Nitric Oxide Generation from Hydroxyurea via Copper-catalyzed Peroxidation and Implications for Pharmacological Actions of Hydroxyurea

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We investigated the generation of nitric oxide ($\cdot$NO) by H_2O_2-dependent peroxidation of hydroxyurea in the presence of copper-containing proteins such as Cu,Zn-superoxide dismutase (Cu,Zn-SOD) or ceruloplasmin as a catalyst. In the reaction mixture of hydroxyurea, Cu,Zn-SOD, and H_2O_2, $\cdot$NO generation was identified by measuring the specific electron spin resonance (ESR) signal of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). The ESR signal of the NO-hemoglobin adduct was also detected in human red blood cells during copper-catalyzed peroxidation of hydroxyurea. The $\cdot$NO production during peroxidation of hydroxyurea was quantified as NO_2^- formation, measured by using the Griess assay, and the amount of NO_2^- was dependent on the concentration of hydroxyurea of the reaction mixture. ESR spin trapping with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) showed hydroxy radical ($\cdot$OH) generation in the reaction of H_2O_2 with either Cu,Zn-SOD or ceruloplasmin. Several $\cdot$OH scavengers, such as ethanol, thiourea, DMPO, and dimethylsulfoxide, and the metal-chelating agent diethylenetriaminepentaacetic acid significantly inhibited $\cdot$NO generation from hydroxyurea. This indicates that $\cdot$NO release from hydroxyurea may be mediated by $\cdot$OH derived from the copper-catalyzed Fenton-like reaction. Incubation of hydroxyurea and Cu,Zn-SOD with xanthine oxidase and hypoxanthine in a system forming $O_2^- \rightarrow H_2O_2$ also resulted in appreciable $\cdot$NO production. These results suggest that $\cdot$NO production from hydroxyurea catalyzed by copper-containing proteins may be the molecular basis of the pharmacological and antitumor action of hydroxyurea.

Key words: Hydroxyurea — Nitric oxide — Superoxide dismutase — Ceruloplasmin — Free radicals

Hydroxyurea is a potent antitumor drug with cytocidal activity against leukemia, malignant lymphoma, and glioblastoma.[1–3] Recently, it was reported to be applicable to treatment of sickle cell anemia, in particular for prevention of the painful crises.[4] It is a potent inhibitor of ribonucleotide reductase, which is an essential enzyme for the synthesis of deoxyribonucleotides.[5] Inhibition of ribonucleotide reductase by hydroxyurea leads to depletion of cellular deoxyribonucleotide pools, resulting in termination of DNA synthesis and repair. This effect of hydroxyurea probably contributes to its potent antitumor action.

In addition, Kwon *et al.* suggested that the cytotoxic action of hydroxyurea may be attributable, at least in part, to nitric oxide ($\cdot$NO) or $\cdot$NO-like species generated from hydroxyurea by an oxidative reaction.[6] However, they employed a reaction system of hydrogen peroxide (H_2O_2) and a free heavy metal ($CuSO_4$), which is unlikely to be relevant to physiological systems.[6] In addition, no direct evidence of $\cdot$NO generation was provided.

In an earlier study, we reported that Cu,Zn-superoxide dismutase (Cu,Zn-SOD) generates hydroxy radical ($\cdot$OH) in the presence of H_2O_2.[7] This $\cdot$OH is highly reactive and mediates a variety of oxidation reactions such as hydrogen atom abstraction and hydroxylation. On the other hand, the high reactivity of $\cdot$NO causes damage to biologically important molecules, for instance, a human endogenous protease inhibitor, as we demonstrated previously.[7] We therefore hypothesized that $\cdot$OH generated from H_2O_2 in the presence of copper-containing proteins such as Cu,Zn-SOD and ceruloplasmin may cause $\cdot$NO generation from hydroxyurea. The present study was designed to test this hypothesis. Direct evidence of $\cdot$NO generation was obtained by an electron spin resonance (ESR) technique using an $\cdot$NO-specific reagent, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO),[8] or using hemoglobin as an endogenous spin trap for $\cdot$NO. In addition, $\cdot$NO generation was quantitatively studied by colorimetric assay with Griess reagents. Lastly, a chemical model reaction for $\cdot$NO production from hydroxyurea was explored by the use of H_2O_2 and copper-containing proteins as an $\cdot$OH generation system.

