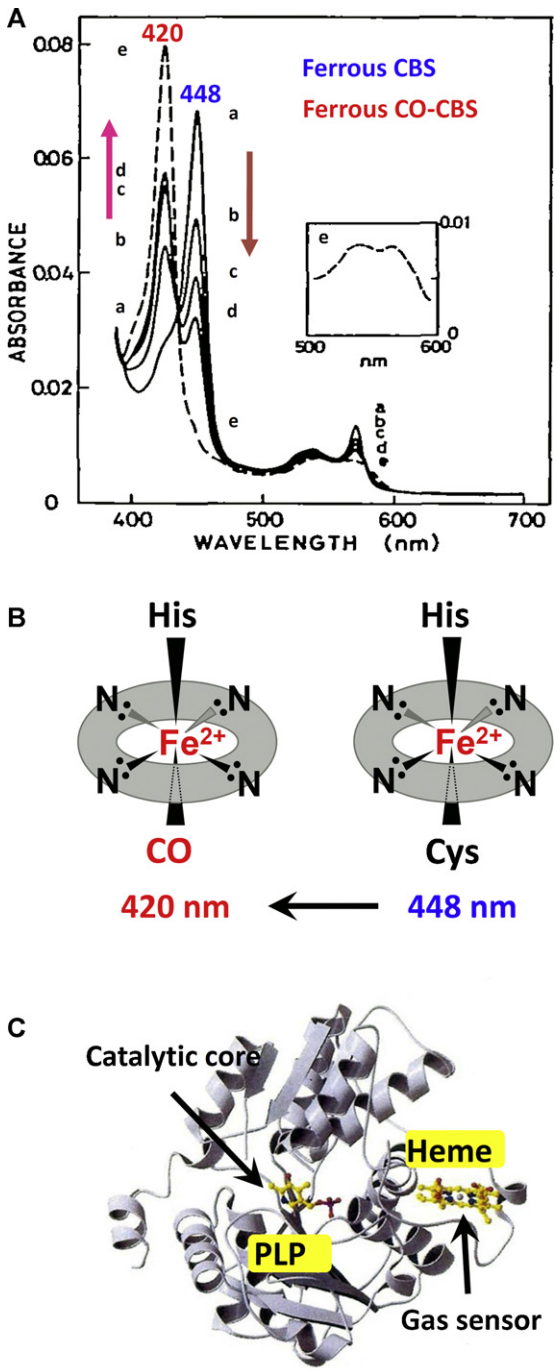

Fig. 2. Cystathionine β-synthase. (A) Spectral change of reduced purified CBS by the addition of CO. Adding CO shifts the absorption peak from 448 to 420 nm. The enzyme solution was first reduced with $Na_2S_2O_4$ and the absorption spectrum was recorded (a). The solution was then bubbled with CO and the absorption spectrum was recorded at 5 min (b), 10 min (c), and 15 min (d) after the CO bubbling. The spectral change was extrapolated to infinite time to depict the expected spectrum of the CO-bound form of the reduced enzyme (e, inset). The spectrum with 420-nm peak is similar to that of the CO-ligated heme–imidazole protein (left, B). The axial ligand at the 5th coordinate position is a thiolated anion, and the 6th position is occupied by histidine (right, B). (C) Crystal structure of the human CBS (PDB 1JBQ). The heme and PLP are presented in a stick model.

(B) Adapted by permission from Omura et al. (1984).

(Kery et al., 1994). H_2S can be generated by the condensation reaction of homocysteine and cysteine catalyzed by CBS (Fig. 2C) (see review by Singh and Banerjee (2011) for comprehensive reactions of H_2S biogenesis). The role of heme of this enzyme has been extensively studied. Original studies (Taoka and Banerjee, 2001; Taoka et al., 1999) using recombinant human CBS indicated that both CO and NO binding to the heme inhibit CBS activity. However, these studies and others using full-length rat CBS (Shintani et al., 2009) showed that the K_i value for NO (~320 μM) was exceedingly higher than that for CO (~5 μM). The result is striking because such a low K_i for CO suggests that CBS acts as a specific CO sensor *in vivo* under physiologic conditions.

In fact, reported values of CO concentrations from the mouse brain are in the range of 1–10 μM (Morikawa et al., 2012; Vreman et al., 2005). In these experiments sulfosalicylic acid was used to liberate CO from the CO-containing heme proteins and the components of the membrane of the tissue homogenates; thus, these values include both free and bound CO. On the other hand, free CO contents in the effluent from the isolated rat perfused liver (Kyokane et al., 2001; Suematsu et al., 1995, 1994) and the cultured medium of the rat hepatocytes (Goda et al., 1998) were determined spectrophotometrically by measuring the formation of the ferrous–CO complex of myoglobin. The steady-state generation of CO was calculated to be ~0.7 nmol/min/g of liver. When the differences in local flow rates between *ex vivo* and *in vivo* systems are considered, it appears that local concentrations of CO in the liver are approximately 1 μM (Suematsu et al., 1995, 1994). By contrast, tissue concentrations of NO are likely to be in the range of 0.1–100 nM (Bellamy et al., 2002; Buerk, 2001; Buerk et al., 2003), much lower than those of CO (see review by Kajimura et al. (2010) for tissue concentrations of gases).

