Cystathionine beta-synthase and PGRMC1 as CO sensors

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Abstract

Heme oxygenase (HO) is a mono-oxygenase utilizing heme and molecular oxygen (O_2) as substrates to generate biliverdin-IX α and carbon monoxide (CO). HO-1 is inducible under stress conditions, while HO-2 is constitutive. A balance between heme and CO was shown to regulate cell death and survival in many experimental models. However, direct molecular targets to which CO binds to regulate cellular functions remained to be fully examined. We have revealed novel roles of CO-responsive proteins, cystathionine beta-synthase (CBS) and progesterone receptor membrane component 1 (PGRMC1), in regulating cellular functions. CBS possesses a prosthetic heme that allows CO binding to inhibit the enzyme activity and to regulate H_2S generation and/or protein arginine methylation. On the other hand, in response to heme accumulation in cells, PGRMC1 forms a stable dimer through stacking interactions of two protruding heme molecules. Heme-mediated PGRMC1 dimerization is necessary to interact with EGF receptor and cytochromes P450 that determine cell proliferation and xenobiotic metabolism. Furthermore, CO interferes with PGRMC1 dimerization by dissociating the heme stacking, and thus results in modulation of cell responses. This article reviews the intriguing functions of these two proteins in response to inducible and constitutive levels of CO with their pathophysiological implications.

Key Words

CO, cystathionine β -synthase (CBS), progesterone receptor membrane component 1 (PGRMC1), neurovascular units, H_2S , cancer chemoresistance

INTRODUCTION

Gases constitute a group of smallest metabolites in biological systems. Most of gases are generated through breaking down fuel molecules such as carbohydrates, amino acids and other organic compounds generated in biological systems. The complexity of biological actions of gases and difficulties in measuring local gas concentrations obscures detailed mechanisms by which gases exert their biological actions, and many questions on direct macromolecular targets that allow gas binding remain unanswered in gas biology [1]. Among putative gas-responsive receptors, heme proteins appear to play central roles in gas-responsive mechanisms.

Among different gases, our attention has long been paid for carbon monoxide (CO) and its reception systems in mammals [2-6]. CO is generated by heme oxygenase (HO) through oxidative degradation of heme. Since gases are too small to handle for exploring specific receptors in biological systems, it had been technically difficult to mine CO-responsive receptors. However, two different strategic approaches, namely, metabolome analyses [7] and chemical biology using affinity nano-beads technology [8], allowed us to show that cystathionine β -synthase (CBS) and progesterone receptor membrane component 1 (PGRMC1) account for CO-responsive receptors [7, 9-11]. This review summarizes usefulness of such strategic approaches for deciphering the gas reception systems and their physiologic implications in mammals.

1. CO reception by heme proteins

1.1. General consideration *in vitro*

Some heme proteins have the ability to serve as sensors of O₂ and gas mediators such as NO, CO and H₂S. Such gas sensors function through the gas reception by heme iron and resultant alterations in protein structure and function of the signal-transducing site of the protein [1]. Determinants of these processes include redox states of the central iron of heme and the binding affinity of gases, the affinity of the axial ligand binding, and conformational changes within the protein triggered by the ligand binding/detachment, and the final outcome of the functional alterations of the protein. Among these determinants, the redox state of the heme iron plays a crucial role for selectivity of the gas ligand binding. The iron atom in the prosthetic heme can exist as either Fe²⁺ (ferrous) or Fe³⁺ (ferric) state that has the ability to form 6 coordinate bonds. Each of the lone pairs on the 4 nitrogen atom of the porphyrin ring can form a

coordinate bond with both forms of iron, holding it at the center of the porphyrin ring. As a result, two more coordinate positions, one above and one below the plane of the porphyrin ring are left. In case of myoglobin or hemoglobin, the globin attaches to one of these positions by utilizing a lone pair of the nitrogen molecule of the histidine residue that is known as proximal histidine. The other position is the point at which a gas forms the sixth coordinate bond [1].

Electronic configurations of the gas ligands such as O₂, CO, NO, and H₂S play crucial roles for binding these gases to heme. All of these gas molecules commonly possess lone pairs of electrons in their outer energy levels, making them active electron-lone-pair donors [1]. In case of myoglobin and hemoglobin, the ferrous form preferentially binds O₂ as the physiologic ligand, and CO and NO with distinctively greater affinities to these proteins. Conversely, the ferric form preferentially binds H₂S and H₂O (12), and anions such as CN⁻, N₃⁻, and OH⁻ (13). Differences in the binding affinity of individual gases to the prosthetic heme also depends on a choice of heme proteins, since structure of the space or pocket around the prosthetic heme that allows the entry of gases differs among different heme proteins [1].

Considering the electron density of the gas and the redox state of the prosthetic heme, CO binds to ferrous heme, but not to ferric heme. The bonding of CO to metal-centered prosthetic group (i.e. heme) is thought to have two steps. The first step is the donation of electron density from CO to the metal, “ π -donation”, while the second step is the back donation from the d π -orbital of the metal to an empty π^* anti-bonding orbital of CO, called “ π -back donation” [14]. The difference in energy between the d-orbital energy of the ferrous iron and the π^* -orbital energy of CO is relatively smaller than that of the ferric iron [1]. Consequently, CO can interact with the ferrous heme, but not with the ferric heme. When carrying out experiments to examine the interaction between CO and heme proteins of interests, researchers need to add dithionite, a reducing reagent that eliminates O₂ and maintains heme iron as a ferrous form in the test tube, since the prosthetic heme becomes ferric under ambient conditions at 21% O₂ in vitro.