MATERIALS AND METHODS

Chemicals 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) of the highest purity, PTIO, and diethylenetriaminepentaacetic acid (DTPA) were obtained from Dojindo Laboratories, Kumamoto. Hydroxyurea was purchased from Nacalai Tesque Inc., Kyoto. Hypoxanthine was obtained from Wako Pure Chemical Industry, Co., Ltd.,

Osaka. All other chemicals were of the highest grade commercially available.

Enzymes and proteins Human recombinant Cu,Zn-SOD (4390 U/mg of protein) was a gift from Nippon Kayaku Co., Ltd., Tokyo. Human ceruloplasmin (50 U/mg of protein) was purchased from Sigma Chemical Co., St. Louis, MO. Xanthine oxidase (XO) from bovine milk was obtained from Boehringer Mannheim GmbH, Mannheim, Germany.

ESR study of ·NO and oxygen radical production Generation of free radical species from H_2O_2 and copper-containing proteins with or without hydroxyurea was examined by use of an ESR technique employing PTIO and DMPO for ·NO and ·OH, respectively, as we have described previously.[8-10] All buffers used in this study were treated with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA) before use to remove any trace metals.

Unless otherwise specified, the reaction was initiated by the addition of H_2O_2 (final concentration: 500 μM) to the reaction mixture containing various concentrations of hydroxyurea, and Cu,Zn-SOD or ceruloplasmin in the presence of either PTIO (500 μM) or DMPO (45 mM) in 200 μl of 10 mM sodium phosphate buffer (pH 7.5). The reaction mixture was transferred to an ESR quartz flat cell (160 μl effective volume) and subjected to X-band ESR measurement at room temperature (JES-RE1X spectrometer, JEOL, Tokyo). For detection of ·NO with PTIO, the reaction mixture was treated with 1 mM potassium ferricyanide for 5 min at room temperature before ESR measurement.

NO-hemoglobin was detected directly by ESR spectroscopy at $-163°C$.[11, 12] Red blood cells from human peripheral blood were incubated in the reaction mixture containing hydroxyurea (1 mM), Cu,Zn-SOD (500 U/ml), and XO (100 mU/ml) with or without hypoxanthine (2 mM) in 10 mM phosphate-buffered 0.15 M NaCl (pH 7.4) for 4 h at 37°C. After the incubation, the samples were centrifuged at 500g for 5 min and the red blood cells were washed with saline, followed by addition of inositol hexaphosphoric acid in hypotonic water. The resultant suspension was transferred to a quartz sample tube and frozen in liquid nitrogen, followed by X-band ESR measurement at $-163°C$ (ESP 380E, Bruker Instrument Inc., Rheinstetten, Germany).

Griess reagent assay Nitrite (NO_2^-) was quantified as described by Green et al.[13] The reaction mixture as just described (500 μl) was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2% H_3PO_4). The solution was incubated for 10 min at room temperature, the optical density at 540 nm due to the diazo compound formed with the Griess reagent in the reaction mixture was measured, and the amount of NO_2^- was determined by using $NaNO_2$ as a standard.