Although the crystallographic structure of CO-ligated forms has yet to be determined, spectroscopic characterization of CO binding and dissociation kinetics to CBS suggest that disruption of a salt bridge between the Cys52 ligand to heme and Arg266 of the enzyme by CO binding is communicated to the active site with concomitant inhibition of enzyme activity (Puranik et al., 2006). While such a regulatory role for the ferrous heme of CBS has been clearly demonstrated *in vitro*, the existence of the ferrous state, of which CO can bind, has been controversial *in vivo* (Singh et al., 2007). Recent study showed the evidence for reversible inhibition of CBS by CO in the presence of a human flavoprotein and NADPH as redox partners (Kabil et al., 2011). These results *in vitro* provide a mechanistic basis for interactions between CO and H_2S *in vivo* discussed in Section 3.2.

2.3. Metabolomics analyses provide evidence for CBS as a CO-sensor in vivo

Differential display of metabolic footprint-profiling is designed to assess the control points by a specific intervention. Changes in patterns of metabolic fluxes on various pathways give a clue for a candidate enzyme responding to a gas. Shintani et al. (2009) applied this method to identify the enzyme on which CO targets by comparing metabolic responses between livers from control mice and those treated with hemin to increase CO production. CO overproduction increases metabolites in the remethylation cycle and simultaneously decreases those in the transsulfuration pathway, which occurs in parallel with a decrease in hepatic H_2S content. Subsequent *in vivo* pulse-chase analysis of ^{15}N-methionine in livers of control mice and hemin-treated mice showed accumulation of ^{15}N-homocysteine and suppression of ^{15}N-cystathionine under the CO-overproducing conditions, suggesting that CO inhibits CBS *in vivo*. The ability of CO to limit CBS activity as a regulator of the transsulfuration pathway may have diverse impacts on biological systems. Such a stress-inducible suppression of H_2S in the liver

stimulates HCO_3^--dependent choleresis (Shintani et al., 2009). The CO-sensitive metabolic adaptation may play a regulatory role in biliary excretion in which it facilitates solubilizing organic anions and/or xenobiotic metabolites in bile under disease conditions or detoxification processes (Fujii et al., 2005; Kyokane et al., 2001; Mori et al., 1999; Norimizu et al., 2003). Mechanisms by which H_2S modulates biliary excretion might involve glibenclamide-sensitive Na^+–K^+–$2Cl^-$ channels in the biliary system, although whether CO directly binds to the channel remains unknown. The ability of CO to interfere with CBS activity as a regulator of the transsulfuration pathway (Takano et al., 2010; Yamamoto et al., 2011) may have diverse impacts on biological systems such as cancer and ischemic diseases. See the recent review by Hishiki for more comprehensive account on this subject (Hishiki et al., 2012).

3. Coordinate actions of CO and H_2S under hypoxia

Recent literature shows that coordinate actions of CO and H_2S mediate acute adaptive responses against a decrease in O_2, (*e.g.* stimulation of breathing (Peng et al., 2010) and cerebral vasodilatation (Morikawa et al., 2012)), proposing a novel signaling of an O_2–CO–H_2S cascade.

3.1. The carotid body

Glomus cells of the carotid body sense O_2 deprivation in the arterial blood and initiate rapid homeostatic responses against hypoxia. The obligatory step in mediating sensory excitation by hypoxia is widely accepted to be an increase in intracellular Ca^{2+} through the opening of the L-type Ca^{2+} channel of glomus cells (Lahiri et al., 2006). Although this Ca^{2+} influx is attributable to cell depolarization *via* the closure of K^+ channels, identity of the effector K^+ channels and/or the mechanism that mediates O_2-sensitive changes in K^+ conductance remained elusive.

Regarding the identity of a K^+ channel, various investigators suggested that the large-conductance Ca^{2+}-activated K^+ (BK) channel is such an effector in glomus cells responsible for O_2-sensitive alteration of K^+ conductance (Lahiri et al., 2006; Peers, 1990; Williams et al., 2004). Li et al. (2010) showed that NaHS, an H_2S donor, induces an increase in nerve activity which is dependent on extracellular Ca^{2+} from the isolated carotid body/sinus nerve preparation which is reversed by a CO donor. As amino-oxyacetic acid, an inhibitor of CBS, impairs the response to hypoxia, these authors suggested that H_2S derived from CBS plays a role in sensory excitation by modulating the activity of the BK channels. Telezhkin et al. (2009, 2010) demonstrated that H_2S depresses K^+ conductance of BK channels on HEK 293 cells stably transfected with the human recombinant BK channel α-subunit and on isolated rat glomus cells using patch-clamp technique.

What might then be the oxygen sensor? What might be the molecular entity that directly couples the oxygen sensor to the effector? Peng et al. (2010) reported that the hypoxic responses of carotid bodies are impaired in mice lacking cystathionine γ-lyase (CSE), the other enzyme producing H_2S. In addition, both hypoxia and pharmacological inhibition of HO-2 evoke H_2S generation. Because HO-2 requires molecular O_2 for its activity, it has been proposed that stimulated action of the carotid body by hypoxia reflects reduced formation of CO which stimulates the BK channel; thus, HO-2 functions as an O_2 sensor (Prabhakar et al., 1995; Williams et al., 2004). Authors, thus, proposed that H_2S mediates O_2 sensing in the carotid body *via* the interaction of HO-2/CO and CSE/H_2S systems. Since CSE does not possess a prosthetic heme, a gas sensor described in Section 2, molecular mechanism by which CSE senses CO, and regulation of its activity remain to be answered.

