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FORMATION OF FREE RADICALS AND NITRIC OXIDE DERIVATIVE OF HEMOGLOBIN IN RATS DURING SHOCK SYNDROME

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Free radicals have been postulated to play an important role as mediators in the pathogenesis of shock syndrome and multiple-organ failure. We attempted to directly detect the increased formation of radicals by Electron Spin Resonance (ESR) in animal models of shock, namely the endotoxin (ETX) shock or the hemorrhagic shock of the rat. In freeze-clamped lung tissue, a small but significant increase of a free radical signal was detected after ETX application. In the blood of rats under ETX shock, a significant ESR signal with a triplet hyperfine structure was observed. The latter ESR signal evolved within several hours after the application of ETX and was localized in the red blood cells. This signal was assigned to a nitric oxide (NO) adduct of hemoglobin with the tentative structur $((\alpha^{2+}\,NO)\beta^{3+})_2$. The amount of hemoglobin-NO formed, up to 0.8% of total hemoglobin, indicated that under ETX shock a considerable amount of NO was produced in the vascular system. This NO production was strongly inhibited by the arginine analog N^G-monomethyl-arginine (NMMA). The ESR signal of Hb–NO was also observed after severe hemorrhagic shock. There are three questions, namely (i) the type of vascular cells and the regulation of the process forming such a large amount of NO during ETX shock, (ii) the pathophysiological implications of the formed NO, effects which have been described as cytotoxic mediator, endothelium-derived relaxing factor (EDRF) or inhibitor of platelet aggregation, and (iii) the possible use of Hb–NO for monitoring phases of shock syndrome.

KEY WORDS: Free radicals, nitric oxide, hemoglobin, ESR, rat, shock, endotoxin, hemorrhage.

ABBREVIATIONS: Hb-NO, nitric oxide adduct of hemoglobin; EDRF, endothelium-derived relaxing factor; ETX, endotoxin, E. coli lipopolysaccharides; NMMA, N^G-monomethyl-L-arginine

As a consequence of severe shock, many patients develop organ dysfunction within two weeks after shock (MOF, multiple organ failure; ARDS, adult respiratory distress syndrome, "shock lung syndrome"), often a lethal complication. The organ failure has tentatively been attributed to a damage of the endothelial cells in the blood cells.[1] During this pathophysiological process, various cellular and humoral "mediator" systems are activated in a complex interacting sequence in which phagocytic and endothelial cells are involved. Free radicals, presumably oxygen radicals and derivatives, have been postulated to be important mediators although no direct evidence for their occurrence and action during shock has been reported.[1,2]

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Therefore, we attempted to directly demonstrate an increased formation of radicals during experimental shock of the rat by using ESR as a specific and sensitive method for the detection of radicals. However, this approach cannot detect very reactive radicals with short life-time (e.g. hydroxyl radical) but only longer-living radicals which accumulate as products of radical chain reactions.

Experimental shock was induced by endotoxin application (ETX shock) or hemorrhage (hemorrhagic shock). Endotoxin shock is a widely used animal model of shock.[3,4] It can be evoked in rats by intravenous application of bacterial lipopolysaccharides and is characterized by hypotension, disseminated intravascular coagulation and multiple-organ system failure. Further symptoms are lethargy, piloerection, chills and tachypnea. Severely involved organs are liver, kidneys (acute tubular necrosis), lungs (shock lung syndrome) and the gastrointestinal tract (mesenteric ischemia). During ETX shock, samples of lung and blood as organs which should be involved in the pathogenesis or ARDS, were frozen and examined by ESR for the formation of radicals. In addition to a small free radical signal in lung tissue, in blood a significant signal of NO adduct of hemeglobin was observed. The latter signal was also detected in the blood of rats after hemorrhagic shock which was investigated as a shock model quite different from ETX shock.

MATERIALS AND METHODS

Animals and endotoxin shock. Male Lewis rats with a weight of about 300 g were used for the experiments. Rats for the sampling of lung-tissue were anaesthesized by pentobarbital (Narcoren®, 50 mg/kg b. w., i.p.) ten minutes prior to the application of ETX. The rats for the blood samples were etherized immediately before the application of ETX. Endotoxin-shock was induced with E. coli lipoplysaccharides, type 055:B5 (Sigma), dissolved in isotonic NaCl just before the experiment. ETX was administered as a single dose via the dorsal penis vein.