RESULTS

In this experiment, the ·NO-specific reagent PTIO was used to determine ·NO generation from hydroxyurea by the ESR technique. PTIO selectively reacts with ·NO, resulting in the formation of a deoxy form of PTIO, i.e., 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI). PTI gives an ESR signal that is clearly distinguished from that of PTIO, and thus we can readily identify ·NO generation by using PTIO. Fig. 1A shows
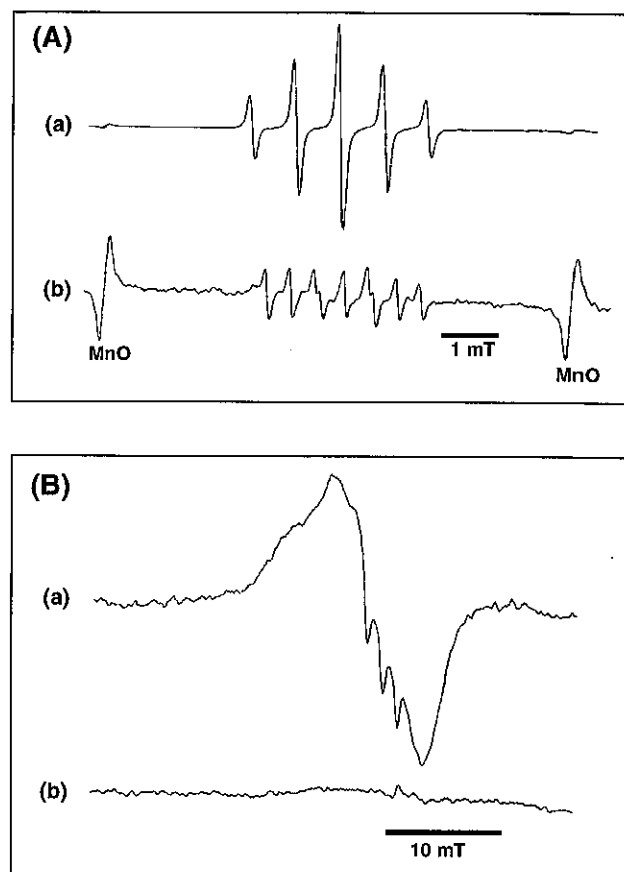

Fig. 1. A, ESR spectra of PTIO: (a) ESR spectrum for the reaction mixture of PTIO with H_2O_2 (5 mM) and hydroxyurea (1 mM); (b) that for the reaction mixture of H_2O_2 (5 mM), hydroxyurea (1 mM), and Cu,Zn-SOD (500 U/ml). The spectra in (A) were recorded at room temperature 120 min after the addition of H_2O_2. B, ESR spectra of NO-hemoglobin formed in human red blood cells during incubation with a reaction mixture of XO, Cu,Zn-SOD, and hydroxyurea. Red blood cells (1×10^7/ml) obtained from a healthy human volunteer were incubated with hydroxyurea (1 mM), Cu,Zn-SOD (500 U/ml), XO (100 mU/ml), and hypoxanthine (2 mM) (a) or without hypoxanthine (b) for 4 h at 37°C.
