Free Radical Biology & Medicine, Vol. 34, No. 12, pp. 1555–1562, 2003

0891-5849/03/$–see front matter

doi:10.1016/S0891-5849(03)00179-5

Original Contribution

EPR ANALYSIS REVEALS THREE TISSUES RESPONDING TO ENDOTOXIN BY INCREASED FORMATION OF REACTIVE OXYGEN AND NITROGEN SPECIES

ANDREY V. KOZLOV,*† LASZLO SZALAY,*‡ FRAZ UMAR,* BRUNO FINK,§ KARL KROPIK,* HANS NOHL,† HEINZ REDL,* and SOHEYL BAHRAMI*

*Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria; †Department of Pharmacology and Toxicology/Institute for Applied Botany, University of Veterinary Medicine, Vienna, Austria; ‡Institute of Surgical Research and Department of Surgery, University of Szeged, Szeged, Hungary; and §Division of Cardiology, Emory University School of Medicine, Atlanta, GA, USA

(*Received* 18 *October* 2002; *Revised* 10 *March* 2003; *Accepted* 14 *March* 2003)

Abstract—The excessive formation of reactive oxygen and nitrogen species (RONS) in tissue has been implicated in the development of various diseases. In this study we adopted ex vivo low temperature EPR spectroscopy combined with spin trapping technique to measure local RONS levels in frozen tissue samples. CP-H (1-hydroxy-3-carboxy-pyrrolidine), a new nontoxic spin probe, was used to analyze RONS in vivo. In addition, nitrosyl complexes of hemoglobin were determined to trace nitric oxide released into blood. By this technique we found that RONS formation in tissue of control animals increased in the following order: liver < heart < brain < cerebellum < lung < muscle < blood < ileum < kidney < duodenum < jejunum. We also found that endotoxin challenge, which represents the most common model of septic shock, increased the formation of RONS in rat liver, heart, lung, and blood, but decreased RONS formation in jejunum. We did not find changes in RONS levels in other parts of gut, brain, skeletal muscles, and kidney. Scavenging of RONS by CP-H was accompanied by an increase in blood pressure, indicating that LPS-induced vasodilatation may be due to RONS, but not due to nitric oxide. Experiments with tissue homogenates incubated in vitro with CP-H showed that $ONOO^-$ and $O_2^{\bullet -}$, as well as other not identified RONS, are detectable by CP-H in tissue. In summary, low-temperature EPR combined with CP-H infusion allowed detection of local RONS formation in tissues. Increased formation of RONS in response to endotoxin challenge is organ specific.

Keywords—Electron paramagnetic resonance, Oxidative stress, Free radicals, Endotoxin, Liver, Heart, Brain, Lung, Muscle, Blood, Intestine, 1-Hydroxy-3-carboxy-pyrrolidine

INTRODUCTION

Reactive oxygen and nitrogen species (RONS) play an important role in inflammatory processes as mediators of injury. They could also be involved in signal transduction [1–3]. Endotoxin (lipopolysaccharide = LPS) is a constituent of the outer membrane of gram-negative bacteria and evokes an inflammatory response by activation of monocytes, macrophages, and endothelial cells [4,5]. The activation of those cells results in the expression of inducible nitric oxide synthase (iNOS) and in an excessive generation of RONS, particularly of superoxide radical ($O_2^{\bullet -}$) and peroxynitrite ($ONOO^-$). iNOS dramatically increases the transformation of L-arginine to nitric oxide (NO). If produced in excess, NO combines with $O_2^{\bullet -}$ to form $ONOO^-$, a cytotoxic oxidant, causing tissue injury during shock, inflammation, and ischemia-reperfusion [6]. Additionally, it has been suggested that $ONOO^-$ may also be a signal transmitter and can mediate vasorelaxation, similarly as NO [7,8]. Apart from $ONOO^-$, other reactive nitrogen species such as nitrogen oxides and nitryl chloride are believed to cause several different pathophysiological events, including inflammation [9,10].

Inflammatory reactions play an important role in endotoxin-induced tissue injury. The latter is mediated by adhesion and migration of leukocytes through the endo-

Address correspondence to: Dr. Andrey Kozlov, Ludwig Boltzmann Institute for Experimental and Clinical Traumatology at the research center of AUVA, Donaueschingenstrasse 13, Vienna A-1200, Austria; Tel: +43 (1) 250 77 4428; Fax: +43 (1) 250 77 4490; E-Mail: andrey.kozlov@vu-wien.ac.at.

thelium, generation of RONS [11], stimulation, and release of several proinflammatory cytokines such as tumor necrosis factor by monocytes/macrophages [12,13]. Local generation of RONS by resident macrophages contributes to tissue injury. Recent studies have demonstrated that activation of the nuclear enzyme poly(ADP-ribose) polymerase-1 by RONS-mediated DNA damage is an important pathway of tissue injury in conditions associated with oxidative stress [14]. The most susceptible organs to a challenge (sepsis, burn, trauma) are heart [11,15], lung [14,16], kidney [12,17], and liver [13,18]. In spite of the consideration of RONS as important mediators of tissue damage triggered by endotoxic shock and by many other diseases, it is still very difficult to determine tissue concentrations of RONS.

Due to the short lifetime, it is difficult to quantitate RONS production precisely in vivo. Application of optical spectroscopy (e.g., fluorescence dyes selectively reacting with RONS) is impossible because tissue is not transparent for optical irradiation. Applications of spin probes and electron paramagnetic resonance spectroscopy (EPR) is limited by fast reduction of spin adducts in vivo. This is mainly due to endogenous reductants and high toxicity because most of the spin traps have to be applied at high concentrations.