Hemorrhagic shock was induced by two arterial hemorrhages of each 1.5 ml/100 g b.w. within 15 minutes. 15 minutes later, an infusion of 3 ml/100 g b.w. of Ringer's solution was given. For hemorrhage, the carotid artery was cannulated, resubstitutions were made by cannulation of the jugular vein.

Ischemia/reperfusion experiments were performed by supracelic aortic clamping according to Bühren *et al.*[5]

Lung tissue samples. After thoractomy of the anaesthesized rat, the left lung was clamped at the hilus and was mobilized. With a freeze-clamp, pre-cooled in liquid nitrogen, a tissue cylinder (2.5 mm ø × 15 mm) was obtained within 5 s after the clamping at the hilus. The estimated freezing time was 0.1–0.2 s. Under liquid nitrogen the frozen tissue cylinder was removed from the freeze-clamp and transferred into an ESR sample tube. Grinding of the frozen tissue was not applied in order to avoid the artifactual generation of radicals.[6,7] In some experiments, immediately before freeze-clamping the lung was perfused with isotonic NaCl via the cannulated caval vein.

Blood samples were taken from the jugular vein of anaesthesized rats. In order to minimize hemodilution, an experimental design was carried out where a maximum of six blood samples (0.5 ml each) was taken from each rat during the experiment. An aliquot of 0.25 ml was immediately frozen in an ESR sample tube in liquid nitrogen. Blood counts were determined with a Coulter® counter.

ESR spectra were recorded with a Varian E-9 spectrometer at 90 K. The spectra have been corrected by subtraction of a spectrum of water. ESR signal intensities were calculated in arbitrary units as double integrals of the corrected spectra.

RESULTS AND DISCUSSION

ESR signals in lung tissue during endotoxin shock

Endotoxin was administered to rats in doses up to 40 mg/kg b. w., and lung tissue was sampled by freeze-clamping without mechanical grinding. Rapidly frozen lung-tissue cylinders from untreated rats showed a very small ESR signal at $g = 2$ (Figure 1). Obviously, our sampling procedures did not generate artifactual radicals which have been reported after mechanical grinding of frozen tissue.[6,7] The ESR signal increased

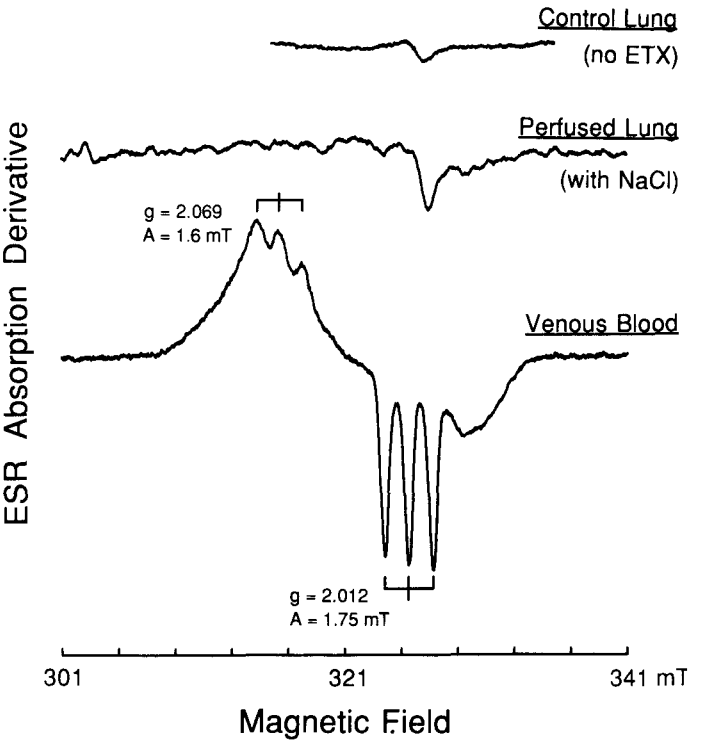

FIGURE 1. ESR signals in lung tissue and blood of rat treated with endotoxin. Lung tissue samples were obtained by freeze-clamping as described in Materials and Methods. The control lung was from a rat which was anaesthesized for 4 hours without exposure to ETX. The perfused lung and venous blood samples were obtained from a rat 4 hours after the application of ETX(40 mg/kg b. w.). After cannulation of the caval vein, the blood was sampled, and the lungs were perfused in situ with isotonic NaCl immediately before the freeze-clamping. The ESR spectrometer settings were microwave power, 10 mW; microwave frequency, 9.20 GHz; modulation amplitude, 8 G; gain, 25,000.

after application of ETX and could be separated into two components (Figure 1). Samples from lung, where blood was removed by perfusion with NaCl solution, showed a small, but significant increase of the ESR signal at $g = 2$ with the appearance of a more axial signal shape (Figure 1). This signal was attributed to lung tissue without blood.