3.2. The brain

The rodent brain generates a substantial amount of CO (~5 to 10 μM) (Vreman et al., 2005) *via* HO-catalyzed reactions using O_2 as a substrate where HO-2 accounts for ~80% of the total rodent brain HO activity (Ishikawa et al., 2005). Although it has been known that CO regulates neuronal transmission (Verma et al., 1993), physiologic roles of CO in the central nervous system (CNS) remain elusive.

Recently Morikawa et al. (2012) reported that the coordinate actions of HO-2 and CBS form a signaling system that mediates hypoxia-induced arteriolar vasodilation. Since the brain is the most susceptible organ to O_2 deprivation, this adaptive response is critical for delivery of O_2 and cellular transport of glucose in brain tissue. Immunohistochemical analysis in the mouse brain reveals expression of HO-2 in neurons and endothelial cells, whereas CBS is expressed predominantly in astrocytes (Fig. 3A and B). In this study hypoxia gives rise to cerebral vasodilation by inhibiting HO-2, which turns out to function as an O_2 sensor in the CNS. This notion of HO-2 as an O_2 sensor is supported by a K_m value of ~15 μM (~11 mmHg) of recombinant mouse HO-2 for O_2 *in vitro*, a suitable K_m value for an O_2 sensor to respond to changes in the brain tissue O_2 concentration (Ndubuizu and LaManna, 2007). As CO physiologically inhibits CBS (see Section 2), the enzyme that forms H_2S, hypoxia reduces the constitutive inhibition of CBS by CO so that increased levels of H_2S are formed which mediate rapid vasodilation of small arterioles (Fig. 3).

Such hypoxia-induced vasodilation of arterioles is attenuated in HO-2-null mice and completely lost in CBS-null mice, but well maintained in CSE-null mice (Fig. 5B), providing compelling evidence for the role of CBS in this mechanism. The observation appears to contradict with the role of CSE of glomus cells in the carotid body. However, the close examination of enzyme distribution may explain this discrepancy. CSE is absent in the cerebral cortex where the vascular response was examined, and is limited to vascular smooth muscle cells surrounding large vessels in the subarachnoid space. Expression levels of CBS and CSE vary between tissues and are differently distributed within the brain (Linden et al., 2008; Morikawa et al., 2012). Spatial relations among the gas-producing enzymes and their receptor systems are certainly an important factor to take into account.

Another important factor is the tissue concentrations of relevant gases. Morikawa et al. further demonstrated the potential for interactions of O_2, CO, H_2S by measuring endogenous CO and H_2S concentrations of the brain exposed varied O_2 concentrations. While hypoxia causes a decrease in CO concentrations and an increase in H_2S, HO-2-null mice do not exhibit such an O_2-dependent alteration of CO and H_2S.

3.3. Inverse relation of H_2S and O_2

Olson et al. (2006) postulated an interesting hypothesis that H_2S catabolism serves as an intrinsic O_2 sensor based on their results that H_2S is inversely related with O_2 in the trout gill chemoreceptors and pulmonary arteries of some mammalian species (Olson and Whitfield, 2010). Olson suggests that the relation of H_2S and O_2 can be analogous to the yin and yang and that the amount of H_2S itself is a universal O_2 sensor.

Not only the production but also degradation of H_2S determines the effective concentration of this gas. Regarding H_2S catabolism, sulfide-quinone reductases (SQR), the disulfide oxidoreductase flavoprotein superfamily, has gained much attention as it contributes to H_2S oxidation by phototrophic bacteria wherein H_2S donates electrons to the respiratory chain (Griesbeck et al., 2000). Whether or not SQR exists and/or plays roles in H_2S metabolism in the mammalian CNS is currently controversial (Ackermann et al., 2011; Lagoutte et al., 2010; Linden et al., 2011). The oxidation of

