The role of thiol and nitrosothiol compounds in the nitric oxide-forming reactions of the iron–*N*-methyl-D-glucamine dithiocarbamate complex

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The object of the present study is to investigate whether the physiologically dominant thiol compounds such as GSH and cysteine or their nitrosothiol compounds affect the formation of the iron–*N*-methyl-D-glucamine dithiocarbamate [(MGD)₂Fe²⁺]–nitric oxide complex. The present study provided experimental evidence that physiological concentrations of GSH (approx. 5 mM) and L-cysteine (approx. 0.5 mM) accelerated the formation of the (MGD)₂Fe²⁺–NO complex from nitrite by two and three times respectively. The rate constants for the reduction of (MGD)₂Fe³⁺ to (MGD)₂Fe²⁺ by GSH and cysteine were calculated as 1.3 and 2.0 × 10² M^{−1} · s^{−1} respectively. Furthermore, depletion of GSH was demonstrated in PC12 cells, and thiol compounds enhanced the formation of reactive oxygen

species by the (MGD)₂Fe²⁺ complex by accelerating its redox turnover. The main effect of the physiological concentration of thiols was the reduction of (MGD)₂Fe³⁺. *S*-nitrosoglutathione spontaneously reacted with (MGD)₂Fe²⁺ to produce the (MGD)₂Fe²⁺–NO complex with a 1 : 2 stoichiometry. In fact, (MGD)₂Fe²⁺ was as good an indicator of nitrosothiols as it was of NO itself. The present study elucidates the difficulties of utilizing the (MGD)₂Fe²⁺ complex for the quantification of NO in biological samples, especially *in vivo*.

Key words: 5,5-dimethylpyrroline-*N*-oxide (DMPO), EPR, reactive oxygen species.

INTRODUCTION

Nitric oxide (NO) has been implicated in many important physiological roles, such as cytotoxic mediation of the immune system, regulation of vasomotor tone in the cardiovascular system and as a neurotransmitter in the central nervous system [1,2]. NO is thought to be identical with the endothelium-derived relaxing factor [1], and its insufficiency is believed to contribute to the pathogenesis of vascular diseases such as atherosclerosis, hypertension and myocardial ischaemia. As a result, much attention has been focused on the potential therapeutic ability of nitrovasodilators (e.g. nitroglycerin and nitroprusside) [3] and the sickle-cell and anticancer drug hydroxyurea [4,5] to release NO.

To understand the mechanisms by which NO, a diffusible free radical with a short biological lifetime, mediates various biological processes, accurate methods for its measurement are required. Several methods for the quantification of NO, such as chemiluminescence [1], methaemoglobin formation [6] and EPR spectroscopy of nitrosyl–metal complexes [7], have been developed [8]. Production of NO can also be assessed indirectly by measuring the NO oxidation product, nitrite, by the Griess reaction [9].

EPR spectroscopy is the only specific general technique available for the detection and measurement of free radical production, but has severe quantum-mechanical limitations for diatomic molecules. EPR methods have been developed, which stabilize NO as a polyatomic adduct using endogenous and exogenous spin traps [10–16]. Conventional nitron- and nitroso-based spin traps are not capable of trapping NO as stable radical

adducts, although nitromethane is an effective spin trap for NO at strongly alkaline pH values [17].

The diethyl dithiocarbamate (DETC)–ferrous complex is a commonly used spin trap for NO [18], resulting in the formation of the (DETC)₂Fe²⁺–NO complex, which has a characteristic triplet EPR signal. Although the (DETC)₂Fe²⁺ complex has been widely used to trap NO from cells and tissues [14,15], quantification of NO using (DETC)₂Fe²⁺ requires complicated procedures to overcome its low solubility in water. In a previous study [16], *N*-methyl-D-glucamine dithiocarbamate (MGD) was used to overcome the poor solubility of (DETC)₂Fe²⁺. The (MGD)₂–Fe²⁺ complex is water-soluble [19] and forms the characteristic triplet EPR spectrum after trapping NO.

Although it is widely assumed that dithiocarbamate–Fe²⁺ complexes specifically react with NO to form dithiocarbamate–Fe²⁺–NO complexes, Hiramoto et al. [20] reported that the coexistence of Fe²⁺–dithiocarbamate complexes and nitrite produced a triplet EPR spectrum, which corresponded to that of the dithiocarbamate–Fe²⁺–NO complex even at physiological pH. In a previous report, we investigated the pathway of NO production from nitrite by the (MGD)₂Fe²⁺ complex under anaerobic conditions at physiological pH [21].