In myocardium an ESR signal at $g = 2.004$ has been reported which has tentatively been attributed to a semiquinone radical.[6-8] This signal decreases during ischemia and increases to about double the amplitude of normal tissue during reperfusion. The

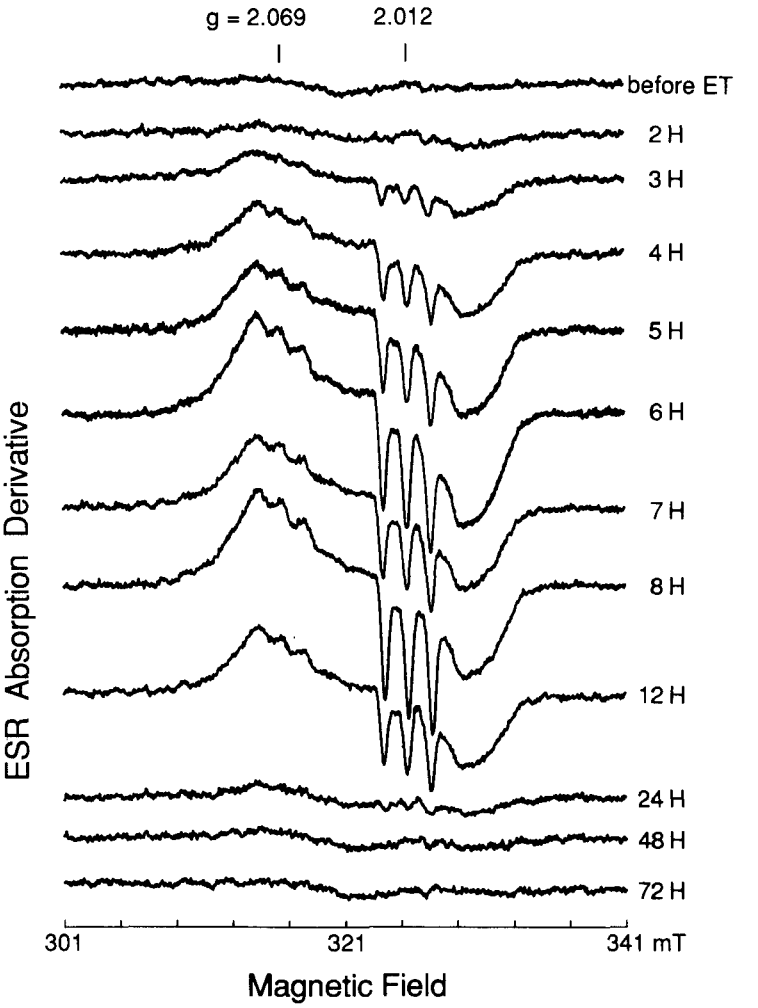

FIGURE 2. Formation of ESR signals in rat blood during endotoxin shock. ETX was administered as a single dose of 7.5 mg/kg b. w. via the dorsal penis vein. Blood samples from the anesthesized rats were taken by puncture of the juglar vein at times as indicated above. The ESR spectrometer settings were as in Figure 1 except modulation amplitude, 5 G.

TABLE I

ESR signal intensities in rat blood and blood counts during ETX shock experiments. The experiments were made as described in Figure 2. Blood counts were determined with Coulter® from diluted blood samples. ESR signal intensities were calculated in arbitrary units as double integrals of the measured spectra. Abbreviations: RBC, red blood cells/μl; WBC, white blood cells/μl; Hb, hemoglobin; Hct, hematocrit.

