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Transfer of Nitric Oxide from the Liver to Erythrocytes – An ESR Study using Nitroglycerin-treated Mice

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Nitric oxide (NO) formation in the liver and blood of the mouse following intraperitoneal treatment with nitroglycerin (glycerol trinitrate, GTN) was determined using electron spin resonance (ESR) spectroscopy. ESR signals of heme–NO complexes were detected at maximum levels within 5 min in the liver, but increased to a maximum level about 15–30 min later in the blood. GTN is not metabolized to release NO *in vitro* in the blood of the mouse. The hepatic microsomes which showed the heme–NO complexes ESR signals were incubated with mouse erythrocytes, with the result that a hemoglobin–NO signal was obtained from the erythrocytes. The activities of microsomal cytochrome P-450, the hepatic level of glutathione, and the reduction rate of nitroxide radicals in the *in vivo* liver, measured using L-band ESR spectroscopy, were temporarily decreased following GTN administration. In conclusion, NO in the liver could be scavenged by circulating erythrocytes, which might minimize NO-induced liver damage.

Keywords: Nitric oxide, nitroglycerin, electron spin resonance, heme–nitrosyl complexes, cytochrome P-450, hemoglobin

Abbreviations NO, nitric oxide; GTN, glycerol trinitrate; ESR, electron spin resonance; DTCS, N-(dithiocarboxy)-sarcosine; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; AU, arbitrary unit

INTRODUCTION

Intrinsic nitric oxide (NO) of the liver is produced mainly in the hepatocytes, endothelial cells and Kupffer cells, and is proposed to play a central role in endotoxin-induced liver damage.[1] NO is characterized by its ability to bind metal-containing proteins or thiol(-SH)-containing antioxidants,[2] and therefore the intrahepatic NO must modulate the activities of iron-containing enzymes such as hepatic cytochrome P-450 or the reduction/oxidation (redox) status of the liver. Both heme–iron in hepatocytes and hemoglobin–iron in erythrocytes easily react with NO and then

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form nitrosyl iron complexes whose signals are determined using electron spin resonance (ESR) spectroscopy.[3] On the other hand, when nitroxide radicals are administered to animals, they are reduced to the corresponding hydroxylamines which are diamagnetic species and lose their ESR signals owing to the redox status of the organ.[4,5] The recent development of L-band ESR spectroscopy has enabled us to measure the reduction rate of nitroxide radicals such as Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) at the hepatic domain of the animal *in vivo*[6]

In the present study, we intended to clarify the correlation between the intensity of NO produced in the liver and the changes in the activity of cytochrome P-450 and redox status of the liver in mice treated with nitroglycerin, that is, glycerol trinitrate (GTN). Furthermore, the role of erythrocytes as a scavenger of NO is discussed in connection with liver damage.

MATERIALS AND METHODS

Reagents

GTN (Millisrol) was provided by Nippon Kayaku Co., Tokyo, Japan. Reagents used were obtained from the following commercial sources: N-(dithiocarboxy)-sarcosine (DTCS) and NOR3 (Dojin Chemical Co., Kumamoto, Japan); Tempol (Wako Pure Chemical Industries, Ltd. Osaka, Japan); Pentobarbital (Dainabot Co., Osaka, Japan); glutathione reductase and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Nacalai tesque Co., Kyoto, Japan); and NADPH and sodium borohydride ($NaBH_4$) (Sigma Chemical Co., St. Louis, USA). Other chemicals used were guaranteed grade and were obtained locally.

Animals

Male ddy mice weighing 10–20 g were obtained from Japan SLC Co., Ltd. Shizuoka, Japan. Each mouse was anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). GTN (10 mg/kg body weight) was administered into the peritoneal cavity of anesthetized mice, and then the organs including the liver were quickly obtained. Blood specimens were taken from the inferior vena cava. Subcellular fractionation of the mouse liver was performed according to the procedure of Hogeboom[7] as follows: 0.25 M sucrose homogenates of the liver were centrifuged at 24,000× *g* for 10 min, and the supernatants thus obtained were further centrifuged at 105,000× *g* for 60 min to obtain the microsomal fraction. Physiological saline solution of 20 mM DTCS with 66.7 mM $FeSO_4$ ($Fe(DTCS)_2$, 10 ml/kg body weight), as an NO-trapping agent,[8] was subcutaneously injected 30 min prior to the administration of GTN.