Recently, two new spin probes, 2-(diethoxyphosphoryl)-2-methyl-3,4-dihydro-2H-pyrrole 1-oxide (DEPMPO) [19,20] and 1-hydroxy-3-carboxy-pyrrolidine (CP-H) [21], have been synthesized and rather successfully used for in vivo studies. The former, DEPMPO, is selective for different types of RONS, but requires high concentrations (10 mmole/kg) and its use is complicated by artifactual formation of sulfur-centered radicals [20]. The latter, CP-H, is not specific for different kinds of radicals. The radicals formed with $O_2^{\bullet -}$ and $ONOO^-$ are not distinguishable, but this spin probe is used at low nontoxic concentrations (200–300 μmole/kg) and its radicals are more stable in vivo than those of DEPMPO. Therefore, this study was performed with CP-H, which is a type of redox probe. The paramagnetic CP-H- radical ($CP^\bullet$ = 3-carboxy-proxyl) appears as a result of a single electron transfer. This spin probe has been used increasingly for RONS detection in blood since it became commercially available. CP-H is used for RONS detection because of its outstanding resistance to endogenous reducing agents and its very low toxicity. CP-H has been shown to detect $O_2^{\bullet -}$ and $ONOO^-$ in in vitro experiments and in blood [22,23].

The aim of this study was to clarify whether CP-H can be used to access the local RONS generation in vivo in selected organs. Following this aim, we performed a three-stage study. Firstly, we adopted EPR as the method for routine ex vivo detection of $CP^\bullet$-radicals in tissues. Subsequently, we applied this assay to the LPS shock model. Finally, we tried to identify the species giving rise to the formation of $CP^\bullet$-radicals.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats weighing 430–460 g (Animal Research Laboratories, Himberg, Austria) were used in this study. The study was approved by the local Committee on Animal Experiments of Vienna, Austria, and all experiments were performed under the conditions described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication NIH 86-23; revised 1985).

In vivo experimental procedures

One group of animals (LPS group, $n = 8$) received 10 mg of endotoxin (*E. Coli* 026:B6, Difco, Detroit, MI, USA)/kg body weight intraperitoneally. The control group ($n = 9$) was treated similarly but received the same volume of saline. Four hours after endotoxin challenge, animals were anesthetized by intramuscular injection of a mixture of ketamine/xylazine (112/15 mg/kg body weight) and maintained under anesthesia with 0.2% of isoflurane for the duration of the acute experiment on a temperature-controlled surgical board (36.0 $\pm$ 0.4°C). A silicone catheter was inserted in the jugular vein for infusion of CP-H (9 mg/kg bolus; 0.225 μg/kg/min infusion) or the vehicle phosphate-buffered solution (PBS) at the same rate. CPH was obtained from Noxygen Science Transfer & Diagnostics (D-79211, Denzlingen). A second silicon catheter was inserted in the femoral artery for measuring blood pressure (Cardiosys, Budapest, Hungary) and withdrawing blood samples. CP-H solution (5 mg/ml) was freshly prepared in 50 mM sodium PBS as described previously [24]. After 120 min CP-H infusion, blood and tissue samples (left cerebral hemisphere without cerebellum, cerebellum, gastrocnemius muscle, heart, kidney, liver, lung, duodenum, jejunum, and ileum) were collected. The samples were placed in 1 ml syringes (B. Braun Melsungen AG), frozen, and kept in liquid nitrogen until they were used for EPR measurements.

Ex vivo experimental procedures

To elucidate the origin of RONS detectable by CP-H in liver and heart, the samples from three control and three LPS-treated animals were homogenized in sucrose buffer (0.25 M sucrose, 10 mM TRIS-HCl, 1 mM EDTA, pH = 7.4) at a ratio of 1:6 liver/buffer (w/v), using Potter-Elvehjem homogenizer. After filtration through three layers of surgical gauze, aliquots of 1 ml were frozen in liquid nitrogen until use in experiments.

Rat liver mitochondria (RLM) were prepared as we described previously [25]. Homogenates or RLM were incubated for 20 min at room temperature with CP-H in a buffer containing 10 mM Hepes, 140 mM KCl, 1.5 mM $CaCl_2$, 4 mM KH_2PO_4. Fifty microliters of homogenate was mixed with 400 μl of buffer. Gas exchange between samples and air was facilitated using a shaking table. At the end of incubation the amount of $CP^{\bullet}$-radicals formed was determined. The experiments on interaction between CP-H and NO or $ONOO^-$ (obtained from Alexis Co. CH-4415, Lausen) solutions were performed anaerobically under nitrogen. Enzyme inhibitors and substrates were used at the following final concentrations: 1 mM N^G-Monomethyl-L-arginine monoacetate (L-NMMA); 2.5 mM L-arginine plus 170 μM BH_4; 200 U/ml superoxide dismutase (SOD). Additionally, the effects of 826 μM desferal (Desferrioxamine B), 900 U/ml catalase, and 300 mM ethanol on the formation of $CP^{\bullet}$-radicals in homogenates were tested in order to elucidate the involvement of iron ions, hydrogen peroxide, and hydroxyl radical, respectively. However, none of those substances had an effect (data are not shown).