Time after ET h	n	ESR signal intensity arb. units	Blood count RBC x 10^6	WBC x 10^3	Hb g/dl	Hct %
before	13	2.5 ± 0.6	8.3 ± 0.4	9.1 ± 0.8	16.2 ± 0.9	45.1 ± 2.3
2	5	3.7 ± 0.8	8.0 ± 0.3	1.6 ± 0.4	15.5 ± 0.5	41.2 ± 1.5
3	5	10.5 ± 1.0	7.8 ± 0.2	2.0 ± 0.4	14.8 ± 0.4	40.4 ± 0.9
4	5	18.4 ± 4.1	7.8 ± 0.2	2.0 ± 0.4	14.8 ± 0.4	40.4 ± 0.9
5	5	23.0 ± 3.3	7.5 ± 0.3	2.4 ± 0.2	14.3 ± 0.3	39.3 ± 1.3
6	10	31.5 ± 4.7	7.6 ± 0.3	2.3 ± 0.6	14.2 ± 0.7	39.7 ± 1.7
7	6	23.6 ± 2.2	7.4 ± 0.5	2.7 ± 0.5	14.2 ± 0.7	38.9 ± 2.1
8	8	29.2 ± 6.4	7.2 ± 0.4	2.7 ± 0.6	13.5 ± 0.6	37.5 ± 1.7
12	4	21.7 ± 2.1	7.0 ± 0.1	6.9 ± 0.9	12.9 ± 0.3	36.2 ± 1.1
24	4	6.3 ± 2.7	7.2 ± 0.4	8.3 ± 1.1	13.6 ± 1.0	36.8 ± 2.5
48	3	3.2 ± 0.1	6.6 ± 0.2	12.6 ± 3.8	12.9 ± 0.6	35.6 ± 1.5
72	3	1.8 ± 0.9	5.7 ± 0.3	16.6 ± 2.3	11.0 ± 0.5	30.6 ± 2.0

signal which we observed in lung tissue showed a lower absolute amplitude which can be explained by the lower density of lung tissue and a lower content of respiratory enzymes in the lung samples. The second difference is, that in ETX shock no hypoxia occurs in the lung tissue and hence no change in the respiratory-dependent redox-state of quinones can be expected. We interpret the increase of the signal in lung tissue during ETX shock as a consequence of radical processes which may result in the accumulation of more stable secondary radicals. The primary, highly reactive radicals would occur at very low steady-state concentrations which cannot be detected by ESR and the applied freezing technique. The low amplitude of the signal and the lack of a resolved structure did not allow an assingment of this signal to a free radical species.

The second component of the ESR signal, with a higher amplitude and a typical triplet structure was observed also in whole blood samples (Figure 1) and was attributed to the blood in the vascular compartment of lung.

ESR signals in blood during endotoxin shock

In the blood of all ETX-treated rats, we detected the ESR signal with the triplet hyperfine structure. The signal appeared 2 to 3 hours after ETX application and increased to maxima at 6 and 8 hours and vanished 24 hours after the ETX application (Figures 2 and 4). Remarkably, the ESR signal showed the same time dependence in all animals during shock although minor individual variations of signal intensity were observed.

All ETX-treated rats showed the formation of ESR signal as well as typical symptoms of ETX shock. Within minutes after ETX application, the rats became lethargic, followed by piloerection, chills and tachypnea. Hypotension and an initial decrease of heart rate were observed (data not shown), the typical hemodynamic alterations as described for ETX shock.[3] The blood count showed acute leukopenia (Table I) which is attributed to the margination in the capillary bed.[1,2] The observed decrease of hemoconcentration may be due to hemodilution by taking the blood samples (0.5 ml/

TABLE II

Localization of the Hemoglobin-NO signal in the red blood cells. Cells were separated into fractions by a Percoll® gradient and centrifugation. The preparation of the fractions took the times as indicated. ESR of the frozen samples was measured at 90 K. Blood counts were determined with a Sysmex® blood counter. For abbreviations see table I.