1.2. General consideration in vivo

Deciphering molecular mechanisms for gas-responsive biological events in vivo has still been a great challenge because of the presence of cell membrane. Biological membranes serve as a diffusion barrier. The size and polarity of the gas molecules are determinants of their availability in and around the sites where the

gas-responsible receptor(s) occur. For example, distinct from CO and NO, H₂S has a greater dipole moment that appears to limit its permeation across the membrane [1]. H₂S possesses an acidic proton with a pK_a of 6.8, rendering this gas the anionic conjugate base like HS⁻ that is the predominant form at the physiologic pH 7.4 [15]

Comparison of the diffusion coefficient (D) allows us to estimate how far a gas molecule travels in a given time in cells and/or tissues. Travelling a 10-mm distance in water at 20°C, O₂ takes 0.021 seconds. On the other hand, H₂S, the D value of which is smaller, takes a slightly longer time, 0.028 s, for travelling the same distance [1]. The presence of gas-responsive receptors or enzymes degrading the gases within such a distance might alter effective diffusion distance. In the presence of hemoglobin in vitro, Longmuir and Roughton [16] compared the D values of CO and molecular nitrogen (N₂), which share comparable molecular weights (i.e., 28.0) and physical properties; measured values of D_{N₂} (2.8 x 10⁶ cm/s) and D_{CO} (3.2 x 10⁶ cm/s) at 20°C in 40% hemoglobin in water were comparable. Le Couteur, et. al. [17] examined CO disposition in the hemoglobin-free rat perfused liver; the studies compared differences in exogenously administered ¹⁴C-sucrose, ³H₂O and CO that were infused into the perfused liver and were measured in the downstream perfusate as a function of time to determine the recoveries and volumes of distribution of these molecules. Results indicated that CO distributes over greater volumes of distribution than sucrose, and even than H₂O, presumably because of the presence of large amounts of heme proteins in the liver. Estimation based on the results also showed that the activation energy for the CO transport across the cell membrane was ~0.6 kcal/mol, which was less than the value reported for O₂ diffusion across erythrocyte membrane [17]. Overall, CO is likely to cross cell membranes by simple diffusion, while possibilities involving unidentified rapid carrier mechanisms or facilitating transport processes for gases should be excluded. Collectively, it is not unreasonable to speculate that CO generated from heme oxygenase appear to bind to its receptor heme proteins and might exert its biological actions through both autocrine and paracrine manners.

2. CBS

2.1. *Homocystinuria and CBS deficiency in human*

Homocystinuria is a metabolic disorder of sulfur amino acids caused by genetic impairment of cystathionine β-synthase (CBS), and is characterized by increased accumulation of homocysteine (Hcy) in cells and plasma. Elevated Hcy results in various vascular and neurological complications. Pathogenic mechanisms by the

increased Hcy have been thought to involve several different processes such as direct Hcy toxicity against proteins which causes homocystinylation, superoxide generation by Hcy oxidation, impairment of cysteine biosynthesis by limiting transsulfuration pathway by functional impairment of CBS [18]. Deficiency of either vitamins B₆ or B₁₂, which supports the catalytic activity or recycling Hcy to methionine, respectively, mimics homocystinemia [18]. Other pathogenic mechanisms are down-regulation of H₂S (downstream effect) through suppression of H₂S-generating activities [7][9] or chemical cancellation of endogenous H₂S by Hcy to generate Hcy persulfides [19], and accumulation of remethylation metabolites and resultant alterations in methyltransferase activities (upstream effect); the latter mechanism might affect methylation of nucleotides and proteins, as discussed later [10].

Present clinical stratagem to suppress cellular and plasma homocysteine levels includes vitamin B₆ intake, dietary restriction of methionine, administration of folate and vitamin B₁₂. While these strategies are inefficient for treatment of homocystinuria, new strategies to treat homocystinuria has been provided. These mainly include enhanced clearance of Hcy from circulation by administration of polyethylene glycol-coated CBS; effectiveness of this translational product suggest that the enzyme replacement therapy for intact CBS is not only a promising approach for treating homocystinuria but also exemplify that such a therapy for metabolic disorders may not necessitate reconstitution of the deficient enzyme into its natural intracellular compartment [20].

CBS gene is encoded with chromosome 21; leukemia in patients with Down syndrome displays chemosensitivity and better prognosis. On the other hand, many studies reported roles of CBS polymorphism in enhanced chemoresistance in malignancy. Mechanistic views will be discussed later in the section 3.4..

