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In Vivo Production of Nitric Oxide in Rats after Administration of Hydroxyurea

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SUMMARY

The metabolism of nitrovasodilators such as glyceryl trinitrate and nitroprusside provides the active moiety of these drugs (that is, nitric oxide). This process is not limited to the known nitrovasodilators, but also occurs with nitroaromatic antimicrobials. Here we report that the administration of hydroxyurea, an antitumor drug, to rats at pharmacological doses formed detectable nitrosyl hemoglobin, which increased with dose. At higher doses, nitrosyl hemoprotein complexes could also be detected in liver tissue. [^{15}N]hydroxyurea was synthesized and compared with [^{14}N]hydroxyurea. These observations verified that nitric oxide detected as nitrosyl hemoglobin or nitrosyl hemoprotein complexes in rats was the result of the metabolism of hydroxyurea. The time course and dose-dependence of nitric oxide generation were also investigated. Hydroxyurea's antineoplastic activity is caused by its direct action on ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis. Because nitric oxide also inhibits ribonucleotide reductase, this metabolite may supplement this action of hydroxyurea. In addition, the known ability of hydroxyurea to ease the pain of sickle cell anemia patients may be the result of vasodilation by the drug-derived nitric oxide.

Hydroxyurea was first synthesized in the 1860s (1). After it was found to be active against a variety of tumors, hydroxyurea was shown to inhibit DNA synthesis (2) by inhibiting ribonucleotide reductase (3,4). As a result, hydroxyurea has become standard therapy for chronic myelogenous leukemia, polycythemia vera, and myeloproliferative disorders, such as essential thrombocythemia (5). It is considered an effective systemic agent for control of severe psoriasis (6) and has been suggested as a radiosensitizer in patients with carcinoma of the cervix and in head and neck cancer (7). Recently, it was reported that hydroxyurea could stimulate fetal hemoglobin synthesis and had been successfully used to treat sickle cell anemia (8, 9). However, the detailed mechanism remains unclear. Hydroxyurea is readily absorbed after oral administration, reaches peak blood levels in 2–4 hr, and is excreted in the urine with a half-life of less than 8 hr (10). It enters cells by passive diffusion and is distributed throughout body water. The structural difference, compared with the functional difference, between hydroxyurea (a hydroxamic acid) and urea suggests that the active moiety of hydroxyurea is the hydroxylated nitrogen atom adjacent to the ketone.

NO is an active nitrogen compound with a high affinity for hemoproteins such as soluble guanylate cyclase, cytochrome P450, and hemoglobin. Interest in NO studies increased exponentially with the discovery that NO plays an important role in endothelium-derived relaxation, inflammation, thrombosis, immunity, and neurotransmission. It has been found that NO, like hydroxyurea, can inhibit the tumor cell ribonucleotide reductase (11). The metabolism of nitrovasodilators, such as glyceryl trinitrate, nitroprusside, (S)-nitrosothiols, azide, sodium nitrite, and hydroxylamine, leads to NO generation (12–16). Other nitrogen-containing compounds, such as quinifur, nitracrine (17), metronidazole (18), and nitroaniline derivatives (19), can also be metabolized to NO. ESR spectroscopy is a specific and sensitive method to detect free radicals. However, NO as a free radical does not have an ESR signal by itself (20), and no undisputed report has shown that it can be detected by using the ESR spin-trapping method with traditional nitrone- and nitroso-based spin traps. Hemoglobin, along with other hemoproteins, can be used for the NO measurement because NO has high affinity for metalloproteins (21). The binding of NO to deoxyhemoglobin or other deoxyhemoproteins produces HbNO or nitrosyl hemoproteins that have characteristic ESR spectra at 77°K. The affinity of deoxyhemoglobin for NO measured at half-saturation of the tetramer is 10^6-fold that of oxygen and 10^3-fold that of CO (22).

$$\text{Hemoglobin-Fe(II)} + \text{NO} \rightarrow \text{hemoglobin-Fe(II)-NO}$$

ABBREVIATIONS: NO, nitric oxide; HbNO, nitrosyl hemoglobin.