Nitrite in biological systems originates as an oxidation product of endogenous NO, as a food component [22] and as the reduction product of nitrate by facultative anaerobic bacteria [23–25]; therefore, the (MGD)₂Fe²⁺–NO production from nitrite by (MGD)₂Fe²⁺ may lead to misinterpretation regarding the actual presence of NO. Moroz et al. [26] reported the production of (MGD)₂Fe²⁺–NO from a NO synthase inhibitor using the

Abbreviations used: DETC, diethyl dithiocarbamate; DMPO, 5,5-dimethylpyrroline-*N*-oxide; GSNO, *S*-nitrosoglutathione; MGD, *N*-methyl-D-glucamine dithiocarbamate.

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(MGD)₂Fe²⁺ complex, and its reaction was facilitated by biological reductants such as cysteine, GSH and ascorbate. The present study suggests that the biological reductants have some effects on the iron–MGD complex.

In the present study, we utilized EPR spectroscopy to explore explicitly the effects of biological thiols and nitrosothiols on the production of the (MGD)₂Fe²⁺–NO complex under aerobic and anaerobic conditions.

MATERIALS AND METHODS

Materials

MGD was synthesized as described previously [19] from *N*-methyl-D-glucamine (Sigma) and carbon disulphide (Sigma). The purity and molecular mass of synthesized MGD were verified with commercially available MGD (OMRF, OK, U.S.A.) by HPLC and MS respectively. *S*-Nitrosoglutathione (GSNO) was prepared by combining equimolar amounts of GSH and nitrite in 0.1 M HCl. The mixture was incubated at 5 °C for approx. 45 min. Then acetone was added to precipitate GSNO, which was then washed with cold water. The formation of GSNO was assessed by measuring the absorbance *A* at 335 nm [27]. ¹⁵N-substituted GSNO was also prepared by using ¹⁵N-substituted sodium nitrite (Cambridge Isotope Laboratories, Andover, MA, U.S.A.).

Sodium nitrite, FeCl₃ · 6H₂O, L-cysteine and GSH were purchased from Sigma. High-purity (99.999 %) FeSO₄ · 7H₂O was obtained from Alfa (Ward Hill, MA, U.S.A.). NO was obtained commercially (National Welders Supply Co., Raleigh, NC, U.S.A.) and purified from higher oxides such as NO₂ and N₂O₃ by passing the gas through a trap containing 1 M KOH solution. NO-saturated aqueous solution was prepared by bubbling NO gas for 10 min through water, which had been previously deoxygenated by bubbling with purified Ar gas for 30 min. Pheochromocytoma PC12 cells were obtained from Daiippon Pharm. Co. (Tokyo, Japan). All other chemicals were of analytical grade.

Preparation of the iron–MGD complex

Stock solutions of FeSO₄ · 7H₂O (0.1 M), FeCl₃ · 6H₂O (0.1 M) and MGD (0.5 M) were prepared only at the time of measurement and were used within a few hours. All solutions were prepared in Ar-purged distilled water. The iron–MGD complex was prepared by adding appropriate amounts of iron and MGD from stock solution into Hepes buffer (pH 7.4), deoxygenated with purified Ar for 30 min.

EPR measurements

All EPR measurements were performed at the room temperature. For anaerobic measurements, samples were directly transferred into an Ar-purged flat cell, which was, in turn, placed in the cavity of the EPR spectrometer. All solutions were mixed before EPR measurement to provide the final concentrations indicated in the Figure legends, and the reactions were initiated by the addition of iron.

A Bruker EMX EPR spectrometer (Bruker, Billerica, MA, U.S.A.) with a TM₁₁₀ cavity and a 17 mm quartz flat cell was employed to collect all EPR spectra except in the isotope experiments of Figure 3. A JEOL EPR spectrometer (JES-TE300; JEOL, Tokyo, Japan) with an ES-UCX2 cavity and a 10 mm quartz flat cell was utilized to perform the NO exchange experiments (Figure 3). Typical instrument conditions for the Bruker instrument were as follows: 20 mW microwave power, 2.0 G modulation amplitude, 0.163 s time constant, 168 s scan

time and 100 G scan range. Spectra were stored on an IBM PC for analysis. Quantification of NO was performed by double integration of the EPR spectrum. The standard for the (MGD)₂Fe²⁺–NO complex was prepared with an NO-saturated aqueous solution (approx. 1.9 mM) [28]. Other individual conditions are described in the Figure legends. Hyperfine coupling constants *a* and spectral simulations were obtained with the program Winsim [29].

Oxygen electrode studies

Changes of oxygen concentration were monitored using a Clark-type electrode, beginning with the addition of 3.6 µl of FeSO₄ and 3.6 µl of MGD from a concentrated stock solution to a reaction chamber containing 1.8 ml of Hepes (20 mM, pH 7.4) to give a final concentration of 0.2 and 1.0 mM respectively. GSH or L-cysteine was added to the chamber 3 min after the addition of FeSO₄. To estimate the oxygen consumption by the addition of these compounds, the initial oxygen concentration in the incubation mixture was assumed to be 254 µM at room temperature [30].

