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KINETICS OF NITRIC OXIDE RELEASE IN NEONATAL AND MATURE RAT BRAIN DURING ENDOTOXEMIA, AS STUDIED BY DIETHYLDITHIOCARBAMATE SPIN TRAPPING

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Nitric oxide belongs to the most important biochemical factors affecting functions of brain and its response to pathological processes. In the beginning of the postnatal development brain reveals a unique plasticity, which is lost during maturation. This ability may influence the response of this organ to endotoxemia, and the related generation of nitric oxide. Using ferrous-diethyldithiocarbamate ($Fe(DETC)_2$) chelate, a lipophilic spin trap for NO detection by electron paramagnetic resonance (EPR) spectroscopy we investigated the kinetics of NO production in brain and liver during endotoxemia induced by lipopolysaccharide (*E. coli*, i.p. 10 mg/kg) in 6- and 30-days-old Wistar rats. The NO-$Fe(DETC)_2$ complex was found to give the characteristic signal, and the amplitude of the 3-rd (high-field) component of its hyperfine splitting was used to quantify the level of NO. The neonatal brains produced NO with a delay, as compared to the mature organs, and the maximal intensity of the process was found 12 hours after LPS injection, *i.e.* twice as late as in the case of the mature organs. In the both groups of the animals, livers revealed similar kinetics to brains, which betrays a systemic character of the phenomenon. Nevertheless, NO generation in the untreated brain varied during the neonatal period, which was, however, not confirmed for the liver. Our results indicate a strong dependence of the dynamics of the rat brain response to LPS on the progress of the postnatal development.

INTRODUCTION

Encephalopathy may be defined as worsening of the mental state initiated by a pathological process situated outside the brain (Bolton, Young & Zochodne, 1993). It is often observed at the onset of sepsis and it correlates with the increased mortality (Titheradge, 1999; Papadopoulos, Davies, Moss, Tighe & Bennett, 2000). Septic encephalopathy is probably a consequence of the action of inflammatory mediators on the brain cells and it may result from their response to these factors (Papadopoulos *et al.*, 2000). Inflammatory processes in the brain during endotoxemia have also been suggested to cause neuronal injury, which manifests itself as long-lasting neurological deficits (Bolton *et al.*, 1993). One of the mediators responsible for encephalopathy induction is lipopolisaccharide (LPS), a component of the wall of gram-negative bacteria (Titheradge, 1999; Papadopoulos *et al.*, 2000). LPS in the brain activates glial cells which in response change their shapes (Hu, Martella, Anderson & Chao, 1994; Moller, Kann, Verkhratsky & Kettenmann, 2000), secrete different inflammatory cytokines (Hu *et al.*, 1994; Utsuyama & Hirokawa, 2002; Cai, Pang, Lin & Rhodes, 2003), and proliferate (Mertsch, Hanisch, Kettenmann & Schnitzer, 2001; Kong, Kristensson & Bentivoglio, 2002).

Another feature of the septic shock is the elevated nitric oxide (NO) production in different tissues (Suzuki, Fujii, Numagami, Tominaga, Yoshimoto & Yoshimura, 1998; Plonka, Chlopicki, Wisniewska & Plonka, 2003). Nitric oxide plays an important role in the nervous system, as being not only an important neurotransmitter and neuromodulator (Krukoff, 1999; Kiss, 2000), but also a crucial element of brain response to different pathological states, such as ischemia (Zhu, Liu, Sun & Lu, 2003), brain injury (Cai *et al*,. 2003; Tuzgen, Tanriover, Uzan, Tureci, Tanriverdi, Gumustas & Kuday, 2003; Armstead, Cines & Al-Roof Higazi, 2004) or infection (Leib, Kim, Black, Tureen & Tauber, 1998; Nahrevanian & Dascombe, 2001). However, its detrimental or beneficial role in the brain pathology has not yet been clearly determined (Iadecola, 1997). Moreover, the function and activity of NO strongly depends on its concentration, cellular source as well as the time course of its production in the tissue (Minghetti & Levi, 1998; Colasanti & Suzuki, 2000). Therefore, any attempts to qualify and quantify the dynamics NO production in brain are justified and valuable.