EPR measurements

The samples were pressed out of the syringes (see above) and moved to a finger-tip liquid nitrogen dewar for EPR measurements. The EPR spectra were recorded at liquid nitrogen temperature with a Bruker EMX EPR spectrometer (BioSpin GmbH Rheinstetten/Karlsruhe, Germany). The general settings low temperature measurements were: microwave frequency 9.431 GHz, modulation frequency 100 kHz, modulation amplitude 5 G; gain 10^5. At room temperature the following settings were applied: microwave frequency 9.776 GHz, modulation frequency 100 kHz, modulation amplitude 2 G; power 5 mW. Other parameters are indicated in figure legends.

Calibration of EPR signals

In order to quantify NO levels in blood, the nitrosyl hemoglobin (NO-Hb) signals observed in blood were compared with those obtained from NO-Hb standards. NO-Hb standards were prepared using NO_2^- solutions reduced by dithionite in the presence of Hb similar to that described by Tsuchiya at al. [26]. Briefly, 100 mg of dithionite was mixed with 400 μl of Hb solution and 10 μl of NO_2^- solution. Calibration was carried out either with an excess of Hb (5mM) and a range of NO_2^- concentrations (0 to 25 μM), or with an excess of NO_2^- (5 mM) and a range of Hb concentrations (0–25 μM). Both calibrations gave identical results. Upon reaction with RONS CP-H is transformed into a stable $CP^{\bullet}$ radical (3-carboxy-proxyl). Therefore, standard solutions of 3-carboxy-proxyl were used to quantify RONS levels in blood and tissues.

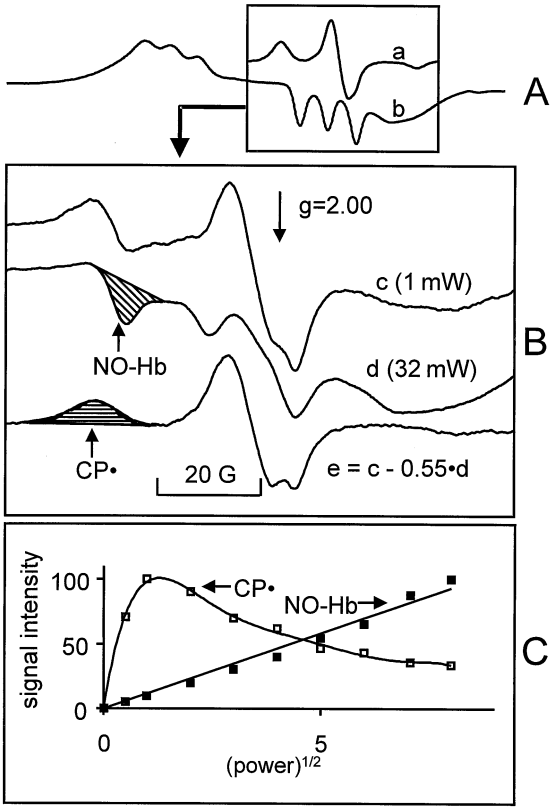

Fig. 1. Detection of $CP^{\bullet}$-radicals and NO-Hb complexes in blood. (A) EPR spectra of $CP^{\bullet}$-radical (spectrum a) and NO-Hb complex (spectrum b) recorded at liquid nitrogen temperature. (B) Separation of NO-Hb and $CP^{\bullet}$-radical signals using power saturation. Pure NO-Hb signal can be obtained at high power (32 mW or more) when $CP^{\bullet}$ signal is saturated (spectrum d). Pure $CP^{\bullet}$ spectrum is obtained by subtraction of spectrum d from mixed spectrum obtained at 1 mW (spectrum c). (C) Power saturation curves of $CP^{\bullet}$ and NO-Hb signals.

Blood parameters

For measurement of pH, base excess, glucose and lactate, heparinized blood samples were analyzed using ABL 625 System (Copenhagen, Denmark).

Statistics

Statistical parameters were calculated using ANOVA (Excel 5.0 software, Microsoft Corp.). The data are presented as mean ± SEM.

RESULTS

Separation of NO-Hb signal and $CP^{\bullet}$ signal by power saturation

At liquid nitrogen temperature the signal of the $CP^{\bullet}$-radical appears as shown in Fig. 1. The middle

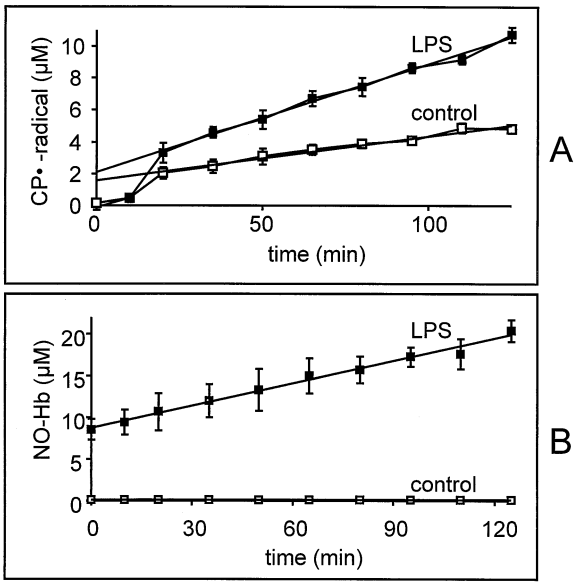

Fig. 2. Generation of NO and RONS in blood of control animals (n = 9) and animals receiving LPS (n = 8). (A) Generation of RONS was followed by CP•-radical signal. The rate of RONS generation in control and LPS challenged animals was 27 ± 3 nM/min and 68 ± 4 nM/min, respectively. (B) Generation of NO was followed by detection of NO-Hb signal. The rate of NO generation in control animals and in animals receiving LPS was 1 ± 3 nM/min and 89 ± 8 nM/min, respectively.