UV–visible spectrophotometer

Thiol-induced changes in the absorption spectra of the (MGD)₃Fe³⁺ complex were monitored by measuring the *A*_{450–700} with an SLM AMINCO DW-2000 spectrophotometer at room temperature. Fe³⁺ (0.5 mM), MGD (2.5 mM) and Hepes buffer (20 mM, pH 7.4) were mixed in the optical quartz cell under anaerobic conditions and then placed into the chamber for measurement. Thiol compounds were introduced into the cell after the formation of the (MGD)₃Fe³⁺ complex.

Growth of PC12 cells and treatment with reagents

The PC12 cells were subcultured on 35 mm polylysine-treated dishes at a density of 1 × 10⁶ cells/dish in RPMI 1640 culture media supplemented with 10 % heat-inactivated horse serum, 5 % foetal bovine serum, streptomycin (50 µg/ml) and penicillin (50 units/ml). After 2 days in culture, cells were washed with oxygenated, prewarmed (37 °C) Krebs–Ringer Hepes buffer (pH 7.4), containing 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂ and 20 mM Hepes. The cells were then incubated in Krebs–Ringer Hepes buffer (37 °C) with or without test compounds for 120 min, and the GSH measurements were performed.

The GSH concentration was determined by the method of Hissin and Hilf with slight modification [31]. The cells were harvested, then centrifuged (1000 *g*) for 5 min at 4 °C to obtain the cell pellet. The pellet was mixed with 0.15 ml of HClO₄ (0.8 M) including 8 mM EDTA, followed by centrifugation (1000 *g*) for 5 min at 4 °C to precipitate the protein. Next, 0.1 ml of the supernatant or standard GSH solution (0–100 µM) was transferred to 2 ml phosphate buffer (0.1 M, pH 8.0) including 5 mM EDTA, and then 0.1 mM *o*-phthalaldehyde (20 mg/ml, diluted with methanol) was added. The GSH concentration was determined by fluorescence intensity (excitation 350 nm; emission 425 nm) with a Hitachi F-3010 fluorescence spectrophotometer. The GSH levels were expressed in terms of 10⁶ cells.

RESULTS

Effects of physiological anions in the formation of the (MGD)₂Fe–NO complex

Paramagnetic NO–iron–dithiocarbamate complexes interact with chloride, phosphate and carbonate anions to modulate their

Figure 1 Enhancement of EPR spectra of the MGD-ferrous-NO complex formation from MGD-ferrous complex and nitrite by thiol compounds

(A) Effect of thiol compounds on the production of the MGD-ferrous-NO complex from nitrite. Measurements were performed using EPR in the absence (○) or presence of thiol compounds. ■, GSH; □, L-cysteine. Each thiol was added to a 0.5 M Hepes buffer (pH 7.4) solution, containing 0.5 mM Fe^{2+} , 2.5 mM MGD and 0.1 mM nitrite. The initial concentrations of GSH and L-cysteine were 5 and 0.5 mM respectively. The concentration of the MGD-ferrous-NO complex was calibrated using a saturated NO solution under the same experimental conditions. (B) Representative EPR spectra of $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex corresponds to (A) at 2 h after the addition of nitrite. (C) Structure of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex. EPR spectrometer instrumental settings were: microwave power, 20 mW; modulation amplitude, 2.0 G; modulation frequency, 50 kHz; sweep time, 1342 s; receiver gain, 100 000.

stability [32]. Therefore we examined whether the formation of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex could be modulated by these anions under our experimental conditions. When 2 μM NO was introduced into 0.1 mM $(\text{MGD})_2\text{Fe}^{2+}$ complex, less than 5% change in the EPR signal intensity of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex was observed in the presence of physiological concentrations of Cl^- (104 mM), phosphate (1 mM) or carbonate (27 mM) anion (results not shown).

EPR measurement of NO formation from nitrite

When 0.1 mM nitrite was added to a solution containing 0.5 mM $(\text{MGD})_2\text{Fe}^{2+}$ complex in 0.5 M Hepes buffer (pH 7.4), a triplet signal characteristic of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex with a nitrogen hyperfine coupling constant of 12.7 G [16] was detected after 2 h incubation under anaerobic conditions (Figure 1B). The intensity of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex increased 3-fold in the presence of 0.5 mM L-cysteine and 2-fold in the presence of 5 mM GSH. The rate of production of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex from 0.1 mM nitrite was calculated to be 1.0×10^{-10} M/s. This value also increased with the addition of L-cysteine (3.1×10^{-10} M/s) and GSH (2.2×10^{-10} M/s) (Figure 1A).