For the *in vivo* (L-band) ESR measurement,[9] an anesthetized mouse was laid prone and gently taped on a plastic board, and was then inserted into the cavity resonator. Tempol was dissolved in sterilized water at a concentration of 280 mM and Tempol (5 ml/kg) was administered through the tail vein.[6] Maintenance of animals and experimental procedures were carried out in accordance with the guidelines of the Japan Council on Animal Care.

ESR Measurements

The liver tissue and whole blood which were frozen in liquid nitrogen (4 mm i.d. × 12 mm long) were analyzed by using an X-band (9.4 GHz) ESR spectrometer (FX2XG, JEOL, Japan). Typical conditions for the X-band ESR measurement of nitrosyl iron complexes were as follows: center of magnetic field, 3250 gauss; modulation amplitude, 4 gauss; microwave power, 10 mW; scan speed, 500 gauss/60 s; time constant, 0.3 s. The concentration (arbitrary unit: AU) of heme–NO, cytochrome P-450 or methemoglobin was determined by double integration of the ESR spectra of each substance.

The ESR spectra from the hepatic domain of mice were obtained using an ESR spectrometer

(FX2XG, JEOL, Japan) equipped with a hand-made L-band microwave power unit (1.2 GHz) and a loop gap cavity resonator (34 mm i.d. × 6.7 mm long). Typical conditions for the *in vivo* ESR measurements were as follows: center of magnetic field, 460 gauss; scan range, 50 gauss; scan time, 60 s; time constant, 0.1 s. The modulation amplitude was adjusted to less than one-third of the line width. The sensitivity curve of the L-band resonator and the position of the hepatic domain of the mouse were described and illustrated in our previous paper.[6]

Chemical Assay

The aniline hydroxylation activity of the microsome was assayed spectrophotometrically by the method of Kato and Gillette.[10] Total glutathione contents of the liver were measured by oxidation of DTNB in a reaction mixture containing 0.25 mM NADPH, 0.1 U/ml glutathione reductase and liver homogenate, using a spectrometer (UV-2100S, Shimadzu, Japan) at 405 nm.[11]

Statistical Analysis

Statistical analysis was performed by ANOVA. Differences were accepted as statistically significant at $p < 0.05$.

RESULTS

ESR Signals of Nitrosyl Iron Complexes of the Organs and Blood obtained from GTN-treated Mice

When GTN was administered into the mouse *in vivo* the ESR signal of heme–NO complexes consisting of a triplet hyperfine structure ($g = 2.02, 2.01, 2.00$) was observed markedly in the liver and the kidney (Figure 1(a)). The other organs such as the heart, spleen, lung and mesenterium had no significant heme–NO ESR signal. The spin-trapping method using $Fe(DTCS)_2$ also proved the formation of NO in the liver and the kidney (Figure 1(b)). The total

