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Spin trapping of nitric oxide produced in vivo in septic-shock mice

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Abstract

A nitric oxide (•NO) spin-trapping technique combined with electron paramagnetic resonance (EPR) spectroscopy has been employed to measure the in vivo production of •NO in lipopolysaccharide (LPS)-treated mice. The in vivo spin-trapping of •NO was performed by injecting into mice a metal–chelator complex, consisting of *N*-methyl-D-glucamine dithiocarbamate (MGD) and reduced iron (Fe^{2+}), that binds to •NO and forms a stable, water-soluble [$(MGD)_2$-Fe^{2+}-NO] complex, and by monitoring continuously the in vivo formation of the latter complex using an S-band EPR spectrometer. At 6 h after intravenous injection of LPS, a three-line EPR spectrum of the [$(MGD)_2$-Fe^{2+}-NO] complex, was observed in the blood circulation of the mouse tail; the [$(MGD)_2$-Fe^{2+}] complex was injected subcutaneously 2 h before EPR measurement. No signal was detected in control groups. Administration of N^G-monomethyl-L-arginine, an •NO synthase inhibitor, caused a marked reduction in the in vivo EPR signal of the [$(MGD)_2$-Fe^{2+}-NO] complex, suggesting that the •NO detected is synthesized via the arginine-nitric oxide synthase pathway. The results presented here demonstrated, for the first time, the in vivo real time measurement of •NO in the blood circulation of conscious, LPS-treated animals.

Key words: Electron paramagnetic resonance; Spin trapping; Nitric oxide; Lipopolysaccharide; N^G-Monomethyl-L-arginine; Septic shock

1. Introduction

Septic shock is an acute cardiovascular collapse resulting from the systemic response to a bacterial infection, and is manifested by hypotension, a lack of response to vasoconstrictors, tissue damage and multi-organ failure [1]. Despite intensive studies, the mechanisms underlying sepsis and septic shock are not yet known.

The overproduction of nitric oxide (•NO), a diffusible free radical, identified in recent years as a potent endogenous vasodilator, has been implicated as the basis for hypotension and the lack of response to vasoconstrictors during septic shock [2,3]. Intravenous administration of lipopolysaccharide (LPS; bacterial endotoxin) produced septic-shock like syndrome in animals and humans [4,5]. Reversal of LPS-induced hypotension in animals was observed following intravenous injection of N^G-monomethyl-L-arginine (NMMA), an inhibitor of nitric oxide synthases [6,7].

In blood, •NO reacts with oxyhemoglobin to yield nitrate and methemoglobin [8]. Elevated plasma and urinary nitrate levels therefore serve as indirect evidence for the overproduction of •NO in cytokine-induced and septic shock in animals and humans [9–11]. Additionally, low-temperature EPR detection of nitrosylated hemoglobin (HbNO) in blood has been used for assessing •NO production in septic shock [12,13] and in allograft rejection [14]. However, none of these methods is suitable for in vivo real time measurement of •NO.

Recently we have described an •NO spin-trapping technique for in vivo measurement of •NO production in the blood circulation of conscious animals [15]. The method is based on the trapping of •NO by a metal-chelator complex consisting of *N*-methyl-D-glucamine dithiocarbamate (MGD) and reduced iron (Fe^{2+}) to form a stable, water-soluble [$(MGD)_2$-Fe^{2+}-NO] complex, whose characteristic three-line spectrum of a mononitrosyl-Fe complex can be detected readily by EPR spectroscopy at ambient temperatures. In the present study, we have used this •NO spin-trapping technique to measure in real time •NO production in conscious, LPS-treated mice.

