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Forum Review

Mechanistic Probing of Gaseous Signal Transduction in Microcirculation

MAKOTO SUEMATSU, KAZUHIRO SUGANUMA, and SATOSHI KASHIWAGI

ABSTRACT

Nitric oxide (NO) and carbon monoxide (CO) serve as activators of soluble guanylate cyclase (sGC) *in vitro,* and the latter serves as a microvascular relaxant for the liver, a major organ for heme oxygenase-dependent heme degradation and gas generation. Another important determinant of local sGC activities is superoxide anion, which scavenges NO and/or activates sGC directly. Altered bioavailability of the oxygen-derived species and its functional outcomes remain unknown, because information on amounts and distribution of these molecules has hardly been examined *in vivo*. Our recent studies provided evidence for such complex actions of multiple gases *in vivo.* Intravital visualization of NO in microcirculation revealed that two distinct sources, NO synthase-1 and -3, play a major role in the maintenance of NO in arteriolar and venular walls, respectively. Besides its vasorelaxing action in the hepatic microcirculation, CO could induce vasoconstriction in the resistant artery where NO is abundantly available; systemic blood pressure was elevated in transgenic mice overexpressing heme oxygenase-1 site-specifically in vascular smooth muscle cells. Such a relationship between the gases has also been demonstrated by mechanistic bioprobing of sGC function using novel monoclonal antibodies. This article aims to provide an overview of advances in visual assessment of the generation and reception of oxygen-derived gaseous mediators *in vivo*. *Antioxid. Redox Signal.* 5, 485–492.

BACKGROUND

MOLECULAR OXYGEN functions primarily as a terminal acceptor of electrons on mitochondrial electron transport. Most of the oxygen consumed in this process is reduced to generate water through the reaction of cytochrome oxidase. Only a small fraction of oxygen is used to generate compounds that exert potent biological actions; such compounds include prostaglandins, oxidized phospholipids, reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radical, and gaseous molecules such as nitric oxide (NO) and carbon monoxide (CO) (Fig. 1) (25). Amounts and localization of all these species depend on supply of molecular oxygen and expression of the generator enzymes, and their functional outcomes after various intracellular signaling cascades were examined by the proximity of receptor proteins to the gas-generating systems. However, such topographic information has not been available because of technical limitations to detect behavior of these small molecules as discernible molecular entities and to visualize functional alterations in the gas signal transducers in vivo. We have attempted to overcome such difficulties to visually prove mechanisms for gaseous signal transduction in cellular components involving endothelial cells, vascular smooth muscle cells, pericytes, mast cells, and platelets and leukocytes in microcirculation. This article for the Forum aims to summarize our methods that have shed new light on mechanisms whereby multiple gases execute their ability to fine-tune functions of microcirculation.

Department of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University, Tokyo 160–8582, Japan.