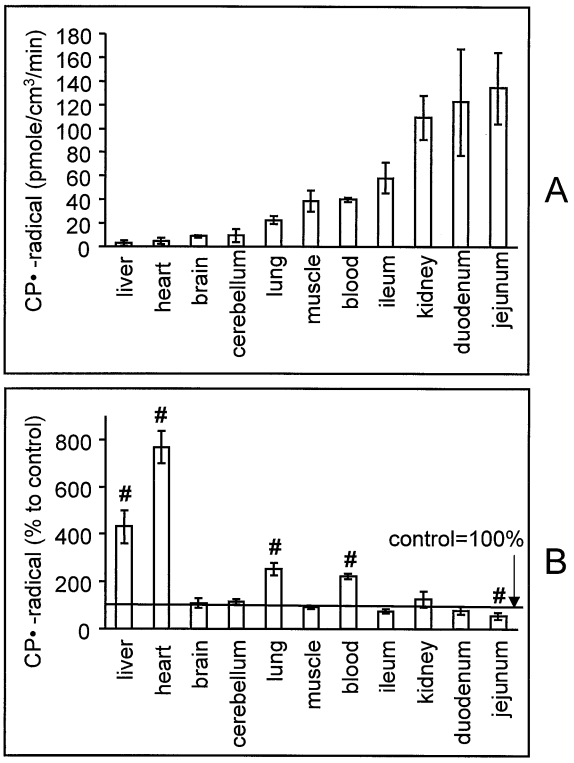

Fig. 3. RONS generation in control rats (n = 9) and LPS-treated rats (n = 8). (A) Absolute concentrations of RONS generated in controls. (B) Effect of LPS challenge on RONS levels in different organs. Control values for each tissue was taken as 100%. # $p < .05$.

component is centered at g = 2.0 and not suitable for estimation of the CP•-radical concentrations because it overlaps with many other free radical signals found in biological systems. Therefore, a low field shoulder has been chosen to quantify the CP•-radical in spite of its lower intensity. Animals subjected to LPS also had an intensive signal of NO-Hb complexes in blood. The triplet characteristic of NO-Hb complexes overlapped with the low field shoulder of CP• signal. To separate these two signals we compared their power saturation characteristics. As shown in Fig. 1 the NO-Hb signal is not saturated below 100 mW. In contrast, the CP• signal is already saturated at 4 mW. Using this difference in saturation, we separated the CP• signal by subtraction of the spectrum obtained at 32 mW (pure NO-Hb spectrum) from the spectrum obtained at 1 mW (CP• signal mixed with NO-Hb signal). The loss of CP• signal intensity after subtraction was less than 3%. The integral intensity of the NO-Hb trough at g = 2.019 (Fig. 1) was taken as the measure of NO concentration and integral intensity of CP• peak at g = 2.021 (Fig. 1) was taken as the measure of the CP• concentration. The absolute concentrations of NO-Hb complexes and CP•-radicals were calculated as described in the Materials and Methods section.

RONS in blood

Four hours after LPS challenge the rate of RONS generation was 2.5 times greater than controls (Fig. 2). The concentrations of NO-Hb complexes in control animals were close to the detection limit of the instrument (100–200 nM of NO). Injection of LPS resulted in approximately 100-fold increase of NO-Hb levels in blood due to the well-known induction of iNOS in this model [27]. Circulating NO levels increased gradually up to the 6th hour after LPS injection (Fig. 2).

RONS in tissue

The analysis of different tissues obtained from control animals revealed that the rate of RONS generation in all organs is sufficient to be measured by low temperature EPR spectroscopy combined with CP-H infusion. We found a great variation of the rates of RONS formation in different organs. The average RONS generation rate increased in the following order: liver < heart < brain < cerebellum < lung < muscle < blood < ileum < kidney < duodenum < jejunum. LPS challenge did not equally influence RONS generation in the organs studied. In heart, liver, and lung, RONS levels were significantly increased (Fig. 3). The most pronounced increase (7-

Table 1. Glucose, Lactate, and Blood Gases of Controls and LPS-challenged Animals Prior to CPH Infusion

	Control (n = 9)	LPS (n = 8)
Glucose (mg/dl)	180 ± 22	118 ± 18*
Lactate (mmol/l)	0.7 ± 0.1	1.2 ± 0.3*
pH	7.30 ± 0.04	7.30 ± 0.03
pCO_2 (mmHg)	46.2 ± 5.7	42.3 ± 5.6
pO_2 (mmHg)	95 ± 17	101 ± 18
sO_2 (%)	94.0 ± 4.8	96.2 ± 2.5
O_2Hb (%)	91.1 ± 4.8	92.7 ± 2.2

* $p < .004$.

fold) was observed in heart. RONS levels were significantly decreased only in jejunum. RONS levels in other organs did not respond to LPS challenge (Fig. 3).

Effect of endotoxin and CP-H on blood pressure

Endotoxin challenge significantly increased plasma lactate levels and lowered glucose concentration without affecting pH, base excess, pO_2, or pCO_2 (Table 1). In the LPS group mean arterial pressure (MAP) decreased gradually before infusion of CP-H. Infusion of CP-H induced an increase in MAP both in control and in endotoxin-treated animals (Fig. 4), but did not influence heart rate (data not shown). In control animals this effect on MAP was temporary, returning to original values after 20 min. CP-H infusion abolished the gradual decrease in blood pressure induced by LPS.