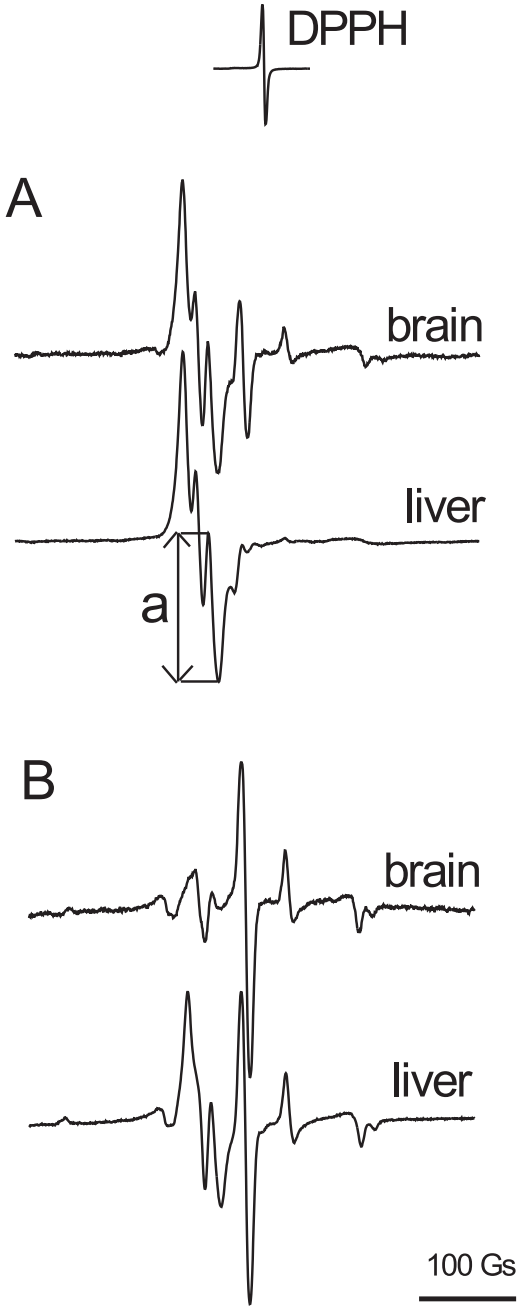

Fig. 1. Representative EPR spectra of frozen rat brain and liver obtained 6 and 24 hours after i.p. LPS injection in 30- (A) and 6-days-old animals (B), respectively. The signal of the MNIC adducts can be appreciated in all the organs. "a" – the 3rd, high-field line (analytical) of the hyperfine splitting. Parameters of assay – see "Material and Methods". DPPH – the free radical standard signal.

Previous studies suggested the stage of postnatal development being one of such essential factors, which determine the biological activity of NO and the corresponding course of inflammatory reaction in the brain (Janeczko, 1994; Anthony, Bolton, Fearn & Perry, 1997; Anthony, Dempster, Fearn, Clements, Wells, Perry & Walker, 1998; Bolton & Perry, 1998). On initial stages of the development brain possesses a unique plasticity, which is lost during maturation. This is assigned by neuronal differentiation, expansion of dendritic trees and establishing neuronal circuits (Blue & Parnavelas, 1983; Bahr & Wolff, 1985; Mrzljak, Uylings, Van Eden & Judas, 1990; Fricker-Gates, Shin, Tai, Catapano & Macklis, 2002), accompanied by differentiation of adjacent glial cells, especially microglia and astrocytes (Dalmau, Finsen, Zimmer, Gonzalez & Castellano, 1998; Domaradzka-Pytel, Ludkiewicz, Jagalska-Majewska & Morys, 2000), as well as by changes in cytokine production profiles (Balasingam, Tejada-Berges, Wright, Bouckova & Yong, 1994; Balasingam, Dickson, Brade & Yong, 1996; Silverstein, Barks, Hagan, Liu, Ivacko & Szaflarski, 1997). These phenomena essentially affect the course of inflammatory reaction in the brain. It has been found that inflammatory reaction in adult brain differs from other tissues and is characterized by the minimal vasculature response, limited neutrophils recruitment, as well as by a delayed influx of monocytes (Anthony et al., 1997; Bolton & Perry, 1998). On the other hand, inflammatory factors injected to the immature brain cause more profound response than in adults, with kinetics and reaction features similar to other tissues (Anthony et al., 1997; Anthony et al., 1998; Bolton & Perry, 1998), i.e. the large neutrophils recruitment and blood-brain barrier breakdown (Anthony et al., 1998; Bolton & Perry, 1998). It is also suggested that the period between day 6 and day 10 of the postnatal development is a "plasticity window " (Mrzljak et al., 1990; Kolb, Gibb & Gorny, 2000), when the results of damages can be compensated for by reorganization of cortical connections (Kolb, Ladowski, Gibb & Gorny, 1996; Kolb, 2003).

