Nitrosothiol Formation Catalyzed by Ceruloplasmin

IMPLICATION FOR CYTOPROTECTIVE MECHANISM IN VIVO*

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Katsuhisa Inoue‡‡, Takaaki Akaite‡‡, Yoichi Miyamoto‡, Tatsuya Okamoto‡, Tomohiro Sawa‡, Masaki Otagiri‡, Shinnichiro Suzuki‡‡, Tetsuhiko Yoshimura‡‡, and Hiroshi Maeda‡‡

From the ‡Department of Microbiology, Kumamoto University School of Medicine, Kumamoto 860-8511, ‡Department of Pharmaceutics, Faculty of Pharmaceutical Science, Kumamoto University, Kumamoto 862-8573, **Department of Chemistry, Graduate School of Science, Osaka University, Osaka 560-0043, and ‡‡Institute for Life Support Technology, Yamagata Technoplos Foundation, Yamagata 990-2473, Japan.

Ceruloplasmin (CP) is a major multicopper-containing plasma protein that is not only involved in iron metabolism through its ferroxidase activity but also functions as an antioxidant. However, physiological substrates for CP have not been fully identified nor has the role of CP been fully understood. The reaction of nitric oxide (NO) with CP was investigated in view of nitrosothiol (RS-NO) formation. First, formation of heavy metal- or CP-catalyzed RS-NO was examined with physiologically relevant concentrations of NO and various thiol compounds (RSH) such as glutathione (GSH). Among the various heavy metal ions and copper-containing enzymes and proteins examined, only copper ion (Cu²⁺) and CP showed potent RS-NO (S-nitrosogluta-thione)-producing activity. Also, RS-NO-forming catalytic activity was evident for CP added exogenously to RAW264 cells expressing inducible NO synthase in culture, but this was not the case for copper ion. Similarly, CP produced endogenously by HepG2 cells showed potent RS-NO-forming activity in the cell culture. One-electron oxidation of NO appears to be operative for RS-NO production via electron transfer from type 1 copper to a cluster of types 2 and 3 copper in CP. Neurological disorders are associated with aceruloplasminemia; besides RS-NO, S-nitrosogluthathione particularly has been shown to have neuroprotective effect against oxidative stress induced by iron overload. Thus, we suggest that CP plays an important catalytic role in RS-NO formation, which may contribute to its potent antioxidant and cytoprotective activities in vivo in mammalian biological systems.

Nitric oxide (NO)¹ may be converted to nitrosoum cation in biological systems through interaction with molecular oxygen (O₂) and thiol compounds (RSH), thus forming nitrosothiols (RS-NO) (1–3). Some of the diverse biological functions of NO appear to be mediated by RS-NO. For example, nitrosylation of various endogenous proteins may modulate intracellular and intercellular signal transduction, including gene transcription (4) and cell apoptosis (5–7). It has been suggested that NO is stored and carried as RS-NO adducts and that S-nitrosoglutathione (GS-NO), among other RS-NO adducts, might be a major reservoir of NO in cells and tissues (8, 9). In addition, NO is readily released from RS-NO via reduction by transition metal ions as well as thiols and ascorbate (10). Thus, the formation of RS-NO not only serves biological functions (endogenous storage and transport of NO) but also preserves the antioxidant effect of NO (NO is protected from reaction with superoxide, O₂⁻, which would lead to formation of a potent cytotoxic peroxynitrite, ONOO⁻) (11, 12). Cytoprotective and antioxidant properties of RS-NO were recently confirmed in studies in vitro and in vivo (13–16).

Ceruloplasmin (CP) is abundant in the plasma of vertebrates and is synthesized in the liver (17), but cells other than hepatocytes, such as macrophages, are also known to produce CP (18–20). In addition, a recent study indicated that this protein is synthesized locally in the central nervous system (21, 22). Furthermore, production of CP is up-regulated at the transcriptional level as an acute-phase reactant in various inflammatory conditions, in cirrhosis of the liver and in acute myocardial infarction (23). In contrast, a patient with aceruloplasminemia has hemosiderosis, diabetes, retinal degeneration, and neurological deficit (24, 25). Some of the pathological consequences in aceruloplasminemia are thought to be caused by impairment of iron uptake due to a loss of ferroxidase activity of CP (26, 27). Although one of the important functions of CP may be its ferroxidase-related activity for iron uptake, the physiological substrate for CP has not been fully identified, nor has the role of CP been fully understood. In the present study, we investigated metal- and CP-catalyzed RS-NO formation occurring under physiological conditions.