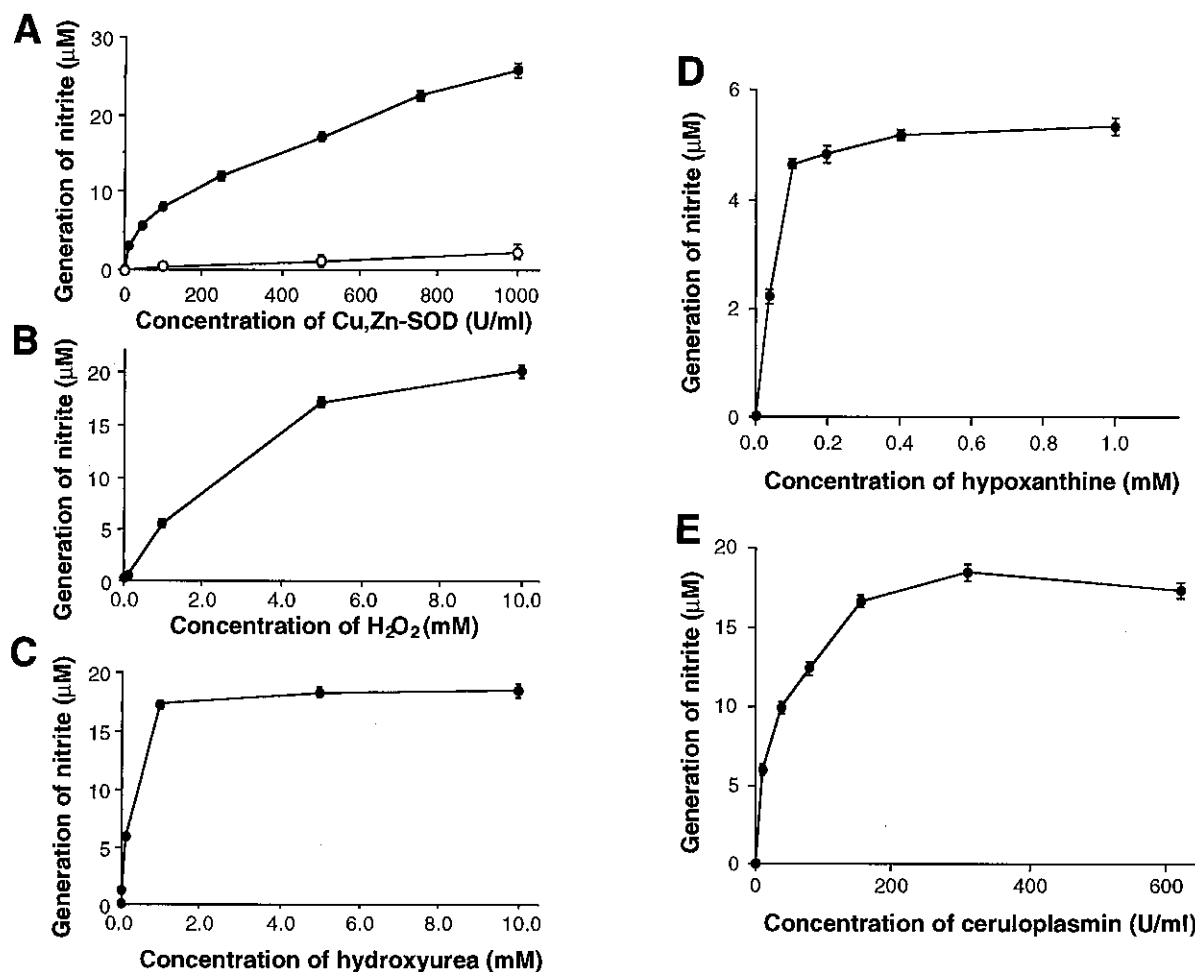
Fig. 2. Generation of nitrite determined by the Griess assay in the reaction system of Cu,Zn-SOD/H$_2$O$_2$/hydroxyurea (A–C), hypoxanthine/XO/Cu,Zn-SOD/hydroxyurea (D), or ceruloplasmin/H$_2$O$_2$/hydroxyurea (E). A, Hydroxyurea (1 mM), H$_2$O$_2$ (5 mM), and different concentrations of Cu,Zn-SOD (10–1000 U/ml); B, hydroxyurea (1 mM), Cu,Zn-SOD (500 U/ml), and various concentrations of H$_2$O$_2$ (0.01–10 mM); C, H$_2$O$_2$ (1 mM), Cu,Zn-SOD (500 U/ml), and different concentrations of hydroxyurea (0.01–10 mM); D, hydroxyurea (1 mM), Cu,Zn-SOD (500 U/ml), XO (100 mU/ml), and various concentrations of hypoxanthine (0.05–1 mM); E, hydroxyurea (1 mM), H$_2$O$_2$ (1 mM), and various concentrations of ceruloplasmin (10–600 U/ml). Open circles in (A) indicate nitrite generation in a complete reaction mixture with 500 μM DTPA. All reaction mixtures were incubated for 120 min at 37°C. Data are expressed as means±SEM ($n=5$).