2.2. CBS as an H₂S-generating enzyme

CBS catalyzes the rate-limiting step in the transsulfuration pathway to generate cystathionine and H₂O through the condensation of Hcy and serine [21] (**Fig.1**). When serine is replaced with cysteine, the CBS reaction generates cystathionine and H₂S. CBS displays varied enzymatic reactions according a choice and combination of substrates [21]. So far as judged from K_m values of substrates, the reaction in the presence of Hcy and cysteine or serine appears to account for a physiologically relevant mechanism for endogenous H₂S generation by this enzyme [22]. The second H₂S-generating enzyme is cystathionine γ -lyase (CSE) that converts cystathionine into cysteine, NH₃ and

α -ketobutylate. CSE also catalyses either cysteine or Hcy to generate H_2S , showing a distinct substrate selectivity from CBS that is unable to use Hcy to generate H_2S [21]. The third enzyme producing H_2S is 3-mercaptopyruvate sulfurtransferase (MST), which uses 3-mercaptopyruvate as a substrate [21]. H_2S generated in cells is degraded through sulfide quinone oxidoreductase (SQR), sulfur or persulfide dioxygenase and sulfite oxidase in mitochondria. Enzymology of H_2S generation and distinct biochemical properties of these enzymes were described in detail in the review by Kabil and Banerjee [21]. Among the three enzymes, CBS possesses prosthetic heme and seems to react with gases, as described in the following section.

2.3. *Identification of CBS as a CO-responsive enzyme in vivo*

Under atmospheric conditions in vitro where O_2 is supraphysiologically high, the prosthetic heme of a majority of heme proteins is ferric and does not allow CO to bind to. On the other hand, in living cells or tissues, many heme proteins experience reductive and oxidative cycles as enzymes or undergo constitutively reductive states in the presence of reducing systems (e.g. hemoglobin reductase for hemoglobin in erythrocytes). In case of CBS, enzymatic oxidoreductases such as methionine synthase reductase in the presence of NADPH appear to serve as the reducing system in vivo [23]. In order to explore CO-responsive heme enzymes under reducing conditions, we used several experimental models using cultured cells or animals in vivo.

Assuming that unidentified CO receptors might involve heme-containing enzymes in cells or tissues, it is not unreasonable to use metabolome analyses in experiments in vivo, where redox states of the prosthetic heme are maintained physiologically. Metabolome analyses in mice or cell culture that are exposed to CO-overproducing states allowed us to list metabolites that are up-regulated, down-regulated or indifferent in response to CO [7]. Paying attention to lists of up-regulated metabolites and those down-regulated, we are able to focus on several candidate metabolic pathways that involve putative enzymes responding to CO. To that effect, we carried out several experimental models, in which CO was overproduced, including acetaminophen-induced liver injury, heme-overloaded liver, and CO-treated cell culture models [7,10]. While patterns of alterations in metabolites in response to CO were different, we found that several metabolites displayed common responses of metabolites; modest increases in remethylation metabolites such as methionine and S-adenosyl-homocysteine (SAH), and decreases in cystathionine and total amounts of glutathione in cells or tissues. These results allowed us to hypothesize the presence of

unidentified CO-responsive enzyme(s) in and around remethylation and trans-sulfuration pathways.

Among enzymes in these pathways, we paid attention to CBS [7]. As discussed in the previous section, CBS is a heme-containing enzyme that was first identified as H-450, in which the addition of CO to its reduced form produced a specific spectral species which resembled that of the reduced CO-complex of a denatured form of cytochrome P450 [24]. Besides homocysteine and serine or cysteine [21], cystine accounts for another important substrate for CBS to generate cysteine persulfide that serves as other poly-sulfur reactive species [25] (**Fig.1**). In the absence of CO, the prosthetic heme of this enzyme is coordinated to histidine and cysteine as axial ligands [7, 26]. Exogenously added CO replaces the axial cysteine ligand to form the specific 6 coordinate structure of the ferrous CBS and inhibits its enzymatic activities. The previous study showing the reactivity of NO[•] to CBS heme showed that NO[•] at supraphysiological levels was able to bind to heme to form 5 coordinate structure, but did not inhibit CBS [26]. But the same group has recently provided evidence showing a rapid binding of NO[•] to CBS-ferrous heme and thus forming the five coordinate structure [27]. Whether such NO binding to CBS and regulation of the catalytic activities might play physiological roles should await further investigation. As seen in the following section of this review, the CO-CBS system constitutes intriguing systems for regulation of cells and organs in vivo. Until roles of endogenous CO in regulation of CBS activity was discovered in vivo, the physiologic roles of the prosthetic heme of CBS had not been unveiled [7, 9, 10].

2.4. CO/CBS/H₂S system regulates biliary function

In vitro studies using recombinant CBS have shown that CO acts as an apparent competitive inhibitor of CBS, with the K_i value of 5 μM, being comparable to physiologic concentrations of the gas [28, 29]. This notion led us to hypothesize that alterations in constitutive and inducible levels of CO from HO might regulate CBS to modulate H₂S. Observation that hepatocytes express both CO-producing HO-2 [4] and H₂S-producing CBS led us to hypothesize that CBS serves as a CO sensor for regulating liver function through endocrine mechanisms.

