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Kinetics of increased generation of $^{\bullet}$NO in endotoxaemic rats as measured by EPR★✪

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Ferrous-diethyldithiocarbamate ($Fe(DETC)_2$) chelate is a lipophilic spin trap developed for $^{\bullet}$NO detection by electron paramagnetic resonance (EPR) spectroscopy. Using this spin trap we investigated the kinetics of $^{\bullet}$NO production in endotoxaemia in rats induced by lipopolysaccharide (*Escherichia coli*, 10 mg/kg). The NO-$Fe(DETC)_2$ complex was found to give a characteristic EPR signal, and the amplitude of the 3rd (high-field) component of its hyperfine splitting was used to monitor the level of $^{\bullet}$NO. We found that in blood, kindey, liver, heart and lung $^{\bullet}$NO production starts to increase as early as 2 h after LPS injection, reaches the maximum 6 h after LPS injection and then returns to basal level within further 12–18 h. Interestingly, in the eye bulb the maximum of $^{\bullet}$NO production was detected 12 h after LPS, and the signal was still pronounced 24 h after LPS. In brief, the highly lipophilic exogenous spin trap, $Fe(DETC)_2$ is well suited for assessment of $^{\bullet}$NO production in endotoxaemia. We demonstrated that the kinetics of increased production of $^{\bullet}$NO in endotoxaemic organs, with the notable exception of the eye, do not follow the known pattern of NOS-2 induction under those conditions. Accordingly, only in early endotoxaemia a high level of $^{\bullet}$NO is detected, while in late endotoxaemia $^{\bullet}$NO detectability is diminished most probably due to concomitant oxidant stress.

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Abbreviations: DETC, diethyldithiocarbamate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EPR, electron paramagnetic resonance; HbNO, nitrosohaemoglobin; LPS, lipopolysaccharide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; SOD, superoxide dismutase.

It is well known that expression of inducible $^{\bullet}$NO synthase (NOS-2) increases in endotoxic shock, and $^{\bullet}$NO produced by NOS-2 contributes to hypotension, vasoplegia, and also, indirectly, *via* peroxynitrite formation, to organ injury in endotoxaemia. Indeed, inhibition of NOS-2 in endotoxaemia reversed hypotension, and restored vascular responsiveness to vasoconstrictor agents (Thiemermann, 1997; Kirkeboen & Strand, 1999). Moreover, numerous reports have demonstrated organ-protective effects of NOS inhibitors, superoxide dismutase (SOD) mimetics, or peroxynitrite ($ONOO^-$) decomposition catalysts (Salvemini *et al.,* 1999).

On the other hand, $^{\bullet}$NO produced by a constitutive isoenzyme, NOS-3, has been shown to play a protective role in endotoxaemia by its vasodilatory (Spain *et al.*, 1994), antiplatelet (Shultz & Raij, 1992) or antileukocyte (Laszlo *et al.,* 1995) activities. Interestingly, it was not long ago demonstrated that overexpression of NOS-3 in mice conferred increased resistance to LPS-induced mortality, further supporting the importance of the cytoprotective role of $^{\bullet}$NO generated by NOS-3 in endotoxaemia (Yamashita *et al.,* 2000). The cytoprotective action of $^{\bullet}$NO is of particular importance in the endotoxaemic lung, as one of us has recently proposed (Chlopicki *et al.,* 2001). Most surprisingly, quite recent papers suggest that even $^{\bullet}$NO synthesized by NOS-2 may be cytoprotective (Kubes, 2000). It might well be that the toxic effect of $^{\bullet}$NO results in the major part from rapid reaction with superoxide anions and peroxynitrite formation while $^{\bullet}$NO itself protects the tissue against injury, mostly *via* cGMP-mediated effects. This hypothesis could be validated by direct measurement of $^{\bullet}$NO and superoxide anions and peroxynitrite formation in endotoxaemia. If the production of $^{\bullet}$NO in endotoxaemia is accompanied by superoxide release, the amount of $^{\bullet}$NO that diffuses to target sites may be much lower than the expected on the basis of high NOS-2 expression. Here we attempted to measure directly $^{\bullet}$NO formation in various organs by electron paramagnetic resonance (EPR) spectroscopy using exogenous spin trap — ferrous diethyldithiocarbamate, $Fe(DETC)_2$.