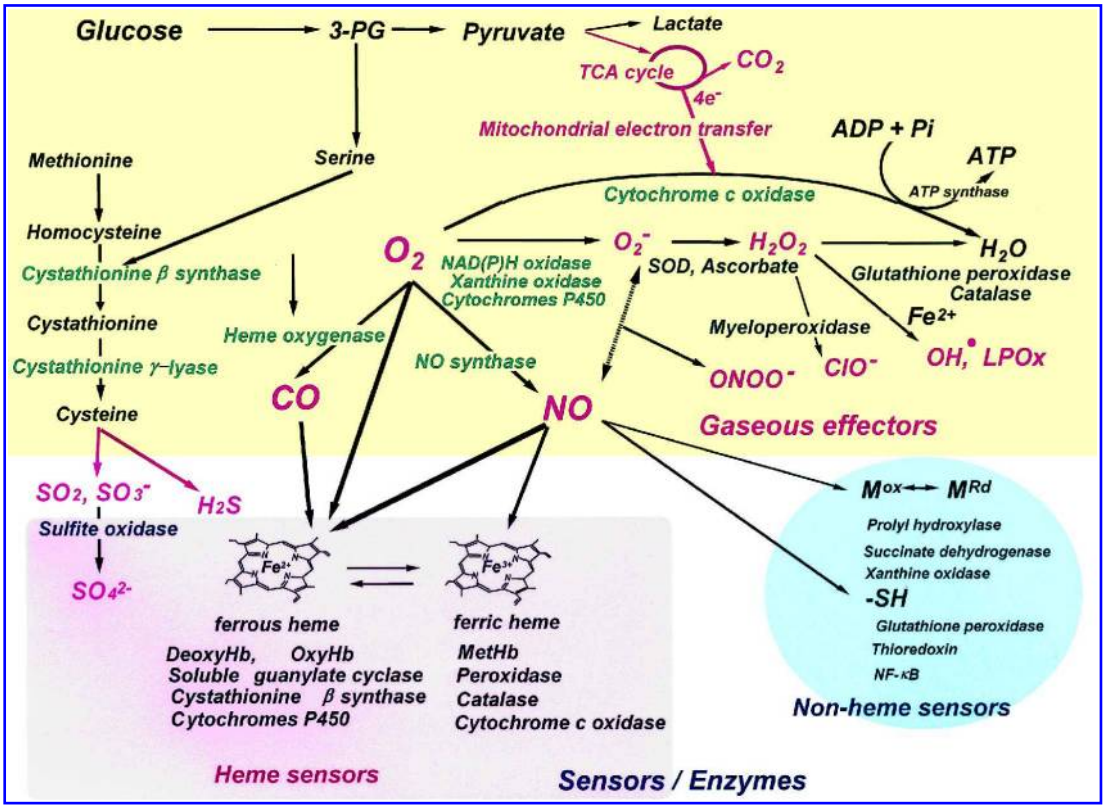

FIG. 1. Metabolic links between cellular energetics and synthesis of gaseous mediators in mammals. Cited from reference 32 with permission.

DETECTING ROS FROM NEUTROPHILS *IN VITRO* AND *IN VIVO*

Neutrophils are one of the major sources that generate large amounts of ROS under stimulated conditions (38). In 1987, we attempted for the first time a real-time visualization of ROS to specify when and where the cells are activated during the process of their tissue recruitment. To this end, we applied an ultrasensitive photon-counting imaging system combined with intravital microscopy: this system enabled us to acquire two-dimensional information on the chemiluminescence emission through oxyradical burst of neutrophils in real time. When neutrophils in culture were stimulated with opsonized zymosan in the presence of luminol, a chemiluminescence probe, they exhibited site-specific photonic burst colocalized with the restricted regions of phagosomes (28). On the other hand, when stimulated with phorbol myristate acetate, which activates NADPH oxidase, the luminol-dependent chemiluminescence was little, if any. The addition of the calcium ionophore A23187, however, induced massive and diffuse elevation of the chemiluminescence (29). As these photonic activities were abolished by catalase or azide or by replacement with the chloride-free medium, a major ROS responsible for this event appeared to derive from the H_2O_2-myeloperoxidase-Cl^- system, such as hypochlorous anion (ClO^-), under the given experimental conditions.

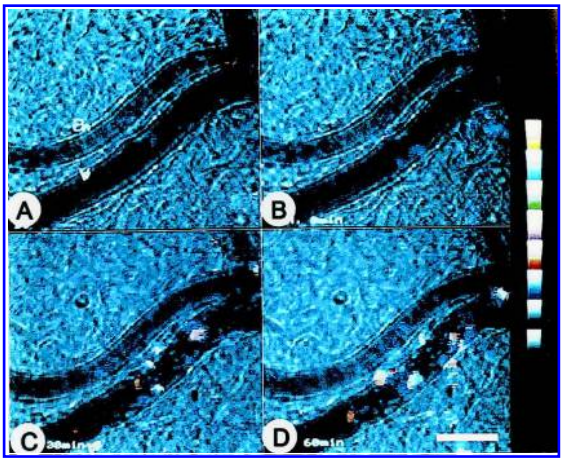

FIG. 2. ***In vivo*** **visualization of oxyradical burst during leukocyte–endothelial cell interactions in postcapillary venules of endotoxemic rats.** (**A** and **B**) Representative micrographs showing basal chemiluminescence activities detected in the absence or presence of the superfusion of luminol, a chemiluminescence probe, on the mesenteric microvessels. (**C** and **D**) Micrographs showing effects of intravenous administration of lipopolysaccharide O-111B4 at 2 mg/kg/h, indicating an increase in photonic burst from adherent leukocytes in the venule (v), but not in the arteriole (a). Bar = 50 μm. Cited from reference 28 with permission.