The 77°-K ESR spectra of HbNO are characterized by three g-values: g_x, g_y, and g_z. The complete assignment of HbNO and other NO-hemoprotein complexes is available (23, 24). There are two different ESR spectra of HbNO. The first type of the ESR spectra has a hyperfine structure of nine lines (triplet of a triplet) arising from an interaction of the unpaired electron with two nitrogen nuclei, one from NO (A_z = 19 ~ 21 G) and one from a nitrogen atom of proximal histidine (A_z = 6.5 G). This type of spectra corresponds to quaternary R (relaxed or oxy) or the six-coordinate structure of HbNO. The other type has a hyperfine structure of three lines (A_z = 16 ~ 17 G) resulting from the interaction of the unpaired electron with the ^{14}N nucleus (with a spin of one) of NO alone. The latter type of ESR spectra was classified as T (tense or deoxy) type or five-coordinate (25). For this type of HbNO, if the nitrogen atom of NO is replaced by ^{15}N with a spin of ½, the hyperfine structure of the ESR spectrum changes from triplet to doublet. This spectroscopic characteristic of the ^{15}N isotope provides a powerful analytical tool to identify the original source of NO production. The naturally existing hemoglobin or hemoproteins can be used to detect NO by measuring the formation of HbNO or nitrosyl hemoprotein complexes, which is especially advantageous for *in vivo* studies. This method has been widely used to detect NO generation in various situations such as endotoxin shock (26, 27) and ischemia reperfusion (28, 29).

Hydroxyurea is known to be decomposed *in vitro* to NO by H_2O and $CuSO_4$ (11), peroxidase (30), and hemoglobin (31), but *in vivo* formation of NO has not been reported. In the experiments described in this article, we used ESR spectroscopy to investigate the possible *in vivo* formation of NO after the administration of hydroxyurea to rats.

Materials and Methods

Animal preparations. Sprague-Dawley rats weighing 350–450 g (Charles River Breeding Laboratories, Portage, Canada) were used throughout the experiments. The rats were anesthetized with Nembutal and administered hydroxyurea (Sigma Chemical, St. Louis, MO) intragastrically. The dosage ranged from 80 mg/kg, which is the equivalent of human therapeutic dose, to 1.02 g/kg. Three hours after the injection, the rats were killed, and the blood was collected from the abdominal vena cava and frozen to 77°K for ESR measurements. For the time-course study of the generation of NO, blood was collected from the tail vein every half hour and measured at 77°K. After the rat was killed, liver tissue was inserted into a quartz tube and frozen to 77°K for ESR measurements in a finger dewar.

Experiments with [^{15}N]hydroxyurea. To verify that NO production resulted from the metabolism of hydroxyurea, we synthesized [^{15}N]hydroxyurea and injected it into the animals and compared the spectra with those obtained from the [^{14}N]hydroxyurea-treated animals. Hydroxyurea with ^{15}N in the hydroxylamine position was synthesized from ^{15}N-labeled hydroxylamine using a modified procedure taken from a Hungarian patent (32). The reaction involves the nucleophilic addition of hydroxylamine to the carbonic group of cyanate. Thus, only the hydroxylamine nitrogen of hydroxyurea had the ^{15}N-label (i.e., $HO^{15}NHCONH_2$). Briefly, approximately 40 g of Dowex-1 (strongly basic) anion exchange resin (Dow Chemical, Midland, MI) was washed with 300 ml of 1 N HCl followed by 300 ml of water. The resin was then washed with 300 ml of a 500 mM solution of potassium cyanate followed by 500 ml of water. Three grams of ^{15}N-labeled hydroxylamine was dissolved in 30 ml of water and added to the resin. The mixture was stirred for 1.5 hr, after which the resin was vacuum filtered. The filtrate and three subsequent 120-ml washes of the resin were allowed to evaporate at 40°. The off-white residue was collected and recrystallized from hot ethanol. The yield of hydroxyurea after recrystallization was about 20%. The NMR spectrum was obtained from a solution of the product (~100 mM) in perdeuterated dimethyl sulfoxide. The proton chemical shift values versus tetramethylsilane were as follows: OH, 8.56 ppm (singlet), $^{15}NHOH$, 8.31, 8.13 ppm (doublet), and NH_2, 6.15 ppm (singlet). The doublet centered at ~8.22 ppm was verified by comparing the NMR spectrum with that obtained from a standard of [^{14}N]hydroxyurea in which the hydroxylamine proton appeared only as a singlet at 8.22 ppm.