MATERIALS AND METHODS

Reagents and drugs. Sodium diethyldithiocarbamate (DETC; $C_5H_{10}NS_2Na$), ferrous sulphate ($FeSO_4 \times 7H_2O$), sodium citrate ($C_6H_5Na_3O_7 \times 2H_2O$), and lipopolysaccharide (LPS) from *Escherichia coli* (serotype 027:B8) were obtained from Sigma Chem. Co. (Baltimore, MD, U.S.A.). Phosphate buffered saline (PBS) was purchased at Wytwórnia Surowic i Szczepionek, BIOMED (Lublin, Poland).

Animals. Male or female Wistar rats weighing 200–250 g (Lod: Wist BR; Animal Laboratory of the Polish Mother's Memorial Research Institute Hospital, Lodz, Poland) were used for the experiments, according to a protocol accepted by the Permanent Committee for Bioethics in Animal Investigation of the Rector of the Jagiellonian University (permission number — 302/97). The animals were kept in community cages, under standard conditions, 12 h day/night regime, and free access to food and water.

Preparation of NO-Fe(DETC)$_2$ solution. Solutions were prepared in PBS bubbled for at least 30 min with nitrogen to remove oxygen. Concentrations are expressed as the final concentrations of reagents in the measured samples. Ferrous sulphate (50 mg/l) dissolved in sodium citrate solution (250 mg/l) was added to a solution of sodium DETC (about 3 mM, i.e. 500 mg/l), and reduced with sodium dithionite (6 g/l). This mixture was supplemented with a solution of $^{\bullet}$NO (0.067 mM) obtained by reduction of a stoichiometric equivalent of sodium nitrite with an excess of sodium dithionite, according to Plonka *et al.* (1999). The solutions were prepared shortly (2–3 min) before experiments, and were mixed just before EPR measurement.

***Experiments* in vivo**. Endotoxaemia was induced by a single i.p. injection of LPS (10 mg/kg body mass, in 0.5 ml PBS). Animals were killed with nitrogen asphyxia 2.5, 4, 6, 8, 12, 18, and 24 h after LPS injection, whereupon blood (about 1 ml, left ventricle) and organs (lungs, liver, kidney, heart and eye bulbs) were isolated. Control animals were injected with 0.5 ml of sterile PBS. Thirty minutes prior to euthanasia, animals were given spin trap (DETC – 500 mg/kg body mass, in 0.2 ml PBS, i.p.), and chelated iron(II) complexes ($FeSO_4 \times 7H_2O$, 50 mg/kg plus sodium citrate – 250 mg/kg body mass, s.c.) in two separate injections. Notably, DETC creates insoluble complexes with iron(II). Accordingly, to prevent their precipitation from injected solution, they must be given separately (Vanin *et al.,* 1989). All animals of the test and control groups were treated identically, so that the time available for the spin trap and iron(II) ions to penetrate tissues and to create insoluble NO-binding complexes was exactly the same – 30 min. The doses were chosen according to Kubrina *et al.* (1992). All the experiments were repeated at least 2–4 times, so as to collect sets of 2–4 independent organ samples per each timepoint.

EPR measurements. Solution of NO-Fe-$(DETC)_2$ was frozen in standard glass pipes (inner diameter 4 mm, length 2 cm) in liquid nitrogen (77 K), immediately after preparation. Tissue and blood samples were prepared in similar pipes directly after isolation, and stored in liquid nitrogen until the EPR measurements. The spectra were recorded at 77 K, using an EPR X-band spectrometer Varian E-3 with a rectangular TE 102 resonant cavity, and a standard quartz Dewar. The EPR signal intensity was expressed as the amplitude of the third (high-field) hyperfine component of the signal (see Fig. 1), because the first and the second component often overlap with a strong line of $Cu(DETC)_2$ complexes (Andriambeloson *et al.,* 1997; Kubrina *et al.,* 1992).