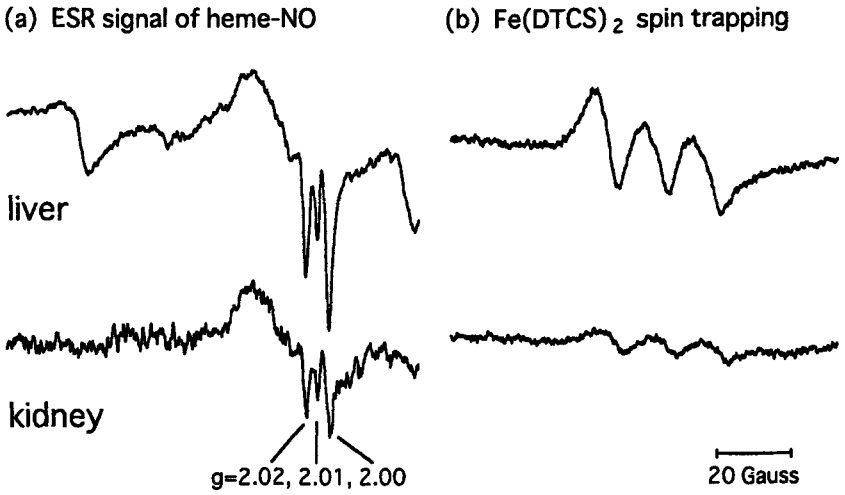

FIGURE 1 ESR signals of (a) heme–NO complexes and (b) $Fe(DTCS)_2$-NO in the liver and kidney of mice 15 min after intraperitoneal administration of GTN (10 mg/kg body weight). A piece of each organ tissue (100 mg wet weight) was put into an ESR quartz tube (4 mm i.d.), and ESR spectra of the sample were measured at 77 K (a) or at 24°C (b). The magnetic field for the ESR measurement of $Fe(DTCS)_2$-NO was 3310 ± 50 gauss. Each ESR signal is derived from a typical experiment; similar results were obtained in three other experiments.

signal intensity reading, calculated by the measured signal intensity multiplied by the weight of the organ, was about 7.5 times larger in the liver than in the kidney. Furthermore, the liver homogenate obtained from mice treated with GTN for 15 min was centrifuged and divided into subcellular fractions; heme–NO signals were detected in the microsomal fraction, but not in the supernatant or mitochondria (data not shown).

The ESR signal of heme–NO complexes in the liver was detected at maximum levels within 5 min after GTN treatment, but decayed rapidly and almost disappeared an hour later (Figure 2(a)). No remarkable signal for non-heme-iron nitrosyl complexes ($g = 2.04$) was detected in the liver. The ferric low-spin peak of cytochromes near $g = 2.26$ transiently decreased at 5 and 15 min, but recovered soon after. In the blood of GTN-treated mice (Figure 2(b)), the ESR signal of hemoglobin–NO complexes increased to a maximum level about 15–30 min later, and thereafter gradually decayed. In addition, the increase in the ferric signal of $g = 6$ which may reflect the methemoglobin was observed after a further 60 min (Figure 3).

ESR Signals of Nitrosyl Iron Complexes of the Liver and Blood after Treatment with GTN, *In Vitro*

GTN or NOR3, a spontaneous NO releasing agent, was added to the liver homogenate and blood of mice, with the result that GTN treatment produced heme–NO complexes ESR signals in the liver homogenate but not in blood. NOR3 treatment, in contrast, produced heme–NO complexes