2. Materials and methods

ICR mice (female, 25–30 g) were supplied by Harlan–Sprague–Dawley (Indianapolis, IN). Animals were allowed free access to water, but not food for 24 h before the experiment. Lipopolysaccharide (LPS; *E. coli* 026:B6) was obtained from Sigma (St. Louis, MO). N^G-monomethyl-L-arginine (NMMA) was from Calbiochem (San Diego, CA). *N*-methyl-D-glucamine and carbon disulfide were obtained from Aldrich (Milwaukee, WI). Pure •NO gas was purchased from Matheson (Joliet, IL) and pure argon gas was obtained from Airco (Murray Hill, NJ). A saturated •NO solution in water was prepared by following the method of Kelm and Schrader [16]. The concentration of the saturated •NO solution at 22°C is 2.0 mM, as verified by an ISO-NO meter from World Precision Instruments (Sarasota, FL). Methoxyflurane was from Pitman-Moore Inc (Mundelein, IL). Sodium pentobarbital was from Anpro Pharmaceutical (Arcadia, CA). NO_2^- was measured by a colorimetric assay [17]. NO_3^- was first converted to NO_2^- by *E. coli* nitrate reductase [18] and measured as described above. *N*-methyl-D-glucamine

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Abbreviations: ·NO, nitric oxide; EPR, electron paramagnetic resonance; MGD, *N*-methyl-D-glucamine-dithiocarbamate; DETC, diethyldithiocarbamate; NMMA, N^G-monomethyl-L-arginine.

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dithiocarbamate (MGD) was synthesized by following the method of Shinobu et al. [19]. Element analysis for $C_8H_{16}NO_5S_2Na \cdot 1\ H_2O$: Calculated: C,30.83; H,5.78; N,4.50; O,30.83; S,20.56; Na,7.39; found: C,31.01; H,6.23; N,4.40; O,30.17; S,19.65; Na,7.57 (Galbraith Lab, Knoxville, TN).

2.1. EPR measurement

The EPR spectra were recorded with an EPR spectrometer, equipped with an S-band microwave bridge and a low-frequency loop-gap resonator with a 4-mm loop, operating at 3.5 GHz [20]. After i.v. injection of LPS (0.6 mg to 6 mg per animal) via the lateral tail vein, mice were anesthetized with methoxyflurane prior to subcutaneous injections with 0.4 ml of the $[(MGD)_2\text{-}Fe^{2+}]$ complex in water (326 mg/kg of MGD and 34 mg/kg of $FeSO_4$) at 0, 2, 4, 6 or 8 h; at least three separate experiments in each group. The mouse housed in a plexiglass restraining tube was transferred to the S-band EPR spectrometer. The tail of the mouse was immobilized by taping down with a thin and narrow plexiglass stick and then placed inside the resonator. The reason that the S-band EPR spectrometer was chosen in this in vivo experiment is because the diameter of the tip region of the mouse tail (about 2–3 mm) fits well with the 4-mm loop of the S-band loop-gap resonator. The animal remained conscious in the restraining tube for in vivo EPR experiments as well as all other experiments reported in this study; no anesthetic agent was used. The in vivo EPR signal was recorded at 2 h after the injection of the $[(MGD)_2\text{-}Fe^{2+}]$ complex. For inhibition experiments, at 6 h after LPS treatment, mice were injected intraperitoneally with an aliquot solution of NMMA in saline (50 mg/kg). Animals were sacrificed and the tissue samples including whole blood, liver and kidney were transferred into quartz tubes (i.d., 2 mm) for EPR measurement at room temperature. Instrument settings include 100 G field scan, 30 s scan time, 0.1 s time constant, 2.5 G modulation amplitude, 100 kHz modulation frequency and 25 mM microwave power [15].

2.2. Surgical preparation

All surgical procedures were approved by the Medical College of Wisconsin Animal Care Committee and the animals were closely monitored to insure that none experienced any undue stress or discomfort. The mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and maintained at a stable depth of anesthesia with supplemental doses and/or (1%) methoxyflurane as required [15]. They were placed on a heated surgical table and chronic indwelling catheters were inserted into the femoral artery and vein. All mice were fully awake and experienced no apparent discomfort when they were returned to the Animal Resource Center.