the ESR spectra of the reaction mixture containing PTIO, hydroxyurea, and H$_2$O$_2$ with or without Cu,Zn-SOD. In the absence of Cu,Zn-SOD, a typical spectrum for PTIO was observed (Fig. 1A-a). On the other hand, addition of Cu,Zn-SOD to the reaction mixture altered the ESR spectrum to that of PTI ($a_N{}^1=0.98$, $a_N{}^3=0.44$ mT) (Fig. 1A-b). These results clearly indicate the generation of ·NO in the reaction system consisting of hydroxyurea, Cu,Zn-SOD, and H$_2$O$_2$.

We further investigated the generation of ·NO by measuring the NO-hemoglobin adduct in red blood cells after incubation with hydroxyurea, Cu,Zn-SOD, and

XO, with or without hypoxanthine. In this system, XO and hypoxanthine were used to supply reactive oxygen species, including H$_2$O$_2$ and O$_2{}^-$. As shown in Fig. 1B, formation of NO-hemoglobin was observed in the reaction mixture containing all of the reagents, but not in the reaction without hypoxanthine, suggesting that ·NO generation requires copper-catalyzed peroxidation of hydroxyurea. The ·NO-generating mechanism described here is physiologically plausible, because all the components occur in living systems.

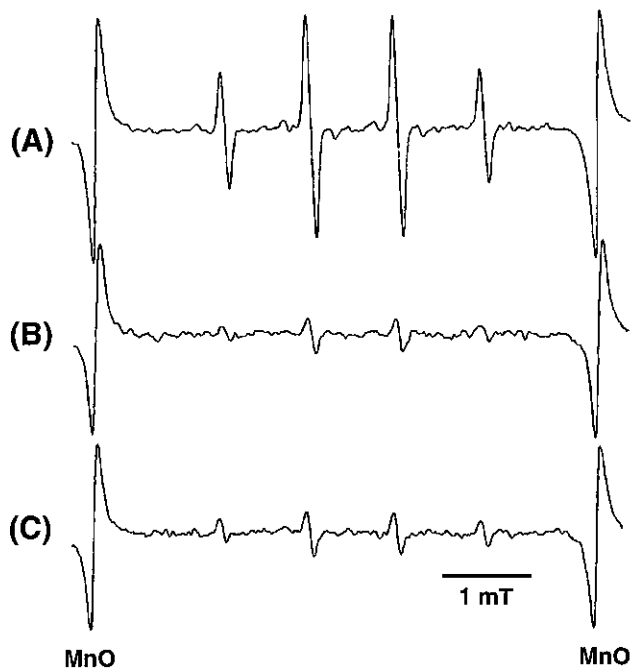·NO generation was examined quantitatively by using a colorimetric assay with Griess reagent and varying the

Fig. 3. ESR spectra of DMPO spin adduct in the reaction system of copper proteins and H_2O_2 or XO/hypoxanthine. A, Cu,Zn-SOD (500 U/ml)/H_2O_2 (500 μM); B, ceruloplasmin (60 U/ml)/H_2O_2 (500 μM); C, Cu,Zn-SOD (500 U/ml)/ hypoxanthine (1 mM) plus XO (50 mU/ml). All spectra were recorded at 120 min after addition of H_2O_2 (A and B) or hypoxanthine (C). ESR spectra were recorded at room temperature under the same conditions as for Fig. 1.

Fig. 4. Inhibitory effect of various 'OH scavengers, a metal-chelating agent, and spin trap agent for 'OH on nitrite generation from hydroxyurea. Inhibition of nitrite generation by various compounds was evaluated in the reaction of Cu,Zn-SOD (500 U/ml), H_2O_2 (5 mM), and hydroxyurea (1 mM). All reaction mixtures were incubated for 120 min at 37°C. DMSO, dimethyl sulfoxide. Data are mean ± SD of three different experiments.

concentrations of Cu,Zn-SOD, H_2O_2, and hydroxyurea (Fig. 2, A, B and C). In all cases, the amount of nitrite formed in the reaction increased in a concentration-dependent manner with respect to each component of the reaction mixture, although the increase of nitrite reached a plateau above 1 mM hydroxyurea. Removal of any component from the reaction mixture resulted in loss of nitrite production. In the reaction mixture containing 1 mM hydroxyurea, 1 mM H_2O_2, and 500 U/ml Cu,Zn-SOD, the amount of nitrite produced was approximately 17 μM within 2 h. Furthermore, addition of DTPA to the reaction mixture strongly inhibited nitrite formation (Fig. 2A), suggesting an important role of the copper ion of Cu,Zn-SOD in the peroxidation of hydroxyurea. The XO plus hypoxanthine system, generating H_2O_2 as well as O_2^-, was also found to be effective for 'NO production from hydroxyurea via copper-catalyzed peroxidation (Fig. 2D).

Cu,Zn-SOD was replaceable by another endogenous copper-containing protein, ceruloplasmin, without any loss of 'NO-generating potency (Fig. 2E). Ceruloplasmin
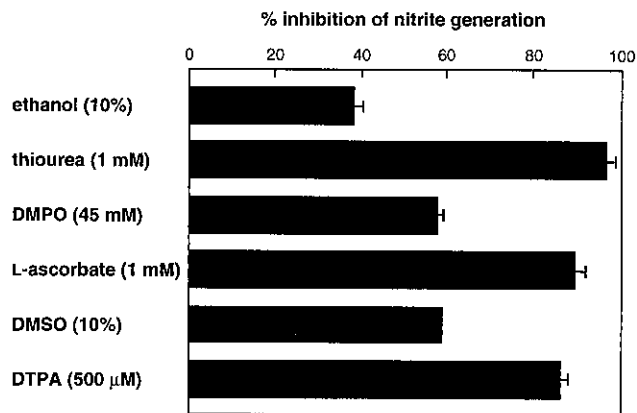
is not only a major copper-containing plasma component, but also one of the well-known acute phase proteins induced in inflammatory tissue and serum.