Fraction	Time min	ESR D.Int.	RBC x 10^6	Hb g/dl	WBC x 10^3	Platelets
Whole blood	1	50.2	8.8	15.3	5.1	10^5
Plasma, platelets	10	7.2 ± 5	0.1	0.5	0	5×10^4
Leucocytes	40	3.3 ± 5	0.1	0.7	2.9	–
Red cells	45	68.2	12.9	22.2	0.4	0
Washed red cells	70	38.7	8.7	15.2	0.3	0

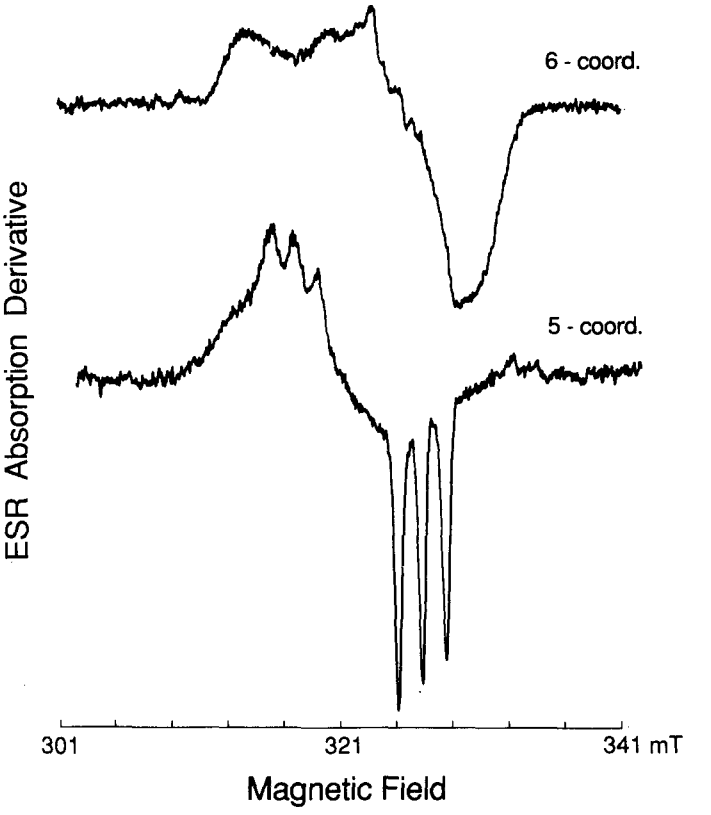

FIGURE 3. *Demonstration of two different types of ESR spectra in rat blood during ETX shock.* The two spectral types were obtained by an iterative subtraction method[12] of spectra measured at different times after ETX application. They have been interpreted as two possible configurations of the heme coordination sphere of Hb–NO: five-coordinate and six-coordinate heme-NO, respectively.

venipuncture). No formation of the ESR signal was observed in saline-treated control animals as well as only a slight decrease in the leukocyte count and in the hematocrit which was attributed to depression by the etherizations and the venipunctures. Necropsy of the ETX-treated rats revealed several abnormalities. Diffuse hyperemia and punctate areas of hemorrhage were evident in the lungs. The bowels were extremely atonic and exhibited segmental ischemia with regions of frank hemorrhage or necrosis; the cecum was invariably infarcted.

The observed ESR signal was localized in the red blood cell fraction. ESR measurements of red and white blood cells and blood plasma which were separated by centrifugation of blood samples, clearly indicated that the ESR signal correlated to the cellular fraction of red blood cells (Table II).

The ESR spectra showed distinct three-line hyperfine splittings of 1.75 mT at $g = 2.012$ and 1.6 mT at g 2.069 which are characteristic of an unpaired electron coupled to nitrogen in a ferrous heme-NO complex.[9,10] A nearly identical ESR spectrum has been reported for a valency hybrid of hemoglobin with nitrosylated ferrous α-subunits $((\alpha^{2+}\,NO)\,\beta^{3+})_2$.[11] This assignment is corroborated by our observation of an ESR signal at $g = 6$ (data not shown) which is typical for the high-spin ferric heme in methemoglobin. Furthermore, the localization of the signal in the red cells and the fact that the NO adduct was formed in concentrations up to 0.8% of the total hemoglobin concentration, makes it very unlikely that this signal was due to NO adducts of other hemeproteins.