HbNO concentration standard preparation. To calibrate the ESR results, standard HbNO samples were prepared and their absorption spectra were measured to determine the HbNO concentration based on the known extinction coefficient of HbNO. Human hemoglobin was prepared from outdated blood acquired from the Red Cross (as described previously) without ion stripping (33). A stock solution of deoxyHb was prepared by reduction of the dissolved O_2 with a 2-fold molar excess of dithionite under an atmosphere of N_2. The excess dithionite and sulfite products of the O_2 reduction were removed by passage over a Sephadex G-25 column equilibrated with N_2-saturated phosphate buffer under an N_2 atmosphere. HbNO solutions were prepared by dilution of deoxyHb stock solution into N_2-saturated buffer followed by exposure of the solutions to NO gas. Concentrations of the HbNO solutions were determined from their optical spectra using an extinction coefficient of 12.6 mM^{-1} cm^{-1} at 545 nm (34). The HbNO solutions were frozen in liquid nitrogen immediately after measurement of their visual spectra. The ESR spectra were then collected from the standard samples with various concentrations of HbNO and the double integration over 350 G encompassing the entire HbNO signal was applied to each spectrum. The calibration chart of the ESR double integration values versus corresponding HbNO concentrations from optical extinction measurements was obtained.

***In vitro* incubations.** *In vitro* incubations of hydroxyurea with blood were carried out in an incubation chamber at 37°. Fresh blood was collected from the abdominal vena cava of a healthy, untreated rat. Blood incubation was done for 4 hr with 10 mM or 100 mM hydroxyurea. After the incubation, the blood was frozen immediately in liquid nitrogen for later ESR measurement.

ESR measurements. All ESR measurements were carried out under liquid nitrogen temperature with samples in a finger dewar. Blood or liver tissue were transferred to a quartz tube (4 mm i.d.) and then frozen to 77°K. All samples including standard samples were made in the length of 25 mm for convenience of quantification. A Bruker 200D spectrometer and TE_{102} cavity (Bruker, Billerica, MA) were employed to collect ESR spectra. Optimized instrumental conditions were used (35). The typical instrument settings were 20 mW of microwave power, 5 G modulation amplitude, 0.32 sec time constant, 8- or 16-min scan time, and 400 G to 2000 G scan range. For quantification, the experimental spectra were obtained at exactly the same conditions as the standard samples of HbNO. The ESR spectra from control samples were subtracted from the spectra obtained from the hydroxyurea-treated animals. The resulting spectra were then double integrated over a range of 350 G. The values of double integration were then converted to concentrations of HbNO or nitrosyl hemoprotein complexes using the calibration chart described above.

Results

HbNO formation in blood. Three hours after the administration of hydroxyurea, HbNO was detected in rat blood taken from the abdominal vena cava. Fig. 1A shows a typical ESR spectrum obtained at 77°K after the intragastric administration of 640 mg/kg [^{14}N]hydroxyurea. This spectrum shows a characteristic three-line hyperfine coupling of ^{14}N nucleus. The ^{14}N hyperfine coupling constant A_z was measured as 16.4 G. Fig. 1B shows an ESR spectrum obtained