Reaction between $(\text{MGD})_2\text{Fe}^{2+}$ complex and nitrosothiol

To investigate whether $(\text{MGD})_2\text{Fe}^{2+}$ can react with nitrosothiol to form the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex, we measured the EPR spectrum of solutions containing the $(\text{MGD})_2\text{Fe}^{2+}$ complex and GSNO under anaerobic conditions. As shown in Figure 2(A), even in the dark, GSNO immediately gave the EPR spectrum of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex. The EPR signal intensity of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex derived from 0.1 mM GSNO was the same as that of 0.1 mM NO (results not shown). The EPR signal intensity for the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex formed by GSNO was directly related to the GSNO concentration (Figure 2A; $r^2 = 0.995$) and was detectable down to 50 nM GSNO, where the signal-to-noise ratio was 2.

Figure 2(B) shows the production of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex versus the ratio $(\text{MGD})_2\text{Fe}^{2+}/\text{GSNO}$ at a GSNO concentration of 0.1 mM. When the ratio $(\text{MGD})_2\text{Fe}^{2+}/\text{GSNO}$ was 2, the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex concentration approached a plateau, and its signal intensity was equal to that obtained for 0.1 mM NO. An excess of $(\text{MGD})_2\text{Fe}^{2+}$ was apparently necessary to capture all the NO [33].

Exchange between iron-bound NO and free NO

Ileperuma and Feltham [34] had reported that the exchange of ^{14}NO with the 5-coordinate complex, namely (dimethyldithiocarbamate) $\text{Fe}^{2+}\text{-}^{15}\text{NO}$, occurred in the organic solvent. To investigate whether the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex also exchanges with free NO, we monitored the changes in the EPR spectrum of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex under the continuous flow of ^{15}NO gas at pH 7.4 in Hepes buffer. The NO gases (^{14}NO and ^{15}NO) for this experiment were synthesized by acid decomposition of 0.5 M nitrite (^{14}N - and ^{15}N -nitrite respectively) with 0.1 M HCl [35] under the Ar flow. The NO was bubbled through a 2 M NaOH solution to remove contaminating HCl, N_2O_3 or other higher oxides of NO before it was introduced into the reaction mixture. As shown in Figure 3, the introduction of ^{15}NO gas into the 0.1 mM $(\text{MGD})_2\text{Fe}^{2+}\text{-}^{14}\text{NO}$ complex changed the triplet spectrum (Figure 3A; $a^{14}\text{N} = 12.7$ G) to a doublet spectrum (Figure 3C) via a mixed EPR spectrum (Figure 3B). The doublet EPR spectrum is that of the $(\text{MGD})_2\text{Fe}^{2+}\text{-}^{15}\text{NO}$ complex (Figures 3C and 3D; $a^{15}\text{N} = 17.8$ G) [36]. The concentrations of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complexes, which were calculated by double integration of the spectra, were equal for each EPR spectrum (Figures 3A–3D).

Oxygen consumption studies

The change of oxygen consumption during the oxidation of the $(\text{MGD})_2\text{Fe}^{2+}$ complex in the presence of thiol compounds at pH 7.4 in Hepes buffer was measured. When 0.2 mM Fe^{2+} was introduced into the oxygen electrode containing 1.0 mM MGD,

Figure 2 Relationship between GSNO concentration and EPR signal intensity of MGD-ferrous-NO complex (A) and effect of initial MGD-ferrous complex/GSNO ratio on the production of the MGD-ferrous-NO complex (B)

(A) Concentration dependence of GSNO (0.2–2.0 μM) on NO formation in a reaction mixture containing 0.1 mM ferrous and 0.5 mM MGD in 0.5 M Hepes buffer (pH 7.4) under anaerobic conditions. The EPR signal intensity was calculated by double integration of each spectrum, and each data point represents the means $\pm$ S.D. for three different experiments. These spectra were recorded after 2 min incubation at room temperature. (B) GSNO (0.1 mM) was introduced into the reaction mixture containing various concentrations of the MGD-ferrous complex (0.05–0.5 mM Fe^{2+} ; $[\text{Fe}^{2+}]/[\text{MGD}]$ ratio, 1:5) in 0.5 M Hepes buffer (pH 7.4) under anaerobic conditions at room temperature. The concentration of MGD-ferrous-NO complex was calibrated by using saturated NO solutions under the same experimental conditions. EPR spectrometer instrumental settings were: microwave power, 20 mW; modulation amplitude, 2.0 G; modulation frequency, 50 kHz; sweep time, 336 s (A) and 168 s (B); receiver gain, 1.0×10^6 (A) and 5×10^3 (B).

rapid but transient oxygen consumption was observed ($-\Delta[\text{O}_2] = 54 \mu\text{M}$). If the $(\text{MGD})_2\text{Fe}^{2+}$ complex completely reduced O_2 to H_2O , then the $\text{Fe}^{2+}/\text{O}_2$ ratio was expected to be 4:1 [37]. This rapid oxygen consumption gave a ratio of 3.7:1, which is in good agreement with this expectation. The oxygen consumption was greatly accelerated upon the addition of 1 mM GSH (at the rate of $0.2 \text{ mM O}_2 \cdot \text{s}^{-1}$) or 1 mM L-cysteine ($2.0 \text{ mM O}_2 \cdot \text{s}^{-1}$) and continued until all of the oxygen was consumed.