The ESR spectra consisted of two spectral components (Figure 3) which evolved with different time dependences during shock (Figure 4). The pure spectra were obtained by a subtraction method[12] and, in accordance with earlier reports,[9,10,12] were assigned to two possible coordination spheres of the heme in the NO adduct: five-coordinate heme with a strong Fe–NO bond and a weak or broken Fe-proximal histidine bond, and six-coordinate heme, with a strong Fe-proximal His bond. The ratio of these two structures is modulated by inositol hexaphosphate,[11] an allosteric effector of hemoglobin.[13] Hence, the observed variation of this ratio during the course of ETX shock (Figure 3) could be explained by varying levels of 2,3-diphosphoglycerate or pH variations in the red cells of the individual animals during the ETX shock. Also more drastic changes of quarternary and tertiary structure of hemoglobin, e.g. by low pH or detergents, produce the five-coordinate heme-NO complex *in vitro*.[9]

The ESR signal of Hb–NO was also observed with higher doses of ETX (40 mg/kg b. w., LD_{100}/4 hours) (Figure 1). Remarkably, this dose generated a higher portion of the five-coordinate complex. It is speculated that five-coordinate Hb-NO might also be a denaturation product of the native six-coordinate hemoglobin-NO. With this dose the Hb–NO signal was detected already 1 h after ETX application and showed a more rapid increase in signal intensity until the death of rats.

The observed time dependence of the Hb-NO signal represented essentially the rate of formation of Hb-NO since the half-life of Hb-NO, as produced by the i.v.-application of NO in saline, was about 60 min (57 ± 15 min, $n = 7$; data not shown).

The NO-production of various cells of the vascular system has been inhibited by the arginine analog NMMA.[14] The application of NMMA during the first 5 hours of ETX shock inhibited the formation of the Hb-NO signal strongly (Table III). This inhibition was partially reversed by L-arginine (data not shown). These findings suggest that NO is produced via the pathway from L-arginine during ETX shock.

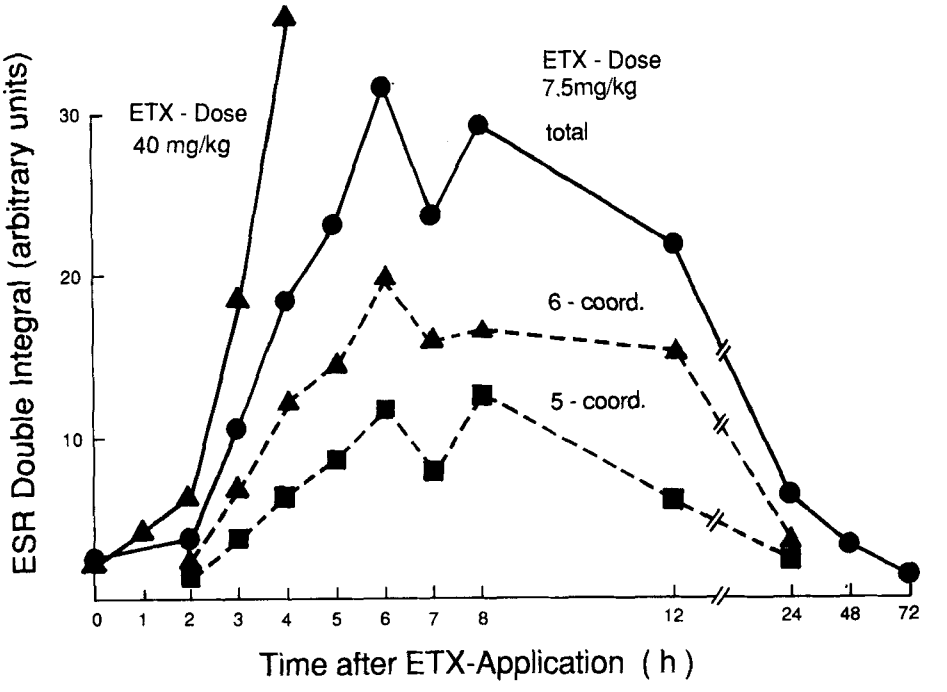

FIGURE 4. Time dependence of ESR signal intensity in rat blood during endotoxin shock. Mean signal intensities (double integrals in arbitrary units) after a single ETX dose of 7.5 mg/kg b. w. (for number of experiments and standard deviations see Table I). The curve "total" displays the intensity of the total signals (corresponding to Figure 2), whereas the curves "5-coord" and "6-coord" give the intensities of the partial spectra of five- and six-coordinate Hb-NO, respectively, which were resolved from the total spectra according to[12] (see figure 3).

For the higher ETX dose of 40 mg/kg b.w. only the mean total signal intensity is shown (number of experiments n $\geqslant$ 4 for each data point).