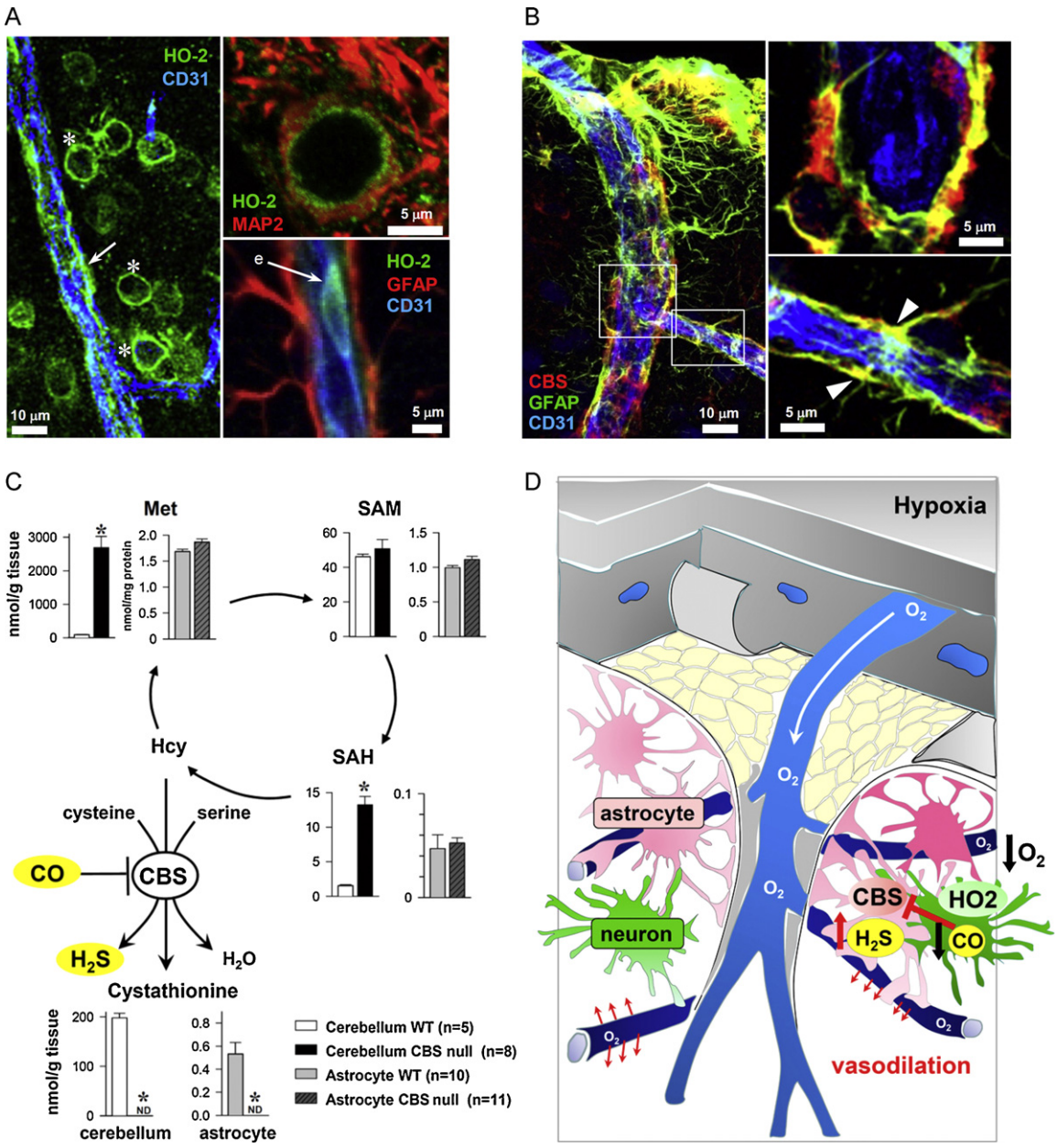

Fig. 3. CO- and H_2S-producing sites in the mouse cerebral cortex. (A) Immunohistochemical localization of HO-2 in the adult mouse cerebral cortex. HO-2 is expressed around nuclei of neurons (asterisks) and in endothelia (arrows). Note the perinuclear regions that exhibit HO-2 immunoreactivity. MAP2, microtubule associated protein 2, a neuronal marker; CD31, an endothelial marker; GFAP, glial fibrillary acidic protein, a glial marker. (B) Immunohistochemical localization of CBS in the adult mouse cerebral cortex. CBS is present in the astrocytic endfeet ensheathing the wall of diving arterioles (top right panel), and pre-capillary arterioles (bottom right panel). (C) Metabolomic analysis reveals that levels of cystathionine, another CBS product, are below the limit of detection in primary astrocytic cultures from CBS-null mice, suggesting the glial generation of H_2S. (D) Schematic depiction of the localization of HO-2 and CBS in the neurovascular unit of the mouse cortex. Astrocytes are well disposed to influence blood vessels, as they possess prominent endfeet directly contacting the vessels. Hypoxia-induced arteriolar vasodilation is mediated *via* a signaling system wherein HO-2 is the O_2 sensor. This drawing is modified from the original drawing of the astroglial relationships to the pia mater and the blood vessels by Reichenbach and Wolburg (2009). (A–C) Adapted by permission from Morikawa et al. (2012).

H_2S on the mitochondrial respiratory chain adds complexity in the O_2–H_2S signaling (Bouillaud and Blachier, 2011) and deserves further investigation.

4. New approaches to elaborate on the complex gas interactions with metabolic systems *in vivo*

What might be the feasible approaches to investigate such complexity and polymodal nature of gas interactions? Here we consider some of the governing factors controlling local gas amounts and actions; these include: (i) substrate and/or cofactor availability; (ii) enzyme control resulting from allosteric control and covalent modification; (iii) spatial distribution of enzyme expression in the tissue; and (iv) temporal regulation of gas generation. One approach is imaging mass spectrometry combined with quantitative metabolomics which satisfy several criteria as it can provide quantitative dynamics of many metabolites simultaneously with spatio-temporal resolution.