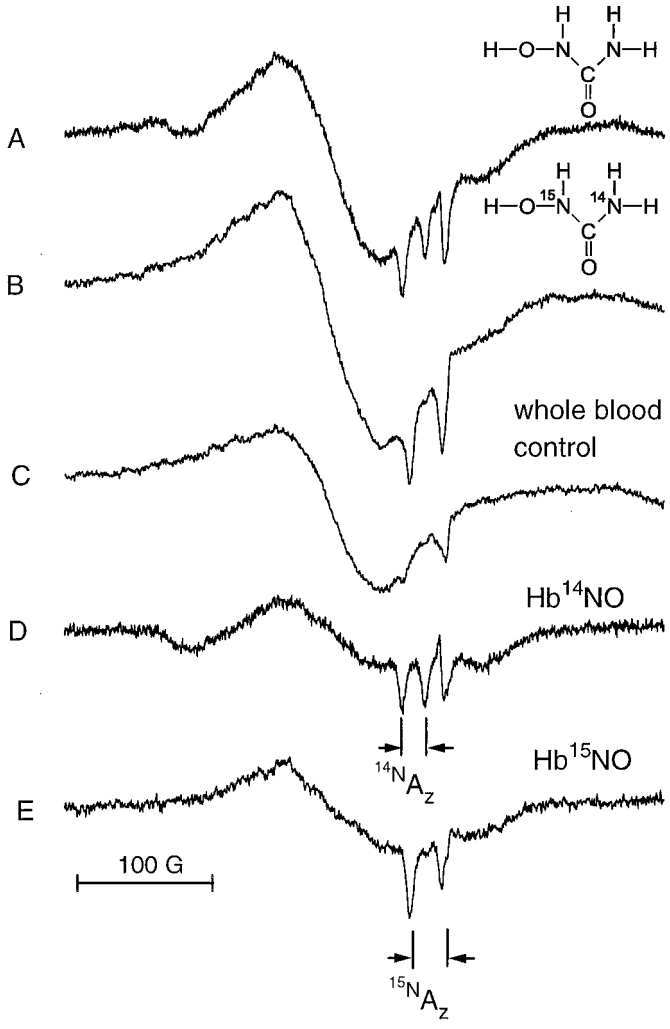

Fig. 1. Formation of HbNO in blood after administration of hydroxyurea. Three hours after the administration of hydroxyurea (640 mg/kg, intragastrically), the rat was killed and the venous blood was collected and transferred to a 4 mm i.d. quartz tube, and was then immediately frozen to 77°K for ESR measurements. A, The blood from the rat treated with 640 mg/kg hydroxyurea. B, The blood from the rat treated with 640 mg/kg ^{15}N-labeled hydroxyurea. C, The blood from the untreated rat. D, Difference spectrum resulting from the subtraction of C from A. E, Difference spectrum resulting from the subtraction of C from B. ESR parameters: 77°K, 700 G scan range, 0.32-sec time constant, 16-min scan, 20 mW microwave power, 5 G modulation amplitude.

under the same conditions but after [^{15}N]hydroxyurea administration (intragastrically). The spectrum shows a clear two-line hyperfine structure, which was caused by the change of nuclear spin from one to one half. Blood has a background signal (Fig. 1C) that was subtracted from the raw data (Fig. 1A and Fig. 1B). The resulting ESR spectrum, Fig. 1D, is typical of five-coordinate [^{14}N]HbNO, which is characterized by a triplet hyperfine structure. The resulting spectrum from the [^{15}N]hydroxyurea experiment, Fig. 1E, is characterized by a doublet with a hyperfine coupling constant of 23.2 G and is assigned to five-coordinate [^{15}N]HbNO. The ESR signals of HbNO detected from the blood provide strong evidence that NO was produced after the administration of hydroxyurea. The result of the ^{15}N isotope experiment further pointed out the original source of NO production was the NOH group of hydroxyurea. At therapeutic dosage (80 mg/kg), the HbNO signal was still easily detectable (Fig. 2). The production of HbNO (therefore, NO) increased when the administered dose increased from 80 mg/kg up to 640 mg/kg (Fig. 3). The time course of the formation of HbNO (Fig. 4) is consistent with the results of pharmacokinetic studies that hydroxyurea reaches its peak value in 2–4 hr after oral administration.

In vitro incubation of the blood with hydroxyurea at 10 mM, a concentration relevant to the doses used in *in vivo* experiments, did not produce detectable HbNO, although incubation at 100 mM hydroxyurea for 4 hr did give the hydroxyurea/hemoglobin complex signal (Fig. 5). The spectrum was recorded over a 2000-G scan range. Fig. 5A is the ESR spectrum of the blood incubated with 100 mM hydroxyurea. Fig. 5B is the signal from the blood incubated with saline. Fig. 5C is the subtraction of 5B from 5A. The signal marked with × in Fig. 5C was assigned to low-spin methemoglobin-hydroxyurea complex as reported previously (31). There was a weak but detectable HbNO signal marked with ○ in Fig. 5C in addition to the methemoglobin-hydroxyurea signal. Using a narrow scan range (500 G) around the area marked with ○ (g = 2.004) and a long scan time, an HbNO signal was obtained (Fig. 6C) that is the result of the subtraction of control (Fig. 6B) from the spectrum of the blood incubated with hydroxyurea (Fig. 6A).

Nitrosyl heme complex formation in liver. Liver tissue from hydroxyurea-treated rat showed a detectable nitrosyl heme complex signal. Fig. 7 represents the result from the liver sample after treatment of 640 mg/kg hydroxyurea for 3 hr. Fig. 7A is the spectrum obtained from the liver sample treated with 640 mg/kg hydroxyurea without further modification. Fig. 7B is the spectrum obtained under the same conditions, except [^{15}N]hydroxyurea was used. The normal liver has an ESR signal as shown in Fig. 7C. Fig. 7D is the result of subtraction of 7C from 7A. The resulting spectrum shows a triplet hyperfine structure with coupling constant of 16.4 G because of ^{14}N and was assigned to the nitrosyl heme complex. Fig. 7E is the result of subtraction of 7C from 7B. The resulting spectrum shows a doublet hyperfine structure with coupling constant of 23.2 G because of ^{15}N. The dosage-dependence of the nitrosyl heme complex