Livers under unstimulated control conditions generate measurable amounts of H₂S constitutively [7][30]. When exposed to acute CO overproduction triggered by acetaminophen or excess heme overloading, livers displayed suppression of constitutive H₂S generation [7]. On the other hand, the livers of heterozygous CBS knockout mice

showed comparable levels of constitutive H₂S generation [7], presumably because of compensatory H₂S generation in hepatocytes by other enzyme systems (e.g. CSE) [21]. When exposed to the CO-overproducing states, the heterozygous CBS-deficient mice exhibited only limited decrease in the constitutive H₂S generation. Considering differences in the substrate specificity between CBS and CSE which were described in the section 2.2., it is not unreasonable to speculate that the CBS deficiency in hepatocytes might cause compensatory Hcy-dependent H₂S generation via CO-insensitive CSE in the same cells. Thus, endogenous H₂S suppression by acutely overproduced CO depends on CBS at least in part [7].

Stress-inducible levels of CO exert its inhibitory action on CBS and thereby suppress endogenous H₂S generation in the liver. What is functional outcome of the liver in response to H₂S suppression ? Prior to our studies of the CO effect on H₂S, we showed that propargylglycine, a potent inhibitor of CSE causes HCO₃⁻-dependent increase in bile excretion [30]. Interestingly, exogenous administration of CO or excess heme results in HCO₃⁻-dependent biliary choleresis (elevated biliary excretion) that mimics effects of propargylglycine [7, 30]. Enrichment of biliary HCO₃⁻ excretion might benefit increased solubility of xenobiotic metabolites and/or many organic anions (e.g. direct bilirubin) in bile [31]. Previous studies showed that HO inhibitors eliminate endogenous CO and increase bile acid-dependent choleresis in perfused rat liver [32], and frequency of bile canalicular contraction in cultured hepatocyte couplets; these events are cancelled by CO supplementation [33]. Stress-inducible CO enriches HCO₃⁻ and glutathione excretion [31], while suppression of CO enriches bile acid excretion in bile. These observations collectively suggest that modulation of endogenous H₂S by stress-inducible CO play a role in a quality control of bile excretion under disease conditions.

2.5. CO/CBS/H₂S system regulates neurovascular units

Besides liver, testes and spleen, brain accounts for one of the largest source of CO derived from HO. HO-2 constitutes approximately 80% of the total HO activities in brain in rodents [34]. As indicated in **Fig.2A**, neurons and endothelial cells constitute the major site of HO-2 expression [9]. To note is that CBS is abundantly expressed in astrocytes; these results led us to hypothesize that neurovascular units constitute an intriguing functional apparatus regulated by multiple gases such as O₂, CO and H₂S [9]. Our previous study showed that constitutive CO generates vasoconstrictor tone in arterioles of neurovascular units, as judged by vasodilatory actions of HO inhibitors and

repression by local CO supplementation. Inhibition of HO-2 or cessation of O₂ as a substrate for CO generation with consequent activation of CBS generates vasodilatory H₂S in neurovascular units [9]. Accordingly, in HO-2-deficient mice, hypoxia fails to induce arteriolar dilatation. Furthermore, H₂S formed by CBS in astrocytes appears to play a crucial role in hypoxic vasodilatory responses of neurovascular units; the event is abolished in intact CBS-deficient mice but not in CSE-deficient mice (**Fig. 2B**)[9]. In these experiments, we utilized CBS-null mice with the C3H/HeJ background that ameliorates lethality of the CBS-null mice with C57BL/6J background, which rarely survive past the weaning age because of severe hepatic steatosis [35]. Maintenance of hypoxic vasodilation in CSE-null mice appears to result from little expression of CSE in cortical neurovascular units, if any, suggesting that CBS, but not CSE, serves as a major H₂S source in cortical neurovascular units [9]. On the other hand, CSE occurs in striatum and hippocampus, and its deficiency plays a critical role for neurodegeneration in Huntington's disease [36]. Considering differences in diffusion coefficient between CO and H₂S, availability of substrates for H₂S generation which were discussed in the sections 1.2. and 2.2., respectively [21], spatial relationship between CBS and CSE and the anatomical proximity of CBS versus heme oxygenases might be determinants of bioavailability of local H₂S and responsiveness to CO.

In the liver microcirculation, hepatic stellate cells, that is, microvascular pericytes express heme-containing soluble guanylate cyclase (sGC) constitutively relax sinusoids through utilizing abundant CO generation from hepatocytes [3,4]. In this organ, heme detoxification is coupled with maintenance of sinusoidal blood flow by CO/sGC system [2,3,4,28]. By contrast, vasotonic effects of CO on cortical microcirculation in brain are distinct from those observed in the liver.

What are metabolic impacts on brain that results from impaired microvascular dilatation in HO-2-deficient mice ? Quantitative imaging mass spectrometry (Q-IMS) allowed us to assess differences in energy metabolism in frozen-dried brain tissues between the wild-type and HO-2 deficient mice (**Fig.3**)[9, 37]. Amounts of AMP, ADP and ATP measured in the whole brain extracts are apparently comparable between the two groups (**Fig.3A**). However, Q-IMS revealed heterogeneity in regional contents of ATP in cortex and hippocampus, where HO-2 is highly expressed: the baseline ATP and energy charge are significantly greater in these regions of HO-2-deficient mice than those of the wild-type mice (**Figs.3B and 3C**). Interestingly, on short-term acute hypoxia with 10% O₂ inhalation, the wild-type mice manage to maintain ATP and energy charge without any significant decreases. On the other hand, HO-2-deficient mice exhibit significantly greater drop of ATP and energy charge. The results raise a

possibility that, under the baseline normoxic conditions, constitutive CO not only blocks CBS/H₂S but also partly limits oxidative phosphorylation, serving as “respiratory lock”. Once hypoxia occurs, the mechanism is unlocked through a decrease in CO that triggers arteriolar dilatation and helps compensatory delivery of O₂ and glucose for energy maintenance. The absence of such an adaptive vasodilatation in HO-2–deficient mice might compromise the ability of brain to compensate ATP levels under acute hypoxia.