RESULTS

A typical EPR signal of the spin adduct NO-$Fe(DETC)_2$ was obtained by mixing $Fe(DETC)_2$ complexes with a solution of •NO. The signal ($g_\perp$ = 2.035; $g_{||}$ = 2.02) with a well-resolved hyperfine splitting at $g_\perp$ is shown in Fig. 1. The third, high-field component of the signal could be distinguished in the spectra from all the investigated organs (see inserts in Fig. 2) and reflected the level of nitrosyl-iron complexes in the measured material.

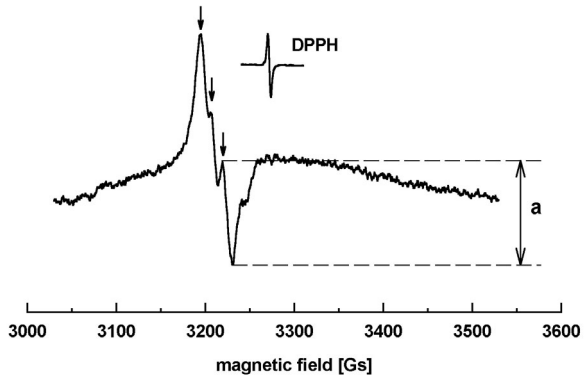

Figure 1. A typical EPR signal of NO-$Fe(DETC)_2$ complexes (•NO – 0.067 mM, $Fe(DETC)_2$ – about 3 mM).

Amplitude of the 3rd hyperfine component was calculated as shown in the scheme. Parameters of assay: magnetic field 3280 ± 250 Gs, modulation amplitude 10 Gs, microwave power and frequency 4 mW and 9.47 GHz, gain 200 000, time constant 0.3 s, scan time 400 s (average of two scans). DPPH marks the position of free radical signal (g = 2.004), arrows – the ^{14}N hyperfine splitting.

Despite differences in the intensity of the signals between tissues (Fig. 2), the time course of changes in the signal intensity of NO-$Fe(DETC)_2$ complex in blood (Fig. 2A), liver (Fig. 2B), lung (Fig. 2C), kidney (Fig. 2D) and heart (Fig. 2E) was similar. The maximum was reached around 6–8 h after administration of LPS, followed by a slow decrease in the signal amplitude, so that 24 h after LPS injection the signal was hardly detectable. In

