A Novel Molecular System for Nitric Oxide Detection with High Sensitivity

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A novel molecular switching system was developed for the direct detection of endogenous nitric oxide. The system is comprised from an Fe(II)–dithiocarbamate complex in which TEMPOL molecule coordinates to the centered iron through a redox interaction. Nitric oxide binds to the iron to liberate the TEMPOL. The system will be potentially useful for the monitoring of endogenous nitric oxide.

Nitric oxide has been recognized as an important messenger in many biological systems. It is known to function as a vasodilator, neuromodulator, effector molecule in the immune system and so on.[1] In spite of intense recent interest in endogenous nitric oxide, there remains ambiguity in its biological actions. While nitric oxide plays some important physiological roles, it is also believed to be related to tissue damage such as ischemia/reperfusion damage,[2] inflammation, and excitatory neuronal cell death.[3] This is probably explained by the fact that nitric oxide reacts with various oxygen species, thiols, and metal proteins to change its chemical form. Among those nitric oxide derivatives, peroxynitrite[4] and *S*-nitrosothiols[5] are the most important. These derivative species have different properties than nitric oxide. It is therefore important that the methods for monitoring endogenous nitric oxide are able to detect nitric oxide directly and to distinguish it from the nitric-oxide-related species. Otherwise, the actions of nitric oxide would be misunderstood in many cases. In addition, the temporal and spatial profile of nitric oxide production should be monitored during the measurement nitric oxide, because the molecule does not have any receptors of its own but instead reveals its bioactivity through chemical reactions.

Many of the ordinary methods of nitric oxide monitoring are indirect and are based on the redox reactions of nitric oxide-derivatives, e.g., the oxidized products of nitric oxide.[6] Some methods can detect the molecule directly, but in these digital imaging, which is necessary for getting the production profile, is not possible.[7] Among the conventional techniques, spin-trapping methods using hemoglobin,[8] Fe(II)–dithiocarbamate complex[9] or PTIO derivatives[10] would be the most promising for satisfying the direct detection of nitric oxide and continuous monitoring for getting the production profile, although the applications using such spin-trapping agents still have limitations due to their low sensitivity for nitric oxide detection. We have designed a new concept, spin-exchange, using Fe(II)–dithiocarbamate complex and TEMPOL, which is a stable water-soluble organic radical, for highly sensitive monitoring of nitric oxide production by modeling the molecular mechanism of guanylate cyclase activation with nitric oxide.[11]

The basic strategy of our probe design is shown in Figure 1. Guanylate cyclase has a heme moiety in the regulation site to which the imidazole group in the histidine residue coordinates. Nitric oxide binds strongly to the heme iron to dissociate the imidazole group. This event makes the enzyme active. In this system, if any probe molecule can play the role of the imidazole moiety,

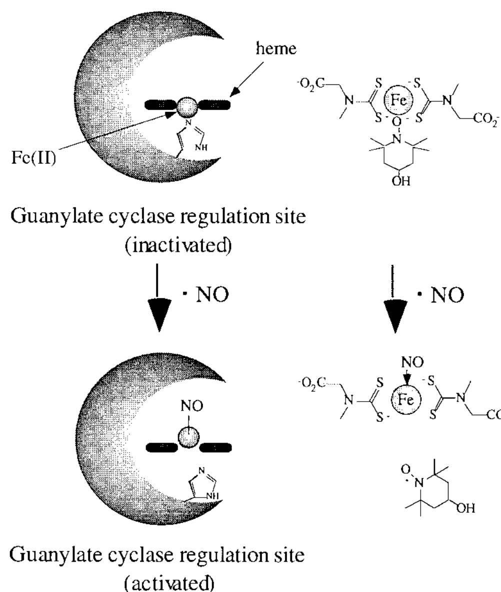

Figure 1. Activation mechanism of guanylate cyclase and the releasing of TEMPOL from the new spin exchange molecular system with nitric oxide.

nitric oxide may release the probe molecule that has a chemical signal from the iron complex. To realize this idea, we used the dithiocarbamate–iron(II) complex and TEMPOL in place of the heme and imidazole, respectively. In this mimic system, TEMPOL, the spin signal, is liberated with the capture of nitric oxide.

When TEMPOL (100 µM) was incubated with dithiocarboxy-sarcosine (DTCS, 5 mM) and iron(II) sulfate (1 mM) in 100 mM phosphate buffer solution (pH 7.4), a gradual disappearance of the EPR signal caused by TEMPOL was observed. This finding indicates that the TEMPOL radical interacts with iron(II) through electronic (redox) interaction producing N–O– and Fe(III). This type of interaction may require a particular electronic state of iron(II) in the dithiocarbamate complex.[12] In fact, other iron(II) complexes with cyclam or salene derivatives did not cause the TEMPOL EPR signal to vanish. The TEMPOL signal did not recover after 1 h of incubation at 37 °C. In contrast, the addition of a spontaneous nitric oxide releasing agent, NOC-7 (100 µM), gradually restored the EPR signal of TEMPOL (Figure 2(a)). Figure 2(b) shows a comparison of the time course of both the release of nitric oxide from NOC-7 and the EPR signal recovery after the addition of NOC-7 in 100 mM phosphate buffer solution (pH = 7.4) at 37 °C. The nitric oxide releasing time course was monitored by absorbance decrease at 260 nm based on NOC-7 decomposition. Both time-courses were completely coincident, indicating that the TEMPOL EPR signal recovery was directly caused by the nitric oxide from NOC-7. On the other hand, the nitrite that is the final product of nitric oxide in aerobic aqueous solution did not bring about any such change in EPR signals, even