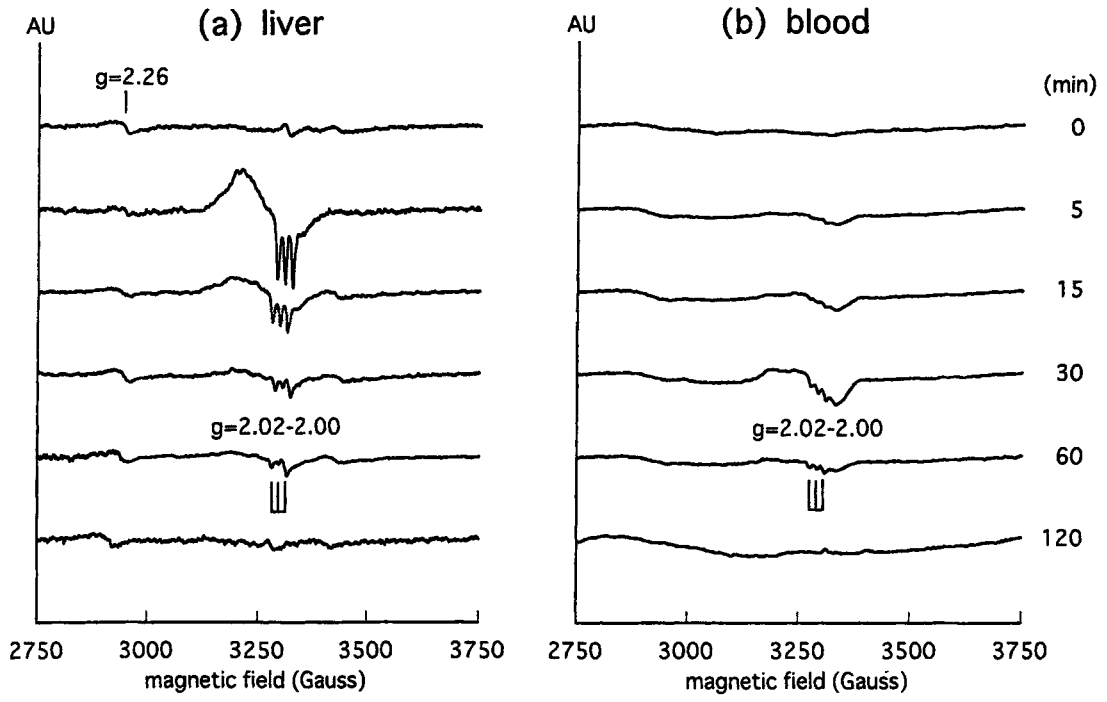

FIGURE 2 Time-course of ESR signals of heme–NO complexes in (a) liver and (b) blood of mice following intraperitoneal administration of GTN (10 mg/kg body weight). The ESR signals of ferric low-spin cytochromes ($g = 2.26$), and nitrosyl iron complexes with heme ($g = 2.02$–2.00) were observed in the liver tissues. The ESR signal of nitrosyl iron complexes with hemoglobin ($g = 2.02$–2.00) was detected in blood. Each ESR signal is derived from a typical experiment; similar results were obtained in three other experiments.

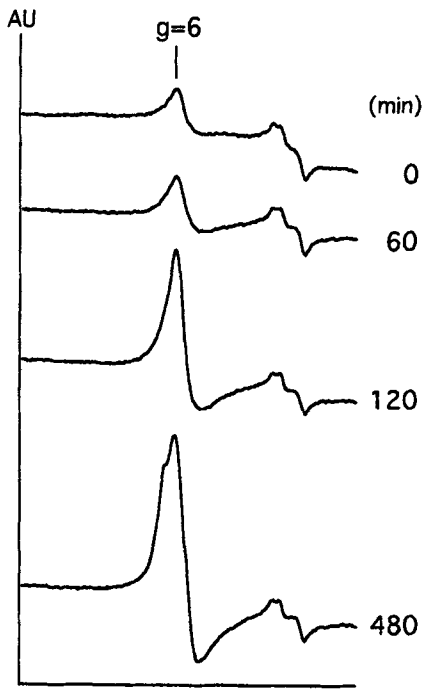

FIGURE 3 Time-course of the methemoglobin ESR signal ($g = 6$) in blood of mice following intraperitoneal administration of GTN (10 mg/kg body weight). The conditions for the ESR measurement were as follows: magnetic field, 1500 ± 1000 gauss; modulation amplitude, 2 gauss; microwave power, 10 mW; scan speed, 500 gauss/60 s; time constant, 1 s. Each ESR signal is derived from a typical experiment; similar results were obtained in three other experiments.

ESR signals in both the liver homogenate and blood (Figure 4).

The amount of heme–NO signals produced in the liver homogenate increased dose-dependently following GTN treatment (0.01–5 μmol/g liver); the signal intensity of produced heme–NO increased to a maximum level about 15–30 min later, and thereafter was stable at 37°C for 3 h (data not shown).

The liver homogenate at an hour after treatment with GTN (1 μmol/g liver) was fractionated into the supernatant containing microsomes and cytosol. After incubating with blood of mice and a brief centrifugation, the supernatant lost the heme–NO complexes ESR signal, which was then detected in the erythrocytes (Figure 5).

Hepatic Cytochrome P-450 Activity and Redox Status of GTN-treated Mice

The time-course of the aniline hydroxylase activity, a marker of cytochrome P-450 activity, was compared with those of ESR signals of cytochrome P-450 and heme–NO complexes using the liver tissues obtained from the GTN-treated mice (Figure 6(a)–(c)). As a result, aniline hydroxylase activity was temporarily decreased in correlation with both the decrease of the cytochrome P-450 ESR signal and the increase of heme–NO signals in the liver. The signal intensity of heme–NO complexes was negatively correlated with aniline hydroxylase activity (data not shown). The time-courses of hemoglobin–NO and methemoglobin in blood are also shown in Figure 6(c) and (d).