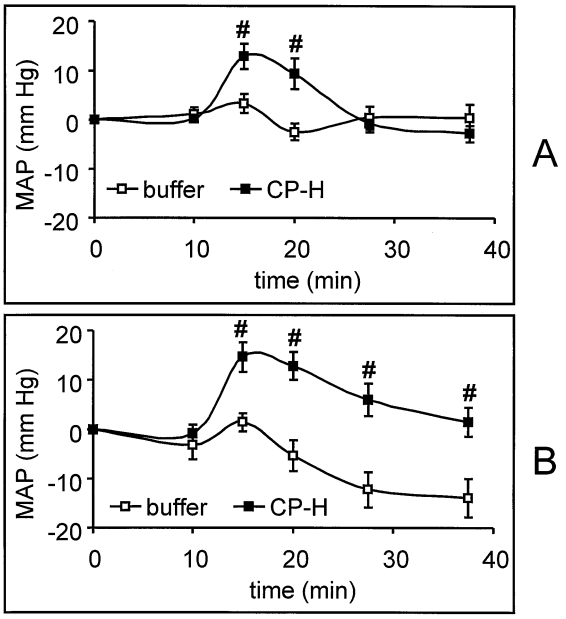

Fig. 4. Effect of CP-H infusion on MAP in control (n = 9; A) and LPS-challenged (n = 8; B) animals. # $p < .05$.

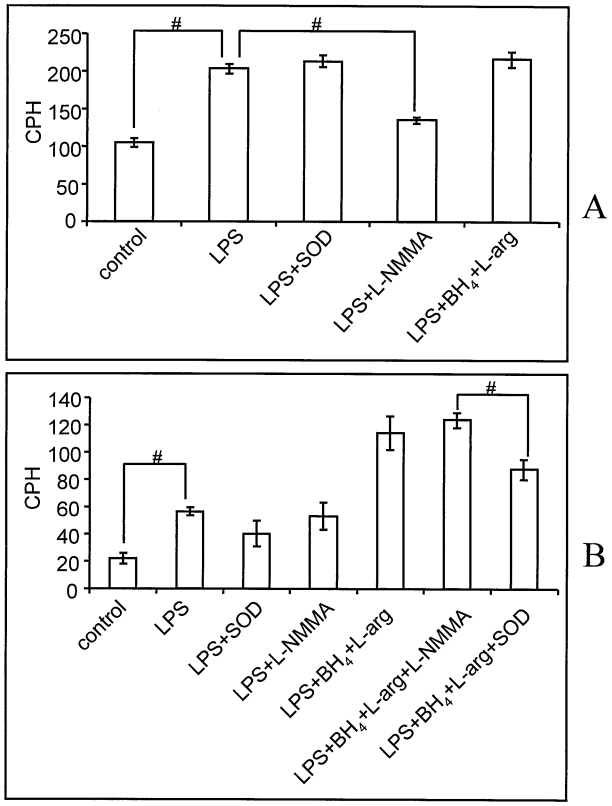

Fig. 5. Effect of NOS inhibitors/stimulators and SOD on the formation of $CP^{\bullet}$-radicals in liver (A) and heart (B) homogenates, obtained from control rats (n = 3) and rats subjected to LPS challenge (n = 3). Homogenates were incubated for 20 min at room temperature with CP-H, then EPR spectra were recorded at room temperature. 1 mM L-NMMA; 2.5 mM L-arginine (L-arg); 170 μM BH_4, and 200 U/ml SOD were used. # $p < .05$.

Elucidation of the nature of RONS resulting in generation of $CP^{\bullet}$-radicals

To elucidate the origin of RONS detectable by CP-H we prepared homogenates from heart and liver of control animals and animals subjected to LPS but without infusion of CP-H (Fig. 5). Incubation of these homogenates in vitro with CP-H resulted in the formation of $CP^{\bullet}$-radicals both in control and in LPS-pretreated homogenates, and the amount of $CP^{\bullet}$ signal was higher in homogenates from LPS animals, reflecting the in vivo situation. In liver homogenates from LPS-treated animals the level of adducts was significantly decreased by L-NMMA, but was not sensitive to BH_4/L-arginine (Fig. 5). In contrast, the inhibitors applied had no effect in heart homogenates from either controls or LPS-treated animals. However, the introduction of BH_4 and L-arginine resulted in almost equal increase of $CP^{\bullet}$ signal in both cases. The signal increase in LPS animals was sensitive to SOD but not sensitive to L-NMMA. In addition to SOD and L-NMMA, we applied the following reagents:

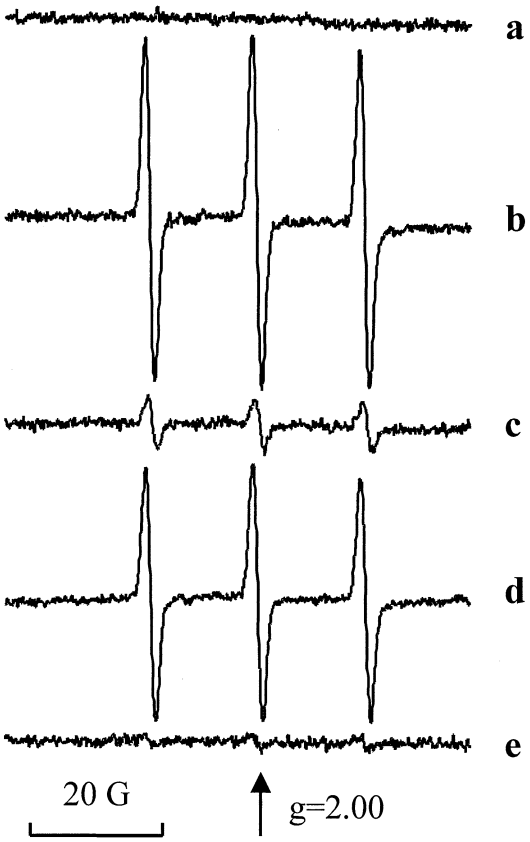

Fig. 6. EPR spectra of $CP^{\bullet}$-radicals detected after 20 min of incubation at room temperature. Spectrum a: CP-H and buffer. Spectrum b: Mitochondria incubated with 2 μg/ml of Antimycin A. Spectrum c: Mitochondria incubated with 2 μg/ml of Antimycin A and 200 U/ml SOD. Spectrum d: CP-H mixed with 20 μM of $ONOO^-$. Spectrum e: CP-H mixed with 20 μM of NO. Spectra a, b, c, and e were recorded at the gain of 2×10^5, spectrum d at the gain of 10^5.

826 μM desferal, an iron chelator, 900 U/ml catalase, and 300 mM ethanol as a scavenger of hydroxyl radicals. However, none of them influenced the levels of detectable RONS (data are not shown).

It is reported that mitochondria represent the main $O_2^{\bullet -}$ source in the cell. Figure 6 shows that CP-H detects $O_2^{\bullet -}$ produced by rat heart mitochondria and that $CP^{\bullet}$ signal is sensitive to SOD. A mixture of CP-H and $ONOO^-$ shows a clear signal. In contrast, a mixture of CP-H and NO shows no signal.

DISCUSSION

Ex vivo low temperature EPR spectroscopy used here for the detection of RONS can be used on animals of any size since it measures tissue/blood samples. The steady state concentration of free radicals in vivo is low. For measurement, signals need to accumulate in tissue until they reach a detectable threshold level. In the frozen samples sensitivity is decreased due to the broadening of the spectrum bands, but can be compensated by time-unlimited accumulation of the signals during measurement. Similar attempts have already been made to detect DEPMPO adducts by low temperature EPR spectroscopy [28].

It has already been shown that the basal level of RONS production in blood can be detected with CP-H using room temperature EPR spectroscopy [24,29]. In this study we found similar results by low temperature EPR, demonstrating that this technique is sensitive enough for in vivo measurements. Using EPR spectroscopy combined with CP-H infusion we have estimated the absolute rates of RONS generation in most rat organs. It has been found that the concentrations of $CP^{\bullet}$-radicals are tissue specific. The highest concentration has been found in intestine and the lowest in heart and liver. Using the calibration procedure described in Materials and Methods, the rates of RONS generation were found in a range of 3 to 100 pmole/cm^3/min. These calculations are based on the assumption that all RONS are transformed to paramagnetic adducts detectable by EPR and all adducts have been detected by EPR. In reality, a portion of RONS reacts with other molecules, and a portion of paramagnetic adducts degrades to EPR silent products. Thus, the real rates of RONS generation are probably even higher. The data presented here are probably the lowest limit of RONS generation rates in control tissue.

LPS is frequently used in experimental models of septic shock initiating a cascade of complications and multiple organ failure. LPS-induced shock is known to be accompanied by excessive RONS formation, however it was not clear whether this increase is tissue specific. Our results show that only three organs, namely heart, liver, and lung responded to LPS challenge with increased RONS generation, while a part of intestine (jejunum) showed even lower RONS generation after LPS challenge compared to the controls. The highest increase of RONS formation was observed in heart. These data suggest that the failure in heart, liver, and lung often reported in septic models is probably mediated by oxidative stress induced by elevated RONS generation in these organs. In contrast, well-documented renal failure is probably not mediated by RONS, since our experimental model did not show any increase in RONS generation.

A substantial amount of literature indicates that LPS activates microglial cells in the brain and that microglia produce RONS, contributing to neurodegeneration [30,31]. These results were obtained in cell culture and in thin tissue slices. In vivo, however, we did not observe any activation of RONS release in brain. This discrepancy could be due to nonadequate in vitro models used in

previous studies or to lower LPS concentrations reaching the brain in in vivo model.

LPS challenge has been shown to decrease MAP [32–34]. A decrease in MAP upon LPS challenge was also observed in our study (Fig. 4). Removal of RONS by infusion of CP-H was accompanied by an increase in MAP. The excessive NO formation is believed to be responsible for low blood pressure during sepsis. In this study we found a strong increase of NO generation resulting in formation of NO-Hb complexes. We also found that CP-H infusion temporarily increases blood pressure in control animals and prevents the decrease in blood pressure in LPS animals. This effect may be due to interaction of CP-H with a species that has a vasodilatory effect. It has been shown that exogenously added $ONOO^-$ induces vasodilation. The vasodilatory effects of exogenously added $ONOO^-$ are, in fact, due to the formation of small amounts of S-nitrosothiols. This could be a good explanation for the effects of CP-H in LPS animals, which produce much more $ONOO^-$ than in controls. However, the effect can also be seen in control animals when $ONOO^-$ is present at very low concentrations, which do not promote vasodilation. Thus one can expect that an unknown substance apart from $ONOO^-$ and S-nitrosothiols may be involved.