2.3. Hemodynamic measurement

Measurement of blood pressure and heart rate were made on conscious mice from 1 to 14 days after surgery [15]. The mice were trained to sit quietly in a plexiglass restraining tube for all blood pressure measurements. Blood pressure was measured using a solid-state pressure transducer (Cobe Laboratories, Lakewood, CO), and a general purpose amplifier. The data were collected at 200 Hz by microcomputer using data acquisition software (DATAQ Instruments Inc., Akron, OH).

3. Results and discussion

The bolus infusion of LPS (6 mg/mouse) into the catheterized mouse femoral vein resulted in a gradual fall in mean arterial pressure from 121 ± 3 mmHg to 85 ± 7 mmHg within 6 h as measured form the catheterized femoral artery. This suggests that the LPS-treated mice were in septic-shock-like conditions after the lethal dose of LPS administration; none of the LPS-treated mice survived over 24 h.

For in vivo EPR experiments, after LPS administration (6 mg/mouse), the mice were injected subcutaneously with an aqueous solution (0.4 ml) containing the spin-trapping reagent, the $[(MGD)_2\text{-}Fe^{2+}]$ complex, at either 0, 2, 4, or 6 h, respectively. The in vivo EPR signal was recorded 2 h after injection of the $[(MGD)_2\text{-}Fe^{2+}]$ complex, i.e. at 2, 4, 6, or 8 h after LPS. The results are shown in Fig. 1. While no signal was detected at 2 or 4 h (Fig. 1a,b), a three-line EPR spectrum of the $[(MGD)_2\text{-}Fe^{2+}\text{-}NO]$ complex (a^N = 12.5 G, and g_{iso} = 2.04), was observed at 6 h (Fig. 1c). The signal intensity was further increased at 8 h after LPS administration as shown in Fig. 1d. No EPR signal was detected in control mice injected only the $[(MGD)_2\text{-}Fe^{2+}]$ (data not shown). A weak EPR signal of the $[(MGD)_2\text{-}Fe^{2+}\text{-}NO]$ complex was detected when the mouse was administrated with LPS at a dose of 0.6 mg per mouse. The dosage of 6 mg per mouse was used for the rest of the data reported in this study. Thus, the results here provide direct in vivo evidence that •NO is produced in LPS-treated conscious mice, as demosntrated in Figure 1c and 1d.

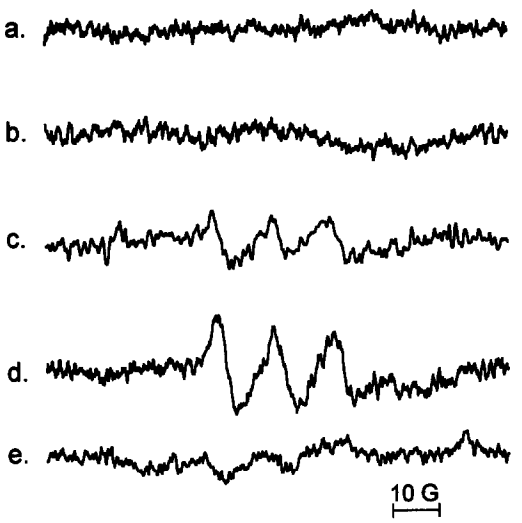

Fig. 1. 3.5 GHz EPR spectra of the $[(MGD)_2\text{-}Fe^{2+}\text{-}NO]$ complex in the circulation of the mouse tail at various times after LPS administration. After intravenous injection of LPS (6 mg; *E. coli* 0.26:B6) through the lateral tail vein, an aliquot (0.4 ml) of $[(MGD)_2\text{-}Fe^{2+}]$ complex in water was injected subcutaneously at (a) 0 h, (b) 2 h, (c) 4 h, and (d) 6 h. (e) Before the injection of the $[(MGD)_2\text{-}Fe^{2+}]$ complex at 6 h as in (d), an aliquot of NMMA in saline (50 mg/kg) was injected intraperitoneally. The concentrations of MGD and $FeSO_4$ in the injected solution were 83.3 mM and 16.7 mM, respectively. All spectra were recorded 2 h after the injection of the $[(MGD)_2\text{-}Fe^{2+}]$ complex. Each of the spectra presented here was an average of nine 30 s scans.