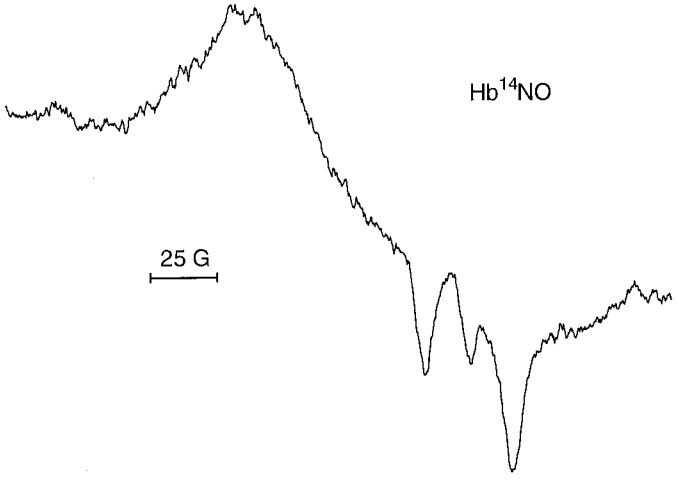

Fig. 2. Formation of HbNO in blood after 80 mg/kg hydroxyurea, intragastrically. ESR parameters: 77°K, 300 G scan range, 0.64-sec time constant, 16-min scan, 20mW microwave power, 5 G modulation amplitude. The spectrum is representative of three experiments.

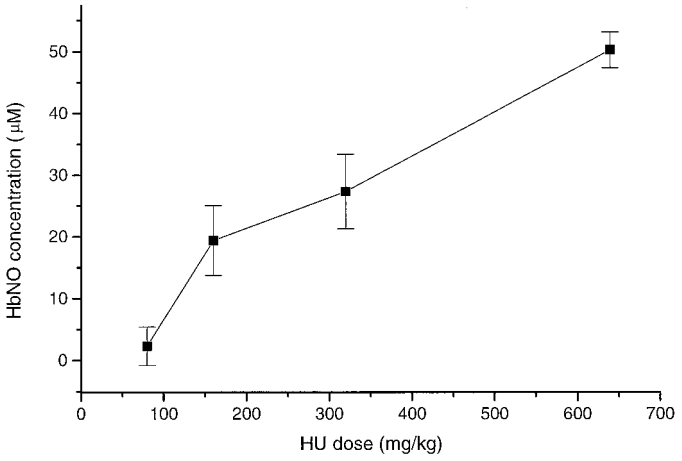

Fig. 3. Hydroxyurea (*HU*) dose dependence of HbNO formation in venous blood at 77°K (three experiments). After subtraction of background signal, ESR spectra were double-integrated and then converted to HbNO concentration. *Error bars*, standard errors.

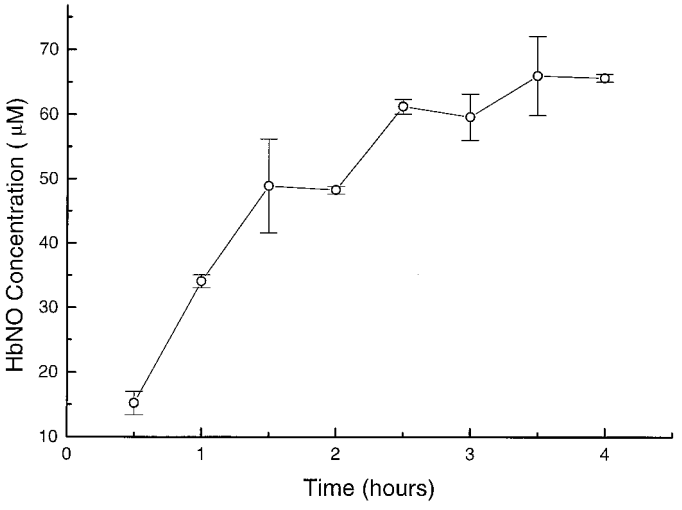

Fig. 4. Time course of the formation of HbNO in blood after hydroxyurea administration. Tail vein blood collected every half hour after administration of 1020 mg/kg hydroxyurea intragastrically. After subtraction of background signals, ESR spectra were double-integrated and then converted to HbNO concentration. *Error bars*, standard errors of three experiments.

concentration in liver (similar to that in blood) was also observed (Fig. 8).