5,5-Dimethylpyrroline-N-oxide (DMPO) spin adducts formed from $(\text{MGD})_2\text{Fe}^{2+}$ complex and thiol compounds

In a previous study [38], we demonstrated the formation of reactive oxygen species from the $(\text{MGD})_2\text{Fe}^{2+}$ complex with molecular oxygen. The fact that thiol compounds stimulated the oxygen consumption indicated the enhanced formation of reactive oxygen species. We conducted spin-trapping experiments

with DMPO to measure the production of reactive oxygen species. When 500 mM DMSO was added to the reaction mixture containing 1 mM $(\text{MGD})_2\text{Fe}^{2+}$ complex (1 mM FeSO_4 + 5 mM MGD) and 100 mM DMPO in Hepes buffer (pH 7.4) under anaerobic conditions, no adducts of DMPO were detected (Figure 4A). However, under aerobic conditions, the DMPO/ $\text{CH}_3\cdot$ spin adduct was observed (Figure 4B) with hyperfine splitting constants $a^N = 16.3 \text{ G}$ and $a = 23.4 \text{ G}$ [39], and this radical adduct formation was attenuated by the addition of catalase [38]. The addition of 1 mM GSH or 1 mM L-cysteine enhanced the formation of the DMPO/ $\text{CH}_3\cdot$ adduct (Figures 4C and 4D). These results clearly indicated that the formation of reactive oxygen species, presumably the hydroxyl radical ($\text{OH}\cdot$) or a closely related species that reacted with DMSO to produce $\text{CH}_3\cdot$ [40], was enhanced by physiological concentrations of thiol compounds.

Absorption spectrum of the iron-MGD complex after the addition of thiol compounds

Figure 5 shows the changes of the absorption spectra of the $(\text{MGD})_3\text{Fe}^{3+}$ complex (Figure 5A) in the presence of thiol compounds in 20 mM Hepes buffer (pH 7.4) under anaerobic conditions at room temperature. The absorption maximum at 515 nm was characteristic of the 6-co-ordinate Fe^{3+} -dithiocarbamate complex [41]. When 5 mM GSH was introduced into the 0.5 mM $(\text{MGD})_3\text{Fe}^{3+}$ complex, the absorption spectrum decreased (Figure 5B), whereas it increased after aeration for 30 s (Figure 5C). An insoluble substance, presumably GSSG, may be responsible for the excess absorption over the control spectrum produced after the introduction of air. Similarly, the addition of 0.5 mM L-cysteine converted the absorption spectrum of the $(\text{MGD})_3\text{Fe}^{3+}$ complex (Figure 5A) into that of $(\text{MGD})_2\text{Fe}^{2+}$ (Figure 5F). The absorption spectrum was restored to that of the $(\text{MGD})_3\text{Fe}^{3+}$ complex by bubbling with air for 30 s (Figure 5E). These results indicate that the $(\text{MGD})_3\text{Fe}^{3+}$ complex was reduced to the $(\text{MGD})_2\text{Fe}^{2+}$ complex in the presence of thiol compounds. The first-order rate constants for the reduction of the $(\text{MGD})_3\text{Fe}^{3+}$ complex by GSH and by L-cysteine were calculated to be 1.3 and $2.0 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively, from the disappearance of the $(\text{MGD})_3\text{Fe}^{3+}$ complex measured at 515 nm. We also measured the self-reduction rate constant for the $(\text{MGD})_3\text{Fe}^{3+}$ complex, because MGD contains thiol groups. The first-order rate constant for the reduction of the $(\text{MGD})_3\text{Fe}^{3+}$ complex by MGD was determined to be $0.15 \text{ M}^{-1} \cdot \text{s}^{-1}$, which was small compared with that of GSH and L-cysteine.

GSH concentration after exposure of PC12 cells to the iron-MGD complex

To examine whether the iron-MGD complex modulates the intracellular GSH level, we exposed cultured PC12 cells to either the $(\text{MGD})_2\text{Fe}^{2+}$ or the $(\text{MGD})_3\text{Fe}^{3+}$ complex for 120 min.