In our previous report, we showed that 'OH formed from Cu,Zn-SOD and H_2O_2 is a potent oxidant of Cu,Zn-SOD itself and of α_1-protease inhibitor.[7] In this context, 'OH generation was investigated in the reaction systems of Cu,Zn-SOD with H_2O_2 or with XO plus hypoxanthine, and of ceruloplasmin with H_2O_2. The 'OH production was examined by the ESR technique using the spin trap DMPO (Fig. 3). In all systems tested, generation of the DMPO-OH adduct was clearly observed, suggesting that 'OH was produced in the reaction of H_2O_2 with copper-containing proteins. To investigate the involvement of 'OH in 'NO generation from hydroxyurea, we examined the effects of 'OH scavengers such as ethanol, ascorbate, dimethyl sulfoxide, thiourea, and DMPO, and the metal-chelating agent DTPA, on 'NO generation from hydroxyurea in the presence of Cu,Zn-SOD and H_2O_2 (Fig. 4). All these reagents markedly inhibited the 'NO generation. Thiourea, L-ascorbate, and DTPA showed especially potent inhibitory effects. These results suggest that 'OH generated via a metal-catalyzed Fenton-like reaction in these systems may be a potent oxidant for hydroxyurea, generating 'NO.

DISCUSSION

We have obtained direct evidence of 'NO generation from hydroxyurea during peroxidation in the presence of

H_2O_2 and copper-containing proteins such as Cu,Zn-SOD and ceruloplasmin. To determine ˙NO generation directly, we employed ESR measurement using the stable organic radical nitronyl nitroxide derivative PTIO, which selectively reacts with ˙NO, resulting in the generation of PTI and ˙NO_2 according to the equation: ˙NO+ PTIO → ˙NO_2+PTI.[8]

The ESR signal of PTI thus generated is clearly distinct from that of PTIO, even in a mixture of the two compounds. We can therefore detect the generation of ˙NO by monitoring the change of the ESR signal of PTIO to that of PTI. This provided unequivocal evidence of ˙NO production by copper-catalyzed peroxidation of hydroxyurea in the presence of H_2O_2 and Cu,Zn-SOD. Moreover, it is known that iron (heme)-containing proteins such as hemoglobin bind ˙NO and give characteristic ESR signals derived from iron-NO adducts. In the present study, we also obtained an ESR signal identical with that of NO-hemoglobin during the incubation of red blood cells with hydroxyurea, H_2O_2, and Cu,Zn-SOD, demonstrating ˙NO generation from hydroxyurea under oxidative conditions. (Fig. 1).

Although the detailed mechanism of ˙NO generation from hydroxyurea remains unclear, the present results suggest the importance of ˙OH as an intermediate species (Figs. 3, 4). The formation of ˙NO from hydroxyurea seems to accompany three-electron oxidation of the compound, and thus ˙OH may act as an oxidant for hydroxyurea. Recently, Fukuto *et al.* reported the generation of ˙NO from N-hydroxylamines, including N-hydroxyl-L-arginine, which is a biosynthetic intermediate in ˙NO generation from ˙NO synthase (NOS).[14] They investigated the effects of various oxidizing agents on ˙NO release from hydroxylamines, which they suggested to be oxidized by ˙OH generated by the Fe(II)-catalyzed Fenton-like reaction with H_2O_2.

Based on our present experiment, the following reaction scheme seems plausible for ˙NO generation from hydroxyurea in the presence of H_2O_2, catalyzed by copper-containing proteins.
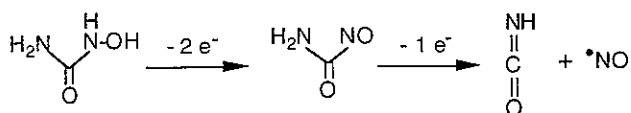
Here, three electrons are abstracted by ˙OH or an ˙OH-like species. The other final reaction product, isocyanic acid (carbimide), is unstable and readily undergoes conversion to NH_3 and CO_2 by incorporating H_2O in aqueous solution. However, this three-electron oxidation reaction of hydroxyurea still requires confirmation.