The ESR signal of Tempol, which is composed of three sharp lines of 1.55 mT hyperfine splitting, decreased in height and rapidly disappeared at the hepatic domain of the liver of the live mouse.[6] The decay of Tempol obeys first order kinetics, so that a semilogarithmic plot of the peak-to-peak heights of the middle ESR signals is a straight line. The kinetic constant (k) was calculated from its slope. The kinetic constant at the hepatic domain was temporarily and significantly smaller in the mice treated with GTN for 15 or 30 min than in the controls (Figure 7(a)). The concentration of total glutathione in the liver was also temporarily decreased in correlation with the decrease of k. Treatment with $NaBH_4$, a reducing agent, revealed the recovery of glutathione concentration (Figure 7(b)).

DISCUSSION

GTN, which has been used in the treatment of ischemic heart diseases, is degraded to the mono- and di-nitrate isomers with the formation of NO.[12] Because we intended to generate NO mainly in the liver, GTN as an NO donor was applied to mice through the peritoneal cavity

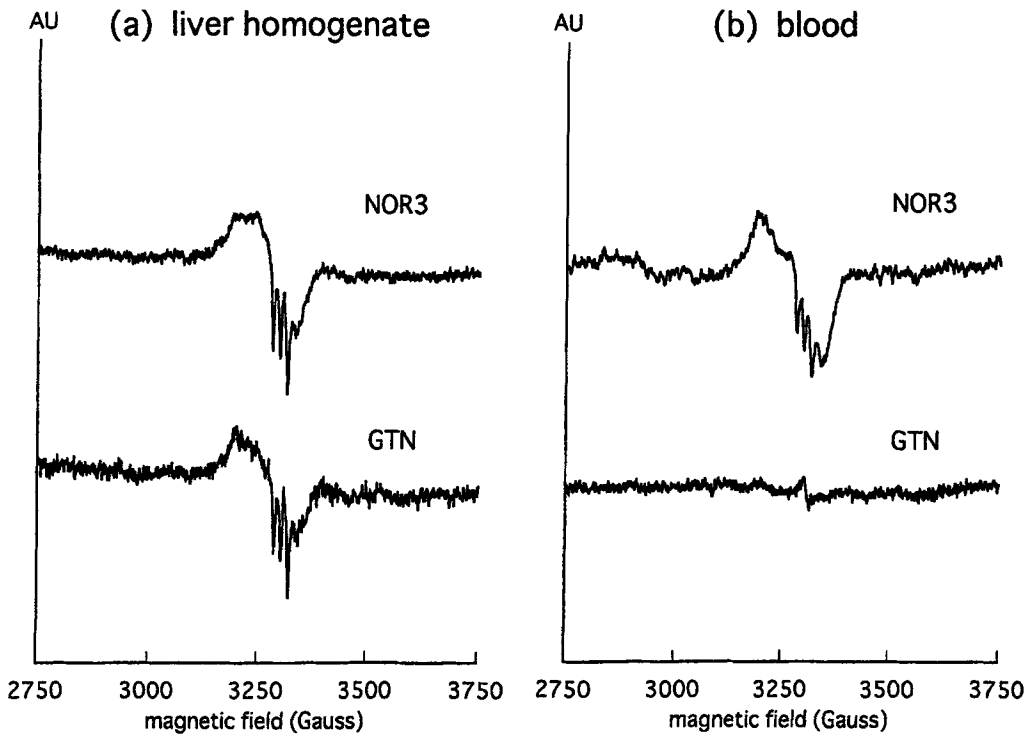

FIGURE 4 ESR signals of (a) the liver homogenate and (b) blood of mice 30 min after treatment with NO donors *in vitro* GTN or NOR3, a spontaneous NO releasing agent, was added to the liver homogenate or blood of mice at a concentration of 1 μmol/g liver homogenate or 1 μmol/ml blood, and the samples were incubated at 37°C for 30 min before ESR measurement. Each ESR signal is derived from a typical experiment; similar results were obtained in two other experiments.

from which GTN must be absorbed into the portal blood and then be directly transported to the liver. In fact, the ESR signals of heme–NO complexes which have a triplet hyperfine structure ($g = 2.02$–2.00)[13] were determined to be much larger in the liver than the other organs; the NO formation in the liver was further confirmed by using spin-trapping with $Fe(DTCS)_2$. Liver fractionation studies also revealed that these ESR signals in the liver tissue were mostly derived from the microsomes.