2.6. *Regulatory roles of CBS for methylation of PFKFB3*

The inhibitory action of CO on CBS activity does not only suppress H₂S generation through trans-sulfuration pathway but also modulate methylation of proteins through regulating remethylation cycle [10]. Serendipitous observation in alterations in carbohydrate metabolomics in cancer cell lines exposed to CO or HO-1 induction allowed us to reveal that glucose biotransformation is shifted from glycolysis towards pentose phosphate pathway through mechanisms involving CO-CBS system [7]. This event results from de-methylation of phosphofructokinase/fructose biphosphate type-3 (PFKFB3), an enzyme to generate fructose 2,6-bisphosphate, an allosteric activator of phosphofructokinase 1 (PFK1) that rate-limits glycolysis. In cancer cells, PFKFB3 exhibits R131/134 arginine methylation that empowers glycolysis as “stable form” to drive Warburg effect [7]. After the cancer cells are exposed to stress conditions that cause HO-1/CO activation, PFKFB3 is de-methylated via inhibition of protein arginine methyltransferase-1, and becomes inactive for glycolytic activation (**Fig.4**) [7]. Once de-methylated, PFKFB3 is poly-ubiquitinated at K142 to be degraded rapidly [7, 38]. To be noted, K142 and R133 located in the same helix with the proximity to R131/134 methylation sites; the arginine methylation at R131/134 strengthens binding of negatively charged fructose 6-phosphate to R133 as the substrate-binding site, and might be less sensitive to ubiquitination at K142 [7] (**Fig.4**). Mechanisms for down-regulating methylation appear to involve up-regulation of S-adenosyl-homocysteine, an inhibitor of methyltransferases, which occurs as a result of CO-mediated CBS inhibition. Such a conversion of the enzyme activity by post-translational methylation/de-methylation switch causes a shift of glucose biotransformation towards pentose phosphate pathway (PPP) under stress conditions. Consequently, cancer cells up-regulate NADPH and acquire anti-oxidative properties through enhancing reduced glutathione (GSH) and increase nucleotide synthesis.

Neural cells are thought to be designed to diminish PFKFB3 through constitutive activation of mechanisms involving anaphase-promoting

complex/cyclosome (APC/C) activator protein Cdh1, a cofactor for poly-ubiquitination that help maintain reduced glutathione against oxidative stress through activation of PPP [39]. On the other hand, astrocytes highly express PFKFB3 to activate glycolysis and to supply lactate towards neurons. PFKFB3 in astrocytes has been reported to benefit attenuation of deposition of amyloid beta oligomer in mice [40]. However, careful investigation should be necessary to determine if the activity of PFKFB3 is determined merely by ubiquitination or by a subtle balance between methylation/de-methylation and ubiquitination in neural systems.

3. PGRMC1

3.1. *PGRMC1: pathophysiological roles and protein interactors*

PGRMC1 was first cloned as one of putative membrane receptors for steroids in human; two genes termed Hpr6.6 and Dg6 were cloned [41]. Hpr6.6 was the human homolog of a previously cloned porcine progesterone binding protein. Hpr6.6 mRNA was reported to occur predominantly in liver and kidney, whereas Dg6 mRNA is preferentially expressed in placenta. Hpr6.6 is located on the X chromosome and was named PGRMC1 afterward. PGRMC1 is expressed in liver, kidney, neural cells, ovary [42], and inner zonation of adrenal cortex [43]. Anti-apoptotic effects of progesterone were reported to depend partly on PGRMC1 signaling in neurons [44] or in immature ovary [45][46], while mechanisms by which PGRMC1 transduces protective signals have long been unknown. Primary structure of PGRMC1 contains heme-binding motif, and actually it allows heme binding [47]; again, functional roles of the heme remained to be solved. Biological significance of PGRMC1 emerged as its binding proteins were revealed: Huges, et. al. showed that PGRMC1 binds to cytochromes P450 to help xenobiotic metabolisms [48]. Ahmed, et. al. discovered it binds to epidermal growth factor receptor (EGFR) to regulate chemoresistance against erlotinib [49]. Since PGRMC1 was shown to be expressed in many of solid tumors [50][51], mechanisms for interactions between PGRMC1 and its target proteins cytochromes P450 and EGFR has attracted great attention of cancer researchers.