To elucidate the origin of RONS trapped in tissues we performed experiments with tissue homogenates. Effects of L-NMMA and L-arginine plus BH_4 in liver homogenates indicate that generation of NO is at least partly involved in formation of $CP^{\bullet}$-related EPR signals. Since NO itself does not produce $CP^{\bullet}$-radicals, one can conclude that derivatives of NO, like $ONOO^-$, are involved. The involvement of $ONOO^-$ is supported by opposite effects of BH_4 and L-arginine and SOD in heart homogenates. In liver from LPS-treated animals excessive formation of RONS is almost completely inhibited by L-NMMA but not by SOD. This fact can indicate either that nitrogen species other than $ONOO^-$ is predominantly formed in liver or that the nitrogen species formed is $ONOO^-$, but that its formation is not limited by $O_2^{\bullet -}$ concentration. In the heart the situation seems to be the opposite. Upon addition of BH_4 and L-arginine, excessive amounts of NO in comparison to $O_2^{\bullet -}$ are produced. This could explain why only SOD but not L-NMMA has an effect on RONS levels under these circumstances. However, a significant part of the $CP^{\bullet}$-signal observed after incubation of homogenates with CP-H is not sensitive to SOD and L-NMMA. We cannot identify the origin of the metabolite responsible for this part of the signal yet. However, the fact that the intensities of signals observed in homogenates were significantly higher than those in tissue under in vivo conditions may indicate that at least a part of this lack of response to inhibitor is produced by homogenization of the tissue and in vitro incubation. In summary, we conclude that CP-H can be used for detection of local RONS levels, but that additional efforts should be performed for the identification of all species detectable by CP-H.

REFERENCES

[1] Gartner, R.; Albrich, W.; Angstwurm, M. W. The effect of a selenium supplementation on the outcome of patients with severe systemic inflammation, burn and trauma. *Biofactors* **14:**199–204; 2001.

[2] Hogg, N. Free radicals in disease. *Semin. Reprod. Endocrinol.* **16:**241–248; 1998.

[3] Taylor, D. E.; Piantadosi, C. A. Oxidative metabolism in sepsis and sepsis syndrome. *J. Crit. Care* **10:**122–135; 1995.

[4] Henneke, P.; Golenbock, D. T. Innate immune recognition of lipopolysaccharide by endothelial cells. *Crit. Care Med.* **30:** S207–S213; 2002.

[5] Schlag, G.; Redl, H. *Pathophysiology of shock, sepsis, and organ failure*. New York: Springer Verlag; 1993.

[6] Szabo, C. The pathophysiological role of peroxynitrite in shock, inflammation, and ischemia-reperfusion injury. *Shock* **6:**79–88; 1996.

[7] Liu, S.; Beckman, J.-S.; Ku, D.-D. Peroxynitrite, a product of superoxide and nitric oxide, produces coronary vasorelaxation in dogs. *J. Pharmacol. Exp. Ther.* **268:**1114–1121; 1994.

[8] Graves, J.-E.; Lewis, S.-J.; Kooy, N.-W. Peroxynitrite-mediated vasorelaxation: evidence against the formation of circulating S-nitrosothiols. *Am. J. Physiol.* **274:**H1001–H1008; 1998.

[9] Heck, D. E. *NO, RSNO, ONOO−, NO+, *NOO, NOx—dynamic regulation of oxidant scavenging, nitric oxide stores, and cyclic GMP-independent cell signaling. *Antioxid. Redox Signal.* **3:**249–260; 2001.

[10] Masuda, M.; Nishino, H.; Ohshima, H. Formation of 8-nitroguanosine in cellular RNA as a biomarker of exposure to reactive nitrogen species. *Chem. Biol. Interact.* **139:**187–197; 2002.

[11] Ben Shaul, V.; Lomnitski, L.; Nyska, A.; Zurovsky, Y.; Bergman, M.; Grossman, S. The effect of natural antioxidants, NAO and apocynin, on oxidative stress in the rat heart following LPS challenge. *Toxicol. Lett.* **123:**1–10; 2001.

[12] Knotek, M.; Rogachev, B.; Wang, W.; Ecder, T.; Melnikov, V.; Gengaro, P. E.; Esson, M.; Edelstein, C. L.; Dinarello, C. A.; Schrier, R. W. Endotoxemic renal failure in mice: role of tumor necrosis factor independent of inducible nitric oxide synthase. *Kidney Int.* **59:**2243–2249; 2001.

[13] Wang, J. H.; Redmond, H. P.; Watson, R. W.; Bouchier-Hayes, D. Role of lipopolysaccharide and tumor necrosis factor-alpha in induction of hepatocyte necrosis. *Am. J. Physiol.* **269:**G297–G304; 1995.

[14] Liaudet, L.; Pacher, P.; Mabley, J. G.; Virag, L.; Soriano, F. G.; Hasko, G.; Szabo, C. Activation of poly(ADP-Ribose) polymerase-1 is a central mechanism of lipopolysaccharide-induced acute lung inflammation. *Am. J. Respir. Crit. Care Med.* **165:**372–377; 2002.

[15] Patten, M.; Bunemann, J.; Thoma, B.; Kramer, E.; Thoenes, M.; Stube, S.; Mittmann, C.; Wieland, T. Endotoxin induces desensitization of cardiac endothelin-1 receptor signaling by increased expression of RGS4 and RGS16. *Cardiovasc. Res.* **53:**156–164; 2002.

[16] Nathens, A. B.; Marshall, J. C.; Watson, R. W.; Dackiw, A. P.; Rotstein, O. D. Diethylmaleate attenuates endotoxin-induced lung injury. *Surgery* **120:**360–366; 1996.

[17] Cunningham, P. N.; Dyanov, H. M.; Park, P.; Wang, J.; Newell, K. A.; Quigg, R. J. Acute renal failure in endotoxemia is caused by TNF acting directly on TNF receptor-1 in kidney. *J. Immunol.* **168:**5817–5823; 2002.