We then applied this system to visualize ROS generated from activated neutrophils *in vivo* (24, 27, 30). Figure 2 illustrates representative micrographs showing spatial and temporal alterations in luminol-dependent chemiluminescence in the mesenteric microvessels of endotoxemic rats (24). As seen, the photonic activities were elevated in parallel with an increase in the number of adherent neutrophils. Most importantly, the activities coincided with venular endothelial walls with adherent cells, whereas those were absent in arteriolar walls or the interstitial space. This suggests that the interface between venular endothelium and adherent leukocytes serves as a primary domain for the oxidant formation during their tissue recruitment. Pretreatment of superoxide dismutase (SOD) in circulation caused elimination of neutrophil adhesion leading to the abolition of the chemiluminescence. This result was consistent with the fact that SOD increases the velocity of venular leukocyte rolling and thereby diminishes their stationary adhesion in response to proinflammatory stimuli or ischemia–reperfusion (35). Other proinflammatory stimuli, such as platelet-activating factor and interleukin-8, also elicited comparable oxidative burst from adherent neutrophils (30, 34), and the SOD administration again attenuated the photonic responses in parallel with a reduction of the adherent cells. Upon exposure to these stimuli, neutrophils adherent to endothelium appeared to exhibit secretagogue activation (*e.g.,* azurophilic degranulation) and utilize ROS to inactivate endogenous proteinase inhibitors and to activate collagenase to enhance proteolytic activities required for cell migration (38). However, such an activation of ROS generation is unlikely a prerequisite for neutrophil migration, because leukotriene B_4-induced leukocyte adhesion and migration did not coincide with any detectable amounts of the chemiluminescence (27). This observation is also consistent with the failure of SOD to attenuate the leukotriene B_4-elicited adhesion response. Mechanisms by which superoxide mediates leukocyte rolling and adhesion have been considered to involve activation of P-selectin expression on venular endothelial cells (16, 22, 31). As NO cancels superoxide, a tenuous balance between these species serves as a determinant of tissue recruitment of neutrophils in postcapillary venules. Thus, establishment of the method to collect spatial and temporal information on NO in microvascular systems became necessary.

MICROVASCULAR DELIVERY OF NO AND REGULATION OF MICROVASCULAR FUNCTION

As NO was first recognized as a critical modulator of venular leukocyte adhesion in 1991 (16), its interactions with ROS and their functional outcomes on adhesion responses of leukocytes in microcirculation have been discussed extensively in varied disease models. Bioavailability of NO in microcirculation depends on expression of NO synthases (NOSs), which are divided into three isoforms; neuronal (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) (19). NO released from the constitutive isozymes has been shown to play important roles for functional integrity of vascular systems under normal conditions. Such roles include maintenance of vascular patency to guarantee ample blood supply, prevention of unnecessary adhesion of platelets and leukocytes (19), stabilization of mast cells (17), regulation of vascular permeability, and modulation of vascular remodeling involving proliferation of vascular smooth muscle cells. When endogenous NO was suppressed by the enzyme inhibitors, apparent ROS generation in microvascular endothelium or in mast cells was markedly elevated and resulted in adhesion of leukocytes through mechanisms involving endothelial P-selectin expression (31).

Until now, most NO available for vascular walls has been thought to derive mainly from endothelial cells, which express NOS3. However, our studies have revealed that the local availability of NO in arterioles and that in venules appears, to depend on distinct enzymes, such as NOS1 and NOS3, respectively, suggesting distinct mechanisms for delivery of NO among different hierarchy of microvessels (12). This concept has been achieved by real-time assessment of microvascular NO generation using 4,5-diaminofluorescein diacetate (DAF-2DA) microfluorography. DAF-2DA is a membrane-permeable fluorescence precursor sensing intracellular NO generation (15). As seen in Fig. 3, the fluorescence occurred comparably in arteriolar and venular walls, and also in mast cells in the interstitium. The immunohistochemistry for NOS1 and NOS3 in the rat mesentery showed that the proximal arterioles express both isozymes, whereas the distal ones with smaller diameters express only NOS1, but little NOS3, if any. This was the case not only in the mesentery, but also in brain. Until now, other studies have suggested that conducting arteries proximal to microvessels mainly use NO derived from its own endothelium expressing NOS3 to maintain their patency to supply blood flow, whereas smaller arterioles could mainly use NO-independent mechanisms (*e.g.,* endothelium-derived hyperpolarizing factor) for vasorelaxation (37). Most of the previous experiments suggesting lesser roles of NO in modulation of arteriolar tone were collected from the *ex vivo* perfused vessels undergoing the denervated and connective tissue-free preparation, and could thus overlook roles of NOS1 around arterioles. Such an underestimation of the paracrine source of NO in arterioles has been revised by our studies.