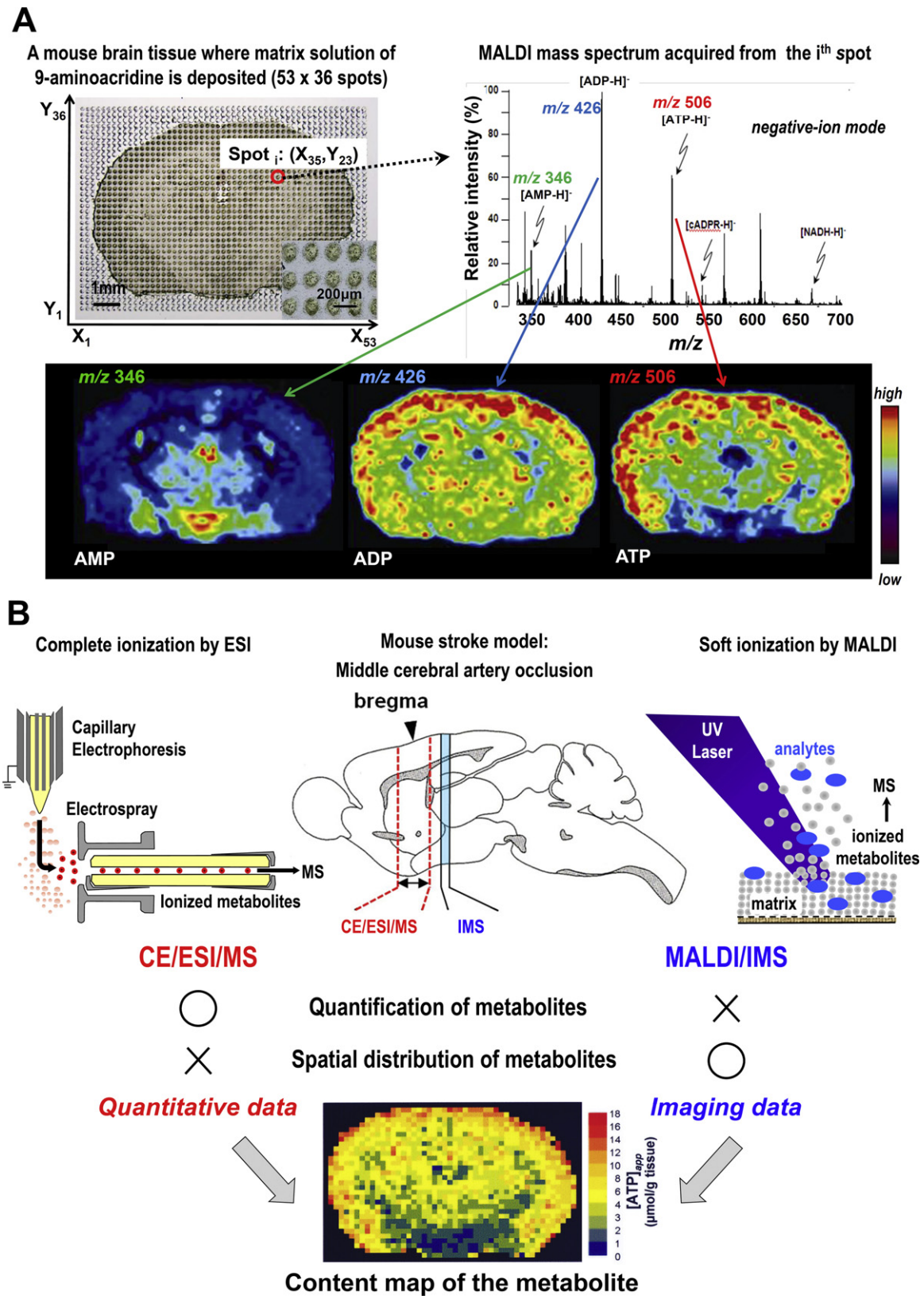

Fig. 4. Imaging mass spectrometry. (A) Technology developed for the spatial analysis of tissue by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS). Frozen brain sections (10-μm thickness) are deposited with an UV-absorbing matrix, in this case 9-aminoacridine, and set in the mass spectrometer. UV laser desorbs and ionizes analytes from the tissue and their *m/z* values are determined using a time-of-flight analyzer. From a raster over the tissue and measurement of the peak intensities over thousands of spots, mass spectrometric images are generated at specific molecular weight values. Images of many different molecules can be constructed from one tissue section. (B) Semi-quantitative IMS combines two advanced mass spectrometry analyses, MALDI-IMS and capillary-electrophoresis/electrospray ionization mass spectrometry (CE/ESI/MS) to estimate apparent content of a specific metabolite at the *i*th spot of tissue to construct a content map. Such content maps whereby abundance of metabolites is assigned in absolute terms, i.e. μmol/g tissue, make quantitative intergroup comparisons of local metabolic responses possible.
(A) Adapted by permission from Hattori et al. (2010).