Besides cytochromes P450 and EGFR, Sigma-2 receptor is a crucial protein that might be necessary to understand PGRMC1 function. Sigma receptors, both Sigma-1(S1R) and Sigma-2 (S2R), are small molecule-regulated, primarily endoplasmic reticulum (ER) membrane-associated sites. A number of drugs bind to sigma receptors, including the antipsychotic haloperidol and (+)-pentazocine, an opioid analgesic. Sigma

receptors are implicated in many central nervous system disorders, in particular Alzheimer's disease and conditions associated with motor control, such as amyotrophic lateral sclerosis. Radio-labeled ligand binding assays were widely used for the pharmacological characterization of S1R and S2R. Methods detailed include a radio-ligand saturation binding assay for defining receptor densities and a competitive inhibition binding assay using [^3H]-1,3-di(2-tolyl)guanidine ([^3H]-DTG), a nonselective sigma receptor ligand, are described for conducting a saturation binding and competitive inhibition assays for the S2R site [52]. Researchers screened structurally different sigma-2 ligands that have a high affinity to S2R. Among them, Xu, et. al. reported that PGRMC1 protein complex serves as the sigma-2 receptor binding site by using newly developed fluorescent probe with high S2R affinity [53]. Recently, evidence showing that PGRMC1 (25 kDa) and S2R (18kDa) are different proteins was provided from 2 groups [54][55]. Both proteins serve as potentially therapeutic targets to control cancer and neuroregenerative disorders [56][57]; Izzo, et. al. has recently revealed synaptotoxic roles of PGRMC1 in Alzheimer's disease [57]. Considering these findings, there remains a possibility that PGRMC1 and S2R forms a complex or interact with each other to regulate cell function. However, structural identification of these proteins or how Sigma-2 ligands affects the receptor function remained largely unknown. Until now, discernible molecular entity and structural information of S2R are lacking.

3.2. *PGRMC1 as a sensor for heme and CO*

Use of metabolome analyses for mining CO-responsive proteins allowed us to identify CBS as a controller of H_2S generation, which contributes to energy management of neurovascular units, and as a regulator of glucose metabolism through switching protein arginine methylation/de-methylation of PFKFB3. However, metabolome analyses allowed us to mine only CO-responsive enzymes and overlook proteins without catalytic activities that become visible by screening alterations in metabolites. To challenge this difficulty, we have applied chemical biology to explore functionally unidentified CO-responsive proteins that are not enzymes. While the direct conjugation of gaseous molecules such as CO to affinity nano-beads to fish receptors seems impossible, we conjugated heme or iron-free protoporphyrin-IX to the affinity nano-beads [8] to mine candidate proteins. Since CO has the ability to bind to the prosthetic heme but not to iron-free protoporphyrin-IX, we chose proteins displaying the affinity to heme alone (**Figs. 5A and 5B**)[11]. Among such proteins with heme-binding affinity, we chose PGRMC1, because its structure-function relationship

remained totally unknown as described in the previous section.

While suppression of PGRMC1 expression inhibits cancer growth as described above, mechanisms by which PGRMC1 facilitates cancer growth has not fully been examined. We succeeded in crystallography of heme-bound PGRMC1 cytosolic domain at 1.95Å (**Figs. 5C-5E**), and revealed that it forms a stable dimer through stacking interactions of two protruding heme molecules (**Fig.5D**)[11]. The heme iron is five-coordinated by Tyr113 of PGRMC1 (**Fig.5E**), and the open surface of the heme mediates dimerization. To be noted is that CO interferes with PGRMC1 dimerization by binding to the sixth coordination site of the heme. In cancer cells, heme-mediated PGRMC1 dimerization is necessary to interact with EGFR (**Fig.6A**)[11]; such interactions with PGRMC1 dimer facilitate the EGF-mediated proliferation signaling (**Fig.6B**), and development of hepatic metastasis in xenograft transplantation model (**Fig.6C**). PGRMC1 knockdown enhances chemosensitivity to erlotinib, and overexpression of the wild-type but not that of Y113F mutant PGRMC1 recovered resistance to erlotinib, an EGFR inhibitor (**Fig.6D**)[11]. Since the site-directed mutagenesis of Y113F blocks heme binding, these results suggest that PGRMC1 dimerization via heme stacking is critical to facilitate EGFR-mediated signaling events. PGRMC1 interacts with CYP3A4, a P450 responsible for inactivation of doxorubicin, and facilitate detoxification into doxorubicinol to acquire chemoresistance [11]. Either CO supplementation or heme deprivation in cancer cells cancels PGRMC1 dimerization, and thereby suppresses the downstream effects via EGFR and P450s (**Fig.7**)[11].

3.3. *Roles of CBS and PGRMC1 for chemoresistance*

As previously shown in our review article elsewhere [58], researchers in the fields of clinical oncology have paid much attention to CBS as a key enzyme of cancer development. Child patients with Down syndrome (DS) with acute myeloid leukemia display greater event-free survival rates versus those with non-DS acute myeloid leukemia [59]; chromosome 21 trisomy is responsive for CBS overexpression in the patients. On the other hand, CBS dysfunction resulting from polymorphism or loss-of-function mutants causes impaired metabolism of remethylation cycle and hyperhomocysteinemia, leading to an elevated risk of head and neck cancer, meningioma, and colorectal adenoma and cancer [60, 61, 62]. As seen in **Fig. 7**, PGRMC1 has the ability to sense both intracellular heme and CO [11]. Roles of heme-associated iron in cancer development attracted attention of clinical oncologists [63]: Excess dietary intake of heme is a risk factor for several types of cancer. Previous

studies showed that deprivation of iron or heme suppresses tumorigenesis [64, 65]. On the other hand, CO interferes with tumor development [66]. Considering the fact that heme is necessary to generate CO from HO, a subtle balance between free heme and CO in and around cancer microenvironments plays a crucial role in cancer development.