ESR signals in blood during hemorrhagic shock

Hemorrhagic shock was examined as a second different shock model for the generation of the ESR signal of Hb–NO in the blood of rats. Two variations of hemorrhagic shock models were employed, namely (i) hemorrhage and subsequent resuscitation with lactated Ringer's solution and (ii) the same procedure but with additional supra-celiac aortic clamping for 15 min.

Figure 5 shows a typical experiment using the first variation of hemorrhagic shock where hemorrhage and resuscitation were carried out in such a way as to achieve longer periods with low mean arterial pressure (Figure 5A). After a lag phase of several hours a Hb-NO signal became detectable which further increased until the death of the rat. The maximal amplitude of the signal was significantly lower than during endotoxin shock. It is interesting to note that the samples taken immediately after the death of the rat (Figure 5B, 6 h 20 min) showed a strong shift to five-coordinate Hb-NO. The lower signal amplitude compared to ETX shock can be explained, in part, by the massive hemodilution caused by the volume substitution with Ringer's solution.

TABLE III
Effect of N^G-monomethyl-L-arginine on the formation of the ESR signal of hemoglobin-NO during endotoxin shock

Time after ETX application	Treatment	ESR Double Integral Arbitrary Units	n
4 hours	ETX[a]	18.7 ± 4.0	6
4 hours	ETX[a] + NMMA[b]	3.1 ± 1.2	3
6 hours	ETX[a]	30.9 ± 5.0	11
6 hours	ETX[a] + NMMA[b]	4.4 ± 1.6	3

[a]ETX, endotoxin, was given as a single i.v.-dose of 7.5 mg/kg b.w. at time 0 h.
[b]NMMA, N^G-monomethyl-L-aginine, was administered as an i.v.-infusion (0.2 ml/min) of a solution of 15 mg/ml in saline: 100 mg/kg b.w. during 10 min. before the ETX application, 50 mg/kg b.w. at 1, 2, 3, 4, and 5 h, respectively.

In the model with additional supra-celiac aortic clamping the Hb–NO signal was also detected at comparable intensity (data not shown), even when the mean arterial pressure did not decrease below 50 mm Hg during the period of hemorrhage. Obviously, ischemia and reperfusion provoked the formation of Hb–NO also at higher blood pressure levels than during pure hemorrhagic shock, indicating a pronounced stimulation of the NO-producing process(es), and an enhanced formation of Hb–NO in the course of ischemia-reperfusion syndrome.

DISCUSSION

Our results, the unequivocal demonstration by ESR of considerable amounts of hemoglobin-NO during shock in rats, raise the question on the origin and the action of the NO part of which is finally trapped by hemoglobin. Historically, two types of processes have been described for the formation of NO or Hb–NO. One type occurs when the organism is suffering from rather drastic conditions, like e.g. acute toxic stress, hypoxia, necrosis and tumors, as well as during the chronic exposure to carcinogenic aromatic amines or N-nitroso-compounds. Under such conditions, ESR signals have been observed in tissue or blood samples and have been attributed to heme-NO-complexes.[15–17] The mechanisms of NO production have been unexplained, except in those cases where the oxidative or reductive metabolism of aromatic amines or N-nitroso compounds, respectively, have been elucidated. Despite the lack of a mechanistic explanation, the occurrence of heme-NO signals was believed to indicate a pathological condition.

The second type of processes, namely the production of NO by cells of the vascular system, has received much attention after the endothelium-derived relaxing factor (EDRF)[18] has been identified as NO.[19] There are several possible sites for NO formation in the vascular system. Phagocytic cells, macrophages[20,21] and polymorphonuclear granulocytes,[22] and endothelial cells[19] have been demonstrated to be capable of producing NO in considerable amounts. Here NO is produced by a specific enzymatic reaction from the guanido group of L-arginine,[21,23,24] which can be inhibited by arginine analogs, such as NMMA in a variety of cell types.[14] The observed inhibition of the formation of Hb–NO during ETX shock by NMMA (Table III) indicated that during ETX shock NO was produced by the specific enzymatic pathway and not by acute toxic stress processes.