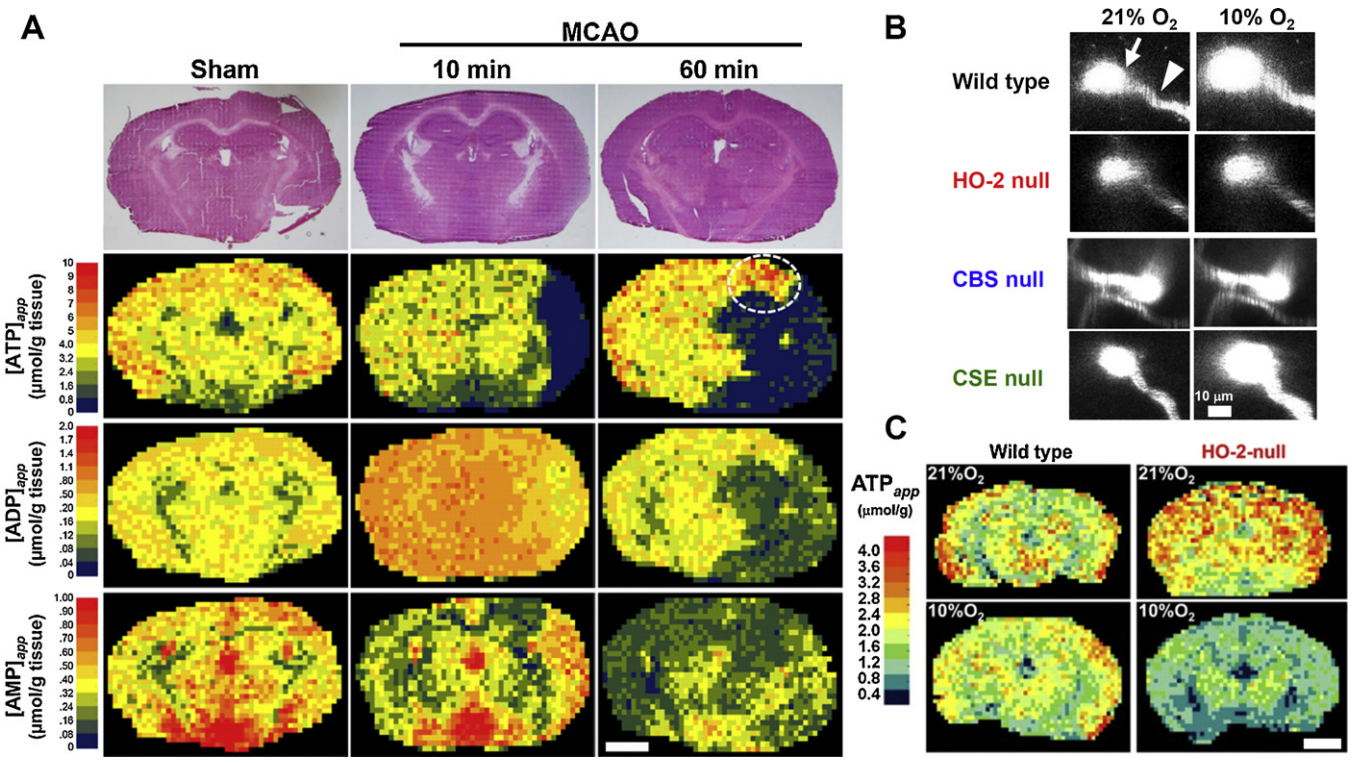

Fig. 5. Spatiotemporal changes in energy metabolism revealed by the semi-quantitative IMS. (A) Focal ischemia induced by a middle cerebral artery occlusion (MCAO) in the mouse brain. Substantial reduction in $[ATP]_{app}$ is seen in the ischemic core, whereas paradoxical elevation in $[ATP]_{app}$ is evident in the penumbra (circle). Sham underwent 60-min without occlusion. (Top) H&E staining after IMS. Scale bar: 2.0 mm. (B) Vasodilatory responses of penetrating (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 is injected *i.v.* to outline the vasculature. (C) Representative semi-quantitative IMS showing impaired ability of HO-2-null mice (right) to maintain ATP levels on exposure to 10% O_2 for 1 min. Note the basal increase in ATP in HO-2-null mice during normoxia (21% O_2 inhalation). Upon hypoxia (10% O_2 inhalation) ATP levels in HO-2-null mice are markedly decreased by 50%, while those in WT mice are maintained. Scale bar: 2.0 mm.

(A) Adapted by permission from Hattori et al. (2010). (B, C) Adapted by permission from Morikawa et al. (2012).

4.1. Semi-quantitative imaging mass spectrometry as a novel tactics to decipher metabolic dynamics of the brain

Hattori et al. (2010) combined two types of mass spectrometry (MS); matrix-assisted laser desorption ionization (MALDI)/MS and capillary-electrophoresis/electrospray ionization (CE/ESI)/MS. Unlike conventional spectroscopic techniques with which chemical profiles are obtained from one selected volume at a time, MALDI/MS has strengths in visualizing multiple metabolites in discrete areas with a single laser ablation (Harada et al., 2009; Kubo et al., 2011; Stoeckli et al., 2001) (Fig. 4A). However, it still requires further efforts to be supported for quantification. By contrast, CE/ESI/MS excels in quantification of metabolites (Kinoshita et al., 2007; Soga et al., 2006) since ESI is efficient in transferring molecules from liquid phase to gas phase. Comparison of transcriptional expression profiles with CE/ESI/MS based metabolomics can be used to reveal novel metabolic pathways (Tian et al., 2005) and their regulatory mechanisms (Kinoshita et al., 2007; Shintani et al., 2009; Tian et al., 2005). However, it removes spatial distribution of molecules due to tissue homogenization to extract metabolites. Combining imaging mass spectrometry (IMS) with CE/ESI/MS complements each other's weakness and enables to transform acquired mass signals of a metabolite in absolute terms such as tissue content in μmol/g. Thus, it is possible to construct maps of small-molecule metabolites whereby abundance of metabolites was assigned in the tissue. Such assignment of contents makes it possible to directly compare patterns of biochemical derangements in the tissue at different time points; which may help determine the multimodal-reaction points of gaseous mediators in the tissue (Fig. 4B).

Applying this technology to a mouse ischemic model using a middle-cerebral artery occlusion, altered energy metabolism is deciphered with spatio-temporal changes in adenylates and other metabolites. Unlike the core where ATP decreased, the penumbra displays paradoxical elevation of ATP despite the constrained blood supply (Fig. 5A). NADH elevated area in the ischemic hemisphere is clearly demarcated by the ATP-depleting core. Results suggest that metabolism in ischemic penumbra does not respond passively to compromised circulation, but actively compensates energy charges.