Millimolar concentrations of H_2O_2 are needed to produce 20–30 μM NO_2^- in the Cu ion-catalyzed oxidation of hydroxyurea (Fig. 2). However, a low micromolar

range of nitrite production was obtained with less than 100 μM H_2O_2. Further, a similar level of nitrite generation was observed in the case of H_2O_2/O_2^- generation by the hypoxanthine/xanthine oxidase system, under physiologically relevant conditions. In this context, Mason's group, in collaboration with one of the present authors (H.M.) has reported that an appreciable level of ˙NO (micromolar concentrations) is released in blood and liver tissue after hydroxyurea administration to rats.[15] Such concentrations of ˙NO are biologically significant, and could contribute to the pharmacological action of hydroxyurea.

Regarding the stoichiometry of the oxidation reaction of hydroxyurea to form ˙NO, different conversion rates of hydroxyurea to ˙NO were noted at various concentrations of hydroxyurea. Specifically, in the reaction mixture with more than 1 mM hydroxyurea, the rate of nitrite formation corresponded to less than 1% of hydroxyurea used for the reaction. In contrast, when the hydroxyurea concentration was below 100 μM, the conversion efficacy was much higher, up to 10%. The peculiar stoichiometry of the ˙NO formation reaction from hydroxyurea may be explained in terms of the reaction scheme proposed above. That is, if a large amount of hydroxyurea exists relative to [˙OH], the one or two electron oxidation products of hydroxyurea can be expected to become the major products of the reaction. Nevertheless, it is noteworthy that ˙NO generation from hydroxyurea proceeds effectively with reaction components available in physiological systems at physiological concentrations. Very recently, Pacelli *et al.* also described possible generation of ˙NO from hydroxyurea by H_2O_2, catalyzed by hemoglobin.[16]

It is now accepted that the cytostatic action of activated murine macrophages against tumor cells is mediated partly by ˙NO as well as by so-called reactive oxygen species.[6] Indeed, ˙NO is known to modulate the activity of a variety of enzymes, particularly those containing ferrous complexes.[17] Among the enzymes susceptible to ˙NO, ribonucleotide reductase appears to be a critical target for the cytostatic action of ˙NO.[18,19] The antitumor action of hydroxyurea is thought to be exerted through inhibition of ribonucleotide reductase.[5] Accordingly, ˙NO release from hydroxyurea seems likely to contribute to the antitumor activity of this drug. Further studies will be needed to identify the major mechanism of cytotoxicity-caused by hydroxyurea.

In addition to the antitumor activity of hydroxyurea, it is used to treat sickle cell anemia.[4] In patients with sickle cell anemia, it increased hemoglobin F levels and significantly attenuated the onset of painful crises.[4] ˙NO also has an important function in control of blood flow via its potent vascular relaxing activity and inhibition of platelet aggregation.[20] Thus, the ˙NO-generating capacity of hy-

droxyurea may play an important role in its therapeutic effects in sickle cell anemia.

In conclusion, our present experiments provide several lines of evidence for 'NO generation from hydroxyurea. In our systems, 'NO was generated from hydroxyurea in the presence of H_2O_2 and endogenous copper-containing proteins, i.e., Cu,Zn-SOD and ceruloplasmin, *via* copper-catalyzed peroxidation. In view of the diverse biological functions of 'NO, the 'NO-generating capacity of hydroxyurea may be important for both its antitumor action and its therapeutic effect in sickle cell anemia.

REFERENCES

1) Bolin, R. R., Robinson, W. A., Sutherland, J. and Hamman, R. F. Busulfan versus hydroxyurea in long-term therapy of chronic myelogenous leukemia. *Cancer*, **50**, 1683–1686 (1982).

2) Horowitz, R. and Wadler, S. Modulation of 5-FU/alpha-interferon with high dose continuous infusion hydroxyurea. *J. Infus. Chemother.*, **5**, 212–214 (1995).