On the other hand, endothelia of capillaries and venules seem to express NOS3. This notion was supported by the observation that 7-nitroindazole (7-NI) induced only modest activation of venular leukocyte adhesion as compared with the same concentration of N^{ω}-nitro-L-arginine methyl ester (L-NAME). As mentioned in the previous section, a tenuous balance between NO and superoxide anion could determine susceptibility of mast cells to degranulation stimuli, and NO serves as a stabilizer of the exocytosis *in vitro* and *in vivo* (17). In both arterioles and venules, sufficient concentrations of NOS inhibitors significantly attenuated the NO-associated fluorescent signals, but only partly; ~40% of the signals remained as the basal activities. Such a low susceptibility to NOS inhibitors raised an important possibility of another source of NO derived from nonenzymatic origins besides locally expressed NOS isozymes (Fig 3). *S*-Nitrosyl hemoglo-

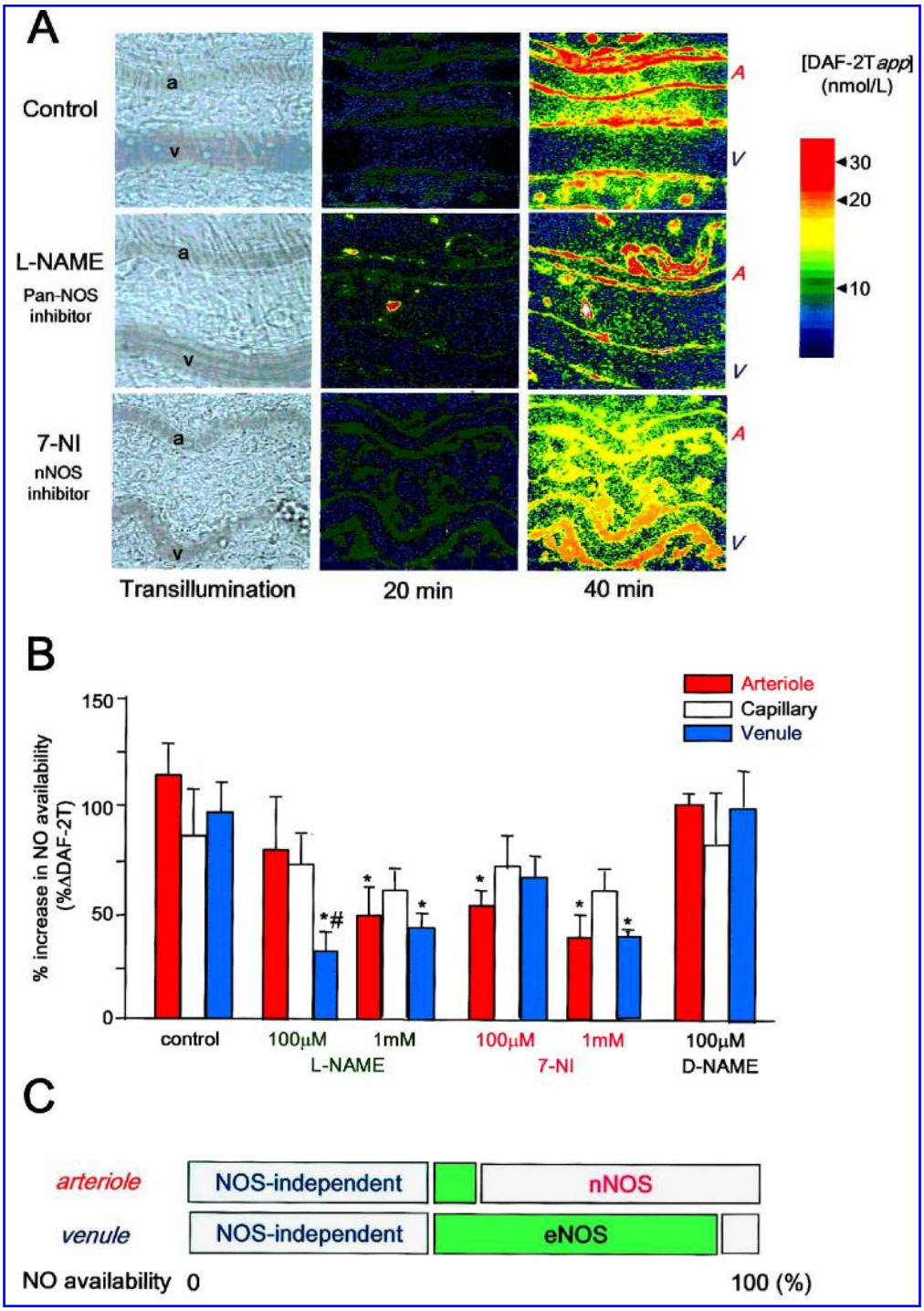

FIG. 3. Representative pictures showing microvascular NO distribution revealed by digital microfluorography using NO⁺-sensitive diaminofluorescein (DAF). (**A**) Pseudocolor representation of DAF fluorescence in the rat mesenteric microcirculation and sensitivity to locally applied NOS inhibitors such as L-NAME and 7-NI, a potent NOS1 inhibitor. *A* and *V* denote a pair of arteriole and venule. (**B**) Semiquantitative analyses of the sensitivity of DAF fluorescence to the NOS inhibitors. Greater susceptibility of arteriolar signals to 7-NI than to L-NAME suggest that NOS1 supports NO availability in arterioles. Note the presence of NOS-independent fraction of the fluorescence even in the presence of sufficient amounts of the inhibitors. (**C**) A schematic diagram of difference in sources of NO between arterioles and venules. A and B are cited from reference 12 with permission.