When cells were exposed to either complex, significant ($P < 0.01$) reduction of the GSH concentration was observed [$1.3 \pm 0.2 \text{ nmol of GSH}/10^6 \text{ cells for } (\text{MGD})_2\text{Fe}^{2+}$ and $0.9 \pm 0.2 \text{ nmol of GSH}/10^6 \text{ cells for } (\text{MGD})_3\text{Fe}^{3+}$ versus $2.6 \pm 0.2 \text{ nmol of GSH}/10^6 \text{ cells for control}$]. However, no significant decrease in GSH was measured with ferrous ($2.6 \pm 0.2 \text{ nmol of GSH}/10^6 \text{ cells for } \text{FeSO}_4$), ferric ($2.4 \pm 0.3 \text{ nmol of GSH}/10^6 \text{ cells for } \text{FeCl}_3$) or MGD ($2.4 \pm 0.4 \text{ nmol of GSH}/10^6 \text{ cells}$) treatment of the PC12 cells. These results clearly indicated that the iron-MGD complex depletes intracellular GSH.

Figure 3 Reaction between iron-bonded NO and free NO

(A) EPR spectrum of 0.1 mM MGD–ferrous complex (0.1 mM Fe^{2+} and 0.5 mM MGD) formed by bubbling with ^{14}NO gas for 20 min, then removing the remaining ^{14}NO gas by bubbling with Ar gas for 10 min at room temperature. (B) Same as (A), but bubbled with the ^{15}NO gas for 5 min after Ar treatment before removing the ^{15}NO gas by bubbling with Ar for 10 min. (C) Same as (B), but bubbled with ^{15}NO gas for 20 min. (D) Same as (A), but with ^{15}NO gas. EPR spectrometer instrumental settings were: microwave power, 20 mW; modulation amplitude, 2.0 G; modulation frequency, 100 kHz; sweep time, 240 s; receiver gain, 25.

Figure 4 Effects of thiol compounds on the production of DMPO/CH_3 radical adduct in the presence of air

(A) 100 mM DMPO with 1 mM Fe^{2+} , 5 mM MGD and 500 mM DMSO in 20 mM Hepes buffer (pH 7.4) under anaerobic conditions. (B) Same as (A), but aerated for 1 min. (C) Same as (B), but in the presence of 1 mM GSH. (D) Same as (B), but in the presence of 1 mM L-cysteine. EPR spectrometer instrumental settings were: microwave power, 20 mW; modulation amplitude, 2.0 G; modulation frequency, 50 kHz; sweep time, 82 s; receiver gain, 50000.

DISCUSSION

Iron–dithiocarbamate complexes such as DETC [14], MGD [16], proline dithiocarbamate [42] and *N*-(dithiocarboxy)sarcosine [43] have been used as spin-trapping agents for NO. Coexistence of these iron complexes and NO leads to a three-line EPR signal at

room temperature. Because of the hydrophobic property of the $(\text{DETC})_2\text{Fe}^{2+}\text{--NO}$ complex, other dithiocarbamate complexes such as MGD–Fe, *N*-(dithiocarboxy)sarcosine–Fe and proline dithiocarbamate–Fe for *in vivo* NO detection have been investigated by several researchers [16,36,43–57]. However, little is known about the redox chemistry of these compounds. Although

Figure 5 Changes of the absorption spectra of the $(\text{MGD})_3\text{Fe}^{3+}$ complex in the presence of thiol compounds

(A) 0.5 mM Fe^{3+} and 2.5 mM MGD. (B) Same as (A), but mixed with 5 mM GSH. (C) Same as (B), but aerated for 30 s. (D) Same as (A), but mixed with 0.5 mM L-cysteine. (E) Same as (D), but aerated for 30 s. (F) 0.5 mM Fe^{2+} and 2.5 mM MGD under the same conditions as (A). All chemicals were mixed in 20 mM Hepes buffer (pH 7.4) under anaerobic conditions at room temperature. Measurements were started within 1 min after the addition of all chemicals.

it is widely assumed that only NO can react with the $(\text{MGD})_2\text{Fe}^{2+}$ complex to form the $(\text{MGD})_3\text{Fe}^{2+}\text{-NO}$ complex (Scheme 1), a previous study has reported that the $(\text{MGD})_2\text{Fe}^{2+}$ complex can react not only with NO but also with nitrite to produce the characteristic triplet EPR signal of $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ [20]. This reaction mechanism was investigated in our previous study [21]. In the present study, we found that the formation rate of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex from nitrite was accelerated in the presence of thiol compounds under anaerobic conditions (Figure 1).