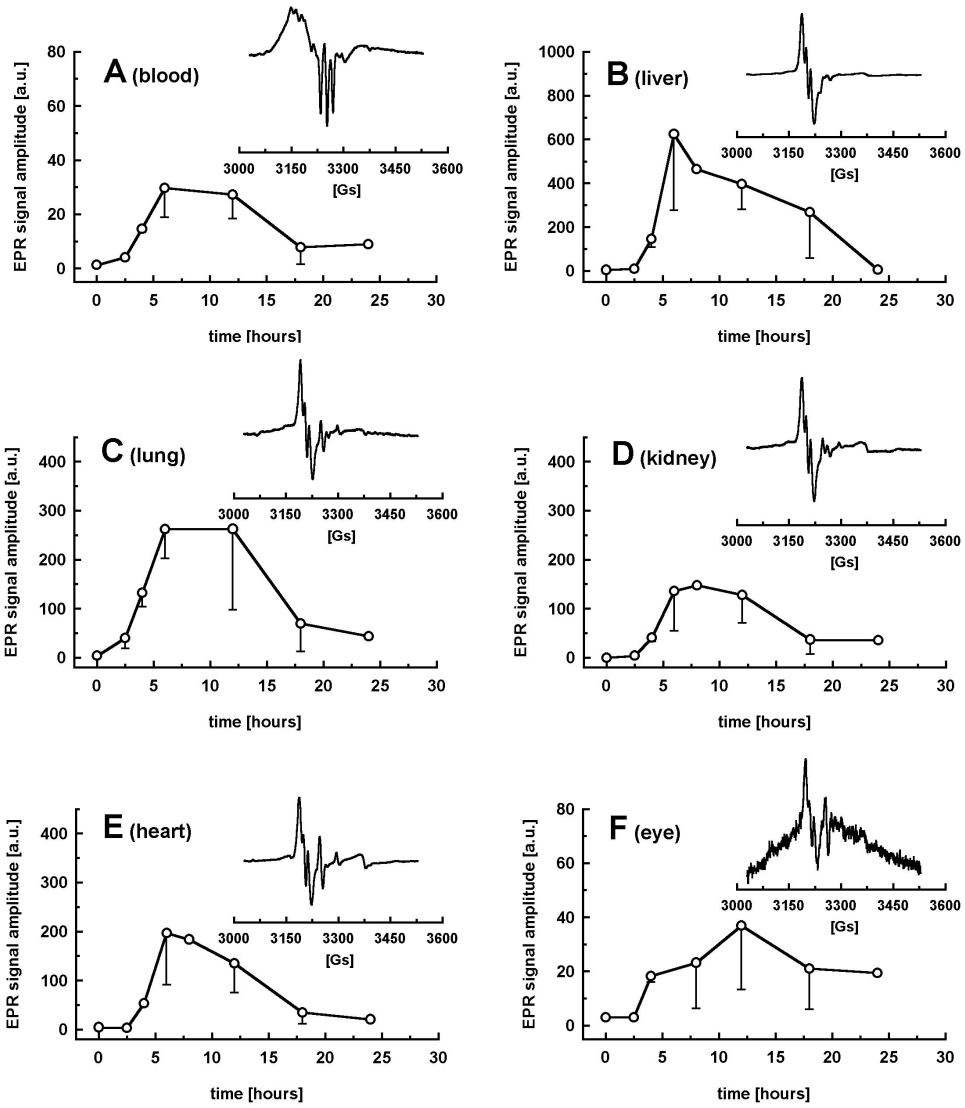

Figure 2. Time course of changes in the amplitude of the 3rd hyperfine component of NO-Fe(DETC)$_2$ signal in blood (A), liver (B), lung (C), kidney (D), heart (E), and eye bulbs (F) of endotoxaemic rats.

Inserts show typical EPR spectra of NO-Fe(DETC)$_2$ complexes – single scans (A–E) or average of two scans (F) – in the tissues 6 (A–E) or 12 (F) hours after LPS, and 30 min after application of the spin trap. Parameters like in Fig. 1 except for received gains – 1250 (liver), 2500 (lung), 4000 (heart), 5000 (kidney), 10 000 (blood) 400 000 (eye bulb). Each point of kinetic curves represents a mean of 2–4 independent organs ±SEM calculated for received gain 200 000. Note variable scaling of the amplitude axes.

blood of endotoxaemic rats the signal had the lowest intensity among all the investigated materials (Fig. 2A), whereas the signal from liver (Fig. 2B) was the strongest. Instead, blood revealed a strong triplet signal of haemoglobin-complexed nitric oxide – nitrosohaemoglobin (HbNO; Fig. 2A) which in other organs was poorly detectable.

In contrast with blood and other organs, in the eye bulbs the maximum could be observed 12 h after induction of endotoxaemia, and 24 h after LPS the signal was still pronounced (Fig. 2F).

DISCUSSION

We analyzed here the formation of $^{\bullet}$NO in rat endotoxaeamia by EPR using exogenous spin trap – Fe(DETC)$_2$. Since DETC *in vivo* of-

ten creates EPR-detectable complexes with copper cations, the signal of which is superimposed on the signal of NO-Fe(DETC)$_2$ adducts, only the 3rd, high field component of the signal remains clearly measureable (Andriambeloson *et al.,* 1997; Kubrina *et al.,* 1992). This component remained detectable in the presence of HbNO signal in blood (Fig. 2A, insert), and in a linear fashion corresponded with •NO concentration (now shown), therefore, it was used by us as the analytical line. Importantly, the weak signal of the spin adducts detectable in blood and strong in solid tissues shows that DETC, a hydrophobic NO-metric spin-trap may be considered a useful NO-metric agent for faithful determination of •NO contents in tissues, as suggested in numerous previous papers (Vanin & Kleschyov, 1998; Kubrina *et al.,* 1992; Mülsch *et al.,* 1992; Vanin *et al.,* 1989). After chelating iron(II) cations, the complex precipitates, which makes it toxic, but which also prevents uncontrolled changes in the trap concentration or trap removal from the tissues (Vanin & Kleschyov, 1998).