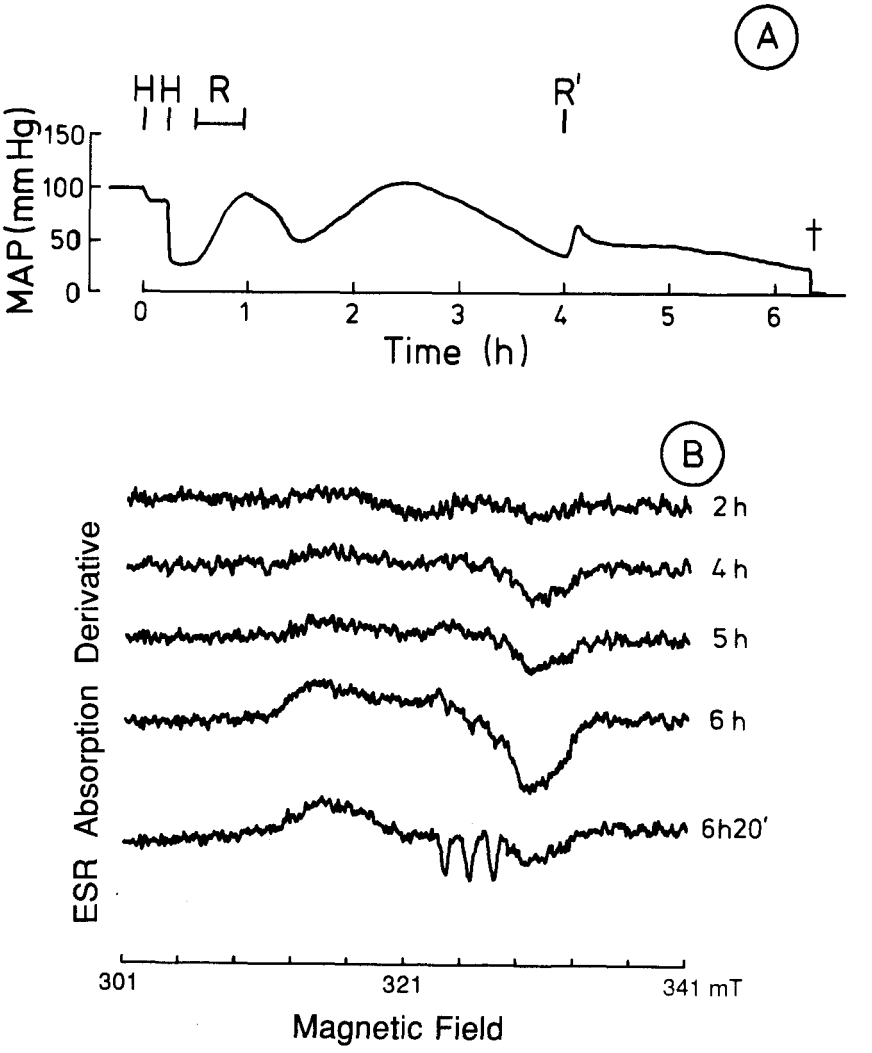

FIGURE 5. Mean arterial blood pressure (A) and ESR signals in rat blood (B) during hemorrhagic shock. The shock was induced by two hemorrhages (H, each 1.5 ml/100 g b. w.). Resuscitation was carried out by infusion of lactated Ringer's solution (R, 9 ml/100 g b. w.; R, 0.5 ml/00 g b. w.) ESR spectra were measured from arterial blood which was sampled at the times as indicated.

Among others, the vascular cells mentioned above play an important role in the pathogenesis of shock. Phagocytic cells become rapidly activated during shock, especially by ETX. Macrophages have been shown to produce NO after stimulation with ETX *in vitro*[21] and appear to be the most likely candiates for the NO-producing cells in ETX shock. In addition, endothelial cells might release NO as EDRF, as a consequence of an increased endogenous bradykinin release and an increased adhesion of various blood cells to the endothelium during shock. NO produced by

free-floating cells in the circulation might be effectively trapped by hemoglobin, which results in the formation of the Hb-NO radical.

However, a large amount of the NO produced by endothelial cells or phagocytic cells adhering to the vascular wall will affect the endothelium. Several effects of NO have been described. NO acts as cytotoxic effector of macrophages,[21] relaxes smooth muscle (vasodilation by NO as EDRF[19]) and inhibits the aggregation of platelets.[25] A vasoprotective effect of NO has been suggested.[26] The role of NO during shock has to be clarified by further studies, as well as the question whether Hb-NO will be a useful signal for monitoring phases of shock.

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