Discussion

Our investigation demonstrated that the metabolism of hydroxyurea in rats leads to generation of NO. This conclusion is based on the detection of characteristic ESR signals of HbNO in blood and nitrosyl hemoprotein complexes in liver from hydroxyurea-treated rats. This is the first time that NO production was observed *in vivo* after administration of hydroxyurea. The hyperfine coupling constant of the ESR signal from nitrosyl complexes was 16.4 G, which is in good agreement with the literature values (25–27). The line shape of the spectra indicated that the majority of the HbNO we observed is in five-coordinate structure, which is reasonable considering that the blood samples were collected from the abdominal vena cava. In venous blood, the oxygen concentration is low, the concentration of deoxyhemoglobin is relatively high, and the HbNO formed is primarily in the five-coordinate structure. Compared with the control rats, the production of HbNO clearly showed that NO was produced after the administration of hydroxyurea. The ^{15}N isotope experiments further confirmed that NO originated from the NOH group of hydroxyurea. Furthermore, the coupling constant of 23.2 G is also expected from the ^{15}N hyperfine coupling constant. This value is approximately 1.41 times the ^{14}N hyperfine coupling constant. The hyperfine coupling is usually contributed by two components, the isotropic and anisotropic interactions. The isotropic hyperfine coupling constant (caused by Fermi contact interaction) and the anisotropic hyperfine coupling (caused by dipole-dipole interaction) are both proportional to the magnetogyric ratio γ_N (36). The values of the magnetogyric ratios γ_N for ^{14}N and ^{15}N are 0.19324 and 0.27107, respectively (36). Therefore, the ratio of 1.41 between the two experimentally determined hyperfine constants (23.2 G and 16.4 G) is very nearly the theoretically expected ratio between these two magnetogyric ratios, which is ~1.40.

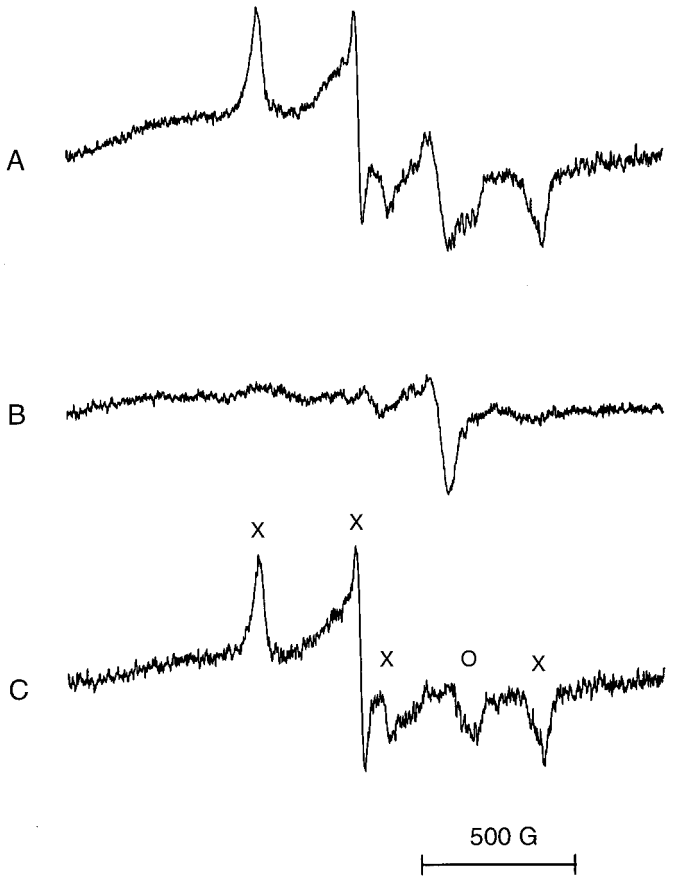

Fig. 5. Hydroxyurea/hemoglobin complex signal after 4-hr *in vitro* incubation of 100 mM hydroxyurea in blood. A, ESR spectrum from hydroxyurea-incubated blood. Hydroxyurea (100 mM) was incubated in blood at 37° for 4 hr. Then the blood sample was transferred to a quartz tube and frozen to 77°K for ESR measurements. B, Blood incubated with saline at the same conditions as the control. C, the difference spectrum resulting from the subtraction of B from A. Scan range was 2000 G. See text for other ESR parameters. The spectra are representative of three experiments.