The heme–NO signals were first determined in the liver but later in the blood after administration of GTN in the live mouse, suggesting that the NO in the liver might be transfered to the blood. It is worth noting that GTN could not be metabolized to release NO in the erythrocytes of mice, and that erythrocytes actually obtained heme–NO signals after the incubation with NO-treated liver microsomes. These findings clearly indicate that the NO formed in the liver transfers to the erythrocytes. Methemoglobin is belived to be produced by a reaction between dissolved NO and oxyhemoglobin[14] or another reaction between hemoglobin–NO and oxygen.[15,16]

The cellular mechanism by which nitrite (NO_2^-) and subsequently NO are produced from GTN has not been elucidated completely; however, previous studies[12,17] have demonstrated that the first step is the enzymatic metabolism of GTN by glutathione S-transferase to produce glutathione sulfenyl nitrite ($GS\text{-}NO_2$). This is followed by the non-enzymatic cleavage of $GS\text{-}NO_2$ by reaction with reduced glutathione to give oxidized glutathione and NO_2^-; the conversion of NO_2^- to NO occurs by an unknown mechanism.[12] Thus the metabolism of GTN is accompanied by the depletion of intracellular

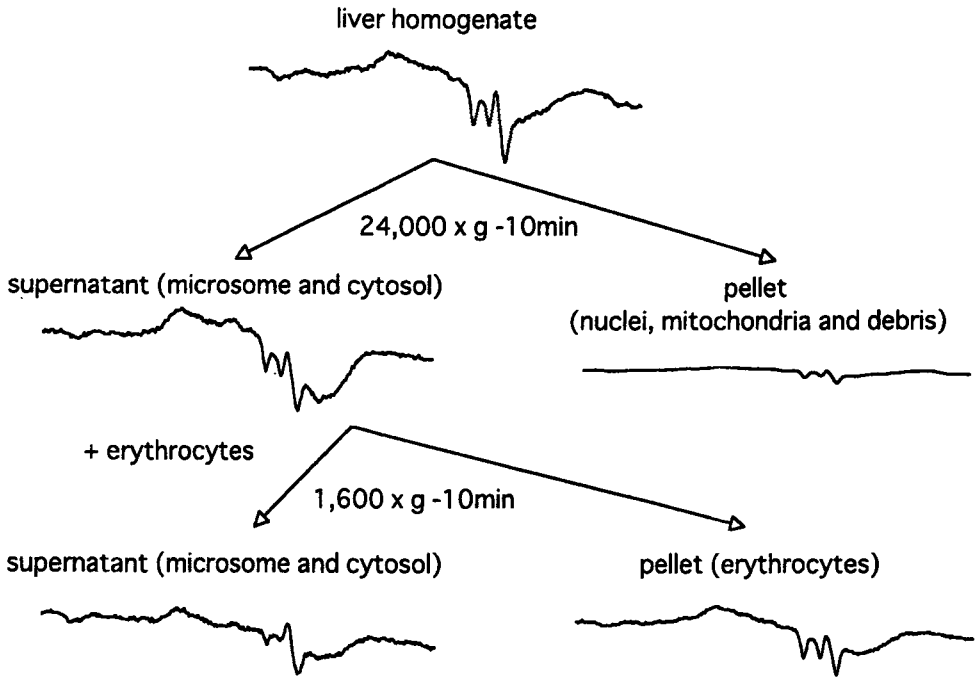

FIGURE 5 Fractionation of liver homogenate treated with GTN (10 mg/kg body weight), and the transfer of nitrosyl iron signals from the microsomal fraction to the erythrocytes. Each ESR signal is derived from a typical experiment; similar results were obtained in two other experiments.

glutathione. Such depletion of glutathione was suggested to reduce the formation of NO.[18] The liver contains a large amount of both glutathione S-transferase and glutathione, and then becomes the major source of plasma glutathione for other organs.[19] In contrast, the erythrocytes or endothelial cells of the blood vessels have lower glutathione concentrations than hepatocytes and then are more easily depleted of glutathione than the liver by GTN metabolism.[20,21] This may be one of the reasons why the mice erythrocytes have a reduced capability for metabolism of GTN. Microsomal cytochrome P-450 has also been shown to participate in the denitration of GTN in the liver.[22] As mentioned above, the present study using ddy mice and GTN provided evidence of the transfer of NO in the liver to erythrocytes, *in vivo* This phenomenon was previously suggested to exist but had not hitherto been directly proved.[23] We have confirmed that the blood of conventional rats (Wistar strain, Japan SLC Co., Ltd. Shizuoka, Japan) and humans is capable of metabolizing GTN and producing a considerable amount of NO, as previously reported.[24]