bin in circulating erythrocytes that could release NO upon a reduction of local oxygen pressure is among such sources taken into account. Considering the recent observation that a drop in the intravascular oxygen tension occurs first in the distal arterioles rather than in capillaries (10), it can be hypothesized that such an alternative source explains the presence of the L-NAME- and 7-NI-insensitive fraction of local NO availability. However, as erythrocytes are thought to consititute a metabolic sink for NO and the gas can readily bind to the hemoglobin-heme in the cells to serve as an allosteric regulator of oxygen transport, roles of *S*-nitrosyl compounds as an NO reservoir should be further examined carefully (40).

MECHANISTIC BIOPROBING OF SOLUBLE GUANYLATE CYCLASE REVEALS INTERACTIONS BETWEEN NO AND CO *IN VIVO*

CO has been considered a gaseous mediator analogous to NO that contributes to signal transduction in neurovascular systems. This gas is one of the reaction products of heme oxygenase (HO), the enzyme that catalyzes protoheme IX into biliverdin-IXα and ferrous iron. Biliverdin-IXα is converted to bilirubin-IXα through the reaction of biliverdin reductase and serves as a potent antioxidant that ameliorates inflammatory responses (8, 36). As described later, in the liver, CO serves as a vasorelaxant for sinusoids that activates soluble guanylate cyclase (sGC) in hepatic stellate cells, liver-specific pericytes; this mechanism explains why this organ can maintain low vascular resistance to ensure ample blood supply from portal venous system (26, 32, 33).