EXPERIMENTAL PROCEDURES

High Performance Liquid Chromatography (HPLC) Flow Reactor Analysis for RS-NO—We recently developed a sensitive and specific RS-NO assay by using HPLC coupled with a flow reactor system (28). This assay used the flow reactor system with mercuric chloride (HgCl₂) and the Griess reagent to detect various peaks of RS-NO eluted from a reverse phase C₁₈ HPLC column. An aliquot (150 μl) was applied to an HPLC column, C₁₈ reverse phase (4.6 × 250 mm; TSK gel ODS-80Ts; Tosoh Co., Ltd., Tokyo, Japan). To separate each RS-NO, the column was eluted with 10 mM sodium acetate buffer (pH 5.5) containing 0.5 mM diethylenetriaminepentaacetic acid with 15% methanol at a flow rate of 0.55 ml/min. The eluate from the HPLC column, connected to a reaction coil, was mixed with a reactant solution containing 1.75 mM H₂O₂.
HgCl₂ (to induce RS-NO decomposition to form NO₃⁻) and Griess reagent;
the mixture was fed through a three-way connector at a flow rate of
0.2 ml/min. The diazo compound obtained was detected at 540 nm by
using a visible light detector (Eicom Co., Ltd., Kyoto) and an integrator
(SYSTEM IV Instruments, Co., Ltd., Tokyo). 

CP and Other Copper Proteins—CP was first isolated by DEAE anion exchange column chromatography
according to the method reported previously (28). In some experiments,
CP was first isolated by DEAE anion exchange column chromatography
followed by purification on an aminomethyl-derivatized Sepharose column chromatography
according to the method reported previously (29). In some experiments,
CP was first isolated by DEAE anion exchange column chromatography
followed by purification on an aminomethyl-Sepharose column. Laccase
from lacquer, ascorbate oxidase from Cucurbita pepo, and azurin and
nitrite reductase from Alcaligenes polyoxidans were purified as
described previously (30–32). A human recombinant Cu,zn-superoxide
dismutase was used provided by Nippon Kayaku Co., Ltd., Tokyo, Japan.
Hemocyanin from keyhole limpets (Megathura crenulata) was
purchased from Sigma. All copper-containing proteins used in this study
were more than 95% pure as judged by SDS-polyacrylamide gel
electrophoresis as described previously (33).

RS-NO Generation in Cell-free Reaction Systems—The NO-releasing reaction was carried out by using propylamine P-NONOate (CH₃N-
(CH₂)₃NH₂) (Genzyme, Cambridge, MA) at 100 units/ml and li-
popolysaccharide (Escherichia coli 026B; Difco) at 10 µg/ml for 12 h
at 37 °C in a CO₂ incubator (5% CO₂, 95% air (v/v)). The culture medium was
then washed, and the cells were resuspended in Krebs-Ringer phosphate
buffer (pH 7.4). Cells were further incubated in the CO₂
incubator with 200 µl of Krebs-Ringer phosphate buffer containing 0.5
mM l-arginine with or without various concentrations of GSH and/or CP
at 37 °C. After incubation for 45 min, the reaction medium was harvested
and mixed with the same volume of pure water containing 10%
methanol and 1 mM diethylenetriaminepentaacetic acid (pH 7.4), fol-
lowed by incubation at 10,000 × g for 30 min at 37 °C. The supernatant
(150 µl) was then applied to the HPLC column for RS-NO analysis just described. All buffers in this experiment were treated
with Chelex 100 resin (Bio-Rad) to remove trace amounts of heavy
metal contaminants.

RS-NO Production by Cells in Culture—RS-NO produced extracellularly in RAW264 cells (a murine macrophage cell line) and HepG2 cells
(a human hepatocyte cell line) was investigated by using the above-
described HPLC-flow reactor system with HgCl₂ and Griess reagent
according to our earlier report (28). RAW264 cells were cultured in
24-well plates (16-mm diameter; Falcon, Lincoln Park, NJ) with a plating density
of 1 × 10⁶ cells/well. After overnight culture, the conflu-
cent monolayer of the cells were rinsed with Krebs-Ringer phosphate
buffer (pH 7.4). The reaction mixture, which inhibited metal-catalyzed degradation of RS-
NO, Aliquots (150 µl) were then applied to the HPLC column for RS-NO analysis just described. All buffers in this experiment were treated
with Chelex 100 resin (Bio-Rad) to remove trace amounts of heavy
metal contaminants.