Because cytochrome 1A1 is more sensitive to NO than cytochrome 1A2,[25] aniline hydroxylase activity, a marker of cytochrome 1A1 activity, was measured in the present study. Consequently, the activity was decreased in correlation with both the decrease of the cytochrome P-450 signal and the increase of the heme–NO signal in the liver, but all these findings returned to their initial levels in a short time thereafter. Thus it is considered that the microsomal dysfunction induced by NO is reversible. As the yields of heme–NO signals were well correlated with the formation of NO in the liver and was negatively correlated with aniline hydroxylase activity, the signal intensity of heme–NO complexes provides an

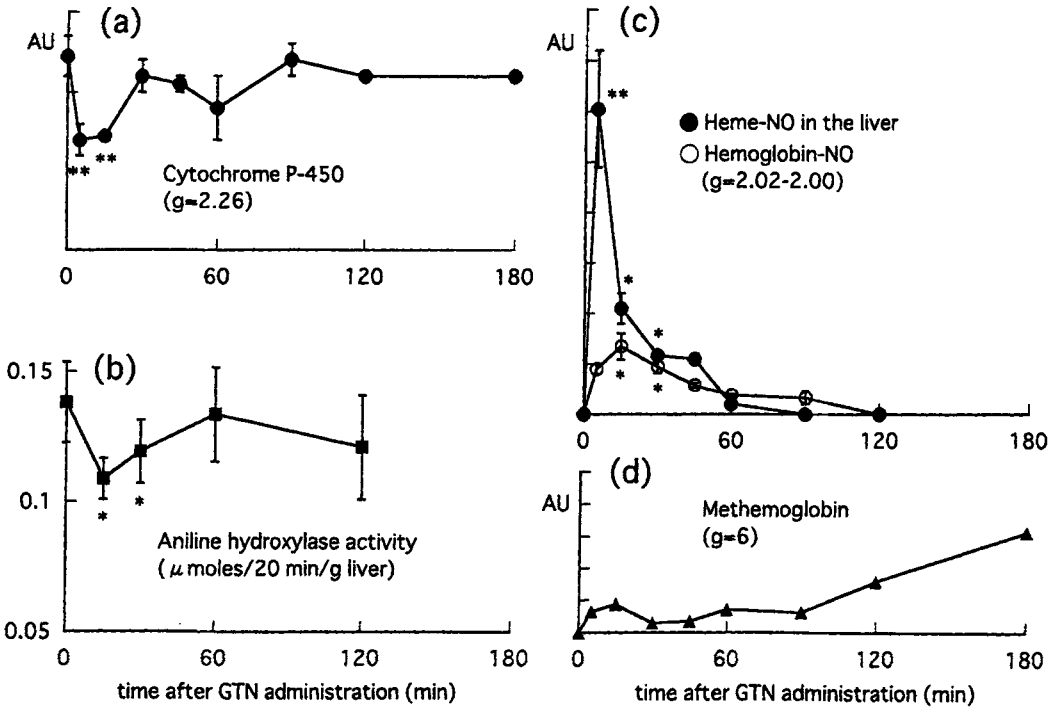

FIGURE 6 Time-course of ESR signals and aniline hydroxylase activity in the GTN (10 mg/kg body weight)-treated mice. (a) Cytochrome P-450 ($g = 2.26$) of the liver, (b) aniline hydroxylase activity of liver homogenates, (c) nitrosyl iron complexes of the liver tissue and blood, and (d) methemogiobin ($g = 6$) of blood ($n = 3$–5, mean ± SE). $*p < 0.05$, $**p < 0.01$, compared to the value at 0 min.

index of not only the formation of NO but also the dysfunction of cytochromes.