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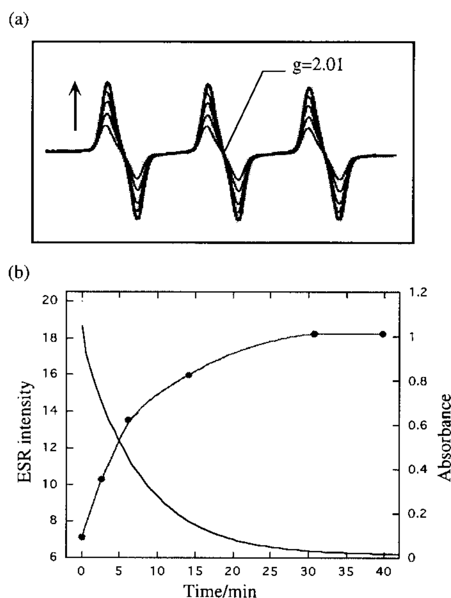

Figure 2. EPR spectral change of the spin exchange system (200 μ M) with nitric oxide released from NOC-7 (100 μ M) (a) and a comparison of the time course of the EPR signal increase (●) and the nitric oxide release from NOC-7 (b). All experiments were performed at 37 ℃ in a 100 mM phosphate buffer (pH 7.4). The release of nitric oxide was monitored by a change of absorbance at 260 nm.

in the presence of 1 mM. SIN-1 (200 μM), which generates peroxynitrite anion, and peroxynitrite itself (200 μM) also did not recover the EPR signal of TEMPOL, indicating that the spin exchange at the center of the iron was caused selectively by nitric oxide.

Simple bisdithiocarbamate–iron(II) complexes are usually used as nitric oxide spin-trapping agents. By the reaction with nitric oxide, they generate iron–nitrosyl complexes, which can be recognized as characteristic three-line EPR signals. However, the sensitivities of the various nitric oxide detection methods are not satisfactory for the monitoring of physiological nitric oxide generation, whose concentration is usually lower than 100 nM. The detection limit of nitric oxide with ordinary iron–dithiocarbamate complexes is in the sub-μM range. In Figure 3, the monitoring of nitric oxide production from NOC-7 (100 μM) by our new molecular system (200 μM) is compared with that performed by the ordinary DTCS2-Fe(II) complex (200 μM). The newly designed spin-exchange system was able to detect nitric oxide with a detection limit of 10 nM, a sensitivity 25-times higher than the ordinary spin-trapping system.

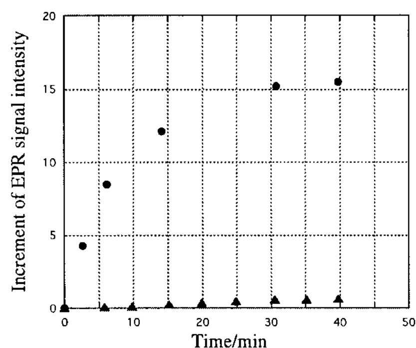

Figure 3. A comparison of the increments of EPR signal intensities between the new molecular system (200-μM) (●) and the ordinary DTCS-Fe(II) complex (200-μM) (▲) with nitric oxide from NOC-7 (100-μM). All experiments were performed at 37℃ in a 100-mM phosphate buffer (pH 7.4).

We then investigated the mechanism of the spin-exchange. It was not clear why the released TEMPOL was not captured by the present excess of DTCS-Fe(II) complex, since five molar excess amount of DTCS-Fe(II) complex to TEMPOL should be formed in the working solution. To elucidate the question, the same experiment for nitric oxide detection using the spin-exchange system and NOC-7 was performed under anaerobic conditions. In this case, the TEMPOL signal did not recover with the nitric oxide generation from NOC-7 (100 μM) (data not shown).[13] In contrast, TEMPOL did not interact with the iron(II) complex of DTCS when both compounds were mixed in an oxygen pre-saturated buffer solution (data not shown).[13] These results indicate that DTCS-Fe(II) complex without TEMPOL tends to bind molecular oxygen at the iron center like a heme in hemoglobin. Once an oxygen molecule binds to the iron, TEMPOL does not interact with the iron anymore. Thus, TEMPOL was not caught up with the DTCS-Fe(II) complex after being released by nitric oxide. In this case, nitric oxide can still give the Fe–nitrosyl signal of DTCS-Fe(II) on EPR spectroscopy. This means that the iron remained as Fe(II) which had not been oxidized. Oxidation of the iron in the complex is probably much slower than could be monitored in the time scale of the above experiments.

EPR methods are useful for endogenous nitric oxide sensing, since these methods detect nitric oxide directly. The spin-exchange system we introduced in the present experiments was much more sensitive than any ordinary spin-trapping agents that are used for the monitoring of endogenous nitric oxide production. The system can detect nitric oxide at a concentration as low as 10 nM. We are currently using our system to detect nitric oxide released from living cells, and our results will be reported in due course.

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13 The interaction between TEMPOL or NO and Fe(II)–DTCS complex can be confirmed by absorption spectra. The absorbance of Fe(II)–DTCS complex at 430 nm increased by the interaction with TEMPOL or NO. NO also decreased the absorbance at 255 nm and 275 nm, but TEMPOL did not. Oxygene did not change the spectrum of Fe(II)–DTCS.