The technique of NO spin trapping with ferrous diethyldithiocarbamate ($Fe(DETC)_2$) complexes has long been applied to measure NO in various tissues including brain using electron paramagnetic resonance (EPR) spectroscopy (Suzuki et al., 1998; Suzuki, Fujii, Tominaga, Yoshimoto, Akaike, Maeda & Yoshimura, 1999). This component creates mononitrosyl-iron complexes (MNIC) with NO, which reveal a characteristic EPR signal ($g_{\perp}$=2.035, g_{II}=2.02) with a hyperfine splitting (A^N=13 Gs) at $g_{\perp}$ (Bune, Shergill, Cammack & Cook, 1995; Vanin & Kleschyov, 1998). It has been proven to be particularly convenient for kinetic studies, and derivatives of DETC turned out good spin traps for *in vivo* NO-metry and EPR imaging. The aim of the present study was to

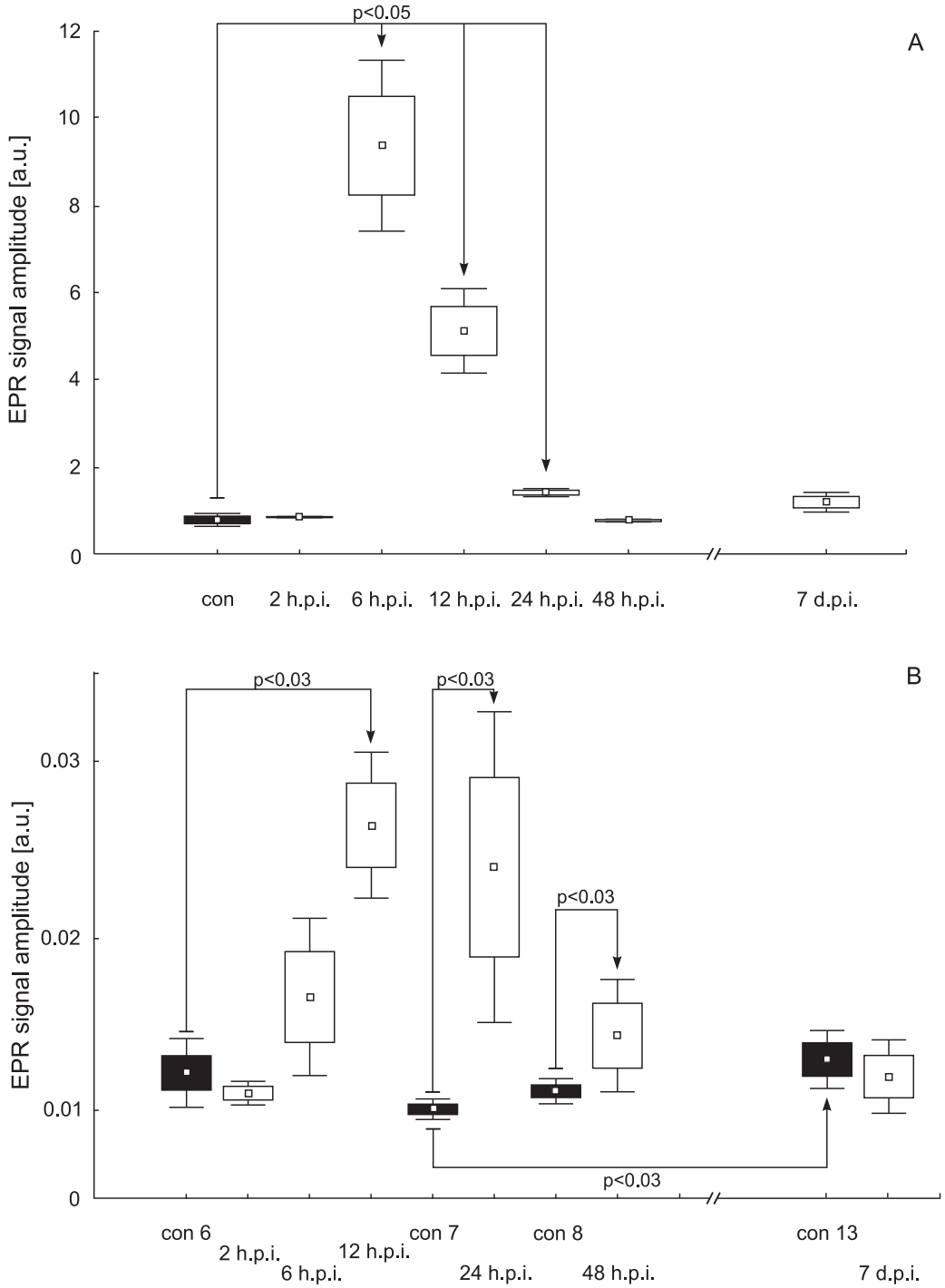

Fig. 2. Kinetics of the NO release in the rat brain following a single i.p. LPS injection (10 mg/kg) in 30- (A), and 6-days-old (B) animals. Each point represents a mean amplitude (n≥3) of the 3rd hyperfine component of the MNIC signal ± SEM (rectangles) and ± SD (error bars). Arrows indicate significant differences between the groups. Parameters of the EPR assay – see Fig. 1.

employ this technique to compare the time course of NO production in the septic brain at two different stages of postnatal development: one week after the birth, and one month later, so as to dissect this way whether there are any differences in NO production in response to inflammatory stimuli determined by the postnatal development.

MATERIALS AND METHODS

Chemicals and Reagents

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 *E. coli* (serotypes 026:B6 and 027:B8) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Phosphate buffered saline (PBS) was purchased at Wytwórnia Surowic i Szczepionek, BIOMED, Lublin, Poland, and chloral hydrate at POCh s.a. Gliwice, Poland.

Animals

All the experimental procedures were performed in accordance with the guidelines instituted by the Bioethical Committee at the Jagiellonian University, Krakow, Poland. Six- and 30-days-old male Wistar rats (from the local animal facility at the Institute of Zoology, Jagiellonian University) used in this study were maintained under conditions of controlled temperature and illumination, standard rodent chow and fresh water available *ad libitum*. Endotoxemia was induced by a single i.p. injection of LPS (10 mg/kg body mass, in PBS). 