The observed 6 h delay for the appearance of the $[(MGD)_2\text{-}Fe^{2+}\text{-}NO]$ complex in the circulation (Fig. 1), agrees well with the time that is required for the induction of the inducible •NO synthase in vascular endothelium after LPS challenge [21–23], as well as with the observed time-dependent drop in mean arterial pressure in the LPS-treated mice as described above. Interestingly, when NMMA (50 mg/kg) was injected at 6 h after LPS administration, the EPR signal intensity was reduced by more than one-half (Fig. 1e), a result further

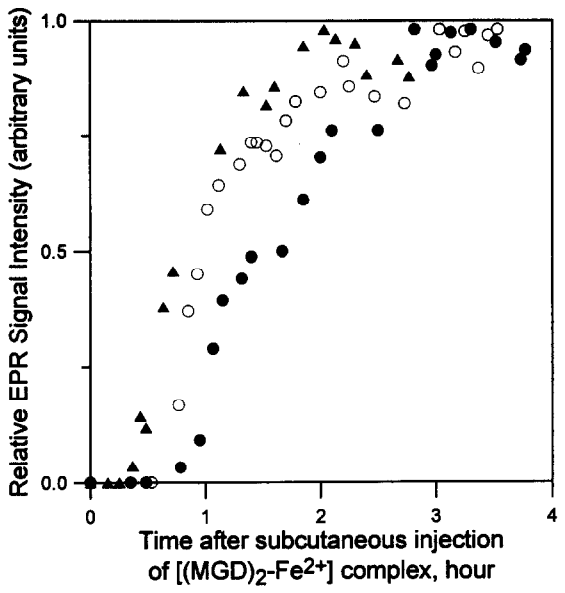

Fig. 2. Time course of in vivo EPR signal intensities of the [$(MGD)_2$-Fe^{2+}-NO] complex in mouse tail circulation during septic shock. Six h after intravenous injection of LPS (6 mg; *E. coli* 026:B6) as described in part (d) of Fig. 1, and aliquot (0.4 ml) of the [$(MGD)_2$-Fe^{2+}] complex in water was injected subcutaneously, and the EPR signal was recorded immediately and followed for a period of 4 h. Normalized data points obtained from three separate experiments (▲, ○, and ■) were shown.

confirming that the observed •NO production was via the arginine-NO synthase pathway [6,7].

Data on serum NO_3^- levels in the LPS-induced shock mice are summarized in Table 1, in which the LPS treatment was shown to produce a nine-fold increase in the blood NO_3^- levels. As expected, the administration of the [$(MGD)_2$-Fe^{2+}] complex, which traps •NO to form a stable [$(MGD)_2$-Fe^{2+}-NO] complex, reduced the blood NO_3^- levels in the LPS-treated mice. The NO_2^- levels in the sera with or without LPS administration were below 1 μM and beyond detection by the Griess reaction (data not shown), which is different from our previous work, in which we reported that the serum of control normal mice contained 11.8 ± 2.2 μM NO_2^- [15]. This difference in NO_2^- level may be ascribed to that in the previous work

Table 1
Effects of LPS and the [$(MGD)_2$-Fe^{2+}] complex on nitrate concentrations in mouse sera[a]

Condition	NO_3^- (μM)
Control	62.6 ± 5.8 (5)[c]
LPS[a]	586.4 ± 47.4 (3)*
LPS + [$(MGD)_2$-Fe^{2+}][b]	336.3 ± 45.9 (3)**

[a] Mice were sacrificed 8 h after intravenous LPS injection (6 mg; *E. coli* 026:B6).
[b] Mice received subcutaneous injection of the [$(MGD)_2$-Fe^{2+}] complex six hours after LPS injection were sacrificed 2 h later. *$P < 0.05$ compared to control. **$P < 0.05$ compared to the LPS- treated group.
[c] The data presented are mean ± S.E.M. (number of mice).

the mice were fed, and in the present study the mice were fasted for 24 h prior to experiments.