We successfully detected the HbNO signal in rats at a dose as low as 80 mg/kg hydroxyurea. The time course experiment demonstrated that the production of NO reached its maxi-

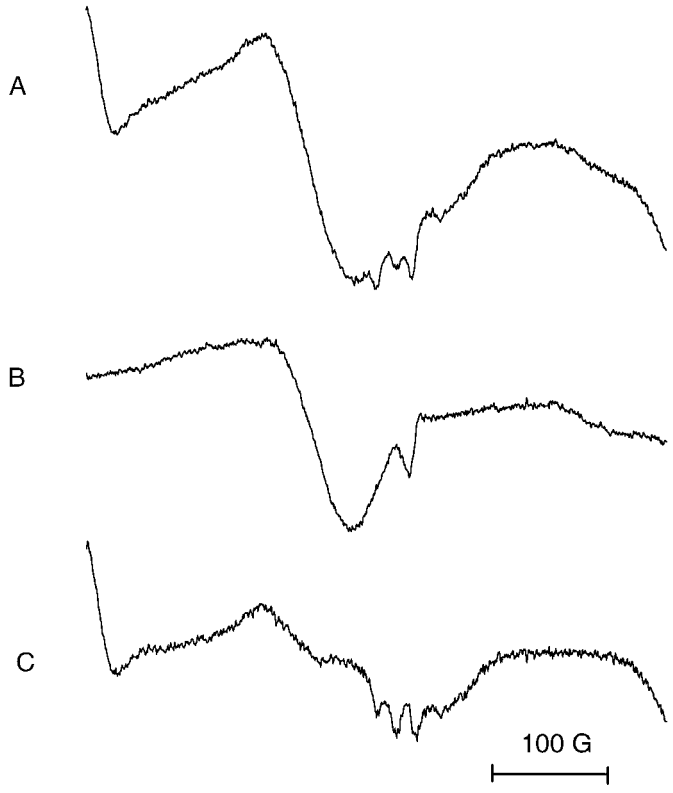

Fig. 6. *In vitro* incubation of hydroxyurea (100 mM) in blood. Same as Fig. 5, except the scan range was 500 G instead of 2000 G. A, 100 mM hydroxyurea incubated in blood for 4 hr. B, Blood incubated with saline as control. C, Result of subtraction of B from A.

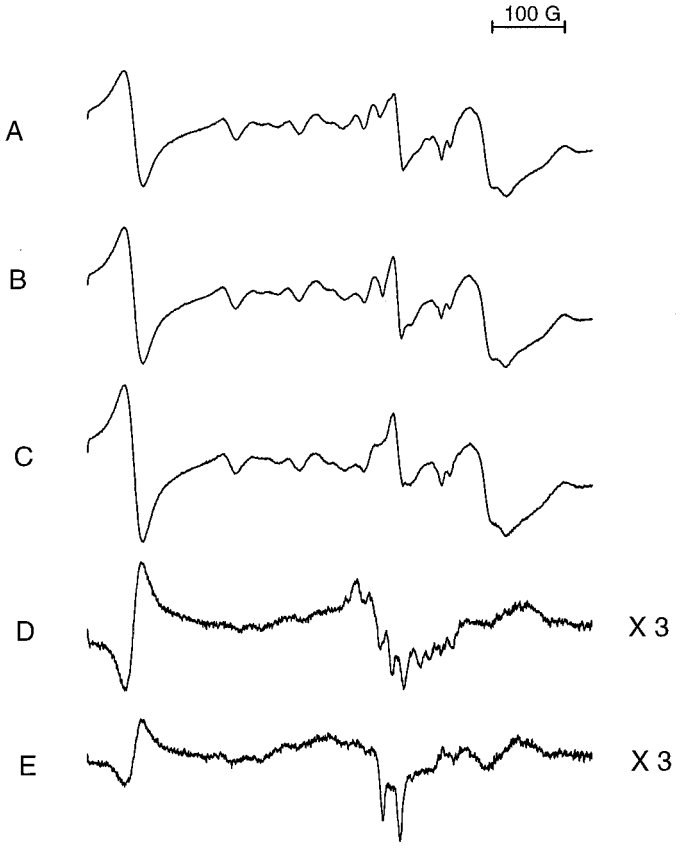

Fig. 7. ESR spectrum of liver tissue of hydroxyurea-treated and untreated rats. A, Liver sample from hydroxyurea-treated (320 mg/kg) rat. B, Liver sample from ^{15}N-labeled hydroxyurea-treated rat (same dose and under the same conditions as in A). C, Liver sample from untreated rat. D, The subtraction of C from A. E, The subtraction of C from B. D and E were multiplied by a factor of 3.

mum in 3–4 hr. This is consistent with the findings that hydroxyurea reaches peak blood levels in 2–4 hr after oral administration. It has been reported that hydroxyurea was taken up by Chinese hamster ovary cells in a linear nonsaturable fashion between 0.01 mM and 100 mM drug (37). Our experiments also showed a continuous increase of NO production when hydroxyurea dose increased from 80 mg/kg to 640 mg/kg.