2, 6, 12, 24, 48 hours (h.p.i.) or 7 days (d.p.i.) after LPS injection animals were given spin trap (DETC — 500 mg/kg body mass, in 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.; Kubrina, Caldwell, Mordvintcev, Malenkova & Vanin, 1992; Vanin, Kubrina, Kurbanov, Mordvintsev, Khrapova, Galagan & Matkhanov, 1989). Similar procedure was carried out with control animals. Thirty minutes after application of the spin trap animals were deeply anesthetized with chloral hydrate and transcardially perfused with saline to rinse out blood from tissues.

EPR measurements and data analysis

To determine whether the observed differences are organ- or rather development stage-specific, we compared the endotoxemia-related NO generation in brain with liver, where this phenomenon is particularly pronounced (Plonka *et al.*, 2003; Bune *et al.*, 1995). The pieces of the organ tissues were collected in glass tubes (inner diameter ca. 4 mm) and frozen in liquid nitrogen immediately after euthanasia to further procedure. EPR measurements were performed in liquid nitrogen (77 K) at X-band (ca. 9.2 GHz) on an ESP300E Brucker spectrometer (30-day animals; field 3330±250 Gs, modulation amplitude 5.26 Gs, conversion time 81.92 s, time constant 20.48 ms, microwave power 16 mW, receiver gain 1×10^4-1×10^5, scan time 83.886 s, resolution 1024 points), and a Varian E-3 spectrometer (6-day animals; magnetic field 3280±250 Gs; modulation amplitude 10 Gs, filter time constant 0.1 s, 1-3 scans averaged, scan time 200 s, resolution 1024 points, microwave power 4 mW; amplification 5-10×10^4 - brains, 10×10^3 - livers).

Received EPR spectra were analyzed with WinEPR software (Brucker WINEPR system ver. 2.11, Brucker-Franzen Analytik GmbH, Germany). The peak-to-peak amplitude of the 3rd (high-field) hyperfine component of the MNIC signal (Fig. 1 A – the liver spectrum – "a") was chosen as representative for the level of the nitrosyl-iron complexes, and indirectly – NO in the measured material (Kubrina *et al.*, 1992; Andriambeloson, Kleschyov, Muller, Beretz, Stoclet & Andriantsitohaina, 1997; Plonka *et al.*, 2003).