Cu²⁺-catalyzed RS-NO Generation in Cell-free Systems—First, to explore the RS-NO-generating system, we examined
RS-NO formation catalyzed by heavy metal ions by using a mixture of glutathione (GSH) and P-NONOate (10 µM), an NO-releasing compound, with or without various heavy metal ions including iron and copper (0.5 µM each) in 0.1 M sodium
phosphate buffer, pH 7.4 (Fig. 1A). Appreciable RS-NO formation
was observed only with Cu²⁺ (CuSO₄) at a concentration of 0.5 µM. Although ferrous iron (Fe²⁺) was reported to catalyze
RS-NO generation via formation of complexes of dinitrosyl iron and
sulfur (37, 38), both Fe²⁺ and Fe³⁺ ions did not show effective RS-NO formation, at least with the low concentration of
NO used in this study.

We then tested the copper-containing protein CP for activity in
RS-NO formation by use of a cell-free NO-releasing system with P-NONOate. CP purified from human plasma catalyzed
significant GS-NO formation in a concentration-dependent fashion, and its activity was comparable to that of free Cu²⁺ ion
at concentrations less than 1.0 µM in the cell-free reaction.
mixture (Fig. 1B). Other mammalian CPs (from rabbits and rats) also showed potent RS-NO-producing activities (Fig. 1C).

It is of considerable interest that an increase in GS-NO formation (by 2-fold) was observed after the addition of physiological concentrations of NaCl (0.1–0.2 M) to the reaction mixture with CP but not to the free Cu²⁺ reaction system (data not shown).

Similar enhancement of the catalytic activity of CP by NaCl was reported for ferroxidase and amine oxidase (35).

For quantitative comparison of the catalytic activity of CP with that of Cu²⁺ for RS-NO production, RS-NO formation was examined as just described by varying the concentration of each reactant, P-NONOate and RSH as substrates and CP and Cu²⁺ as catalysts, for RS-NO generation. In these experiments, both GSH and N-acetyl-L-cysteine (NAC) were used as low molecular weight thiol substrates for S-nitrosylation. As shown in Fig. 2A, an almost linear increase in each RS-NO adduct (GS-NO and NAC-NO) was observed, in parallel to the concentration of CP or Cu²⁺ in the presence of RSH (10 μM) and P-NONOate (10 μM). RS-NO formation as catalyzed by CP was much higher than that catalyzed by Cu²⁺. The amount of RS-NO generated reached a plateau or declined, however, when CP or Cu²⁺ concentrations exceeded 2 μM. Similarly, CP showed more effective RS-NO generation than did free Cu²⁺ ion in the presence of various concentrations of NaCl or GSH (data not shown). Very little RS-NO was produced without CP or Cu²⁺ (Fig. 2A). The percentage of RS-NO yield from free NO ranged from 20 to 40% and from 50 to 70% (RS-NO/NO) for GS-NO and NAC-NO, respectively. Maximum production of RS-NO was achieved with NAC. 69.1 ± 3.3% NO was converted to NAC-NO in the reaction of 2.5 μM P-NONOate and 10 μM NAC in 0.1 M phosphate buffer plus 0.1 M NaCl (pH 7.4) in the presence of 2.0 μM CP. An appreciable amount of NO was converted to RS-NO by CP even at the nanomolar range of NO, whereas very little RS-NO formation was observed in the absence of CP (data not shown). In general, RS-NO formation was greater with NAC-NO than with GS-NO. These results suggest that CP works effectively for RS-NO formation with NO and RSH. When GS-NO and NAC-NO are formed, however, they appear to be partly degraded by Cu²⁺ or actually by Cu¹⁺ as reported previously (10). Accordingly, CP and Cu²⁺ can catalyze both synthesis and degradation of RS-NO, with RS-NO synthesis catalyzed by CP predominating compared with degradation under these physiological reaction conditions. In fact, CP decomposed RS-NO much less effectively than did Cu²⁺ ions in the presence of RSH (Fig. 2B).