In vitro hydroxyurea incubation with blood did not result in a detectable ESR signal when the hydroxyurea concentration was 10 mM, but did produce an HbNO signal when the hydroxyurea concentration was 10 times higher. It has been reported that at this high concentration, hydroxyurea reacts with oxyhemoglobin, resulting in the production of the MetHb-hydroxyurea complex. It was further proposed that this low-spin ferric adduct can further produce NO by oxidative degradation. Thereafter, the NO binds to deoxyhemoglobin and produces an ESR-detectable HbNO signal. However, the reaction is slow and the yield is low. The *in vivo* results show that the HbNO signal is strong and can be detected even at a low dose. In addition, no MetHb-hydroxyurea complex signal was detected *in vivo*. For these reasons, the pathway of NO production from MetHb-hydroxyurea degradation is unlikely to be dominant in rats.

Although the similarity of the structures between hydroxyurea and *N*-hydroxy-L-arginine, an intermediate of NO biosynthase, prompts the similar mechanism of NO production, the detailed mechanism of how hydroxyurea is metabolized to NO *in vivo* is not yet clear. *In vitro* results demonstrate that hydroxyurea can be oxidized to a nitroxide and ultimately NO.

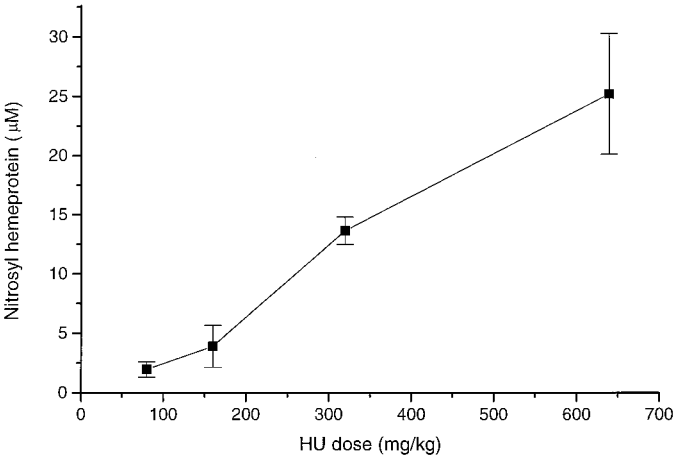

Fig. 8. Hydroxyurea (*HU*) dose-dependence of nitrosyl heme complexes in liver. After 3 hr, liver samples were taken and frozen to 77°K. The ESR spectrum from untreated rat liver was subtracted from hydroxyurea-treated rat liver and then double integrated. The results of double integration were then converted to nitrosyl heme complex concentrations. *Error bars*, standard error of three experiments.

The formation of NO from hydroxyurea by peroxidase oxidation was recently reported (38). It has also been reported that the NOH group of hydroxyurea can be oxidized by lipoxygenase (39), peroxidase/H_2O_2 (30), and tyrosyl radical from ribonucle-

otide reductase (40) to form its corresponding nitroxide. At this point, further experiments need to be carried out to determine which pathway is the major pathway of hydroxyurea metabolism to produce NO *in vivo*.

The signals found in liver tissue support the conclusion of NO production from the metabolism of hydroxyurea. The triplet (for [^{14}N]hydroxyurea) or doublet (for [^{15}N]hydroxyurea) hyperfine structure was assigned to the nitrosyl heme protein complex. It is not clear, however, whether NO was originally produced in the liver and then transferred to the blood or if it was produced both in blood and in liver simultaneously.

Hydroxyurea and NO have some similar biochemical properties, such as inhibiting ribonucleotide reductase and binding to hemoglobin. Hydroxyurea-derived NO may play a subsidiary role in the anticancer activity of hydroxyurea. The relaxation effect of NO on blood vessels could also be a possible mechanism to ease the pain of sickle cell crisis. The comparison of the functions and the structures between the hydroxyurea and urea points to the hydroxylated nitrogen atom adjacent to the ketone as the active moiety of hydroxyurea. Our *in vivo* studies provided strong evidence that NO is produced by metabolism of the NOH group of hydroxyurea. This result suggests that hydroxyurea-derived NO may be the active agent of hydroxyurea in at least some cases. The inhibition of ribonucleotide reductase by the hydroxyurea metabolite NO and the possible beneficial effects of NO-dependent vasodilation of the arterioles of sickle cell patients both warrant further investigations.

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