The quantitative results were expressed as mean EPR signal amplitudes of 3-6 animals ± SEM and ± SD. As the supposition on the normal distribution of the results cannot be maintained due to the heterogeneity and putative variability of the NO-generating cell population in developing brain, statistical significance of the differences between experimental and control groups was estimated using the nonparametric Mann-Whitney – *U* test.

RESULTS AND DISCUSSION

MNIC signals in the rat endotoxemic brain and liver

We analysed here the time-dependent formation of NO in rat endotoxemic brain and liver by EPR using exogenous spin trap – $Fe(DETC)_2$. Since DETC *in vivo* often creates EPR-detectable complexes with copper II cations, the signal of which is partially superimposed on the signal of NO-$Fe(DETC)_2$ adducts, only the 3rd, high field component of the signal remains clearly measurable (Kubrina *et al.*, 1992; Andriambeloson *et al.*, 1997) and in the linear fashion corresponds with NO concentration, therefore, it was used by us as the analytical line. Both in endotoxemic livers and brains the EPR MNIC signal of various intensities could be detected besides the $Cu(DETC)_2$ adduct signal, and its high field component was pronounced enough to carry out the kinetic comparisons (Fig. 1).

Kinetics of NO release in endotoxemia as a function of the type of organ

The results of this study show some essential features of nitric oxide production in endotoxemic neonatal and mature animals. Its kinetics is more or less the same in the both studied organs (compare