In mammals, HO exists in two forms: HO-1 and HO-2 (36). HO-1 is induced by stressors such as cytokines, heavy metals, hypoxia and ROS. Excess NO could also cause the HO-1 induction (26). By contrast, HO-2 appears to be constitutive. The HO/CO system may regulate function of heme proteins and enzymes in several ways: in the liver, cytochrome P450 (but not other heme enzymes such as catalase) contains ferrous heme under normoxic conditions and thus is susceptible to inhibition by CO (18). By contrast, cytochrome oxidase in mitochondria is unlikely to be affected by CO, because its prosthetic heme is predominantly in the ferric state; thus, the susceptibility of each heme protein to this gas greatly depends on its redox states (26). HO catalyzes prosthetic heme of cytochromes P450 and could reduce the holoenzymes. The HO reaction requires reducing equivalents supplied by NADPH, and thus its competition for the electron pool could interfere with other enzyme reactions. CO shares several heme proteins as signal transducers with NO and could fine-tune functions of the receptors through distinct mechanisms. One such example is the effect of these gases on hemoglobin allostery: as widely known, CO stabilizes the six-coordinated form of the prosthetic heme and increases the affinity of molecular oxygen in other subunits, whereas NO binds to the α subunit of the heme and breaks the proximal histidine-Fe bond, forming a five-coordinated nitrosyl heme complex to decrease the affinity of oxygen in β subunits.

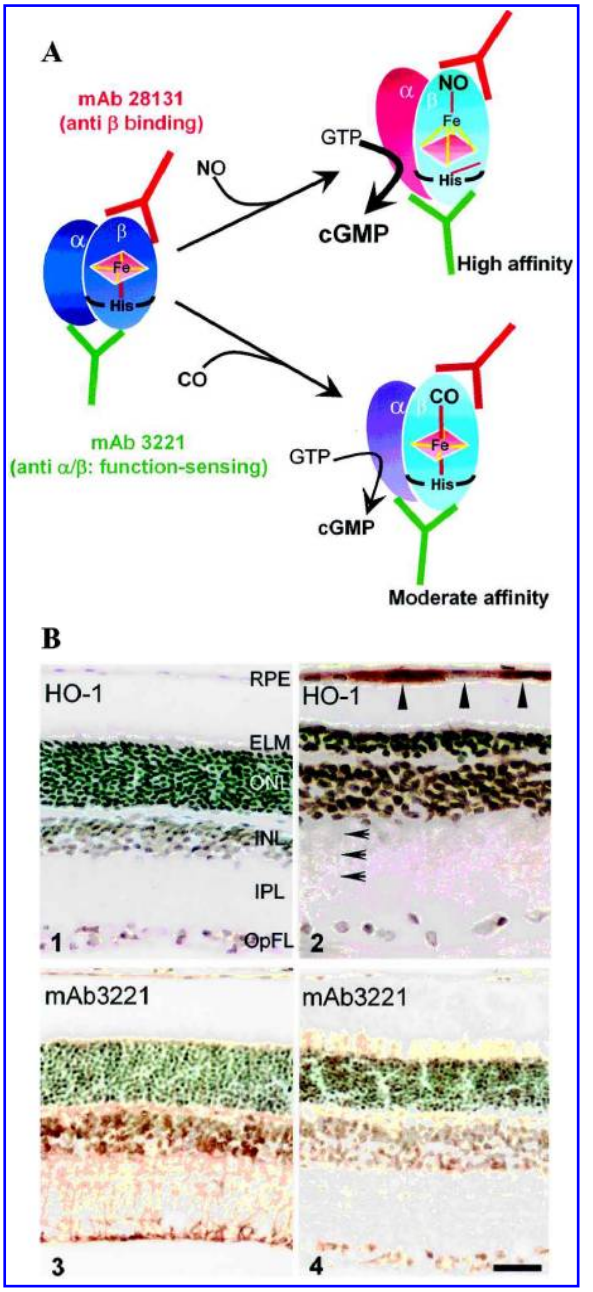

FIG. 4. Mechanistic bioprobing of gas-dependent regulation of sGC by mAb3221 and mAb28131. (**A**) Functional epitopes responsible for detecting the activation of the enzyme by NO and CO. (**B**) Application of the antibody 3221 to immunohistochemistry of rat retina exposed to sustained light exposure. The light exposure causes stress responses of HO-1 throughout the entire retinal cell layers (panel **2**). Distinct patterns of sGC activation among the layers are evident (panel **4**). The HO-1 induction caused an inhibition of the sGC activities in the inner plexiform layer (IPL) where ample NO is available. By contrast, it causes activation in the external limiting membrane (ELM) where NO is limited. INL, inner nuclear layer; ONL, outer nuclear layer; OpFL, optic nerve fiber layer; RPE, retinal pigment epithelium. Bar = 30 μm. Panel B was cited from reference 11 with permission.