**CP-catalyzed RS-NO Production in the Cells in Culture**—To further examine the production of RS-NO as catalyzed by CP in biological systems, the murine macrophage cell line RAW264...
NAC or GSH (Fig. 3 increased RS-NO production, even in the presence of exogenous RSH, according to our method. Typical elution profiles of RS-NO generation were shown in Fig. 3 (HPLC flow reaction). Very little extracellular GS-NO was formed with the addition of CP or CuSO₄, in the presence of NAC or GSH in 50 μM of Krebs-Ringer phosphate buffer, after which the amount of RS-NO in the culture medium was determined. A, elution profiles of various RS-NOs and NO₂ formed in the supernatant of the culture medium by the addition of P-NONOate (10 μM) to RAW264 cells. B, the concentration of CP or CuSO₄ was varied in the presence of 50 μM NAC or GSH; in C, various concentrations of GSH were used with either CP or CuSO₄. CP, interferon-γ and lipopolysaccharide-stimulated cells without CP or CuSO₄ addition. Data are expressed as the means ± S.E. of four experiments.

was studied in culture with or without CP. RAW264 cells were stimulated with interferon-γ and lipopolysaccharide to express inducible NO synthase (28). Then, extracellular RS-NO generation was measured in the supernatant of the culture medium with or without the addition of RSH, according to our method (HPLC flow reaction). Typical elution profiles of RS-NO generated in the culture supernatant were shown in Fig. 3A. Very little extracellular GS-NO was formed without the addition of CP and RSH, except that low levels of GS-NO production (approximately 100 nm) was observed without CP addition in the culture with 50 μM GSH (Fig. 3B). RS-NO formation increased in linear fashion, in parallel to the concentration of CP. In contrast, the addition of Cu⁺⁺ ion resulted in only marginally increased RS-NO production, even in the presence of exogenous NAC or GSH (Fig. 3B). Significantly high levels (>2 μM) of GS-NO generation from endogenous GSH by RAW264 cells became apparent when CP (2 μM) was added to the cell culture without exogenous GSH, and its formation increased in parallel to the concentration of GSH added to the cell culture together with CP (Fig. 3C). RS-NO formation was greater with NAC than with GSH, indicating that NAC is a better substrate than GSH for CP-catalyzed RS-NO production in cell culture. This result is consistent with RS-NO formation in the cell-free system as described above. The great efficacy of RS-NO generation from NAC may be due to the stability of NAC-NO; in a separate experiment, NAC-NO was less susceptible than GS-NO to Cu⁺⁺-catalyzed decomposition. It is of considerable interest that the concentration of NAC-NO formed reached one-third of the total concentration of NO-oxidized products (NO₂, NO₃, and RS-NO) in the reaction system of inducible NO synthase-expressed cells plus NAC and CP (data not shown).

Moreover, GS-NO was formed efficiently by CP produced endogenously by HepG2 cells in culture. Specifically, GS-NO formation was observed clearly in the culture medium of the cells containing various concentrations of GSH, when NO was generated in the medium by the addition of P-NONOate (10 μM) (Fig. 4). CP release from the cells into the medium was assessed by Western blotting and densitometrical analysis, and its time profile was correlated with that of the GS-NO-forming activity (Fig. 4A). The amounts of CP produced in the medium were 80 ± 16 nM and 144 ± 17 nM (n = 4; means ± S.E.) for 24- and 48-h culture of the cells, respectively. These values are almost consistent with those reported previously (17, 39). The GS-NO-producing activity recovered in the culture medium was increased in a time-dependent fashion after initiating cell culture (Fig. 4B) and inhibited strongly by treatment of the medium with anti-CP antibody (by more than 90%) (Fig. 4C) but not with nonprimed antibody (not shown), indicating that CP synthesized de novo by HepG2 cells in culture catalyzes RS-NO formation. The GS-NO-generating potential of CP produced by the cultured HepG2 cells is almost comparable with that of purified human CP; we found in a separate experiment that 100 nM purified CP generated almost 200 nM GS-NO from 10 μM P-NONOate and 100 μM GSH, and a similar range of GS-NO production was observed with the culture medium of HepG2 cells obtained after a 48-h culture, as shown in Fig. 4, B and C. CP-dependent GS-NO formation was reduced when P-NONOate was administered directly to the HepG2 cell culture (Fig. 4B). This reduction seems to be due to intracellular incorporation (or transnitrosylation) of GS-NO formed extracellularly via the catalytic reaction of CP, because we observed a similar decrease in the concentration of authentic GS-NO that was given exogenously to the cell culture (not shown). It is also important that GS-NO formation from endogenous GSH was observed appreciably in the culture medium without addition of GSH (Fig. 4C), suggesting that GS-NO could be readily formed by CP produced by the cells with concomitant NO generation under physiological conditions.