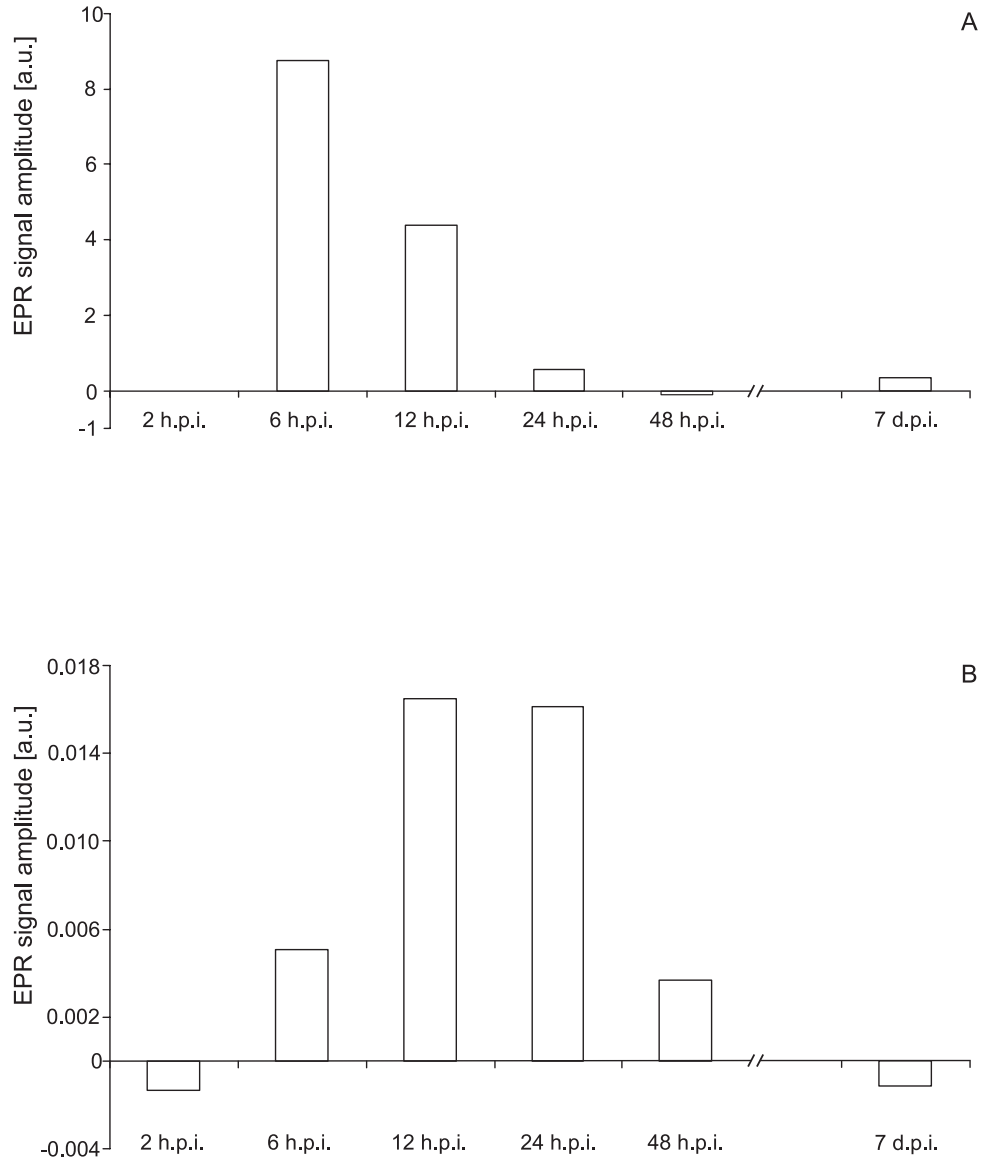

Fig. 3. Comparison of the NO release in the rat brain in response to the LPS challenge in the 30- (A), and 6-days-old animals (B), after subtraction of the appropriate control values in untreated animals: A – on day 30, B – on day 6, 7, 8 and 13 after birth, which corresponds to days 1, 2 and 7 after LPS injection in the parallel test groups (see Fig. 2 B). For the EPR assay parameters – see Fig. 1.

Fig. 2 and 3 with Fig. 4 and 5). It is known that during endotoxemia NO synthesis in the liver outgrows other organs (Kozlov, Szalay, Umar, Fink, Kropik, Nohl, Redl & Bahrami, 2003; Plonka et al., 2003), while the profile of NO production tends to stay similar in various organs and tissues, including liver, blood, lung, kidney or heart (Plonka *et al.*, 2003). Our study confirmed this tendency, which means that observed changes of NO level in the brain was both the reflection of the real nitric oxide production in this organ, and a part of the systemic reaction to LPS. This may be of a particular importance in regard to other processes putatively inducing NO synthesis in the brain, like brain injury, for which we expect the EPR technique to be reliable and useful, as well.

Kinetics of NO release in endotoxemia as a function of the animal postnatal development

Comparison of the absolute (Fig. 2 A and 4 A), as well as the relative (Fig. 3 A and 5 A) kinetics of NO release in the 30-days-old brain and liver with the 6-days-old organs (panels B of Fig. 2-5, respectively) revealed a striking disproportion in NO production in neonatal and mature organs. While the level of NO in the mature liver was