The time course for the appearance of the in vivo EPR signal of the [$(MGD)_2$-Fe^{2+}-NO] complex in the blood circulation of the 6 h LPS-treated mice is shown in Fig. 2. No signal was detected until about 45 min after subcutaneous injection of the [$(MGD)_2$-Fe^{2+}] complex, and then the signal incrased with time and reached a plateau at about 4 h, at which the equilibrium appeared to be reached between the formation and the clearance of the [$(MGD)_2$-Fe^{2+}-NO] complex in the blood. All EPR data reported in this study were recorded at 2 h after subcutaneous injection of the [$(MGD)_2$-Fe^{2+}] complex. To investigate the tissue distribution of the [$(MGD)_2$-Fe^{2+}-NO] complex, we sacrificed the 6 h LPS-treated mice and recorded the EPR signal in various tissues. The [$(MGD)_2$-Fe^{2+}-NO] complex was shown to be present in liver, kidney and whole blood (Fig. 3), which is in agreement with the data reported previously by other investigators using diethyldithiocarbamate (DETC) as a spin-trapping reagent in which they detected the presence of the [$(MGD)_2$-Fe^{2+}-NO] complex in various tissues [24]. Among all tissues examined thus far, the liver tissue retained the highest EPR signal intensity of the [$(MGD)_2$-Fe^{2+}-NO] complex (Fig. 3 and Table 2). The concentration of the [$(MGD)_2$-Fe^{2+}-NO] complex in Fig. 3a was about 20 μM. The presence of the signal in isolated whole blood also confirms that the EPR signals in Fig. 1c and d are due to the [$(MGD)_2$-Fe^{2+}-NO] complex circulating in the blood rather than trapped in the tail region, although it is possible that some of the detected signal is attributable to the [$(MGD)_2$-Fe^{2+}-NO] complex formed in the endothelium and smooth muscle of the tail region.

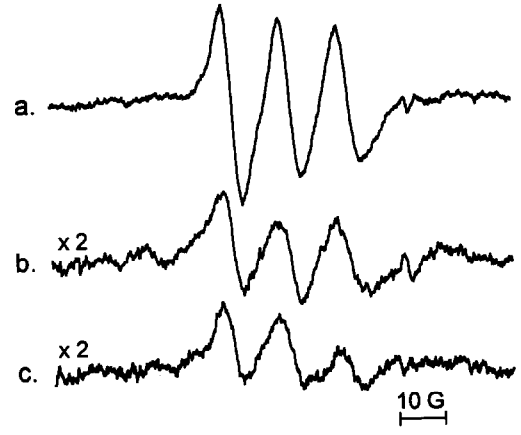

Fig. 3. 3.5 GHz EPR spectra of the [$(MGD)_2$-Fe^{2+}-NO] complex in various tissues of the septic-shock mouse. The experimental conditions were the same as described in Fig. 2, except that septic-shock mice were sacrificed 8 h after LPS administration. Isolated (a) liver tissue (89 mg, wet weight), (b) kidney tissue (74 mg, wet weight) and (c) heparinized whole blood (100 μl) were transferred into quartz tubes (i.d. 2 mm) for EPR measurement at 22°C. Each of spectra presented here was an average of nine 30 s scans.