**Molecular Mechanism of CP-catalyzed RS-NO Formation**—Further studies were executed to elucidate the molecular mechanism of NO oxidation and RS-NO generation by CP. We first investigated the interaction of NO with various types of copper ion in the CP molecule by EPR spectroscopy at 110 K. Human purified CP gives EPR spectra derived from type 1 and 2 copper, clearly identified on the basis of a characteristic four-line hyperfine pattern at gᵣ regions. When human CP was reacted with NO derived from P-NONOate in a cell-free system under anaerobic conditions, the EPR signal intensity at both gᵣ and g₂ regions was attenuated, and the hyperfine structure of the type 1 signal was abolished, but an appreciable type 2 copper signal remained unaffected (data not shown). This is consistent with previous results and indicates that NO can react and bind with type 1 copper selectively (40). These data may be interpreted to suggest that an electron on the NO molecule that bound with the type 1 copper may be integrated into the electron orbit of the copper atom.

Also, as shown in Fig. 5A, RS-NO formation catalyzed by CP was significantly suppressed by various type 2 and type 3 copper binding inorganic compounds such as azide, cyanide, and fluoride (41, 42). Thus, it seems that not only type 1 but also type 2 and 3 clusters may be critically involved in the
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FIG. 4. GS-NO formation in HepG2 cell culture. A, after 24- or 48-h culture of the HepG2 cells in 500 μl of DMEM + nonessential amino acids, CP production was examined by Western blotting with the use of a specific anti-CP antibody. B, control (CP Std), purified human CP (100 ng). An aliquot (10 μl) of each culture medium or purified CP solution was subjected to SDS-polyacrylamide gel electrophoresis and followed by immunoblotting. The immunoreactive band for CP (132 kDa) is indicated by an arrow. B, time profile of GS-NO generation in the control medium. To the supernatant of the medium or directly to the HepG2 cell culture P-NONOate and GSH were added at final concentrations of 10 and 100 μM, respectively, followed by GS-NO formation and quantification. C, effect of GSH concentration and anti-CP treatment on GS-NO formation in the medium of the cells. The culture medium was harvested after a 48-h culture, and its supernatant was incubated with P-NONOate (10 μM) and various concentrations of GSH. Medium + anti-CP Ab, GS-NO formation in the medium treated with anti-CP antibody (immunoprecipitation). Control medium, medium without HepG2 cell culture. Data are the means ± S.E. (n = 4).

FIG. 5. Analysis for catalytic reaction of CP producing RS-NO. A, effect of various type 2 and 3 copper inhibitors on GS-NO formation catalyzed by CP. The reaction mixture contained human CP (0.5 μM), GSH (10 μM), and P-NONOate (0.2 or 10 μM) in the presence or absence of NaN3, NaCN, or NaF (1 mM each) in 0.1 M sodium phosphate buffer (pH 7.4) plus 0.1 M NaCl. The reaction proceeded at 37 °C for 30 min. Data are the means ± S.E. (n = 4). B, effect of ONOO− treatment of human CP on amine oxidase and GS-NO-producing activities. CP was treated with ONOO−, followed by measurement of the amine oxidase activity and GS-NO-producing potential. Data are the means ± S.E. (n = 4).

| Copper type (number of copper) | % Conversion (RS-NO/NONO) |
|-------------------------------|---------------------------|
| GS-NO                          | NAC-NO                    |
| Control                       | 0.27 ± 0.03               | 0.43 ± 0.02 |
| CuSO4                         | 5.61 ± 0.55               | 16.14 ± 0.24 |
| Human CP                      | 28.90 ± 2.38              | 50.03 ± 1.83 |
| Laccase                       | 10.42 ± 0.50              | 6.97 ± 0.83 |
| Ascorbate oxidase             | 6.03 ± 1.14               | 3.19 ± 0.15 |
| Azurin                        | 2.15 ± 0.24               | 1.53 ± 0.23 |
| Cu,Zn-SOD                     | 1.97 ± 0.01               | 1.91 ± 0.16 |
| Hemocyanin                    | 5.70 ± 0.23               | 2.75 ± 0.03 |
| Nitric oxide                   | 1.06 ± 0.05               | 2.85 ± 0.16 |

DISCUSSION

RS-NO can be generated in the presence of RSH during O2-dependent autooxidation of NO (which forms N2O3, a strong nitrosating substance). However, the reaction of physiological concentrations of NO (ranging from nm to low μM) with O2 occurs very slowly in solution at neutral pH under ambient conditions (44). Moreover, H2O2, which is available in excess in biological systems, will compete in the reaction of N2O3 with RSH (44). Therefore, RS-NO formation (thiol nitrosylation) in biological systems may require yet unspecified nitrosating systems occurring in vivo. In this context, our current study revealed for the first time a unique function of CP: biologically relevant RS-NO formation mediated through the oxidation reaction of NO catalyzed by CP.