Identical structural differences in the prosthetic heme also explain the distinct ability of NO versus CO to activate sGC, a common signal transducer for both gases (14, 26).

In biological systems, multiple gases might share the individual signal transducer for fine-tuning cell functions, but little evidence for demonstrating this hypothesis has been available. In other words, development of molecular probes that can sense the ligand binding and/or specific conformational changes allowed us to examine the behavior of gaseous mediators *in vivo*. Newly generated monoclonal antibodies (mAbs) against sGC, mAb28131 and mAb3221, have made it possible to examine the activation state of the enzyme regulated by NO and CO in the rat retina, where two gases and one transducer might play a role in cyclic GMP-dependent regulation of the tissue function. mAb3221 recognizes a regiospecific structure determined by the two subunits of a native sGC protein. Characterization of mAbs by surface plasmon resonance technology revealed that mAb3221 increases its affinity to the antigen by 100- or 10-fold in the presence of NO or CO, respectively. These results suggest that mAb3221 recognizes a regiospecific epitope formed by α and β subunits of the enzyme that could be altered by binding of gas mediators to the heme (Fig. 4A). On the other hand, the affinity of mAb28131 to the enzyme remained constant regardless (11). The results conform to our idea that comparison of immunoreactivities between the two distinct mAbs enables us to assess local sGC regulation by NO and CO *in vivo* using immunohistochemical approaches.

In these studies, we used rat retina as an experimental system because its well defined anatomical layers consisting of specific cell types enabled us to examine spatial relationships between NO- and CO-generating enzymes and their receptor protein, sGC. In this tissue, sGC was localized mainly in Mueller's glia cells, which constitute the tissue-supporting interface between microcirculation and the neural system, and partly in on-type bipolar cells, the second neuron for phototransduction. Direct detection of the sGC function with mAb3221 led us to propose a novel hypothesis to explain the long-standing controversy concerning synergistic or antagonistic interactions of the two diatomic gases to regulate sGC activities *in vivo*: The effect of CO on modulating sGC activity is not static, but dynamic in that low tissue NO makes CO a stimulatory modulator of sGC, whereas high tissue NO makes CO an inhibitory one. Administration of L-arginine enhanced, and that of the NOS inhibitor attenuated, mAb3221 immunoreactivities. Blockade of HO by the competitive inhibitor zinc protoporphyrin-IX elicited marked enhancement of the immunoreactivities, suggesting that endogenous CO plays a modulatory role in NO-mediated sGC activation.

It should be noted that sustained exposure to visible light caused HO-1 induction throughout the entire retina, but resulted in heterogenous responses of sGC activation among different layers. HO-1-derived CO suppressed sGC in inner plexiform and nuclear layers, while activating it in external limiting membrane (Fig. 4B). This event appeared to result from heterogenous availability of NO among the layers. NO could be ample in inner plexiform and nuclear layers, because these layers are proximal to NO-generating enzymes in microvessels and amacrine cells. On the other hand, external limiting membrane is located far from the NO-generating site.

Approaches for mechanistic probing of signal transduction by gases are going to unravel how multiple gases regulate cellular and tissue functions *in vivo*. Although physiologic roles of CO in neural tissues are unknown, retina could benefit from this nonradical sGC agonist to maintain housekeeping cyclic GMP without causing potential degradation of retinoids through a radical agonist such as NO. Such a way to utilize CO appears to be the case in stress-induced spermatogenic control or in relaxation of hepatic stellate cells for increasing sinusoidal blood flow where NO-breakable DNA or vitamin A is heavily stored, respectively (6, 21, 33). These findings enable a new understanding of the link between multiple gases and the function of transducers *in vivo*.

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ABBREVIATIONS

CO, carbon monoxide; DAF, diaminofluorescein; HO, heme oxygenase; mAb, monoclonal antibody; L-NAME; N^{ω}-nitro-L-arginine methyl ester; 7-NI, 7-nitroindazole; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SOD, superoxide dismutase.

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Address reprint requests to:
Makoto Suematsu, M.D., Ph.D.
Professor and Chair
Department of Biochemistry and Integrative Medical Biology
School of Medicine, Keio University
35 Shinanomachi, Shinjuku-ku
Tokyo 160-8582, Japan

E-mail: msuem@sc.itc.keio.ac.jp

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