4.2. Regulatory roles of gases in energy metabolism on hypoxia

With semi-quantitative IMS, physiologic consequences of HO-2 loss in the CNS are in part unraveled. Namely, basal ATP content in the brain is increased by the deletion of HO-2, suggesting that CO marginally suppresses ATP production under a normoxic condition. Once the tonic inhibition is liberated by hypoxia, it gives way to the rise in dynamic strength of compensatory ATP maintenance. The cortex of HO-2-null mice whose neurovascular units lacking such a tonic inhibitory system cannot compensate ATP levels on hypoxia (Fig. 5C). The observation is consistent with previous studies indicating that pharmacological inhibition of HO increases the basal O_2 consumption in the liver (Sano et al., 1997) and that an increase in endogenous CO by the enzyme induction inhibits cellular respiration through its inhibitory effects on cytochrome *c* oxidase (D'Amico et al., 2006). Further investigation is required to reveal gas-mediated metabolic interactions among neuron, glia and microvasculature at cellular levels.

Goubern et al. (2007) showed that mitochondria of human colon adenocarcinoma cell lines utilize H_2S as an energetic substrate. Indeed it becomes increasingly evident that sulfide oxidation is coupled to ATP synthesis in not only bacteria but also mammalian cells under certain conditions (Bouillaud and Blachier, 2011; Lagoutte et al., 2010). Using murine vascular smooth muscle cells (SMCs) from mesenteric arteries, Fu et al. (2012) showed that CSE translocates from the cytosol to mitochondria upon the exposure to a calcium ionophore leading to an increase in the mitochondrial ATP production. These authors also demonstrated that exogenous H_2S improves ATP synthesis upon hypoxia, but not under normoxia, raising a possibility for a regulatory role of H_2S on energy production. Such a possibility deserves further investigation.

4.3. A monobromobimane-based assay for detecting a trace amount of endogenous H_2S in the tissue sample

Among O_2, CO, and H_2S, the determination of H_2S concentration in biologic samples appears to be the most challenging case due to the nature of this gas that is reversibility converted into different molecular entities of its related species. Reported values for H_2S concentration are highly variable in the last decade (Whitfield et al., 2008). However, current consensus is that H_2S concentration could be very low (Furne et al., 2008; Singh and Banerjee, 2011).

Monobromobimane, an electrophilic reagent typically used to analyze thiols, undergoes HS^--dependent sulfhydration to form a bis-S-bimane derivative (Shen et al., 2011; Togawa et al., 1992; Wintner et al., 2010). This thiol-specific reaction combined by mass spectrometry to detect the derivative is found to be sensitive enough to measure a trace amount of endogenous HS^- (Wintner et al., 2010). It should be noted that the method cannot differentiate free sulfide from the sulfide bound to various molecular entities such as persulfide (Wintner et al., 2010). Nevertheless, this method made it possible to measure endogenous HS^- of the mouse brain tissue under the condition where no exogenous substrates are added (Morikawa et al., 2012) (Fig. 6), which has been otherwise difficult to detect.

4.4. Experimental caution conducting 'gas biology' research

Gas dynamics is a direct function of tissue metabolisms, and *vice versa*. Because of experimental ethics, studies discussed in this