To examine the effects of NO on the redox status of the *in vivo* liver, the decay of Tempol in the hepatic domain of the live mouse was measured using our *in vivo* ESR system.[6] Because our previous study with isolated rat hepatocytes[26] suggested that the decay rate of nitroxides was dependent on the cellular content of glutathione, the changes in the amount of hepatic glutathione were also estimated. As we had expected, both the decay rate of Tempol and the glutathione content temporarily and simultaneously decreased in the liver after GTN administration. The fact that treatment with $NaBH_4$, a reducing agent, returned the glutathione content to normal suggested the formation of an S-nitroso adduct of glutathione in this period.

The previous *in vitro* study[27] using isolated microsomes treated with NOC7, a spontaneous NO releasing agent, has indicated that NO reversibly binds to the heme moiety of cytochrome P-450, but that it irreversibly inactivates cytochrome P-450 via the thiol modification pathway. By contrast, the present *in vivo* study showed that cytochrome P-450 activity, glutathione concentration and the redox status in the liver decreased only temporarily following treatment with GTN and were time-dependently restored to each of initial levels. It, however, could not be denied that minor changes remained in cytochrome P-450.

In conclusion, the present study has clearly demonstrated the transfer of NO formed in the liver to the erythrocytes in the *in vivo* mouse, whose blood hardly metabolized GTN to NO.

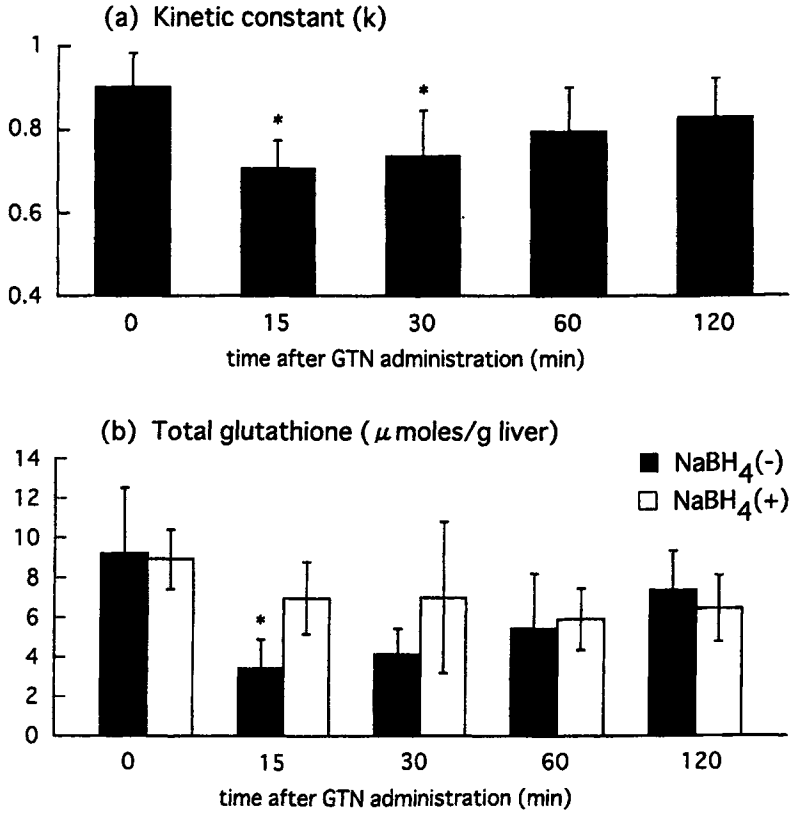

FIGURE 7 Time-course of (a) the kinetic constant (k) of the decay of Tempol and (b) total glutathione concentration of the liver of mice following GTN (10 mg/kg body weight) treatment ($n = 3$–4, mean ± SE). $*p < 0.05$, compared to the value at 0 min. The procedure for L-band ESR measurement of the decay of Tempol was described in our previous paper[6] in detail. Liver homogenate was incubated with 0.1 M $NaBH_4$ at 37°C for 5 min to break the S-nitroso band.[28] The sample was acidified and centrifuged to remove excess $NaBH_4$, and was then applied to measure the glutathione concentration.

In addition, it has been revealed that the hepatic cytochrome P-450 function and the redox status of the liver were transiently changed by NO; these changes were reversible. The NO-scavenging property of the hemoglobin in erythrocytes must therefore play a role in minimizing liver damage induced by NO.

Acknowle dgment s

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