Thus, careful discussion should be required for addressing anti-proliferative effects of CO on cancer cells. Sensitivity of cancer cells to erlotinib or doxorubicin is enhanced with acute heme deprivation by succinyl acetone, a potent inhibitor of heme synthesis, or exogenously applied CO that interferes with PGRMC1 dimerization [11]. In the study using human-derived ovarian cancer, PGRMC1 knockdown renders cancer cells less sensitive to cisplatin [67]. On the other hand, CO inhibits CBS and contributes to a shift of glucose biotransformation towards PPP that enhances NADPH/GSH system [10]. Through this mechanism, cancer cells acquire chemoresistance against cisplatin, too [10, 68]. Recent studies revealed that EGFR interacts with system xc(-) (xCT/CD98hc complex) and benefits accelerated cystine uptake to maintain GSH for cancer survival [69], while it remains unknown whether CO-mediated dissociation of PGRMC1 blocks this process. For cancer cells under quiescent phases, CO-mediated shift towards PPP might help biomass synthesis for nucleotides [38]. Cancer cells incorporate cysteine for generating GSH through xCT as well as trans-sulfuration delivery from methionine pool [70]. Actions of the heme-HO-CO system on CBS and PGRMC1 in cancer deserve further studies provided for evidence that regional characteristics of heme and/or thiols serves as a metabolic niche for cancer development in specific organs including liver, brain and bone marrow, where CO and its receptors might function as regulatory mechanisms for tumor dormancy or cancer stemness.

4. Future outlook: Mind CO sensors in brain

Critical role of endogenous CO for a bioregulator was first discovered as a neurotransmitter [71]. Our discovery of actions of endogenous CO as a vasodilator in the liver shared the same soluble guanylate cyclase [2, 3]. Many previous studies showed that CO and NO may act as retrograde messengers for long-term potentiation in hippocampus [72], while HO-2-deficient mice appeared to display no detectable phenotypes of memory disturbance [73]. CO may also contribute to chemoreception and neural regulation of O₂ sensing in carotid body [74]. CO has the ability to regulate conductance of potassium channels and serves as an alternative mechanism for vasorelaxation or O₂ sensing besides soluble guanylate cyclase, while molecules to

which CO directly bind remain unknown [75]. In these days, researchers had believed that CO might be a gaseous mediator that substitutes or modestly mimics activation of soluble guanylate cyclase by NO. However, understanding differences in chemical properties to react with heme proteins between NO and CO [76] led researchers to inquire if any CO receptors which are not triggered by NO might exist. At the same time, in parallel with progresses of CO biology [77, 78], we asked a question as to why brain generates huge amounts of CO through HO activity. As mentioned above, protective roles of PFKFB3 have been reported in astrocytes to attenuate amyloid beta deposition and resultant cell death [40]. There are several studies suggesting that HO-1 plays a protective role in deposition of amyloid beta oligomer in neural systems [79][80]. As mentioned earlier, PGRMC1 was reported to mediate the oligomer binding and synapto-toxicity [37]. Emerging evidence for CO-responsive CBS and PGRMC1 will allow researchers to build a new hypothesis that deserves further studies to solve the gas mystery and hopefully to envision clinical solution of intractable neural diseases.

Acknowledgments

This work was supported by JST, ERATO, Suematsu Gas Biology Project, Tokyo 160-8582. Y.K. is the leader of CREST, JST. The contents of this article were presented in the 10th World Congress for Microcirculation on September 27, 2015 in Kyoto.

The present affiliation of M.S. is President, Japan Agency for Medical Research and Development (AMED) with no conflict of interests to be declared. PGRMC1 crystallography was partly supported by Platform for Drug Discovery, Informatics, and Structural Life Science from MEXT, Japan (T.K.).

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Figure Legends

Figure 1. CO generation by heme oxygenase (HO)-1 and -2 and its effects on CBS activities. Met; methionine, SAM; S-adenosyl-methionine, SAH; S-adenosyl-homocysteine, Cys-SSH; cysteine persulfide, PFKFB3; phosphofructokinase/fructose biphosphatase type-3, PPP; pentose phosphate pathway, GSH; reduced glutathione, GSSG; oxidized glutathione.