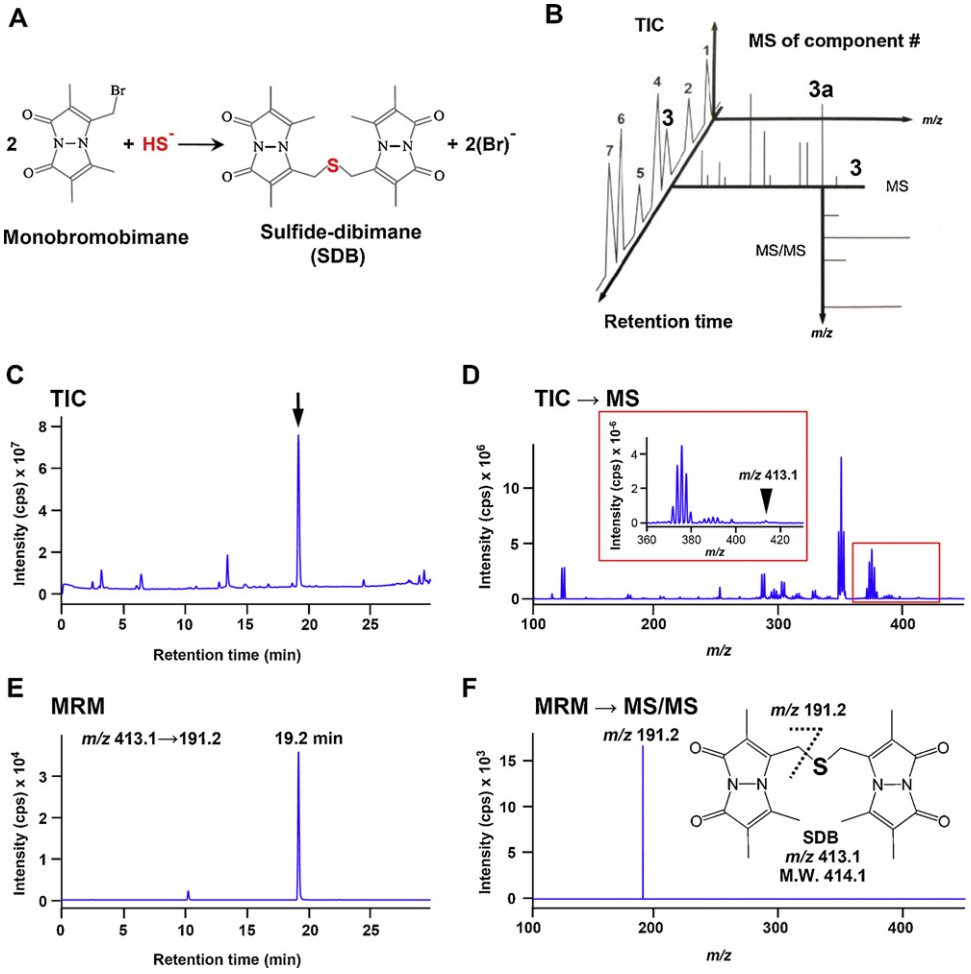

Fig. 6. Determination of endogenous H_2S production in the mouse brain tissue by LC/ESI/MS/MS using a monobromobimane-based assay. (A) The derivatization of hydrogen sulfide (hydrosulfide anion) with monobromobimane forming sulfide-dibimane (SDB). (B) Multiple reaction monitoring (MRM). Triple quadrupole instrument enables MRM operation as they combine tandem MS operation and fast switching of *m/z*. Here, compound *3* is chosen to illustrate the effect of selectively measuring the fragmentation of one precursor ion, *3a*, defined by its *m/z*. (C) An unresolved total ion chromatogram (TIC) in full-scan mode using brain extracts prereacted with monobromobimane. The arrow indicates a peak containing impurities and the target compound, SDB. (D) Negative-ion mass spectrum (MS) of the peak extracted from the TIC with 19.0- to 19.4-min retention time (arrow in C). (Inset) An enlarged spectrum between *m/z* 360 and 430, where the spectrum of the $[M-H]^-$ ion of SDB is indistinguishable (arrowhead). (E) MRM is conducted on the triple quadrupole to detect SDB. (F) MS/MS spectrum of $[M-H]^-$ ion at *m/z* 191.2 is defined as SDB. The chemical structure shows the SDB fragment assignment. Note the higher signal-to-noise ratio compared with the spectrum shown in *C*.

(A) Adapted by permission from Shen et al. (2011). (B) Adapted by permission from Gross (2011). (C–F) Adapted by permission from Morikawa et al. (2012).

article are conducted under anesthesia. General anesthesia evidently affects metabolism including O_2 consumption, so that experimental caution should be taken to interpret the results in the literature. See the review (Lindahl, 2008) for holistic view on this issue.

Whether the anesthesia impacts the CO generation is an intriguing issue from two points. First, changes in O_2 contents due to anesthesia might cause changes in HO activity as O_2 is a substrate for HO. Second, use of anesthesia might decrease intracellular NADPH concentration utilized by the HO reaction. The HO reaction starts with the formation of the ferric heme–HO complex. Subsequently ferric heme–iron is then reduced to a ferrous state by the first electron donated from NADPH-cytochrome P450 reductase. Since anesthesia including urethane, α-chloralose, and isoflurane are known to be metabolized by various cytochromes P450 systems (Restrepo et al., 2009), it is not unreasonable to speculate that use of anesthesia attenuates CO generation independently of O_2 content but rather due to a reduced NADPH availability caused by a competition for this electron donor between HO and P450 reductase. These points should be investigated in the future.

5. Future directions: where does 'gas biology' stand now?

While we are still waiting for new tools for visualizing and measuring of gaseous molecules *in situ*, the field of *Gas Biology* has added several cutting-edge technologies. Historically, it has not been easy to evaluate the brain tissue pO_2 especially in conscious unanesthetized animals as nicely reviewed by Ndubuizu and LaManna (2007). Recently the principle of O_2-dependent phosphorescence quenching of a newly engineered porphyrinic probe, platinum porphyrin-coumarin-343, combined with a two-photon approach revealed the PO_2 in the brain tissue and in the vasculature with high spatial and temporal resolution in three dimension (Sakadzic et al., 2010). Although currently limitted to the detection of Ag-halide clusters, unique development potentially offers the high resolution H_2S tissue map (Akahoshi et al., 2012). The method exploits high affinity of silver atom for sulfur and time-of-flight–secondary ion mass spectrometry (TOF–SIMS) for high sensitivity to detect trace elements. The tissue section is brought on the surface of nano-sized silver particles deposited on the silicon plates for the silver to react with tissue-derived H_2S. Furthermore, when combined with metabolome analysis, large-scale computational biosimulation of metabolism turned out to be a useful strategy to develop hypotheses on regulatory mechanisms for metabolic systems, as demonstrated by the study to predict novel roles of hemoglobin to trigger hypoxia-induced glycolytic activation through multiple enzymes (Kinoshita et al., 2007). High-performance affinity latex beads (Sakamoto et al., 2009) could offer a powerful method to elucidate gas-sensitive proteins in various experimental conditions.

Now that many biochemical investigations have made sound bases for the interactions of gas mediators at the level of purified enzymes, our hope is to bridge accumulated knowledge *in vitro* to solving problems *in vivo*. With the help of cutting-edge technologies, we should be able to gain new insights into the complexities of gas interactions and translate experimental work into new therapies to treat human diseases.

Author disclosure statement

No competing financial interests exist.

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