[18] New, K. J.; Eaton, S.; Elliott, K. R.; Spitz, L.; Quant, P. A. Effect

of lipopolysaccharide and cytokines on oxidative metabolism in neonatal rat hepatocytes. *J. Pediatr. Surg.* **36:**338–340; 2001.
[19] Karoui, H.; Hogg, N.; Frejaville, C.; Tordo, P.; Kalyanaraman, B. Characterization of sulfur-centered radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite. ESR-spin trapping and oxygen uptake studies. *J. Biol. Chem.* **271:** 6000–6009; 1996.
[20] Liu, K. J.; Miyake, M.; Panz, T.; Swartz, H. Evaluation of DEPMPO as a spin trapping agent in biological systems. *Free Radic. Biol. Med.* **26:**714–721; 1999.
[21] Dikalov, S.; Skatchkov, M.; Bassenge, E. Spin trapping of superoxide radicals and peroxynitrite by 1-hydroxy-3-carboxy-pyrrolidine and 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine and the stability of corresponding nitroxyl radicals towards biological reductants. *Biochem. Biophys. Res. Commun.* **231:**701–704; 1997.
[22] Dikalov, S.; Skatchkov, M.; Bassenge, E. Quantification of peroxynitrite, superoxide, and peroxyl radicals by a new spin trap hydroxylamine 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine. *Biochem. Biophys. Res. Commun.* **230:**54–57; 1997.
[23] Dikalov, S.; Skatchkov, M.; Fink, B.; Bassenge, E. Quantification of superoxide radicals and peroxynitrite in vascular cells using oxidation of sterically hindered hydroxylamines and electron spin resonance. *Nitric Oxide* **1:**423–431; 1997.
[24] Fink, B.; Dikalov, S.; Bassenge, E. A new approach for extracellular spin trapping of nitroglycerin-induced superoxide radicals both in vitro and in vivo. *Free Radic. Biol. Med.* **28:**121–128; 2000.
[25] Kozlov, A. V.; Staniek, K.; Nohl, H. Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett.* **454:** 127–130; 1999.
[26] Tsuchiya, K.; Takasugi, M.; Minakuchi, K.; Fukuzawa, K. Sensitive quantitation of nitric oxide by EPR spectroscopy. *Free Radic. Biol. Med.* **21:**733–737; 1996.
[27] Kozlov, A. V.; Sobhian, B.; Duvigneau, C.; Gemeiner, M.; Nohl, H.; Redl, H.; Bahrami, S. Organ specific formation of nitrosyl complexes under intestinal ischemia/reperfusion in rats involves NOS-independent mechanism(s). *Shock* **15:**366–371; 2001.
[28] Dambrova, M.; Baumane, L.; Kalvinsh, I.; Wikberg, J. E. Improved method for EPR detection of DEPMPO-superoxide radicals by liquid nitrogen freezing. *Biochem. Biophys. Res. Commun.* **275:**895–898; 2000.
[29] Bassenge, E.; Sommer, O.; Schwemmer, M.; Bunger, R. Antioxidant pyruvate inhibits cardiac formation of reactive oxygen species through changes in redox state. *Am. J. Physiol. Heart Circ. Physiol.* **279:**H2431–H2438; 2000.
[30] Chang, R. C.; Rota, C.; Glover, R. E.; Mason, R. P.; Hong, J. S. A novel effect of an opioid receptor antagonist, naloxone, on the production of reactive oxygen species by microglia: a study by electron paramagnetic resonance spectroscopy. *Brain Res.* **854:** 224–229; 2000.
[31] Liu, B.; Du, L.; Hong, J. S. Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. *J. Pharmacol. Exp. Ther.* **293:**607–617; 2000.
[32] Goff, J. M.; Jackson, M. R.; Taher, M.; Cutting, M. A.; Alving, B. M.; Krishnamurti, C. Physiologic responses of cross-linked hemoglobin in endotoxin-treated rats. *Artif. Cells Blood Substit. Immobil. Biotechnol.* **27:**11–21; 1999.
[33] Greenberg, S. S.; Xie, J.; Zatarain, J. M.; Kapusta, D. R.; Miller, M. J. Hydroxocobalamin (vitamin B12a) prevents and reverses endotoxin-induced hypotension and mortality in rodents: role of nitric oxide. *J. Pharmacol. Exp. Ther.* **273:**257–265; 1995.
[34] Iwase, M.; Yokota, M.; Kitaichi, K.; Wang, L.; Takagi, K.; Nagasaka, T.; Izawa, H.; Hasegawa, T. Cardiac functional and structural alterations induced by endotoxin in rats: importance of platelet-activating factor. *Crit. Care Med.* **29:**609–617; 2001.

ABBREVIATIONS

BH_4—(6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride
$CP^{\bullet}$—3-carboxy-proxyl (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy)
CP-H—1-hydroxy-3-carboxy-pyrrolidine (1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine)
DEPMPO—2-(diethoxyphosphoryl)-2-methyl-3,4-dihydro-2H-pyrrole 1-oxide
EPR—electron paramagnetic resonance
L-NMMA—N^G-Monomethyl-L-arginine monoacetate
LPS—lipopolysaccharide
MAP—mean arterial pressure
NO-Hb—nitrosyl hemoglobin
$O_2^{\bullet -}$—superoxide radical
$ONOO^-$—peroxynitrite
RONS—reactive oxygen and nitrogen species
SOD—Superoxide dismutase