Next, we studied the reaction between GSNO and $(\text{MGD})_2\text{Fe}^{2+}$ under anaerobic conditions. *S*-Nitrosothiol compounds are known to be unstable under physiological conditions [58], with the rate of NO liberation depending on light or the presence of transition metals [59–64]. However, in the absence of light and transition-metal ions, *S*-nitrosothiols are stable at physiological pH and temperature [65]. Other authors have also reported that at pH 7.4, the spontaneous decomposition of *S*-nitrosothiols was slow [66], and that 1 mM GSNO was stable for at least 90 min under aerobic conditions [67].

As shown in Figures 2 and 3, the reaction between GSNO and the $(\text{MGD})_2\text{Fe}^{2+}$ complex was complete within 2 min under anaerobic conditions even in the dark. Since GSNO does not decompose to NO spontaneously, $(\text{MGD})_2\text{Fe}^{2+}$ must react with GSNO to form the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex (Scheme 1).

Arnell et al. [68] proposed a mechanism of *S*-nitrosothiol decomposition by DETC. In contrast, our present results do not support the proposal that GSNO was decomposed by DETC itself. As shown in Figures 2(A) and 3, the reaction between $(\text{MGD})_2\text{Fe}^{2+}$ and GSNO was completed within 2 min, whereas the liberation of NO from GSNO by DETC was slow under anaerobic conditions [68]. Probably, GSNO had reacted with $(\text{DETC})_2\text{Fe}^{2+}$ formed by trace iron. Furthermore, if the production of NO from GSNO was independent of the $(\text{MGD})_2\text{Fe}^{2+}$ complex, the production of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex from GSNO would not be complete at the ratio of $(\text{MGD})_2\text{Fe}^{2+}/\text{GSNO} = 2$, since $[\text{MGD}] \gg [\text{Fe}^{2+}]$ (Figure 2B). This result clearly indicates that $(\text{MGD})_2\text{Fe}^{2+}$ was responsible for the decomposition of GSNO.

Experiments using an isotope-labelling technique clearly indicate that the NO molecule of the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex exchanges with free NO (Figure 3):

It seems that the $(\text{MGD})_2\text{Fe}^{2+}\text{-NO}$ complex can dissociate and reassociate with the NO molecule in the presence of free NO.

We considered the possible formation of *S*-nitrosothiols from nitrite and thiol compounds under the experimental conditions of Figure 1 and examined whether the formation of the

Scheme 1 Possible reaction pathways of $(\text{MGD})_2\text{Fe}^{2+}$ in biological systems

(MGD)₂Fe²⁺–NO complex from nitrite at neutral pH could be attributed to the formation of *S*-nitrosothiol. By using an HPLC method, Goldman et al. [69] reported that no GSNO formation was observed from the mixture containing sodium nitrite and GSH at neutral pH values. This result was also supported by calculations. The third-order rate constants between nitrite and GSH or L-cysteine were reported as 1080 and 456 M⁻²·s⁻¹ respectively [70]. According to the rate constants mentioned above, the spontaneous formation rates of GSNO and L-CysNO were calculated as 2 × 10⁻¹¹ and 9 × 10⁻¹³ M·s⁻¹ respectively, under the experimental conditions of Figure 1(A). These values were smaller than the observed values of (MGD)₂Fe²⁺–NO formation (2.2 × 10⁻¹⁰ and 3.1 × 10⁻¹⁰ M·s⁻¹ respectively). This means that the contribution of *S*-nitrosothiol formation from nitrite would be negligible.

In general, the mechanism for autoxidation of Fe²⁺ is often described according to the following reactions:

By adding Eqns. (2–5), the following reaction was obtained:

On the basis of Eqn. (6), an Fe²⁺/O₂ concentration ratio of 4:1 is expected. The Fe²⁺/O₂ consumed ratio of 3.7:1 at the early stage of the reaction (0–2 min after the addition of Fe²⁺) was in good agreement with the expected stoichiometry [38].

GSH and L-cysteine are important intracellular reductants [71]. Intracellular concentrations of GSH are reported to be 0.5–10 mM [72–74], and the L-cysteine content of the liver is submillimolar [75]. The addition of thiol compounds accelerated the oxygen consumption by (MGD)₂Fe²⁺ solutions, with the O₂ consumption rate in the presence of L-cysteine being greater than that in the presence of GSH. To investigate the formation of reactive oxygen species (Scheme 1), we adopted the spin-trapping method using DMPO. As shown in Figure 4, in the presence of thiol compounds and DMSO, an increase in the DMPO/CH₃· radical adduct concentration was observed, and the effect of L-cysteine was greater than that of GSH (Figures 4C and 4D). When catalase was added to the reaction mixture, the DMPO/CH₃· radical adduct was decreased (results not shown), as reported previously [38]. These results indicate that the coexistence of thiol compounds accelerated the formation of reactive oxygen species such as OH· or a OH·-like species [76], which reacts with DMSO to produce CH₃· [40,77].