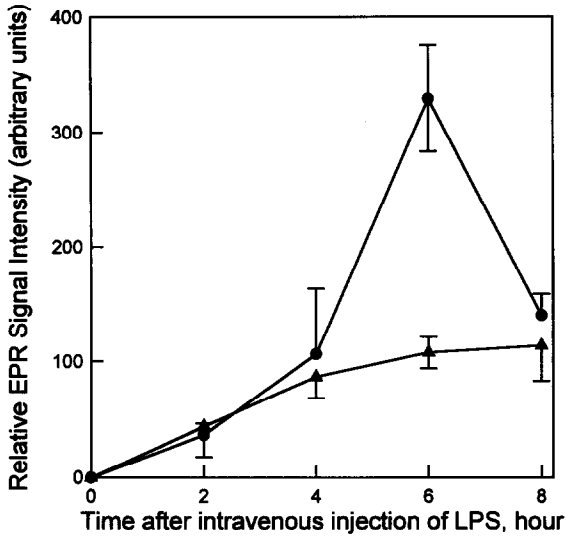

Fig. 4. Time course of EPR signal intensities of the [$(MGD)_2$-Fe^{2+}-NO] complex in isolated liver and kidney tissues after intravenous injection of LPS. After intravenous injection of LPS (6 mg; *E. coli* 026:B6), the mice were injected subcutaneously with an aliquot (0.4 ml) of the [$(MGD)_2$-Fe^{2+}] complex at 0, 2, 4, 6, or 8 h. The animals were sacrificed two hours after each time point of the injection of the complex, and the liver (●) and kidney (▲) tissues were transferred to quartz tubes for EPR measurement. The averaged wet weights of liver and kidney tissue sample were 88 ± 3 and 81 ± 4 mg, respectively, which correspond to 59 and 61 mg after corrections for the blood present in these tissues [26]. The data presented are averages of three separate experiments.

Fig. 4 shows the time-dependent EPR signal intensities of the [$(MGD)_2$-Fe^{2+}-NO] complex in the liver and kidney tissues after i.v. injection of LPS and subcutaneous injection of the [$(MGD)_2$-Fe^{2+}] complex. The signal intensity in the liver appeared to peak at 6 h after LPS administration, and then decreased with time (Fig. 4, closed circles). This is consistent with a 6 h delay in the LPS-induced NO_3^- generation in vivo [21–23], and the time course of the in vivo EPR signals as shown in Fig. 1. On the other hand, the signal intensity in the kidney tissue increased slowly with time; no maximum was noted (Fig. 4, closed triangles). Previously, using sodium nitroprusside (SNP) as an •NO dodnor and the [$(MGD)_2$- Fe^{2+}] complex as an •NO spin-trapping reagent, we showed that the in vivo EPR signal of the [$(MGD)_2$-Fe^{2+}- NO] complex formed in the blood circulation of the mouse increased with time and reached a maximum at 19 ± 3 min, and then decayed with a $t_{1/2}$ of about 15 min [15]. In contrast, in the present study, the in vivo EPR signal of the [$(MGD)_2$-Fe^{2+}-NO] complex detected in the blood circulation of the LPS-induced shock mouse as shown in Fig. 2 increased over at least 4 h and plateaued for several additional hours or until the animal died. This apparent discrepancy may be due to the differences in ways by which the [$(MGD)_2$-Fe^{2+}] complex was injected. In the previous study, the [$(MGD)_2$-Fe^{2+}] complex was injected intravenously, where it reacted with •NO released from SNP to form the [$(MGD)_2$-Fe^{2+}-NO] complex; the latter was then cleared from the circulation within an hour [15]. We also injected the [$(MGD)_2$-Fe^{2+}- NO] complex intravenously into the normal control mice, and found that the complex was excreted into the urine within 30 min as detected by EPR spectroscopy (Komarov and Lai, unpublished data). On the other hand, in the present study, the [$(MGD)_2$-Fe^{2+}] complex was injected subcutaneously, where it may diffuse slowly from the site of injection into the capillary bed, and some of the complex binds to •NO generated from inducible •NO synthase in the vascular endothelium and smooth muscle cells to form the [$(MGD)_2$-Fe^{2+}-NO] complex prior to entering the blood circulation. Its sustained accumulation (see Fig. 2) may at least in part be due to such a slow release of the [$(MGD)_2$-Fe^{2+}] complex into the circulation.