Our data show that in the majority of organs, •NO complexes are detectable as early as two hours after LPS injection. We also show that the intensity of •NO production reaches the maximum about 6 h after administration of LPS and then either stays at approximately the same level until 12 h and then decreases (kidney, lung, blood), or starts to diminish already 12 h after LPS (heart, liver). In all cases, 24–48 h after LPS the signal intensity returned to the basal level. Similar kinetics were observed for HbNO in endotoxaemic rat blood (Plonka *et al.,* 1999; Westenberger *et al.,* 1990), as well as lung (Plonka *et al.,* 1999).

Notably, during endotoxaemia in rats *in vivo* the induction of NOS-2 as measured at the mRNA and protein level follows a different pattern. NOS-2 protein level reaches the maximum 12–24 h after LPS and is present at almost the same level 30 h after LPS (Brandes *et al.,* 1999) while mRNA reaches the maximum after 12 h and is no longer detectable 30 h after LPS administration. Also in isolated macrophages stimulated with LPS or cytokines both NOS-2 gene promotor activity, and NOS-2 protein reach the maximum 24 h after stimulation with LPS but not earlier (Cieslik *et al.,* 2002). This is consistent with the hypothesis that production of •NO reaches its maximum early in endotoxaemia, while later, due to concomitant increase in superoxide generation, peroxynitrite is formed. This concept may be further supported by the fact that a single dose of 10 mg of LPS *in vivo* in rats leads to a marked increase in the expression and activity of NADPH oxidase and xanthine oxidase attaining its peak 12–30 h after LPS (Brandes *et al.,* 1999). Accordingly, late endotoxaemia is associated with increased superoxide anion production, increased generation of $ONOO^-$ and nitrotyrosine formation leading to dysfunction of various organs (Salvemini *et al.,* 1999). Although without detailed measurements of superoxide and peroxynitrite production this hypothesis posesses only the value of speculation, we suggest that it is the most probable explanation of our results.

A different time course of •NO production in endotoxaemia is observed in the eye bulbs, in which the maximum of the signal of NO-Fe(DETC)$_2$ complexes was seen 12 h after LPS and even 24 h after LPS it was still well detectable. The kinetics of the NO-Fe(DETC)$_2$ signal in the eye corresponds better than in other organs with the well known time course of the expression of NOS-2 protein in endotoxaemia. Accordingly, one could suggest that inflammatory formation of free radicals is limited in endotoxaemic eye (Rocha *et al.,* 1997) and does not interfere with detection of •NO. This hypothesis may find its rationale in the fact that eye is an immunologically priviledged organ, with a unique pattern of inflammatory response (Streilein & Stein-Streilein, 2000; Taylor *et al.,* 1998). On the other hand, the low intensity of the EPR signals detectable in the eye bulb, as compared to other organs and tissues, may be accounted

for by poorer penetration of iron(II) ions or DETC through the non-vascularized parts of the organ and their slightly altered metabolism.

Spin trapping of $^{\bullet}$NO by a highly lipophilic exogenous spin trap – $Fe(DETC)_2$, which traps $^{\bullet}$NO at the site of $^{\bullet}$NO synthesis, has turned out to deliver congruent information on the magnitude of $^{\bullet}$NO production in various tissues. However, additional studies are needed to clarify the basis for the incompatibility between the known kinetics of NOS-2 expression and the kinetics of $^{\bullet}$NO production, assessed here by EPR technique, in lung, liver, heart, kidney, as well as for the exceptional kinetics of $^{\bullet}$NO production in endotoxaemic eye. Progress towards a better picture of the time course of oxidative and "nitrosative" stress accompanying endotoxaemia in various parts of the organism will be achieved by supplementary *ex vivo* or *in vivo* kinetic measurements using DETC derivatives, such as *N*-(dithiocarboxy)sarcosine (Plonka *et al.*, 2003). Direct and parallel measurements of superoxide and peroxynitrite production in endotoxaemic tissues are mandatory to explain the early drop in the level of $^{\bullet}$NO despite the persistence of NOS-2 expression. How to rescue $^{\bullet}$NO activity in the late endotoxaemia to take advantage of its cytoprotective properties without perpetuating its hypotensive effects presents a challenge for future investigations.

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