To demonstrate further that the thiol compounds act as a reductant of the (MGD)₃Fe³⁺ complex, we measured the absorbance change of (MGD)₃Fe³⁺ in the presence or absence of thiol compounds. As shown in Figure 5, these thiol compounds reduced (MGD)₃Fe³⁺ to the (MGD)₂Fe²⁺ complex. Second-order rate constants between (MGD)₃Fe³⁺ and GSH or L-cysteine were calculated to be 1.3 × 10² and 2.0 × 10² M⁻¹·s⁻¹ respectively. Moreover, these absorption spectra of the reduced (MGD)₂Fe²⁺ complex were returned to that of the oxidized complex by air oxidation (Figures 5C and 5E). From the results in Figure 5 and the fact that the (MGD)₂Fe²⁺ complex produces reactive oxygen species after air oxidation (Figure 4) [38], we expect that GSH or L-cysteine contributes to the formation of reactive oxygen species through the reduction of the (MGD)₃Fe³⁺ complex in the presence of molecular oxygen (Scheme 1). We

Table 1 Rate constants of iron–dithiocarbamate complexes

Iron complex	Reactant	Rate constants	Reference
(MGD) ₃ Fe ³⁺	L-Cys	2.0 × 10 ² M ⁻¹ ·s ⁻¹	This work
(MGD) ₃ Fe ³⁺	GSH	1.3 M ⁻¹ ·s ⁻¹	This work
(MGD) ₃ Fe ³⁺	MGD	0.15 M ⁻¹ ·s ⁻¹	This work
(MGD) ₂ Fe ²⁺	NO ₂ ⁻	4.84 ± 0.13 M ⁻² ·s ⁻¹	[21]
(MGD) ₂ Fe ²⁺	NO ₂ ⁻	1.46 ± 0.08 M ⁻² ·s ⁻¹	[21]
(MGD) ₂ Fe ²⁺	O ₂	5 × 10 ⁵ M ⁻¹ ·s ⁻¹	[38]
(MGD) ₂ Fe ²⁺	NO	(1.21 ± 0.53 × 10 ⁶) M ⁻¹ ·s ⁻¹	[56]

then utilized PC12 cells to determine if this generation of reactive oxygen species caused oxidative stress in a living system. Depletion of intracellular GSH concentration was observed due to the following reactions:

The resulting superoxide will also oxidize GSH through the formation of peroxynitrite [78], and the hydrogen peroxide formed from the superoxide will oxidize GSH through the action of glutathione peroxidase. Regardless of the mechanisms, MGD–iron complexes can deplete intracellular GSH.

In our previous reports, we demonstrated that the (MGD)₂Fe²⁺ complex (1) reacts with molecular oxygen to form reactive oxygen species, which are capable of oxidizing some nitrogen-containing compounds to NO under aerobic conditions [38], and (2) reduces nitrite to form (MGD)₂Fe²⁺–NO at physiological pH value under anaerobic conditions [21]. We have now demonstrated that physiological thiol compounds such as GSH and L-cysteine reduce (MGD)₃Fe³⁺ to the (MGD)₂Fe²⁺ complex, which facilitates the formation of reactive oxygen species, and that (MGD)₂Fe²⁺ reacts not only with NO but also very rapidly with *S*-nitrosothiols to form the same complex as NO.

In the utilization of (MGD)₂Fe²⁺ for the detection of NO *in vitro* [79,80], *in vivo* and *ex vivo* [81,82], one should carefully consider its diverse reaction properties (Scheme 1). Which of these reactions will dominate *in vivo* will depend, to a large extent, on the relative concentrations of thiol compounds, molecular oxygen, nitrite, *S*-nitrosothiol and NO as well as the various reaction rates of the iron–MGD complex (Table 1). The presence of thiol compounds facilitates the formation of the (MGD)₂Fe²⁺–NO complex from nitrite. In addition, thiol compounds promote the formation of reactive oxygen species, which cause oxidative stress, or they react with nitrogenous compounds such as hydroxyurea to produce NO [38]. The very rapid reaction of (MGD)₂Fe²⁺ with GSNO to form (MGD)₂Fe²⁺–NO clearly demonstrates the non-specific character of NO analysis based on the formation of the (MGD)₂Fe²⁺–NO complex. Under our conditions, the detection limit of GSNO by the (MGD)₂Fe²⁺ complex (50 nM; Figure 2A) partially overlaps with the physiological concentration of GSNO (20–200 nM) [83]. This is approximately the same detection limit as for NO itself [46]. In any case, special attention to these facts is needed to interpret the detection of the EPR signal of the (MGD)₂Fe²⁺–NO complex from biological samples containing thiol compounds, nitrite and/or *S*-nitrosothiols.

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