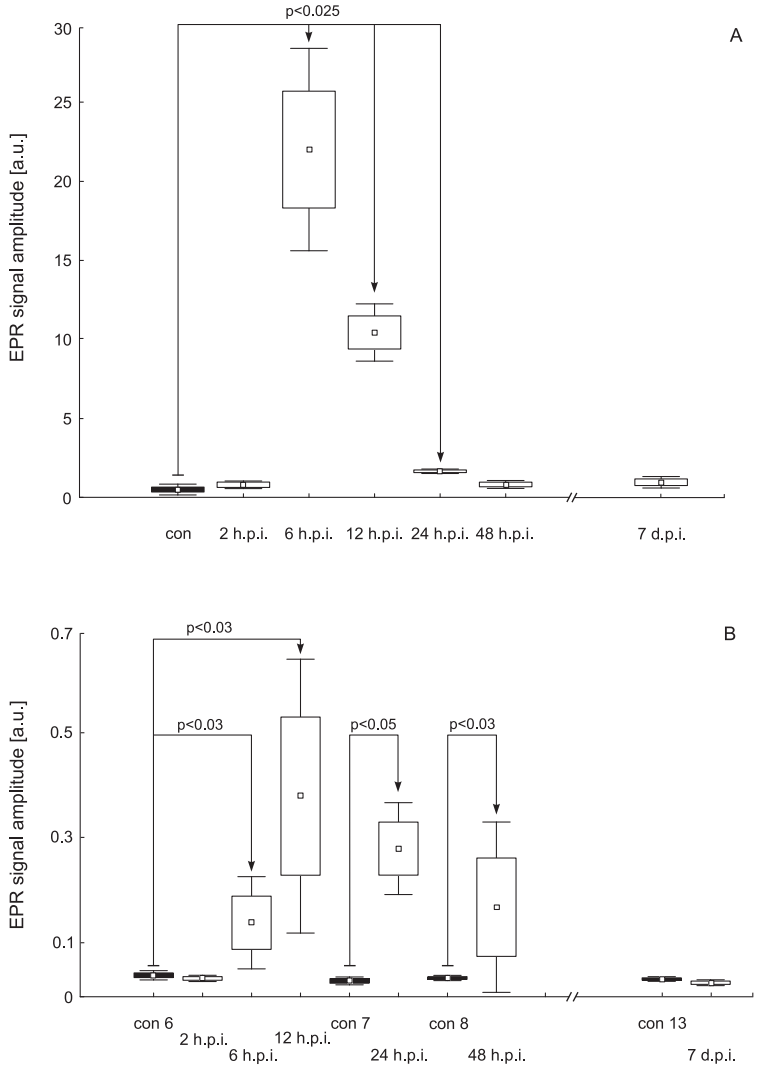

Fig. 4. Kinetics of the NO release in the rat liver following a single i.p. LPS injection (10 mg/kg) in 30- (A), and 6-days-old (B) animals. Each point represents a mean amplitude ($n \geq 3$) of the 3rd hyperfine component of the MNIC signal ± SEM (rectangles) and ± SD (error bars). Arrows indicate significant differences between control (filled boxes) and experimental groups (open boxes). Parameters of the EPR assay – see Fig. 1.

about twice as big as in the brain (cf. Fig. 2 and 3 A with 4 and 5 A), in the neonatal animals this difference was about 20 times larger (cf. Fig. 2 and 3 B with 4 and 5 B). This finding primarily indicates that in relation to the liver (here - a kind of the positive control) the reaction of the neonatal brain to the LPS challenge is to some degree suppressed as compared to the mature organ, which must be a result of developmental processes specific to the brain.

The age-dependent differences in the profile of the NO release following LPS application can be appreciated from a direct comparison of panels A (30 days) with B (6 days) of Fig. 2-5. In the 30-days-old rat brain and liver, nitric oxide production started shortly after endotoxemia induction, and reached the maximum ca. 6 h.p.i. During the next 6 hours (until 12 h.p.i.) the level of NO decreased, but it remained still higher than in the normal brain tissue. On the 1 d.p.i. the NO content was imperceptibly higher than in control brains and in the following days it reached the values characteristic of unaffected brains (see Fig. 2 and 3 A).

A different temporal profile of nitric oxide production was found for the neonatal rat brain (Fig. 2 and 3 B). Here we observed a gradual increase of NO level starting as late as 6 h.p.i. (Fig. 2-3 B). The highest NO concentration was found 12 h.p.i., i.e. twice as late as in the 30-days-old rats. Moreover, this high NO level was maintained till 24 h.p.i., whereupon it gradually got lower within the next several days (Fig. 2-3 B). A similar profile could be observed for the neonatal liver (Fig. 4-5 B).

Variable level of nitric oxide in the normal neonatal brain

It is noteworthy that there were differences in NO concentration in the control, untreated rat brains during the second week of postnatal development (Fig. 2 B) which prompted us to prepare separate control groups for each time point fol-