Figure 2. Microanatomical orientation of HO-2 and CBS and hypoxic vasodilatation of arterioles in neurovascular units of mice. Citation from Morikawa, et al [9]. **A.** Immunohistochemistry of HO-2 and CBS in the neurovascular units of neonatal mouse cerebellar cortex. *Upper panels:* HO-2 is abundantly expressed in Purkinje cells (asterisks), while CBS, an H₂S-producing enzyme, occurs predominantly at the ascending processes of Bergmann glia or astrocytes. *Lower panels:* Magnified images show that HO-2 immuno-reactivities surround microvessels and overlap with CD31, an endothelial marker. On the other hand, CBS occurs in radial processes of astrocytes (arrowhead), as evidenced by the colocalization with GFAP. e, endothelium. **B.** Hypoxia-induced vasodilation of diving and pre-capillary arterioles in vivo is attenuated in HO-2-deficient mice (red) and abolished in CBS-null mice (blue). *P < 0.05 compared with WT mice. Hypoxia-induced vasodilation of precapillary arterioles is abolished in CBS-null mice (blue), but not in CSE-null mice (Green).

Figure 3. Impaired ability of HO-2-null mice to maintain ATP levels on exposure to acute short-term hypoxia at 10% O₂. Citation from Morikawa, et al [9]. **A.** Alterations in AMP (AMP_{whole}), ADP (ADP_{whole}), ATP (ATP_{whole}), and energy charge (EC_{whole}) in the whole brain. The concentrations of adenylates were determined by CE-MS. *P < 0.05 compared with the wild-type (WT) normoxia; †P < 0.05 compared with HO-2-deficient normoxia. **B.** Quantitative imaging mass spectrometry (Q-IMS) showing spatial distribution of apparent ATP concentrations (ATP_{app}) and regional energy charge (EC_{reg}). Note the basal increase in ATP in HO-2-deficient mice. cx, cortex; hp, hippocampus. **C.** Quantitative analysis of regional ATP concentrations and energy charge in WT and HO-2-deficient mice. *P < 0.05 as compared with WT normoxia; †P < 0.05 as compared with HO-2-null normoxia.

Figure 4. A schematic diagram for regulation of directional glucose metabolism via PFKFB3 methylation by CO/CBS system. Citation from Yamamoto T, et al.

[10]. **A:** Stress-induced CO generation inhibits the CBS activity, and results in de-methylation of PFKFB3. Protein arginine methylation of PFKFB3 (R131/134) stabilizes the enzyme to accelerate glycolysis through the up-regulation of F-2,6-BP, an allosteric activator of PFK1. Demethylation of PFKFB3 is subject to polyubiquitination at K142, causing the ubiquitin/proteasome pathway-dependent degradation of the enzymes. As a result, methylated PFKFB3 is decreased to shift glucose biotransformation towards PPP. **B:** Structural proximity of R131/134 with R133, the substrate binding site, and K142, the ubiquitination site.

Figure 5. Chemical biological exploration of PGRMC1 and its crystallographic analyses. Citation from Kabe Y, et al. [11]. **A.** High-performance affinity nanobeads conjugated with heme and iron-free protoporphyrin-IX (PP-IX). Beads contain ferrite particles. **B.** Gel chromatography showing proteins fished by the heme-conjugated nanobeads. **C.** Crystal of PGRMC1-heme complex. **D.** Structure of the PGRMC1 dimer formed through stacked hemes at 1.95 Å resolution. Two PGRMC1 subunits (blue and green ribbons) dimerize via stacking of the heme molecules. **E.** Heme coordination of PGRMC1 with Tyr113.

Figure 6. PGRMC1 dimer interacts with EGFR to facilitate cancer growth. Citation from Kabe Y, et al. [11]. **A.** FLAG-PGRMC1 wild-type (wt) and Y113F mutant proteins (a.a.44-195), in either apo- or heme-bound form, were incubated with purified EGFR and co-immunoprecipitated with anti-FLAG antibody-conjugated beads. Input and bound proteins were detected by Western blotting. **B.** HCT116 cells expressing control shRNA or those knocking down PGRMC1 (PGRMC1-KD) were treated with EGF or left untreated, and components of the EGFR signaling pathway were detected by Western blotting. HCT116 cells were also treated with or without EGF, RuCl₃ and CO-releasing molecule (CORM3). Phosphorylation of downstream components of the EGFR signaling pathway was detected by Western blotting. **C.** Xenograft transplantation showing tumor-bearing livers of super-immunodeficient NOG mice at 10 days after intrasplenic injection of HCT116 (control) or PGRMC1-knockdown cells. The cancer cells were fluorescent because of Venus expression. **D.** Effects of PGRMC1 knockdown (KD) and exogenous expression of the wild-type (WT) and Y113F mutant PGRMC1 on sensitivity of HCT116 cells to Erlotinib. HCT116 (control) cells, PGRMC1-KD cells or PGRMC1-KD cells expressing shRNA-resistant PGRMC1 wt or Y113F. The data represent mean±s.d. of 4 separate experiments. *P<0.01 using ANOVA with Fischer's LSD test.

Figure 7. Schematic diagram for the regulation of PGRMC1 functions. Citation from Kabe Y, et al. [11]. Apo-PGRMC1 exists as a monomer. On binding to heme, PGRMC1 forms a dimer through stacking interactions between the heme moieties, which enables PGRMC1 to interact with EGFR and cytochromes P450, leading to an enhanced proliferation and chemoresistance of cancer cells. CO interferes with the stacking interactions of the hemes and thereby inhibits PGRMC1 functions for EGFR and P450s.

Fig 1

Fig.2

Fig.3

A **B**

Fig. 4

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Fig.6

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