When the [$(MGD)_2$-Fe^{2+}] complex was injected intravenously into the LPS-induced shock mice, the EPR signal of the [$(MGD)_2$-Fe^{2+}-NO] complex was detected in both liver and kidney tissues as shown in Fig. 5a and b, respectively, but no signal was detected in blood (Fig. 5c). The lack of the detectable signal in blood as shown in Fig. 5c may be attributable to the inability of the intravenously injected [$(MGD)_2$-Fe^{2+}] complex to compete efficiently for •NO with hemoglobin (20 mM) in red cells, which is known to react rapidly with •NO ($\sim 10^7$ $M^{-1} \cdot s^{-1}$) to produce nitrate and methemoglobin [25]. On the other hand, the [$(MGD)_2$-Fe^{2+}] complex may compete for •NO in tissues where there are far fewer scavenging reactions of •NO, as demonstrated in Fig. 5a and b. Thus, the different routes by which the [$(MGD)_2$-Fe^{2+}] complex was administrated seemed to affect the detectability of the [$(MGD)_2$-Fe^{2+}-NO] complex in the circulation of LPS-treated mice: while the subcutaneous injection produced the detectable [$(MGD)_2$-Fe^{2+}-NO] signal (Fig. 1), the intravenous injection did not (Fig. 5c).

In summary, we have demonstrated the in vivo real time measurement of •NO produced in the circulation of the LPS-treated mice by using a newly developed •NO spin-trapping method and S-band EPR spectroscopy

Table 2
Quantitation of the amounts of the [$(MGD)_2$-Fe^{2+}-NO] complex present in various tissues of the LPS-induced shock mice

Tissue	[$(MGD)_2$-Fe^{2+}-NO] (nmol/g)[a]
Liver	36.8 ± 5.4 (3)[b]
Kidney	10.9 ± 1.6 (3)
Whole blood	5.4 ± 1.4 (3)

[a] The amounts of the [$(MGD)_2$-Fe^{2+}-NO] complex present in various tissues were calculated by comparing the signal intensities obtained from the tissues to the signal intensity of a standard solution containing 0.1 mM of the [$(MGD)_2$-Fe^{2+}-NO] complex in saline solution.
[b] The data presented are mean ± S.E.M. (number of mice); the amounts of [$(MGD)_2$-Fe^{2+}-NO] complex present in the blood of liver and kidney tissues as well as the amounts of the blood presented in the wet weights of liver and kidney tissues have been corrected [26].

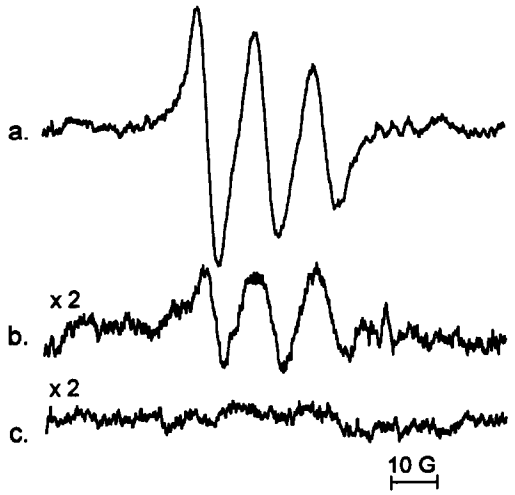

Fig. 5. 3.5 GHz EPR spectra of the [$(MGD)_2$-Fe^{2+}-NO] complex in various tissues of the septic-shock mice after intravenous injection of the [$(MGD)_2$-Fe^{2+}] complex. Six hours after intravenous injection of LPS (6 mg; *E. coli* 026:B6) through the lateral tail vein, and aliquot (0.2 ml) of the [$(MGD)_2$-Fe^{2+}] complex in saline was injected intravenously through the lateral tail vein. The mice were sacrificed 30 min after the complex injection, and the isolated (a) liver tissue (88 mg, wet weight), (b) kidney tissue (83 mg, wet weight), and (c) heparinized whole blood (100 μl) were transferred into capillary tubes for S-band EPR measurement at 22°C. Each of spectra presented here was an average of nine 30 s scans.

[15]. The approach described here should be useful for studying the pathogenesis of cytokine-induced and septic shock [2]. This new •NO spin-trapping reagent may also be suitable for functional EPR imaging or functional magnetic resonance imaging of whole animals in various pathophysiological conditions where •NO is overproduced.

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