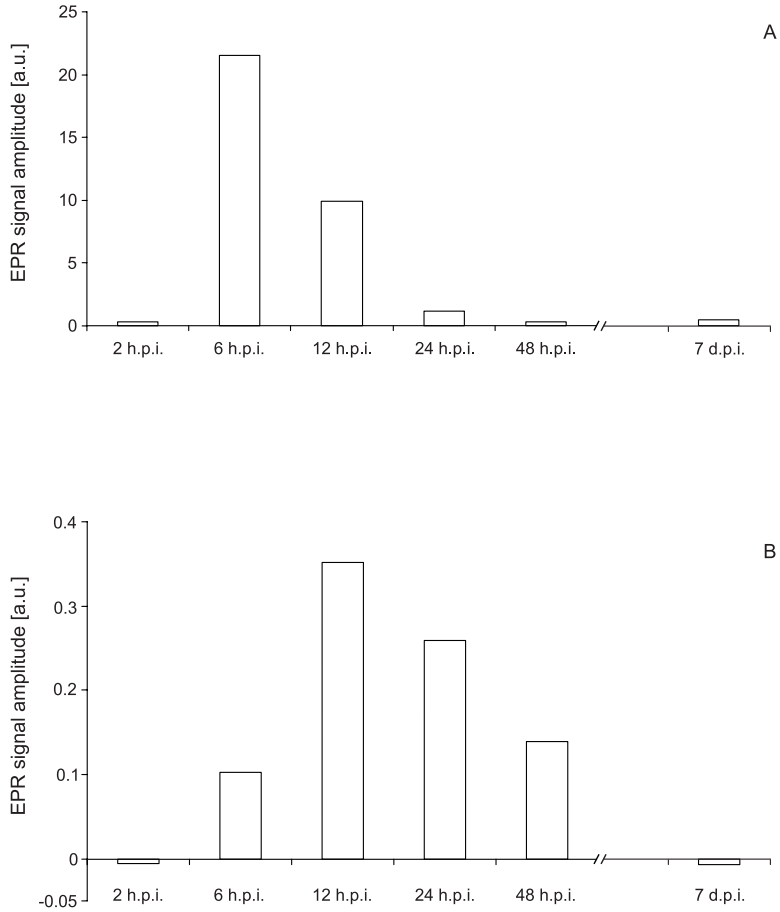

Fig. 5. Comparison of the NO release in the rat liver in response to the LPS challenge in the 30- (A), and 6-days-old animals (B), after subtraction of the appropriate control release in untreated animals (see Fig. 4 B, and 3). For the EPR assay parameters – see Fig. 1.

lowing LPS application (see Fig. 2-5 B). There was a statistically significant rise of the concentration of NO-Fe(DETC)$_2$ complexes in the normal brain on postnatal day 13 in comparison to day 7. This phenomenon is probably connected to the process of nervous system growth during this stage of development, with a considerable reorganization and maturation of the brain cells and blood–brain barrier. As this phenomenon concerns relatively very weak signals of the control tissue, it may be to some degree attributed to the constitutive production of NO by the neuronal isoenzyme of the nitric oxide synthase (NOS 1 or nNOS) (Kozlov, Biagini, Tomasi & Zini, 1995), which is believed to play an important role in controlling the process of brain development (Ogilvie, Schilling, Billingsley & Schmidt, 1995; Boissel, Schwarz & Forstermann, 1998). Multiple transcripts resulting from alternate splicing of the NOS 1 pre-mRNA may be translated to various forms of NOS 1 protein of so far unknown biological functions, and the profile of these variants of NOS 1 may be time- and tissue-specific (Alderton, Cooper & Knowles, 2001). Some of these enzymes may be unable to produce NO, but the ability to reduce dioxygen to superoxide can be preserved (Shimizu, Ishii, Momose & Yamamoto, 1998). The fact that similar differences were not observed in the control livers (cf. Fig. 4 B) may additionally support this hypothesis.

CONCLUSIONS

Our study convincingly shows that the production of nitric oxide in the nervous system, and its responsiveness to lipopolisaccharide is age-dependent. Astrocytes and microglia are the main type of brain cells suspected of the expression of inducible nitric oxide synthase (iNOS, NOS 2) in response to the LPS challenge, which leads to the elevation of NO release in the brain tissue (Simmons & Murphy, 1992). However, it is known that astrocytes as well as microglial cells display different activity, dependent on the stage of the nervous tissue development (Hatten, Liem, Shelanski & Mason, 1991; Ling & Wong, 1993; Dalmau et al., 1998; Acarin, Gonzalez, Castro & Castellano, 1999; Acarin, Gonzalez, Hidalgo, Castro & Castellano, 1999; Streit, Walter & Pennell, 1999). It was previously affirmed that inflammatory reactions in adult and neonatal brains differ. In adult brain there is a little influx of the neutrophils, and a delay as for monocytes (Anthony et al., 1997; Bolton & Perry, 1998). A more intense re-

sponse takes place in the brains of young animals where the inflow of neutrophils from the blood is larger, and, moreover, where the amoeboid microglial cells are still present and act as macrophages in the tissue (Anthony et al., 1997; Anthony et al., 1998; Bolton & Perry, 1998). Hence, a different time-course and level of NO production must be related to the age-dependent differences of NOS 2 expression by glial cells, especially microglia. The role of blood-borne cells as a source of inflammatory cytokines influencing other cells should not be ruled out, either. Moreover, we cannot exclude that the processes observed in this study are not only the result of age-dependent differences in NOS 2 expression, but also in generation of superoxide and peroxynitrite, especially in late stages of endotoxemia (Plonka *et al.*, 2003; Brandes, Koddenberg, Gwinner, Kim, Kruse, Busse & Mugge, 1999). This justifies further investigation in this field, employing EPR and NO spin trapping as a convenient and valuable research technique towards a better understanding of the dynamics of brain development and activity.

Acknowledgements

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