3) Levin, V. A. Chemotherapy of primary brain tumors. *Neurol. Clin.*, **3**, 855–866 (1985).

4) Schecheter, A. N. and Rodgers, G. P. Sickle cell anemia — basic research reaches the clinic. *N. Engl. J. Med.*, **332**, 1372–1374 (1995).

5) Nyholm, S., Thelander, L. and Gräslund, A. Reduction and loss of the iron center in the reaction of the small subunit of mouse ribonucleotide reductase with hydroxyurea. *Biochemistry*, **32**, 11569–11574 (1993).

6) Kwon, N. S., Stuher, D. J. and Nathan, C. F. Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.*, **174**, 761–767 (1991).

7) Sato, K., Akaike, T., Kohno, M., Ando, M. and Maeda, H. Hydroxyl radical production by H_2O_2 plus Cu,Zn-superoxide dismutase reflects the activity of free copper released from the oxidatively damaged enzyme. *J. Biol. Chem.*, **267**, 25371–25377 (1992).

8) Akaike, T., Yoshida, M., Miyamoto, Y., Sato, K., Kohno, M., Sasamoto, K., Miyazaki, K., Ueda, S. and Maeda, H. Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/'NO through a radical reaction. *Biochemistry*, **32**, 827–832 (1993).

9) Sato, K., Akaike, T., Kojima, Y., Ando, M., Nagao, M. and Maeda, H. Evidence of direct generation of oxygen free radicals from heterocyclic amines by NADPH/cytochrome P-450 reductase *in vitro*. *Jpn. J. Cancer Res.*, **83**, 1204–1209 (1992).

10) Akaike, T., Sato, K., Ijiri, S., Miyamoto, Y., Kohno, M., Ando, M. and Maeda, H. Bactericidal activity of alkyl peroxyl radicals generated by heme-iron-catalyzed decomposition of organic peroxides. *Arch. Biochem. Biophys.*, **294**, 55–63 (1992).

11) Westenberger, U., Thanner, S., Ruf, H., Gersonde, K., Sutter, G. and Trents, O. Formation of free radicals and nitric oxide derivative of hemoglobin in rats during shock syndrome. *Free Radical Res. Commun.*, **11**, 167–178 (1990).

12) Kosaka, H., Sawai, Y., Sakaguchi, H., Kumura, E., Harada, N., Watanabe, M. and Shiga, T. ESR spectral transition by arteriovenous cycle in nitric oxide hemoglobin of cytokine-treated rats. *Am. J. Physiol.*, **266**, C1400–C1405 (1994).

13) Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138 (1982).

14) Fukuto, J. M., Wallace, G. C., Hszieh, R. and Chaudhuri, G. Chemical oxidation of N-hydroxyguanidine compounds — release of nitric oxide, nitrosyl and possible relationship to the mechanism of biological nitric oxide generation. *Biochem. Pharmacol.*, **43**, 607–613 (1992).

15) Jiang, J., Jordan, S. J., Barr, D. P., Gunther, M. R., Maeda, H. and Mason, R. P. *In vivo* production of nitric oxide in rats following administration of hydroxyurea. *Mol. Pharmacol.*, **52** (1997), in press.

16) Pacelli, R., Taira, J., Cook, J. A., Wink, D. A. and Krishna, M. C. Hydroxyurea reacts with heme proteins to generate nitric oxide. *Lancet*, **347**, 900 (1996).

17) Rubbo, H., Darley-Usmar, V. and Freeman, B. A. Nitric oxide regulation of tissue free radical injury. *Chem. Res. Toxicol.*, **9**, 809–820 (1996).

18) Lepoivre, M., Fieschi, F., Coves, J., Thelander, L. and Fontecave, M. Inactivation of ribonucleotide reductase by nitric oxide. *Biochem. Biophys. Res. Commun.*, **179**, 442–448 (1991).

19) Lepoivre, M., Flaman, J.-M., Bobé, P., Lemaire, G. and Henry, Y. Quenching of the tyrosyl free radical of ribonucleotide reductase by nitric oxide. *J. Biol. Chem.*, **269**, 21891–21897 (1994).

20) Moncada, S. and Higgs, A. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.*, **329**, 2002–2012 (1993).