CP is a multicopper enzyme consisting of three domains (45, 46). Three different types of copper, i.e. types 1, 2, and 3, are classified according to their coordinate binding structure and unique spectroscopic characteristics. These Cu2+ ions are located in different sites in the CP domains. Type 1 copper produces a blue color and an EPR signal with narrow hyperfine splitting. Type 2 has an EPR signal typical of regularly coordinated tetragonal copper complexes. Type 3 is an EPR-silent antiferromagnetically coupled binuclear Cu2+–Cu2+ unit. Type 1 copper is localized in each of three domains of CP. Other copper ions, i.e. types 2 and 3, form triclusters of one type 2 and two type 3 between domains 1 and 3, as revealed by x-ray crystallography (47).

It is well accepted that type 1 copper in multicopper enzymes functions as an electron acceptor, as was also revealed by the present EPR study; four electrons are transferred from type 1 copper to the types 2 and 3 cluster, where O2 is reduced to form H2O. Such an effective electron transfer reaction with reduction of oxygen to water by CP is coupled to one-electron oxidation of a variety of substances such as ferrous iron and various organic amines (48, 49). Therefore, similar to the ferroxidase reaction of NO with CP to form RS-NO as from CP and inactivate its oxidase activity (43), the RS-NO-generating activity of CP was significantly abolished (Fig. 5B). Therefore, the electron appears to be transferred from NO at the site of type 1 copper to O2 around the tri-copper cluster of CP along the same electron track as in amine oxidation by CP.

It is of paramount importance that RS-NO-generating activity was observed predominantly with multicopper proteins, which contain types 1, 2, and 3 copper such as CP as well as laccase. In contrast, the efficacy of RS-NO formation by other copper-containing proteins was lower than that of free copper ion (CuSO4), indicating type 1, 2, or 3 copper per se is not so potent in catalyzing RS-NO generation. Overall, CP is the most efficient RS-NO-generating protein among the multicopper enzymes and proteins tested (Table I).
and amine oxide reactions catalyzed by CP, one-electron oxidation of NO by CP to form NO\(^+\) coupled with four-electron reduction of O\(_2\) may operate in CP-catalyzed RS-NO generation from NO. In fact, we found that oxygen consumption is occurring in the reaction of NO and CP together with RSH (data not shown). The intramolecular electron transfer during CP-catalyzed RS-NO formation is shown schematically in Fig. 6.

CP exists abundantly in plasma (2–3 \(\mu\)M), and its production is highly up-regulated under inflammatory conditions. For example, the concentration of CP in the bronchoalveolar lavage fluid of adult respiratory distress syndrome patients increased up to approximately 1 \(\mu\)M (50). It is also known that a high concentration of GSH (400–600 \(\mu\)M) exists in the alveolar spaces of the lung (51, 52), although it is significantly decreased (30 \(\mu\)M) in adult respiratory distress syndrome (52), whereas the plasma contains only low \(\mu\)M levels of GSH in normal human subjects. Accordingly, the concentrations of CP and GSH used in our study should be physiologically conceivable so that we could understand the biological significance of CP-catalyzed RS-NO formation.

CP is synthesized mainly by hepatocytes and secreted to the plasma, and murine astrocytes as well as a human monocye cell line (U937 cells) are also reported to express CP (19, 21, 22, 34). Although CP expression by RAW264 cells remains clarified by RS-NO formation, which we verify with the HepG2 cells, more importantly, we found that GS-NO formation is catalyzed by RS-NO formation. In this context, astrocytes in the brain tissues have been shown to express a unique membrane-bound form CP (21, 22), and thus, lack of CP is suggested to cause oxidative stress and neuronal degeneration as seen in aceruloplasminemia (24, 25). In addition, our preliminary data show that GS-NO formation was clearly observed in the cultured rat brain slices without any addition of CP. All these results seem to imply possible involvement of CP-catalyzed RS-NO generation in cytoprotective actions of CP in vivo.

In conclusion, CP may play an important role not only in copper transport but also as a multicopper oxidase to oxidize NO, thus generating RS-NO in the presence of RSH. In the latter case, CP might function to protect cells and tissues by generating RS-NO from highly toxic free radicals and reactive nitrogen oxides such as hydroxyl radical and peroxynitrite.

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